The lipoprotein NlpD in Cronobacter sakazakii responds to acid stress and regulates macrophage resistance and virulence by maintaining membrane integrity

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ABSTRACT

Cronobacter sakazakii, an emerging opportunistic pathogen, is implicated in severe foodborne outbreak infections in premature and full-term infants. Generally, acid tolerance is vital for the pathogenesis of foodborne pathogens; however, its role in C. sakazakii virulence remains largely unknown. To screen out acid-tolerance determinants from transposon mutants, anovel counter-selection method using gentamicin and acid was developed. Using the counterselection method and growth assay, we screened several acid-sensitive mutants and found that nlpD encodes an acid-resistance factor in C. sakazakii.

Compared to the wild-type strain, the nlpD mutant exhibited attenuated virulence in a rat model. Using macrophage THP-1 cells and a pH probe, we verified that nlpD enables bacteria to resist macrophages by resisting acidification. Finally, we confirmed that nlpD maintains C. sakazakii membrane integrity in acid using propidium iodide permeabilization assays via flow cytometry. Our results confirm that nlpD is a novel virulence factor that permits C. sakazakii to survive under acid stress conditions. Considering that NlpD is a conserved lipoprotein located in the bacterial outer membrane, NlpD could be used as a target for drug development.

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Introduction

Cronobacter sakazakii (formerly Enterobacter sakazakii) is an emerging opportunistic foodborne pathogen that causes life-threatening sepsis, meningitis, and necrotizing enterocolitis in premature and full-term infants [1–3]. Since the first report describing an association between C. sakazakii infection and consumption of a powdered infant formula (PIF) in Tennessee in 2001 [4], the risk of infection due to the use of PIF in the neonatal health-care setting has been known. Since then, C. sakazakii has been widely studied around the world.

The virulence traits and pathogenic mechanisms of C. sakazakii remain largely unknown, although initial research has begun to identify specific genes that are involved in the infection process [5–7]. This initial research has revealed that C. sakazakii is able to form biofilms [8], possesses iron acquisition genes [9], and persists in human macrophages [10].

Acid resistance and desiccation resistance are very important for C. sakazakii infection; extraordinary desiccation resistance enables bacteria to survive in PIF, which is the main vehicle of C. sakazakii infection [8]. In addition, C. sakazakii can adapt to a low-pH environment [11]. This characteristic may be associated with the fact that the organism encounters an acidic environment during its passage through the stomach and during its survival within macrophages.

The gastric pH of infants varies from 2.9 to 5.8 after feeding [11,12]. In addition, it has been reported that some organic acids present in the stomach may have antibacterial effects [13]. C. sakazakii can grow at pH values as low as 4.1 [14] and has been shown to tolerate exposure to low pH for long periods [15]. The uptake of invading microorganisms by macrophages is an intrinsic defense response used to kill harmful bacteria. Phagocytes engulf bacteria and form phagolysosomes. The phagolysosomes fuse with lysosomes containing acid hydrolases and free radicals that kill phagocytized bacteria. It has also been reported
that acidification may play an important role in killing bacteria [16,17]. C. sakazakii has been shown to survive and even to multiply within macrophages [10,18].

Studies have shown that the acid determinants of bacteria also affect bacterial virulence. For example, deletion of the acid-tolerance response gene dsrA of Salmonella enterica results in defective invasion efficacy and renders the bacterium unable to cause gut inflammation in mice [19]. In addition, the yaeB mutation in Salmonella typhimurium results in increased acid sensitivity and decreased survival within macrophages [20]. Moreover, acid-adapted bacteria demonstrate increased virulence. For example, compared to the nonacid-adapted wild-type Listeria monocytogenes, the acid-adapted strain and a constitutively acid-tolerant mutant showed more invasion of enterocyte-like cells and greater survival within activated macrophages [21]. However, little information is available on the association between acid tolerance and virulence in C. sakazakii.

The objective of this study was to investigate genes putatively associated with acid tolerance-mediated resistance to macrophages and with pathogenicity. We found that nlpD rendered C. sakazakii resistant to acid stress in the stomach and within macrophages by maintaining the bacterial pH. NlpD is a novel virulence factor of C. sakazakii and could be a drug target in the future.

