

# Contribution of the thermotolerance genomic island to increased thermal tolerance in *Cronobacter* strains

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## **Abstract**

*Cronobacter* spp. are opportunistic pathogens associated with serious infections in neonates. Increased stress tolerance, including the thermotolerance of some *Cronobacter* strains, can promote their survival in production facilities and thus raise the possibility of contamination of dried infant formula which has been identified as a potential source of infection. Some *Cronobacter* strains contain a genomic island, which might be responsible for increased thermotolerance. By analysis of *Cronobacter* sequenced genomes this determinant was found to be present in only 49/73 *Cronobacter sakazakii* strains and in 9/14 *Cronobacter malonaticus* strains. The island was also found in 16/17 clinical isolates originating from two hospitals. Two configurations of the locus were detected; the first one with the size of 18 kbp containing the *thrB-Q* genes and a shorter version (6 kbp) harbouring only the *thrBCD* and *thrOP* genes. Strains containing the thermotolerance island survived significantly better at 58°C comparing to a *C. sakazakii* isogenic mutant lacking the island and strains with the longer version of the island were 2-10 times more tolerant than those with the shortened sequence. The function of the genomic island was further confirmed by its cloning into a low-copy vector and transforming it into the isogenic mutant. Different levels of *rpoS*, encoding for stress-response sigma factor, expression were also associated with variability in strain thermotolerance.

**Keywords:** *Cronobacter* spp., heat stress, *rpoS*, thermotolerance, thermotolerance island

## Introduction

*Cronobacter* spp. are Gram-negative, rod shaped bacteria from the family *Enterobacteriaceae*. They were formerly known as *Enterobacter sakazakii* and were classified as a separate genus in 2007 (Iversen et al. 2007). Recently the genus *Cronobacter* has undergone a number of revisions and currently contains seven species (Iversen et al. 2008; Joseph et al. 2012; Forsythe et al. 2014; Stephan et al. 2014). *Cronobacter* strains are opportunistic pathogens that can cause serious infections in neonates, including meningitis, necrotising enterocolitis and sepsis with low frequency, but high lethality rate (Hunter and Bean 2013; Holy and Forsythe 2014). Infections in adults also have been reported, in particular among the elderly and immunocompromised patients (Patrick et al. 2014; Alsonosi et al. 2015).

Whereas *Cronobacter* strains are ubiquitous and have been isolated from various foods and environments (Turcovsky et al. 2011; Holy and Forsythe 2014) the main vehicle for their transmission in neonatal infections is rehydrated powdered infant formula (PIF) (Yan et al. 2012; Holy and Forsythe 2014). In comparison with other *Enterobacteriaceae*, members of the genus *Cronobacter* are considerably resistant to desiccation (Caubilla-Barron and Forsythe 2007; Osaili and Forsythe 2009; Walsh et al. 2011; Jaradat et al. 2014) and this property contributes to their survival in PIF. Thermal tolerance is another important factor for survival of *Cronobacter* in food (Dancer et al. 2009; Jaradat et al. 2014; Huertas et al. 2015). It has been shown that *Cronobacter* strains differ in their tolerance to heat stress. Edelson-Mammel and Buchanan (2004) obtained decimal reduction times at 58°C (D<sub>58</sub>) in the range from 0.5 to 9.9 min and the *Cronobacter* strains fell into two heat resistance phenotypes, thermosensitive and thermotolerant. A protein marker unique for thermotolerant *Cronobacter* strains was later identified by mass spectrometry (Williams et al. 2005). In our previous work, we found that this thermotolerance marker is encoded by a gene localised on a genomic island which contained a cluster of conserved genes encoding several stress response proteins (Gajdosova et al. 2011). We observed that an isogenic mutant lacking the thermotolerance island was defective in survival at increased temperatures both in exponential and stationary growth phase (Orieskova et al. 2013).

The aim of the present study was to compare the composition of the thermotolerance genomic island in genome sequenced *Cronobacter* strains and to correlate the genomic differences with their phenotypes.

## Materials and Methods

**Bacterial strains used in the study.** *Cronobacter* collection strains were obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM/LMG) and from Nottingham Trent University UK. *C. sakazakii* JG-05 was prepared from *C. sakazakii* ATCC 29544<sup>T</sup> by deletion of the whole genomic island (Orieskova et al. 2013). Clinical *Cronobacter* strains were isolated during 2012-2013 from two hospitals in the Czech Republic (Alsonosi et al. 2015). Strains and plasmids used in the study are listed in Tables S1 and S2. All strains were maintained in LB medium supplemented with 15% glycerol at -80°C and routinely cultured on LB medium/LB agar at 37°C.

