Determination of genes involved in heat resistance response of *Cronobacter sakazakii*

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RESUMEN

Cronobacter sakazakii is considered an emerging opportunistic pathogen causing meningitis, septicaemia and enterocilitis in neonates, related to the use of contaminated Powdered Infant Formula (PIF). *C. sakazakii* has an unusual surviving ability under dry conditions and has been suggested to be one of the most thermotolerant members of the *Enterobacteriaceae*. It is necessary to understand the molecular mechanisms underlying the thermal resistance of *C. sakazakii* and may ultimately be useful in the development of control strategies in PIF factories. In the current study, a transposon mutagenesis approach was used to identify the genes involved in heat resistance. A total of 23 mutants were found corresponding to 12 different defective genes. Heat resistance of selected mutants were determined with the use of the thermoresistometer Mastia. Only 2 mutants had a greater sensitivity to heat compared with the heat resistance of the wild type. Genes identified to be involved in the cellular response to thermal treatments were Ribosome maturation protein RimP and Outer membrane Porin L (OmpL). The results suggest that the novo protein synthesis, and the use of cysteine for the formation of disulfide bonds for stabilization of proteins against denaturation during thermal treatments are key processes in the resistance against heat stress.

Palabras clave: *C.* sakazakii**,** Heat resistance; Transposon mutagenesis; Heat response; Weibull distribution

1. Introdution

Cronobacter sakazakii is a gram-negative rod belonging to the family *Enterobacteriaceae*. It is also considered an emerging opportunistic pathogen causing meningitis, septicaemia and enterocilitis in neonates, related to the use of contaminated Powdered Infant Formula (PIF). Preterm, low-birth-weight or immune-compromised infants exposed to *C. sakazakii* are at particular risk. Mortality rates of 10%-80% have been described and survivors often suffer from neurological sequel [1][2]. *C. sakazakii* has an unusual surviving ability under dry conditions, but the thermal tolerance of this microorganism strains may differ [2]. Some authors suggested that *C. sakazakii* is one of the most thermotolerant members of the *Enterobacteriaceae*, having a D-value of 4.2 min at 58ºC. On the other hand, recent studies revealed that some *C. sakazakii* strains do not show such thermotolerance [3]. Indeed, D-values at 58ºC have been reported to range from 0.27 to 9.87 min [3][4]. Some studies show that *C. sakazakii* can survive to thermal stress (either during manufacture, or when the PIF is reconstituted) [4][5][6]. At home, manufacturers recommend to prepare the formula just before each feeding using boiled water. The FAO-WHO (2004) recommended the use of hot water (70º-90ºC) during the reconstitution of powdered, due the heat resistance of this microorganism.

A greater understanding of the molecular mechanisms underlying the thermal resistance of *C. sakazakii* is required and may ultimately be useful in the development of control strategies in PIF factories. In the current study, a transposon mutagenesis approach was employed to identify the genes involved in heat resistance.

2. Materials & Methods

2.1 Bacterial strain and culture conditions: *Cronobacter sakazakii* DPC6529 strain used in this study was obtained from the University College of Cork culture collection. The culture was routinely grown in Luria-Bertani (LB) medium.

2.2 Screening of a *C. sakazakii* DPC6529 transposon mutagenesis library: A transposon mutagenesis library constructed in a previous study [7] by using the EZ-Tn5<KAN 2>Tnp transposome kit (Epicentre, Madison, USA) was tested.

Selection: Thermal resistance determinations were done at 60ºC, in an eppendorff heating-block. Pre-selected mutants, in a previous sep, were those showing no growth or slow growth in the pre-selection. Pre-selected mutants were grown overnight in LB broth + kanamycin and incubated overnight (~16 h) at 37ºC. Sterile eppendorfs with 990 mL of ¼ strength Ringer's solution were pre-heated at 60ºC, were inoculated with 10 µL of the overnight to reach a concentration of approx. 10^6 cells/mL. Samples were collected at preset times. Samples were properly diluted and plated in LB agar + kanamycin.