Results

Screening for acid-resistance genes of C. sakazakii

Transposon mutagenesis can efficiently produce a large number of near-random mutations of the bacterial genome; however, subsequent selection of stress-sensitive mutants generally requires a large number of single colony screenings since the rate of transposon insertion is quite low. Here, we developed a counterselection method that relies on the antagonism of an acidic environment to bactericidal antibiotics that specifically kill actively replicating bacteria. Gentamicin is a quick-acting bactericidal antibiotic that is most efficient against rapidly growing bacteria [22]. Therefore, exposure to a moderately acidic pH that inhibits the growth of acid-sensitive mutants should protect these strains from the bactericidal effect of gentamicin, while wild-type strains and mutants that are able to grow under moderately acidic conditions should be killed quickly. Three concentrations of gentamicin were tested for bactericidal activity against C. sakazakii (Fig S1A). Strong bactericidal activity was observed at all three gentamicin concentrations. At 200 mg/L, gentamicin reduced the number of viable C. sakazakii to below the detection level within 60 min. To further select a suitable acidic pH, an LB medium adjusted to various pH values using hydrochloric acid was investigated for its inhibitory effects on the growth of C. sakazakii. The dnaK deletion mutant of C. sakazakii was generated as an acid-sensitive control. Compared to neutral pH, pH 3.0 showed a significant inhibitory effect on the growth of ΔdnaK C. sakazakii compared to its effect on the growth of wild-type C. sakazakii (WT) after culture for 60 min (Fig S1B).

In order to establish a counterselection method that depends on the antagonistic effects of acidic pH on the bactericidal activity of gentamicin, the survival of C. sakazakii was studied after exposure to a combination of acidic pH and gentamicin. The acid-sensitive ΔdnaK mutant strain was capable of withstanding the killing of gentamicin after growth inhibition by acidic pH (Fig S1C). By contrast, rapid killing of the acid-resistant wild-type strain due to gentamicin was observed; the number of viable bacteria of the resistant strain was below the detection threshold after 60 min. Therefore, it appears that the combination of an acidic pH and gentamicin has the desired counterselective effect on acid-sensitive strains of C. sakazakii.

Using this method, we screened several acid-sensitive mutants and identified the insertion sites of their mutations. The phenotype of acid sensitivity was further verified by knocking out the open reading frames of the genes. Finally, we identified five genes, sidA, hmsP, recA, rpfR, and nlpD, that enable C. sakazakii to resist acidic environments (Figure 1) but that do not affect the growth rate of the bacterium in neutral medium (data not shown). NlpD, an extracellular membrane protein, is thought to be related to biofilm formation [23], but it has not previously been reported to be related to acid tolerance. Therefore, the role of nlpD in the acid tolerance and virulence of C. sakazakii was investigated in this study.

nlpD knockout reduces bacterial virulence

During the process of C. sakazakii infection, the pathogen is exposed to a variety of acidic environments, including the gastric acid environment, the intestinal acid environment, and the acidic environment inside macrophage phagocytic vesicles. We have verified that
nlpD contributes to acid stress induced by hydrochloric acid, which is the main component of gastric acid. However, whether nlpD acts similar under organic acid stress that exists in intestinal and macrophage remains unknown. Therefore, the survival of wildtype, nlpD mutant, and complement strains was tested at low pH adjusted by a diversity of organic acids. Compared with wild-type strain, knockout showed significant growth disadvantages in medium acidified regardless of the pH regulated by acetic acid, butyric acid, or lactic acid (figure S2). These results clearly demonstrated that nlpD confers C. sakazakii with resistance to both inorganic acid stress and organic acid stress.

As an acid-resistance factor, nlpD may affect the pathogenicity of C. sakazakii. Three groups of 3-d-old rats were orally infected with WT, ΔnlpD, and ΔnlpD complemented strains of C. sakazakii, and the survival of the animals was monitored for more than 5 d. Rats infected with WT bacteria or with strains in which the mutations were complemented began to die at 12 hours, and 80% of the animals had died by 72 hours. In contrast, rats infected with ΔnlpD bacteria began to die at 48 hours, and 80% of them survived for at least 72 hours after infection. The significant difference between the ΔnlpD and WT strains was identified by a survival curve (Figure 2a). Whether nlpD affects the colonization of C. sakazakii in mice was also investigated. The bacterial load of ΔnlpD bacteria in blood (Figure 2b), liver (Figure 2c), and spleen (Figure 2d) was 8- to 20-fold lower than that of WT bacteria. In addition, intestinal tissue from rats infected with the WT strain showed dilatation and necrosis by 48 h post-infection, accompanied by perforation and destruction of villi, whereas intestinal tissue damage was significantly less severe in ΔnlpD-infected rats (Figure 2e and figure 2f). These results indicate that nlpD knockout decreases the virulence of C. sakazakii.

nlpD does not affect bacterial adhesion

NlpD is a lipoprotein that is thought to be involved in biofilm formation [24]. We next tested the effect of nlpD on bacterial adhesion. A gentamicin protection test was conducted to study the role of nlpD in colonization by C. sakazakii of the small intestinal epithelial cell line Caco-2 and the human brain microvascular endothelial cell line HBMEC. No significant differences in colonization by WT, ΔnlpD, or ΔnlpD-C C. sakazakii were observed in either of these cell lines (Figure 3). Therefore, it is clear that nlpD does not affect colonization by C. sakazakii in vitro.