**Detection of genes from the thermotolerance island in *Cronobacter* genomes.** *Cronobacter* genomes deposited in the PubMLST database (<http://pubmlst.org/cronobacter/>) were used as the source of genomic sequences for analyses (Jolley and Maiden 2010; Forsythe et al. 2014). Genes *thrB-Q* from the *C. sakazakii* ATCC 29544<sup>T</sup> thermotolerance island (FR714908) and *thrOP* from *C. sakazakii* NTU701 were Blast searched against the database and hits with sufficient coverage and similarity (both up to 90% on nucleotide level) were scored as positive.

**Indirect assays for the assessment of RpoS status.** The catalase activity of *C. sakazakii* strains was measured according to Alvarez-Ordenez et al. (2012) by dropping 20 µl of 6% hydrogen peroxide onto fresh colonies that were grown on LB agar for 24 h at 37°C. Intensity of bubbling indicated level of catalase activity. Yellow pigmentation of colonies was assessed using cultures grown on LB-PIF agar, which was prepared by mixing of one volume of 2-times concentrated LB-agar and one volume of reconstituted PIF. LB-PIF plates were grown at 37°C for 24 h in a dark incubator followed by 48 h further incubation in daylight at room temperature.

**Determination of thermotolerance.** 100 µl overnight bacterial culture (approximately 18 h at 37°C) was mixed with 900 µl fresh pre-heat LB medium and incubated at 58°C. Aliquots were withdrawn into sterile 200 µl thin-wall microtubes and chilled on ice for 1 min and the numbers of surviving cells were determined by the plate-count procedure. Each value was determined in triplicate for each culture.

**Cloning of the thermotolerance island.** The island was amplified from genomic DNA using CloneAmp Hifi PCR Premix (Clontech) and primers pThr2118F-HindIII (ataagcttacggatcggtgccattattacg) and pThr17274R-HindIII (acgaagcttgccctgtcgaacctatggat) for *C. sakazakii* ATCC 29544<sup>T</sup> strain and pThr2118F-HindIII and pCs701-R1-HindIII (ccaagcttctgtaggggtcggttaggtc) for *C. sakazakii* NTU696 and *C. sakazakii* NTU701 strains. Vector DNA (origin, Cm cassette) was amplified from the pACYCDuet1 (Novagen) with primers VecD2f-Thr2f (cgatccgtaagcttatagaagcacacgggtcacactg) and VecD2r-Thr2r (acagggcaagcttcggttcaaccagtcagctcctt) or with VecD2f-Thr2f and VecD2r-Thr701R (ccctacagaagcttggttcaaccagtcagctcctt) respectively. The In-Fusion® HD EcoDry™ Cloning Kit (Clontech) was used for direct joining of the resulting fragments in recombinant plasmids pACYCThr2-ATCC29544, pACYCThr2-NTU696 and pACYCThr2-NTU701 (Fig.1, Table S2). The PCR product obtained by pACYCDuet1 amplification with primers VecD2f-Thr2f /VecD2r-Thr2r digested by HindIII was ligated to prepare control plasmid pACYCK2.

**Cloning of rpoS gene.** The gene was amplified from *Escherichia coli* KL53, an uropathogenic strain, with primers RpoSF (agcgctagcatgagtcagaatacgtgaaagttc) and RpoSR (gatgaattcttactcgcggaacagcg). The PCR product was digested with EcoRI and NheI and cloned into vector prepared by digestion of the pBAD-GFPuv plasmid with the same enzymes (Table S2). The same primers were used for *rpoS* sequencing in *C. sakazakii* NTU696.

## Results and Discussion

### Comparison of thermotolerance islands in *Cronobacter* strains

The aim of this study was to characterise the thermal tolerance which is mediated by the genomic island present in some *Cronobacter* strains. The island from the *C. sakazakii* ATCC 29544<sup>T</sup> type strain, which is 18 kbp long and harbours *thrB-Q* genes (Fig. 1, Table S3), was described in our previous studies (Gajdosova et al. 2011; Orieskova et al. 2013). Recently, genomes of fourteen *Cronobacter* strains covering seven species were published (Joseph et al. 2012) and a comparison with *C. sakazakii* ATCC 29544<sup>T</sup> showed that the genes from the thermotolerance island were found in two from five *C. sakazakii* (NTU696 and NTU701) and in both *C. malonaticus* (LMG23826<sup>T</sup> and NTU507) genomes from this study. *C. malonaticus* strains contained thermotolerance islands of similar size and organisation as that present in *C. sakazakii* ATCC 29544<sup>T</sup>. However, the genomes of *C. sakazakii* NTU696 and *C. sakazakii* NTU701 possessed a version of the island shortened to 6 kbp, having only *thrBCD* and *thrOP* genes from, respectively, the beginning and the end of the original version (Fig 1). The similarity of the *thrBCD* gene products from the long and shortened islands was high (93%-99% at the amino acid level), but the *thrOP* gene products showed only moderate similarity (76% and 77% at the amino acid level for ThrO and ThrP respectively).

