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Transposon mutagenesis reveals genes involved in osmotic stress and drying in *Cronobacter sakazakii*

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ABSTRACT

This study characterizes the growth in hyperosmotic media and the resistance to desiccation of a collection of fifteen *Cronobacter sakazakii* strains. *C. sakazakii* strains showed similar abilities to grow/persist under osmotic stress conditions to strains from other related *Enterobacteriaceae*, i.e. *Cronobacter muytjensii*, *Cronobacter malonaticus*, *Enterobacter gergoviae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, and *S.* Typhimurium. Nevertheless, some degree of heterogeneity among *C. sakazakii* strains could be observed, and in general strains isolated from clinical sources showed the greatest robustness. A transposon mutagenesis approach was used to identify genetic systems involved in the response of *C. sakazakii* DPC 6529 to hyperosmotic conditions. We obtained evidence that de novo protein synthesis, repair of damage in macromolecules and maintenance of the structure and integrity of the cellular envelope are essential processes for the cell under osmotic stress. Moreover, some metabolic activities are also important, including the synthesis of glutamine as a compatible solute and the regulation of nucleotide and nucleoside pools. The Cpx system, known as an envelope stress response regulator, and the sigma factors RpoN and RpoS seem to be the main signals regulating the bacterial response to hyperosmotic conditions. Among the identified salt-sensitive mutants, only those disrupted in *dnaK* and *dnaJ*, encoding two molecular chaperones, were important for *C. sakazakii* survival under desiccation. This suggests that the systems and proteins involved in the desiccation response differ from those responsible for growth under hyperosmotic conditions, at least under the conditions tested in the current study.

1. Introduction

Cronobacter (formerly classified as *Enterobacter* sakazakii) is a recently described genus comprised of seven Gram-negative, rod-shaped bacterial species (i.e. *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis*, *C. universalis* and *C. condimenti*) that can cause necrotizing enterocolitis, meningitis and bacteraemia in infants, with mortality rates of up to 40–80% (Healy et al., 2010; Norberg et al., 2012; Yan et al., 2012). *Cronobacter* spp. have been isolated from a wide variety of food sources, with powdered infant formula (PIF) being the most common vehicle involved in newborn infections (Beuchat et al., 2009; Farber, 2004; Friedemann, 2007; Osaili & Forsythe, 2009). Since standard pasteurization practices seem to be effective for the complete inactivation of *Cronobacter* spp. during PIF manufacturing (Breeuwer, Lardeau, Peterz, & Joosten, 2003), plant-derived ingredients added to PIF without any prior heat treatment or thermally sensitive ingredients used for PIF production have been regarded as a potential source of contamination (Healy et al., 2010). In addition, the survival and/or persistence capabilities of the microorganism in dry factory environments may facilitate post-pasteurization contamination (Riedel & Lehner, 2007). Alternatively, contamination from utensils used during the reconstitution of PIF, such as blenders or spoons, could also be responsible for transmission (Caubilla-Barron et al., 2007; van Acker et al., 2001).

Cronobacter spp. have been shown to be more resistant to osmotic stressful conditions than other *Enterobacteriaceae* (Breeuwer et al., 2003; Caubilla-Barron et al., 2007). This may explain their capacity to survive in low water activity environments such as that found in PIF (aw ~0.2–0.5) (Gurtler & Beuchat, 2007). In fact, the organisms have been shown to persist for long periods in PIF samples, with some capsulated strains surviving up to 2.5 years (Barron & Forsythe, 2007). It is generally acknowledged that bacteria respond to osmotic stress by means of a bi-phasic response in which the stimulation of potassium uptake (and its counter-ion glutamate) is considered a primary response. This is followed by a dramatic increase in the cytoplasmic concentration (either by synthesis and/or uptake) of compatible solutes (e.g. glycine-betaine, carnitine, ectoine, choline, proline and trehalose) as the secondary response (reviewed in Sleator & Hill, 2002). An in silico analysis recently performed by