C. sakazakii requires nlpD for survival in macrophages

Previous studies have found that C. sakazakii resists killing by macrophages [18,25]. Phagocytes mainly
use acidic environments, ROS, and lysozyme to kill intracellular bacteria. Based on our discovery that \( nlpD \) is an acid-resistance factor in \( C. sakazakii \), \( nlpD \) may be involved in the tolerance of this bacterium to killing by macrophages. To determine whether \( nlpD \) affects the macrophage tolerance of \( C. sakazakii \), non-activated and IFN-\( \gamma \)-activated macrophages derived from human THP-1 cell lines were challenged with WT, \( \Delta nlpD \), or \( \Delta nlpD-C \) strains. All of the strains survived and replicated in non-activated macrophages (Figure 4a). In IFN-\( \gamma \)-activated macrophages, the CFU of the WT and \( \Delta nlpD-C \) strains remained basically unchanged after the addition of gentamicin (Figure 4b); however, the number of viable \( \Delta nlpD \) bacteria showed a continuous decline after the addition of gentamicin, and after 8 hours, the CFU of \( \Delta nlpD \) bacteria was below the detection threshold. Thus, the \( \Delta nlpD \) strain did not resist killing by macrophages; therefore, \( C. sakazakii \) needs \( nlpD \) to survive in macrophages.

Figure 2. The acid determinant \( nlpD \) is involved in the pathogenicity of \( Cronobacter sakazakii \).
(A) Survival of rat pups during the 120-hour period following oral infection with various bacterial strains. Combined data from three independent experiments are shown (log-rank test; ***, \( P < 0.001 \)). (B–D) Colony-forming units (CFU) present in the blood, liver, and spleen of infected rats at 24 hours post-infection. Blood was collected from the facial vein, and the liver and spleen were harvested and homogenized in ice-cold PBS for use in colony enumeration. (E) Histopathologic examination of intestinal tissues of uninfected, WT-infected, and \( \Delta nlpD \)-infected rats. Scale bars, 100 \( \mu \)m. (F) Intestinal sections obtained at 24 hours post-infection were graded as grade 0 (normal) to grade 4 (severe) by a pathologist blinded to the groups according to morphological changes (\( n = 4 \)). \( Salmonella enterica \) serovar Typhimurium and \( E. coli \) DH5a serve as positive and negative controls, respectively.
Cronobacter sakazakii requires nlpD to maintain its pH in macrophages

To determine whether the reduced macrophage survival of nlpD was due to the acidic environment inside the macrophage, the pHluorin2 gene, which encodes an enhanced, ratiometric, pH-sensitive green fluorescent protein, was cloned into the pUC57 vector and transformed into *C. sakazakii*. Fluorescence images were generated using a confocal microscope, and the pH of the bacterial cells was analyzed. In IFN-γ-activated macrophages, the pH of the WT strain was observed to be above 6, while that of the ΔnlpD strain was below 5 (Figure 5a and 5b). Therefore, the presence of nlpD maintains the internal pH of the bacteria in macrophages. However, it is surprising that a pH equal to that within macrophages does not kill the ΔnlpD strain. ΔnlpD still grew at a pH of 4.5, similar to the pH within lysosomes, although it showed slower growth than the WT strain (Figure 5c). Therefore, the hypothesis that the synergistic action of low pH and other factors present in macrophages can kill ΔnlpD was proposed. The synergistic effect of pH 4.5 and ROS was verified by the marked reduction in the *in vitro* survival of the ΔnlpD mutant in H₂O₂ at pH 4.5 (Figure 5e); this reduction in survival did not occur when the mutant was exposed to H₂O₂ at a neutral pH (Figure 5d). These results clearly indicate that nlpD indeed facilitates resistance to the synergistic effect of bactericidal acidity and reactive oxygen species in macrophages.