The number of sequenced genomes is steadily increasing and 108 *Cronobacter* genome sequences representing all seven species of the genus have been deposited in the *Cronobacter* PubMLST database (<http://pubmlst.org/cronobacter/>) at the time of analysis (May 2015) (Forsythe et al. 2014). In agreement with our previous observations, the thermotolerance island was present only in *C. sakazakii* (in 49 from 73 strains) and in *C. malonaticus* (in 9 from 14 strains) deposited in the database (Table S4). Twenty-three of them (14 *C. sakazakii* and all 9 *C. malonaticus*) contained the full length version of the island, while thirty-five *C. sakazakii* strains possessed the shortened one. We observed high sequence conservation in the locus; the similarity at the DNA level reached 99.5-100% between strains with the same type of the island. By cluster analysis sequences of the long islands were separated into two groups: the first contained the majority of the *C. malonaticus* strains; *C. sakazakii* and the one remaining *C. malonaticus* strain belonged to the second cluster (Fig. S1). Strains containing the short island were separated into several smaller groups in agreement with their origin and MLST sequence type (Fig. S2).

A genomic region with a high degree of sequence similarity was recently described also in thermotolerant *Klebsiella pneumoniae* strains responsible for nosocomial infections due to increased resistance to disinfection protocols (Bojer et al. 2010) and in several heat resistant *E. coli* strains isolated from beef samples (Mercer et al. 2015).

Thermotolerant *Cronobacter* strains originated from various sources, mainly from clinical samples (73%), PIF and formula production facilities (14%) and from the environment (13%). By comparing the genomic island occurrence in the MLST sequence types we observed that the majority of the strains belonged to the ST4 (61%), which is a virulent clonal lineage responsible for most of the meningitis cases in newborns (Joseph and Forsythe 2011; Forsythe et al. 2014). Several other positive *C. sakazakii* strains were classified as ST8, ST12 and ST13, which are also associated with infections in neonates and adults. For example, the *C. sakazakii* ATCC 29544<sup>T</sup> type strain, which belongs to ST8, was isolated from human throat and the ST12 and ST13 strains were isolated during an NICU outbreak in France in 1994 (Caubilla-Barron et al. 2007); during this epidemic ST12 strains were associated with necrotizing enterocolitis cases (Forsythe et al. 2014; Masood et al. 2015). The nine *C. malonaticus* strains containing the thermotolerance island belonged to five different sequence types. However, four ST7 strains were the most prevalent found among these strains and this sequence type is connected with

adult infections, especially in immunocompromised and elderly patients (Forsythe et al. 2014; Alsonosi et al. 2015).

### Thermotolerance in selected strains

Subsequently, the thermal tolerance of *C. sakazakii* and *C. malonaticus* strains obtained from the study described by Joseph et al. (2012) were compared with that of the *C. sakazakii* type strain ATCC 29544<sup>T</sup>. The mutant *C. sakazakii* JG-05, prepared from *C. sakazakii* ATCC 29544<sup>T</sup> by deletion of the whole thermotolerance island and described in our previous study (Orieskova et al. 2013) was used as a control (Fig. 2). We observed that strains containing the thermotolerance island survived significantly better at 58°C compared with the deletion mutant and that *C. sakazakii* ATCC 29544<sup>T</sup> possessed the highest thermotolerance of all the strains, which is in agreement with our previously reported results (Gajdosova et al. 2011). Strains with the longer version of the island (*C. sakazakii* ATCC 29544<sup>T</sup>, *C. malonaticus* LMG23826<sup>T</sup> and NTU507) were 2-10 times more thermotolerant than strain *C. sakazakii* NTU701 containing the sequence shortened to the *thrBCD* and *thrOP* genes. This observation confirmed the results of Bojer et al. (2013), who studied *K. pneumoniae* strains containing a similar thermotolerance region and showed that thermotolerance could differ according to the presence of the longer or shorter version of the locus. The *C. sakazakii* NTU696 strain was the only exception, as it contained a thermotolerance island with an almost identical sequence to that of *C. sakazakii* NTU701, but its survival ability was lower than that of the deletion mutant *C. sakazakii* JG-05 (Fig. 2). We concluded that the lowered heat stability of *C. sakazakii* NTU696 must be caused by some genetic defect occurred outside from the thermotolerance island (as shown below).

