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Heat resistance of *Cronobacter sakazakii* DPC 6529 and its behavior in reconstituted powdered infant formula

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ABSTRACT

Cronobacter sakazakii is an opportunistic pathogen in neonates which can cause meningitis, septicaemia and enterocolitis related to the consumption of contaminated Powdered Infant Formula (PIF). *C. sakazakii* has an unusual ability to survive under dry conditions and it could be among the most thermotolerant members of the *Enterobacteriaceae*. Little is known about how *Cronobacter* species respond to heat stress and the mechanisms involved in the process. In the current study we determined the heat resistance of a particularly stress tolerant *C. sakazakii* strain, *C. sakazakii* DPC 6529, and monitored the behavior of a lux-tagged derivative under different reconstitution and handling scenarios in a commercial brand of PIF. Some of the molecular mechanisms involved in the heat stress response were investigated using a transposon mutagenesis approach. Survival curves of *C. sakazakii* DPC 6529 in Luria-Bertani (LB) broth and PIF at various temperatures (58, 60, 62 and 64 °C) displayed an upward concavity and were fitted to the non-linear Weibull model. While at the highest treatment temperatures heat resistance was lower in PIF than in LB broth, at lower temperatures no significant differences in heat resistance were observed. Experiments in real time with artificially inoculated PIF reconstituted at different water temperatures (50, 55, 60, 65, 70 °C) and cooled at different rates confirmed that *C. sakazakii* can survive for long time periods in powdered formula, and is capable of proliferating after reconstitution. The use of water at temperatures between 50 and 65 °C for reconstitution did not provide a significant inactivation of *C. sakazakii* cells. Reconstitution at 70 °C reduced the bacterium to levels below the detection limit, although survivors were able to proliferate and reached dangerous levels when the reconstituted product was stored for a long time at room temperature. The cooling rate had an important impact on survival and subsequent growth of *C. sakazakii*, which makes it advisable to avoid rapid cooling of baby formula. Transposon mutagenesis allowed the identification of some of the molecular mechanisms involved in the response of *C. sakazakii* DPC6529 to heat stress. Genes identified included the Ribosome Maturation Protein RimP and Outer Membrane Porin L (OmpL). Results suggest that *de novo* protein synthesis, and the uptake of cysteine for the formation of disulfide bonds for protein stabilization, are key processes.

1. Introduction

Cronobacter sakazakii is a Gram-negative rod bacteria belonging to the family *Enterobacteriaceae*. It is also considered to be an opportunistic pathogen causing meningitis, septicaemia and enterocolitis in neonates, usually related to the consumption of contaminated Powdered Infant Formula (PIF). Preterm, low-birth-weight or immune-compromised infants exposed to *C. sakazakii* are at high risk. Mortality rates of 40%-80% have been described and survivors often suffer from neurological sequelae (Bowen & Braden, 2006; Friedemann, 2009; Holy & Forsythe, 2014).

C. sakazakii is considered a ubiquitous microorganism, and it has been isolated from a wide variety of sources including foods, dry blended raw materials, water waste and thermal spring water, soil, and several environments in houses, food production lines and hospitals (Norberg et al., 2012). PIF has been epidemiologically linked to *C. sakazakii* disease outbreaks. PIF is basically a non-sterile product which can be a good medium for growth of microorganisms once rehydrated. A number of surveys for *Cronobacter* spp. (formerly *Enterobacter sakazakii*) in PIF for newborns and other infant foods have revealed their presence at variable frequencies, ranging from 0% to 14%

