Function of thermotolerance genomic island in increased stress resistance of *Cronobacter sakazakii*

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Summary
Bacteria belonging to genus *Cronobacter* are opportunistic pathogens associated with serious infections in neonates. Powdered infant formula has been identified as a potential source of infection in several cases. In the present study, a deletion mutant of *C. sakazakii* ATCC 29544 lacking the 18 kbp thermotolerance genomic island was prepared. The mutant and wild-type strain had the same maximum growth rate at optimal conditions. However, the mutant strain was defective in survival at increased temperatures both in exponential and stationary growth phase. Besides the increased thermotolerance, the protection effect of the genomic island was observed also for osmotic stress, but not for desiccation. The increased stress tolerance of *Cronobacter* strains containing the thermotolerance island can promote their survival in production facilities and in powdered infant formula.

Keywords
Enterobacteriaceae; *Cronobacter*; food; stress; λ Red recombination

Cronobacter spp. are opportunistic pathogens that can cause serious infections in neonates, including meningitis, necrotizing enterocolitis and septicemia, with fatality rates as high as 80% [1–4]. Infections in adults have also been reported, in particular among the elderly and immunocompromised patients [5]. The genus *Cronobacter* was established by a polyphasic taxonomic approach as a re-classification of *Enterobacter sakazakii* and other strains. Currently, seven species and three subspecies have been classified in *Cronobacter* genus [6, 7].

Although these bacteria are widely distributed in the environment and in various foods [8–10], powdered infant milk formula was implicated as a vehicle of transmission in many clinical manifestations [1–4]. Accurate knowledge of the growth condition range of *Cronobacter* strains is necessary to minimize the risk of contamination during the production and preparation of rehydrated infant formula. In comparison with other Enterobacteriaceae, *Cronobacter* is considerably resistant to desiccation [11–13] and this property contributes to its survival in powdered infant formula. Thermal tolerance is another important factor for *Cronobacter* survival in food [14]. It was shown that *Cronobacter* strains differ in their tolerance to heat stress. Edelson-Mammel and Buchanan [15] obtained decimal reduction times at 58 °C (D_{58}) in the range from 0.5 min to 9.9 min and the *Cronobacter* strains fell into two heat resistance phenotypes, namely, thermosensitive and thermotolerant (including *C. sakazakii* ATCC 29544). The protein marker unique for thermotolerant *Cronobacter* strains was later identified by mass spectrometry [16]. In our previous work, we found out that the thermotolerance marker is encoded by a gene localized on a genomic island that contained a cluster of conserved genes. Many of them, among others Hsp20 small heat-shock protein, ClpK ATPase with chaperone activity, M48 and DegP2 peptidases and thioredoxin, are involved in

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Tab. 1. Primers used in the study.

Name	Sequence	Used in
thrBF	ttttcacgctcgatgaattg	real-time PCR (*thr*B gene)
thrBR	tgatgcactgatcgatttcac	
thrDF	atgtcggtccagagcatttc	real-time PCR (*thr*D gene)
thrDR	gaccaccttgctgacctgtt	
thrIF	gcacagccacaggtagatga	real-time PCR (*thr*I gene)
thrIR	ccttggtcttgtttgcatca	
thrLF	gcagagagctttgggaaatg	real-time PCR (*thr*L gene)
thrLR	aattgccgcttgatcttgtc	
tufAF	ccgcagactcgtgagcacat	real-time PCR (*thr*A gene)
tufAR	agcagctcttcgtcatcaacca	
pDelBF	caaggggctgcgggcactcggctacaacgtgaggatcggttgtaggctggagctgcttcg	construction of deletion mutant
pDelQR	aaactcccggttgtagttcc acaagcatctcgcgagtcttattccggggatccgtcgacc	
pOrfABF	gccgactggatcaaactcat	
pOrfABR	accgatcctcacgttgtagc	
pOrfQF	cttgtggaactacaaccgggagtt	
pOrfQR	aagtgcgagcgaaggtagag	

some type of stress response, including heat, oxidation and acid stress. The thermotolerance DNA region was present in nine out of 73 *C. sakazakii* strains and two out of ten *C. malonaticus* strains in screening of 99 *Cronobacter* strains. The same genomic island was also detected in strains belonging to the genera *Enterobacter*, *Citrobacter* and *Escherichia*, and its presence positively correlated with increased thermotolerance [17].

