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Analysis of Osmotic Tolerance, Physiological Characteristics, and Gene Expression of *Salmonella enterica* subsp. *enterica* Serotype Derby

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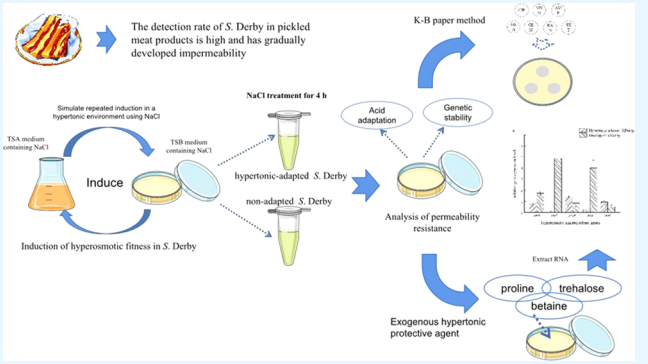
ABSTRACT: *Salmonella* is an important foodborne pathogen, and recent epidemiological studies have shown high infection rates of *Salmonella enterica* subsp. *enterica* serotype Derby (*S*.Derby) in poultry in western China and other regions. *S*.Derby presents increasing concerns with the development of resistance to hypertonic environments; however, there are few reports investigating the mechanism of resistance. Therefore, in this study, we examined hypertonic adaptation in *S*.Derby at the physiological and molecular levels. The K-B paper method, wiping glass bead method, crystal violet staining, and RT-PCR combined with comparative genomics analysis were employed to characterize virulence, drug resistance, biofilm formation, and changes in gene expression of genes related to hypertonic adaptation in *S*.Derby. Hypertonic-adapted *S*.Derby exhibited resistance to OXA, AMP, PEN, and CEP antibiotics, and biofilm-forming ability was 1.25 times that of nonadapted *S*.Derby. RT-PCR results showed that compared with nonadapted *S*.Derby, the expression of virulence-related genes in hypertonic-adapted *S*.Derby increased by 2−3 times, that of biofilm-related genes increased by 2−4 times, and that of OXA, AMP, PEN, and CEP-related drug resistance genes was relatively high. Four hypertonic tolerance-related genes (*otsA, proV, proW, omsV*) were preliminarily identified in *S*.Derby. The expression of *proW* was always relatively high in hypertonic-adapted *S*.Derby, the expression of *otsA* gradually became higher than that of *proW* with increasing time of osmotic stress, and the expression of *proV* and *omsV* was only high in non-hypertonic-adapted *S*.Derby.



1. INTRODUCTION

Salmonella is an important foodborne zoonotic agent worldwide that infects multiple hosts, including humans and other animals, and causes various diseases. The most common disease manifestation is gastroenteritis, which can cause nausea, vomiting, abdominal pain, and other symptoms. A high level of infection can endanger human safety and even lead to death.[1] Statistically, more than 100 million people worldwide are infected with *Salmonella* every year. In China, nearly 70−80% of bacterial food poisoning cases are caused by *Salmonella* infections.[2] *Salmonella enterica* subsp. *enterica* Derby is an important serotype that can adapt to pigs.[3] *Salmonella* in pork is the second largest source of *Salmonella* infection. The main *Salmonella* serotypes in pigs reported in 26 countries, including Europe, mainly include Typhimurium, Delphi, and Enteritidis, with the detection rate of *S*.Derby also increasing. In recent years, various regions in China have reported *S*.Derby in pork.[4] *Salmonella* outbreaks not only cause huge economic losses to the food manufacturing industry, but they also pose a growing threat to the safety of consumers.

The environment in which bacteria live is often extreme or harsh. *Salmonella* is subject to cross-synergism of a variety of stress factors in its external environment, resulting in changes in its tolerance. In food processing and storage, high temperatures, organic acids, NaOH, disinfectants, and other sterilization measures are often used. Some studies have shown that the tolerance of *Salmonella* increases after treatment at 45 °C for 30 min (sublethal). When *Salmonella* is in a relatively dry environment, its thermal adaptability increases, and it

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Table 1. Criteria for Determining Bacterial Susceptibility to Drug

mechanism	category	name	abbreviation	content/μg	judgment standard (diameter of bacteriostatic ring)/mm		
					R	*I*	*S*
inhibition of cell wall synthesis	penicillins	oxacillin	OXA	20	≤13	14−16	≥17
		ampicillin	AMP	20	≤13	14−16	≥17
		penicillin	PEN	20	≤13	14−16	≥17
	glycopeptides	polymyxin B	PB	20	≤8	8−11	≥12
	cephalosporins	cefothiophene	CEP	20	≤14	15−17	≥18
inhibiting protein synthesis	aminoglycosides	kanamycin	KAN	20	≤13	14−17	≥18
		gentamicin	GEN	20	≤13	14−17	≥18
	tetracyclines	minocycline	MNO	20	≤14	15−18	≥19
inhibition of nucleic acid synthesis	quinolones	ciprofloxacin	CIP	20	≤15	15−21	≥21
		enrofloxacin	ENR	20	≤15	15−21	≥21
		norfloxacin	NOR	20	≤15	15−21	≥21
	sulfonamides	compound sulfamethoxazole	SXT	20	≤10	11−15	≥16

exhibits a strong tolerance to ultraviolet radiation.[5] After sublethal high-temperature stress, expression of the *Salmonella* virulence gene changes, and the infection rate of the host increases. During storage, owing to the cross-synergism of many stress factors, the tolerance of *Salmonella* to its environment changes. In addition, the survival rate of *Salmonella* with acid adaptability under temperature stress and in a hypertonic environment is higher than that of ordinary *Salmonella*, but it is more sensitive to sodium hypochlorite stress.[6]

The aim of this study was to evaluate hypertonic-adapted vs nonadapted *S*.Derby in terms of (1) the effect of hypertonic conditions on hypertonic adaptability; (2) changes in toxicity, drug resistance, and biofilm synthesis; and (3) changes in expression patterns of hyperosmotic tolerance-related genes over time of hyperosmotic stress.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Culture Media. The *Salmonella enterica* subsp. *enterica* ser. Derby (*S*.Derby; CMCC50719) used in this study was purchased from China Medical Microorganism Culture Collection Center. Bacteria were activated in tryptic soy broth (TSB) medium or on TSA (TSB plus 2% agar) slopes, grown at 37 °C for 18 h, and then stored at −80 °C as a stock culture. TSB was used as the hypertonic acclimation and induction medium throughout the experiment.

2.2. Induction of Hyperosmotic Fitness in *S*.Derby. *2.2.1. Acid Adaptation.* For hypertonic acclimation, *S*.Derby was first activated, and then a single colony was picked, inoculated in TSB liquid medium, and cultured at 37 °C and 180 rpm for 18 h. Subsequently, 0.1 mL of the bacterial suspension was streaked and inoculated into solid medium containing NaCl TSA and incubated at 37 °C for 24 h. Ten rounds of hypertonic induction were repeated in this manner. After hypertonic induction, the nonadapted strains were inoculated into TSB liquid medium and cultured at 37 °C and 180 rpm for 18 h. The initial concentrations of the above strains were obtained using the plate colony counting method. More specifically, 1 mL of each culture solution was centrifuged at 120 rpm for 3 min, the supernatant was discarded, and the pelleted cells were resuspended with 1 mL of saturated NaCl solution. Bacterial solutions were treated at room temperature for 2, 4, and 8 h, diluted 10-fold with sterile normal saline, and plated, and colonies were counted at the appropriate dilution. The survival rate was calculated according to formula 1 to evaluate the tolerance of the hypertonic-adaptable strain of *Salmonella* Derby.

