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Functional Screening of the *Cronobacter sakazakii* BAA-894 Genome reveals a role for ProP (ESA_02131) in carnitine uptake

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Keywords: carnitine, *Cronobacter*, osmolytes, osmotolerance, stress

Cronobacter sakazakii is a neonatal pathogen responsible for up to 80% of fatalities in infected infants. Low birth weight infants and neonates infected with *C. sakazakii* suffer necrotizing enterocolitis, bacteraemia and meningitis. The mode of transmission most often associated with infection is powdered infant formula (PIF) which, with an a_w of ~0.2, is too low to allow most microorganisms to persist. Survival of *C. sakazakii* in environments subject to extreme hyperosmotic stress has previously been attributed to the uptake of compatible solutes including proline and betaine. Herein, we report the construction and screening of a *C. sakazakii* genome bank and the identification of ProP (ESA_02131) as a carnitine uptake system.

Introduction

The neonatal pathogen *Cronobacter sakazakii* is a Gram negative, rod shaped, motile, non-spore-forming, facultatively anaerobic bacterium belonging to the Enterobacteriaceae family.[1] *C. sakazakii* is associated with neonatal infections and can cause bacteraemia and sepsis, cerebro-spinal and peritoneal fluid accumulation, brain abscesses, cyst formation, necrotizing enterocolitis (NEC), meningitis and intracerebral infarctions.[2] While *C. sakazakii* is ubiquitous in the environment,[3,4] the ability of the organism to survive in extremely low water environments is a unique stress survival strategy which has significantly raised the profile of this foodborne pathogen in recent years.[5]

Survival of *C. sakazakii* for extended periods in powdered infant formula has been observed, with some capsulated strains surviving for up to 2.5 years.[6] Indeed, a 2004 study found that 8 out of 9 PIF factories investigated were contaminated with *C. sakazakii*.[7] Given that pasteurisation effectively inactivates *C. sakazakii*, the presence of the pathogen in powdered infant formula is attributed mainly to post-processing environmental contamination, such as the addition of contaminated ingredients and/or the use of non-sterile equipment.[8] Previous studies also suggest that reformulation; specifically the addition of non-essential substitutes, such as carnitine and proline, may act as protective compounds, facilitating increased survival of *C. sakazakii* and other osmotolerant pathogens in PIF.[9-11]

The global standard for the composition of infant formula[12] lists the compositional requirements proposed by The Codex Alimentarius Commission of Food and Agriculture Organization of the United Nations and the World Health Organization. This standard states that other ingredients may be added to ensure that the infant formula is adequate as the sole source of nutrition or to provide other benefits that are comparable with breastfed babies. In particular protein is recognized as an essential nutritional requirement and the global standards suggest a minimum guideline of 1.8 g/100 kcal for cow's milk protein and hydrolysed cow's milk protein and 2.25 g/100 kcal for Soy protein isolates. Formula is also supplemented with a large variety of amino acids to provide a more nutritionally complete and balanced food source.[12] Examples of free amino acids include carnitine, tyrosine, cysteine and taurine. Carnitine is proposed as a compositional requirement in infant formula by the FAO/WHO, wherein a minimum quantity of 1.2 mg/100 kcal is proposed with no maximum quantity specified. A previous study, carried out by the Scientific Committee on Food and the Life Sciences Research Office of the American Society for Nutritional Sciences, proposed a maximum level of 2 mg/100 kcal, based on the usual range found in human milk, in the absence of indications of any untoward effects of higher L-carnitine intake in infants.[13] By contrast, the International Expert Group concluded that no maximum level is required.[12] Carnitine is therefore present in many brands of powdered infant formula at varying levels.

Carnitine is most commonly found in foods of animal origin.[14,15] In prokaryotes carnitine has been identified as an important osmoprotectant; allowing the cell to overcome significant increases in osmolality.[16,17] In particular, carnitine has been identified as an essential osmoprotectant in the foodborne pathogen *L. monocytogenes* and has been shown to play a significant role in pathogenesis.[18,19] Smith[20] demonstrated that 1 μM

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carnitine results in an approximate doubling of the growth rate of *L. monocytogenes* at 4% NaCl.[21] Cells grown in defined and complex media demonstrate a high rate of carnitine uptake, suggesting that the listerial carnitine transport system is constitutively expressed rather than induced by a high ionic or non-ionic solute concentration.[22] Furthermore, carnitine uptake in *L. monocytogenes* is ATP-driven and varies depending on the growth phase of the cell and the availability of other osmolytes such as betaine or proline.[14] The principal carnitine uptake system in *L. monocytogenes* is encoded by the *opuC* operon which encodes an ATP binding protein (OpuCA), an extracellular substrate binding protein (OpuCC) and 2 membrane associated proteins (OpuCB and OpuCD).[18] This ATP-binding transport system has also been demonstrated to transport proline and glycine betaine, further highlighting the importance of this compatible solute in cellular osmoregulation.

