

Letter to the Editor



Examine the Correlation between Heat Shock Protein IbpA and Heat Tolerance in *Cronobacter sakazakii**

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We used a proteomic approach to identify IbpA in *Cronobacter sakazakii* (*C. sakazaki*), which is related to heat tolerance in this strain. The abundance of IbpA in *C. sakazakii* strains strongly increased after heat shock. *C. sakazakii* CMCC 45402 *ibpA* deletion mutants were successfully constructed. The *C. sakazakii* CMCC 45402 Δ *ibpA* and wild-type strains could not be distinguished based on colony morphology on LB agar plates or biochemical assays. The growth of the *C. sakazakii* CMCC 45402 Δ *ibpA* mutant in heat shock conditions was indistinguishable from that of the isogenic wild-type, but showed greater heat resistance than *E. coli* O157:H7 strain CMCC 44828. This study suggests that the absence of a single *ibpA* gene has no obvious effect on the phenotype or heat resistance of the strain *C. sakazakii* CMCC 45402.

Cronobacter spp. (formerly *Enterobacter sakazakii*) are Gram-negative opportunistic foodborne pathogens that cause life-threatening infections, such as meningitis, sepsis, bacteremia, and necrotizing enterocolitis in neonates, children, immunocompromised adults, and older persons^[1]. The mortality rate for neonatal *Cronobacter sakazakii* (*C. sakazaki*) infections has been reported to be as high as 80%^[2]. Presently, little is known about the pathogenicity mechanisms of *Cronobacter* species, and it is generally accepted that *Cronobacter* spp. are relatively thermoresistant^[1]. Virulence factors and heat tolerant mechanisms of *Cronobacter* are still not elucidated fully, and data on the virulence properties of *Cronobacter* in food from China have not yet been reported. Thus, in the present study, a proteomic approach was used to investigate the putative heat-tolerance associated proteins in *C. sakazakii*, and to identify any correlation between IbpA and the heat tolerance of *Cronobacter*. The strain in the present study, *C. sakazakii* CMCC 45402, is the first

genome-sequenced *C. sakazakii* strain in China^[3], which was originally isolated from milk samples.

To investigate the proteomic responses and identify possible differences in gene expression under heat stress conditions, we first compared the proteomic profiles of *C. sakazakii* CMCC 45402 grown at low and at high temperatures. Cultures were incubated at either 37 °C for the duration of growth or were shifted to 45 °C for the final 2 h. These two samples were then analyzed by two-dimensional-PAGE (Figure 1 A&B). The protein expression patterns of the cultures grown at the high temperature were very similar to those grown at the low temperature, and many landmark spots had counterparts. However, based on quantitative differences in relative volume determined by image analysis, we identified spots whose expression was altered between the different growth temperatures. The most striking observation was one spot, located at the bottom of the gel (pH 4-7), which appeared to be overexpressed upon exposure to the high temperature compared with its expression in the sample at low temperature (Figure 1 C&D). The corresponding protein was identified by MALDI-TOF MS analysis as IbpA (pI: 5.45; MW: 15,805).

IbpA belongs to the family of the small heat shock proteins (sHsps), which are expressed in many bacteria, animals, plants, and even archaea, and exert various functions in cells. The most critical function of sHsps is to protect and restore the structure of proteins upon heat shock^[4]. However, to the best of our knowledge, the sHsps system in *C. sakazakii* has not yet been studied in detail, and the relationship between IbpA and heat tolerance has not been investigated in *C. sakazakii*.

To investigate the functional properties of the IbpA protein in *C. sakazakii* cells, we performed Western blot analysis of *C. sakazakii* strains CMCC 45401 and 45402, along with control strains

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Escherichia coli O157:H7 CMCC 44828 and *Salmonella typhi* CMCC 50098. All strains were incubated for 12 h at 37 °C for the normal samples, and for 10 h at 37 °C and then 45 °C for 2 h for the heat treatment samples. Expression of IbpA in *C. sakazakii* cells with and without heat shock was examined. We observed an increased level of the 15.8-kD protein in the heat-shocked *C. sakazakii* cells (CMCC 45401 and 45402) by Western blotting analysis. The abundance of the IbpA in the two *C. sakazakii* strains strongly increased after heat shock. No obvious bands were detected in the normal samples of the two *C. sakazakii* strains. Additionally, no expression was detected in the cell extracts of *E. coli* O157:H7 CMCC 44828 or *S. typhi* CMCC 50098 under either of the growth conditions.