nlpD activation depends on EnvZ

The EnvZ/OmpR two-component system has been reported to be involved in signal transduction during acid stress [26,27]. In *C. sakazakii*, we also found that ΔevnZ and ΔevnZΔompR showed attenuated acid resistance compared to WT (Figure 6a and 6b). Considering that NlpD is a lipoprotein located in the outer membrane and that the two-component system can widely activate the expression of outer membrane proteins [28–30], we speculated that the EnvZ/OmpR two-component system might affect the expression of nlpD. To determine whether the absence of EnvZ affects the expression of nlpD, RT-qPCR was used to measure the level of transcription of nlpD in the WT and ΔevnZ strains. In neutral medium, ΔevnZ showed a similar level of nlpD transcription compared to the WT strain (Figure 6c). In the acidic environment, a significant reduction in nlpD transcription in ΔevnZ was observed compared with the WT strain (Figure 6d), suggesting that nlpD transcription is regulated by EnvZ. At the same time, the results showed that nlpD transcription was significantly higher in an acidic pH environment than at neutral pH, suggesting that nlpD transcription is activated by acidic environments (Figure 6e). In addition, western blotting was used to directly detect the expression of His6-tagged NlpD. The NlpD level in ΔevnZ was significantly lower than that in the WT strain under acidic conditions, but no difference was observed at neutral pH (Figure 6f). These results indicate that the activation of nlpD in acidic environments is dependent on the presence of EnvZ.

![Figure 3](image-url)
Next, we explored the molecular mechanisms responsible for the function of nlpD that were observed above. We have disclosed that in addition to nlpD, four other genes rpfR, hmsP, sdiA, and recA also conferred C. sakazakii acid resistance (Figure 1). Moreover, acid environment could promote the transcription of hmsP, sdiA, and recA in C. sakazakii (Fig S3 B-D). However, the transcription of rpfR, hmsP, sdiA, and recA was hardly changed in nlpD-knockout strain in neither neutral pH nor pH 3.0 (Fig S3), suggesting nlpD provides acid resistance but independent of rpfR, hmsP, sdiA, and recA.

A low-pH environment can affect the function of proteins, including the proteins of intermediate metabolism and the bacterial envelope. Previous studies have shown that a low pH can affect the function of the bacterial membrane, including increasing the sensitivity of bacteria to hydrophobic drugs, the loss of mobility, and reducing the ability to form biofilm [31], suggesting that a low pH can cause bacterial membrane damage. As a membrane protein, we speculate that nlpD could maintain the membrane integrity of C. sakazakii at a low pH. Consistent with this conjecture, we observed an increase in the permeability of propidium iodide to C. sakazakii at pH 3.0 compared
with pH 7.0 (Figure 7a). However, the nlpD mutant significantly increased the permeability of propidium iodide in an acidic environment (Figure 7b), suggesting that nlpD could maintain the integrity of the bacterial cell membrane in an acidic environment.

**Discussion**

The low pH of gastric secretions is considered the body’s first line of defense against food-borne pathogens. The ability of bacteria to resist being killed by acid during passage through the stomach increases their likelihood of colonizing the intestines and causing an infection [32]. Thus, foodborne pathogens have evolved a variety of acid-tolerance genes for survival in these acidic environments [33–36]. Although many studies have identified a series of acid determinants in vitro, only a few recent reports have linked bacterial acid-tolerance genes to bacterial virulence. In our work, we found that the acid-tolerance gene nlpd of *C. sakazakii* confers tolerance to macrophages and is a virulence factor. Our study suggests that more attention should be paid to the search for virulence factors from the perspective of acid-tolerance genes.

Our study demonstrated for the first time that *C. sakazakii* can grow at pH 3.0, while previous investigations have found that *C. sakazakii* can withstand an acidity of pH 3.5 [37]. In fact, *C. sakazakii* exists widely in the environment and in food, where the environmental pH changes over time [38]. Therefore, it is not surprising that *C. sakazakii* has extraordinary acid tolerance.

NlpD was found to confer macrophage tolerance as well as tolerance of low pH. Previous studies have found that macrophage removal aggravates *C. sakazakii* infection [25], suggesting that macrophages provide protection against *C. sakazakii* infection in mice. Moreover, we found that macrophages can kill nlpD-knockout bacteria efficiently, indicating that these cells do indeed play a bactericidal role, but nlpD provides macrophage tolerance. Therefore, the macrophage tolerance of *C. sakazakii* has an important impact on the pathogenicity of the bacterium.