The formula for calculating the survival rate is as follows

$$\text{survival rate}\% = \frac{\text{logarithmic number of colonies after treatment}}{\text{logarithmic number of colonies before treatment}} \times 100 \quad (1)$$

2.2.2. Genetic Stability of Hypertonic-adapted S.Derby. The hypertonic adaptation of *S*.Derby was carried out for multiple passages, and the 5th and 10th generation strains were tested for hypertonic tolerance using a saturated NaCl solution. Specifically, 1 mL of bacterial solution was added to a sterilized tube and centrifuged, and the supernatant was discarded. The pelleted cells were incubated in saturated NaCl for 2, 4, and 8 h, and then gradient dilution was performed. Nonadapted strains were used as controls, and the bacterial survival rate was calculated using the colony counting method.

2.3. Susceptibility of Hypertonic-Adapted *S*.Derby to Antibiotics. After the hypertonic-adapted and nonadapted *S*.Derby were activated and cultured in TSB, 1 mL of each sample was placed in a sterilized centrifuge tube and centrifuged at 120 rpm for 3 min, and the supernatant was discarded. The precipitate was resuspended in 1 mL of normal saline, shaken to mix evenly, and then subjected to gradient dilution before coating a TSA plate. Thereafter, three antibiotic drug-sensitive tablets were placed equidistant in a triangular position in the plate, and the samples were cultured at 37 °C for 18 h. Vernier calipers were used to accurately measure the diameter of the anti-sensitivity ring and formulate Table 1, according to the National Committee for Clinical Laboratory Standards (NCCLS)[17] to determine strain sensitivity.

2.4. Effect of Hypertonic Adaptation of S.Derby on Biofilm Synthesis. For the wipe-glass bead method, a food-grade 304 stainless steel sheet was used as the carrier for the formation of *Salmonella* biofilm. Hypertonic-adapted and nonadapted *S*.Derby were inoculated into TSB containing the stainless steel sheets and cultured at 37 °C for 24, 48, 72, 96, and 120 h. Adherent bacteria on the surface of the steel sheets were collected by wiping with cotton balls, and the cotton balls were then placed in centrifuge tubes filled with 10 mL of sterile physiological saline and vortexed fully. The cells

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Table 2. Primers Used in the Evaluation of Gene Expression by qPCR

gene	function	primer
stn	enterotoxin protein	F:GGTCGGTCCCACTTTCTTTTG
		R:GGTCAGTCAGGATGCCCAA
invA	secretory/invasion protein	F:AAAGAAGGGTCGTCGTTAGGA
		R:GTAGACGCTCCGCAAGTT
sseL	toxic protein	F:TAATCTGGAAGGGGCATGTAG
		R:GACCTGGGGCATAGAGT
hliA	transcriptional regulator	F:CGATGCTCTTTATCTTCCGACA
		R:CGTTCTGGTCATCCTGCTC
mgtC	Mg^{2+} transporter protein	F:GCAGCGTATAAATCAACTCCC
		R:CGAACCTAACCCTTGTAAACAG
fliA	sigma F factor of RNA polymerase	F:CCTGTTGCGGAGTATCGTCA
		R:CGTTAACACCAGTTGCTCGC
pipA	pathogenicity island encoded protein	F:CAGAGTGTGTGGTGGAGTACC
		R:GAAGTGTTCCTGAGCCACGG
gyrA	helicase gene	F:ACGTACTAGGCAATGACTGC
		R:AGAGTCGCCGTCGATAGAAC
tetA	tetracyclines	F:TGGTCCGGAGGCCAGACGTG
		R:TTCCGAGCATGAGTGCCCGC
parC	topoisomerase gene	F:CTATGCGATGTCAGAGCTGG
		R:TAACAGCAGCTCGGCGTATT
aadA	aminoglycoside drugs gene	F:TTTGCTGGTTACGGTGAC
		R:GCTTCCATTGCCCAGTCG
cmlA	chloramphenicol drugs gene	F:GGCCTCGCTCTTACGTCATC
		R:GCGACACCAATACCCACTAGC
blamte	*β*- lactam drugs gene	F:TTGCTCACCCAGAAACGCTGGT
		R:GCCGAGCGCAGAAGTGGTCC
qnr	fluoroquinolones gene	F:CACCGCTTGCACATTCATTGGC
		R:ACCGTCGAGTTCGGCGTGG
suI	sulfonamides gene	F:TTTCCTGACCCTGCGCTCTAT
		R:GTGCGGACGTAGTCAGCGCCA
floR	chloramphenicol	F:GACGCCCGCTATGATCCAAC
		R:GAGCATCGCCAGTATAGCCAA
bcsA	adhesion gene	F:ATGAGCGCCCTTTCCCG
		R:TCATTGTTGAGCCTGAGCCA
ropE	sigma inducible factor	F:ATGAAAAACAAATTGTTATTTATGATGTT
		R:TTAGCGTTGGGTGACGC
nahA	$Na^{+}/H^{+/}$ antiporter	CAGGCGTCATTGTCGGTTTC
		AATCGTGACGCCCTGAAGAG
fxsA	suppresses F exclusion of bacteriophage	F:CCATGTGCTTGGTGTCCTGA
		R:GAGCAGTAAGCCAGCGATGA
csrA	membrane protein synthesis gene	F:ATGCTGATTCTGACTCGTCG
		R:TTAGTAACTGGACTGCTGGGA
pspC	phage shock protein	F:ACTGGTGCGTATTCTGGTCG
		R:TACGGTATCCAGCAGTTCGC
sirA	membrane protein synthesis gene	F:TTGATCAACGTTCTTCTTGTTGA
		R:TCACTGGCTTGTTAACGTCTC
ompR	membrane protein synthesis gene	F:ATGCAAGAGAATTATAAGATCTGGTG
		R:TCATGCTTTAGAACCGTCCG
ybfA	putative periplasmic protein	F:AGACTATCCAGCGCATGTCA
		R:GACCAGACACGGTGCAGATA
yjbO	phage shock protein G	F:CTGATGTTAATGGTGACGGGC
		R:AGCCACGGTAACAGCTTGAT
marT	putative transcriptional regulatory protein	F:GCAGCCACAGCATCAGACTA
		R:TTGTTGTGAGACGACGGAGG
otsA	haizatang Synthase	F:GCTGGACGCCGCTCTATTAT
		R:CATGACGCGAAATACGCTCG
proW	aminobetaine/L-proline ABC transporter	F:TTACCCTGGCGCTGGTATTG
		R:CAGGATCGTCAGGCGTACAA
proV	proline/betaine ABC transporter osmase	F:ACAGGTACTGATTGACGGCG
		R:GCCCGCTAATTCCATACCGA
omsV	osmotic protectant ABC transporter ATP binding	F:CAGCGCATTGGTGTGATACG