To assess the biological function of IbpA in the heat tolerance of *C. sakazakii*, a Δ *ibpA* mutant was constructed. *ibpA* was deleted using a modified version of the λ -Red recombination system^[5]. A *C. sakazakii* CMCC 45402 Δ *ibpA* strain was constructed by allelic exchange *via* replacement of the *ibpA* gene with a kanamycin resistance cassette.

The fragment containing the upstream and downstream regions of *ibpA* separated by a

kanamycin resistance gene was amplified, and the 2.5-kb linear fragment was then introduced into competent *C. sakazakii* 45402 cells by electroporation. In the plasmid pKD46, a Δ *ibpA* allelic exchange mutant, designated 45402 Δ *ibpA*, was constructed. The 45402 Δ *ibpA* strain was verified by assessing kanamycin resistance, ampicillin sensitivity, and by PCR analysis.

To verify the genotype of the Δ *ibpA* mutant, the corresponding gene regions of the mutant were amplified by PCR and compared with the gene regions of the wild-type strain. Because there was no kanamycin resistance gene in the wild-type strain, no products were amplified when primers specific for the kanamycin resistance cassette were used, while corresponding products were detected from the Δ *ibpA* strain. Primers specific for *ibpA* failed to yield a product from the Δ *ibpA* mutant. This confirmed that the deletion event occurred at the *ibpA* locus.

The Δ *ibpA* mutant and the wild-type strain were streaked onto LB agar plates and incubated overnight at 37 °C to observe the primary colony morphology. Biochemical tests were conducted using Phoenix systems panel type NMIC/ID-4 (BD Diagnostic Systems, Sparks, MD, USA). *C. sakazakii*