### Cloning of the thermotolerance island

The function of the thermotolerance island was further confirmed by cloning it into a low copy plasmid vector. Three constructs, pACYCThr2-ATCC29544, pACYCThr2-NTU696 and pACYCThr2-NTU701, were prepared and transformed into the *C. sakazakii* JG-05 deletion mutant (Fig. 1, Table S2). We observed significantly higher survival of all three constructs at 58°C compared with a control containing the vector only (Fig. 3a), which further proved the role of this region in increased thermal tolerance. The pACYCThr2-ATCC29544 plasmid enhanced the thermal tolerance of *C. sakazakii* JG-05 seventeen times. However, by comparing with the parent strain *C. sakazakii* ATCC 29544<sup>T</sup>, the complemented mutant *C. sakazakii* JG-05 pACYCThr2-ATCC29544 showed four times reduced survival. We propose that lowered thermotolerance of the complemented mutant was caused either by polar effects or by impaired regulation of gene expression as excessive production of heat shock proteins could be toxic for the cell (Battesti et al. 2011). Similar to the results with the original strains, the *C. sakazakii* JG-05 complemented with the shortened version of the island (pACYCThr2-NTU696 or pACYCThr2-NTU701) showed increased survival comparing to the non-complemented strain but it was not as thermotolerant as the strain transformed with the full version of the island (Fig. 3a). We observed that, unlike the parent strain *C. sakazakii* NTU696, the pACYCThr2-NTU696 plasmid was able to increase thermotolerance of the complemented strain to a similar extent as the construct pACYCThr2-NTU701. These data strongly suggest that the difference in thermal tolerance between *C. sakazakii* NTU696 and NTU701 was caused by genetic determinants outside of the island. The constructed plasmids were also transformed into *E. coli* DH5 $\alpha$  (Fig 3b). We observed better survival of the *E. coli* control strain compared to *C. sakazakii* JG-05 but the presence in this strain of the thermotolerance constructs also significantly (seven times) increased heat tolerance. It is also noteworthy that no difference between the plasmids containing short and long versions of the island was seen in *E. coli* (Fig 3b). By analysis of thermotolerance in *Klebsiella* strains (Bojer et al. 2013) found out that resistance mediated by the *clpK* (*thrD*) gene alone is dependent on the presence of a *clpP*

background, but *clpP* was not necessary for thermotolerance mediated by the entire heat tolerance locus encompassing *clpK*. In our screening, 104/108 *Cronobacter* genomes deposited in the MLST database (May 2015) possessed the *clpP* gene. In the study by Huertas et al. (2015), a *Cronobacter clpP* mutant was found to be attenuated in thermotolerance by screening of a transposon library originating from an environmentally resistant strain. However, it is not known if the thermotolerance island is present in this strain. The presence of the long version of the thermotolerance island correlated with heat resistance in *E. coli* and complementation generating thermotolerance was achieved only if the entire genomic island was cloned into a sensitive *E. coli* strain and thus the thermoresistance was dependent on the entire genomic island, and not on the function of a single protein (Mercer et al. 2015).

### **Complementation of *rpoS* gene**

According to the study by Alvarez-Ordóñez et al. (2012), *Cronobacter* strains expressed variable levels of *rpoS* gene encoding for stress-response sigma factor ( $\sigma^{38}$ ) and this variability was responsible for differences in many phenotypic properties between strains. As the resistance to heat stress is also influenced by the level of RpoS we checked the status of this gene in our strains. We measured production of catalase and yellow pigmentation as markers of RpoS activity (Alvarez-Ordóñez et al. 2012) and found out that the RpoS level in *C. sakazakii* NTU701 was apparently high because of high production of oxygen from peroxide and yellow pigmentation of colonies. However, zero activity was detected in *C. sakazakii* NTU696 by these two tests suggesting non-functional *rpoS*. We detected that a premature stop codon in the *rpoS* gene (G443A mutation) was responsible for this property. By transformation of an *E. coli rpoS* mutant with the thermotolerance island plasmids we observed increased thermotolerance in transformed strains but the cell survival was lower than that of the same constructs in wild-type *E. coli* (data not shown). This experiment confirmed our previous observations that genes from the thermotolerance island are expressed in both exponential and stationary phases but exponentially grown bacteria are not as tolerant as stationary bacteria (Gajdosova et al. 2011; Orieskova et al. 2013). Subsequently, a recombinant plasmid containing *rpoS* from *E. coli* under the control of the arabinose promoter was constructed and used to complement the *C. sakazakii* NTU696 strain. We observed that the heat survival of *C. sakazakii* NTU696 pBAD-*rpoS* reached the level of the *C. sakazakii* NTU701 containing the same thermotolerance island and the functional *rpoS* gene (Fig. 4) and thus we confirmed the role of *rpoS* mutation in the lowered survival of *C. sakazakii* NTU696 at high temperatures.

