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Title	Investigation of growth and stress tolerance characteristics of Cronobacter spp.
Authors	Flaherty, Claire
Publication date	2012-12
Original Citation	Flaherty, C, 2012. Investigation of growth and stress tolerance characteristics of Cronobacter spp.. PhD Thesis, University College Cork.
Type of publication	Doctoral thesis
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Download date	2026-11-16 09:50:11
Item downloaded from	https://hdl.handle.net/10468/995



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Investigation of Growth and Stress Tolerance Characteristics of *Cronobacter* spp.



Ollscoil na hÉireann, Corcaigh

THE NATIONAL UNIVERSITY OF IRELAND, CORK

A Thesis presented to the National University of Ireland for the Degree of Doctor of Philosophy by

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December 2012

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Declaration

I hereby declare that the research presented in this thesis is my own work and effort and that it has not been submitted for any other degree, either at University College Cork or elsewhere. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

This work was completed under the guidance of Prof. Colin Hill and Dr. Máire Begley at Food for Health Ireland, Biosciences Institute (Microbiology Department), University College Cork.

Signature:…….

Date:……

Thesis Abstract

Cronobacter spp. are opportunistic pathogens which can be isolated from a wide variety of foods and environments. They are Gram negative, motile, non-spore forming, peritrichous rods of the *Enterobacteriaceae* family. This food-borne pathogen is associated with the ingestion of contaminated infant milk formula (IMF), causing necrotizing enterocolitis, sepsis and meningitis in neonatal infants.

The work presented in this thesis involved the investigation and characterisation of a bank of *Cronobacter* strains for their ability to tolerate physiologically relevant stress conditions that are commonly encountered in the gastrointestinal tract. While all strains were able to endure the suboptimal conditions tested, noteworthy variations were observed between strains. A collection of these strains were Lux-tagged to determine if their growth could be tracked in IMF by measuring bioluminescence. The resulting strains could be easily and reproducibly monitored in real time by measuring light emission. Following this a transposon mutagenesis library was created in one of the Lux-tagged strains of *Cronobacter sakazakii*. This library was screened for mutants with affected growth in milk. The majority of mutants identified were associated with amino acid metabolism. The final section of this thesis identified genes involved in the tolerance of *C. sakazakii* to the milk derived antimicrobial peptide, Lactoferricin B (Lfcin B). This was achieved by creating a transposon mutagenesis library in *C. sakazakii* and screening for mutants with increased susceptibility to Lfcin B.

Overall this thesis demonstrates the variation between *Cronobacter* strains. It also identifies genes required for growth of the bacteria in milk, as well as genes needed for antimicrobial peptide tolerance.

Chapter I

Applications of milk derived antimicrobials in the dairy industry

Literature Review

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1. Abstract

Milk is a polyphasic secretion of the mammalian gland containing approximately 5% lactose, 3.2% protein, 4% lipid and 0.7% mineral salts, but this varies between species. It is a complex physiological liquid that provides an array of nutrients and bioactive components, including antimicrobials. These antimicrobials can be derived from the proteins, oligosaccharides and lipids present in milk. There is enormous potential for the discovery of new and enhanced antimicrobials originating from milk. This could be of major significance to the dairy industry especially in the area of food safety and functional foods. This review outlines the applications of a number of milk derived antimicrobials that are currently in use or those that are of potential use for the dairy industry.

2. Introduction

Milk is a valuable and highly complex biological material and is one of the most extensively studied foods. Its composition within any mammalian species is distinctive of the neonatal requirements of its offspring, providing the optimal composition of nutrients necessary during the newborn period of that species (Séverin *et al*., 2005). In addition to its nutritional role, milk contains hormones and growth factors that affect development, as well as agents that modulate immune function such as anti-inflammatory components (Field, 2005). Furthermore, milk contains an array of "bioactive factors" which provide the infant with a safeguard from infection with various microorganisms. Much research has been undertaken on the antimicrobial potential of proteins, oligosaccharides and lipids in milk. These antimicrobials have several mechanisms of action; they may either directly kill the pathogen or inhibit the pathogen binding to receptors in the gut (bacterial lectins) (Newburg *et al*., 2005). The bacteria are subsequently swept away, leaving the host mucosa untouched (Mills *et al*., 2011). They may also stimulate the immune system and indirectly deter the pathogen by promoting the growth of commensal bacteria; e.g. human milk oligosaccharides (HMO) encourage proliferation of *Bifidobacterium* and *Lactobacillus* species (Boehm *et al*., 2007). The present review will give an overview of the antimicrobial components of milk, and describe the current and potential applications of these milk-derived antimicrobials for the dairy industry.

3. Milk derived antimicrobials

3.1 Milk proteins

In regards to bovine milk proteins, caseins (insoluble) are the main (80%) proteinaceous component of milk and can contain different levels of phosphate (α_{S1}, α_{S2}-, β- and κ-casein). Whey proteins (soluble) comprise approximately 20% of total milk proteins; they consist of α-lactalbumin, β-lactoglobulin, lactoferrin, transferrin, bovine serum albumin, and immunoglobulin. In human milk protein, the casein to whey protein ratio varies throughout lactation. It can range from ~20:80 in early lactation to 50:50 in late lactation (Lönnerdal, 2003).

Peptides encrypted within the sequence of the parent proteins have displayed antimicrobial activity and need to be released in order to become active. This can be achieved by hydrolysis of caseins and whey proteins by digestive proteases in the gastrointestinal tract (Clare *et al*., 2000), or by fermentation with selected proteolytic bacteria (e.g. lactic acid bacteria; LAB, *Bacillus* spp.) (Kent *et al*., 2012). Both of these methods can also be performed *in vitro* to create new peptides. Milk acidification following heat treatment has also been reported to generate active peptides (Zucht *et al*., 2005). The majority of milk derived antimicrobial peptides (AMPs) discovered so far are of bovine and human origin (Table 1). However, AMPs derived from ovine and caprine proteins have also been identified (Corrêa *et al*., 2011, López-Expósito *et al*., 2006a Almaas *et al*., 2011). The minimum inhibitory concentration (MIC) of milk-derived AMPs have been shown to vary greatly; e.g. Cp1 α_{s1}-casein f(99-109) has an MIC of 125μg/mL while Cr5 bovine

α_{s2}-casein f(164-207) has an MIC of 4.8µg/mL against *Listeria monocytogenes* (McCann *et al*., 2006, McCann *et al*., 2005).

Milk derived AMPs are typically relatively short (12 to 100 amino acids) and do not have conserved sequences. However, many of them have conserved characteristics. For example, the majority have a positive charge and are amphiphilic (Jenssen *et al*., 2006). These characteristics have been proposed to facilitate interaction between the positively charged peptide and the negatively charged bacterial membrane (Martin *et al*., 1995).

Information is lacking regarding the precise mode of action of milk derived AMPs. Lfcin B is one of the most highly researched milk derived AMPs. It has been shown to have pore forming ability (Hwang *et al*., 1998) and has been proven to inhibit the DNA, RNA and protein synthesis of *Escherichia coli* (Ulvante *et al*., 2004). Tu *et al*., (2011) identified Lfcin B intracellular targets using an *E. coli* proteome chip. They found phosphoenolpyruvate was a target suggesting one of its mechanisms of action may be associated with pyruvate metabolism. Pyruvate assays showed an abnormal accumulation of pyruvate when *E. coli* was exposed to Lfcin B (Tu *et al*., 2011) and this could inhibit growth (Webb, 1968). However, further research is required to elucidate the intracellular targets of other milk derived AMPs.

3.2 Milk oligosaccharides

Human milk contains 10-20 g/L oligosaccharides, representing the third most abundant solid component in milk after fat and lactose (Séverin *et al*., 2005). The composition of HMO is very intricate in comparison to bovine milk (Boehm *et al*., 2007). Currently, up to 200 different structures have been defined for HMO (Ninonuevo *et al*., 2006). Infants are unable to digest HMO (Gnoth *et al*., 2000),

which indicates their presence is connected to functions outside basic nutrition. Milk oligosaccharides and glycans are formed with the same types of glycosyltransferases that synthesise human cell surface glycans. Therefore, it is conceivable they would have structural moieties in common (Newberg *et al*., 2005). The milk glycans could competitively inhibit the ability of pathogens to bind to receptors in the gut. Alternatively, HMO may act as "receptor decoys" when pathogens bind to human milk glycans (oligosaccharides) instead of to the host cell surface glycans. Other functions of HMO include immunomodulatory effects and brain development (Kunz *et al.,* 2000).

3.3 Milk lipids

Human milk contains approximately 4% lipids, which do not initially have antimicrobial activity but can become antiviral, antibacterial or antiprotozoal following digestion in the gastrointestinal tract (Issacs *et al*., 1990). The lipid fraction of human milk consists primarily of triglycerides, which are composed of three fatty acids covalently bound to a glycerol molecule by ester bonds. Microbial killing by milk lipids is primarily due to free fatty acids (FFA) and monoglycerides, which are released from milk triglycerides by both milk derived bile-salt-stimulated lipase and lipolytic activity in the gastrointestinal tract of the breast-fed infant (Tormar *et al*., 1987). Viruses appear to be the most susceptible to antimicrobial lipids and unsaturated fatty acids (oleic and linoleic acids) are the most antiviral of the lipids in human milk (Thormar *et al*., 2007, Isaacs *et al*., 1990). The exact mode of action of the lipid fraction of human milk has not been fully determined, but antimicrobial activity is thought to be exerted through disruption of the lipid bilayer (Isaacs *et al.,* 1994). Milk gangliosides have also been shown to reduce adherence of pathogens and promote growth of *Bifidobacteria* (Bode *et al.,* 2004).

4. Applications of milk derived antimicrobials

4.1 Food safety

Food safety is a major issue in society. Microorganisms have adapted to changes in food production, processing and preservation techniques, resulting in a number of new and re-emerging foodborne pathogens (Skovgaard, 2003). Unknown agents of foodborne illness (yet to be identified or not detected) account for approximately 81% of foodborne illness and hospitalizations (Woteki *et al*., 2003). In addition, an increasing proportion of our food is imported, chronic sequelae (e.g. Guillain Barre Syndrome) remain a significant problem and antibiotic resistant strains are constantly emerging.

Parallel to the importance of food safety, consumer demand for more natural and minimally processed food has become an increasingly recognised market driver. To restrict the level of foodborne pathogens and spoilage bacteria, several approaches to food preservation have been developed; these include cold storage temperatures, drying, pH adjustment, fermentation, thermal processing and chemical preservatives. Many of these methods are severe and affect the overall quality, nutritional content and organoleptic properties of the product (Szczepaniak *et al*., 2011). Therefore, there is substantial interest in naturally produced antimicrobial agents as food preservatives -especially in the dairy industry, where there is the problem of spoilage from spore forming bacteria.

Bacteriocins are antibacterial peptides produced by bacteria that kill or inhibit the growth of other bacteria. Nisin is currently the only bacteriocin widely used as a food preservative (Cleveland *et al*., 2001); however, its practical application is

limited because of its low stability, reduced activity at high pH and poor efficacy in certain food matrices (Pol *et al*., 2000). Thus, there is a need for more enhanced preservatives which could include development of a promising milk derived antimicrobial.

Examples of peptides that may improve the safety of food, particularly infant milk formulae (IMF), are Caseicin A and Caseicin B (Hayes *et al*., 2006). These peptides were generated following fermentation of sodium caseinate with *Bacillus* isolates (Kent *et al*., 2012). They are active against *Cronobacter sakazakii*, a bacterium regarded as a significant problem in powdered IMF (Healy *et al*., 2010). The CODEX Alimentarius Commission (CAC/RCP 66-2008) has a zero tolerance policy for *C. sakazakii* in IMF. In addition, IMF is not sterile and bacterial contamination with naturally residing bacteria can occur if rigorous hygienic good manufacturing practices (GMP) are not maintained. An assessment of the ability of Caseicin A and B to kill *C. sakazakii* spiked in reconstituted milk formula found that the fermentate reduced pathogen numbers by >4 log cfu/ml, comparing favourably with the positive control monocaprylin (Hayes *et al*., 2009). A spectrum of activity against other infant formula pathogens, *Listeria innocua* and *Pantoea agglomerans* was also shown. This research reinforces the potential application of addition of the fermentate to infant formula during manufacture for use as a preservative to inhibit *C. sakazakii.*

L. monocytogenes is a food borne pathogen associated with high rates of morbidity and mortality (Mead *et al*., 1999), especially in expectant mothers and infants. It has been the cause of considerable commercial loss, responsible for 71% of all recalls in food products between 1993 and 1998 (Wong *et al*., 2000). In addition, there has been an increase in the incidence of listeriosis (Goulet *et al*., 2008). The authors also

hypothesized that the recently reduced salt content in ready-to-eat products may allow growth of the organism if present as a contaminant. This could increase the likelihood of infection when these products are consumed by susceptible individuals. The discovery of a milk derived antimicrobial active against *L. monocytogenes* would also be a significant boost to food safety. An example is the novel antibacterial peptide (Cp1) which exhibited an MIC of 125µM against both strains of *L. monocytogenes* used in the study (McCann *et al*., 2006). However further research is required regarding the feasibility of addition of this milk derived AMP to food.

Lactoferrin (LF) is an iron-binding glycoprotein found in milk, saliva, tears, seminal fluids, mucins, and the secondary granules of neutrophils (Naidu, 2002). Its ability to bind Fe^{3+} ions facilitates its antibacterial, antiviral and antiparasitic activity (Adlerova *et al*., 2008). Activated lactoferrin (ALF) is a form of lactoferrin, and the 'activation' is a patented process. ALF is sprayed on meat surfaces to aid prevention of bacterial contamination during processing and is considered GRAS (Generally Recognized As Safe) by the FDA (Food and Drug Administration) (Naidu, 2002). The efficacy of ALF and LF to detach collagen-bound *E. coli* O157:H7 was measured by an *in vitro* adhesion blocking assay. The bacterial detachment efficacy of ALF was 2.7 log higher than the LF treatment. Activated lactoferrin was also shown to display activity against a myriad of foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes, Salmonella* spp., *Campylobacter* spp., *Vibrio* spp. *Staphylococcus aureus, Bacillus* spp. yeasts and mould as well as DNA and RNA viruses (Naidu, 2002). There is potential for the application of this antimicrobial protein as a food safety preservative.

Lactoperoxidase (LPO) is a glycoprotein naturally present in milk, which in itself has no antibacterial effect, but in combination with thiocyanate (SCN^-) and hydrogen

peroxide (H_2O_2) forms a potent antimicrobial system (lactoperoxidase-thiocyanate-hydrogen peroxide system) against a variety of microorganisms (bacteria, fungi and viruses). The LPO system has been recognized as important in the preservation of raw and pasteurized milk (Marks *et al*., 2001) by inhibiting various spoilage and pathogenic microorganisms (Seifu *et al*., 2005). Numerous investigations have been carried out on its potential application as a natural food preservative. For example, the LPO system has been used to inactivate or inhibit *Salmonella* Enteritidis in tomato juice, carrot juice, milk, eggs and chicken skin extract at various pH's and temperatures. It was found to be an effective preservative in fruit and vegetable juices, reducing cell numbers by up to 5.4 orders of magnitude. However, it was less effective in animal products (Touch *et al*., 2004). The LPO system has also been shown to inactivate *E. coli* and *Shigella* spp. in acidic fruit and vegetable juices by >5 orders of magnitude (Van Opstal *et al*., 2006), further highlighting its potential to act as an effective preservative in acidic food.

Research on antimicrobial peptides derived from milk fat globule membrane (MFGM) fractions is also underway. Xanthine oxidase is a well-characterized iron-sulphur molybdenum flavoprotein that is present in MFGMs in mammary epithelial cells (Spitzberg, 2005), but is not present in IMF (Stevens *et al*., 2000). The enzyme can weakly generate superoxide and hydrogen peroxide from nitrates and nitrites (Millar *et al*., 1998) and displays antibacterial activity (Stevens *et al*., 2000). Hancock *et al*., (2002) showed that human and bovine milk inhibited *E. coli* metabolism and this activity was dependent on xanthine oxidase activity and the presence of nitrite. Clare *et al*., (2008) screened MFGM fractions against *E. coli* O157:H7, *L. monocytogenes*, *S.* Typhimurium, *P. fluorescence*, *B. cereus*, *Lactobacillus acidophilus* and *Lactobacillus gasseri*. Antibacterial activity was

displayed against each of the pathogens, while no activity was observed against the *Lactobacillus* strains. Therefore, there is application potential for the addition of xanthine oxidase to infant formula or creation of a fraction from MFGM with higher antibacterial activity.

"High energy" sports drinks can be quite acidic and this may lead to dental erosion (Mathew *et al*., 2002), especially in athletes with a stressed immune system. Raising the pH of the drinks may solve this problem, but simultaneously increases the risk of spoilage or occurrence of pathogenic bacteria. The bacteriocin enterocin AS-48 (produced by *Enterococcus faecalis*) is an example of an antimicrobial peptide that may act as a natural preservative against these bacteria in less acidic sports drinks (Viedma *et al*., 2009). This study adjusted commercial energy drinks to pH 5, subjected the drinks to *L. monocytogenes*, *S. aureus*, *Bacillus cereus* and *Bacillus licheniformis* and stored them at 37°C. Addition of enterocin AS-48 (1mg/ml) rapidly inactivated *L. monocytogenes* in all the drinks tested, while the rest of the bacteria were killed with higher levels of enterocin AS-48 (12.5mg/ml). Discovery of a milk derived antimicrobial with the same function is possible. It could be added as a preservative to reduce the risk of dental caries.

4.2 Functional Foods

There are many definitions for functional foods and these are constantly evolving. A commonly used definition is a food which "besides nutritional effects has demonstrated a benefit for one or more functions of the human organism improving the state of health or well being or reducing the risk of disease" (Diplock *et al*., 1999). There are numerous prospects for the addition of milk-derived antimicrobials to food products due to their wide variety of properties (evoking nutritional,

hormonal, immunological, neurological and nutritional responses). This may provide a variety of functions in that it could aid in limiting or reducing severity of the disease or act as a prophylaxis. Incorporation into the diet as a functional food therefore has the potential to decrease morbidity, mortality and healthcare costs related to intestinal infections (Gardiner *et al*., 2009). The remainder of this review will focus on examples of potential functional food applications in different areas of the dairy industry.

4.2.1 Dental Hygiene

Streptococcus mutans is one of the causative agents in the development of dental caries (Huo *et al*., 2011), which is a highly prevalent disease. Once *S. mutans* adheres to the buccal surface of the tooth, acid formed by the bacteria due to fermentation of sugars accumulate in plaque on the teeth thereby leading to tooth decay (Loesche, 1986). The acid-forming and acidophilic properties of *S. mutans*, along with its ability to generate extracellular glucans, are important factors in the progression and maintenance of cariogenic biofilms (Loesche, 1986). *Porphyromonas gingivalis* is a black pigmenting oral anaerobe and is one of the primary aetiological agents of periodontal disease, a disorder of the tissues surrounding and supporting the teeth (periodontium).

Malkoski *et al*., (2001) demonstrated that CMP (caseinomacropeptide), a C-terminal fragment of bovine milk κ-casein, inhibits the growth of oral pathogens *P. gingivalis* and *S. mutans*, as well as *E. coli*. This group also identified the active component as nonglycosylated Ser(*P*)^{149} κ-casein-A (106-169), and designated this novel peptide kappacin. Huo *et al*., (2011) investigated the antimicrobial activity of a peptide fragment of human lactoferrin (hLF1-11) against *S. mutans* which displayed a

median MIC of 23.28μM. Halpin *et al*., (2008) investigated the ability of dairy powders and individual milk proteins to inhibit adhesion of *S. mutans* to hydroxylapatite (an analogue of tooth enamel). Whey protein concentrate 80 (sweet and acid), sodium caseinate and the casein fractions α-, β-, and κ-casein were found to have high levels of anti-adhesive activity. In addition to kappacin and hLF1-11, these proteins may have potential benefit in a functional food, such as soft cheeses and milk. This would be of particular importance to young children and the elderly who may not have sufficient dental hygiene.

Other potential uses would be in oral care products such as toothpaste, mouth wash and chewing gum. One major advantage of these is there is straightforward accessibility to the oral cavity if supplemented as a functional food or an oral care product. A patent has been filed (Warner-Lambert Company) to use CPP (caseinophosphopeptide), a phosphorylated casein-derived peptide, in chewing gum compositions to promote anticariogenicity (Aimutis, 2004). In addition, there are examples of toothpastes (e.g. BioXtra® and Oralbalance®) that contain the lactoperoxidase system, lysozyme and lactoferrin (Tenovuo, 2002) to improve dental health. These products could aid in lowering the vast number of people affected by dental caries and chronic periodontitis. In addition to these studies, there is the potential for the discovery of antimicrobials in milk with improved activity against dental pathogens.

4.2.2 Infant Formula/Diseases

Formula-fed infants have been shown to have higher levels of gastroenteritis than breast-fed infants (Quigley *et al*., 2007); therefore, there are opportunities for the use of IMF as a functional food, in order to limit the incidence of gastroenteritis in

bottle-fed infants. The mother's breast milk is widely known to constitute the best nourishment for neonates and infants. However, various situations exist where breast feeding is either not possible (HIV positive mother, illness, etc) or is not chosen, and synthetic preparations are required. IMF has been extensively used as an alternative for infants instead of bovine milk. The nutritional material of milk shows significant disparity among species in regards to the individual components. Thus, the use of modified or "humanized" bovine milk in infant formula, designed to simulate human milk as a substitute or supplement, is an important area of research. Modifications to infant formulae are regularly being made as the components of human milk are characterized and the nutrient requirements of diverse groups of infants are identified e.g. soy-based, lactose free and preterm IMF (Carver, 2003; Carver, 2001).

IMF are increasingly enriched with plant oligosaccharides and enzymatically linked galactooligosaccharides, which have prebiotic activity. However, they lack the intricacy and variety of natural HMOs, and are therefore unlikely to successfully imitate the structure-specific effects of HMO (Fanaro *et al*., 2005). HMOs have been shown to bind to the intestinal membrane guanylin cyclase receptor, which in turn blocks binding by the stable toxin (STa) of enterotoxigenic *E. coli* (Crane *et al*., 1994). A fucosylated oligosaccharide (α-1,2-linked) from human milk inhibited binding of invasive strains of *Campylobacter jejuni* to its host cell (Ruiz-Palacios *et al*., 2002). These studies suggest HMO have potential applications in the development of IMF as a functional food to facilitate tackling gastrointestinal disease. However, the disadvantages of HMO addition to IMF include difficulty in reproduction of the complex composition and structure of HMO.

Rotavirus is a major infant pathogen which is a significant cause of severe dehydration diarrhoea in infants, 95% of all children are infected before the age of 5

resulting in approximately 600,000 deaths annually (Glass *et al.*, 2006). Milk-fat globule membrane (MFGM) proteins, MUC1, butyrophilin and lactoadherin have been demonstrated to inhibit rotavirus replication in MA104 and Caco-2 cell lines (Kvistgaard *et al*., 2004). This study illustrated that lactoadherin possesses rotavirus inhibitory activity, as it considerably reduced rotavirus infections *in vitro*. Perez-Cano *et al*., (2008) showed supplementing rats with whey protein concentrate (WPC) modulated the immune response against the virus and reduced the severity of infection. WPC has also been found to reduce diarrheal induced symptoms in suckling mice (Wolber *et al*., 2005). Therefore, there is potential for addition of a milk derived anti-rotaviral to IMF or dairy products (cheese or yogurt) as a functional food. The dairy products could be given to young children of crèche going age and may potentially decrease the frequency and severity of the illness.

4.2.3 Sports Drinks

Whey protein supplements, including purified α-lactalbumin have been added to sport drinks due to their high protein content. While moderate exercise enhances immunity (Davis *et al*., 2004), intense athletic training has been shown to stress the immune system (Mackinnon, 2000). Bioactive components found in whey (e.g. IgA, glutamine and lactoferrin) may potentially benefit athletes by improving immune function, gastrointestinal health and exhibiting anti-inflammatory activity (Marshall, 2004).

4.2.4 Gastrointestinal Diseases

Improving gastrointestinal health is a key issue in today's society and the addition of a milk-derived antimicrobial to improve health or act as a prophylaxis against infection would be of interest to the dairy industry. Sprong *et al*., (2001) investigated

the efficacy of bovine milk lipids (digestion products of bovine milk triglycerides and membrane lipids) in potentially inhibiting gastroenteritic bacteria through bactericidal well diffusion assays. The pathogens tested were *C. jejuni*, *S. enteritidis* phage type 4, *E. coli* O157:H7, *Clostridium perfringens* and *L. monocytogenes*. The bactericidal activity of fatty acids depended on chain length; $C_{10:0}$ and $C_{12:0}$ fatty acids were toxic to all of the test pathogens. The authors hypothesised if these fatty acids are released during gastric digestion of milk lipids they may inhibit gastrointestinal infections. However, the feasibility issues of adding too much fatty acids or monoglycerides for antimicrobial activity include increased cost and impact on taste.

Table 1 lists the antimicrobial peptides from human and bovine milk discovered to date. Many of these have activity against pathogens in the gastrointestinal tract; e.g. β-casein f(184-210) has antibacterial activity against *E. coli* and *Salmonella* species as well as *S. aureus, Enterobacter faecium*, and *Yersinia enterocolitica* (Minervini *et al*., 2003). Utilizing these peptides as components of a functional food in order to improve gastrointestinal health and reduce severity of illness is very promising.