Recent *in silico* analysis of the *C. sakazakii* genome identified 2 copies of *opuC*,[5] suggesting that *C. sakazakii* may indeed utilize carnitine in a similar way to *L. monocytegenes*. In the same study we identified 7 copies of the ProP homolog, originally annotated as a proline uptake system, and suggest that these ProP homologues are the primary drivers of *C. sakazakii* osmotolerance.[23] Furthermore, as has been previously reported in other bacteria,[24] we suggest that the presence of a C-terminal coiled coil domain of one of the ProP homologues (ESA_02131) is essential for optimal transporter activity.[25]

Herein, we describe a functional genomic screen of *C. sakazakii* BAA-894; revealing a novel role for the ProP homolog (ESA_02131) as a carnitine uptake system. Indeed, carnitine uptake via ProP allows growth of the pathogen at salt concentrations far in excess of that afforded by proline; a finding which has significant food safety implications, given that carnitine is often added to infant formula to boost its nutritional benefit.[26]

Material and Methods

Media, chemicals and growth conditions

Bacterial strains and plasmids used in this study are listed in **Table 1**. *Escherichia coli* strains were grown at 37°C in either Luria-Bertani (LB) medium or M9 minimal media[27] containing 0.5% glucose, 0.04% arginine, 0.04% isoleucine, 0.04% valine (Sigma-Aldrich Co.) to support the growth of *E. coli* MKH13.[28] *Cronobacter sakazakii* was grown in either LB or chemically defined media. Carnitine was filter sterilised and added to M9 minimal media to a final concentration of 1 mM (Sigma-Aldrich Co.). Ampicillin was made up as a concentrated stock solution (100 mg/ml) and added to media at the required levels. Electrotransformed cells were recovered using SOC media prepared as described by Sleator et al.[29] Osmolality of the defined and complex media was adjusted by the addition of NaCl. All strains were grown at 37°C with shaking at 200 rpm, unless otherwise stated. Where solid media was required, agar was added at 1.5%.

DNA manipulations and sequence analysis

Restriction enzymes, RNase, shrimp alkaline phosphatase and T4 DNA ligase were obtained from Roche Diagnostics and used according to the manufacturers' instructions. Bacterial genomic DNA was isolated as described previously.[30] Plasmid DNA was isolated using the High Pure Plasmid Isolation kit (Roche Diagnostics). PCR reactions were carried out using the high fidelity Velocity DNA polymerase Kit (Bioline) in accordance with manufacturers' instructions. PCR products were purified using the Roche plasmid purification kit (Roche Diagnostics). Oligonucleotide primers (listed in **Table 2**) used for PCR and sequence analysis were synthesized by MWG (MWG Operon, Germany and GATC, Germany). Nucleotide analysis was performed using the Simplicity™ platform[31] (nSilico Ltd., Cork, Ireland). Electrocompetent *E. coli* DH5α, obtained from New England Biolabs and *E. coli* MKH13 were made electrocompetent using the method outlined by Sambrook et al.[27] Electrotransformation was carried out using standard protocols.

Creation of *C. sakazakii* BAA-894 genome bank

A DNA library, consisting of *C. sakazakii* BAA-894 genomic DNA, was created by partially digesting the genomic DNA with Sau3A to yield genomic fragments, ranging in size from 300 bp to 3000 bp. The pUC18 plasmid was digested with BamH1 and dephosphoylated with shrimp alkaline phosphatase. The genomic fragments were ligated to the digested plasmid using T4 DNA ligase (Roche). The resulting recombinant plasmids were then purified and transformed into restriction-deficient E. coli DH5α. LB plates containing ampicillin (50 μg/ml), IPTG (isopropyl-1-thio-β-D-galactopyranoside) (1 mM), and X-Gal (5-bromo-4-