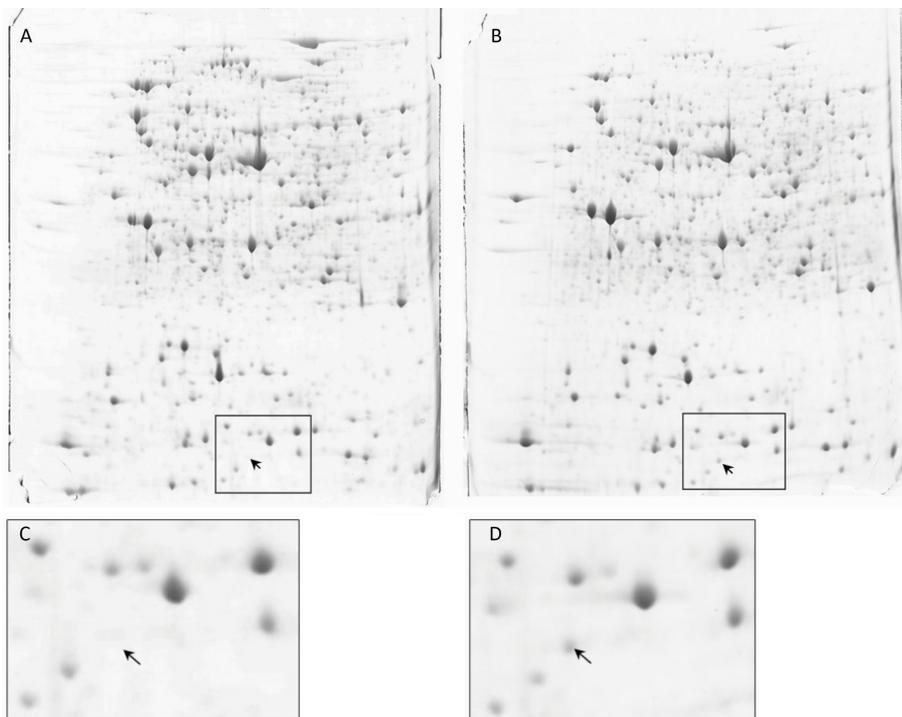


Figure 1. Two-dimensional gel electrophoresis patterns of the whole-cell proteins of *C. sakazakii* CMCC 45402 grown at low temperature (A) and at high temperature (B). The differentially expressed protein spot in the low temperature sample (C) and in the high temperature sample (D) was identified by MALDI-TOF MS analysis as *IbpA*.

CMCC 45402 $\Delta ibpA$ and wild-type strains were indistinguishable based on colony morphology and biochemical assays. Both strains were characterized by dull colonies with a slightly irregular shape and yellow pigment on LB agar plates. Both isolates possessed similar biochemical characteristics as *Cronobacter* with a confidence value of 99%.

The LB plates were incubated at different temperatures from 37 °C to 54 °C and the overnight heat-treated plates were then removed and incubated at room temperature overnight for recovery. Bacterial colonies from each plate were counted and compared. The survival test at different treatment temperatures showed that the growth of the *ibpA* mutant was indistinguishable from that of the isogenic wild type, and the maximum growth temperature of the *ibpA* mutant and wild type *C. sakazakii* CMCC 45402 was at least 48 °C with no reduction in cell count. However, the *E. coli* O157:H7 strain CMCC 44828 was more sensitive to these relatively high temperatures. When the treatment temperature was raised to 52 °C, a clear growth

defect was observed in *E. coli* O157:H7 CMCC 44828, for there were no bacterial colonies from any dilution on LB plates, whereas the $\Delta ibpA$ and the wild type *C. sakazakii* CMCC 45402 showed many colonies on LB plates with a reduction in cell counts of 2 log (cfu/mL) in the same dilution at the same temperature. The results showed a stronger thermal resistance of the wild type *C. sakazakii* CMCC 45402 and $\Delta ibpA$ strains than the control strain *E. coli* O157:H7 CMCC 44828.

C. sakazakii CMCC 45402 wild-type and $\Delta ibpA$ strains, as well as control strain *E. coli* O157:H7 CMCC 44828, were heat-shocked at 45 °C, 48 °C, or 50 °C. As shown in Figure 2A, the growth curves of the *C. sakazakii* CMCC 45402 wild-type and $\Delta ibpA$ strains remained similar at all three heat-stress temperatures. All three strains grew slower at 48 °C than at 45 °C. Severe growth inhibition of *E. coli* O157:H7 CMCC 44828 was observed when the temperature was raised to 50 °C, with no living cells recovered at 3 h post-exposure to the heat stress. The cell density of the two *C. sakazakii* strains decreased over time at 50 °C (Figure 2A-C).