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Table 2. continued

gene	function	primer
		R:CGTTCTGTCCTGACCGACAA

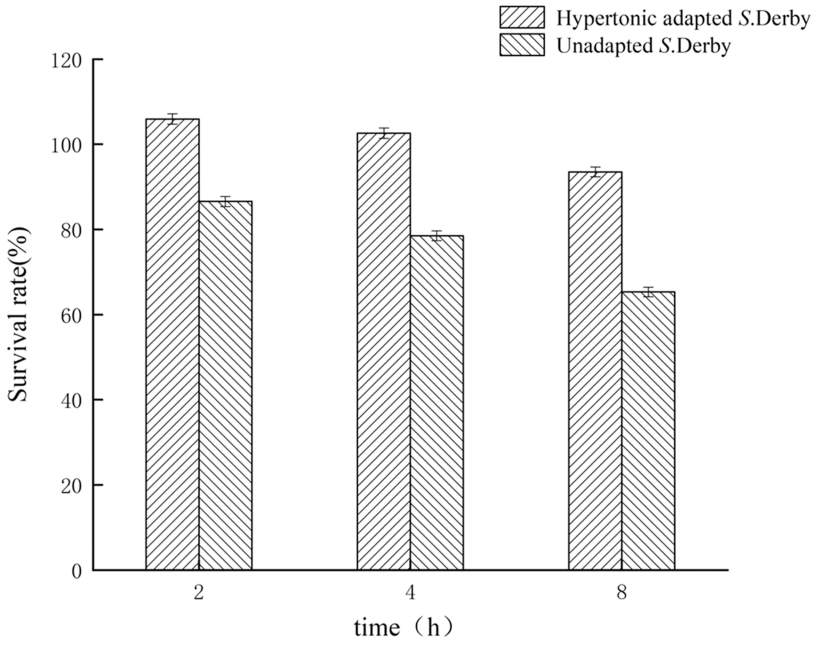

Figure 1. Survival rate of osmotic tolerant strains of *S*.Derby under saturated NaCl stress. After 10 rounds of repeated acclimation in TSA medium containing NaCl, 1 mL of bacterial suspension with hypertonic adaptability and non-hypertonic adaptability was treated with sterilized saturated NaCl for 2, 4, and 8 h. Data represent the means of three independent experiments ± standard deviations; an * above the bar represents a significant difference in survival rate in one experiment ($P < 0.05$).

on the cotton ball were fully detached, and the absorbance of the solution was measured at OD_{570} nm.

For crystal violet staining, *Salmonella* was inoculated into a sterilization test tube containing 9 mL of TSB and cultured at 37 °C for 24, 48, 72, 96, and 120 h, and then the bacterial solution was discarded. After washing with sterile distilled water three times, the cells were stained with 0.1(w/v)% crystal violet solution for 20 min and then decolorized with a corresponding amount of 95% ethanol for 10 min. The absorbance of the solution was measured at OD_{570} nm. Three parallel experiments were conducted for each sample, and blank culture medium was used as the control.

2.5. Hypertonic Resistance in the Presence of Added Betaine, Trehalose, or Proline. We verified the hypertonic adaptive response of *S*.Derby in the presence of osmotic regulators (betaine, trehalose, and proline). Hypertonic-adapted and nonadapted *S*.Derby was cultured in TSB medium at 37 °C for 18 h. Thereafter, 1 mL of culture solution was centrifuged at 120 rpm for 3 min, the supernatant was discarded, and 1 mL of saturated NaCl solution was added to resuspend the bacteria, which were then incubated at room temperature for 4 h. Betaine, trehalose, or proline (20 nmol/mL each) was then added to the TSA medium, and an appropriate dilution of the cell suspension was used to coat TSA plates for counting after 1, 2, and 4 h.

2.6. Gene Expression Analysis. *2.6.1. RNA Extraction and cDNA Synthesis.* To study the effect of hypertonic adaptation induction on gene expression, hyperosmotic-adapted and nonadapted (control) *S*.Derby cells were quickly transferred into RNase-free tubes and centrifuged at 12 000*g* at 4 °C for 5 min. RNA was extracted from the pelleted cells using TRIzol reagent (Vazyme, Nanjing, China). DNase (OMEGA) treatment was used to eliminate genomic DNA contamination. The purity and concentration of RNA were determined before cDNA synthesis was performed using a 1st strand cDNA Synthesis Kit (Vazyme), and reverse transcription was performed as recommended by the manufacturer.

2.6.2. Primer Design. Table 2 shows the quorum sensing, toxicity, drug resistance, biofilm synthesis-related, and hypertonic adaptability-related genes used for quantitative fluorescence detection. Primers were designed using Primer Premier 5 software. Routine PCR and agarose gel electrophoresis were used to determine primer specificity. Amplification of cDNA was performed using a StepOne ¢ Real-Time PCR System (Applied Biosystems). Primer efficiency was estimated using standard curves based on serial dilutions of the cDNA template, and three technical replicates were analyzed for each concentration. Serial dilutions of cDNA were amplified by real-time PCR using gene-specific primers on the same PCR instrument as used for the gene expression experiment. The amplification efficiencies of the target and reference genes were calculated based on the slope of the standard curve using the formula (representative slope of the standard curve). The qPCR assay was performed unless the amplification efficiency of primer pairs was between 80 and 100%.

2.6.3. Real-time Quantitative PCR (qRT-PCR). cDNA template (1 μL) was mixed with 12.5 μL of Master Mix (High ROX Premixed; Vazyme), 8.5 μL of water, and 1 μL each of 100 μM forward and reverse primers per PCR reaction. Thermocycling was initiated at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, and 60 °C for 60 s. The

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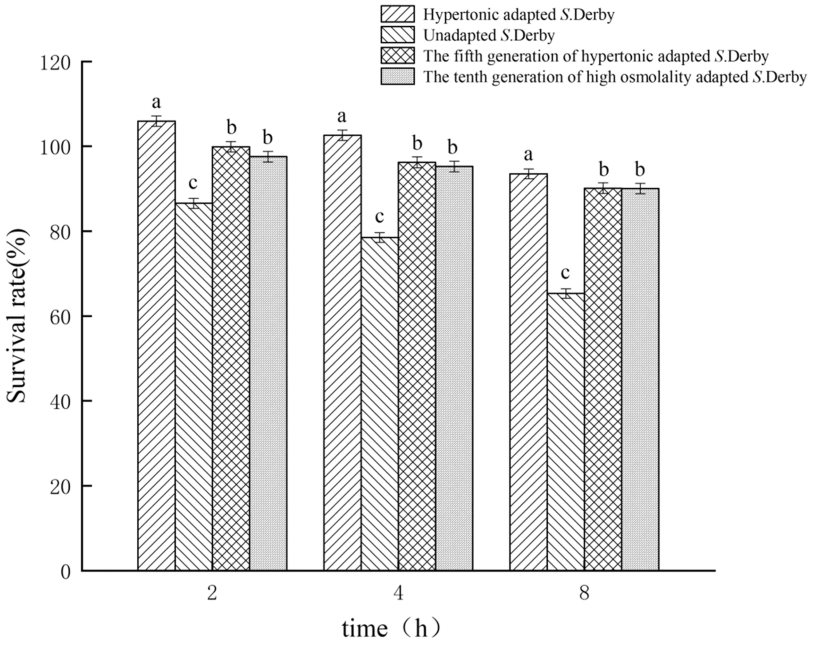

Figure 2. Validation of genetic stability of *S*.Derby with hypertonic-adapted *S*.Derby was subcultured many times. The 5th and 10th generation strains were selected to measure the survival rate after 2, 4, and 8 h of NaCl stress. The untreated hypertonic-adapted and nonadapted *S*.Derby were used as the control.

dissociation curve was used to ascertain the presence of a single peak. The experiments were repeated three times, with three technical replicates for each gene. qRT-PCR results were calculated using the comparative $2^{-\Delta\Delta CT}$ method, and the 16SrRNA gene was used as a reference to determine the relative transcription levels of the target genes.