### **Thermotolerance of clinical *Cronobacter* strains**

In the last part of the present study we characterised the thermotolerance of clinical *Cronobacter* strains. Seventeen strains isolated during 2012-2013 in two hospitals in the Czech Republic and described previously (Alsonosi et al. 2015) were used in the experiments. The presence of three genes (*thrD*, *thrI*, *thrJ*) from the genomic island was tested in these strains by PCR. A positive signal for all three markers was obtained for ten *C. sakazakii* ST4 and six *C. malonaticus* ST7 strains. The single *C. sakazakii* ST64 strain tested was negative for all three markers. In next step, strain survival was tested at 58°C (Fig. 5). In agreement with our previous experiments, we observed that strains with the thermotolerance island survived 2-11 times better compared with the *C. sakazakii* JG-05 deletion mutant. At the same time, thermotolerant *C. sakazakii* strains survived better than clinical isolates belonging to *C. malonaticus* and thus confirmed our data obtained with culture collection strains.

The frequency of the thermotolerance island in hospital isolates was quite high compared with its occurrence in strains from other environments. For example only up to 10% (6/76) of *C. sakazakii* and *C. malonaticus* isolated from food in our previous studies were positive for the *thrI* marker (Turcovský et al. 2011, Gajdošová et al. 2011, Gičová et al. 2013).

The island is also frequently present in genomes of ST4 and ST7 strains which are *Cronobacter* clones possessing increased virulence (Joseph and Forsythe 2011; Forsythe et al. 2014). Previously, *K. pneumoniae* strains containing genes homologous to the genomic island were described to cause nosocomial outbreaks as they were able to survive routine disinfection processes (Bojer et al. 2010). We suppose that similar mechanisms could be responsible for spreading of the thermotolerant *Cronobacter* strains in hospitals. Another interesting observation was that *Cronobacter* strains lacking the thermotolerance genomic island are significantly less tolerant to heat stress compared with *E. coli* (Fig. 3a and 3b) and therefore we propose that a relatively high frequency of this genetic element in *C. sakazakii* and *C. malonaticus* presents a compensation mechanism for this defect.

## Conclusion.

In this study, the contribution of the thermotolerance genomic island to the increased thermal tolerance in some *Cronobacter* strains was confirmed. We observed that the full-length island containing the *thrB-Q* genes was more efficient than its shortened version. Different levels of *rpoS* expression were found to contribute to variability in thermotolerance. The presence of the thermotolerance island likely improves the ability of positive *Cronobacter* strains to survive in the PIF, food production facility and hospital environments.

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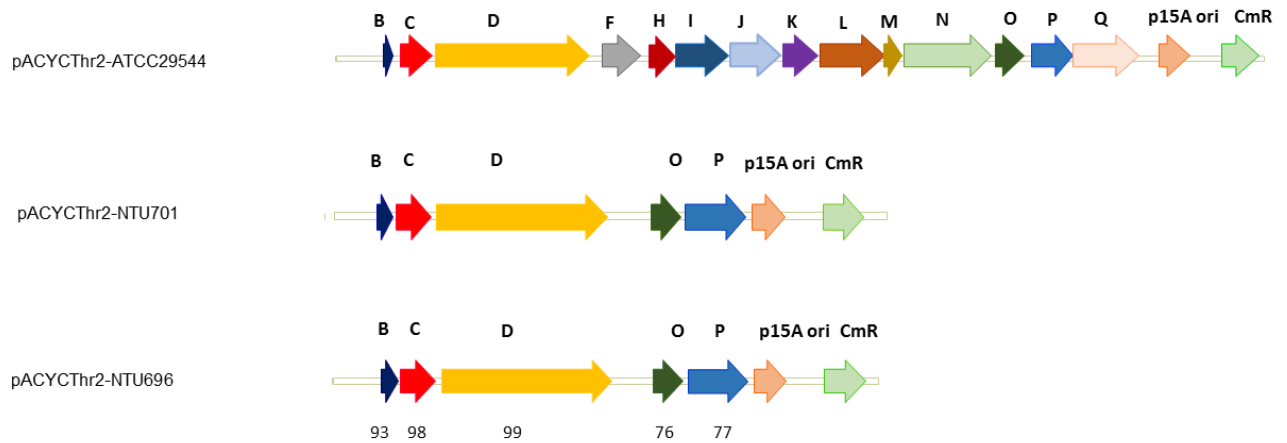