In regards to non-gastrointestinal infections, human lactoferrin has been found to interfere with the development of the HSV-1 infection at several steps of the viral replication cycle and with HSV-1 cell-to-cell spread in a dose and virus strain dependent approach (Välimaa *et al*., 2009). Isaacs *et al*., (2004) created a microbicide combining a milk-derived lipid-ether and a synthetic antimicrobial peptide and found it reduced both HSV-1 and HSV-2 by at least 1,000 fold more than the sum of the inactivations produced by the lipid-ether and the peptide alone. Therefore, there are possibilities for use of this microbicide as a functional food to aid limiting the occurrence and duration of cold sores (HSV-1).

Table 1 Milk derived antimicrobial peptides discovered to date (human and bovine)

Name	Size (amino acids)	Actual or Theoretical Mw (Da) Charge	Method of discovery	Activity Spectrum	MIC	Reference
Casein derived AMPs						
α_{S1}-caseins						
Isracidin (bovine α_{S1}-casein), (α_{S1}-CN f(1-23)	$_{1}$RPKHPIKHQGLPQEVLNENLLR F$_{23}$	2770 + charge	Chymosin digestion of bovine α_{s1}- casein	*S. aureus, L. monocytogenes, E. coli, Candida albicans*	0.1-1 mg/ml for Gram positives 0.059mM for *E. coli*	Lahov *et al.*, 1996 Hayes *et al.*, 2006
Cp1 (α_{S1}-casein), (α_{S1}-CN f(99-109)	$_{99}$LRLKKYKVPQL$_{109}$	1386 + charge	Pepsin hydrolysate of bovine sodium caseinate	*B. cereus, L .innocua, L. monocytogenes, Citrobacter freundii, Enterobacter aerogenes, E. coli, S. enteritidis, S.* Typhimurium	125-1000µg/ml	McCann *et al.*, 2006
Caseicin A	Residues 21-29 of bovine α_{S1}-casein $_{21}$IKHQGLPQE$_{29}$	1049 + charge	Degradation of sodium caseinate by *Bacillus* isolates	*E. coli, C. sakazakii*	0.05mM	Hayes *et al.*, 2006 Kent *et al.*, 2012
Caseicin B	Residues 30-37 of bovine α_{S1}-casein $_{30}$VLNENLLR$_{37}$	970 neutral	Degradation of sodium caseinate by *Bacillus* isolates	*E. coli, C. sakazakii*	0.22 mM	Hayes *et al.*, 2006 Kent *et al.*, 2012
Caseicin C	Residues 195-208 of α_{S1}-casein $_{195}$SDIPNPIGSENSEK$_{208}$	1486.56 + charge	Degradation of sodium caseinate by *Bacillus* isolates	*E. coli,* and minor activity against *Listeria innocua*	1mM	Hayes *et al.*, 2006 Kent *et al.*, 2012
α_{s2}-Caseins:						
Casocidin-I (bovine milk)	Residues 165-203 of α_{S2}-casein $_{165}$KTKLTEEKNRLNFLKKISQRY QKFALPQYLKTVYQHQK$_{203}$	4870 + charge	Isolated from bovine milk	*E. coli* and *Staphylococcus carnosus*	3mm zone of inhibition with10µl of sample	Zucht *et al.*, 1995
Cationic bovine α_{s2}-CN f183-207	$_{183}$VYQHQKAMKPWIQPKTKVIP YVRYL$_{207}$	3115 + charge	Pepsin hydrolysate of α_{s2}-casein	*E. coli, L. innocua, B. cereus, Micrococcus flavus, Streptococcus thermophilus*	25-99µM	Reiko *et al.*, 1999
Cationic bovine α_{s2}-CN f164-179	$_{164}$LKKISQRYQKFALPQY$_{179}$	2011	Peptic hydrolysate of α_{s2}-	*E. coli, L. innocua, B. cereus, M. flavus, S.*	8-16µM	Reiko *et al.*, 1999

		+ charge	casein	*thermophilus*		
β-Caseins						
β-CN f(184-210) human β-casein	$_{184}$QELLLNPTHQYPVTQPLAPVH NPISV$_{210}$	3133 + charge	Hydrolysis of sodium caseinate with a proteinase of *Lb. helveticus*	*E. coli, S. aureus, Enterobacter faecium, Y. enterocolitica, Salmonella* species, *Lactobacillus* species, *Bacillus megaterium* and *L. innocua*	50-100µg/ml	Minervini *et al.*, 2003
Casecidin 15 bovine β-casein(193-207)	$_{193}$YQEPVLGPVRGPFPI$_{207}$	1668 No charge	Naturally found in bovine colostrum	*E. coli*	0.4-0.5mg/ml	Birkemo *et al.*, 2008
Casecidin 17 bovine β-casein (193-209)	$_{193}$YQEPVLGPVRGPFPIIV$_{209}$	1881 No charge	Naturally found in bovine colostrum	*E. coli*	0.4-0.5mg/ml	Birkemo *et al.*, 2008
κ-Caseins						
Caseinomacropeptide (CMP) (bovine milk)	Residues 106-169 of bovine milk κ-casein AIPPKKNQDKTEIPTINTIASGEP-TSTPTTEAVEST-VATLEDSPEVI-ES-PPEINTVQ-VTSTAV	7678 + charge	Hydrolyses of κ-casein by chymosin	Influenza, *Vibrio cholerae, Streptococcus mutans, P. gingivalis, E. coli*	1.7mg/ml for *S. mutans*, 3.8mg/ml for *P. gingivalis* 4.3mg/ml for *E. coli*	Malkoski *et al.*, 2001
Kappacin	Residues 106-169 of bovine κ-casein $_{106}$AIPPKKNQDKTEIPTINTIASG EP-TSTPTTEAVEST-VATLEDSPEVI-ES-PPEINTVQ-VTSTAV$_{109}$	7678 Anionic phosphopeptide	RP-HPLC of Caseinomacropeptide	*S. mutans*	59µg/ml	Birkemo *et al.*, 2008, Malkoski *et al.*, 2001
Bovine κ-casein f(18-24)	$_{18}$FSDKIAK$_{24}$	807 + charge	Hydrolyses of κ-casein by pepsin	*L. innocua, E. coli, S. carnosus*	MIC not determined	López Expósito *et al.*, 2006b
Bovine κ-casein f(30-32)	$_{30}$YVL$_{32}$	393 Neutral	Hydrolyses of κ-casein by pepsin	*L. innocua, Serratia marcescens, S. carnosus*	MIC not determined	López Expósito *et al.*, 2006b
Bovine κ-casein f(139-146)	$_{139}$VESTVATL$_{146}$	818 - charge	Hydrolyses of κ-casein by pepsin	*L. innocua, E. coli, S. carnosus*	MIC not determined	López Expósito *et al.*, 2006b
Bovine κ-casein f(34-75)	$_{34}$PAAVRSPAQILQ$_{75}$	1250 + charge	Hydrolyses of κ-casein by pepsin	*E. coli*	MIC not determined	López Expósito *et al.*, 2006b
Bovine κ-casein f(28-30)	$_{28}$IQY$_{30}$	422 neutral	Hydrolyses of κ-casein by pepsin	*E. coli*	MIC not determined	López Expósito *et al.*, 2006b

Bovine κ-casein f(42-49)	$_{42}$YYQQKPVA$_{49}$	955 + charge	Hydrolyses of κ-casein by pepsin	*E. coli, S. carnosus*	MIC not determined	López Expósito *et al.,* 2006b
Bovine κ-casein f(25-29)	$_{25}$EIPT$_{29}$	657 - charge	Hydrolyses of κ-casein by pepsin	*E. coli, S. marcescens*	MIC not determined	López Expósito *et al.,* 2006b
Bovine κ-casein f(118-121)	$_{118}$RINKK$_{121}$	529 + charge	Hydrolyses of κ-casein by pepsin	*E. coli*	MIC not determined	López Expósito *et al.,* 2006b
Human κcasein f(63-117)	$_{63}$YQRRPAIAINNPYVRTYYANP AVVRPHAQUIQRQYLPNSHPPT VVRRPNLHPSF$_{117}$	6430 + charge	Hydrolyses of κ-casein by pepsin	*S. carnosus*, Gram positive and Gram negative bacteria and yeast	MIC not determined	Liepke *et al.,* 2001
Miscellaneous						
Casecidins	amino acid sequence not published	Data not shown + charge	Chymosin digestion of caseins	*S. aureus, Sarcinia, Bacillus subtilis, Diplococcus pneumoniae, Streptococcus pyogenes* and some *Lactobacillus*	MIC not determined	Lahov *et al.,* 1996
Bovine α_{s1}-casein f(24-33)	$_{24}$FVAPFPEVFG$_{33}$	1109 + charge	Isolated from water soluble extract of cheese	*E. coli, B. megaterium, L. innocua, S. aureus, Salmonella spp., Y. Enterocolitica, Lactococcus lactis* and *Lactobacillus* species	70-250 µg/ml	Rizzello *et al.,* 2005
181-207 Cr1 Bovine α_{s2}-casein	$_{181}$KTVYQHQKAMKP-WIQPKTKV-IPYVRYL$_{207}$	3346 + charge	Chymosin digest of bovine sodium caseinate	*B. cereus, B. subtilis, L. innocua, L. monocytogenes, E. coli, S.* Enteritidis, *S.* Typhimurium	>84ug/ml for *B. cereus* 21µg/ml for *B. subtilis, L. mono, L. innocua* and *S.* Typhimurium >168µg/ml for *E. coli,* >68µg/ml for *S.* Enteritidis	McCann *et al.,* 2005
180-207 Cr3 Bovine α_{s2}-casein	$_{180}$LKTVYQHQKAMKPWIQPKT KV-IPTVRYL207	3458 + charge	Chymosin digest of bovine sodium caseinate	Data not shown	MIC not determined	McCann *et al.,* 2005
175-207 Cr4 Bovine α_{s2}-casein	$_{175}$ALPQYLKTVYQHQKA-MKPWIQP-KTKVIPYV-RYL$_{207}$	4031 + charge	Chymosin digest of bovine sodium caseinate	*B. subtilis, L. innocua, L. monocytogenes, E. coli, S.* Typhimurium	10.7µg/ml for *B. subtilis, L. innocua, L. monocytogenes,* >171.2µg/ml for *E. coli,* 21.4µg/ml for *S.* Typhimurium	McCann *et al.,* 2005
164-207 Cr5/Cr6 Bovine α_{s2}-casein	$_{164}$LKKISQRYQKFALPQYLKTV YQHQKAMKPWIQPKTKVIPYV RYL$_{207}$	5453 + charge	Chymosin digest of bovine sodium caseinate	*B. subtilis, L. innocua, L. monocytogenes, E. coli*	4.8µg/ml against *B. subtilis, L. innocua, L. mono,* >76.2µg/ml for *E. coli*	McCann *et al.,* 2005
172-207 Cr7 Bovine α_{s2}- casein	$_{172}$QKFALPQYLKTVYQHQKAM KPWIQPKTKVIPYVRYL$_{207}$	4436 + charge	Chymosin digest of bovine sodium caseinate	Data not shown	MIC not determined	McCann *et al.,* 2005

Whey derived AMPs						
β-Lactoglobulin f(15-20) LGDT2	$_{15}$VAGTWY$_{20}$	700 - charge	Hydrolysis of bovine β-lactoglobulin with pepsin	*B. subtilis, M. luteus, S. aureus, Staphylococcus epidermidis, S. lentus*	MIC not determined	Pellegrini *et al.*, 2001 Benkerroum, 2010
β-Lactoglobulin f(25-40) LGDT4	$_{25}$AASDISLLDAQSAPLR$_{40}$	1630 + charge	Hydrolysis of bovine β-lactoglobulin with pepsin	*B. subtilis, S. lentus, S. zooepidemicus*	MIC not determined	Pellegrini *et al.*, 2001 Benkerroum 2010
β-Lactoglobulin f(78-83) LGDT1	$_{78}$IPAVFK$_{83}$	670 - charge	Hydrolysis of bovine β-lactoglobulin with pepsin	*B. subtilis, S. lentus, S. zooepidemicus*	MIC not determined	Pellegrini *et al.*, 2001 Benkerroum, 2010
β-Lactoglobulin f(92-100) LGDT3	$_{92}$VLVLDTDYK$_{100}$	1070 amphipathic	Hydrolysis of bovine β-lactoglobulin with pepsin	*B. subtilis, M. luteus, S. aureus, S. epidermidis, S. lentus*	MIC not determined	Pellegrini *et al.*, 2001 Benkerroum, 2010
Bovine α-lactalbumin f(1-5) LDT1	$_{1}$EQLTK$_{5}$	620 - charge	Hydrolysis of bovine α-lactalbumin with trypsin	*Bordetalla bronchiseptica, B. subtilis, M. luteus, S. epidermidis, S. luteus, S. zooepidemicus*	MIC not determined	Pellegrini *et al.*, 1999 Benkerroum, 2010
Bovine α-lactalbumin f(17-31) S-S (109-114) LDT2	GYGGVSPLEWVCTTF ALCSEK	2250 - charge	Hydrolysis of bovine α-lactalbumin with trypsin	*B. subtilis,Klebsiella.pneumoniae, M. luteus,S. aureus, S. epidermidis, S. luteus, Streptococcu zooepidemicus*	MIC not determined	Pellegrini *et al.*, 1999 Benkerroum, 2010
Bovine α-lactalbumin f(61-68)S-S(75-80) LDC	CKDDQNPH ISCDKF	1650 - charge	Hydrolysis of bovine α-lactalbumin with chymotrypsin	*B. bronchiseptica, K.pneumoniae P. aeruginosa, B. subtilis,S. aureus, S. epidermidis, S. luteus, S. zooepidemicus*	MIC not determined	Pellegrini *et al.*, 1999 Benkerroum, 2010
Lactophoricin derived from sequence of bovine milk component-3 of protease peptone	$_{1}$NTVKETIKYLKSLFSHAFEVVK T$_{23}$	2380 + charge	Synthesized by Synergy 423 peptide synthesizer	*S. aureus, L. innocua, Salmonella* spp., *Pseudomonas aeruginosa, E. coli*	>300µM	Campagne *et al.*, 2004
Lactoferricin B	$_{17}$FKCRRWQWRMKKLGAPSITC VRRAF$_{41}$	3125 + charge	Pepsin enzymatic cleavage of lactoferrin	Broad spectrum against of activity Gram positive and Gram negative including *L. monocytogenes* and *E. coli*	0.6-45µg/ml	Haukland *et al.*, 2001, Bellamy *et al.*, 1992
Lactoferrampin Derived from N1 domain of bovine lactoferrin	WKLLSKAQEKFGKNKSR	2047 + charge	Synthesized by Fmoc-chemistry on a MilliGen 9050 peptide synthesizer	*B. subtilis, E. coli, P. aeruginosa*	2.1-25 µM	Van der Kraan *et al.*, 2004

5. Discussion

This review discusses the potential applications of milk derived antimicrobials in the dairy industry, including their applications as functional foods. The market potential for functional foods is increasing rapidly; in 2008 the entire functional food market was worth an estimated US$80 billion (Vergari *et al*., 2011) and this figure is rapidly increasing with Global Industrial Analysts (GIA) estimating it will reach US$130 billion by 2015. This market could include the addition of milk derived antimicrobials for use as a functional food due to their potential to reduce the severity of an infection or act as a prophylaxis.

Milk derived antimicrobials display the advantage of having a broad spectrum of activity. In addition, they originate from a safe and inexpensive resource. For example, whey protein is a by-product of cheese making; consequently, there is enormous capacity for its use in the dairy industry. More research is required to determine the viability of integrating the antimicrobials discovered so far into food. There is clearly a large spectrum of milk derived antimicrobials, but more investigations need to be performed to unearth antimicrobials with higher MICs against pathogenic bacteria and greater/varying spectrums of activity. This is of particular significance at a time where the problem of antibiotic resistance is overwhelming (Cantón *et al*., 2011) and people are becoming more susceptible to infectious diseases (Rocourt *et al*., 2003). Another consideration is that antimicrobial factors in milk have been shown to work synergistically, thereby further enhancing their activity. The total antibacterial effect in milk is greater than the sum of the individual components. For example, López-Expósito *et al*., (2008) investigated the

synergistic effect of two milk derived peptides, bovine lactoferricin (Lfcin B) (lactoferrin f(17-41)) and bovine α_{s2}-casein f(183-207), in conjunction with nisin or lactoferrin. Lfcin B is a potent milk derived antimicrobial peptide corresponding to a fragment of lactoferrin. In combination with lactoferrin it revealed a synergistic effect against *E. coli* and *S. epidermidis*. Bovine lactoferrin and nisin both increased their antimicrobial activity when used in combination with α_{s2}-casein f(183-207).

5.1 Identifying novel milk derived antimicrobials

Identification of novel milk antimicrobials (Figure 1) focuses on the co-ordinated dissection of milk to uncover a new array of bioactive substances with the potential to positively impact on human health. This includes microbial fermentation with proteolytic bacteria or enzymatic hydrolysis with digestive enzymes such as trypsin and pepsin. In addition, knowledge in bioinformatics tools is required to identify milk derived antimicrobials. Metagenomic analysis may be necessary to determine the effects the milk components have on the bacteria in the human gut. Other sophisticated cell- and 'omics'- based technologies, e.g. proteomics, glycomics, glycoproteomics and lipidomics, may be utilized to identify fractions with prospective bioactivity and to clarify their mechanism(s) of action. Proteomics is the study of the whole set of proteins encoded by a genome and encompasses protein expression, structure and function. It has the ability to scientifically prove the safety of new products. Proteomic research offers opportunities for the dairy industry in process optimization and monitoring, quality and traceability, safety and nutritional evaluation (Pedreschi *et al*., 2011).

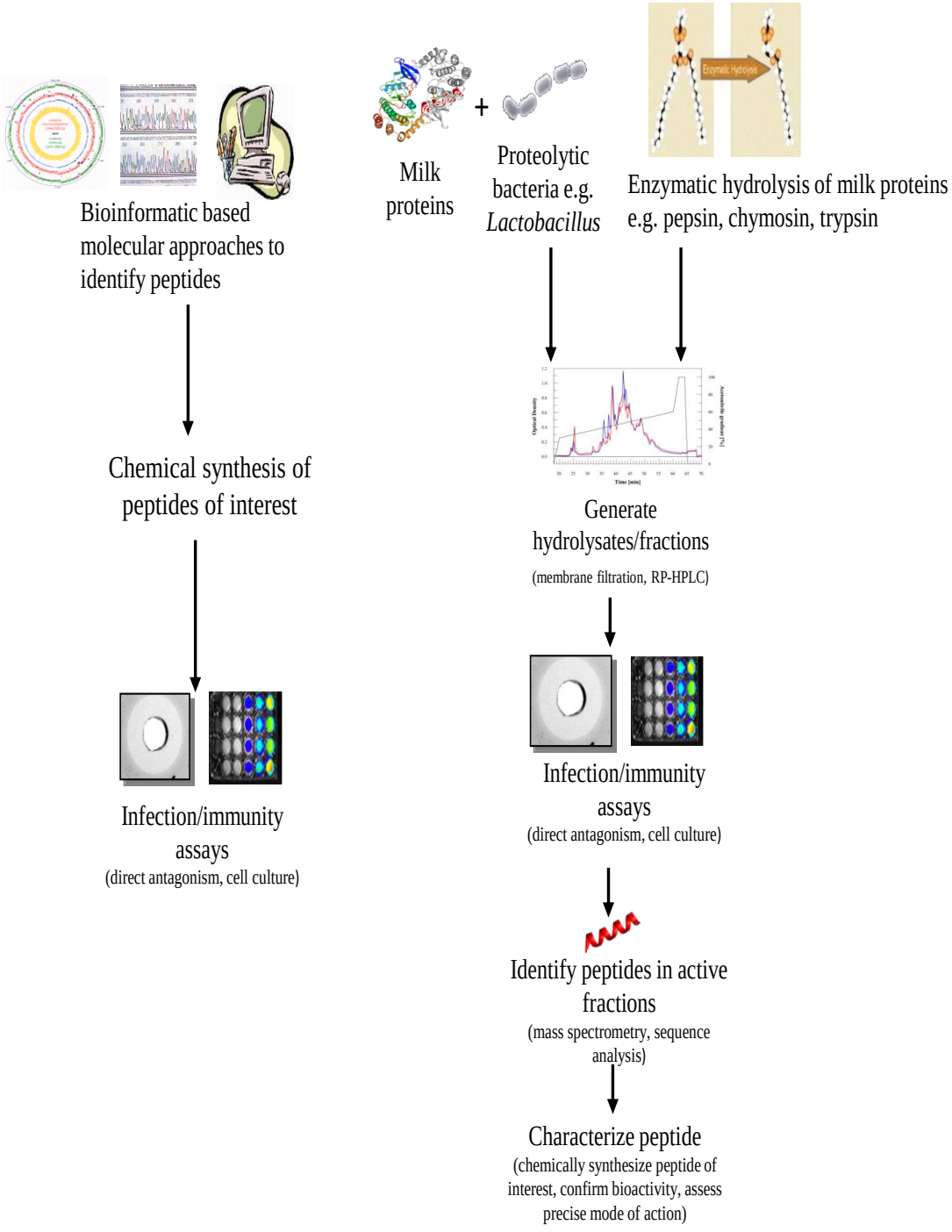


Fig.1 A schematic diagram of the processes involved in identification of novel milk derived antimicrobials

5.2 Technologies required for the production of food containing milk derived antimicrobials

Until recently, the production of milk derived antimicrobials was limited due to a lack of appropriate large scale technologies (Korhonen *et al*., 2006). Novel techniques such as mass spectrometry, chromatographic techniques and membrane separation technology help solve this problem through enriching peptides with a specific molecular weight range (Korhonen *et al*., 2007). Mass spectrometry based techniques enable the characterization of human and animal milk components in fresh and processed milk (Casado *et al*., 2009). Ion exchange membrane chromatography can be used for the enrichment of peptide fractions from protein hydrosylates.

Separation technologies are used to concentrate foods, as well as extract the relevant minor components. Spray drying is essential in order to convert fluid materials into solid or semi-solid particles (Murugesan *et al.,* 2011) and it is the most often used encapsulation technique utilized by the food industry (Reineccius, 2004). To ensure accurate delivery of antimicrobials their bioavailability needs to be optimized. The formulation and stability of the peptide in certain food matrices and during digestion is vital to guarantee successful human delivery of the most promising milk derived bioactives. Microencapsulation or nanoencapsulation may provide solutions in enhancing stability of the peptide. Microencapsulation ensures protection of unstable, sensitive materials from environmental conditions, allowing controlled and targeted release of the materials. It can mask the odour or taste, is easier to handle and has better processing ability (Kuang *et al.,* 2010). For example, enzymatic digestion can be used effectively to prepare bioactive preparations for formulation in infant functional food. However, it is necessary to inactivate the enzyme after a

certain time, which causes impurities in the final product. A fungal protease encapsulated with nanoparticles was shown to be a simple, rapid and efficient method for generating peptides from casein. Also, the use of sonication enhanced the production of the peptides by shortening the process (Madadlou *et al.*, 2011). This study suggested it may help industrialization of enzymatic production of functional products from casein and milk. Microencapsulation is used to guarantee drug delivery in the pharmaceutical industry and these technologies could certainly be adapted for use in the dairy industry.

Membrane–based technology such as nanofiltration, ultrafiltration and diafiltration procedures are also utilized to produce ingredients which contain specific bioactive peptides based on casein or whey protein hydrolysates (Korhonen, 2009). Nanofiltration and ultrafiltration concentrate the protein and diafiltration generates whey protein concentrate (WPC) (Casado *et al.*, 2009). High throughput assays, for example Lux-tagging *C. sakazakii* so its growth can be easily monitored in IMF (Morrissey *et al.*, 2011), and a diverse array of expertise in the latest cutting-edge technologies are needed to enhance production of new milk derived antimicrobials.

A study design investigating the *in vitro* and *in vivo* models needs to be set up to validate health claims. This is imperative in order to satisfy the regulatory organizations. Regulatory issues are an important factor to consider in particular with reference to health claims. Finally, consumer acceptance is another issue of paramount importance. The health or wellness benefit must be very clear, convenient and contain fewer additives, taste must not be affected and price is also an issue.

6. Conclusion

This review discusses the potential applications of milk derived antimicrobials for the dairy industry. These include the areas of food safety and functional food. It is evident there are a considerable number of promising applications. However, further research into the feasibility of utilizing these antimicrobials is essential. In addition progress in the development of new technologies will enhance discovery and production of new milk derived antimicrobials for the dairy industry.

Thesis Introduction

This thesis was written with a view to publishing as many sections as possible. As several comprehensive reviews of Cronobacter spp. are available in the literature (Norberg et al., 2012, Yan et al., 2012 Healy et al., 2010), it was decided instead to focus the literature review on milk-derived antimicrobials. Short overviews of Cronobacter spp. and Lux-technology are also provided.

Introduction - 1; *Cronobacter* spp.

Cronobacter spp. (previously known as *Enterobacter sakazakii*) are Gram-negative, catalase positive, oxidase negative, motile by peritrichous flagellae, rod shaped, non-spore forming bacteria belonging to the *Enterobacteriaceae* family. This ubiquitous micro-organism has been associated with severe neonatal infections; these include meningitis, meningoencephalitis, sepsis, and necrotising enterocolitis. It is also associated with serious sequelae including brain abscess and impaired sight and hearing (Muytjens *et al*., 1983). The bacterium was first implicated in a case of neonatal meningitis in 1958 when an outbreak resulted in the death of two infants in England (Urmenyi *et al*., 1961). Although the frequency of infection generally tends to be low, the prognosis is poor with case-mortality rates varying from 33-80% among infected infants (Lai, 2001). Consequently, *Cronobacter* spp. have been ranked as a 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration' by the International Commission for Microbiological Specifications for Foods, which places this pathogen in the same grouping as *Listeria monocytogenes, Cryptosporidium parvum* and *Clostridium botulinum* types A and B (ICMSF).