The aim of the present work was to construct a deletion mutant of *C. sakazakii* ATCC 29544 lacking the whole thermotolerance island to be able to further elucidate the role of this region in *Cronobacter* survival during stress conditions.

MATERIALS AND METHODS

Bacterial strains and cultivation

C. sakazakii ATCC 29544, a clinical isolate, was obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG Bacteria Collection, Ghent, Belgium). All *Cronobacter* and *E. coli* strains were cultured in Luria-Bertani (LB) medium at 37 °C. Strains were stored for long periods in 15% glycerol solution at –70 °C.

Determination of gene expression by Reverse Transcriptase Real-time PCR

Gene expression was measured for bacterial cells cultured in LB medium at four different conditions:

1. Exponential cells grown at 37 °C with aeration up to optical density 0.3–0.5 at 600 nm (OD_{600});
2. Stationary phase bacteria cultured for 18–20 h at 37 °C with aeration;
3. Microaerobically grown stationary cells that, after overnight growth with aeration, were incubated for next 1 h at 37 °C without mixing;
4. Thermally stressed cells that, after overnight growth with aeration at 37 °C, were incubated for next 1 h at 54 °C without mixing.

Total RNA was isolated from 1 ml of bacterial culture using RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA (0.5–1 μg) was reverse transcribed with GoTaq 2-Step RT-qPCR System (Promega, Madison, Wisconsin, USA) and the same kit was used in real-time PCR. Gene expression was quantified by amplification of cDNA samples with orf-specific primers: thrBF/thrBR, thrDF/thrDR, thrIF/thrIR, thrLF/thrLR, tufAF/tufAR (Tab. 1) using ABI Prism 7900 HT Fast (LifeTechnologies, SanDiego, California, USA) apparatus. The transcription levels in samples were calculated according to calibration curves obtained with decimal dilutions of genomic DNA. For all primers, the slope of the calibration curve reached values from –3.4 to –3.7, *thr*D with the slope –4.9 being an exception. Transcription values in different conditions were compared with the level in exponentially grown cells using *tuf*A gene as endogenous control for normalization.

Preparation of deletion mutants

Isogenic mutants of *C. sakazakii* ATCC 29544 lacking the thermotolerance island were prepared

by modified λ Red recombination method [18, 19]. Linear PCR product containing kanamycin (Kn) resistance cassette surrounded by fragments with homology to deletion region was made by overlapping PCR. The Kn resistance cassette was prepared by amplification of the template plasmid pKD13 with primers pDelBF/pDelQR (Tab. 1), which contained 40 bp long 5´extensions homologous to DNA sequence flanking *C. sakazakii* ATCC 29544 thermotolerance region (Acc. No. FR714908, National Center for Biotechnology Information, Bethesda, Maryland, USA). Two surrounding regions of *C. sakazakii* ATCC 29544 genome were amplified by primers pOrfABF/pOrfABR and pOrfQF/pOrfQR. Finally, all three PCR products were used as templates in PCR with primers pOrfABF/pOrfQR, which resulted in a DNA molecule possessing homologous sequences of about 500 bp on both ends. Parent strain *C. sakazakii* ATCC 29544 was transformed with plasmid pKD46 encoding for λ Red recombinase proteins. Cells were grown at 30 °C in presence of 0.2% arabinose for induction of λ Red genes. Prepared linear mutagenic fragment was transformed into electrocompetent cells and clones, in which recombination between the thermotolerance region and Kn cassette occurred, were selected on an antibiotic-containing medium. Helper plasmid was cured from cells by cultivation at 37 °C. Successful recombination was confirmed by PCR and the resulting clone was named *C. sakazakii* JG-01. Next, the Kn cassette was removed from the construct by Flip recombinase encoded by thermosensitive helper plasmid pCP20, strain *C. sakazakii* JG-05 being prepared by this procedure.