2.7. Statistical Analysis. All data were analyzed using analysis of variance (ANOVA) in SAS, and Duncan's new multiple range test levels ($P < 0.05$) were considered statistically significant.

3. RESULTS

3.1. Hyperosmotic Tolerance of Hypertonic-Adapted *S*.Derby. The survival percentage of *S*.Derby challenged in saturated NaCl was used to indicate bacterial hyperosmotic tolerance. The survival rate of hypertonic-adapted *S*.Derby was significantly higher than that of nonadapted *S*.Derby with prolonged hypertonic treatment time ($P < 0.05$) (Figure 1). After 2 h of saturated NaCl stress treatment, the survival rate of hyperosmotic-adapted *S*.Derby was 105.94%. After 8 h of stress treatment, the survival rate of hypertonic-adapted and nonadapted *S*.Derby was 93.53 and 65.32%, respectively, representing the largest difference in survival rate. These results showed that *S*.Derby possessed hypertonic tolerance after being domesticated in a 4% NaCl stress environment. Similar to the results of Liu Mi et al.,[7] cyclic acclimation in BMM medium containing 4% NaCl enhanced the ability of *Salmonella* to resist hyperosmotic stress.

3.2. Genetic Stability of Hypertonic-Adapted *S*.Derby. To verify whether hypertonic-adapted *S*.Derby exhibited good genetic stability, the strains were subcultured several times. Genetic stability was expressed as the survival rate of the fifth- and tenth-generation strains (Figure 2). With the prolongation of saturated NaCl treatment time, the survival rate of hypertonic-adapted *S*.Derby was significantly higher than that of nonadapted *S*.Derby ($P < 0.05$). The survival rate of the fifth- and tenth-generation strains of activated culture was similar to that of the first generation of hypertonic-adapted *S*.Derby. When treated with saturated NaCl for 8 h, the survival rate was ≥ 85%, while the survival rate of nonadapted *S*.Derby was < 70%. The above results indicate that the hypertonic-adapted *S*.Derby possessed good genetic stability.

3.3. Antibiotic Susceptibility of Hypertonic-Adapted *S*.Derby. To determine whether the sensitivity of hypertonic-adapted *S*.Derby to antibiotics changed with treatment, 12 types of drug-sensitive tablets were tested (Table 3). The antibiotic sensitivity of hypertonic-adapted and nonadapted *S*.Derby showed that nonadapted *S*.Derby was resistant only to MNO and PB (glycopeptides and tetracyclines, respectively) and PEN, OXA, and AMP (penicillins). Hypertonic stress enhanced the drug resistance of hypertonic-adapted *S*.Derby, which showed resistance to PEN, OXA, AMP (penicillins), and CEP (cephalosporins). MNO and PB act as mediators. Nonadapted *S*.Derby was sensitive to seven antibiotics: GEN, KAN, SXT, NOR, CIP, ENR, and CEP (aminoglycosides, sulfonamides, quinolones, and cephalosporins), whereas hypertonic-adapted *S*.Derby was sensitive to six antibiotics, GEN, KAN, SXT, NOR, CIP, and ENR (aminoglycosides, sulfonamides, quinolones).

Table 3. Analysis of Drug Resistance of Osmotic Resistant *S*.Derby Strain Under Hypertonic Stress[a]

sample	antibiotic sensitivity		
	S	I	R
hypertonic-adapted *S*.Derby	GEN, KAN, SXT, NOR, CIP, ENR	PB, MNO	OXA, AMP, PEN, CEP
non-hypertonic-adapted *S*.Derby	GEN, KAN, SXT, NOR, CIP, ENR, CEP	PEN, OXA, AMP	MNO, PB

[a]S: high sensitivity; I: intermediary; R: drug resistance.

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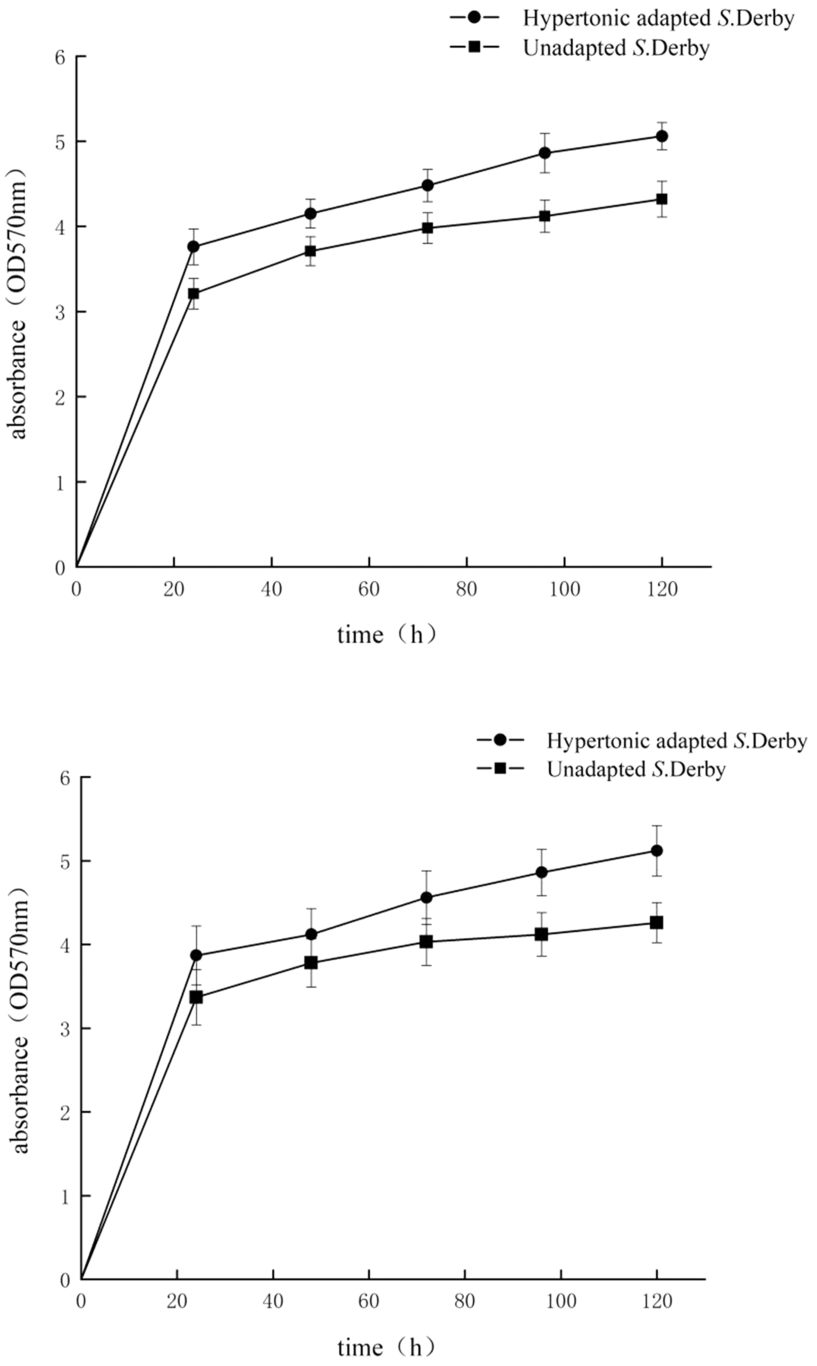

Figure 3. Determination of the biofilm-forming ability of hypertonic-adapted *S*.Derby. After the culture of *S*.Derby was circularly induced to hypertonic-adapted in NaCl-mediated solution, it was transferred to TSB medium for 120 h, and the absorbance at different time periods was measured. The untreated nonadapted *S*.Derby were used as the control.