Taxonomy

Cronobacter spp. was defined as 'yellow pigmented *Enterobacter cloacae*' until 1980, when it was designated a new species, *E. sakazakii* by Farmer *et al*., (1980). The reclassification was based on differences in DNA-DNA hybridization, biochemical reactions, pigment production and antibiotic susceptibility. Analysis of both partial 16S ribosomal DNA (rDNA) and *hsp60* housekeeping gene sequences revealed that *E. sakazakii* consists of at least four distinct clusters and it was hypothesized that clusters 2, 3 and 4 may represent original species (Iverson *et al*., 2004b). Based on DNA-DNA hybridisation and phenotyping, *E. sakazakii* was subsequently proposed to be reclassified into a new genus, *Cronobacter*, composed of six distinct species: *Cronobacter sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinesis* and a *Cronobacter* genomospecies group 1 (Iversen *et al*., 2008). Two new species were recently added to the genus; *C. universalis* and *C. condimenti*, which replaced the original genomospecies 1 (Joseph *et al*., 2012a).

Reservoir

The natural environment of *Cronobacter* is not known, but these appear to be widely distributed microorganisms. They have been isolated from a number of environments including hospitals, dust from households and food production lines and soil (Kandhi *et al*., 2004, Khan *et al*., 1998). In addition, *Cronobacter* has been isolated from foods such as fruits, vegetables, herbs, cereals grains, cheese, fermented bread, tofu, sour tea, cured meat, minced beef, sausage meat and water (Friedemann, 2007, Iverson *et al*., 2004c Farmer *et al*., 1985). Schmid *et al*., (2009) characterized two *C. sakazakii* isolates from plant roots and the root colonization behaviour of nine

Cronobacter strains originating from clinical and plant sources was assessed. The results indicated that plants may be the natural habitat of *Cronobacter* spp.

Growth in infant milk formula (IMF)

Powdered IMF has been identified as one of the major contamination sources and transmission vehicles. This in particular poses a serious risk of infection for immature neonates whose immune systems are not fully developed (Mullane *et al*., 2006). Low birth weight neonates (<2.5kg) and infants less than 28 days old are at increased risk compared to more mature infants (Caubilla-Barron *et al*., 2007). Definitive links between the presence of *C. sakazakii* in IMF and an outbreak of infection has been reported (CDC, 2002).

Infant formulae are pasteurised during manufacturing and *Cronobacter* does not survive such heat treatment (Nararowec-White *et al*., 1997b). However IMF is not sterile and contamination with *Cronobacter* can occur if strict microbiological standards are not maintained. Two main routes of IMF contamination are considered: external and intrinsic contamination. External contamination is caused by poor handling; e.g., the reconstitution of IMF using equipment contaminated with *Cronobacter*. In intrinsic contamination, the ingredients added during processing, drying or packaging are contaminated. It has been suggested that plant-originated IMF ingredients that are not heat treated are potential sources of contamination (Healy *et al*., 2010).

Voluntary recalls of IMF contaminated with *C. sakazakii* has occurred in the United States, Europe and Asia which has led health care facilities, manufactures and governing bodies to improve hygienic practices and maintain higher microbiological standards (Chenu *et al*., 2009).

Detection methods

Traditional methods for isolation and detection of *Cronobacter* from IMF have been shown to be quite time-consuming. Detection requires sequential growth steps in broth and selective agar media that can take 6-7 days to complete (FDA, 2002). There are also problems with specificity, especially when discriminating between *Cronobacter* spp. and other *Enterobacter* species (Lampel *et al*., 2009). Recently, the FDA has devised a new method for detection of *Cronobacter* in milk which is undergoing validation (Figure 1). This involves a real time PCR assay and chromogenic agars (Lampel *et al*., 2009). Lampel *et al*., (2009) tested the new method and found it drastically shortened the detection time to 24-48 hours. Sensitivity and specificity were also increased using the new method.

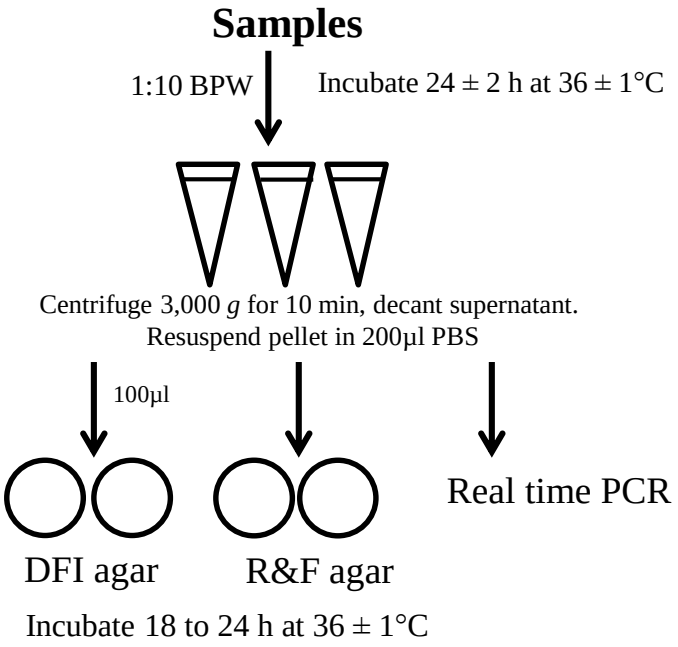


Fig 1. Schematic summary of new FDA method for isolating *Cronobacter* from IMF (adapted from Lampel *et al.*, 2009). BPW (buffered peptone water), PBS (phosphate buffered saline), DFI (Druggan, Forsythe, Iversen), R&F® (*Enterobacter sakazakii* Chromogenic Plating Medium).

Cronobacter spp. physiology

Cronobacter spp. have the capability to adhere to hydrophilic and hydrophobic surfaces, produce extracellular polysaccharides and engage in cell-to-cell signalling with other bacteria in the form of biofilm production. Dancer *et al.*, (2009b) illustrated *Cronobacter* spp. to be one of the most stress tolerant bacteria of all the *Enterobacteriaceae* with respect to pH, heat and low a_w. However, the stress

tolerance is strain and growth phase dependent. This innate stress tolerance may explain its presence in desiccated infant powder (Hunter *et al*., 2008). Iversen *et al*., (2004d) showed *Cronobacter* spp. grew between 6 to 45°C, with an optimal range of 37-43°C.

The bacterium has also been shown to persist under desiccated conditions in infant formula for over two years (Edelson-Mammel *et al*., 2005). Riedel *et al*., (2007) employed proteomics to identify differentially expressed proteins in response to two different osmotic stresses: desiccation and growth in hyperosmotic media. The results indicated that under high osmolarity conditions the central metabolic pathways, including amino acid biosynthesis and transport protein production, were shut down along with the downregulation of the motility apparatus. In contrast, an accumulation of structural proteins was observed which may function to preserve the proper functioning and integrity of the cell (Healy *et al.*, 2009).

Pathogenesis

Information is lacking regarding the mechanisms of *Cronobacter* spp. pathogenesis compared to other food borne pathogens. The exact infectious dose is unknown, but experiments with neonatal CD-1 mice by Richardson *et al*., (2009) demonstrated that the infectious dose for the strain used in that study was 10^2 CFU.

Similar to other foodborne pathogens the bacteria is ingested and eventually reaches the gastrointestinal tract. Infants have a higher gastrointestinal pH than adults (Maffei *et al*., 1975) and this may facilitate survival of *Cronobacter* (Pagatto *et al*., 2009). Following this the microorganism must translocate across the gastrointestinal epithelial cells. In mammalian tissue culture, the microorganism can attach to intestinal cells and survive internally in macrophages (Pagotto *et al*., 2003), therefore

permitting the bacteria to avoid host immune responses. However, the specific bacterial adhesions and host cell receptors involved in these processes are not known. Some strains of *C. sakazakii* produce capsular material but how this material contributes to macrophage evasion remains to be determined.

Similar to *Citrobacter diversus* or *Citrobacter freundii*, *Cronobacter* appears to have a tropism for the central nervous system. *C. sakazakii* have been shown to adhere and invade human brain microvascular endothelial cell lines (HBMEC), highlighting their ability to cross the blood brain barrier (Townsend *et al*., 2008), which could result in meningitis, formation of brain abscesses and other neurological complications.

Several studies have suggested that OmpA contributes substantially to the virulence potential of *Cronobacter* spp. (Kim *et al*., 2010, Mittal *et al*., 2009, Mohan Nair *et al*., 2007, Sinamsetty *et al*., 2008, Mohan Nair *et al*., 2007). Mohan Nair *et al*., (2009) described how OmpA binds fibronectin, facilitating the invasion of brain endothelial cells. Mittal *et al*., (2009) demonstrated that *ompA* expression affected the onset of meningitis in new born rats. OmpA positive *Cronobacter* isolates successfully crossed the intestinal barrier, multiplied in the blood and were able to subsequently transverse the blood brain barrier, whereas OmpA negative isolates could not bind to intestinal epithelial cells.

Genomics

The genome sequence of *C. sakazakii* BAA-894 was published in 2010 (Kucerova *et al.,* 2010). This revealed the genome is composed of one chromosome (4.36Mb) and two plasmids (pESAK2 31kb, 51% GC and pESAK3 131kb, 56% GC). Automated annotation of the genome identified 4392 genes covering 87% of the chromosome,

38 genes covering 83% of pESAK2 and 127 genes covering 87% of pESAK3. Two further *C. sakazakii* genome sequences have since been published; *C. sakazakii* E899 (Chen *et al*., 2011) and *C. sakazakii* ES15 (Shin *et al*., 2012). In addition, the genome sequence of *C. turicensis* has also been published (Stephan *et al*., 2011). Additional *C. sakazakii* genome sequencing projects are in progress (http://www.ncbi.nlm. nih.gov/genome/genomes/1170).

Sequencing of these genomes will aid identification of virulence genes. Kucerova *et al*., (2010) identified genes predicted to be involved in invasion of HBMEC. They found a copper and silver resistance efflux system unique to *Cronobacter* spp. but homologous to a copper and silver resistance efflux system in *E. coli*, which may allow bacteria to invade HBMEC (Franke *et al*., 2003). Genomic sequencing will also assist in the comparative genomics of *Cronobacter* strains of varying virulence. *Cronobacter* spp. differ in their virulence potential with regards to invasion of intestinal cells, survival in macrophages and serum resistance (Townsend *et al*., 2008, Townsend *et al*., 2007). Characterisation of the lipopolysaccharide (LPS) genes and resulting O-antigen could be significant in developing an identification system based on serotyping (Kucerova *et al*., 2010).

A multilocus sequencing typing scheme has been established for the entire *Cronobacter* genus (Joseph *et al*., 2012c). One sequence type of particular relevance is *C. sakazakii* ST4, which appears to have a high propensity for causing neonatal meningitis (Joseph *et al*., 2011). Joseph *et al*., (2012b) compared the genome sequences of the seven *Cronobacter* species. Sets of universal core genes and accessory genes unique to each strain were identified. They found *C. sakazakii* to be unique in the *Cronobacter* genus due the presence of genes encoding the utilization of exogenous sialic acid. However, further improvement in draft genomes is needed

to identify virulence traits of strains with similar modes of infection (Joseph *et al*., 2012b).

Treatment and antimicrobial resistance

C. sakazakii is naturally resistant to all macrolides, linomycin, clindamycin, streptogramins, rifampicin, fusidic acid and fosfomycin. It is susceptible to some antibiotics, including tetracyclines, aminoglycosides, several β-lactams, chloramphenicol, antifolates and quinolones (Stock *et al*., 2002). A combination of ampicillin and gentamycin or ampicillin and chloramphenicol has traditionally been used for the treatment of *Cronobacter* infection (Drudy *et al*., 2006). The acquisition of transposable elements, the production of β-lactamases and the presence of multiple antibiotic resistance genes may allow *C. sakazakii* to become resistant to ampicillin, gentamycin and chloramphenicol (Girlich e*t al*., 2001).

It is clear that *Cronobacter* spp. represents a serious risk to immature neonates fed with contaminated IMF. Future research on this pathogen should involve discovering approaches for limiting the presence of *Cronobacter* in IMF. This will include improving the detection and isolation methods, uncovering further information regarding its pathogenicity and finding inhibitors of *Cronobacter* spp.

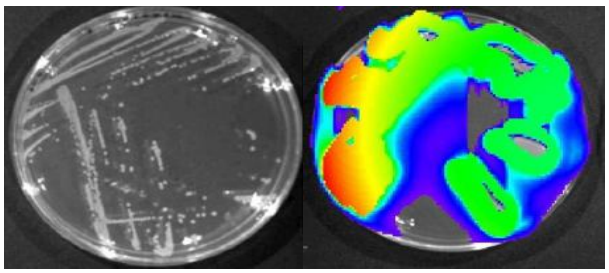
Introduction – 2; Lux-tagging

Bioluminescence is the manufacture of light by living organisms. The light emitted as a result of an enzymatic reaction catalyzed by the enzyme luciferase is encoded by *lux* genes. These genes have been identified in bacteria, fungi, crustaceans, jellyfish, worms and beetles (Widder, 2010). One of the most commonly employed bioluminescent systems is the Lux system from the Gram negative bacterium *Photorhabdus luminescens* (Waidmann *et al*., 2011). In the presence of oxygen the luciferase enzyme catalyzes the oxidation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde to yield flavin mononucleotide (FMN) and long chain fatty acid. This results in the emission of light at a wavelength of 490nm (Baker *et al*., 1992) (Figure 2A). The luciferase enzyme is encoded by *luxAB* genes and the fatty acid reductase complex for production of the aldehyde is encoded by the *luxCDE* genes (Meighen *et al*., 1991). These *lux* genes from *Photorhabdus luminescens* can be transferred and expressed in other bacteria. The resulting Lux-tagged bacteria emit light (Figure 2B).

A

$$FMNH_2 + O_2 + R\text{-}CHO \xrightarrow{\text{Luciferase (}lux\text{)}} FMN + R\text{-}COOH + H_2O \ (+ \text{LIGHT } 490nm)$$

B



Non Lux-tagged strain Lux-tagged strain

Fig 2. Schematic of the *lux* reaction (**A**). Non Lux-tagged and Lux-tagged strain on LB agar viewed with a Xenogen IVIS 100 Imager (**B**).

The production of reduced flavin mononucleotide requires a functional electron transport chain, therefore only metabolically active cells can emit light (Alloush *et al*., 2006). In addition, bioluminescent readings are instantaneous and the output can be measured in real time. This is an advantage as it provides a less labour intensive

and quicker method compared to traditional techniques such as performing viable plate counts.

Lux-tagged bacterial strains have many potential food related applications such as studying the microbial ecology of food to determine if they can support bacterial growth, investigating injury and survival of pathogenic and spoilage microorganisms, reporters of virulence gene expression (e.g. toxins) in food, detection of pathogens, ability to form biofilms and biocide efficiency, identifying probiotic bacteria capable of reaching the gastrointestinal tract and monitoring starter culture activity (Griffiths, 2000). It is evident Lux technology provides a major advantage to the food industry due to its fast, sensitive and real-time ability to monitor the presence of bacteria in food.

Aims of the thesis

The aims of this thesis were to characterise a bank of *Cronobacter* strains for their ability to tolerate to the sub-optimal conditions of the gastrointestinal tract. A second aim was to examine growth of the pathogen in milk, employing Lux technology. The third aim of this thesis was to create a transposon mutagenesis library in a Lux-tagged *C. sakazakii* strain and screen this library for mutants affected in growth in milk. The final aim was to identify genes involved in the tolerance of *C. sakazakii* to the milk derived antimicrobial peptide Lactoferricin B.

Chapter II

Tolerance of *Cronobacter* strains to physiologically relevant stress conditions

A manuscript based on this chapter is in preparation

Abstract

Food-borne pathogens must overcome many barriers in the human gastrointestinal tract in order to infect and cause disease. Some *Cronobacter* strains can cause opportunistic infections in infants, and powdered infant formula is considered to be the primary transmission vehicle. The aim of the present study was to examine the ability of a collection of *Cronobacter* strains to tolerate sub-optimal conditions that may be encountered in the gastrointestinal tract. Strains were exposed to low pH, bile and salt. While all strains were capable of tolerating physiologically relevant conditions, noteworthy variations in tolerance was observed. Antibiotic disk assays revealed that all strains were sensitive to the antibiotics traditionally used to treat *Cronobacter* infections (ampicillin, gentamycin and chloramphenicol).

Introduction

Cronobacter spp. (previously known as *Enterobacter sakazakii*) are a group of Gram-negative, motile, peritrichously flagellated, rod shaped, non-spore forming bacteria belonging to the *Enterobacteriaceae* family. They are catalase positive, oxidase negative, facultative anaerobes (Nazarowec-White *et al*., 1997a). Based on DNA-DNA hybridisation and phenotyping the genus *Cronobacter* was proposed to be composed of six distinct species; *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinesis* and a *Cronobacter* genomospecies group 1 (Iversen *et al*., 2008). A new species was recently added to the genus; *C. condimenti,* and *C. universalis* now replaces the original *Cronobacter* genomospecies group 1 (Joseph *et al*., 2012a). To date, only strains of *C. sakazakii, C. malonaticus* and *C. turicensis* have been associated with neonatal infection.

C. sakazakii is an opportunistic pathogen than can cause life threatening infections and powdered infant formula is considered to be the primary transmission vehicle. Outcomes of infection include meningitis, meningoencephalitis, sepsis, and necrotising enterocolitis (Muytjens *et al*., 1983). Although the incidence of *C. sakazakii* infection generally tends to be low, the prognosis is poor with case-mortality rates varying from 33-80% (Lai, 2001). The exact infectious dose is unknown, but experiments by Richardson *et al*., (2009) with neonatal CD-1 mice demonstrated that the infectious dose for the strain used in that study was 10^2 CFU.

To date, the majority of research related to survival of *C. sakazakii in vivo* has focused on adhesion and invasion of cell lines and its ability to cross the blood brain barrier (Mohan Nair *et al*., 2009, Mittal *et al*., 2008, Singamsetty *et al*., 2008,

Townsend *et al*., 2008, Mange *et al*., 2006). The ability of the organism to tolerate conditions encountered in the gastrointestinal tract has not been investigated in great detail. In order to survive in this environment *C. sakazakii* must endure an array of sub-optimal conditions. The low pH of the stomach is the initial major barrier encountered by the pathogen. Once in the intestine the organism encounters low oxygen levels, elevated salt conditions, intestinal bile salts and weak acids. While some studies that investigate the ability of *Cronobacter* to tolerate environmental stress are available in the literature, these usually use a collection of strains but focus on one stress condition (e.g. Osaili *et al.*, 2009) or use only a single *C. sakazakii* strain and examine several stresses (e.g. Hsiao *et al*., 2010). To our knowledge a single study that investigates the tolerance of a collection of *Cronobacter* strains to various physiologically relevant stresses is not available. Therefore, the aim of the present study was to address this lack of information using a collection of *Cronobacter* strains (thirteen *C. sakazakii* strains, one *C. malonaticus* and one *C. muytjensii*). For comparison purposes four *Enterobacter* strains (two *E. cloacae*, one *E. gergorviae* and one *E. aerogenes*) were also included in experiments. Antibiotic disk assays were carried out to examine the antibiotic tolerance of the strains.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains used in this study are listed in Table 1. *Cronobacter, Enterobacter, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa* and *Salmonella* Typhimurium strains were routinely grown in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) statically at 37°C. *Listeria monocytogenes* and *Listeria innocua* strains were grown in BHI (Brain Heart Infusion) (Oxoid, UK) statically at 37°C. *Lactococcus lactis* HP was grown in M17 broth (Oxoid) supplemented with 0.5% glucose (Sigma-Aldrich) statically at 30ºC. For certain experiments Brilliance *Enterobacter sakazakii* (DFI, Druggan, Forsythe and Iversen) agar (Oxoid, UK), TSA (Tryptic Soya Agar) (Oxoid), Mueller Hinton (Oxoid) broth, Mueller Hinton agar and M9 (minimal media) (Sambrook *et al*., 2001) were used. For solid media 1.5% agar was added.

DNA extraction and manipulations

Strains were grown overnight (~16 hrs) in LB broth. DNA was extracted from 1ml of overnight cultures as described by Hoffmann *et al*., (1987). PCR products were purified using the Invitrogen PCR purification kit. The concentration of nucleic acids was determined using an Invitrogen Qubit fluorometer (Eugene, Oregon, USA). PCR amplification was carried out using a G-storm cycler (Essex, UK). PCR products were separated on 1% (m/v) agarose gels and visualised with the DNR Bio-Imaging System (Jerusalem, Israel).

Table 1. Strains used in this study.

Strain		Isolated from	Source	Growth conditions
DPC6522*	*Cronobacter sakazakii*	Blood	DPC	LB at 37°C
DPC6523*	*Cronobacter sakazakii*	CSF	DPC	LB at 37°C
DPC6524*	*Cronobacter sakazakii*	Stool	DPC	LB at 37°C
DPC6525*	*Cronobacter sakazakii*	Urine	DPC	LB at 37°C
DPC6526*	*Cronobacter sakazakii*	Blood	DPC	LB at 37°C
DPC6527*	*Cronobacter sakazakii*	Blood	DPC	LB at 37°C
DPC6528*	*Cronobacter sakazakii*	CSF	DPC	LB at 37°C
DPC6529*	*Cronobacter sakazakii*	Tracheal aspirate	DPC	LB at 37°C
DPC6530*	*Cronobacter sakazakii*	Bronchial alveolar lavage	DPC	LB at 37°C
DPC6531*	*Cronobacter malonaticus*	Brain Tumour	DPC	LB at 37°C
ATCC51329	*Cronobacter muytjensii*		DPC	LB at 37°C
NCTC8155	*Cronobacter sakazakii*	Tin of dried milk powder	DPC	LB at 37°C
ATCC12868	*Cronobacter sakazakii*		DPC	LB at 37°C
ATCC29004	*Cronobacter sakazakii*		DPC	LB at 37°C
ATCC29544	*Cronobacter sakazakii*	Child's throat	DPC	LB at 37°C
NCTC11434	*Enterobacter gergorviae*	Human urinary tract	DPC	LB at 37°C
NCTC11590	*Enterobacter cloacae*	unknown	DPC	LB at 37°C
NCTC11933	*Enterobacter cloacae*	Human/clinical isolate	DPC	LB at 37°C
NCTC10006	*Enterobacter aerogenes*	sputum	DPC	LB at 37°C
LO28	*Listeria monocytogenes*		UCC	BHI at 37°C
EGDe	*Listeria monocytogenes*		UCC	BHI at 37°C
HP	*Lactococcus lactis*		UCC	GM17 at 30ºC
	Listeria innocua		UCC	BHI at 37°C
O157:H7	*Escherichia coli*		UCC	LB at 37°C
DH5-α	*Escherichia coli*		UCC	LB at 37°C
	Bacillus cereus		UCC	LB at 37°C
	Pseudomonas aeruginosa		UCC	LB at 37°C
UK-1	*Salmonella* Typhimurium		UCC	LB at 37°C

Strains were obtained from the DPC, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland and UCC, University College Cork culture collections.

*These strains were originally obtained from M. Ardino, Centers for Disease Control and Prevention, Atlanta, United States. These strains were originally isolated as being *C. sakazakii* strains. *rpoB* genes were sequenced to confirm their correct identity.

rpoB sequencing

PCRs were performed on ten *C. sakazakii* strains using *rpoB* primers (Table 2). PCR products were purified with the Invitrogen PCR purification kit and sequenced by MWG Biotechnologies (Ebersberg, Germany). Homology searches were performed using BLAST on the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.gov).

Oxidase test

An oxidase test was carried out on isolated colonies that were freshly grown overnight on TSA. An oxidase strip (Oxoid) was laid on the moist lid of the petri dish. A loopful of each strain was transferred to a strip. A positive result was indicated by the appearance of a deep purple colour within 10-30 seconds. Lack of a colour change indicated the colonies were oxidase negative. *Pseudomonas aeruginosa* (oxidase positive) and *E. coli* DH5-α (oxidase negative) were employed as controls.

Pigment production

The production of a yellow pigment was examined by streaking strains onto TSA and incubating at 25°C for 48-72 hrs (FDA, 2002). The presence of pigment genes was examined by PCR using primers based on the *ESA_03347* gene (NC_009778.1) that has been shown to be involved in pigment production (Johler *et al*., 2010). Primer sequences are provided in Table 2.

Bacterial capsule formation

The presence of capsule genes was examined by PCR using primers based on *ESA_03359* (NC_009778.1) a homologue of *kpsM* which is involved in capsule biosynthesis (Healy *et al.*, 2009). Primer sequences are provided in Table 2.

Table 2. Primers and PCR conditions used in this study.

Primer	Sequence 5'→3'	Size of PCR product
ESA_03347 F	CATGGATAACGCCCTGCT	259 bp
ESA_03347 R	AACTGCGAAGTTTATGGCG	
ESA_03359 F	GTATTGAGCGAATACATCACG	206 bp
ESA_03359 R	GCGCTGCTGGCCATGACA	
ESA_01288 F	AGCGTCTGTCCTCTGGTCTGC	271bp
ESA_01288 R	CAGACGCTGAGTGATTTCCG	
rpoB F	AACCAGTTCCGCGTTGG	760bp
rpoB R	CCTGAACAACACGCTCGGA	

Catalase test

Strains were grown overnight on TSA agar and a loopful of colonies was transferred onto a glass slide. Two drops of H_2O_2 (hydrogen peroxide) (35% w/v) were added, the presence of bubbling indicated the strain was catalase positive. *L. lactis* HP (catalase negative) and *E. coli* DH5-α (catalase positive) were used as controls.