Determination of growth curve

A 1% inoculum of the overnight culture was prepared in a final volume of 20 ml LB medium and cultured aerobically at 37 °C with shaking. Growth of the culture was monitored at 1 h intervals by measuring the optical density at 600 nm on Saphire II spectrofluorimeter (Tecan, Grödig, Austria) in 200 μl sample volumes.

Determination of thermal and osmotic tolerance

Survival at various temperatures: 10 μl bacterial culture was mixed with 990 μl fresh pre-heated LB medium and incubated statically at 37 °C, 42 °C, 48 °C, 50 °C and 54 °C for 20 h. Numbers of survived cells were determined by cultivation of decimal dilutions on LB agar medium.

Thermotolerance at 58 °C: 50 μl bacterial culture was mixed with 450 μl fresh pre-heated LB medium and incubated statically at 58 °C. Samples were withdrawn from the thermoblock in 1 min intervals, chilled on ice for 1 min and numbers of survived cells were determined by the plate-count procedure.

Osmotic and desiccation stress: 1 ml bacterial overnight culture was centrifuged and the cell sediment was resuspended in 1 ml 40% saccharose, in 4 mol·l^{-1} NaCl or stored without any solution. Prepared samples were incubated at 37 °C and numbers of survived cells were determined by the plate-count procedure in one-day intervals.

RESULTS AND DISCUSSION

The aim of our study was to investigate molecular mechanisms of increased heat tolerance of some *Cronobacter* strains mediated by the presence of 18 kb genomic island [17]. The region contained conserved genes (*thr*B-*thr*Q) with significant homologies to bacterial stress response proteins. In the present study, we further characterized the genomic island and assessed its contribution to cell survival under various conditions.

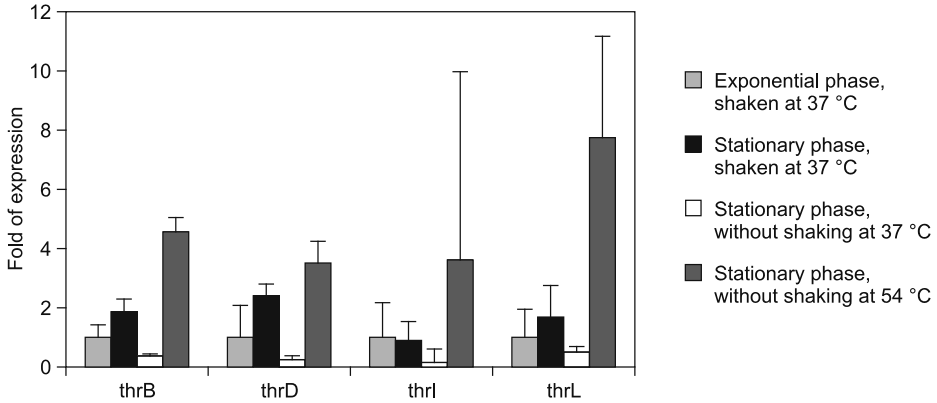

Fig. 1. Detection of gene expression in thermotolerance island from *C. sakazakii* ATCC 29544 by real-time PCR.