3.4. Biofilm Formation by Hypertonic-Adapted *S*.Derby. The results of the two biofilm detection methods were similar and revealed that the biofilm formation rate of hypertonic-adapted *S*.Derby was higher than that of the control strain (Figure 3). At 24 h, the adhesion of hypertonic-adapted *S*.Derby (3.76 Log CFU/cm^2) was significantly higher than that of the control strain (3.21 Log CFU/cm^2). Maximal adhesion (5.06 Log CFU/cm^2) was reached at 120 h in hypertonic-adapted *S*.Derby. Biofilm formation tended to stabilize after 24 h in hypertonic-adapted *S*.Derby, whereas the amount of biofilm formation by the control strain tended to stabilize after 72 h. Thus, both the hypertonic-adapted and control strain of *S*.Derby adhered to the stainless steel surface and formed a biofilm; however, hypertonic-adapted *S*.Derby formed a biofilm faster than did the control strain, and the amount of adhesion was higher than that of the control strain.

3.5. Hyperosmotic Resistance in the Presence of Added Betaine, Trehalose, or Proline in Hypertonic-Adapted *S*.Derby. The survival rate of hypertonic-adapted and nonadapted cultures of *S*.Derby was measured after 1, 2, and 4 h of NaCl stress when the culture medium was supplemented with trehalose, proline, or betaine (4%, w/v; Figure 4). Hypertonic-adapted *S*.Derby exhibited higher survival rates under NaCl stress when trehalose, proline, or

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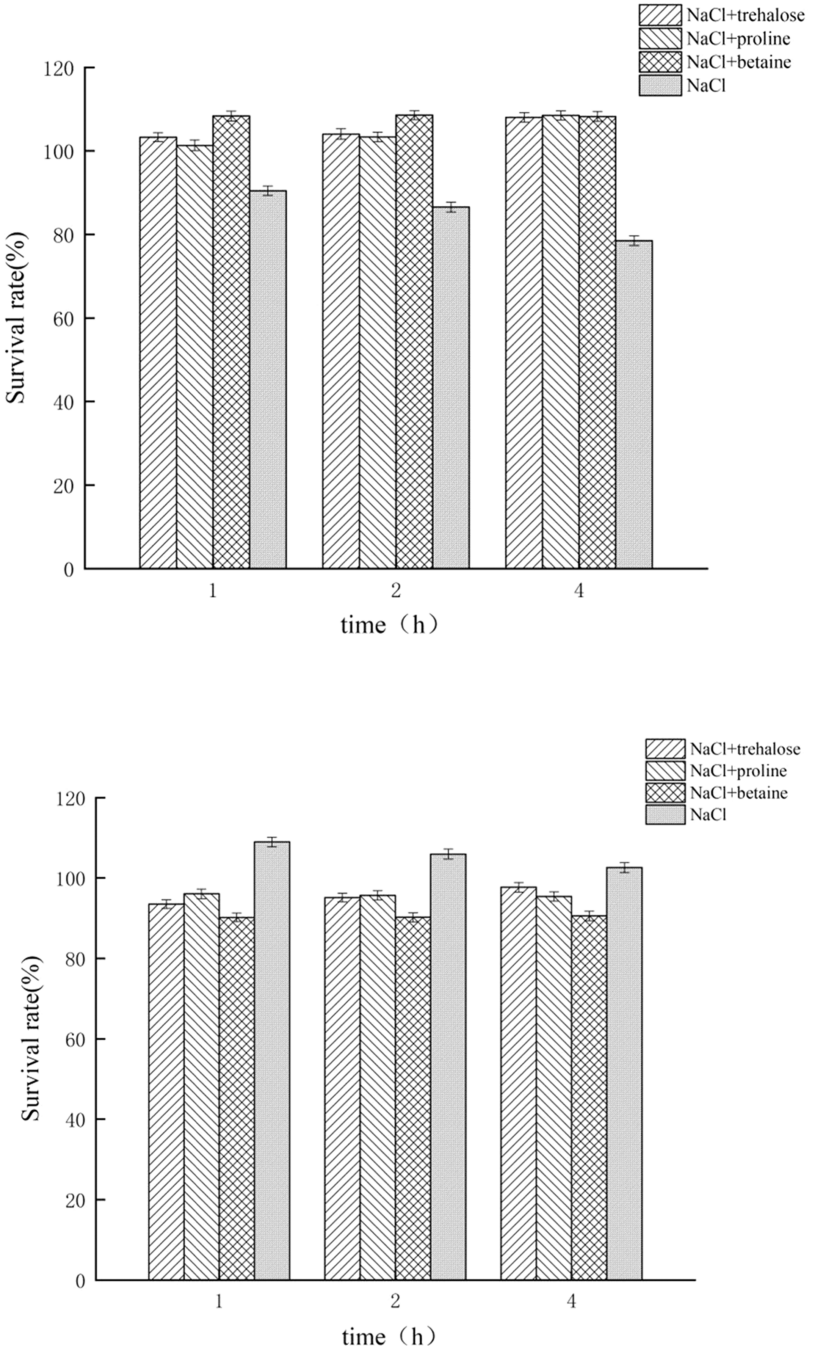

Figure 4. Effects of betaine, trehalose, and proline on the survival rate of *S*.Derby. The survival rate of hypertonic-adapted *S*.Derby under the protection of betaine, trehalose, and proline was analyzed. Each group was divided into three experiments to calculate the average value and error value in parallel; * represents the significant difference in the survival rate of the two strains at the same time ($P < 0.05$).

betaine was not included in the culture medium. The addition of trehalose resulted in a 15% decrease in the survival rate, whereas betaine resulted in a significant decrease of 18%. However, nonadapted *S*.Derby survived better when trehalose, proline, or betaine was added to the culture medium than in their absence. Under NaCl stress for 1, 2, and 4 h, the survival rate of nonadapted *S*.Derby increased 1.13-, 1.21-, and 1.38-fold in the presence of trehalose; 1.12-, 1.19-, and 1.38-fold in the presence of proline; and 1.2-, 1.25-, and 1.38-fold, respectively, in the presence of betaine.