Motility assay

Motility was examined at 37°C using motility medium (tryptone 10g/l (Sigma Aldrich, Germany), NaCl 5g/l, and agar 5g/l) (Iversen *et al.*, 2007). The media was stab inoculated with 5µl of overnight culture and incubated at 37°C for 48 h. The swimming behaviour of the cells was inspected visually and the zones of growth on

the plates were measured. Any zone bigger than 10 mm was considered positive. The presence of flagella genes was examined by PCR using primers based on *ESA_01288/fliC* (NC_009778.1) a putative flagellin gene.

Protease assay

Strains were grown overnight in LB broth and streaked onto skim milk agar i.e. LB agar supplemented with 1% (w/v) reconstituted skim milk. LB agar was autoclaved at 121°C for 15 min, while 10% (w/v) milk powder solution was autoclaved at 110°C for 10 min. The autoclaved milk solution was mixed with LB agar to a final concentration of 1% while still hot (Jones *et al*., 2007). Streaked plates were incubated for 3 days at 37°C. Any strain giving a clear halo was considered positive. *Bacillus cereus* was used as a positive control and *E. coli* DH5-α was used as a negative control.

Gram stain

Gram staining was performed on the strains where Gram positive bacteria stain purple and Gram negative bacteria stain pink. A loopful of overnight culture was stained with 1% crystal violet, washed with H_2O, subsequently flooded with Gram's iodine, washed with 95% alcohol and counterstained with safranin. *E. coli* DH5-α (Gram negative) and *Listeria innocua* (Gram positive) were used as controls.

H_2O_2 assay

The sensitivity of each of the strains to H_2O_2 (hydrogen peroxide) (35% w/v) was tested. Strains were grown overnight in LB broth, diluted 1:10 in ¼ strength Ringer's solution (Merck, Darmstadt, Germany) and swabbed onto LB agar with sterile swabs. Sterile paper disks were subsequently placed on the surface of the plates.

10µl of H_2O_2 was pipetted onto each disk. The plates were incubated overnight at 37°C and zones of inhibition were measured with a digital Vernier Calipers (Fisherbrand).

Growth curves and survival assays

Strains were grown in triplicate in LB broth and incubated overnight (~16 hrs) at 37°C. 1ml of cultures were centrifuged at 8000 x *g* for 7 min, the supernatants were removed and cell pellets were resuspended in 1ml of ¼ strength Ringer's solution (Merck, Darmstadt, Germany). 20µl of washed cells was added to 1ml LB broth, vortexed to mix and 200µl was transferred to a well of a 96-well plate (Genetix, UK). Growth at 37°C was monitored by measuring optical density (OD) at 620nm using a temperature controlled automatic plate reader (Thermo Scientific Multiscan FC). When required, viable plate counts were performed by serially diluting cultures in ¼ strength Ringer's solution and enumeration on LB agar. Growth curves were performed in LB adjusted to pH 5.5, LB adjusted to pH 6.5, LB containing 0.3M NaCl (Sigma Aldrich, Germany), LB containing 5% NaCl and M9 (minimal media). The pH of the medium was lowered using 5M Lactic acid and the pH was determined using a pH meter (ORIEN 3 STAR, Thermo Scientific, Singapore). The pH was adjusted prior to autoclaving at 121°C for 15 min. A growth curve was performed in LB under anaerobic conditions using a GasPak 100 anaerobic system (BD, Sparks, MD, USA) and Anaerocult A sachets (Merck). Growth in LB containing 0.3% porcine bile (Sigma Aldrich, Germany) was also examined. Porcine bile was added before autoclaving at the required level and viable plate counts were taken after 3 h by serially diluting cultures in ¼ strength Ringer's solution and enumeration on LB agar.

For survival assays, strains were grown statically at 37°C for approximately 16 hrs. 1ml of overnight cultures were centrifuged at 8000 x *g* for 7 min, the supernatants were discarded and the cell pellets were resuspended in 1ml ¼ strength Ringer's solution. 20µl was added to 1ml of test broth and incubated statically at 37°C for 1 h. Viable plate counts were performed by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar in triplicate. Test broths included LB supplemented with 5% porcine bile, LB supplemented with 10% NaCl and LB adjusted to pH 3.2. LB broth containing bile was vortexed well before and after autoclaving at 121°C for 15 min. When required, the pH of the medium was adjusted using HCl (Hydrochloric acid) (1M) before autoclaving. Results are presented as % survival, this was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero, i.e. immediately prior to treatment.

Antimicrobial susceptibility assay

A disk diffusion assay was carried out following the instructions of the Clinical and Laboratory Standards Institute (CLSI, 2005). The antimicrobial susceptibility test disks (Oxoid, UK) included amoxicillin/clavulanic acid (30µg), ampicillin (10µg), cefotaxime (30µg), ceftriaxone (30µg), chloramphenicol (30µg), ciprofloxacin (5µg), gentamycin (10µg), nalidixic acid (30µg), kanamycin (30µg), tetracycline (30µg), cefalothin (30µg), trimethoprim-sulfamethoxazole (25µg) and cefuroxime (30µg). *Cronobacter* strains were streaked on TSA (Tryptic Soya Agar) and incubated at 37ºC for 24 hrs. Colonies were suspended in 3ml of Mueller Hinton broth. The suspensions were swabbed onto the entire surface of Mueller Hinton agar with sterile swabs after which the relevant antimicrobial disks were placed onto the surface of the plate with a sterile forceps. The plates were incubated upright at 37ºC

for 24 hrs under aerobic conditions. The diameters of zones of inhibition were measured using a digital Vernier Callipers (Fisherbrand) and interpreted according to the CLSI guidelines for *Enterobacteriaceae* (*Staphylococcus* spp. for erythromycin) (Clinical and Laboratory Standards Institute 2005).

Results and Discussion

rpoB sequencing

The *Cronobacter* and *Enterobacter* strains used in this study are listed in Table 1. Of the *Cronobacter* strains, five were obtained from culture collections and ten were isolated from clinical samples. In order to confirm that the clinical strains were indeed *Cronobacter* spp., *rpoB* genes were partially sequenced as performed by Stoop *et al*., (2009) and homology searches were performed using BLAST (Table 3). Of the ten strains, nine were identified as *C. sakazakii* and one was identified as *C. malonaticus* (DPC6531).

Table 3. *rpoB* sequencing of the ten clinical *Cronobacter* strains. Homology searches were performed using BLAST on the National Centre for Biotechnology Information (NCBI) website.

Strain	Identity	Accession Number
DPC 6522	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6523	100% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6524	100% to *C. sakazakii* ATCC 25944	FJ717657
DPC 6525	100% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6526	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6527	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6528	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6529	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6530	99% to *ESA_03690 C. sakazakii* BAA-894	CP000783.1
DPC 6531	100% to *C. malonaticus* LMG23826	FJ717659

Characterisation tests

As expected, all of the strains were Gram negative, oxidase negative and catalase positive. When PCRs were performed with primers for the pigment gene (*ESA_03347*), products were amplified for all 15 *Cronobacter* strains. When plated onto TSA one of these strains, DPC6523 did not produce a yellow pigment. It is possible that the amount of pigment produced by DPC6523 is too low to be detected by visual inspection or that pigment is not produced by this strain under the conditions tested. As expected, the four *Enterobacter* strains (NCTC11434, NCTC11590, NCTC11933 and NCTC10006) did not produce yellow pigment on TSA agar and PCR products were not amplified with *ESA_03347* primers.

When PCRs were performed with primers for the capsule gene (*ESA_03359*), products were amplified for 14 of the 15 *Cronobacter* strains. Products were not amplified for strains ATCC29544 (Table 4). Two of our strains have previously been examined for capsule production by Hurrell *et al*., (2009). In agreement with the findings of that study we obtained a PCR product for ATCC12868 but not for ATCC29544. PCR products were not amplified for any of the *Enterobacter* strains with the *ESA_03359* primers.

PCR products were amplified with primers for the motility gene (*ESA_01288*) for the 15 *Cronobacter* strains. 14 of the *Cronobacter* strains were positive on motility agar, ATCC51329 appeared to be non-motile on motility agar. This suggests that motility genes may not be expressed for this strain under the conditions used. For the four *Enterobacter* strains, PCR products were not amplified for NCTC10006 and phenotypic examination of two strains (NCTC11590 and NCTC10006) suggested

they were non motile on motility agar. This indicates NCTC10006 may be non-motile as it was negative for the molecular and phenotypic tests.

A clear halo was obtained for all *Cronobacter* strains when streaked onto skim milk agar suggesting that they may be protease positive. Halos were not observed for any of the four *Enterobacter* strains. Strains were streaked on Brilliance *Enterobacter sakazakii* agar (DFI) (Oxoid) which contains 5-bromo-4-chloro-3-indolyl-α-D-glucopyranoside, a compound that *Cronobacter* can hydrolyse with the enzyme α-glucosidase to produce blue/green colonies (Iversen *et al*., 2004a). On DFI agar all the *Cronobacter* strains produced blue/green colonies but the four *Enterobacter* strains did not. Iversen *et al*., (2004a) showed that none of the 31 *E. cloacae* strains and six *E. aerogenes* strains tested in their study pigmented blue/green on DFI.

Table 4. Characterisation of the *Cronobacter* and *Enterobacter* strains used in this study.

Strains	Gram stain	Oxidase	Catalase	Capsule *ESA_03359*[a]	Pigment production		Motility		Protease production	Colony colour on DFI agar
					TSA at 25°C	*ESA_03347*[b]	Motility agar	*ESA_01288*[c]		
DPC 6522	−	−	+	+	+	+	+	+	+	Blue green
DPC 6523	−	−	+	+	−	+	+	+	+	Light green
DPC 6524	−	−	+	+	+	+	+	+	+	Blue green
DPC 6525	−	−	+	+	+	+	+	+	+	Blue green
DPC 6526	−	−	+	+	+	+	+	+	+	Blue green
DPC 6527	−	−	+	+	+	+	+	+	+	Blue green
DPC 6528	−	−	+	+	+	+	+	+	+	Blue green
DPC 6529	−	−	+	+	+	+	+	+	+	Blue green
DPC 6530	−	−	+	+	+	+	+	+	+	Blue green
DPC 6531	−	−	+	+	+	+	+	+	+	Blue green
ATCC51329	−	−	+	+	+	+	−	+	+	Blue green
NCTC8155	−	−	+	+	+	+	+	+	+	Blue green

ATCC12868	−	−	+	+	+	+	+	+	+	Blue green
ATCC29004	−	−	+	+	+	+	+	+	+	Blue green
ATCC29544	−	−	+	−	+	+	+	+	+	Blue green
NCTC11434	−	−	+	−	_	−	+	+	−	Yellow
NCTC11590	−	−	+	−	−	−	−	+	−	Yellow
NCTC11933	−	−	+	−	−	−	+	+	−	Yellow
NCTC10006	−	−	+	−	−	−	−	−	−	Yellow

[a]= Result of PCR performed with *ESA_03359* primers.

[b]= Result of PCR performed with *ESA_03347* primers.

[c]= Result of PCR performed with *ESA_01288* primers.

DFI= Brilliance *Enterobacter sakazakii* agar (Druggan Forsythe Iversen).

pH conditions

Following consumption, the acidity of the stomach is the first major barrier encountered by pathogens. The fasting gastric pH of the adult stomach is 1.5, which increases between 3.0 and 5.0 during feeding (Hill *et al*., 2002). Neonates are likely to exhibit higher gastric pH levels than adults, often reaching values above pH 4.0 (Maffei *et al*., 1975). A range of pHs may also be encountered in the intestinal lumen (Kitagawa *et al*., 1966).

In order to investigate the pH tolerance of strains a growth curve was performed in LB adjusted to pH 5.5 (Fig. 1a). All strains were capable of growth but variation in generation times was observed amongst the strains ranging from 27.36 min for DPC6524 to 42.59 min for ATCC29544 (see also Fig 2a and 2b). The shortest overall generation time was 21.32 min for the *Enterobacter* strain NCTC10006. Generation times for the remaining three *Enterobacter* strains ranged from 29 to 32.66 min. A growth curve was also performed in LB adjusted to pH 6.5 which also showed variation between the strains (Fig 2c). When exposed to low pH (LB adjusted to pH 3.2, Fig. 1b) a variation in survival was observed for *Cronobacter* strains; the lowest survival rate was 1.89% for ATCC51329 and the highest survival rate was 72.29% for DPC6529. The *Enterobacter* strain NCTC10006 which had the shortest generation time for growth at pH 5.5 also displayed the highest tolerance at pH 3.2 as cell numbers were unaffected. Survival of the remaining *Enterobacter* strains ranged from 4% to 46%. Dancer *et al.*, (2009b) demonstrated that *C. sakazakii* was more acid tolerant than most closely related enteric pathogens. Variability in acid tolerance between the strains was also noted. Survival of other gastrointestinal pathogens (*L. monocytogenes, E. coli* and *S. Typhimurium*) at pH 3.2 was also investigated (Fig. 7a). The majority of strains were more acid sensitive than

the other the gastrointestinal pathogens tested in this study. Strain DPC6529, and the *Enterobacter* strains NCTC11590 and NCTC1006 had similar survival rates to the gastrointestinal pathogens tested.

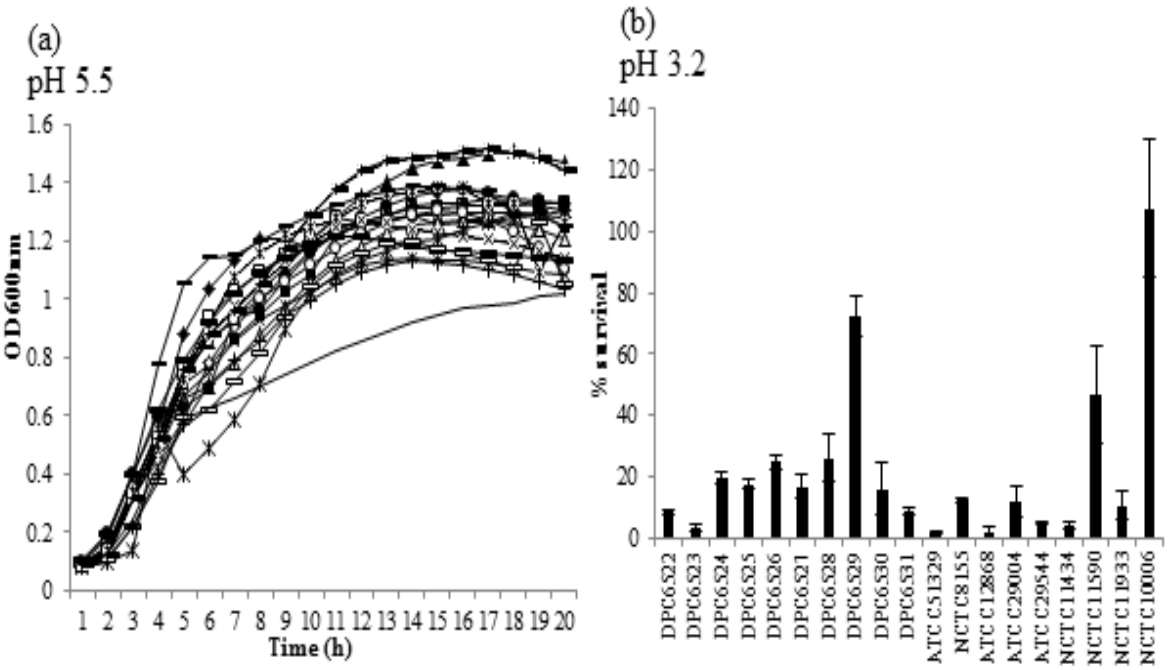


Fig. 1 (a) Growth of strains in LB adjusted to pH 5.5 DPC6522 (■) DPC6523 (□) DPC6524 (♦) DPC6525 (◊) DPC6526 (▲) DPC6527 (Δ) DPC 6528 (●) DPC6529 (○) DPC6530 (X) DPC6531 (⊠) ATCC51329 (▬), ATCC8155 (▭) ATCC12868 (+) ATCC29004 (⊞) ATCC29544 (═) NCTC11434 (⁕), NCTC11590 (⊠) NCTC11933 (▄) and NCTC10006 (▭). The presented graphs are representative of three independent experiments. Error bars were omitted for clarity **(b) Survival of strains in LB adjusted to pH 3.2.** Viable plate counts were performed following one hour incubation by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar. % survival was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero, i.e. immediately prior to treatment.

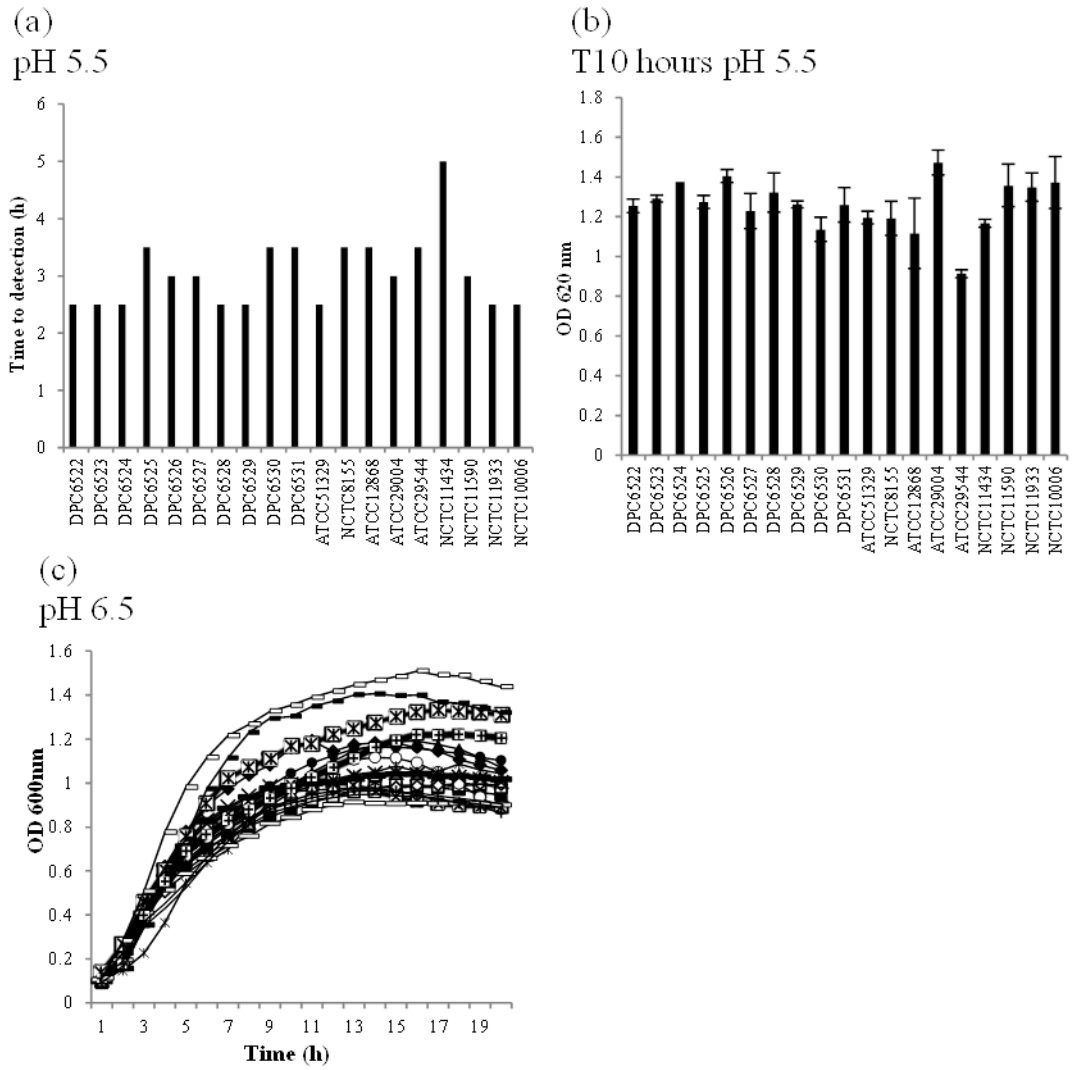


Fig. 2 (a) Time to detection (TTD) in LB adjusted to pH5.5. TTD is the time (h) at which the culture reaches an OD_{620nm} of 0.5. **(b)** OD_{620nm} at T10 hours in LB adjusted to pH5.5. **(c) Growth of strains in LB adjusted to pH 6.5.** DPC6522 (■), DPC6523 (□), DPC6524 (♦), DPC6525 (◊), DPC6526 (▲), DPC6527 (Δ), DPC6528 (●), DPC6529 (○), DPC6530 (X), DPC6531 (⊠), ATCC51329 (▬), ATCC8155 (▭), ATCC12868 (+), ATCC29004 (⊞), ATCC29544 (=), NCTC11434 (✳), NCTC11590 (⊠), NCTC11933 (▭) and NCTC10006 (▬). The presented graph is a representative of three independent experiments. Error bars were omitted for clarity.

NaCl

Gastrointestinal pathogens must tolerate exposure to high osmolarity. Growth of strains in LB containing 0.3M (1.7%) NaCl was investigated as this concentration is considered an approximate surrogate for the level encountered in the upper small intestine (Sleator *et al*., 2007). Fig. 3a shows that all strains were capable of growth, however, differences were observed between the strains. For the *Cronobacter* strains the generation times ranged from 23.9 min for DPC6522 to 37.9 min for DPC6531. Of all of the strains, *Enterobacter* strain NCTC11933 had the longest generation time of 42.1 min. The generation times for the other *Enterobacter* strains ranged from 28 min to 34.95 min (see also Fig. 4a and 4b). Growth in LB containing 5% NaCl was also investigated (Fig. 4c). All strains were able to grow under this condition but there was disparity in growth between each of the strains. When exposed to 10% NaCl a variation in survival rates was observed between the strains (Fig. 4b). For the *Cronobacter* strains, the lowest overall survival rate was 19.7% for ATCC12868 and the highest survival rate was 72.92% for DPC6530. For the *Enterobacter* strains survival ranged from 36% to 57%. Overall, the *C. sakazakii* strains displayed a higher level of salt tolerance than concentrations approximating those that may be encountered in the small intestine. The survival capabilities of the majority of the collection of strains were similar to the other gastrointestinal pathogens tested (*L. monocytogenes, E. coli* and *S. Typhimurium*; Fig. 7b).

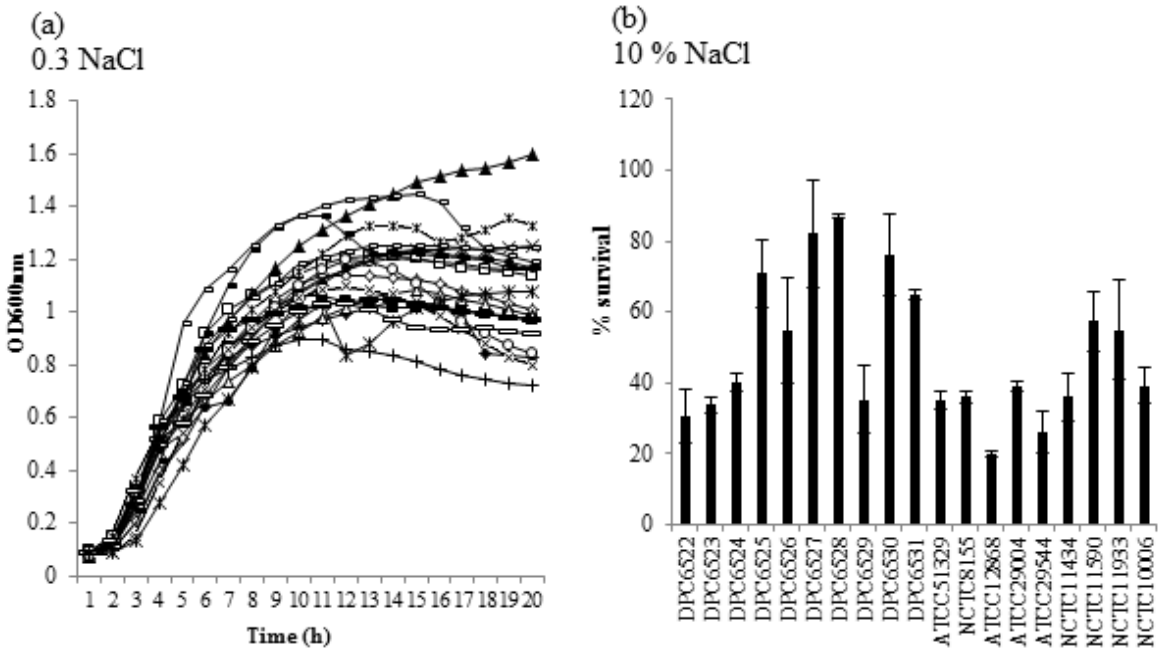


Fig. 3 (a) Growth of strains in LB containing 0.3M NaCl. DPC6522 (■), DPC6523 (□), DPC6524 (♦), DPC6525 (◊), DPC6526 (▲), DPC6527 (Δ), DPC 6528 (●), DPC6529 (○), DPC6530 (X), DPC6531 (⊠), ATCC51329 (▬), ATCC51329 (▬), ATCC8155 (▭), ATCC12868 (+), ATCC29004 (⊞), ATCC59244 (=), NCTC11434 (✳), NCTC11590 (⊠), NCTC11933 (■) and NCTC10006 (▭). The presented graph is a representative of three independent experiments. Error bars were omitted for clarity. (b) **Survival of strains in LB containing 10 % NaCl**. Viable plate counts were performed following one hour incubation by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar. % survival was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero.