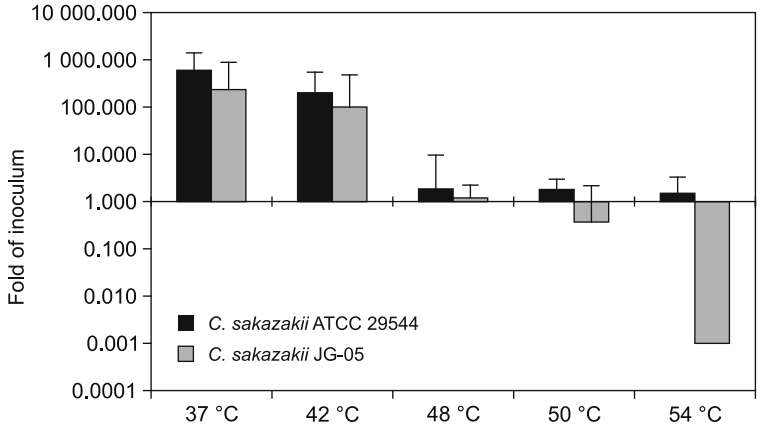

Fig. 2. Survival of *C. sakazakii* ATCC 29544 and its deletion mutant *C. sakazakii* JG-05 at various temperatures.

Average values of three independent experiments are shown, bars show standard deviation.

Expression of genes from the thermotolerance island

Previously, high level of transcription from the thermotolerance island was estimated [17]. We decided to quantify this transcription in various conditions by reverse transcription real-time PCR. High expression levels of *thr*B, *thr*D, *thr*I and *thr*L genes in both exponential and stationary phase of *C. sakazakii* ATCC 29544 growth were observed. According to our estimation, levels of expression were as high as that of *tuf*A endogenous control. In agreement with the function of genes in the protection against heat damage, a 12- to 20-fold increase in the transcription of thermotolerance genes was observed when cells were transferred from 37 °C to 54 °C (Fig. 1).

Construction of isogenic mutant lacking the thermotolerance island

The role of the thermotolerance island in cell survival during stress conditions was assessed by comparing the thermotolerant *C. sakazakii* ATCC 29544 with an isogenic mutant lacking this region. Several methods for allelic replacement in bacteria were developed in recent years [19]. The frequently used method designed by Datschenko and Wanner [18] exploits linear DNA molecule with short (40 bp) ends with homology to the exchanged chromosomal region as a substrate for homologous recombination mediated by Red proteins from λ bacteriophage. In our first experiments, we also used linear DNA fragment with 40 bp homologies, however, no recom-

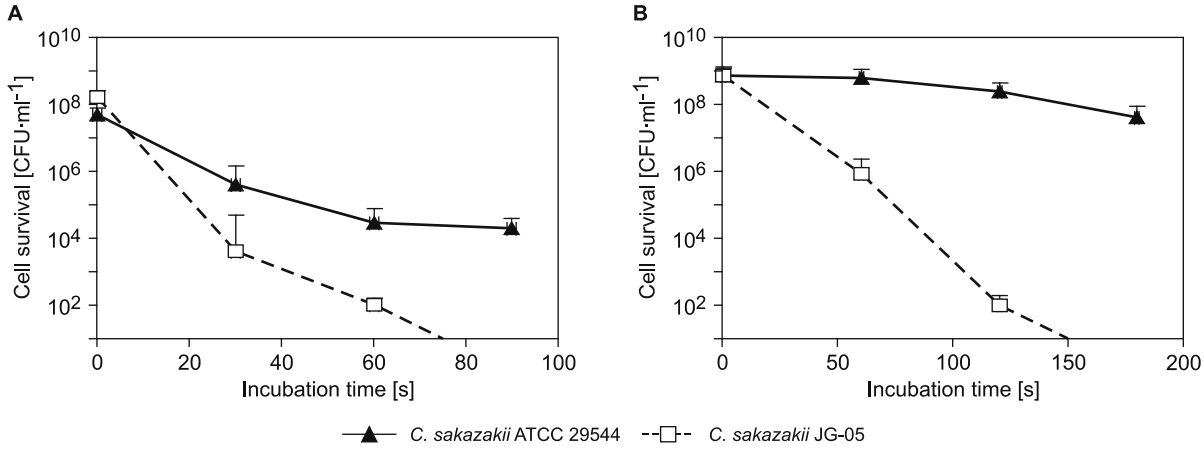

Fig. 3. Inactivation of *C. sakazakii* ATCC 29544 and its deletion mutant *C. sakazakii* JG-05 at 58 °C.