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Feeney and Sleator (2011) demonstrated that the *C. sakazakii* BAA-894 genome contains homologs of all the main osmotolerance loci of *Escherichia coli*. They also showed the presence of multiple copies of several of these loci (*proP* and *opuC*). However, the physiological response of *Cronobacter* spp. to osmotic stress has not yet been fully investigated. The role of trehalose as a compatible solute important for *Cronobacter* spp. survival under dry conditions has been highlighted by Breeuwer et al. (2003), while Riedel and Lehner (2007) have identified differentially expressed proteins in response to desiccation and growth in hyperosmotic media by using a proteomics approach. Proteins up-regulated in response to both stresses fell into three main categories: DNA/RNA stabilizing enzymes, molecular chaperones and oxidative stress related proteins.

A greater understanding of the molecular mechanisms underlying the osmotic tolerance of *Cronobacter* spp. is required and may ultimately be useful in the development of control strategies in PIF factories. In the current study, growth under hyperosmotic stressful conditions and resistance to desiccation of a collection of *C. sakazakii* strains was examined. In addition, a transposon mutagenesis approach was employed to identify genes involved in osmotic stress tolerance.

2. Materials and methods

2.1. Bacterial strains, media, chemicals and growth conditions

Bacterial strains used in this study are listed in Table 1. In total, fifteen *C. sakazakii* strains were included in the study. In addition, strains from related *Enterobacteriaceae* were also used — i.e. *C. muytjensii*, *C. malonaticus*, *E. gergoviae*, *E. cloacae*, *E. aerogenes*, *E. coli* and *S.* Typhimurium. Master stocks of all strains were maintained in cryovials in the presence of a final concentration of 40% glycerol as cryoprotectant and stored at −80 °C. For resuscitation, bacteria were inoculated into tubes containing 10 ml of Luria–Bertani (LB) broth (Merck), incubated at 37 °C for 24 h, and cultures were streaked on LB agar plates, which were subsequently incubated under the same conditions and stored at 4 °C. Working stationary-phase cell suspensions were obtained by the inoculation of fresh LB broth with an isolated colony from the stock LB agar plates and were then incubated overnight at 37 °C. For certain experiments bacteria were cultured in the minimal medium M9 (Flucka) supplemented with 0.5% glucose, 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$. The reagents sodium chloride, potassium chloride, and glutamine were purchased from Sigma, and used to supplement the culture media in some specific experiments. The antibiotic kanamycin was made up as concentrated stock and added to media at 50 μg/ml where necessary. For solid media, agar was added to 1.5%.

Table 1
Bacterial strains included in the study.

	Strain	Origin	Source*
#1	*C. sakazakii* ATCC BAA-894	American Type Culture Collection (ATCC)	DPC
#2	*C. sakazakii* NCTC08155		DPC
#3	*C. sakazakii* ATCC12868	American Type Culture Collection (ATCC)	DPC
#4	*C. sakazakii* ATCC29004	American Type Culture Collection (ATCC)	DPC
#5	*C. sakazakii* DSM4485		DPC
#6	*C. sakazakii* NCTC11467		DPC
#7	*C. sakazakii* DPC 6522	Blood	DPC
#8	*C. sakazakii* DPC 6523	Cerebrospinal fluid	DPC
#9	*C. sakazakii* DPC 6524	Stool	DPC
#10	*C. sakazakii* DPC 6525	Urine	DPC
#11	*C. sakazakii* DPC 6526	Blood	DPC
#12	*C. sakazakii* DPC 6527	Blood	DPC
#13	*C. sakazakii* DPC 6528	Cerebrospinal fluid	DPC
#14	*C. sakazakii* DPC 6529	Tracheal aspirate	DPC
#15	*C. sakazakii* DPC 6530	Bronchial alveolar lavage	DPC
#16	*C. muytjensii* ATCC51329	American Type Culture Collection	DPC
#17	*C. malonaticus* DPC 6531	Brain tumor	DPC
#18	*E. gergoviae* NCTC11434		DPC
#19	*E. cloacae* NCTC11590		DPC
#20	*E. cloacae* NCTC11933		DPC
#21	*E. aerogenes* NCTC10006		DPC
#22	*E. coli* DH5α ATCC67876	American Type Culture Collection	DPC
#23	*S.* Typhimurium UK-1		DPC

* All strains were obtained from DPC, Dairy Products Research Centre, Fermoy, Co. Cork, Ireland.