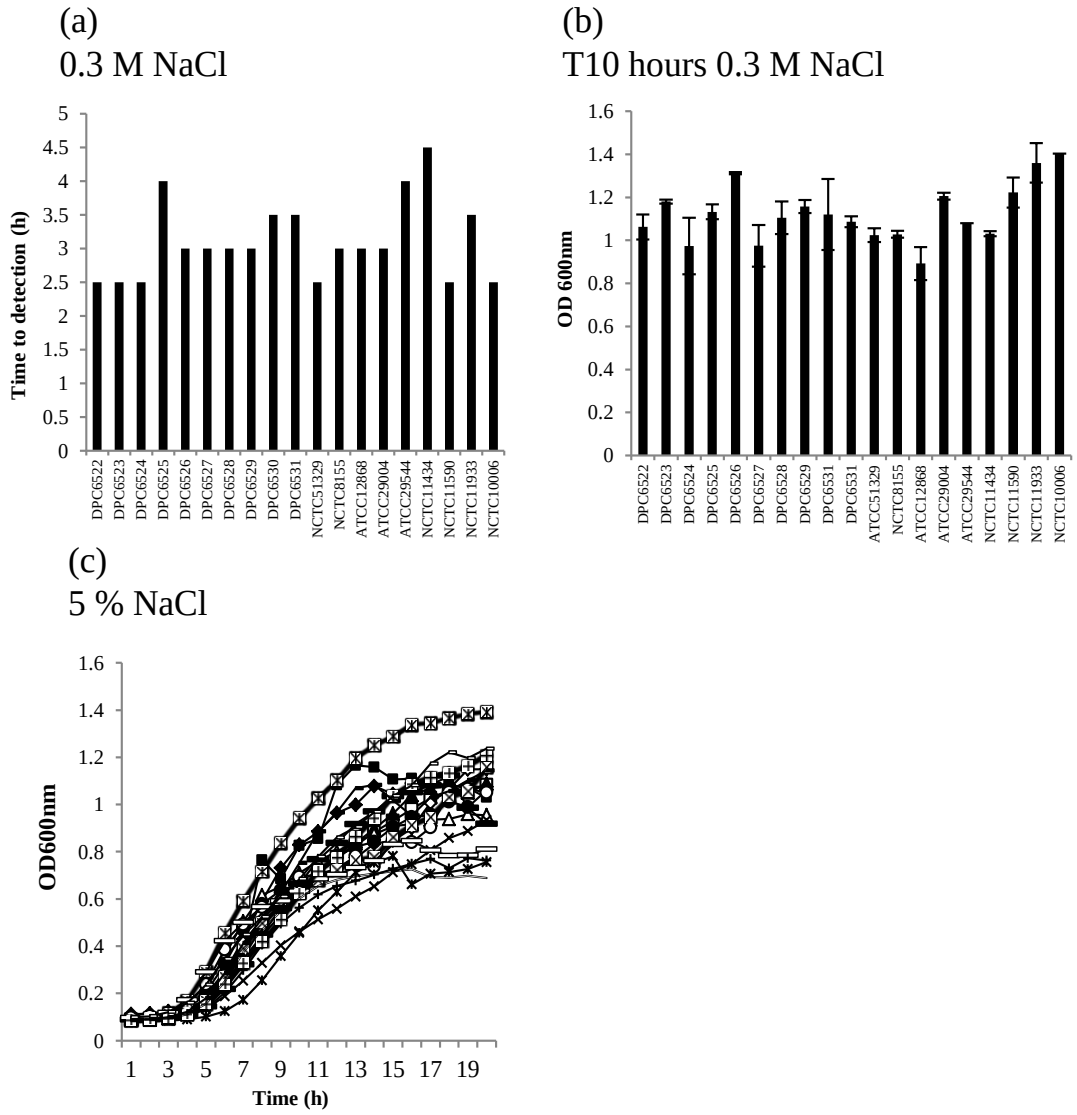


Fig. 4 (a) Time to detection in LB containing 0.3M NaCl. TTD is the time (h) at which the culture reaches an OD_{620nm} of 0.5. **(b)** OD_{620nm} at T10 hours in LB containing 0.3M NaCl. **(c) Growth of strains in LB plus 5% NaCl.** DPC6522 (■), DPC6523 (□), DPC6524 (♦), DPC6525 (◊), DPC6526 (▲), DPC6527 (Δ), DPC 6528 (●), DPC6529 (○), DPC6530 (X), DPC6531 (⊠), ATCC51329 (▬), ATCC8155 (▭), ATCC12868 (+), ATCC29004 (⊞), ATCC29544 (=), NCTC11434 (✳), NCTC11590 (⊠), NCTC11933 (▬) and NCTC10006 (▭). The error bars represent the standard deviation of triplicate experiments.

Bile conditions

Bile is a digestive secretion that plays a key role in the emulsification and solubilisation of lipids (Begley *et al*., 2005). It functions as part of the body's defence system as bile salts have potent antimicrobial activity through their ability to emulsify the phospholipids, cholesterol and proteins of cell membranes causing cells to lyse. Therefore the ability of pathogens to tolerate bile is essential for their survival and colonization (Begley *et al*., 2005). Initially strains were streaked on agar containing 0.3% porcine bile (Sigma); this is a concentration which was chosen to approximate the average levels of bile encountered *in vivo* (Begley *et al*., 2005). All strains grew on agar containing 0.3% porcine bile with colony morphologies similar to growth on LB agar alone. Growth of the *Cronobacter* strains in LB broth supplemented with 0.3% bile after 3 h was also investigated. All strains were capable of growth; after 3 h the increase in log cfu/ml ranged from a 1.08 log increase for DPC6523 to a 1.63 log increase for DPC6524 (Fig. 5a). For the *Enterobacter* strains the lowest overall increase in log cfu/ml was 0.46 for NCTC11434, the numbers of the remaining *Enterobacter* strains increased by ~1.5 log.

When exposed to LB containing 5% porcine bile there was variation in survival rates between all the strains (Fig. 5b). For the *Cronobacter* strains the lowest survival rate was 24.98% for DPC6524. However, cell numbers were not affected for DPC6527 and DPC6528. Survival of other gastrointestinal pathogens (*L. monocytogenes, E. coli* and *S. Typhimurium*) in LB containing 5% porcine bile was also investigated (Fig 7c). The survival capabilities of the majority of the collection of strains were similar to the gastrointestinal pathogens. To our knowledge there is only one study available in the literature that has examined the bile tolerance of *C. sakazakii*. As part of a larger study, Hsiao *et al*., (2010) showed that the *C. sakazakii* strain under

investigation (strain BCRC13988) was capable of growth in 0.5% and 2% bile solution (oxgall) over a 12 hour period. In our experiments, variation in bile tolerance was also observed for the *Enterobacter* strains tested; survival ranged from 36% to 66.6% following exposure to 5% porcine bile.

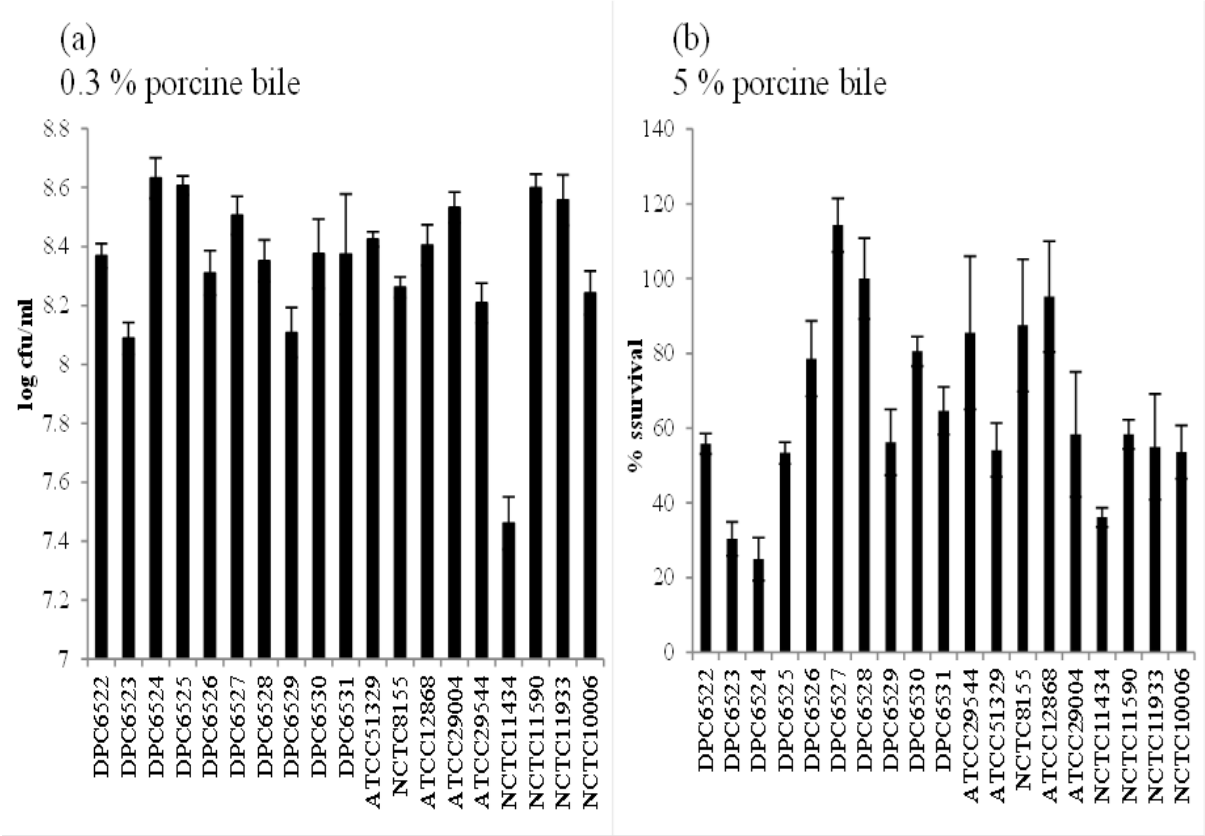


Fig. 5 (a) Growth of strains in LB containing 0.3% bile. Plate counts were performed after three hours. (b) **Survival of strains in LB containing 5% porcine bile**. Viable plate counts were performed following the one hour incubation by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar. The error bars represent the standard deviation of triplicate experiments % survival was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero.

Other physiologically relevant stress conditions

In M9 (minimal media) variation in growth of strains was observed (Fig 6a). We also compared growth both aerobically and anaerobically (Fig 6b). The absence of O_2 affected the progress of the strains with an OD_{620nm} approximately 50% that of the same strains grown anaerobically. For the H_2O_2 assay the strains were inhibited at various levels with zones ranging from 37-45.23mm (Table 5).

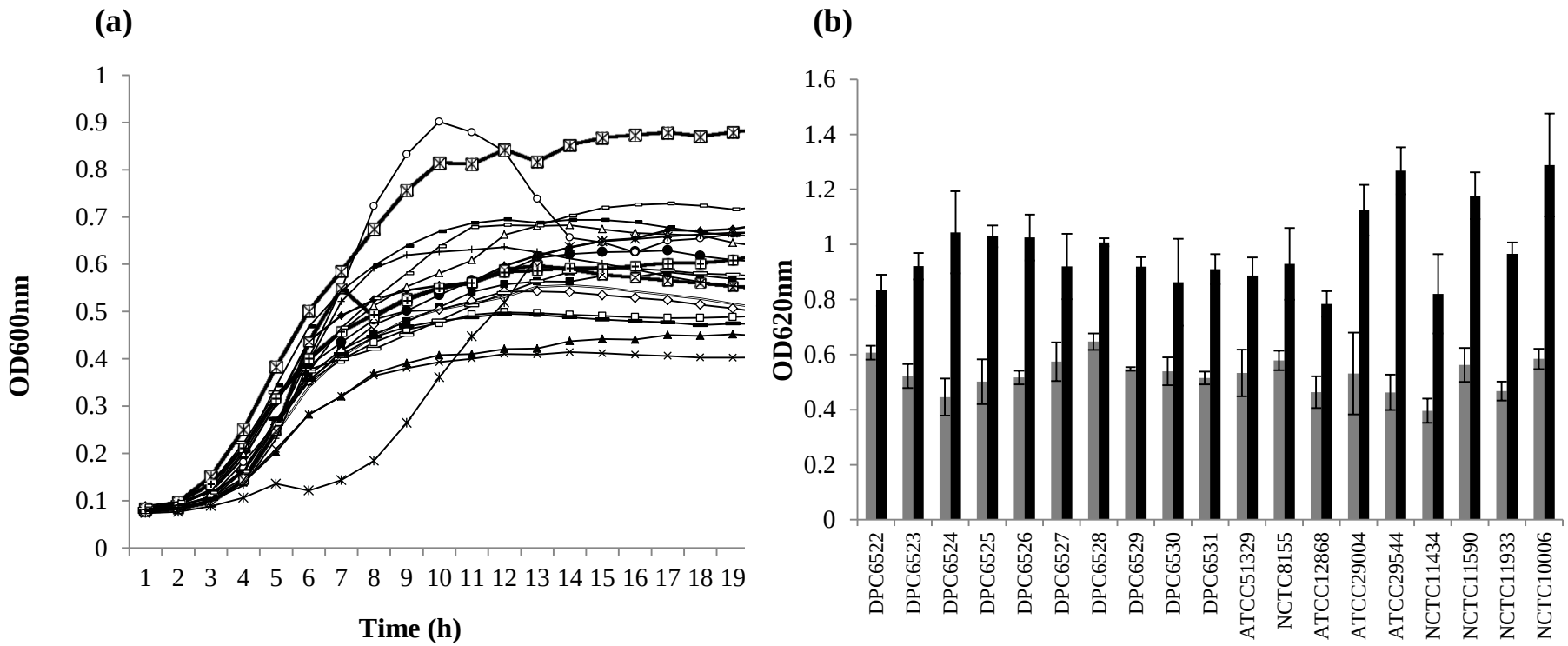


Fig. 6 (a) Growth of strains in M9 minimal media. DPC6522 (■), DPC6523 (□), DPC6524 (♦), DPC6525 (◊), DPC6526 (▲), DPC6527 (Δ), DPC 6528 (●), DPC6529 (○), DPC6530 (X), DPC6531 (⊠), ATCC51329 (▬), ATCC8155 (▭), ATCC12868 (+), ATCC29004 (⊞), ATCC29544 (=), NCTC11434 (✳), NCTC11590 (⊠), NCTC11933 (■) and NCTC10006 (▭). The presented graphs are representative of three independent experiments. Error bars were omitted for clarity. **(b) Growth of strains incubated aerobically (■) and anaerobically (■).** OD_{620nm} was taken after 16 hour incubation at 37°C. The error bars represent the standard deviation of triplicate experiments.

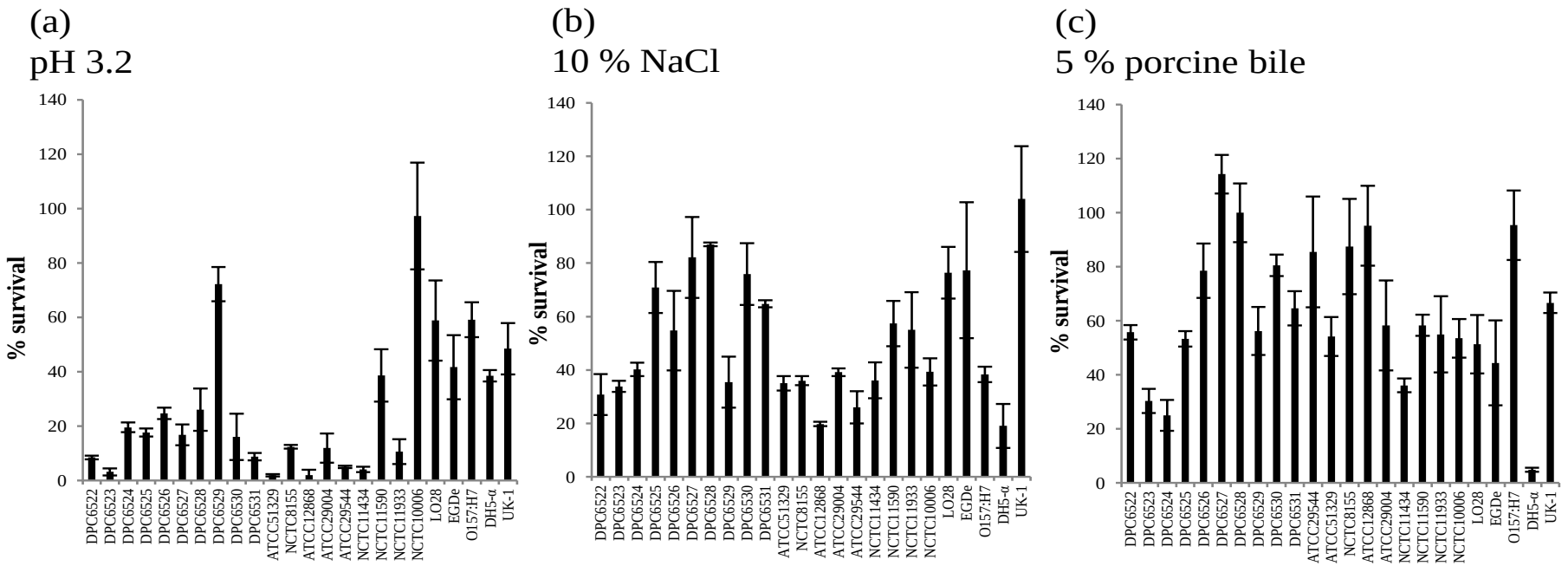


Fig. 7. Survival of *Cronobacter* strains, *L. monocytogenes* LO28, *L. monocytogenes* EGDe, *E. coli* O157:H7, *E. coli* DH5-α, and *Salmonella enterica* serovar Typhimurium UK-1 **(a)** in LB adjusted to pH 3.2 **(b)** LB plus 10 % NaCl and **(c)** LB plus 5% porcine bile**.** Viable plate counts were performed following the one hour incubation by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar. % survival was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero.

Table 5. H_2O_2 disk assays. Zones were measured using a digital Vernier callipers. Results are in millimetres, and expressed as averages of triplicate experiments ± the standard deviation.

Strains	Zone size (mm)
DPC6522	37.5±0.3
DPC6523	38.3±0.4
DPC6524	37.6±0.9
DPC6525	38.0±3.8
DPC6526	39.9±1.1
DPC6527	37.3±2.1
DPC6528	40.7±0.3
DPC6529	37.8±2.8
DPC6530	41.2±2.4
DPC6531	37.0±1.9
ATCC51329	36.5±0.9
NCTC8155	46.1±1.4
ATCC12868	37.4±1.2
ATCC29004	40.8±0.2
ATCC29544	38.6±1.1
NCTC11434	45.2±0.9
NCTC11590	42.1±1.1
NCTC11933	43.0±1.2
NCTC10006	38.6±3.0

Antimicrobial disk assays

A combination of ampicillin and gentamycin or ampicillin and chloramphenicol has traditionally been used for the treatment of *C. sakazakii* infection (Drudy *et al*., 2006). The acquisition of transposable elements, the presence of β-lactamases and the incidence of numerous antibiotic resistance genes may allow *C. sakazakii* to display resistance to ampicillin, gentamycin and cefotaxime (Girlich e*t al*., 2001). Therefore it is important to investigate the use of other antimicrobials for the treatment of *C. sakazakii* infection. The results of the antibiotic disk assays of the strains are listed in Table 6. All of the *Cronobacter* strains were classified as sensitive to ampicillin. Three *Enterobacter* strains (NCTC11590, NCTC11933 and NCTC10006) displayed resistance to ampicillin according to the CLSI standards. All strains were sensitive to gentamycin. None of the *C. sakazakii* strains tested showed resistance to ampicillin or gentamycin and DPC6528 was the only *Cronobacter* strain to have intermediate sensitivity to chloramphenicol. Therefore the traditional treatment employed for *C. sakazakii* infection would be satisfactory for these strains. The other antibiotics tested were trimethoprim-sulfamethoxazole, ceftriaxone, cefotaxime, tetracycline, nalidixic acid, cefalothin, cefuroxime, ciprofloxacin, amoxicillin-clavulanic acid and kanamycin (Table 2). *E. cloacae* NCTC11590 displayed resistance to ampicillin and amoxicillin-clavulanic acid and intermediate sensitivity to three other antimicrobials. *E. cloacae* have an intrinsic resistance to ampicillin and exhibit a high-frequency of mutation of resistance to extended spectrum and broad spectrum cephalosporins (Then, 1987).

All of the *Cronobacter* isolates tested by Stock *et al*., (2002) displayed resistance to macrolides, linomycin, clindamycin, streptogramins, rifampicin, fusidic acid and fosfomycin. The study also found the *Cronobacter* strains were susceptible to some

antibiotics, including tetracyclines, aminoglycosides, several β-lactams, chloramphenicol, antifolates and quinolones. Kim *et al*., (2008) tested 113 *E. sakazakii* isolates for their antibiotic resistance and 31.8% were resistant to ampicillin and all of the isolates were resistant to at least one antibiotic. Al-Nabulsi *et al*., (2011) illustrated the *C. sakazakii* strains they tested were sensitive to gentamycin, kanamycin, ciprofloxacin and amoxicillin but displayed resistance to tetracycline. This paper noted strain variation in their antimicrobial susceptibilities, our study also illustrated differences between strains.

Table 6. Antibiotic disk assay. Zones were measured using a digital Vernier callipers. Results are in millimetres, and expressed as averages of triplicate experiments ± the standard deviations.

Strains	AMP 10µg	CN 10µg	CTX 30µg	TE 30µg	NA 30µg	C 30µg	STX 25µg	KF 30µg	CXM 30µg	CIP 5µg	AMC 30µg	K 30µg	CRO 30µg
DPC 6522	27.4±2.0	17.8±0.6	27.2±4.2	21.9±0.3	22.0±0.7	24.7±1.0	30.0±1.0	14.3±0.4*	22.6±0.4	25.0±1.3	28.4±1.5	16.5±1.7*	25.2±0.6
DPC 6523	20.4±0.7	22.4±0.2	29.7±1.4	22.6±0.9	28.3±0.5	28.4±1.0	31.7±0.9	11.3±0.6*	20.5±0.1	20.8±1.0	25.0±1.0	17.6±0.9	22.9±1.3
DPC 6524	26.4±0.3	18.2±0.9	31.0±1.1	31.00±1.1	18.2±0.8	22.7±2.2	33.8±0.5	24.1±0.2	20.4±0.9	21.7±0.4	29.7±0.6	16.8±0.7*	20.8±2.6
DPC 6525	21.5±0.2	16.6±1.0	28.3±0.5	28.3±0.5	24.9±0.5	24.4±1.43	31.9±0.9	25.4±0.4	20.9±0.2	17.6±0.3*	26.0±0.5	16.1±0.3*	21.0±0.9
DPC 6526	22.4±1.4	18.3±0.9	26.9±1.0	22.2±0.6	18.3±0.9	24.2±1.9	28.4±0.9	24.8±1.0	21.5±0.1	22.4±0.7	24.4±0.6	16.5±0.4*	24.7±0.4
DPC 6527	26.8±0.3	19.4±0.3	27.0±1.0	24.3±1.3	24.5±1.0	25.0±1.7	29.1±1.3	26.3±0.3	20.5±0.4	21.3±1.0	29.0±0.9	16.4±1.2*	24.7±0.3
DPC 6528	16.2±0.8	19.2±0.7	29.4±0.5	18.6±2.1	20.9±0.9	16.6±1.4*	24.9±1.4	22.6±0.4	17.8±0.6	17.8±0.4*	27.2±0.2	16.0±0.2*	24.7±0.7
DPC 6529	17.0±0.4	18.6±1.1	27.4±0.6	25.0±0.3	24.4±0.0	23.4±0.2	23.9±0.06	22.9±1.1	18.1±1.1	20.5±2.4	23.0±0.2	16.4±0.8*	23.6±1.3
DPC 6530	19.7±0.2	17.6±1.2	29.0±0.9	21.1±1.7	21.2±0.6	20.2±1.0	25.3±2.0	22.8±1.1	19.4±0.5	21.0±1.4	24.3±0.1	17.3±0.9	26.1±0.9
DPC 6531	21.6±0.8	18.4±1.7	29.7±1.1	19.9±2.33	22.6±1	25.0±1.0	32.4±0.4	26.5±1.1	21.4±0.5	25.3±1.4	29.3±0.8	18.8±1.2	24.5±1.2
ATCC51329	27.9±0.8	18.4±1.0	30.6±1.0	22.5±1.0	19.4±1.3	21.0±0.3	30.9±0.8	23.3±0.4	24.5±1.0	27.9±1.2	30.9±0.5	20.3±0.7	27.0±0.1
NCTC8155	22.4±0.5	19.3±0.9	33.8±1.4	23.3±1.2	25.3±0.7	26.4±1.5	27.8±0.3	29.1±0.8	25.2±0.1	29.4±0.9	25.4±0.7	18.6±0.6	27.4±2.2
ATCC12868	21.7±0.9	18.6±0.5	29.8±1.3	24.6±2.0	27.6±0.5	23.5±1.2	26.0±0.3	26.0±0.4	22.3±0.1	35.8±1.0	24.6±0.8	18.9±0.8	26.2±0.6
ATCC29004	21.3±0.8	18.0±0.4	29.1±0.7	20.3±0.0	21.0±1.3	23.3±1.5	24.6±1.4	21.9±0.7	21.9±0.4	30.4±0.8	25.1±0.7	18.8±0.1	28.5±0.5
ATCC25944	26.8±1.3	20.7±1.1	32.4±0.9	26.4±0.0*	24.0± 0.7	23.2±0.3	28.5±1.3	27.2±0.3	22.8±0.6	30.8±0.8	29.7±0.3	19.9±0.6	29.4±0.8
NCTC11434	17.5±0.9	19.8±0.4	18.3±1.0*	22.7±0.5	13.5±0.5*	17.7±1.2	23.4±0.6	23.0±0.4	16.4±0.3*	29.5±0.5	20.0±0.1	21.2±1.2	20.2±1.1
NCTC11590	**5.9±0.0**	17.3±1.0	22.8±0.3	16.1±0.6*	20.9±0.5	24.5±0.4	22.1±2.2	14.8±0.4*	23.3±0.6	25.4±0.4	**9.4±0.2**	15.6±0.8*	20.6±0.5
NCTC11933	**8.6±1.1**	17.6±0.8	29.4±2.2	15.4±1.7*	22.5±0.9	19.3±0.6	25.5±0.2	20.8±0.9	18.5±0.2	27.3±0.6	**9.9±0.3**	16.4±0.8*	24.5±1.1
NCTC10006	**5.9±0.0**	15.8±0.6	28.2±2.0	16.6±0.4*	20.9±0.2	22.7±1.3	21.3±0.4	17.1±0.6*	18.3±1.6	23.4±0.9	**12.6±0.2**	16.3±0.5*	27.1±0.2

AMP=ampicillin, CN=gentamycin, CTX=cefotaxime, TE=tetracycline, NA=nalidixic acid, C=chloramphenicol, STX=trimethoprim-sulfamethoxazole, KF=cefalothin, CXM=cefuroxime, CIP=ciprofloxacin, AMC=amoxicillin-clavulanic acid, K=kanamycin, CRO=ceftriaxone

Zone sizes were interpreted according to the CLSI guidelines for Enterobacteriaceae (CLSI, 2005). Results classified as "resistance" are highlighted in bold and those classified as "intermediate sensitivity" are indicated by an asterix.