A – cultures in exponential growth phase, B – cultures in stationary growth phase.
Average values of three independent experiments are shown, bars show standard deviation.

binants resistant to kanamycin were obtained. Similarly to other authors constructing mutants in non-*E. coli* strains [20, 21], we extended the homologous region up to 500 bp and we successfully prepared strains *C. sakazakii* JG-01 and *C. sakazakii* JG-05 lacking the whole thermotolerance island from the *thr*B to *thr*Q genes by this approach.

Comparison of thermotolerant strain with its isogenic mutant

Determination of growth rates at optimal conditions did not show any difference between *C. sakazakii* ATCC 29544 and its deletion mutant JG-05 (data not shown). However, when the survival of cells at different temperatures was examined, better survival of the parent strain compared with the deletion mutant at temperatures higher than 48 °C was observed (Fig. 2). The difference in numbers of live cells increased with the rising temperature and almost no *C. sakazakii* JG-05 cells were able to survive at 54 °C. When inactivation rates at 58 °C were measured, protection of the thermotolerance genes was observed in both exponential and stationary growth conditions (Fig. 3), but the protective effect on cells during stationary phase was more pronounced. We detected a three-times decrease of decimal reduction time at 58 °C D_{58} (from 27 s to 10 s) in *C. sakazakii* JG-05 mutant comparing with wild-type *C. sakazakii* ATCC 29544 during exponential growth. This value reached an eightfold difference (from 145 s to 17 s) for stationary phase cells. The higher resistance of stationary phase bacterial cells to stress conditions is well known and it was documented for *Cronobacter* previously [22]. The value of D_{58} for *C. sakazakii* JG-05 stationary cells is in the range obtained for thermosensitive *Cronobacter* strains [17] and thus our results proved the role of the thermotolerance island in cell protection against the heat stress. Our results are in agreement with similar experiments with *Klebsiella pneumoniae* possessing homologous cluster of thermotolerance genes encoded on a conjugative plasmid [21, 23]. Also in *Klebsiella*, the thermotolerance island had no influence on growth rate in permissive temperature, but considerably increased cell survival at elevated temperatures.

In further experiments, the influence of the thermotolerance island on *Cronobacter* survival during desiccation and osmotic stress was examined (Fig. 4). No effect of the island on desiccated strains was detected, but the survival of thermotolerant cells in a highly osmotic environment was considerably enhanced in comparison to the isogenic mutant. The protection effect was observed in both NaCl and saccharose solutions.