3.6. Changes in Gene Expression in Hypertonic-Adapted *S*.Derby. *3.6.1. Changes in Expression of the Virulence Gene.* The expression levels of genes encoding toxic protein products were high in hypertonic-adapted *S*.Derby (Figure 5). The expression levels of *hilA, invA, sseL*, and *pipA* on *Salmonella* pathogenicity island 1 (SPI) of the type III protein secretion system (T3SS) were 8.22, 2.89, 1.06, and 0.82, respectively. The expression levels of genes encoding the enterotoxin *Stn*, virulence protein *Mgtc*, and RNA polymerase factor FliA were 2.91, 2.71, and 5.39, respectively. Therefore, it can be seen that the pathogenicity of *S*.Derby induced by NaCl is significantly improved in terms of toxicity, invasiveness, etc. This may be because the *Salmonella* virulence factor is an outer membrane protein. When *Salmonella* invades macrophages,

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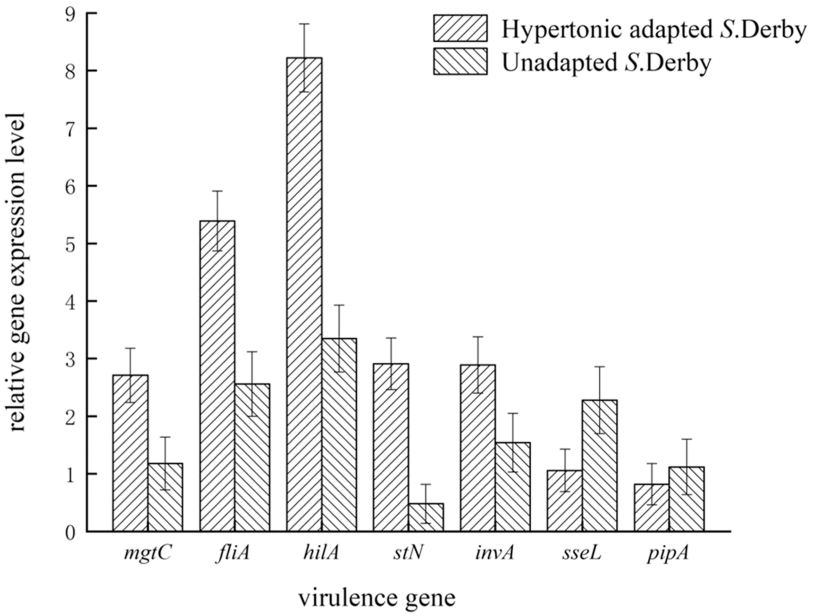

Figure 5. Analysis of results of fluorescence quantitative PCR for toxic genes. Expression patterns of quorum sensing-related genes under hyperosmotic stress. Hypertonic-adapted *S*.Derby was treated with saturated NaCl stress for 4 h. Total RNA was extracted. The 16S gene was used as the internal reference gene, and the gene expression was measured by the $2^{-\triangle\triangle}$ method.

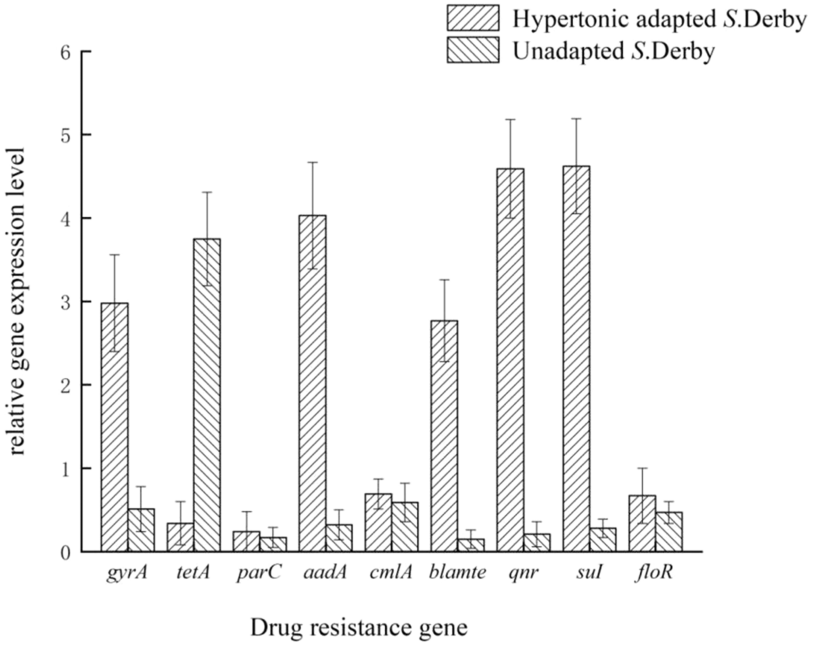

Figure 6. Analysis of drug resistance gene by fluorescence quantitative PCR. Expression patterns of quorum sensing-related genes under hyperosmotic stress. Hypertonic-adapted S.Derby was treated with saturated NaCl stress for 4 h. Total RNA was extracted. The 16S gene was used as the internal reference gene, and the gene expression was measured by the $2^{-\triangle\triangle}$ method.

vesicles are formed, and effector proteins encoded by genes of SPI-2 prevent *Salmonella*-containing vacuole from fusing with lysosomes. However, the osmotic pressure balance inside and outside of the cell is destroyed in a hypertonic environment, which causes the loss of substances inside the cell. Therefore, by increasing the expression of virulence island genes related to the cell membrane, the levels of membrane proteins are increased.

3.6.2. Changes in Expression of Drug Resistance Genes. The expression levels of *gyrA, tetA, aadA, blamte, qnr*, and *suL* changed in response to hyperosmotic treatment, which changed the drug resistance of *S*.Derby (Figure 6). In hypertonic-adapted *S*.Derby, *gyrA, aadA, blumte, qnr*, and *suL* showed high expression levels with log 2 (FC) values of 2.98, 4.03, 2.77, 4.59, and 4.62, respectively, while the control strain showed low expression. The AcrAB-TolC efflux system can mediate the efflux of many kinds of antibiotic drugs, such as penicillin, tetracycline, sulfonamides, and aminoglycosides, as well as organic solvents, including disinfectants, imparting multidrug resistance. The expression of transcription factors was stimulated or inhibited by hypertonic induction of

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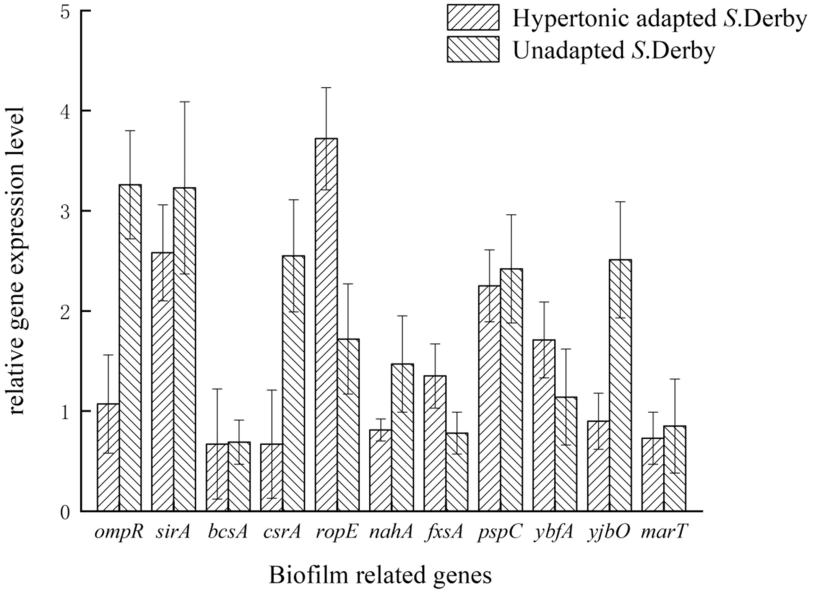

Figure 7. Analysis of fluorescent quantitative PCR results of synthetic genes in biofilm. Expression patterns of quorum sensing-related genes under hyperosmotic stress. Hypertonic-adapted *S*.Derby was treated with saturated NaCl stress for 4 h. Total RNA was extracted. The *16RNA* gene was used as the internal reference gene, and the gene expression was measured by the $2^{-\triangle\triangle}$ method.

Salmonella Derby, which led to a change in the expression of drug-resistant genes.