Macrophages rely on acidity, ROS, and other substances to kill intracellular bacteria [39]. Bacteria have

**Figure 5.** *nlpD* deletion attenuates bacterial survival within IFN-γ-activated macrophages. Transmitted images with overlays of bacteria in green (left), fluorescent bacteria (center), and pseudocolor images of the 410:470 excitation ratio (right) of nonactivated (A) and IFN-γ-activated (B) macrophages 24 h after infection with WT, the Δ*nlpD* mutant, and the complemented mutant are shown. Scale bar, 10 μm. (C) Sensitivities of *C. sakazakii* strains to pH 4.5. (D) Sensitivities of *C. sakazakii* strains to 200 mM hydrogen peroxide (H₂O₂) and pH 4.5. To initiate experiments, *C. sakazakii* strains were cultured in Luria-Bertani (LB) overnight at 37°C. In all cases, the estimated initial bacterial densities were 0.1 optical density at 600 nm. Optical densities or CFU counts of *C. sakazakii* were determined immediately before (time zero) and at the indicated time points. **All data represent the mean and standard deviation of three biological repeats.**
evolved multiple mechanisms to resist killing by macrophages. For example, the molecular chaperone DnaK can repair proteins that have been damaged by macrophages and contributes to the survival of *Salmonella* within macrophages [40]. The efflux pump EmrKY contributes to the survival of *Shigella* within macrophages [41]. In addition, a family of surface-exposed virulence factors termed “macrophage infectivity potentiators” (MIPs) have been described in intracellular microorganisms, and these virulence factors are necessary for the survival of *Neisseria gonorrhoeae* [42] and *Legionella pneumophila* [43] within macrophages. Recently, the *nlp*-like gene *fkpA* of *C. sakazakii* has been shown to play a role in macrophage resistance, but its role in virulence has not been elucidated [10]. Previous studies have mostly considered that *nlpD* is related to envelop integrity and iron acquisition [44–46]. However, a few studies reported that *nlpD* affects virulence in the phytopathogens *Xanthomonas* [47] and *Yersinia pestis* [48]. Except in *Yersinia pestis*, however, the virulence mechanism of *nlpD* remains poorly understood. Our results reveal for the first time the relationship between *nlpD* and acid resistance and demonstrate that *nlpD* leads to macrophage tolerance and virulence based on an acid-tolerance mechanism.

Similar to the observation in *Vibrio cholerae*, *Salmonella dublin*, and *Escherichia coli*, *rpoS* in *C. sakazakii* was identified to locate downstream of *nlpD* as well. *rpoS* has been verified to be associated with the resistance to various environmental stresses, such as oxidative stress, carbon starvation, UV irradiation, and acidic conditions. The adjacent location of *nlpD* and *rpoS* clusters potentially indicated the synergistic effect on environmental stress responses. However, the stop codon introduced by the point mutation C994A in *rpoS* of *C. sakazakii* leads to a premature termination of the sequential translation. The defect of *rpoS* in *C. sakazakii* could potentially pose an emphatic participation of *nlpD* in bacterial acid resistance and pathogenicity.

Two-component systems are widely used by bacteria to sense and respond to environmental changes. We found that *C. sakazakii* can regulate the expression of the acid stress gene *nlpD* through the EnvZ-OmpR two-component system. Similarly, the acid response in Group B Streptococci is regulated by the CovS/CovR two-component system [49]. In fact, EnvZ-OmpR is a two-component system that exists widely in bacteria and is believed to be related to biofilm formation and osmotic tolerance [50–52]. EnvZ has also been widely reported to regulate the expression of a variety of outer membrane
proteins [53–55]. Our experiments demonstrate that EnvZ can sense external acidic environments and can regulate the level of the outer membrane protein NlpD, thereby affecting the acidic tolerance of bacteria.

NlpD is a conserved protein present in bacteria that plays an important role in pathogenicity and may serve as a target for future drug development.

**Materials and methods**

**Bacterial strains, plasmids, and growth conditions**

The strains and plasmids used in this study are listed in Table 1, and the primers are listed in Table 2. Bacteria were stored in LB broth containing 15% glycerol (Biosharp, China) at −80°C. To initiate all experiments, strains were revived in LB broth (Oxoid, UK). When necessary, antibiotics were added at final concentrations of 100 μg mL⁻¹ ampicillin (Sangon, China) or 50 μg mL⁻¹ kanamycin (Sangon, China). *E. coli* DH5α (Weidi, China) was used as the host for the preparation of plasmid DNA, and *E. coli* S17 lambda pir (Weidi, China) was used for preparation of the pCVD442 suicide vector [56]. Plasmid construction was performed according to standard protocols with the minor modification that cloning of PCR fragment into the linearized vector was accomplished using a commercial seamless cloning and assembly kit (Vazyme, China). pCVD442 (Miaolingbio, China) was linearized by PCR using the primer pair pCVD442-fwd and pCVD442-rev, and the upstream and downstream fragments of genes to be knocked out were amplified by PCR using the primers listed in Table 2. In-frame deletion mutants were generated using the pCVD442 suicide vector method described previously [57]. Deletion mutants were complemented using the low-copy vector pACYC184 (Miaolingbio, China). ΔnlpD complementation was performed using the pACYC184 plasmid containing an *nlpD* gene fragment amplified using the primer pair *nlpD*-comp-fwd and *nlpD*-comp-rev. *nlpD*(his) complementation in the ΔnlpD mutant was performed using the pACYC184 plasmid containing an *nlpD*(his) gene fragment amplified using the primer pair *nlpD*(his)-fwd and *nlpD*(his)-rev. Transformation and selection of *C. sakazakii* were performed using the method described previously.