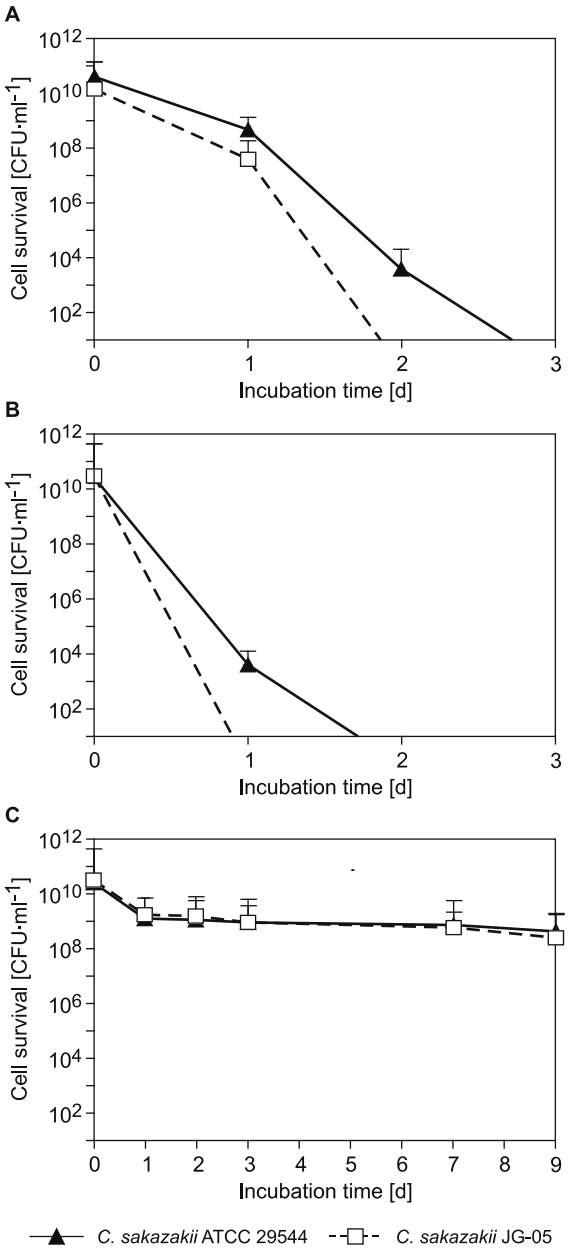

Fig. 4. Survival of stationary growth phase *C. sakazakii* ATCC 29544 and its deletion mutant *C. sakazakii* JG-05 under osmotic and desiccation stress.

A – 4 mol·l^{-1} NaCl, B – 40% saccharose, C – after desiccation. Average values from three independent experiments are shown, bars show standard deviation.

Our observations are in agreement with previous experiments showing that some proteins of the thermotolerance island were induced and translocated by osmotic stress and in desiccated cultures [24]. On the other hand, there are also other properties that are helpful for *Cronobacter* survival in unfavourable conditions. The activity of alternative sigma factor RpoS was recently described as

an important agent causing differences in cell survival during osmotic, pH, heat or oxidative stress [25]. The increased desiccation and osmotic resistance of *Cronobacter* was explained also as a result of presence of multiple homologues of *E. coli* osmoprotection genes in *C. sakazakii* BAA-894 genome [26]. Another important factor responsible for efficient survival in desiccated environments is production of a bacterial capsule [11, 13].

CONCLUSION

Cronobacter infections are rare, but may be life-threatening, affecting sensitive persons especially neonates, where powdered infant milk formulae was recognized as a source of infection. Spread of this pathogen in food is connected to its high resistance to various types of environmental stresses, e. g. resistance to dry and osmotic environment, heat resistance and tolerance to low pH [3, 4, 11–14]. In our study we showed that increased thermotolerance of some *Cronobacter* strains is facilitated by the unique genomic region and the same genes also improve cell survival in highly osmotic conditions. This thermotolerance island is present in about 10% of *C. sakazakii* and *C. malonaticus* strains [17] and it was recently detected in two *C. sakazakii* and two *C. malonaticus* sequenced genomes [27]. A multilocus sequence typing (MLST) scheme has been established for *Cronobacter* spp. [28] and it was shown that one particular sequence type, the clonal lineage *C. sakazakii* ST4, is responsible for majority of neonatal meningitis cases around the world [28, 29]. *C. sakazakii* ST4 is also frequently isolated from powdered formulae as well as from manufacturing facilities. By comparision of the current *Cronobacter* Multi Locus Sequence Typing (MLST) Database [30] with our previous thermotolerance screening [17], the thermotolerance genomic region was detected in two *C. sakazakii* ST4 strains (ATCC 29004 and 201206/19). A part of the thermotolerance island (*thr*BCD genes) is also present in genome of *C. sakazakii* 701 that is the strain isolated from a fatal case of neonatal meningitis belonging to the ST4 type. The *thr*D gene encodes for ClpK ATPase with chaperone activity and its presence (in both *thr*BCD or *thr*B-Q arrangement) was proved to increase the thermotolerance [17, 21, 23]. It may be supposed that the thermotolerance island can influence survival of some ST4 strains in powdered infant milk formulae or environment. Therefore, increased stress resistance of some *Cronobacter* strains due to the thermotolerance island should be taken into consideration in sanitary measures during infant formula production, storage and preparation.

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