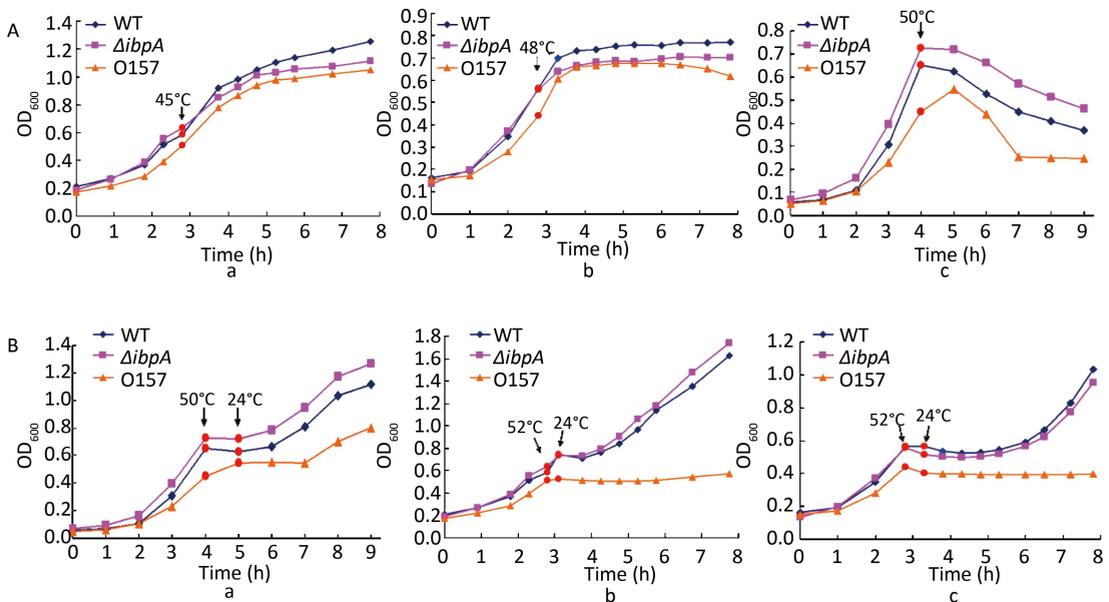


Figure 2. Growth of *C. sakazakii* CMCC 45402 wild-type and $\Delta ibpA$ strains, as well as control strain *E. coli* O157:H7 CMCC 44828, under different prolonged heat stress conditions (Panel A) and transient severe heat stress conditions (Panel B) in liquid medium. *C. sakazakii* CMCC 45402 wild-type (WT), $\Delta ibpA$, and *E. coli* O157:H7 CMCC 44828 cultures were grown to OD₆₀₀ = 0.4-0.7 at 24 °C and then shifted to 45 °C (A-a), 48 °C (A-b), or 50 °C (A-c) for 5 h. *C. sakazakii* CMCC 45402 wild-type (WT), $\Delta ibpA$, and *E. coli* O157:H7 CMCC 44828 cultures were grown to OD₆₀₀ = 0.4-0.7 at 24 °C and then heat-shocked for 1 h at 50 °C (B-a), 15 min at 52 °C (B-b), or 30 min at 52 °C (B-c). Following heat shock, cells were incubated at 24 °C for recovery. Data represents the average of two independent measurements.

We next compared the ability of *C. sakazakii* CMCC 45402 wild-type and Δ *ibpA* strains to tolerate transient exposure to severe heat stress (1 h at 50 °C, 15 min at 52 °C, or 30 min at 52 °C) (Figure 2B). Under the three conditions, the growth of the Δ *ibpA* mutant was indistinguishable from that of the isogenic wild-type. However, a clear growth defect was observed for *E. coli* O157:H7 CMCC 44828 under all three heat-stress conditions. Specifically, one hour after heat stress at 50 °C for 1 h, the cell density of the *C. sakazakii* mutant and wild-type cultures began to recover, while *E. coli* O157:H7 CMCC 44828 grew slowly for an additional 1 h. Similar delays in recovery of the *E. coli* strain compared with the two *C. sakazakii* strains were observed for the other two stress conditions. Under the heat stress condition of 52 °C for 15 min, less than one hour was required for the recovery of *C. sakazakii* CMCC 45402 wild type and Δ *ibpA* cultures, following which, cell density began to increase. However, cell density of the *E. coli* O157:H7 CMCC 44828 remained unchanged for approximately 3 h after heat stress at 52 °C for 15 min. Under the heat stress condition of 52 °C for 30 min, more than 2 h were required for the *C. sakazakii* CMCC 45402 wild type and Δ *ibpA* cultures before the cell density slowly rose. In contrast, the *E. coli* O157:H7 CMCC 44828 exhibited a prolonged recovery phase of 5 h even until the end of the experimental period. These results clearly demonstrated the general heat resistance of *C. sakazakii* strain CMCC.