2.2. Assessment of growth ability in hyperosmotic media

For testing bacterial growth in hyperosmotic media, overnight stationary phase cultures were inoculated into LB broth containing various concentrations (4%; 6%; 8%; 10% w/v) of NaCl or KCl (inoculation level of 1%). Growth was measured spectrophotometrically in 96-well culture plates (Genetix) by determining the optical density at 600 nm (OD_{600}) using a temperature-controlled automatic plate reader (Multiscan FC; Thermo Scientific). The parameters "time to detection" (TTD), chosen as the time (in hours) to reach an OD_{600} of 0.2, and "growth rate" (in h^{-1}) were determined for each strain under the different conditions tested. Due to the high detection limits of OD readers (~10^7 viable cells) and to the progressively slower proliferation rate of cells when they reach their maximum cell densities, growth rates may be underestimated by using OD values. For this reason, TTD measurements are often used in combination (Biesta-Peters, Reij, Joosten, Gorris, & Zwietering, 2010; Salih, Mytilinaios, Schofield, & Lambert, 2012). The threshold value of OD_{600} 0.2 for the TTD parameter was chosen because it is well above the detection limit of the optical reader, and such a threshold helps avoid false-positive growth samples due to fluctuations in optical density close to the detection limit. High TTD values reflect a delay in bacterial growth rather than differences in growth rates.

2.3. Assessment of bacterial sensitivity to dehydration

Sensitivity to dehydration was monitored as previously described (Alvarez-Ordóñez, Begley, & Hill, 2012). Briefly, 96-well plates filled with 100 μl aliquots of the working stationary-phase cell suspensions were kept without lids at 25 °C for air drying. Subsequently, plates were incubated at room temperature for up to 12 days. Bacterial survival was monitored after rehydrating the samples with 100 μl of PBS and plating 10-fold serial dilutions in triplicate on LB agar plates. Survivors were enumerated following incubation of the plates at 37 °C for 48 h (longer incubation times did not have any influence on the count).

2.4. Construction and screening of a transposon mutagenesis library

A transposon mutagenesis library was constructed for the strain *C. sakazakii* DPC 6529 by using the EZ-Tn5<KAN-2>Tnp Transposome kit (Epicentre, Madison, WI) in accordance with protocols provided by the supplier. Briefly, 1 μl of transposon DNA was added to 50 μl of electrocompetent cells, prepared following conventional procedures. After electroporation at 25 μF of capacitance, 2.5 kV of voltage and 200 Ω of resistance, cells were transferred to 1 ml of LB broth and incubated for 1 h at 37 °C for recovery. 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. Afterwards, single colonies were picked in 96-well plates containing 150 μl/well of LB broth supplemented with 7.5% glycerol and 50 μg/ml of kanamycin. After incubation at 37 °C for 24 h, 96-well plates were stored at −80 °C. −80 °C master plates were thawed and transposon mutants were screened for a defect in growth in hyperosmotic media by replica plating from stock plates onto screening plates (LB agar plates supplemented with 7% NaCl) using 96-pin replicators (Genetix). Mutants were replicated onto LB agar plates as positive control plates. Growth of mutants was monitored after incubation of screening plates for 48 h at 37 °C. Defects in growth were characterized spectrophotometrically by monitoring the growth of salt-sensitive mutants in LB broth and LB broth supplemented with 7% NaCl or 8% KCl. Moreover, the resistance to drying of those mutants impaired