3.6.3. Changes in Expression of Biomembrane Synthesis Genes. Among the genes related to biofilm synthesis, the expression of *ompR* (outer membrane protein); *bcsA* (cellulose synthase); *sirA* (invasion transporter); *csrA* (carbon-regulated transport protein); and *fxsA, pspC, ybfA, yjbO*, and *marT* (various outer membrane proteins) was affected by hypertonic stress, with expression levels of 1.07, 0.67, 2.58, 0.67, 1.35, 2.25, 1.71, 0.9, and 0.73, respectively (Figure 7). Because the difference in internal and external osmotic pressure in a hypertonic environment will lead to the loss of intracellular substances, to improve their own survival rate, *Salmonella* will promote the expression of *ompR* encoding an outer membrane protein. At the same time, it can be seen from the expression of genes encoding toxic proteins that the genes in the SPI island of the T3SS system are in a high expression state; therefore, the expression of invasion transporters is also increased. RNA polymerase factor encoded by *rpoS* is the main transcription factor used by many pathogenic bacteria to cope with hypertonic, acidic, and other extreme environments; the expression of *rpoS* was 3.72, as determined using real-time quantitative PCR (qRT-PCR). On the one hand, this supports the accuracy of the results of PCR analysis, and on the other hand, it also shows that the transcription level of *Salmonella* Delphi changed to varying degrees in a hypertonic environment.

3.6.4. Expression of Hyperosmotic Tolerance Genes. Through comparative genomic analysis, 156 strains of *S*.Derby were obtained from the NCBI database and laboratory sequencing, and genome comparative analysis was carried out. Representative hypertonic adaptive genes (*otsA, proV, proW*, and *omsV*) were selected, and their gene expression patterns were determined in *S*.Derby in response to different times of hypertonic stress treatment (Figure 8). The outer membrane protein gene *ompC* was used for all comparisons. When NaCl stress was applied for 1 h, only *proW* was rapidly transcribed in hypertonic-adapted *S*.Derby, and the expression level increased by 1.42-fold. *otsA, proV*, and *omsV* were rapidly transcribed in nonadapted *S*.Derby, and their expression levels were 1.74-, 4.89-, and 4.03-fold, respectively. When NaCl stress was applied for 2 h, the expression levels of *otsA, proW*, and *ompC* in hypertonic-adapted *S*.Derby increased 1.54-, 1.83-, and 2.11-fold, respectively, while the expression levels of *otsA, proV, omsV*, and *ompC* in nonadapted *S*.Derby increased 2.41-, 2.59-, 2.55-, and 1.06-fold, respectively. When NaCl stress was applied for 4 h, the expression levels of *otsA, proW*, and *ompC* in hypertonic-adapted *S*.Derby increased 4.73-, 1.78-, and 1.96-fold, respectively, while the expression levels of *otsA, proV*, and *omsV* in nonadapted *S*.Derby increased 1.52-, 2.34-, and 1.64-fold, respectively.

With the extension of stress time, the surface levels of the four genes showed an increase or decrease to varying degrees. Based on their increased expression levels in response to NaCl stress, *otsA* and *proW* appeared to play a significant role in the hyperosmotic tolerance of hypertonic-adapted *S*.Derby, while *proV* and *omsV* appeared to play a major role in nonadapted *S*.Derby; however, the transcription levels of the latter two genes decreased gradually.

4. DISCUSSION

Salmonella is widely distributed worldwide and is one of the pathogens that cause bacterial food poisoning. Between 2015 and 2019, *Salmonella* was reported as a major pathogen causing zoonosis.[8] About 40% of bacterial food poisoning cases in China are caused by *Salmonella*, and worldwide, *Salmonella* accounts for ~80.3 million food poisoning incidents every year, resulting in ~155 000 deaths. A total of 2500+ serotypes of *Salmonella* have been isolated worldwide, with ~300 serotypes in China. After *Salmonella* infects the human body, the incubation period is short, and the onset of disease is sudden, resulting in diarrhea, fever, and other symptoms. In severe cases, bacteremia can occur. About 85.6% of global *Salmonella* cases are the result of foodborne infections.[9] With the development of society, food requirements and preferences are no longer limited to nutrition; rather, the taste of the food

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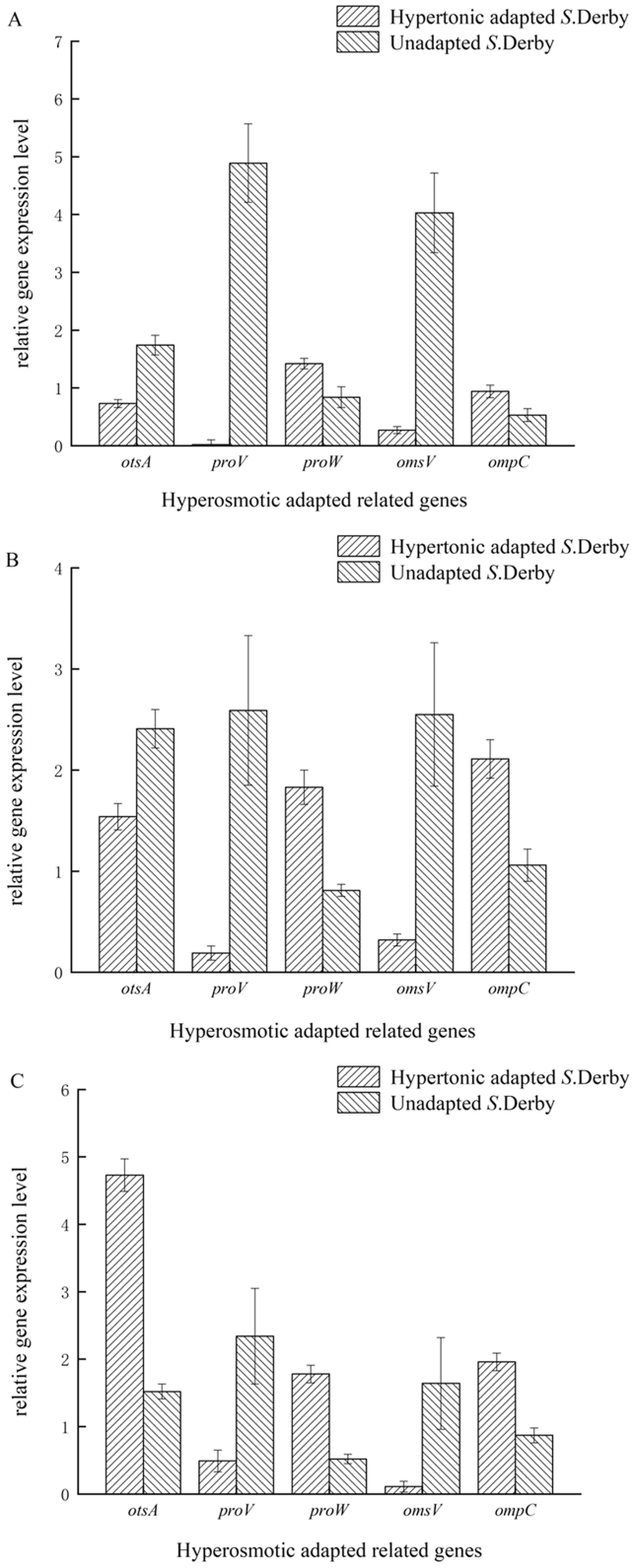