**Random mutagenesis and screening**

The transposon mutagenesis in the strain *C. sakazakii* was performed according to protocols described previously.
Table 1. Bacterial strains and plasmids used in this study.

| Strains, plasmids          | Description                                                                 | Reference, source |
|----------------------------|-----------------------------------------------------------------------------|-------------------|
| Cronobacter sakazakii WT   | Wild-type Cronobacter sakazakii BAA-894                                       | [64]              |
| ΔdnaK                      | Markerless deletion mutant ΔdnaK                                              | This study        |
| ΔsidA                      | Markerless deletion mutant ΔsidA                                              | This study        |
| ΔhmsP                      | Markerless deletion mutant ΔhmsP                                              | This study        |
| ΔrcfA                      | Markerless deletion mutant ΔrcfA                                              | This study        |
| ΔprP                      | Markerless deletion mutant ΔprP                                               | This study        |
| ΔnlpD                      | Markerless deletion mutant ΔnlpD                                              | This study        |
| ΔenvZ                      | Markerless deletion mutant ΔenvZ                                              | This study        |
| ΔenvZΔompR                 | Markerless deletion mutant ΔenvZΔompR                                         | This study        |
| ΔnlpD                      | nlpD complementation in ΔnlpD                                                  | This study        |
| ΔnlpD-nlpD(this)           | nlpD(this) complementation in ΔnlpD                                           | This study        |
| ΔenvZ-nlpD(this)           | nlpD(this) complementation in ΔenvZ                                           | This study        |
| WT-nlpD(this)              | nlpD(this) complementation in WT                                              | This study        |
| WT-pHluorin2               | WT harboring pACYC184-pHluorin2                                              | This study        |
| ΔnlpD-pHluorin2            | ΔnlpD harboring pACYC184-pHluorin2                                           | This study        |
| ΔnlpD-C-pHluorin2          | ΔnlpD-C harboring pACYC184-pHluorin2                                         | This study        |
| Escherichia coli S17 lambda pir | Strain for construction and controls                                         | [65]              |
| S17 lambda pirΔΔnaK        | S17 lambda pir harboring pCVD442-ΔdnaK                                        | This study        |
| S17 lambda pirΔsidA        | S17 lambda pir harboring pCVD442-ΔsidA                                        | This study        |
| S17 lambda pirΔhmsP        | S17 lambda pir harboring pCVD442-ΔhmsP                                        | This study        |
| S17 lambda pirΔrcfA        | S17 lambda pir harboring pCVD442-ΔrcfA                                        | This study        |
| S17 lambda pirΔprP         | S17 lambda pir harboring pCVD442-ΔprP                                         | This study        |
| S17 lambda pirΔnlpD        | S17 lambda pir harboring pCVD442-ΔnlpD                                        | This study        |
| S17 lambda pirΔenvZ        | S17 lambda pir harboring pCVD442-ΔenvZ                                        | This study        |
| S17 lambda pirΔenvZΔompR   | S17 lambda pir harboring pCVD442-ΔenvZΔompR                                   | This study        |
| Salmonella enterica        | Standard/reference strain used as control                                     | [66]              |
| Listeria monocytogenes     | Standard/reference strain used as control                                     | [67]              |
| Listeria monocytogenes EGD-e | Low-copy plasmid                                                               | [64]              |
| Plasmids                   |                                                                             |                   |
| pACYC184                   |                                                                             |                   |
| pACYC184-nlpD              |                                                                             |                   |
| pACYC184-nlpD(this)        |                                                                             |                   |
| pACYC184-pHluorin2         |                                                                             |                   |
| pCVD442                   |                                                                             |                   |
| pCVD442-ΔdnaK              |                                                                             |                   |
| pCVD442-ΔsidA              |                                                                             |                   |
| pCVD442-ΔhmsP              |                                                                             |                   |
| pCVD442-ΔrcfA              |                                                                             |                   |
| pCVD442-ΔprP               |                                                                             |                   |
| pCVD442-ΔnlpD              |                                                                             |                   |
| pCVD442-ΔenvZ              |                                                                             |                   |
| pCVD442-ΔenvZΔompR         |                                                                             |                   |