Table 1. Bacterial strains and plasmids

Strain or Plasmid	Relevant Genotype or Characteristics	Source or Reference
Plasmids		
pUC18	Amp^r, lacZ′, pMB9 replicon	40
pUC18::BAA894	pUC18 containing 300–3000bp fragment from C. sakazakii genome	This study
Strains		
Cronobacter sakazakii BAA-894	C. sakazakii strain isolated from powdered formula associated with neonatal intensive care unit	41
Escherichia coli DH5α	Intermediate cloning host.supE44 ΔlacU169(80lacZΔM15)R17 recA1 endA1 gyrA96 thi-1 relA1.	Invitrogen
E. coli MKH13	MC4100Δ(putPA)101Δ(proP)2Δ(proU)	42
E. coli MKH13::pUC18	Host strain harbouring the empty pUC18 plasmid. Amp^r	This study
E. coli MKH13::ProP (ESA_02131)	Host strain harbouring pUC18 containing ProP (ESA_02131) from the C. sakazakii genome. Amp^r	This study

Table 2. Primers

Primer name		Primer sequence (5′ to 3′)	Length	Characteristics
pUC18 MCS Check	F	CATTAGCTCACTCATTAGGCACC	23	pUC18 insert check
	R	CATTGTAAAACGACGGCCAGTG	22	pUC18 insert check

chloro-3-indolyl-β-D-galactopyranoside) (40 μg/ml) were used for blue/white screening as outlined by Sambrook et al.[27] Complemented plasmids were isolated from E. coli DH5α and purified prior to electrotransformation into the osmotically sensitive *E. coli* MKH13 strain. *E. coli* MKH13 strains expressing the plasmid were selected on media containing ampicillian. Growth on high osmolality media containing added carnitine, followed by PCR of transformants and subsequent sequencing, confirmed successful transformation. Transformants were pooled and stocked at −80°C.

Osmotolerance assay

The genome bank was screened on defined media containing 1 mM carnitine in the presence of NaCl (>4%) for positive selection of osmotolerant clones. Overnight cultures of *E. coli* MKH13 clones possessing a putative osmotolerance gene of interest were grown at 37°C with shaking at 200 rev min^{-1} in 10 ml M9 minimal media containing 0.5% glucose, 0.04% arginine, 0.04% isoleucine, 0.04% valine (Sigma-Aldrich Co.). Cells were centrifuged at 5,000 g, before being washed and re-suspended in ¼ strength Ringer's solution. The cell suspension (200 ul) was added to 1800 ul of defined growth media containing carnitine and varying concentrations of NaCl (0–10%). Optical density (OD_{600}) readings were taken every hour, over a 48 hour period. Readings were taken in triplicate and graphs were constructed using SigmaPlot version 11.0.

Results and Discussion

A *C. sakazakii* BAA-894 genome bank was created using a random shot gun approach, with partial Sau3A digestion. Genome fragments of various sizes (300–3000 bp) were cloned into the *E. coli* DH5α, a restriction deficient intermediary cloning host, followed by MKH13, an osmosensitive secondary host, for further phenotypic characterization. Following transformation of the pUC18::BAA-894 genome bank into MKH13, successful transformants were selected on minimal medium containing 4% added NaCl and 1 mM carnitine. No colonies appeared following a control transformation with the empty vector (pUC18 alone), while transformation efficiencies of approximately 70 CFU/μg of DNA were achieved from the plasmid bank, with colonies appearing after 36 h at 37°C. Plasmid DNA isolated from a representative colony was retransformed into MKH13 to confirm complementation. Sequence analysis revealed the cloned insert to be ProP (ESA_02131)

E. coli MKH13 harbouring ProP (ESA_02131) was grown in minimal media containing 0–10% NaCl and 1 mM carnitine. Growth was monitored each hour over a 48 hour period at an optical density of 600 nm. The transformed cells demonstrated significant osmotolerance, with carnitine as the only compatible solute present, while the control *E. coli* MKH13::pUC18 strain showed little or no grow above 4% NaCl. Notably, E. coli MKH13 expressing ProP (ESA_02131) grew to an OD of 0.55 in media containing 10% NaCl (**Fig. 1**). Previous work in our lab demonstrated that ProP (ESA_02131) encodes a proline/glycine betaine uptake system.32 However, this is the first study to show that the membrane bound transporter also functions as a carnitine uptake system. Furthermore, the growth kinetics recorded in the presence of carnitine are significantly higher than those observed when proline was supplied as the sole compatible solute. *E. coli* MKH13 transformed with ProP (ESA_02131) reached an optical density of 0.25 at 9% NaCl with 1 mM proline, compared to 0.57 at the equivalent salt concentration with 1 mM carnitine.