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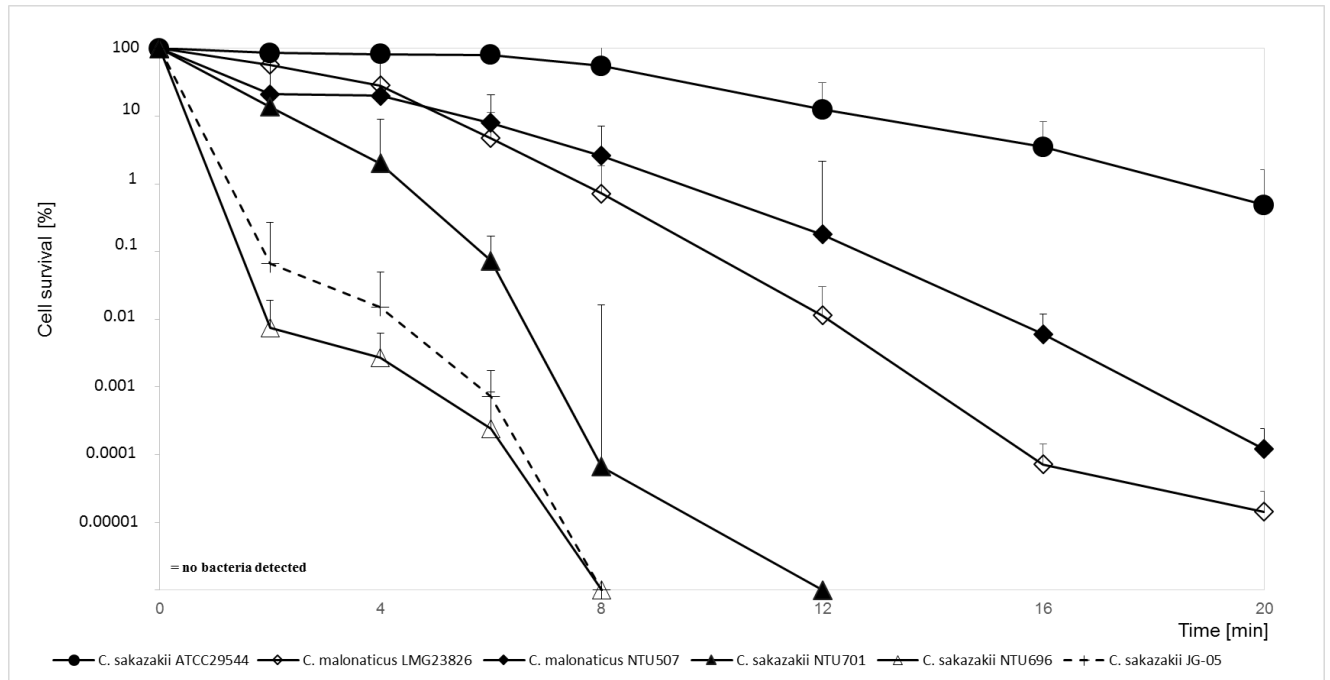
**Fig 1. Plasmid constructs containing the thermotolerance islands from *Cronobacter* strains**

Genes B-Q originated from the thermotolerance islands of the strains *C. sakazakii* ATCC 29544<sup>T</sup>, *C. sakazakii* NTU696 and *C. sakazakii* NTU701. p15A origin and chloramphenicol resistance gene (CmR) were from pACYC-Duet. Numbers in bottom line represent protein similarity between *C. sakazakii* NTU696 and *C. sakazakii* ATCC 29544<sup>T</sup>. Similarity of proteins from *C. sakazakii* NTU701 and *C. sakazakii* NTU696 is 99.5-100%. Predicted gene functions are listed in Table S3.