Surprisingly, despite the predicted role of IbpA in tolerance of heat stress, a growth curve analysis demonstrated that the Δ *ibpA* mutant showed no obvious difference from the isogenic wild-type strain under heat stress in liquid medium in the current study. These results differ from those of a previous study^[6], in which the *Pseudomonas putida* *ibpA* deletion mutant exhibited a severe growth defect under heat stress conditions and reduced survival during recovery, implying a critical role of IbpA in heat tolerance. However, the results described in this paper may not be typical in terms of the heat resistance of *C. sakazakii* in general. The strain used in the current study was isolated from milk products, and may therefore be typical in terms of its survival capabilities, perhaps accounting for the unexpected result. Thus, it remains uncertain whether IbpA is involved in the heat resistance of *C. sakazakii*. Another point worth noting is that IbpA and IbpB are molecular chaperone proteins^[7]. IbpB and IbpA both play a role in protecting enzymes from heat inactivation^[8]. IbpA cooperates with IbpB in the

stabilization of thermally aggregated proteins^[9]. It has been reported that *ibpA* transcripts preferentially accumulate in *E. coli* under heat shock conditions, and subsequently, IbpA might regulate the expression of *ibpB*^[10]. Regulation of *ibpA* and *ibpB* expression is complex, and is not yet fully understood. Our study indicated that the expression of *ibpA* may be temperature-controlled. Although the intricate interplay between IbpA and IbpB remains elusive in *C. sakazakii*, it is conceivable that IbpB might accumulate compensatively in the *ibpA* knockout strain. This may explain the lack of an obvious difference in growth between the mutant and wild-type strains under heat stress conditions.

Our results suggest that the absence of a single *ibpA* gene may have no obvious effect on the phenotype or heat resistance of the strain *C. sakazakii* CMCC 45402, but the findings of this research provide a better understanding of IbpA, and provide the basis for further work on the heat tolerance of this species. It will be challenging to obtain deeper mechanistic insights into the role of IbpA in the heat resistance of *C. sakazakii*.

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REFERENCES

1. Yan QQ, Condell O, Power K, et al. *Cronobacter* species (formerly known as *Enterobacter sakazakii*) in powdered infant formula: a review of our current understanding of the biology of this bacterium. *J Appl Microbiol*, 2012; 113, 1-15.
2. Lai KK. *Enterobacter* infections among neonates, infants, children, and adults: case reports and a review of the literature. *Medicine (Baltimore)*, 2001; 80, 113-22.
3. Zhao Z, Wang L, Wang B, et al. Complete genome sequence of *Cronobacter sakazakii* strain CMCC 45402. *Genome Announc*, 2014; 2, e01139-13.
4. Vishnyakov IE, Levitskii SA, Manuvera VA, et al. The identification and characterization of IbpA, a novel α -crystallin-type heat shock protein from mycoplasma. *Cell Stress Chaperones*, 2012; 17, 171-80.

5. Mosberg JA, Lajoie MJ, Church GM. Lambda red recombineering in *Escherichia coli* occurs through a fully single-stranded intermediate. *Genetics*, 2010; 186, 791-9.
6. Krajewski SS, Nagel M, Narberhaus F. Short ROSE-like RNA thermometers control IbpA synthesis in *pseudomonas* species. *PloS One*, 2013; 8, e65168.
7. Allen SP, Polazzi JO, Gierse JK, et al. Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in *Escherichia coli*. *J Bacteriol*, 1992; 174, 6938-47.
8. Kitagawa M, Miyakawa M, Matsumura Y, et al. *Escherichia coli* small heat shock proteins, IbpA and IbpB, protect enzymes from inactivation by heat and oxidants. *Eur J Biochem*, 2002; 269, 2907-17.
9. Matuszewska M, Kuczyńska-Wiśnik D, Laskowska E, et al. The small heat shock protein IbpA of *Escherichia coli* cooperates with IbpB in stabilization of thermally aggregated proteins in a disaggregation competent state. *J Biol Chem*, 2005; 280, 12292-8.
10. Gaubig LC, Waldminghaus T, Narberhaus F. Multiple layers of control govern expression of the *Escherichia coli* *ibpAB* heat-shock operon. *Microbiology*, 2011; 157, 66-76.