In summary, the present study was initiated to examine the capability of sixteen *Cronobacter* strains to tolerate the physiologically relevant conditions of the gastrointestinal tract (low pH, salt and bile). While all strains were able to tolerate the conditions examined, noteworthy variations in tolerance were observed. It is therefore important that future studies aimed at investigating the mechanisms used by *Cronobacter* to survive within the gastrointestinal tract should include a collection of strains and not just focus on a single strain.

Chapter III

Investigation of the use of a cocktail of Lux-tagged *Cronobacter* strains for monitoring growth in infant milk formulae

Claire Flaherty, Máire Begley and Colin Hill

Manuscript submitted

Abstract

The objective of the present study was to create a collection of Lux-tagged *Cronobacter* strains to determine whether bioluminescence could be used to monitor growth in infant milk formulae (IMF). Nine *Cronobacter* strains (seven *C. sakazakii*, one *C. malonaticus* and one *C. muytjensii*) were transformed with plasmid p16S*lux* and integration of the plasmid at the desired site on the chromosome was confirmed by PCR. The integrated plasmid was shown to be stable in the absence of antibiotic selection and growth of the Lux-tagged strains was similar to their non-tagged counterparts. Growth of Lux-tagged strains was monitored in real-time in ten commercial brands of IMF by measuring light emission using a luminometer. While all of the IMF tested were able to support the growth of the *Cronobacter* strains, differences were observed between the brands. We also noted variations in the amount of light emitted by individual *Cronobacter* strains. Monitoring light emission by a combination of two strains that produced higher and lower than average RLU readings was found to be a good surrogate for the entire collection of Lux-tagged strains.

Introduction

Cronobacter is a genus within the family Enterobacteriaceae and consists of seven species; *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinesis*, *C. universalis* and *C. condimenti* (Iversen *et al*., 2008, Joseph *et al*., 2012a). *Cronobacter* spp. have been isolated from a variety of environments and foods including water (Farmer *et al*., 1985), soil (Khan *et al*., 1998) dust from households and food production lines (Khandhi *et al*., 2004), fruits, vegetables, herbs, cereals and grains (Friedemann, 2007).

C. sakazakii is an opportunistic infant pathogen that causes necrotizing enterocolitis, sepsis and meningitis (Chenu *et al*., 2009). Although the frequency of infection generally tends to be low, the prognosis is poor with case-mortality rates varying from 33-80% (Lai, 2001). Powdered infant milk formula (IMF) is thought to be the primary transmission vehicle of *C. sakazakii* (Healy *et al*., 2010). IMF is not manufactured as a sterile product but conforms to international microbiological specification guidelines. According to the Codex Alimentarius Commission (CAC) guidelines issued in 2008 each batch of IMF must be tested to ensure that it is free from *C. sakazakii* by testing thirty 10g samples (CAC). However, *C. sakazakii* has been isolated from retailed IMF by several research groups. For example, Iversen *et al*., (2004c) investigated the presence of *Cronobacter* in IMF and other food products and isolated the bacterium from 2 of 82 IMF tested (2.4%). Nazarowec-White *et al*., (1997b) analyzed 120 cans of IMF from 5 different companies in Canada and found 6.7% contained *Cronobacter* spp. Muytjens *et al*., (1998) isolated *Cronobacter* from 20 of 141 IMF (14%) tested. It has been noted that *Cronobacter*

spp. are heterogeneously distributed throughout batches of IMF (Jogenburger *et al*., 2011) which may explain why *Cronobacter* may not always be detected by IMF manufacturers prior to sale.

Schmid *et al*., (2009) suggest that plants may be the natural habitat of *Cronobacter* spp. and that plant-derived IMF supplements (e.g. starches and proteins), particularly those that are not subjected to an additional heating step, are potential sources of contamination in the factory setting. Furthermore equipment employed in the IMF manufacturing facility (dryers, bag filling platform, packing vacuums, blenders, production and particularly air filters) are also possible sources of contamination (Mullane *et al*., 2008, Mullane *et al*., 2007).

IMF manufacturers may be interested in developing novel compositions or ingredients that limit or inhibit the growth of *C. sakazakii.* As IMF is opaque, bacterial growth cannot be examined by measuring optical density and is usually analyzed by performing direct plate counts (Nair *et al*., 2004) or indirectly by measuring pH changes (Chang *et al*., 2009) or alterations in electrical impedance (Yang *et al*., 2008). These processes can be time-consuming and laborious. Morrissey *et al*., (2011) transformed a *C. sakazakii* strain with luciferase (*lux*) genes using plasmid p16S*lux* and monitored growth of the bacterium in milk and infant formulae by measuring bioluminescence. Reproducible results were obtained in real-time even when low initial inoculums were used. Light could also be detected in the presence of competing bacteria. Overall the study suggests that this method of following growth of *C. sakazakii* offers advantages to the conventional methods that are routinely used and may be utilized in high-throughput studies aimed at identifying anti-*C. sakazakii* agents or developing novel IMF compositions (Morrissey *et al*., 2011).

Similar to other bacterial genera, not all strains of *C. sakazakii* are genetically or phenotypically identical (Caubilla-Barron *et al*., 2007, Healy *et al*., 2009, Ye *et al*., 2011). Therefore, it is important that studies aimed at developing novel IMF should not focus solely on one strain but should include a collection of strains from different sources. These strains must be individually Lux-tagged if the previously mentioned bioluminescence-based experiments are to be employed. As *Cronobacter* strains are not naturally competent, methods such as electroporation or $CaCl_2$ transformation are required to transform strains with plasmids. Differences in outer cell structure of *Cronobacter* strains (e.g. the presence of a capsule) may affect the uptake of plasmids. It is therefore possible that not all strains may be as easy to manipulate as the strain employed by Morrissey *et al*., (2011) or that plasmid p16S*lux* may not be stable in all strains.

The primary aim of this study was to Lux-tag a collection of *Cronobacter* strains from a variety of environmental sources and determine if their growth could be monitored in IMF by measuring bioluminescence. A second aim was to examine whether growth of a cocktail of strains would be a good indicator of the average growth of *Cronobacter* strains in IMF.

Materials and Methods

Bacterial strains and culture conditions

The *Cronobacter* strains used in this study are listed in Table 1. Strains were routinely grown in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) statically at 37°C. For solid media 1.5% agar was added. Lux-tagged *Cronobacter* strains were grown with shaking at 37ºC in LB broth supplemented with 500µg/ml erythromycin. *Escherichia coli* DH10B::p16S*lux* was grown at 30°C shaking in LB supplemented with 500µg/ml erythromycin. Erythromycin (Sigma, Germany) was made up as a concentrated stock in ethanol and added to cooled autoclaved media. For certain experiments Brilliance *Enterobacter sakazakii* (DFI, Druggan, Forsythe and Iversen) agar (Oxoid, UK) was used.

Table 1. Bacterial strains used in this study.

Strain		Relevant Information	Source/ reference
DH10B::p16S*lux*	*E.coli*	Erythromycin resistant	Riedel *et al*., 2007
NCTC8155	*Cronobacter sakazakii*	Isolated from a tin of dried milk powder	
DPC6522	*Cronobacter sakazakii*	Isolated from blood	M Ardino
DPC6526	*Cronobacter sakazakii*	Isolated from blood	M Ardino
DPC6527	*Cronobacter sakazakii*	Isolated from blood	M Ardino
DPC6529	*Cronobacter sakazakii*	Isolated from tracheal aspirate	M Ardino
DPC6531[a]	*Cronobacter malonaticus*	Isolated from a brain tumor	M Ardino
ATCC51329[b]	*Cronobacter muytjensii*		
ATCC12868	*Cronobacter sakazakii*		
ATCC29004	*Cronobacter sakazakii*		
ATCC29544	*Cronobacter sakazakii*	Isolated from a child's throat	
NCTC8155::p16S*lux*	*Cronobacter sakazakii*		Morrissey *et al*.,
DPC6522::p16S*lux*	*Cronobacter sakazakii*		This study
DPC6526::p16S*lux*	*Cronobacter sakazakii*		This study
DPC6527::p16S*lux*	*Cronobacter sakazakii*		This study
DPC6529::p16S*lux*	*Cronobacter sakazakii*		This study
DPC6531::p16S*lux*	*Cronobacter malonaticus*		This study
ATCC51329::p16S*lux*	*Cronobacter muytjensii*		This study
ATCC12868::p16S*lux*	*Cronobacter sakazakii*		This study
ATCC29004::p16S*lux*	*Cronobacter sakazakii*		This study
ATCC295445::p16S*lux*	*Cronobacter sakazakii*		This study

[a]This strain was classified as *C. sakazakii* but *rpo*B sequencing revealed it was a *C. malonaticus.*

[b]This strain was deposited as *C. sakazakii* but was re-classified as *C. muytjensii* by Iversen *et al*., (2008).

All strains were obtained from the DPC, Dairy Products Research Centre, Moorepark, Fermoy, Co. Cork, Ireland. Strains DPC6522, DPC6526, DPC6527, DPC6529 and DPC6531 were originally obtained from M. Ardino, Centers for Disease Control and Prevention, Atlanta, United States.

Creation of competent *Cronobacter* cells and electroporations

Strains were grown statically overnight in LB for approximately 16 hrs. A 1% inoculum was transferred to fresh LB broth and incubated with shaking for approximately 3 hrs at 37°C until an OD_{620nm} of ~0.6 was reached. Cultures were chilled on ice for 15 min after which they were centrifuged at 900 x *g* for 10 min at 4°C. The supernatants were discarded, and the cells were resuspended in ice cold sterile distilled water and re-centrifuged. This was repeated three times, after which the cells were resuspended in 10% ice cold glycerol and re-centrifuged. The supernatants were removed and cells were resuspended in 500µl of 10% glycerol. 50µl aliquots were transferred to cold sterile 1.5ml microcentrifuge tubes and stored at -80°C. For electroporations, 2µl of p16S*lux* plasmid preparation (~1µg) was added to 50µl of competent cells in a 2mm cuvette (Molecular BioProducts, San Diego CA). Cells were shocked using a Harvard Apparatus ECM 630 Electro Cell Manipulator (MA, USA) with settings of 2.5kV, 200Ω and 25µF. The cells were resuspended in 1ml LB and incubated shaking at 30°C for 1 h. Transformants were selected by plating onto LB agar containing erythromycin (500µg/ml). Plates were incubated at 30°C for 48 hrs. Colonies were checked for light emission using the Xenogen IVIS100 imaging system (Xenogen, Alameda, CA). Bioluminescence was measured in relative light units (photons/sec/cm) with a binning of sixteen and an exposure time of one min. In order to integrate the plasmid into the chromosome, strains were grown overnight in LB containing 500µg/ml erythromycin at 30°C, diluted 1:1000 into fresh LB containing erythromycin and grown overnight at 42°C. Dilutions were subsequently plated on LB agar containing 500µg/ml erythromycin and incubated at 42°C overnight.

Molecular biology experiments

Plasmid p16S*lux* was extracted from its *E. coli* host strain using the Invitrogen Quick plasmid miniprep kit. It was subsequently alcohol precipitated using Pellet Paint Co-Precipitant (Novagen, Merck). Integration of p16S*lux* into the chromosome of *Cronobacter* strains was confirmed by PCR using primers 16S_fwd_new (ACACTGGAACTGAGACACGGTCCAGACTCC) and 16S_int_rev (TTGTAAAACGACGGCCAGTGAGCGCGCG). Amplification of the target region was achieved with 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 min. PCR amplification was carried out using a G-storm cycler (Essex, UK). A 1,163-bp PCR product was amplified when p16S*lux* was integrated at the correct site in the chromosome. The amplified PCR products were separated on 1% (m/v) agarose gels and visualized with the DNR Bio-Imaging System (Jerusalem, Israel).

Monitoring growth of *Cronobacter* strains in LB broth by measuring optical density and in IMF by measuring bioluminescence

Strains were grown in triplicate in LB broth and incubated overnight (~16 hrs) at 37°C. Cells were centrifuged at 8000 x *g*, washed and resuspended in 1ml ¼ strength Ringer's solution (Merck, Darmstadt, Germany). 20µl of washed cells was added to 1ml of LB broth, vortexed to mix and 200µl was transferred to a 96-well plate (Genetix, UK). Growth was monitored by measuring optical density (OD) at 620nm using a temperature controlled automatic plate reader (Thermo Scientific Multiscan FC). Viable plate counts were performed at selected time points by serially diluting samples in ¼ strength Ringer's solution and enumeration on LB agar. Ten brands of IMF were purchased in a local supermarket. IMF was aseptically weighed and added to sterile distilled water (10% wt/vol) and mixed by swirling manually for 5 min. In

order to monitor bioluminescence, a 2% inoculum of washed Lux-tagged cells was added to fresh IMF and 200µl of this was transferred to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). Bioluminescence was measured every hour in relative light units (RLU) using a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). When viable plate counts were required, cultures were serially diluted in ¼ strength Ringer's solution and enumerated on DFI agar.

Plasmid stability

The Lux-tagged *Cronobacter* strains were grown overnight (~16 hrs) in duplicate in LB broth containing erythromycin (500µg/ml) at 37°C. 1ml of overnight was centrifuged at 8000 x *g* for 7 min, washed and resuspended in 1ml ¼ strength Ringer's solution (Merck, Darmstadt, Germany). 200µl was transferred into 10ml of fresh LB broth without antibiotic. Tubes were incubated at 37°C for approximately 8 hrs after which 200µl of culture was transferred into 10ml of fresh broth and incubated at 37°C overnight (~16 hrs). This passaging was repeated for a further three days. At every passage 100µl aliquots were serially diluted in ¼ strength Ringer's solution and 100µl was spread onto LB agar. Plates were incubated at 37°C. 50 random colonies from each passage were scored for antibiotic resistance by patching them onto LB agar and LB agar supplemented with 500µg/ml erythromycin.

Results

Growth of *C. sakazakii* NCTC8155::p16S*lux* in IMF

Growth of the Lux-tagged *C. sakazakii* strain employed by Morrissey *et al*., (2011) NCTC8155::p16S*lux* was examined in ten different brands of IMF. As previously observed, growth of the strain could be easily monitored by measuring bioluminescence. Variations in RLU readings were observed amongst the different brands of IMF (Fig. 1). For example at T24 hrs the readings obtained for IMF7 was five times higher than IMF3 (102.1 RLU versus 18.16 RLU, respectively).

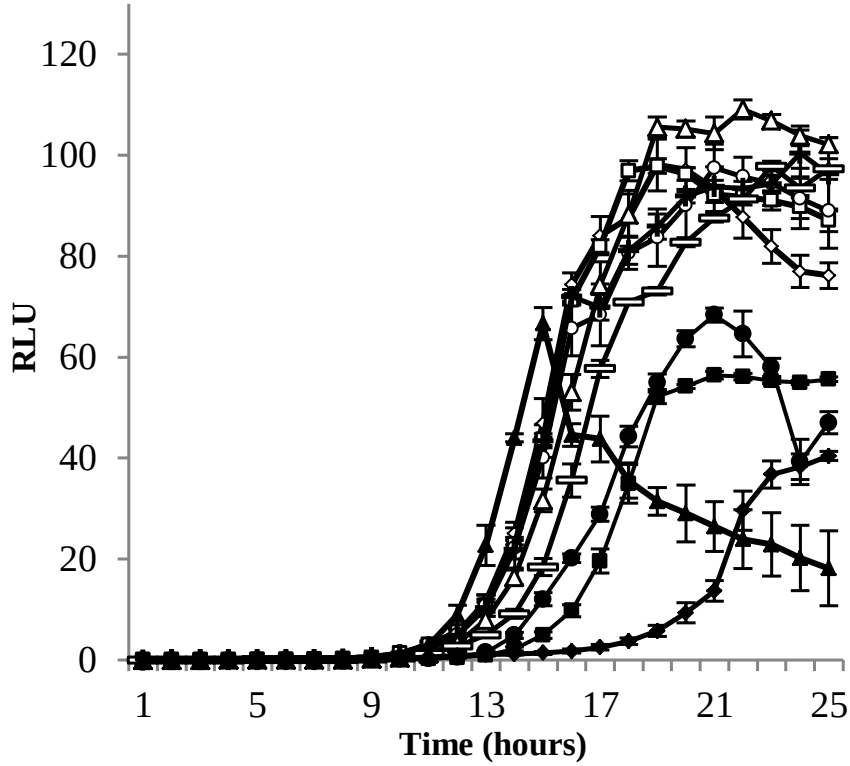


Figure 1. Growth of NCTC8155::p16S*lux* in ten different powdered infant formulae: IMF1 (◊), IMF2 (□), IMF3 (▲). IMF4 (●), IMF5 (○), IMF6 (+), IMF7 (Δ), IMF8 (■), IMF9 (▭) and IMF10 (♦). The initial inoculum was approximately $1x10^4$ cfu/ml with RLU taken over a 24 h period at 37°C. Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Creation and analysis of Lux-tagged *Cronobacter* strains

Plasmid p16S*lux* was used to Lux-tag nine strains of *Cronobacter* (seven *C. sakazakii*, one *C. malonaticus* and one *C. muytjensii*) (Table 1). This plasmid contains the Lux operon derived from *Photorhabdus luminescens* under the control of a constitutive promoter, P_{help} (highly expressed *Listeria* promoter) (Fig. 2A). It facilitates homologous recombination based integration into a 16S rRNA gene of Gram negative bacteria (Riedel *et al*., 2007). After initial transformation with p16S*lux*, bacteria were maintained under permissive conditions (30°C), which allowed for plasmid replication. Changing to the non-permissive temperature (42°C) selects for strains in which p16S*lux* integrates into the bacterial chromosome. Using primers based on the integration site, PCR amplification of a 1,163 bp product in the Lux-tagged strains confirmed integration of p16S*lux* into the correct location (Fig. 2B). Lux-tagged strains emitted light when viewed with the IVIS100 imaging system confirming expression of the *lux g*enes. The Lux-tagged *C. sakazakii* strain created in Morrissey *et al*., (2011) is also included (NCTC8155::p16S*lux*) (Fig. 2C).

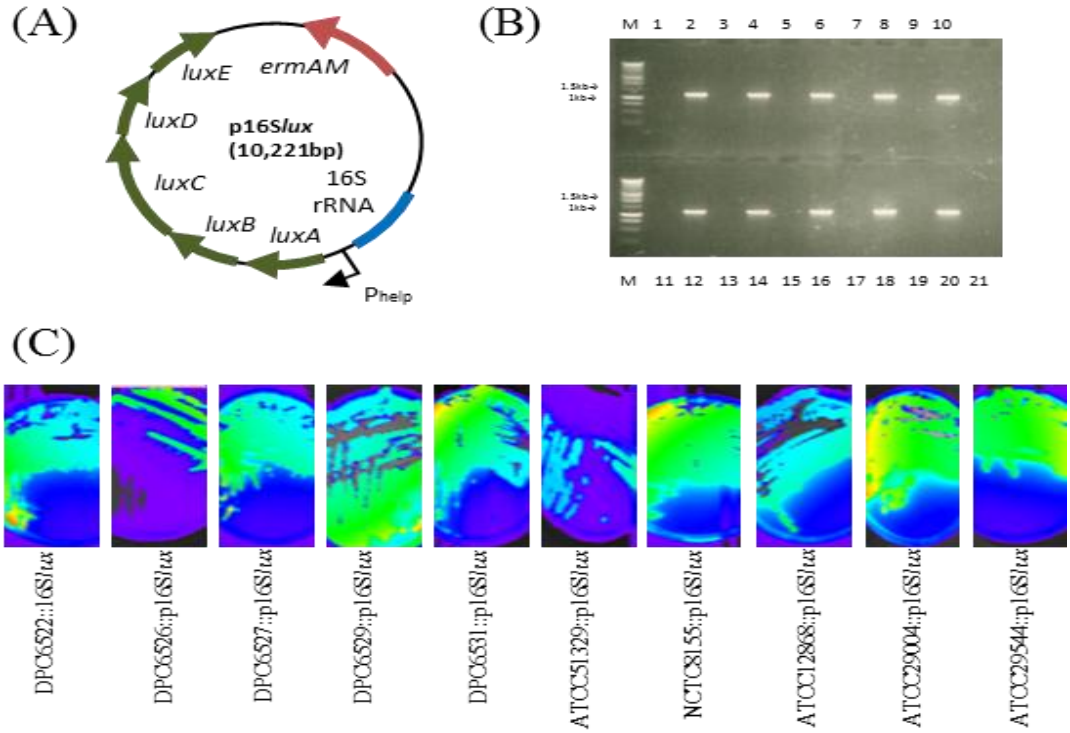


Figure 2. (**A**) Map of p16S*lux* showing the P_{help} promoter, DH10B 16S rRNA gene and Lux operon (*lux* A-E)**.** (**B**) Gel picture of PCR reactions performed to ensure integration of p16S*lux* into the chromosome. M molecular marker (1Kb, Promega); Lane 1, DPC6522; Lane 2, DPC6522::p16S*lux*; Lane 3, DPC6526; Lane 4, DPC6526::p16S*lux*; Lane 5, DPC6527; Lane 6, DPC6527::p16S*lux*; Lane 7 DPC6529; Lane 8, DPC6529::p16S*lux*; Lane 9, DPC6531; Lane 10, DPC6531::p16S*lux*; Lane 11, ATCC51329; Lane 12, ATCC51329::p16S*lux*; Lane 13, NCTC8155; Lane 14, NCTC8155::p16S*lux*; Lane 15, ATCC12868; Lane 16, ATCC12868::p16S*lux*; Lane 17, ATCC29004; Lane 18, ATCC29004::p16S*lux*; Lane 19, ATCC29544; Lane 20, ATCC29544::p16S*lux*; Lane 21, negative control (water). (**C**) *C. sakazakii* Lux-tagged strains streaked onto LB plus 500µg/ml erythromycin agar plates and viewed with an IVIS imager.

Growth curves of the non-tagged strains and their Lux-tagged counterparts were carried out in LB broth to examine if integration of the plasmid affected growth under standard laboratory conditions (Fig 3 A-J) and generation times are given in Table 2. Of the ten strains examined, statistically significant differences in generation times were observed for two strains. The generation times of Lux-tagged *C. sakazakii* strains DPC6526 and DPC6529 were longer than their non-Lux-tagged counterparts (Table 2). Although statistically significant it was noted that these differences were slight, so overall, integration of plasmid p16S*lux* does not have a major impact on the growth of strains.