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(Kandhai et al., 2010; Muytjens, Roelofs-Willemse, & Jaspar, 1988). For example, Iversen and Forsythe (2004) investigated the presence of *Cronobacter* spp. in PIF and other food products and isolated the bacterium from 2 of 82 samples tested. Nazarowec and Farber (1997) analyzed 120 cans of PIF from five companies in Canada, and 6.7% of the cans contained *Cronobacter* spp. Muytjens et al. (1988) isolated *Cronobacter* spp. from 20 of 141 PIF samples tested. These authors also reported that *Cronobacter* spp. are normally only present in PIF at very low levels of <1 CFU g^{-1}. Pan et al. (2014) found 49 of 399 samples of commercial PIF and follow up formulae in China to be contaminated with *Cronobacter* spp. On the other hand, Li et al. (2014) tested 195 food samples, including 48 PIF samples, for presence of *Cronobacter* spp. in China and did not detect the microorganism. Sonbol, Jospeh, McAuley, Craven, and Forshythe (2013) have recently characterized by multilocus sequence typing (MLST) *Cronobacter* spp. strains isolated between 1988 and 2009 from surveys at 14 countries. Their results showed that 85% of strains belonged to *C. sakazakii*. The remaining strains were *C. turicensis* (10%), *C. malonaticus* (4%), and *C. muytjensii* (1%). No strains of *C. dublinensis*, *C. universalis* or *C. condimenti* were identified in their study. They reported that 25% of isolates were in the clinically significant ST4 clonal complex, involved in neonatal meningitis cases, although many of these isolates were single representatives of pulsotypes and therefore this does not mean that 25% of strains from PIF factories are clonal complex ST4.

Contamination of PIF by *Cronobacter* spp. can be intrinsic or extrinsic. With intrinsic contamination the organism is introduced into PIF during the manufacturing process (Asakura, Morita-Ishihara, Yamamoto, & Igimi, 2007). It is generally accepted that *Cronobacter* spp. do not survive the pasteurization treatments applied during manufacture of PIF. Therefore, it has been suggested that intrinsic contamination probably occurs following heat processing (Iversen & Forsythe, 2004). Nonetheless, *Cronobacter* spp. have been shown to survive during industrial drying processes (Arku, Mullane, Fox, Fanning, & Jordan, 2008) and several studies have illustrated their ability to survive in dry environments for long periods (Mullane, Whyte, Wall, Quinn, & Fanning, 2007; Terragno et al., 2009). Some strains can even survive for periods of up to 2.5 years in contaminated PIF (Barron & Forsythe, 2007). Extrinsic contamination may result from the use of contaminated utensils (such as blenders and spoons), in the preparation of formula and bacteria have been isolated from such utensils in the past (Noriega, Kotloff, & Schwalbe, 1990).

Cronobacter spp. have an unusual ability to survive under dry conditions, which may provide a competitive advantage in dry environments such as those found in PIF and in manufacturing plants. However, there is no consensus on whether *Cronobacter* spp. are also particularly more tolerant to heat treatments than other non-spore forming bacteria. It has been suggested that *Cronobacter* spp. are one of the most thermotolerant members of the *Enteriobacteriaceae* (Dancer, Mah, Rhee, Hwang, & Kang, 2009; Nazarowec & Farber, 1997). However, Asakura et al. (2007), Arroyo, Condón, and Pagán (2009), Al-Holy, Lin, Abu-Ghoush, Al-Qadiri, and Rasco (2009) and Osaili, Shaker, Al-Haddaq, and Holley (2009) showed that the heat resistance varies widely among strains. Previous studies have shown that the *D*-values of *Cronobacter* spp. in PIF and laboratory media range from 8.58 to 85.50, 0.12 to 15, and 0.01 to 0.13 min at 50 °C, 58 °C and 65 °C, respectively; and z-values range from 3.1 to 10.86 °C (Al-Holy et al., 2009; Arroyo et al., 2009; Asakura et al., 2007; Breeuwer, Lardeau, Peterz, & Joosten, 2003; Chang, Chiang, & Chou, 2009; Dancer et al., 2009; Nazarowec & Farber, 1997; Osaili et al., 2009; Walsh et al., 2011; Yemiş, Pagotto, Bach, & Delaquis, 2011, 2012). It is difficult to compare D-values obtained for identical strains by different researchers, as many factors (e.g. type of cultivation media, bacterial growth phase, previous heat shock treatment) can influence thermotolerance (Gajdosova et al., 2011). Measurements performed in PIF at 58 °C by Edelson-Mammel and Buchanan (2004) suggest that *C. sakazakii* strains can be classified into two distinct thermal resistance phenotypes.