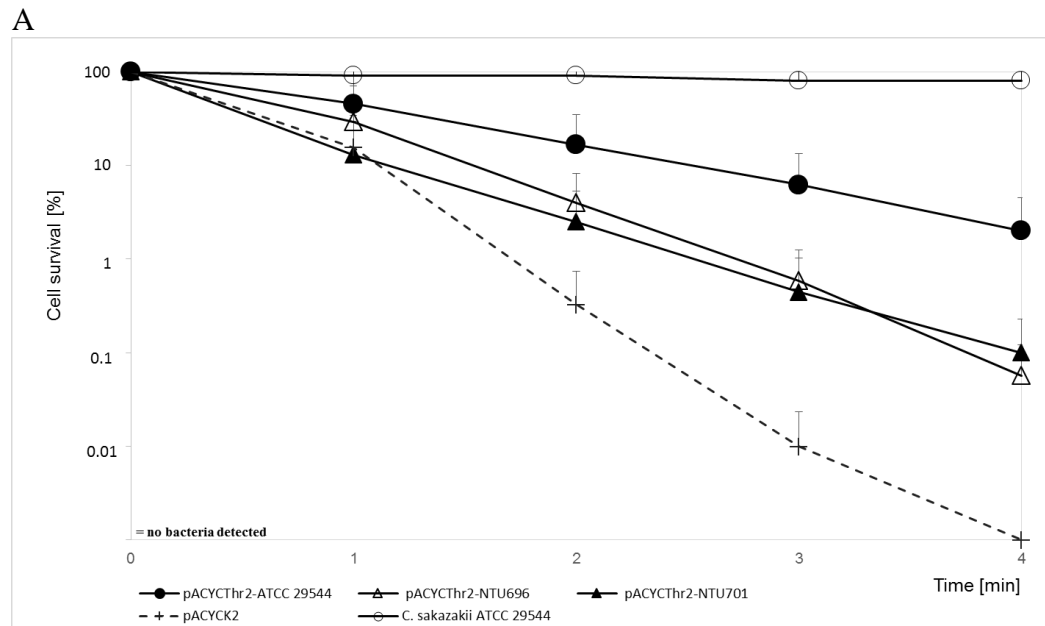
## Fig 2. Thermotolerance of *Cronobacter* strains

Labelling of the strains: *C. sakazakii* ATCC 29544<sup>T</sup> (filled circles), *C. sakazakii* NTU701 (filled triangles), *C. sakazakii* NTU 696 (empty triangles), *C. sakazakii* JG-05 (crosses), *C. malonaticus* LMG 23826<sup>T</sup> (empty diamonds), *C. malonaticus* NTU507 (filled diamonds). Survival of stationary phase cells was determined at 58°C.

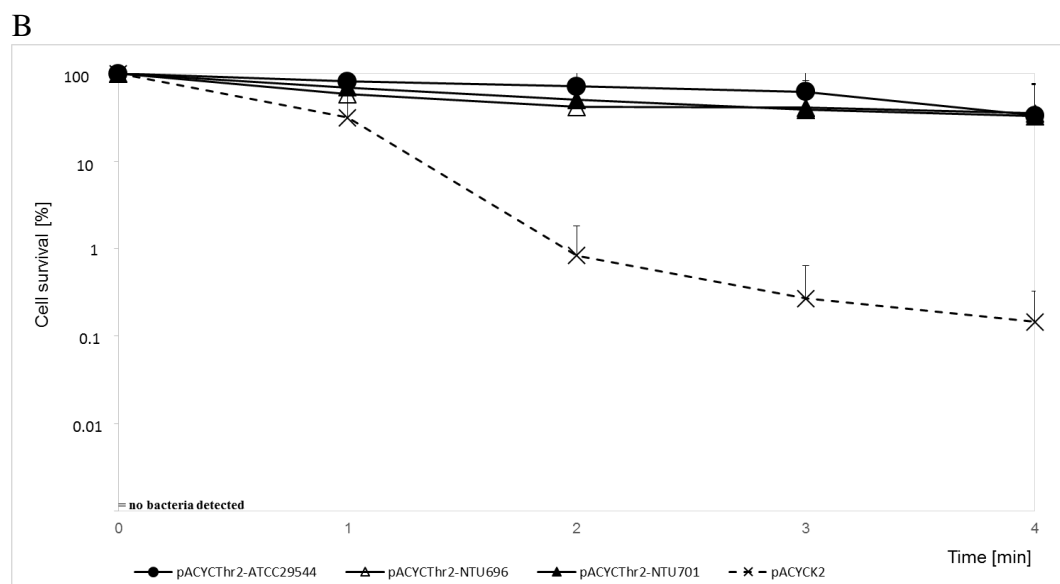


**Fig 3. Survival of *E. coli* and *C. sakazakii* strains complemented with plasmids encoding for the thermotolerance islands**

A) *C. sakazakii* JG-05 (a deletion mutant lacking the thermotolerance island) was transformed with plasmids pACYC-ATCC29544 (filled circles), pACYC-NTU701 (filled triangles) and pACYC-NTU696 (empty triangles) encoding for the thermotolerance islands from the respective *Cronobacter* strains (Fig. 1). *C. sakazakii* ATCC 29544<sup>T</sup> (empty circles) and *C. sakazakii* JG-05 transformed with empty vector pACYCK2 (crosses) were used as positive and negative controls, respectively. Survival of stationary phase cells was determined at 58°C.