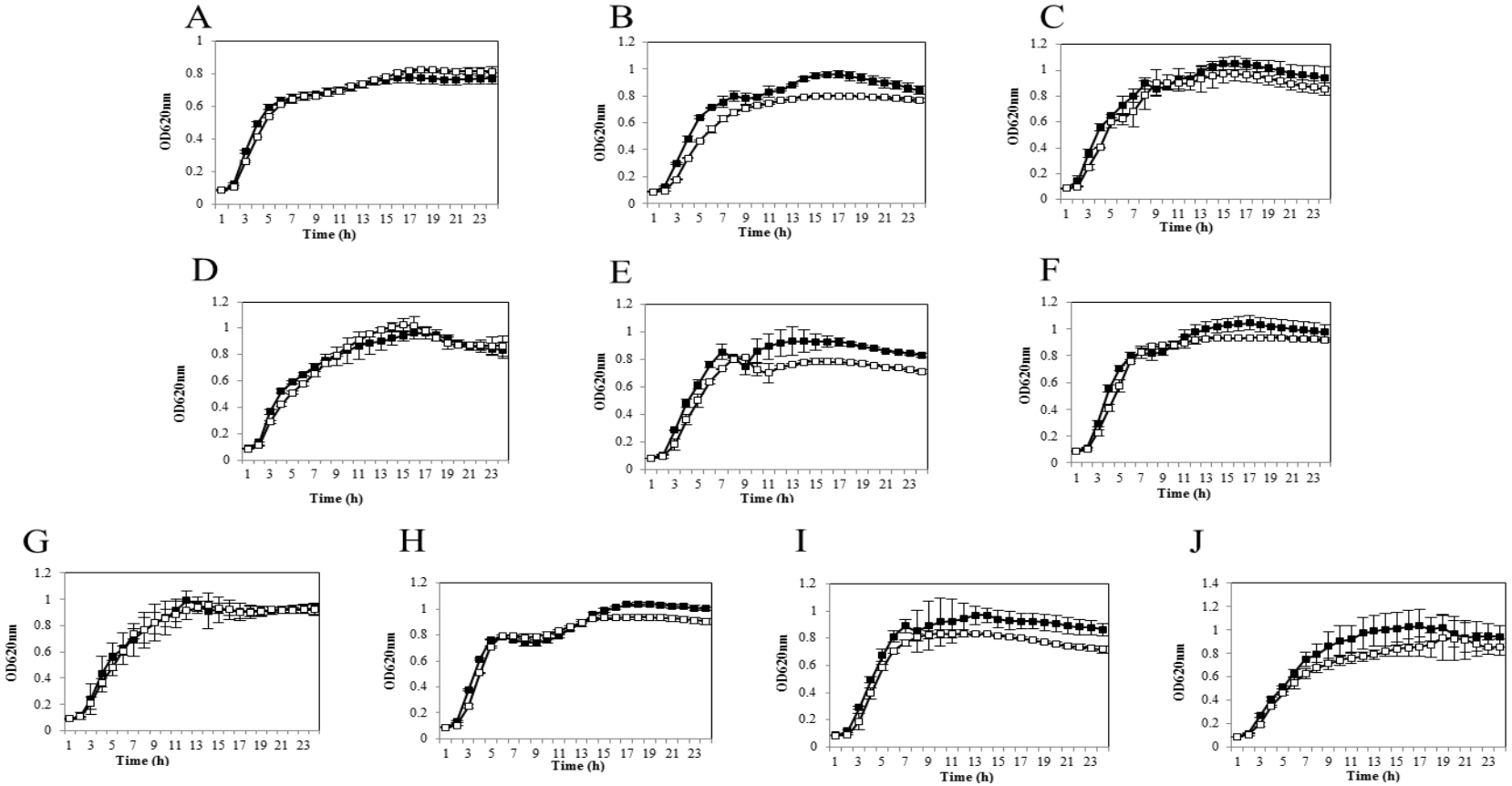


Figure 3. Growth curves in LB broth at 37°C over 24 hours (A) DPC6522::p16Slux (□) and DPC6522 (■), (B) DPC6526::p16S*lux* (□) and DPC6526 (■), (**C**) DPC6527::p16S*lux* (□) and DPC6527 (■), (**D**) DPC6529::p16S*lux* (□) and DPC6529 (■), (**E**) DPC6531::p16S*lux* (□) and DPC6531 (■), (**F**) ATCC51329::p16S*lux* (□) and ATCC51329 (■), (**G**) NCTC8155::p16S*lux* (□) and NCTC8155 (■), (**H**) ATCC29004 (□) and ATCC29004::p16S*lux* (■) (**I**) ATCC12868::p16S*lux* (□) and ATCC12868 (■), (**J**) ATCC29544::p16S*lux* (□) and ATCC29544 (■). Data are represented as mean OD_{620nm} ± standard deviations for three biological repeat.

Table 2. Generation times of non-tagged versus Lux-tagged strains. Results are expressed as averages of triplicate experiments ± the standard deviation.

Strain	Generation time (min)	Lux-tagged strain	Generation time (min)	p value
DPC6522	28.23 ± 0.93	DPC6522::p16S*lux*	30.94 ± 0.94	0.053
DPC6526*	31 ± 1.68	DPC6526::p16S*lux*	33.11 ± 0.77	0.02
DPC6527	29.94 ± 4.2	DPC6527::p16S*lux*	35.95 ± 2.5	0.117
DPC6529*	27.4 ± 0.3	DPC6529::p16S*lux*	28.6 ± 0.4	0.019
DPC6531	35.77 ± 1.1	DPC6531::p16S*lux*	34.81 ± 1.9	0.49
ATCC51329	35.682 ± 1.4	ATCC51329::p16S*lux*	36.4 ± 0.85	0.48
NCTC8155	28.11 ± 2.8	NCTC8155::p16S*lux*	32 ± 1.49	0.51
ATCC12868	35.06 ± 1.6	ATCC12868::p16S*lux*	37.39 ± 0.8	0.1189
ATCC29004	38.58 ± 1.15	ATCC29004::p16S*lux*	37.404 ± 2.11	0.46
ATCC29544	32.4 ± 1.06	ATCC29544::p16S*lux*	30.2 ± 3.2	0.36

* According to the student t-test for these strains the p value is <0.05, therefore the difference in growth rates is statistically significant. Analysis of the scientific data was carried out using basic statistical software in Excel.

The stability of p16S*lux* was examined by continuously passaging the Lux-tagged strains in LB broth in the absence of erythromycin and scoring for antibiotic resistance. The plasmid was shown to be 100% stable over two transfers for all of the strains and 98% stable over four transfers for all of the strains (Fig. 4). Some instability was observed over longer time periods. For example, while ATCC29544::p16S*lux* was 97% stable over six transfers, DPC6531::p16S*lux* was only 74% stable over the same time period (Fig. 4).

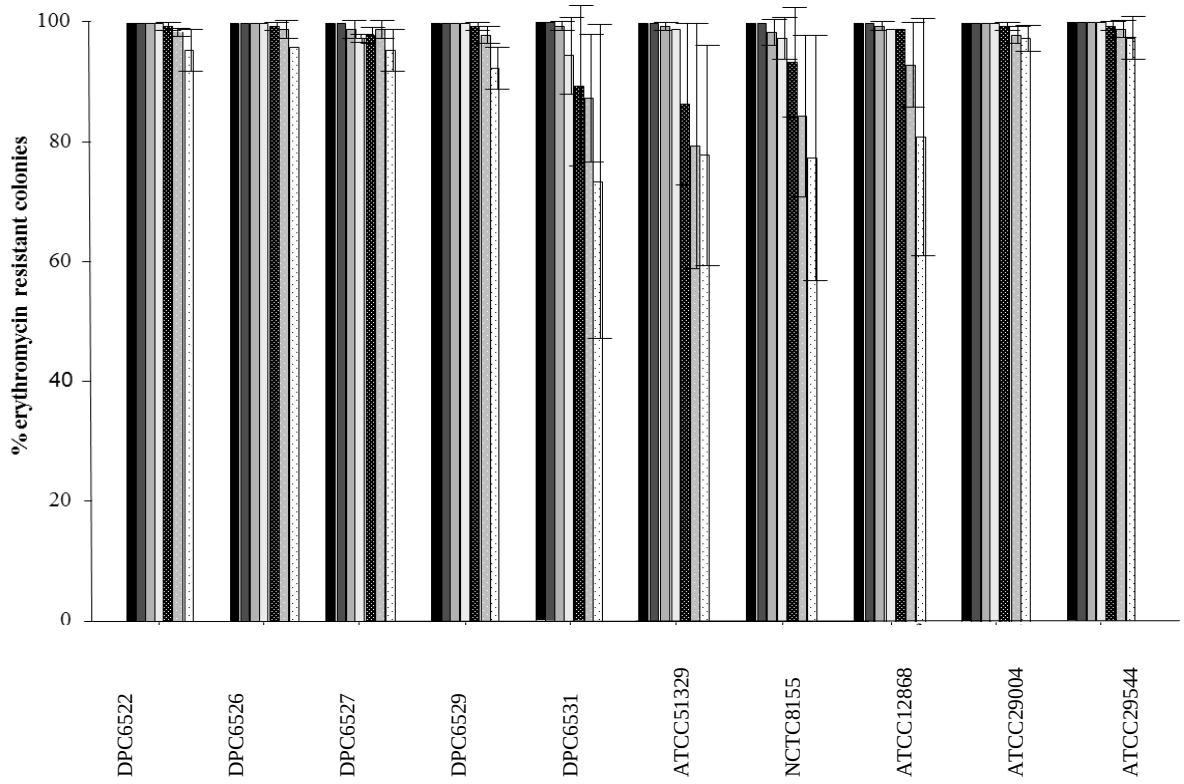


Figure 4. Stability of p16S*lux* for each strain. Percentage of erythromycin resistant colonies following daily passaging of Lux-tagged strains in the absence of antibiotic selection. Overnight (■), passage 1 (■), passage 2 (■), passage 3 (□), passage 4 (▣), passage 5 (▩) and passage 6 (⚄). Data are represented as mean % of erythromycin resistant colonies ± standard deviations for three biological repeats.

Growth of Lux-tagged strains in IMF

Growth curves were performed with the ten Lux-tagged strains in two IMF, IMF5 and IMF8. These two IMF were chosen as in earlier experiments with NCTC8155:p16S*lux* higher RLU readings than average were obtained for IMF5 and lower than average RLU readings were obtained for IMF8 (Fig. 1). These two IMF brands are produced by different manufacturers. While lowest RLU readings and plate counts were obtained for NCTC8155:p16S*lux* in IMF10 this IMF was not selected for further experiments as it is a lactose free formula and growth of *Cronobacter* is limited. Growth of Lux-tagged strains in IMF5 and IMF8 is portrayed in Fig. 5A and Fig. 5B, respectively. Similar to the results obtained with NCTC8155::p16S*lux*, growth of the Lux-tagged strains in both IMF could be easily and reproducibly monitored by measuring bioluminescence.

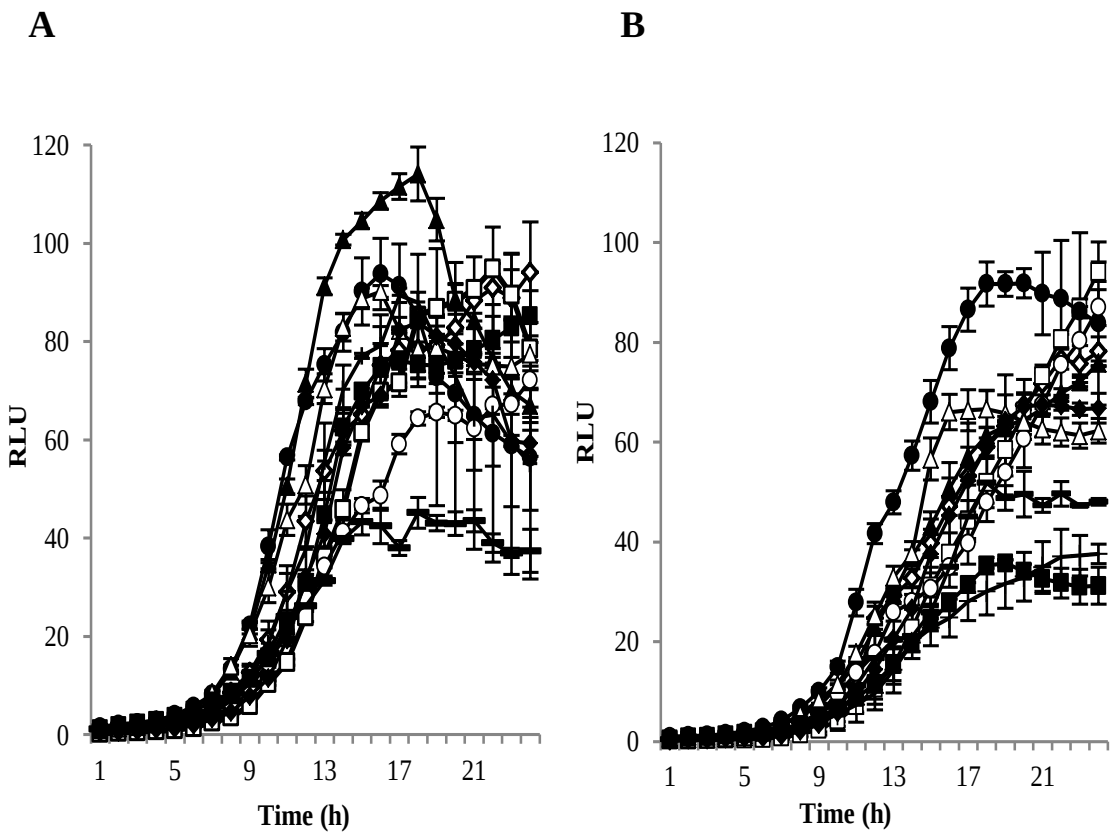


Figure 5. Growth curves of Lux-tagged strains in IMF5 (**A**) and IMF8 (**B**) DPC6522::p16S*lux* (◊), DPC6526::p16S*lux* (□), DPC6527::p16S*lux* (▲) DPC6529::p16S*lux* (=), DPC6531::p16S*lux* (○), ATCC51329::p16S*lux* (+), NCTC8155::p16S*lux* (Δ), ATCC12868::p16S*lux* (■), ATCC29004::p16S*lux* (▬) and ATCC29544::p16S*lux* (♦) at 20°C over 24 h. Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Growth of individual Lux-tagged *Cronobacter* strains and a cocktail of Lux-tagged strains in IMF8

Growth of the ten Lux-tagged *Cronobacter* strains in IMF8 was reanalyzed and the average RLU of all ten strains was calculated (Fig. 6A and 6B). Plate counts were performed at 16 hrs for DPC6522::p16S*lux* for which RLU were higher than the average of the ten strains (~94 RLU) and ATCC12868::p16S*lux* for which RLU were lower than the average of the ten strains (~40 RLU). Cell numbers for both strains were ~1x10^7 cfu/ml.

In addition to the individual Lux-tagged strains a cocktail of the ten strains was included (Fig. 6A). This was carried out in order to determine if growth of a collection of *Cronobacter* strains (cocktail) could be used as an approach to predicting *Cronobacter* growth as an alternative to employing ten strains individually. The RLU values obtained for the cocktail of the Lux-tagged strains was similar to the average RLU obtained for the ten individual strains.

The experiment was repeated with DPC6522::p16S*lux* alone, ATCC12868::p16S*lux* alone, a cocktail of all 10 Lux-tagged strains and a mix of DPC6522::p16S*lux* and ATCC12868::p16S*lux* (Fig. 6B). The cocktail of the ten strains and the cocktail of the two strains were comparable. Therefore these two strains could potentially be used instead of the cocktail of ten Lux-tagged *Cronobacter* strains as an accurate indicator of the bacterial growth in IMF.

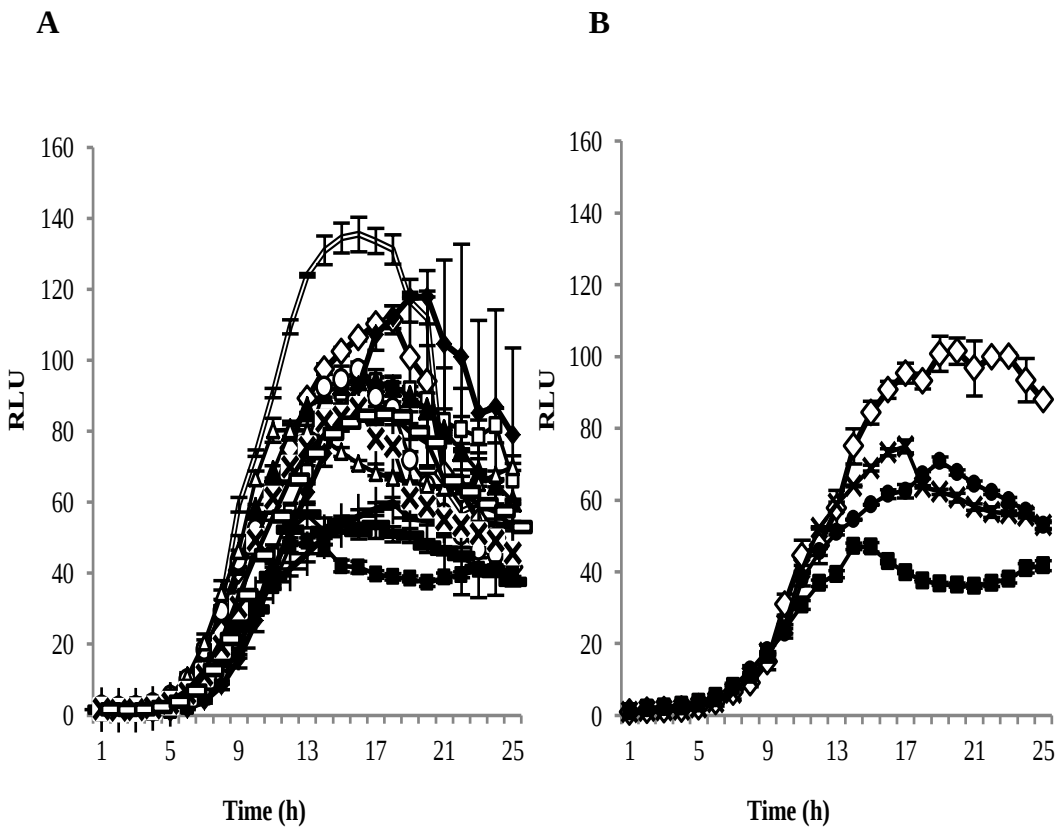


Figure 6. (A). Growth of Lux-tagged *Cronobacter* strains at 20°C over 24 h in IMF8. Also included are a cocktail of the ten strains and the average of the ten Lux-tagged *Cronobacter* strains. DPC6522::p16S*lux* (◊), DPC6526::p16S*lux* (□), DPC6527::p16S*lux* (▲) DPC6529::p16S*lux* (=), DPC6531::p16S*lux* (○), ATCC51329::p16S*lux* (+), NCTC8155::p16S*lux* (Δ), ATCC12868::p16S*lux* (■), ATCC29004::p16S*lux* (—), ATCC29544::p16S*lux* (♦), cocktail of the ten Lux-tagged strains (×) and average RLU of the 10 *lux*-tagged strains (▭). **(B)** Using a cocktail of two stains was also investigated DPC6522::p16S*lux* (◊). ATCC12868::p16S*lux* (■), mix of DPC6522::p16S*lux* and ATCC12868::p16S*lux* (●) and a cocktail of the ten Lux-tagged strains (×). Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Discussion

Initial experiments examined growth of *C. sakazakii* NCTC8155::p16S*lux*, the Lux-tagged strain used by Morrissey *et al*., (2011), in ten different brands of IMF produced by four different manufacturers. It was found that growth of the strain could be easily and reproducibly monitored in real-time by monitoring light emission. Differences in RLU readings were observed between formulae and differences in viable plate counts were also noted (data not shown). The lowest RLU readings were obtained for IMF10 which was the only lactose free IMF. In addition to variations in nutrient composition between formulae it is also possible that the growth of *C. sakazakii* could be affected by the resident microflora in IMF. This microflora consists mainly of *Bacillus* spp. which may outgrow *C. sakazakii* or produce inhibitory substances such as bacteriocins.

Healy *et al*., (2009) employed microarray comparative genome indexing (CGI) to analyze the genomic content of 78 *Cronobacter* strains and highlighted the genomic similarities and differences between strains. The phenotypic diversity of *Cronobacter* strains has also been demonstrated (Caubilla-Barron *et al*., 2007, Ye *et al*., 2011); therefore not all strains of *Cronobacter* are identical and may proliferate at different rates in different IMF. As IMF manufacturers strive to create new and improved formulae, it is likely that they will want to investigate the extent to which these new compositions support the growth of *C. sakazakii*. It is important that more than one strain is included in these experiments so if bioluminescence-based assays are to be used a collection of Lux-tagged *Cronobacter* strains will be required. While attempts by previous researchers have failed to Lux-tag *C. sakazakii* (Forsythe,

2009), we were able to successfully Lux-tag all seven *C. sakazakii* strains we selected at random from our culture collection using plasmid p16S*lux*. A *C. malonaticus* strain and a *C. muytjensii* strain were also successfully tagged. While integration of the plasmid had a slight effect on the generation times of two of the ten stains, expression of the luciferase and accessory proteins and the process of light emission did not seem to be a huge burden on bacterial metabolism. The integrated plasmid was stable when cells were cultured in the absence of antibiotic over short time periods.

It was observed that growth of the Lux-tagged strains could be easily monitored in real-time in IMF by measuring bioluminescence. Differences in RLU readings were observed between strains and these differences did not always directly correlate with differences in cell counts. For example in IMF8 after 16 hrs the RLU reading for DPC6522::p16S*lux* was twice the reading for ATCC12868::p16S*lux* (~94 RLU vs ~40 RLU) while cell counts for both strains were the same (~$1x10^7$ cfu/ml). The light reaction involves the oxidation of reduced riboflavin phosphate ($FMNH_2$) the concentration of which depends on the metabolic activity of the cell (Bachmann *et al.,* 2007, Meighan, 1991). It is possible that the different *Cronobacter* strains used in our study produce different amounts of $FMNH_2$ which affects RLU output. Therefore, our experiments show that the growth of Lux-tagged *Cronobacter* strains can be easily followed by measuring light emission and bioluminescence-based experiments can be employed to determine if an infant formula composition supports their growth. Although RLU readings obtained for different strains cannot be directly translated into viable cell numbers uniformly for all strains, cell numbers and RLU readings for individual strains can be directly correlated.

Experiments with mixes of strains demonstrated that a cocktail of two Lux-tagged *Cronobacter* strains provides a good representation of the average growth of all ten strains in IMF. This means that rather than performing future growth analyses in IMF with all ten strains, the use of two strains would reduce the amount of work and samples required.

In conclusion, we have successfully Lux-tagged a collection of *Cronobacter* strains and demonstrated that growth in IMF could be monitored by measuring bioluminescence. In addition to examining the effects of various infant formula compositions on growth of *Cronobacter*, a bank of Lux-tagged strains may have other potential applications. For example, the strains may be of use in examining biofilm formation in experiments similar to those performed in *L. monocytogenes* (Begley *et al*., 2009). Milk components have been shown to influence biofilm formation by *C. sakazakii* (Dancer *et al*., 2009a) and biofilms contribute to persistence of the pathogen on food contact surfaces (Iversen *et al*., 2004d). Lux-tagged strains may also be employed to investigate biocide efficiency or examine growth and survival in *in vitro* (i.e. cell culture) and *in vivo* models of infection (Griffiths, 2000).

Chapter IV

Identification of *Cronobacter sakazakii* mutants with impaired growth in milk

Manuscript submitted

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Abstract

Cronobacter sakazakii is a rare cause of neonatal meningitis, septicaemia and enterocolitis. Contaminated infant milk formulae (IMF) have been epidemiologically linked as a source of *C. sakazakii* infection in several clinical cases. Lux-tagged *C. sakazakii* strains emit light and their growth can be monitored in real-time in IMF by measuring bioluminescence. A transposon mutagenesis library was created in a Lux-tagged strain of *C. sakazakii*. This library was screened for mutants with impaired growth in milk. It was found the majority of genes disrupted in these mutants were associated with amino acid metabolism (valine, isoleucine, glutamic acid, methionine and arginine). Addition of the relevant amino acid fully restored growth of the mutant to wild type levels of growth in milk. Complementation of mutants with an intact copy of the disrupted gene on a plasmid also resulted in restoration of growth in milk. Our results highlight the importance of genes involved in amino acid metabolism for growth in this environment. A better understanding of how the bacterium grows in milk may ultimately aid in the development of more effective preservation techniques to limit growth of this pathogen in milk.

Introduction

Cronobacter sakazakii is an opportunistic pathogen, associated with the ingestion of contaminated powdered infant milk formula (IMF). It is a Gram negative, peritrichously motile, non-spore forming, facultative anaerobic bacteria. Although rare, infections by this organism have a case fatality rate between 33-80% (Lai, 2001). The organism is ubiquitous and can survive for long periods of time (up to two years) in a desiccated state (Edelson-Mammel *et al*., 2005). These features may provide *C. sakazakii* with a competitive advantage, contributing to its presence in dry environments such as IMF. In addition, IMF is not sterile therefore contamination with *C. sakazakii* can occur if strict microbiological guidelines are not adequately followed. Isolation of the pathogen from commercially available IMF can range from 2.4 to 14% (Iversen *et al*., 2004c, Nazarowec-White *et al*., 1997b, Muytjens *et al*., 1988). The FDA method for detection of *C. sakazakii* in IMF has been revised to include molecular biology tests as well as microbiological analyses (Lampel *et al*., 2009). Future studies with this more sensitive method may provide more accurate data on the true incidence of *C. sakazakii* in IMF.

However, it is evident that contamination of IMF with *C. sakazakii* occurs. In November and December of 2011, four infants were infected with *C. sakazakii* resulting in two deaths in the United States (CDC, 2012). This resulted in a voluntary recall of suspect IMF. Therefore *C. sakazakii* is undoubtedly of major concern to infant formula manufacturers and public health.

Much of the research on growth of bacteria in milk focuses on lactic acid bacteria (LAB) such as *Streptococcus thermophilus* and *Lactococcus lactis.* These studies include investigation of the proteomic signature of LAB in milk (e.g. Herve-Jimenez *et al*., 2008, Gitton *et al*., 2005) or identifying genes essential for growth of LAB in milk (e.g. Arioli *et*

al., 2009, Dudley *et al*., 2001). Less information is available concerning the growth of pathogenic bacteria in milk. Examples of such studies include investigation of the gene expression profiling of *L. monocytogenes* in milk (Liu *et al*., 2008) and examining proteomic changes of *Escherichia coli* in milk compared to laboratory media (Lippolis *et al*., 2009). To our knowledge, genes required for growth of *C. sakazakii* in milk have not yet been identified. A better understanding of the molecular mechanisms this pathogen utilizes to grow in milk may assist in developing more effective preservation techniques.