[58] Shortly, a transposon insertion library was constructed by using the EZ-Tn5< KAN-2> Tnp Transposome kit (NovoBiotec, China) according to the manufacturer’s instruction. To select for transposon insertion clones, 100 µl aliquots were plated onto LB agar plates containing 50 µg/ml kanamycin, and plates were incubated for 24 h at 37°C. Afterward, single colonies were rinsed three times with PBS. The bacterial suspension was centrifuged at 12,000 rcf for 1 minute. The supernatant was discarded, and the residue was washed twice with LB broth (pH 3.0). Aliquots (1 mL) were diluted 10-fold in LB broth (pH 3.0). After half an hour, 5-mL aliquots of the dilution were subcultured in 5 mL LB broth (pH 3.0) containing 100 mg/L gentamicin at 37°C with shaking for 1 hour. The strains were then serially diluted and plated on LB agar (pH 7.0). The colonies growing on LB agar were further tested in acidic challenge assays to determine their acid sensitivity. The randomness of insertions was verified according to the previously described method [59]. Genomic DNA was analyzed from individual mutants by Southern blotting using a digoxigenin probe against the kanamycin-resistance cassette contained within the transposon to confirm single transposon insertions.

**Bacterial survival assays**

The bacterial survival assays were performed according to protocols described previously with small changes [60]. In the activation assays, 200 mM H2O2 and pH 3.0 were used.

**Macrophage survival assay**

Macrophage survival was analyzed as described previously with minor changes [10], namely, that THP-1 cells were differentiated into macrophages using phorbol 12-myristate 13-acetate (Aladdin, China) (PMA)
| Primers used in this study | Sequence (5’-3’) |
|----------------------------|-----------------|
| **For Construction**       |                 |
| pCVD442-fwd                | GGCTGACAGACAGATATATATATATATATATATTAGTG |
| pCVD442-rev                | GCAGATACCTTCCTTTCTTAATATTTAGACCATTTATCAGGTTATTG |
| ΔdnaK-A                    | GAAAAGGAAAGTATCTCGCGATATACTTTAGATTG |
| ΔdnaK-B                    | CGCATTTACACTTACGCTTACTACGCTTTGCATCTGAGTTG |
| ΔdnaK-C                    | CGTTTAGGTTGTTAATTCGCGCTTATCGAGGTTAAATAC |
| ΔdnaK-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔsidA-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔsidA-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔsidA-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔsidA-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔhmsP-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔhmsP-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔhmsP-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔhmsP-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔrecA-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔrecA-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔrecA-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔrecA-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔrpfR-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔrpfR-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔrpfR-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔrpfR-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔnlpD-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔnlpD-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔnlpD-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔnlpD-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔenvZ-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔenvZ-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔenvZ-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔenvZ-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔompR-A                    | GAAAAGGAAAGTATCTCGCGAATACTTTAGATTG |
| ΔompR-B                    | AGATAGGTTACACTTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| ΔompR-C                    | CGTACATGCTCAAGGCGATATACTTTAGATTG |
| ΔompR-D                    | GATAAATTCAAGGCGCTTTCTTGATTCGACCTGACGTTAATAC |
| **For sequencing confirmation** |                 |
| ΔdnaK-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔdnaK-F                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔsidA-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔsidA-F                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔhmsP-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔhmsP-F                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔrecA-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔrecA-F                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔrpfR-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔrpfR-F                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔompR-E                    | GTGCGACAGGCGTGCCGAAAATC |
| ΔompR-F                    | GTGCGACAGGCGTGCCGAAAATC |
| **For RT-PCR**             |                 |
| nlpD RT-F                  | GGTATGCAAGGCGTGCCGAAAATC |
| hmsP RT-F                  | GTGCGACAGGCGTGCCGAAAATC |
| hmsP RT-R                  | GTGCGACAGGCGTGCCGAAAATC |
| sdiA RT-F                  | GTGCGACAGGCGTGCCGAAAATC |
| sdiA RT-R                  | GTGCGACAGGCGTGCCGAAAATC |
| recA RT-F                  | GTGCGACAGGCGTGCCGAAAATC |
| recA RT-R                  | GTGCGACAGGCGTGCCGAAAATC |
| rpfR RT-F                  | GTGCGACAGGCGTGCCGAAAATC |
| rpfR RT-R                  | GTGCGACAGGCGTGCCGAAAATC |
| 16s rRNA-F                 | GTGCGACAGGCGTGCCGAAAATC |
| 16s rRNA-R                 | GTGCGACAGGCGTGCCGAAAATC |
treatment at 60 ng/mL for 48 hours, and interferon-γ (ProteinTech, USA) (IFN-γ) was used to activate macrophages for enhanced microbial killing. C. sakazakii strains were added to THP-1 cells at an MOI of 100. Salmonella enterica serovar Typhimurium and DH5α at the same MOI were used as positive and negative controls, respectively.