Kets et al.[33] was one of the first to report on the osmoprotective effects of carnitine in bacteria; showing increased growth of *Lactobacillus plantarum* in media containing between 0.5–1 M NaCl. Beumer et al.[34] also described the osmoprotective effective of carnitine on *L. monocytogenes* grown at elevated osmolarities. Carnitine has since been identified as playing a central role in bacterial osmoprotection, transported from the external environment rather than being synthesized endogenously.[35] The opuC operon, 2 copies of which were previously identified on the *C. sakazakii* BAA-894 genome,[5] encodes a carnitine transporter found in many Gram positives, including *Bacillus subtilis* and *L. monocytogenes*. However, in addition to carnitine, OpuC has

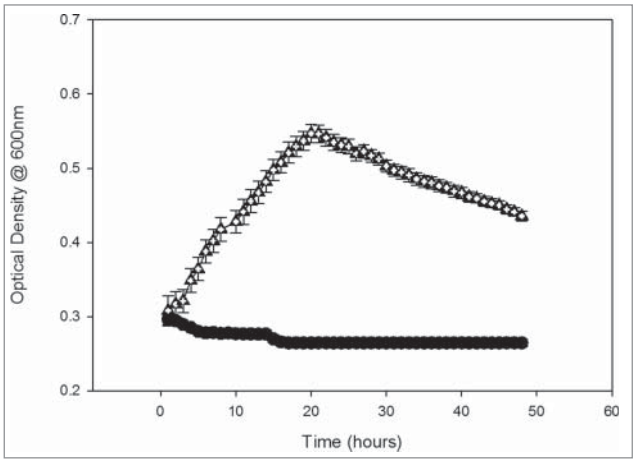

Figure 1. Growth of *E. coli* MKH13::ProP (ESA_02131), triangles and *E. coli* MKH13::pUC18, circles, in M9 minimal media with 1 mM carnitine and 10% NaCl.

also been shown to transport other compatible solutes such as ectoine, crotonobetaine, choline, proline betaine and glycine betaine.

Likewise the ProP transporter of *E. coli*, although originally identified as a proline porter, also exhibits carnitine uptake at Km and Vmax values of 200–250 uM and 1.2 nmol min^{-1}, respectively.[36] The results obtained in the present study demonstrate that ProP (ESA_02131) likely plays a similar role to its homolog in *E. coli*; facilitating the uptake of carnitine as well as proline and glycine betaine. Indeed, the greater osmotic protection afforded to *C. sakazakii* by carnitine, versus proline, would suggest that ProP might be more appropriately regarded as a carnitine uptake system, rather than simply a proline porter, in *C. sakazakii*. Indeed, we failed to link either of the 2 OpuC homologues (ESA_01738–01741 and ESA_01108–ESA_01111) to carnitine uptake, using the functional screen described in the current study; though this might simply be due to limitations of the existing bank.

The use and concentration of carnitine in PIF is diverse; varying widely between manufacturers. In 2003 the Scientific Committee on Food approved L-carntine as a nutrient for inclusion in infant formulae based on soy protein and hydrolysed protein but did not consider it necessary to add L-carnitine to cows' milk-based formula.[37] However, many brands of PIF continue to add carnitine as a non-essential supplement. The specific function of carnitine in PIF is as a nutrient supplement. Carnitine is a quaternary amine which is required for the transport of long chain fatty acids across the mitochondrial membrane to be oxidised for energy at the target tissue or for storage as a triglycerol.[38] However, the results of this study demonstrate that *C. sakazakii*, like many other osmotolerant pathogens, can utilize carnitine as a compatible solute to aid survival in this dry environment. Thus, while carnitine may be a beneficial nutritional supplement in PIF, its use also contributes to survival of pathogens such as *C. sakazakii*, with mortality rates of up to 80% in infected infants.

Conclusion and Future Prospects

The Irish dairy industry produces approximately 15% of the entire global exports of infant milk formula and is therefore a major contributor to the global PIF industry. Much of the PIF produced in Ireland contains carnitine as a nutrient supplement. While the nutritional benefits of carnitine are important for healthy growth and development of the infant,[38] the presence of carnitine in PIF may also facilitate the survival of the neonatal pathogen *C. sakazakii*. This study demonstrated that ProP (ESA_02131), previously identified as a proline/glycine betaine transporter,[32] can be further characterized as a carnitine uptake system. Furthermore, carnitine appears to have a greater osmoprotective effect than proline when accumulated via ProP (ESA_02131). Thus, while reversing the policy of adding carnitine to infant formula, to boost its nutritional value, is an obvious first step in reducing the burden of *C. sakazakii*, ProP (ESA_02131) itself is also an obvious target for the control of the pathogen. This approach involves smugglin technology; the use of antimicrobial compounds which structurally mimic carnitine, killing the cell rather than protecting it. This represents a viable alternative to antibiotic therapy and may lead to effective control of the pathogen in high risk foods such as PIF.[10,39]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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