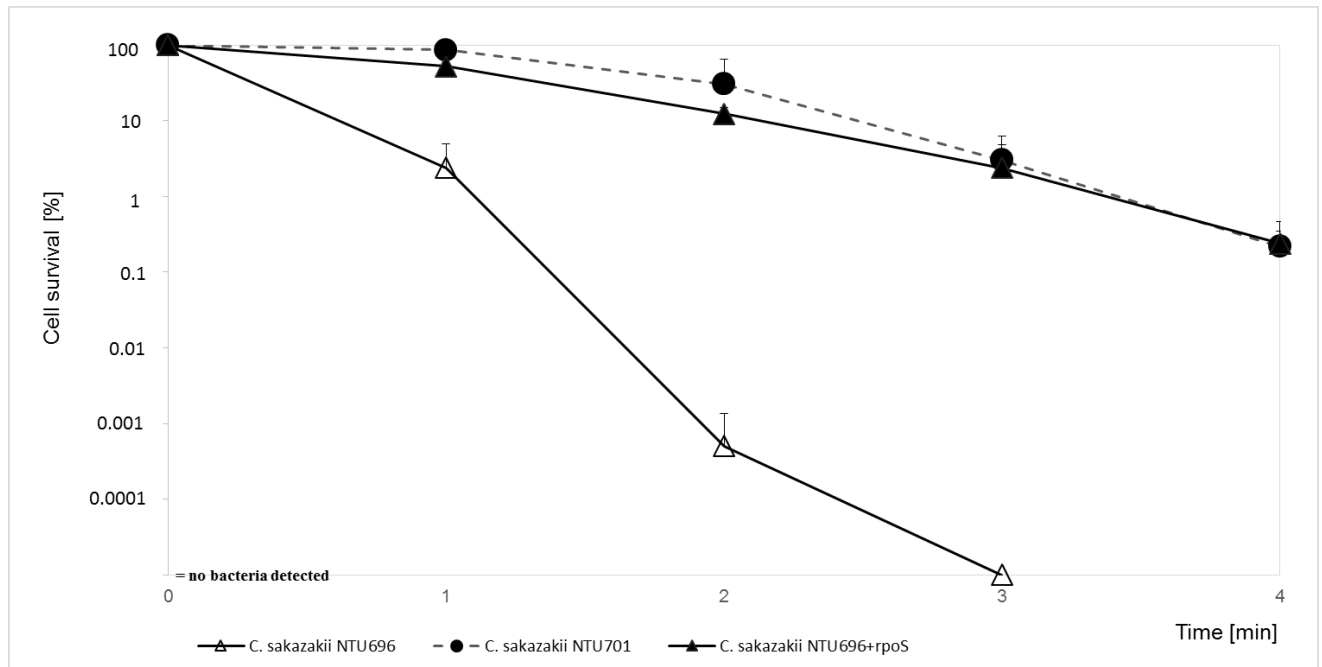


B) *E. coli* DH5 $\alpha$  was transformed with plasmids pACYC-ATCC29544 (filled circles), pACYC-NTU701 (filled triangles) and pACYC-NTU696 (empty triangles) encoding for the thermotolerance islands from the respective *Cronobacter* strains (Fig. 1). Empty vector pACYCK2 (crosses) was used as negative control. Survival of stationary phase cells was determined at 58°C.



#### Fig 4. Influence of *rpoS* expression on thermotolerance

*C. sakazakii* NTU696 was transformed with plasmid pBAD-*rpoS* (filled triangles) encoding for the *rpoS* gene from *E. coli* KL53 under arabinose promoter. *C. sakazakii* NTU701 (filled circles) and *C. sakazakii* NTU696 (empty triangles) were used as positive and negative controls, respectively. Survival of stationary phase cells was determined at 58°C.



### Fig 5. Thermotolerance of *Cronobacter* clinical isolates

Labelling of the strains: clinical *C. sakazakii* strains with the thermotolerance island (solid black lines), clinical *C. malonaticus* strains with the thermotolerance island (solid grey lines), the only clinical *C. sakazakii* strain lacking the thermotolerance island (217, solid black line with circles). *C. sakazakii* ATCC 29544<sup>T</sup> (dot chain line) and *C. sakazakii* JG-05 (dot line) were used as positive and negative controls, respectively. Survival of stationary phase cells was determined at 58°C.