The transposon insertion sites were identified by modified single-primer PCR, as done by Alvarez-Ordoñez et al., 2014. The location of the transposon insertion was revealed with the help of BLAST (Basic Local Aligment Search Tool) analysis (www.ncbi.nlm.nih.gov/BLAST/).

2.3 Heat resistance: Heat resistance determinations were carried out in a thermoresistometer Mastia [8]. Three separate experiments per condition were performed. Viable counts were based on duplicate counts, from appropriate dilutions in L.B agar for *C. sakazakii* DPC6529 and LB agar + kanamycin for *C. sakazakii* DPC6529 transposon mutants. Plates were incubated for 24 h at 37ºC for *C. sakazakii* DPC6529 and for 48 h at 37ºC for transposon mutants.

2.4 Data analysis: Survival curves were obtained by plotting the logarithm of CFU/mL vs the treatment time.

Survival curves showed an upward concavity (presence of a tail). The cumulative form of the Weibull distribution function, as proposed by Mafart et al (2002), was used (Eq. 1):

$$\log N_t = \log N_0 - (t/\sigma)^p \quad (1)$$

Where N_t is the final population, N_0 the initial population, δ represents the time for the first decimal reduction (min), and p is the shape parameter. A single p value for all survival curves corresponding to each strain was used as proposed by Esteban et al. (2013). Calculations were estimated with the GInaFiT application (version 1.6) for Microsoft Excel [10].

3. Results and Discussion

The mutant library used has about 2400 mutants. After the pre-selection 140 mutants showed some sensitiveness to heat treatment. From these 140 mutants, the defective gene can be involved in heat resistance or in growth or other metabolic functions. Therefore a selection step was done, in which the heat resistance of the 140 mutants was compared to the heat resistance of the wild type strain by using a simple thermal resistance determination method. The selection enabled to shift out the mutants in which the transposon was inserted in genes involved in heat resistance, from those that was not. Heat resistance determinations were primarily carried out in an eppendorff heating-block due it quickness and easiness to analyze a large amount of samples in a short time. The use of the thermoresistometer mastia to analyze the selected mutants was due to the higher accuracy of the thermoresistometer on the heat resistance determination [11]

After theselection step, 28 mutants were found to show a significant decrease in the heat resistance compared to the wild type. From the 28 sensitive mutants 5 were not able to be sequenced. Transposon insertion sites were mapped for the rest (Table 1). Using standard homology searches, the insertion sites were mapped in the genome of *C. sakazakii ES15*, and functions were assigned to the defective genes.

In the thermoresistometer the thermal resistance comparisons were done at a temperature of 58ºC in LB broth. Survival curves from the tested mutants showed tailing phenomena (p<1), except from the mutant 7 that showed a slight shoulder (p>1) (Table 2). Mutants showed slight differences in thermal resistance when they were compared to the wild type, except for mutants 7 and 10, which showed a greater sensitivity to heat. Table 2 shows the heat resistance values (δ value), the determination coefficients (r2), and the time needed for a 5 log cycle reduction, for the wild type and the mutants. It is necessary 2.25 min at 58ºC to reduce 5 log cycles the initial population of *C. sakazakii* DPC6529 (wild type), while only 1.70 and 1.86 min are necessary to achieve the same level of reduction in the population of mutants 7 and 10, respectively.

Genes identified for Mutant 7 and 10 were Ribosome maturation protein RimP and Outer membrane Porin L (OmpL), respectively (Table 1). RimP has an important function in ribosome