Some studies have shown that *Cronobacter* spp. can survive the thermal stress imposed when PIF is reconstituted with warm water (Asakura et al., 2007; Osaili et al., 2009). The Food and Agriculture Organization (FAO) and World Health Organization (WHO) of the United Nations have recommended reconstitution of PIF with water at $\geq$70 °C to reduce the potential risk of *Cronobacter* spp. (FAO/WHO, 2004). This action would be expected to result in a 4–6 $\log_{10}$ reduction of *Cronobacter* spp., depending on the type of product (Osaili et al., 2009). Despite this, PIF reconstitution recommendations vary considerably among producers and countries. Chap et al. (2009), who performed an international survey of *Cronobacter* spp. in infant foods, observed that many countries do not follow the FAO/WHO recommendations. Only Korea, among the countries analyzed by these authors, clearly recommended the use of water at temperatures exceeding 70 °C to reconstitute PIF. Recommendations in other countries varied considerably, and included non-specified temperatures (e.g. use of warm water) or the use of a temperature of 40 °C, close to the optimum growth temperature of *Cronobacter* spp. Manufacturers normally recommend boiling the water and then cooling down to 50 °C before the addition of measured PIF.

The objectives of this study were to (i) evaluate the heat resistance of a particularly stress tolerant *C. sakazakii* strain, *C. sakazakii* DPC 6529; (ii) assess the behaviour of a lux-tagged *C. sakazakii* DPC 6529 strain in real time in a commercial brand of PIF under various reconstitution scenarios and (iii) elucidate some of the molecular mechanisms involved in the management of heat stress by *C. sakazakii* DPC 6529 using a transposon mutagenesis approach.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Cronobacter sakazakii DPC6529 used in this study was obtained from the University College Cork culture collection. This strain was originally isolated from a tracheal aspirate and has been previously used by Alvarez-Ordóñez, Begley et al. (2014), Alvarez-Ordóñez, Cummins et al. (2014) and Flaherty, Begley, and Hill (2013) to evaluate its resistance to acid and osmotic stress and its behaviour and growth in infant formula. A lux-tagged derivative of this strain constructed in a previous study (Flaherty et al., 2013) was also used. The cultures were routinely grown overnight (~16 h) at 37 °C in Luria-Bertani broth (LB, Merck) and in LB supplemented with erythtromycin (Sigma) (500 μg/mL).

2.2. Heat resistance determinations

Heat resistance determinations were carried out in a thermoresistometer Mastia (Conesa, Andreu, Fernández, Esnoz, & Palop, 2009). The vessel of the thermoresistometer was filled with 400 mL of the heating medium, LB broth or PIF. LB was sterilized before bacterial inoculation by heating it at 135 °C for 2 minutes in the thermoresistometer and then was cooled to the treatment temperature (58, 60, 62 and 64 °C). When PIF was used as heating medium, the instrument was sterilized with distilled water, cooled, emptied, immediately filled with PIF and heated to the treatment temperature. Once the treatment temperature was reached and stabilized, the medium was inoculated with 0.2 mL of the microbial suspension containing ~5×10^9 cells/mL. Samples were collected into sterile test tubes at preset intervals. Samples were then appropriately diluted, plated and plates were incubated. Three separate experiments per condition were performed. Viable counts were based on duplicate counts, from appropriate dilutions plated in LB agar for *C. sakazakii* DPC6529, LB agar supplemented with kanamycin (Sigma) (50 μg/mL) for *C. sakazakii* DPC6529 transposon mutants and LB agar supplemented with erythromycin for the *C. sakazakii* DPC6529 lux tagged strain. Plates were incubated for 24 h at 37 °C for *C. sakazakii* DPC6529 and for 48 h at 37 °C for the transposon mutants and the lux-tagged strain.