Figure 8. Analysis of the results of fluorescent quantitative PCR for hypertonic regulatory genes. Expression patterns of quorum sensing-related genes under hyperosmotic stress. Hypertonic-adapted *S.* Derby was treated with saturated NaCl stress for 1, 2, and 4 h. Total RNA was extracted. The 16S gene was used as the internal reference gene, and the gene expression was measured by the $2^{-\triangle\triangle}$ method.

has become increasingly important. Novel food-processing methods are also gradually evolving and include extreme environmental conditions for bacteria. To adapt to these harsh environments, bacteria have evolved complex coping mechanisms, which are closely related to the large number of small RNAs (sRNAs) in bacteria.[10] When bacteria receive signals of environmental changes, they activate transcription of sRNAs in response to the population effect.[11] sRNA can in turn regulate gene expression. Bacteria regulated by sRNAs exhibit changes in their pathogenicity, including enhanced resistance to some antibiotics and improved resistance to disinfectants commonly used in food processing, such as organic acids. This can result in incomplete sterilization. In recent years, *Salmonella* Derby has been detected at a high rate in pickled meat products in China, in which the environment is hypertonic. Xu et al.[12] found that sigma factors rope and ROPS in *Salmonella* typhimurium can cooperate to regulate gene expression in an attempt to cope with the stress of a hypertonic environment. In the current study, a NaCl solution was used as a hypertonic induction medium to study the hyperosmotic tolerance of *S.*Derby under hypertonic stress.[13]

We cultivated Del *Salmonella* using hypertonic adaptation through multiple rounds of hypertonic acclimation. We verified that the survival rate of hypertonic-adapted *S.*Derby was ≥ 90%, and much higher than that of control, nonadapted *S.*Derby after 2, 4, and 8 h of saturated NaCl treatment. During food processing, the environment of cured meat products is basically at the level of saturated NaCl, and *S.*Derby in meat products is highly likely to be impermeable, resulting in enhanced tolerance and bacterial food poisoning. Therefore, we need to accurately understand changes related to toxicity, drug resistance, and biofilm synthesis in hypertonic-adapted *S.*Derby to reduce the chances of food poisoning.

The emergence of bacterial resistance is primarily due to the long-term use of antibiotics. Bacterial antibiotic resistance is a serious problem in medicine. This property can spread at the gene level between different types of bacteria, which makes it difficult to control. The multidrug resistance of bacteria is mainly the result of the actions of the AcrAB-TolC efflux system, which exists in a variety of pathogenic bacteria, including *Salmonella*, *Staphylococcus aureus*, *Campylobacter*, *Shigella*, and other Gram-negative bacteria.[14,15] The AcrAB-TolC efflux system can mediate the efflux of many types of antibiotic drugs, such as penicillin, tetracycline, sulfonamides, and aminoglycosides, as well as disinfectants, resulting in multidrug resistance.[16] TolC is a membrane protein and an essential component of the AcrAB-TolC efflux pump system. There are two main ways for drugs to be discharged from bacteria by the AcrAB-TolC efflux pump: one is to combine with drugs and directly pass through the cell membrane to be discharged, and the other is to combine with drugs in the periplasmic space and then cooperate with TolC to discharge the drugs. Overexpression of efflux system components usually reduces the accumulation of antibiotics in bacteria and reduces the sensitivity of bacteria to antibiotics. Anelall[16] and Zhao[17] found that *Salmonella* exhibits high resistance to ampicillin and sulfa drugs. The overuse of ampicillin in livestock and poultry, where it was one of the earliest antibiotics used, is one of the reasons for *Salmonella* resistance. In addition, drug resistance genes are important factors in determining the type of drug resistance in bacteria.[18]

The biofilm formation rate of impermeable strains of *S.*Derby was significantly higher than that of control strains.

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The highly complex and organized functional form of biofilms depends on the mutual cooperation between molecules in the matrix, which shape the spaces between biofilm bacteria, provide mechanical stability, and create a living environment for the bacteria. Biofilms are conducive to nutrient acquisition.[19] When bacteria are subjected to external environmental stress, they tend to enhance their own adhesion, further enhancing biofilm synthesis in an attempt to better survive, multiply, and adapt to their environmental changes.[20]

The main reason foodborne pathogenic bacteria threaten human health is that virulent pathogenic bacteria can invade host cells and reduce or even stop the activity of these cells. The toxicity of many Gram-negative bacterial pathogens originates from the T3SS, which can deliver toxic protein (effects) to host cells and change their original translated protein, thus preventing host cells from producing an immune response to the pathogen. The generation of an immune response in host cells is mainly regulated through ribonucleic acid protein S3 (RPS3) that guides NFkB subunits to specific kB sites to form the IKBad protein.[20−22] The NFkB regulatory pathway can regulate host cell proliferation and immune capacity and is synthesized by activated B cells.[18] B-cell lymphoma-2 (BCL2) associated agonist of cell death (BAD) proteins can control the apoptosis of host cells and eliminate invading cells, preventing the spread of pathogenic bacteria. In pathogenic cells, T3SS encodes *sseL*, which forms the SPI virulence island. Expression of *sseL* on SPI-2 can reduce the nuclear abundance of RPS, inhibit the synthesis of NFkB, promote the degradation of IKBAD protein, weaken the immune response of host cells to the pathogen, and promote their own invasion.[23] The environment within the human body varies; the acidity in the stomach, bile salts in the intestines, and changes in Fe^{2+} and Mg^{2+} content can all influence pathogenic bacteria toxicity.[24]

There have been many studies on the resistance of *Salmonella enteritidis* and *Salmonella typhimurium* under environmental stress, as well as on the adaptability of *Salmonella Heidelberg* in acidic environments and the multidrug resistance of *Salmonella duck*. Some studies have found that ethanol, NaOH, NaClO, UV light, etc., are all effective sterilization methods against *Salmonella*, but the resistance of *S*.Derby to the above conditions has significantly increased. However, the survival ability of hypertonic-adapted *S*.Derby decreased significantly in response to bile salt (2−3 times), high temperature (4−5 times), and low pH stress (9−12 times). The reason for this difference may be related to the transcription factors in *Salmonella*. The RpoS transcription regulator is a factor that stabilizes pressure in *Salmonella*, regulates the expression of specific genes, and plays a crucial role in bacteria.[25] In high temperature, hypertonic, bile salt, and acidic conditions, *rpoS* expression was upregulated.[26,27] This suggests that RpoS induced by hypertonic adaptation may participate in the cross-resistance of *Salmonella* to high temperature, bile salt, and/or low-pH environments.

Cell osmotic resistance is related to the amount and type of osmotic protective agents, such as betaine, proline, and trehalose, and the induced expression of outer membrane proteins related to osmotic pressure.[28,29] Most foodborne pathogens maintain pH stability via the amino acid decarboxylase system, mainly involving lysine, arginine, and glutamic acid.[30] Glutamine and lysine in the cell walls of bacteria can effectively improve the resistance to bile salt.[31] Bacteria perceive temperature using a signal transport exchange system and an RNA-mediated feedback channel.[32] The ability of *Salmonella* to adapt to the above four environmental stresses is related to the expression of gene regulatory proteins. RpoS is a type of RNA polymerase, which is an important enzyme in DNA reverse transcription for the synthesis of RNA translation proteins.[33] Therefore, *RpoS* is likely a transcription factor that plays a major role in regulating bacteria under environmental stress. The regulatory mechanism of *RpoS* requires further study, but the protein appears to play a crucial role in controlling the development of bacterial tolerance and, hence, the complete elimination of foodborne pathogens with environmental resistance, which is also of great significance for biological research.

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