assembly at high temperature. Nord, Bylund & Lövgren (2009), reported that in *Escherichia coli* the RimP protein facilitates the maturation of the 30S subunits so that translational competent. OMPs include integral membrane proteins as well as lipoproteins that are anchored to the outer membrane via N-terminally attached lipid. Nutrients too large to transverse general porin channels enter through specific porins [13][14][15]. Outer membrane porin L (OmpL) protein, allows an efficient diffusion of low-molecular-weight solutes such as small sugars and tetraglycine [16]. Datigalongue, Nikaido & Raina (2000), showed that OmpL allowed rapid permeation of cysteine (Cys) and reduced glutathione at rates expected from the sizes of these compounds. Cysteine is an essential amino acid that performs vital functions in the catalytic activity and structure of many proteins. The formation of disulfide bonds between cysteine are needed for proper folding and stability of some proteins, particularly those found in extracytoplasmic compartments [17]. Considering the above together with the results of this study, it can be suggested that *C. sakazaki*i use disulphide bonds to stabilize proteins against denaturation during thermal treatments. In the other hand the deficiency of the RimP protein produces a dearth in mature 30S subunits and an absence of ribosome assembly at high temperatures, affecting predicted roles in translation and protein synthesis, leading the cell unable to recover from the damage caused by thermal treatments**.**

4. Conclusion

Our study sheds light on some of the molecular mechanisms involved in the cellular response of *C. sakazakii* to thermal treatments. Our findings suggest that the novo protein synthesis, and the use of cysteine for the formation of disulfide bonds for stabilization of proteins against denaturation during thermal treatments are key processes in the resistance against heat stress. Further studies are therefore needed in order to better characterize the response of *C. sakazakii* to thermal treatments

5. Acknowledgments

The financial support of this research was provided by the Ministry of Science and Technology of the Spanish Government and European Regional Development Fund (ERDF) through Project AGL- 2010-19775. J.P Huertas is grateful to the Spanish Ministry of Science and Innovation for his fellowship (BES-2011-046580). We acknowledge the funding received by Food for Health, Ireland under the grant number CC20080001 by Enterprise Ireland.

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Tables and Figures

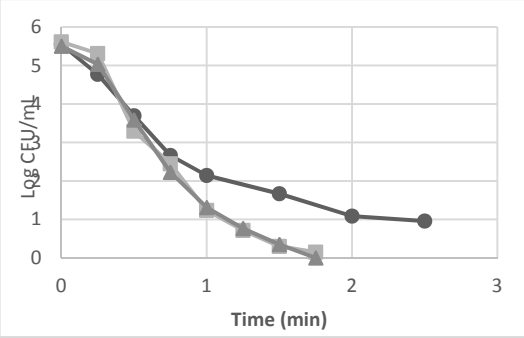

Figure 1. *Thermal resistance of C. Sakazakii DPC6529 (●), mutant 7 (■) and mutant 10 (▲) at 58ºC in LB broth*

Table 2. Heat resistance values for *C. sakazakii* DPC6529 and sensitive mutants obtained at 58 ºC in LB broth, as derived from the Weibull model.

Mutant	Gene	Locus Tag
1	Putative pili assembly Chaperone (4)	ES15_0052
2	Thiosulfate sulfur transferase GlpE (4)	ES15_0248
3	Hypothetical Protein (Membrane Protein; Homologue to a type VI Secretion System-Associated protein) (4)	ESA_03930
4	Lipopolysacaride biosynthesis protein WZZE (1)	ES15_3698
5	Transposon protein TonB (1)	CSSP291_07485
6	RNA-23s ribosomal RNA (1)	ES15_4326
7	Ribosome maturation protein *RimP* (1)	ES15_3516
8	ATP-dependent Clp protease proteolytyc subunit ClpP (3)	ES15_2946
9	(Dimethylallyl) Adenosine Trna methylthiotransferase (1)	ES15_2748
10	Outer membrane Porin L *OMPL* (1)	ES15_0029
11	Hypothetical (Family Methyltransferase; Adomet_Mtase - Domain) (Tellurite Resistance Protein) (1)	ES15_3416
12	Hypothetical Protein (1)	

	p	δ	R2	5D±S.D
Wild Type	0.788	0.292	0.921	2.25 ± 0.126
Mutant 7	0.943	0.293	0.943	1.695 ± 0.078
Mutant 10	1.182	0.471	0.966	1.863 ± 0.023

Table 2. Heat resistance values for *C. sakazakii* DPC6529 and sensitive mutants obtained at 58 ºC in LB broth, as derived from the Weibull model.