Intracellular pH measurement

Intracellular pH measurement assays were performed as described previously [61], except that the gene pHluorin2, which encodes an enhanced, ratiometric, pH-sensitive green fluorescent protein, was used in the pACYC184 vector and transformed it into C. sakazakii. Images were acquired using a Leica SPE confocal microscope (Leica, Germany) with dual excitation at 405 nm and 485 nm and an emission filter of 535 nm.

RT-PCR

RT-PCR was performed in a Bio-Rad CFXConnect™ system (Bio-Rad, USA) using SYBR® green mix (NEB, USA) according to standard methods. The level of transcription was assessed through qPCR and normalized with internal control 16s rRNA using specific primers listed in Table 2. Thermocycling conditions for qPCR were as follows: initial denaturation at 92°C for 3 min and then denaturation at 92°C for 5 s, annealing at 56°C for 5 s, extension at 72°C for 5 s, and melt curve analysis at 65–95°C. Amplification of the single PCR product was confirmed by monitoring the dissociation curve followed by melting curve analysis.

Rat virulence assay

Rat infections were conducted as described previously with minor changes [62]. Salmonella enterica serovar Typhimurium and E. coli DH5α served as positive and negative controls, respectively. Uninfected group was treated with a single dose of LB (100 μL) and infected groups with bacterial culture (10⁷ CFU) by oral gavage. Then, the animals were maintained atypical condition with mother. The survival of the infected animals was recorded at defined time intervals. To analyze bacterial colonization of organs, the rats were sacrificed 24 hours after infection. Organs were homogenized in PBS, the homogenates were serially diluted, and the number of bacteria was determined by plating the dilutions on LB agar.

Adhesion assay

Bacterial adhesion was measured as described previously with small modifications [63]. C. sakazakii was prepared and applied to the Caco-2 cell monolayer at an MOI of 100. After 45 min incubation, the plates were washed three times with PBS, and the cells were lysed in 1% Triton X-100 (Abcone, China). The cell suspensions were serially diluted and plated on LB agar for enumeration of adherent bacteria. Salmonella enterica serovar Typhimurium was included as a positive control for adhesion to Caco-2. Listeria monocytogenes EGD-e was used as a positive control for adhesion to HBMECs. E. coli DH5α served as a negative control for adhesion to both Caco-2 and HBMECs.

Immunoblot analysis of His6-tagged proteins

Immunoblot analysis was performed as described previously with small modifications [31]. Briefly, bacterial samples were disrupted by sonication (TissueLyser, China). The cell lysates were subsequently centrifuged, and the precipitates were transferred to membrane protein dissolution buffer (Sangon Biotech). The dissolved samples were used in western blotting. Protein concentration was determined using a BCA protein assay kit (Abcam, China). The proteins were transferred to nitrocellulose membranes (Bio-Rad, USA), and the membranes were blocked with 3% (w/v) milk (BD, USA) solution before incubation with monoclonal HRP-conjugated anti-6× His antibodies (Abcam, UK) diluted in 5% (w/v) BSA (Sangon Biotech). Protein signals were detected by HRP Substrate (Bio-Rad, USA) and Enhanced ECL Chemiluminescent Substrate (MBio, China).

Propidium iodide (PI) assay

The PI assay was performed as described previously with small modifications [58]. Briefly, C. sakazakii were grown in LB broth up to the mid logarithmic phase, harvested, washed, and adjusted to 10⁶ CFU/ml in LB at pH 3.0 or in LB at pH 7.0. The cells were incubated at 37°C for 2 hours. Subsequently, the cells were washed in PBS buffer and incubated with PI (1.3 µg/ml) at 37°C for 20 min in dark. A total of 10,000 cells were acquired for each flow cytometry analysis using a flow cytometer (Becton Dickinson).

Ethics statement

The study was approved by the Ethics Committee of our department, and written informed consent was obtained from all participants before the study.
Statistical analysis

Statistical analysis of all data was conducted using the GraphPad Prism program GraphPad (version 8.3). Significant differences were identified using Student’s two-tailed unpaired t-test (*, P < 0.05; **, P < 0.01; ***, P < 0.001, ns, not significant).

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Disclosure statement

The authors have no competing interests to disclose.

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