As milk is opaque it does not lend itself to growth measurements by optical density. The traditional processes used to measure bacterial growth in milk can be lengthy and time-consuming (e.g. direct plate counts, monitoring pH variation and changes in electrical impedance). Morrissey *et al*., (2011) transformed a *C. sakazakii* strain with luciferase (*lux*) genes employing the plasmid p16S*lux* and monitored growth of the bacterium in milk and infant formulae by measuring bioluminescence (light emission). This process provides real time tracking of *C. sakazakii* growth in milk and monitoring growth of the Lux-tagged *C. sakazakii* strain was shown to be reproducible.

The aim of the current study was to create a transposon mutagenesis library in a Lux-tagged strain of *C. sakazakii* and screen this library for mutants affected in their growth potential in milk.

Materials and methods

Bacterial strains and culture conditions.

Strains used in this study are listed in Table 1. They were routinely grown in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) statically at 37°C for ~16 hours unless otherwise stated. For solid media 1.5% agar was added. For certain experiments Brilliance *Enterobacter sakazakii* (DFI, Druggan, Forsythe and Iversen) agar (Oxoid, UK) was used. Reconstituted skim milk (RSM) (Carbery) was prepared by dissolving (10% wt/vol) powder in distilled water and autoclaving at 110°C for 10 minutes. IMF was aseptically weighed and added to sterile distilled water (10% wt/vol) and mixed by swirling manually for 5 min. If required antibiotics were added at the following final concentrations: erythromycin, 500µg/ml, kanamycin, 50µg/ml and chloramphenicol, 10µg/ml. Erythromycin (Sigma, Germany) and chloramphenicol (Sigma) were made up as a concentrated stock in ethanol. Kanamycin (Sigma) was filter sterilized and made up as a concentrated stock in sterile distilled water. When necessary antibiotics were added to cooled autoclaved media. The amino acids; glutamic acid (Sigma), isoleucine (Sigma), valine (Sigma), arginine (Sigma) and methionine (Sigma) were made up as concentrated stocks in sterile distilled water and added to cooled autoclaved media. Stocks of glutamic acid were filter sterilized. Stocks of isoleucine, valine, arginine and methionine were autoclaved at 121°C for 15 min.

Table 1. Bacterial strains, plasmid and transposon used in this study

Strain or plasmid	Relevant genotype or characteristic(s)	Source or reference
WT*	*Cronobacter sakazakii* 6529::p16S*lux* Ery^r	Chapter 3
DH5-α	*E. coli*	UCC culture collection
pNZ44	*E. coli-L. lactis* high-copy-number shuttle vector, Cm^r containing constitutive P44 promoter from *L. lactis* chromosome	McGrath *et al*., 2001
EZ-Tn5 <kan-2> (Tn)	Km^r, mini-Tn5 transposon	Epicentre, Madison, WI
M1	WT with an insertion in *ESA_03607* Km^r Ery^r	This study
M2	WT with an insertion in *ESA_00653* Km^r Ery^r	This study
M3	WT with an insertion in *ESA_03781* Km^r Ery^r	This study
M4	WT with an insertion in *ESA_01561* Km^r Ery^r	This study
M5	WT with an insertion in *ESA_03810* Km^r Ery^r	This study
M6	WT with an insertion in *ESA_03820* Km^r Ery^r	This study
M7	WT with an insertion in *ESA_00904* Km^r Ery^r	This study
M1Comp	M1 harbouring pNZ44+*ESA_03607* Cm^r	This study
M3Comp	M3 harbouring pNZ44+*ESA_03781* Cm^r	This study
M5Comp	M5 harbouring pNZ44+*ESA_03810* Cm^r	This study
M6Comp	M6 harbouring pNZ44+*ESA_03820* Cm^r	This study

*WT=wild type

Ery^r = Erythromycin resistance

Cm^r= Chloramphenicol resistance

Km^r = Kanamycin resistance

Transposon mutagenesis

A transposon mutagenesis library was constructed using the EZ-Tn5 <KAN-2>Tnp Transposome kit (Epicentre, Madison, WI) according to protocols provided by the manufacturer. Briefly, *C. sakazakii* DPC6529::p16S*lux* was grown overnight in LB plus 500µg/ml erythromycin for approximately 16 h statically. A 1% inoculation was transferred to fresh LB incubated shaking for ~3 hours at 37°C until an OD of ~0.6 was reached. The cells were centrifuged at 900 x *g* for 10 minutes at 4°C, the supernatant was discarded, and cells were resuspended in ice cold sterile distilled water and re-centrifuged. This was repeated three times, after which the cells were resuspended in 10% ice cold glycerol and centrifuged again. The supernatant was removed and cells were resuspended in 500µl of ice cold 10% glycerol. 50µl aliquots were transferred to cold sterile 1.5ml centrifuge tubes and stored at -80°C.

For electroporations, 1µl of transposon DNA was added to 50µl of competent cells on ice in a 2mm cuvette (Molecular BioProducts, San Diego CA). The electroporation conditions were 2.5kV, 200Ω and 25µF using a Harvard Apparatus ECM 630 Electro Cell Manipulator (MA, USA). To aid recovery the cells were resuspended in 1ml super optimal broth with catabolite repression (SOC) and incubated shaking for 1 hour at 37°C. Cells were diluted 1:100 in prewarmed SOC medium. To select for transposon insertion clones, aliquots of 100µl were plated onto LB plates containing 50µg/ml kanamycin and plates were incubated overnight at 37°C. Single mutants were picked and transferred to individual wells of 96-well plates (Genetix, UK), containing 150µl/well of LB supplemented with 7.5% glycerol and 50µg/ml kanamycin. Plates were incubated overnight at 37°C for approximately 16 hours and subsequently stored at -80°C.

Screening of transposon mutants

The -80°C stock plates were thawed and replicated into 96-well plates (Genetix) containing LB broth plus 50µg/ml kanamycin using a 96-pin replicator (Genetix). These were incubated

at 37°C for approximately 16 hours. Subsequently mutants were serially diluted in ¼ strength Ringers solution and an initial inoculum of ~1x10^5 cfu/ml was added to RSM in a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). Bioluminescence was measured every hour in relative light units (RLU) using a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). 1046 transposon mutants were screened for their growth progress in milk. When viable plate counts at selected time points were required, cultures were serially diluted in ¼ strength Ringer's solution and enumerated on LB agar. When required, light emission was examined using the Xenogen IVIS100 imaging system (Xenogen, Alameda, CA). Bioluminescence was measured in relative light units (photons/sec/cm) with a binning of sixteen and an exposure time of one minute.

DNA extraction and manipulations

Strains were grown overnight (~16 hrs) in LB broth. DNA was extracted from 1ml of overnight cultures using the Invitrogen PureLink Genomic DNA Mini Kit following the manufacturer's instructions. Plasmids were extracted using the Invitrogen Quick plasmid miniprep kit. PCR products were purified using the Invitrogen PCR purification kit. Colony PCRs were performed following lysis of cells with IGEPAL CA-630 (Sigma). The concentration of nucleic acids was determined using an Invitrogen Qubit fluorometer (Eugene, Oregon, USA). PCR amplification was carried out using a G-storm cycler (Essex, UK). PCR products were separated on 1% (m v^{-1}) agarose gels and visualised with the DNR Bio-Imaging System (Jerusalem, Israel). Enzymes and respective buffers were obtained from Roche (Basel, Switzerland).

Identification of transposon insertion sites

The transposon insertion site for each mutant was identified by single primer PCR. DNA fragments were amplified from chromosomal DNA of the transposon mutants using primer Tn5PCRR (Table 2) with the reaction conditions described by Karlyshev *et al.*, (2000). The

conditions were 1 min at 94°C, 20 cycles of 94°C for 30s, 50°C for 30s and 72°C for 3 min, 30 cycles of 94°C for 30s, 30°C for 30s and 72°C for 2 min, 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 2 min, followed by a 7 min extension at 72°C. PCR products were purified and sequenced using primer KAN-2 RP1 (Table 2) by MWG Biotechnologies (Ebersberg, Germany).

BLASTn (Basic Local Alignment Search Tool) searches were performed with the obtained sequences (http://blast.ncbi.nlm.nih.gov/) to identify homologous genes. Homologous regions in the genome of the sequenced *C. sakazakii* BAA-894 strain (accession number NC_009778) were analysed and the information obtained was used to generate Figure 4. Proteins were analysed for the presence of conserved domains using the conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/).

Table 2 Primers used in this study

Primer	**Sequence 5'→3'**
Kan-2 RP1	GCAATGTAACATCAGAGATTTTCAG
Tn5PCRR	CGAGCAAGACGTTTCCCGTTG
ESA_03607 F	**GCTCTAGA**AGGCGCAGTAAGAGGTCAC*
ESA_03607 R	**AACTGCAG**AACGTCAGACGAAACGGTC†
ESA_03781 F	**GGGGTACC**GACACGGACAACATCACGAG‡
ESA_03781 R	**GCTCTAGA**GGGTGGGTAGTGAAACG*
ESA_03810 F	**GCTCTAGA**CTGAGGTCTGTCAACAGTG*
ESA_03810 R	**AACTGCAG**CATCCACCGTGATATTCAGG†
ESA_03820 F	**GCTCTAGA**ACGACACAGCGATTGATG*
ESA_03820 R	**GGGGTACC**GTCATCCTCGCAATAGCA‡
pNZ44 F	CTAATGTCACTAACCTGCCCCGTTAG
pNZ44 R	GGCTATCAATCAAAGCAACACGTG

Recognition sites of restriction enzymes are highlighted in bold and underlined.

* *XbaI*

† *PstI*

‡ *KpnI*

Monitoring growth of selected mutant strains in LB broth by measuring optical density and in IMF by measuring bioluminescence.

Mutants were grown in triplicate in LB broth and incubated overnight (~16 h) at 37°C. Cells were centrifuged at 8000 x *g*, washed and resuspended in ¼ strength Ringer's solution (Merck, Darmstadt, Germany). For growth curves, the washed cells were serially diluted and an initial inoculum of ~1x10^5 cfu/ml was added to LB broth, 200µl of this was transferred to a 96-well plate (Genetix, UK). Growth was monitored by measuring optical density (OD) at 620nm using a Thermo Scientific Multiscan FC plate reader where a reading was taken once an hour for 24 h. Viable plate counts were performed at selected time points by serially diluting samples in ¼ strength Ringer's solution and enumeration on LB agar. In order to monitor bioluminescence in IMF washed cells were serially diluted in ¼ strength Ringer's solution and an initial inoculum of ~1x10^5 cfu/ml was added to fresh IMF, 200µl of this was transferred to a 96-well white plate (Nunc, Thermo Fisher Scientific, Roskilde, Denmark). Bioluminescence was measured every hour in relative light units (RLU) using a Luminoskan luminometer automatic plate reader (Thermo Fisher Scientific, Roskilde, Denmark). When viable plate counts were required, cultures were serially diluted in ¼ strength Ringer's solution and enumerated on DFI agar.

Complementation of transposon mutants

For complementation of the mutants, the functional genes from *C. sakazakii* DPC6529::p16S*lux* were amplified by PCR using primers listed in Table 2. An annealing temperature of 50°C and extension time of 1 min was used. PCR products were subsequently purified and digested with the relevant restriction enzymes. Each PCR product was ligated to similarly digested pNZ44 (McGrath *et al*., 2001). The resulting ligation mix was transformed into electrocompetent *E. coli* DH5α cells. Transformants were selected on LB agar containing 10µg/ml chloramphenicol and incubated overnight at 37°C. Colony PCRs

were performed using pNZ44 forward and reverse primers. Selected colonies were grown overnight in LB supplemented with 10µg/ml chloramphenicol. Plasmids were extracted and electroporated into electrocompetent *C. sakazakii* cells. Transformants were selected on LB agar containing 10µg/ml chloramphenicol and incubated overnight at 37°C.

Results

Creation and screening of the transposon mutant library

A *C. sakazakii* DPC6529::p16S*lux* transposon mutant library was successfully created. The library was screened for mutants affected in their growth potential in milk using a bioluminescence based approach (Figure 1).

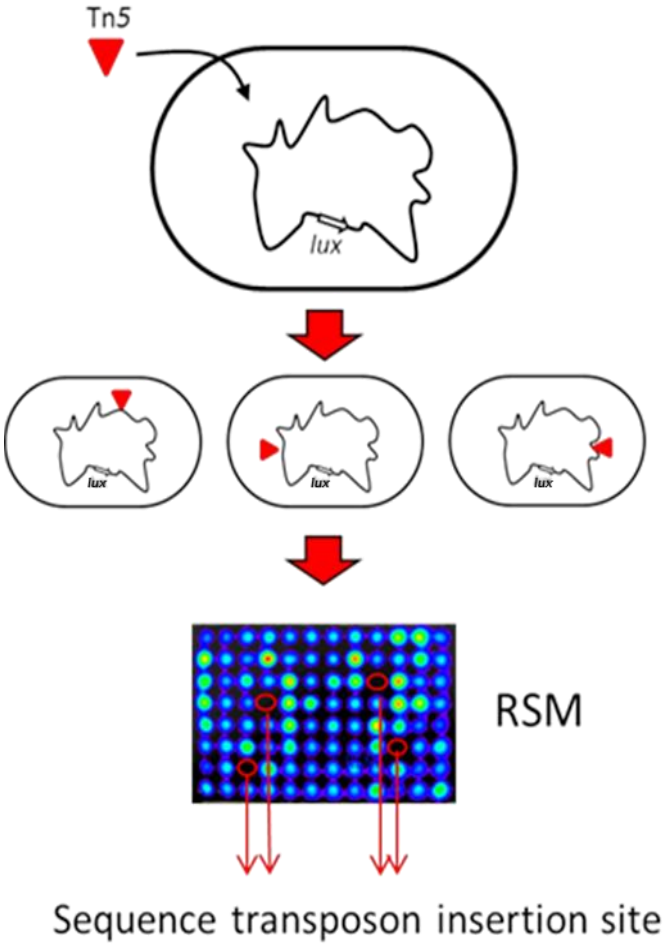


Fig 1. Method used to create and screen the Transposon mutagenesis bank.

Of 1046 mutants screened 35 were chosen for further analysis due to the lack or relatively low amount of light emitted by these strains in RSM in the initial screen. The number of mutants was further reduced in three ways. Firstly, the amount of light emitted by the mutants during growth in LB broth was examined. Any mutants emitting no or very low amounts of light were excluded from further investigations. Secondly, these mutants were streaked on LB agar and the colonies were checked for light emission using the Xenogen IVIS100 imaging system. Some mutants emitted no light or some light after a 5 minute exposure. These mutants were again omitted from further analyses. Thirdly, additional elimination of the mutants involved measuring growth (OD_{620nm}) of each mutant in LB broth in order to determine if growth of these mutants was impaired in standard laboratory medium. Out of the 35 mutants, 28 were eliminated by this process.

Growth analyses of mutants from initial screen

The remaining seven mutants were selected for further analysis. These mutants emitted approximately the same amount of light as the wild type in LB broth but emitted lower amounts of light than the wild type in RSM (Figure 2 A and B). In addition, all seven mutants displayed growth similar to the wild type strain when grown in LB broth at 37°C, indicating that disruptions of the genes in questions were not essential for growth under these conditions (data not shown). Plate counts after six hours in LB and RSM corroborated these results. The transposon mutants had lower plate counts in RSM than the wild type and the same plate counts as the wild type in LB. In RSM plate counts at T6 were: WT (~$2x10^8$ cfu/ml), M1 (~$9x10^7$ cfu/ml), M2 (~$9x10^7$ cfu/ml), M3 (~$5x10^7$ cfu/ml), M4 (~$13x10^7$ cfu/ml), M5 (~$9x10^7$ cfu/ml), M6 (~$4x10^7$ cfu/ml) and M7 (~$3.5x10^7$ cfu/ml).

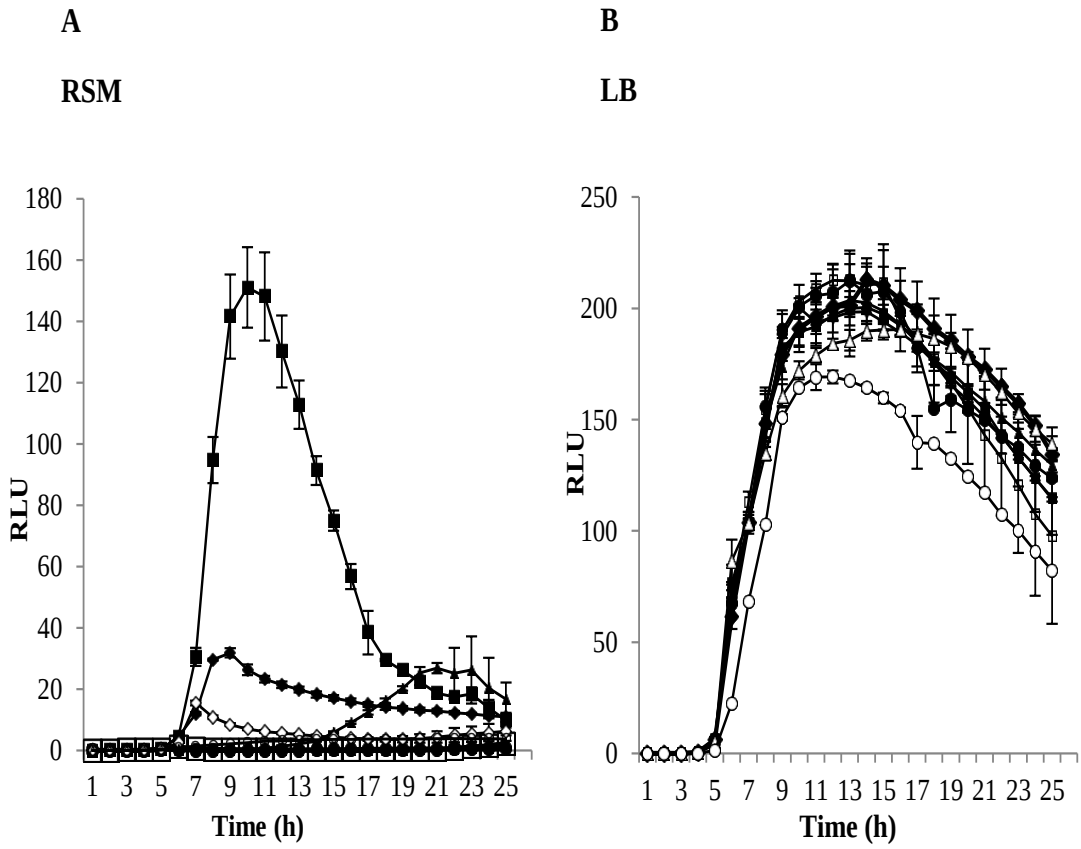


Fig 2. (A) Growth of mutants in RSM. (B) Growth of mutants in LB. WT (■), M1 (♦), M2 (Δ), M3 (●), M4 (□), M5 (◊), M6 (Δ) and M7 (○). Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Growth of mutants in IMF

The seven mutants were grown in different infant milk formulae (IMF2 and IMF6) produced by two different manufacturers (Figure 3 A and B). Growth of five of the mutants was negatively affected in IMF compared to the wild type. M7 emitted more light in both IMF than was previously observed in RSM. However, growth still appeared to be delayed for this mutant compared to the wild type indicated by a longer lag phase. M4 emitted similar amounts of light to the wild type in IMF6 and plate counts were also the same as the wild type. However, this was not the case for this mutant in IMF2 where emission of light was similar to its previous growth in RSM.

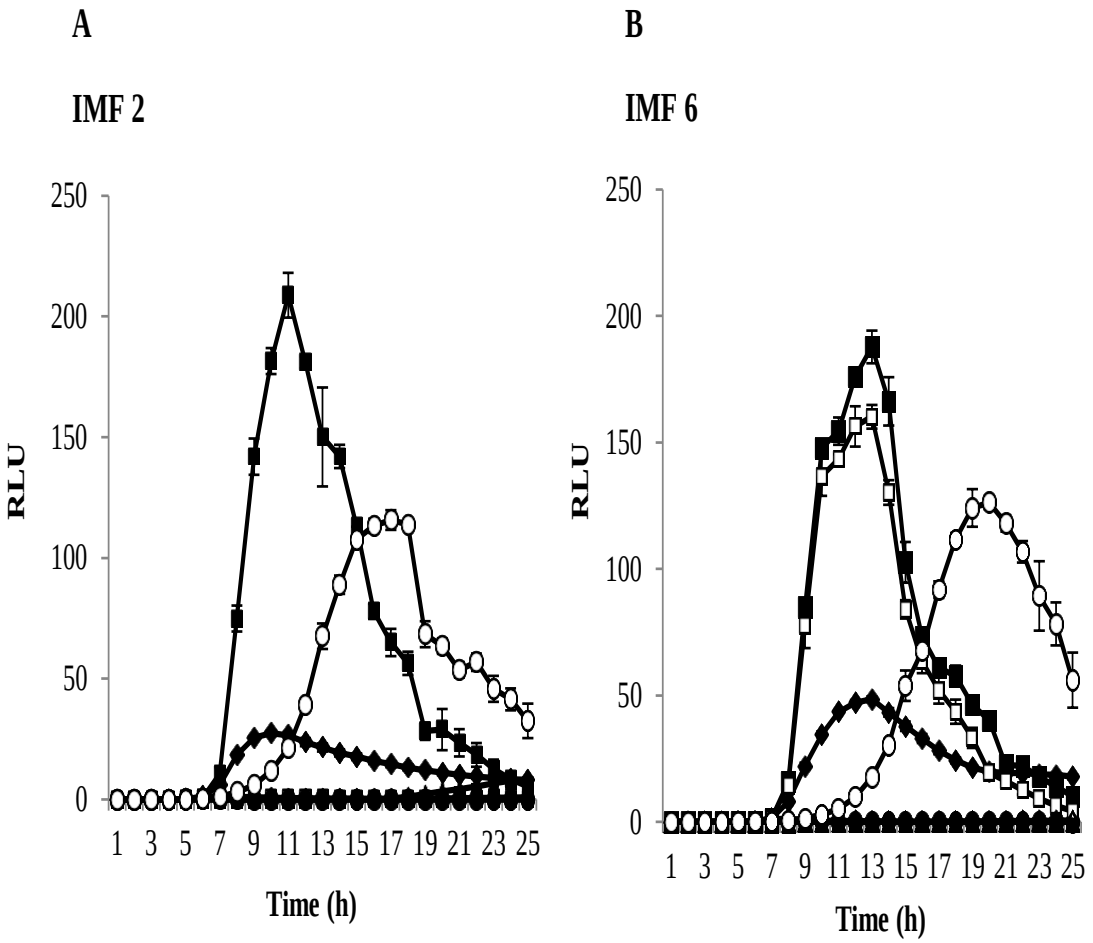


Fig 3. Growth of mutants in IMF 2 (A) and IMF 6 (B). WT (■), M1 (♦), M2 (Δ), M3 (●), M4 (□), M5 (◊), M6 (Δ) and M7 (○). Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Bioinformatic analysis

The site of the transposon insertion was identified by performing a single primer PCR followed by sequencing of the resultant products (Table 3). Locations of transposon insertion sites are shown in Figure 4. Conserved domains of each of the proteins are illustrated in Figure 5.

Table 3. Overview of the transposon mutants with inhibited growth in RSM

Tn Mutant	Tn insertion site	Protein accession number, Size of protein	Proposed function of encoding protein
M1	*ESA_03607*	YP_001439653 472 aa	GltD, NADPH-dependent glutamate synthase, it catalyzes the single-step conversion of L-glutamine and α-ketoglutarate into two molecules of L-glutamate.
M2	*ESA_00653*	YP_001436772 356 aa	Hypothetical protein, phosph-2-dehydro-3-deoxyheptonate aldolase, it catalyzes the formation of 3-deoxy-D-arabino-hept-2-ulosonate 7 phosphate from phosphoenolpyruvate and D-erythrose 4-phosphate.
M3	*ESA_03781*	YP_001439809 491 aa	IlvC, ketol-acid reductoisomerase, it catalyzes the formation of (R)-2,3-dihydroxy-3-methylbutanoate from (S)-2-hydroxy-2-methyl-3-oxobutanoate in valine and isoleucine biosynthesis.
M4	*ESA_01561*	YP_001437651 531 aa	TrpD, bifunctional glutamine amidotransferase/anthranilate phosphoribosyltransferase, TrpD forms a heterotetramer with TrpE and the complex catalyzes the formation of anthranilate from chorismate and glutamine; also catalyzes the formation of N-(5-phospho-D-ribosyl)-anthranilate from athranilate and 5-phospho-alpha-D-ribose 1-diphosphate, it functions in tryptophan biosynthesis
M5	*ESA_03810*	ABU78996 381 aa	ArgE, acetylornithine deacetylase, it catalyzes the formation of L-ornithine from N(2)-acetyl-L-ornithine in arginine biosynthesis
M6	*ESA_03820*	YP_001439842 301 aa	MetF 5,10-methylenetetrahydrofolate reductase, this is reduced to 5-methyltetrahydrofolate by methylenetetrahydrofolate reductase, a cytoplasmic, NAD(P)-dependent enzyme. 5-methyltetrahydrofolate is utilized by methionine synthase.
M7	*ESA_00904*	ABU76174 505 aa	Amidophosphoribosyltransferase, it catalyzes first step of the de novo purine nucleotide biosynthetic pathway.

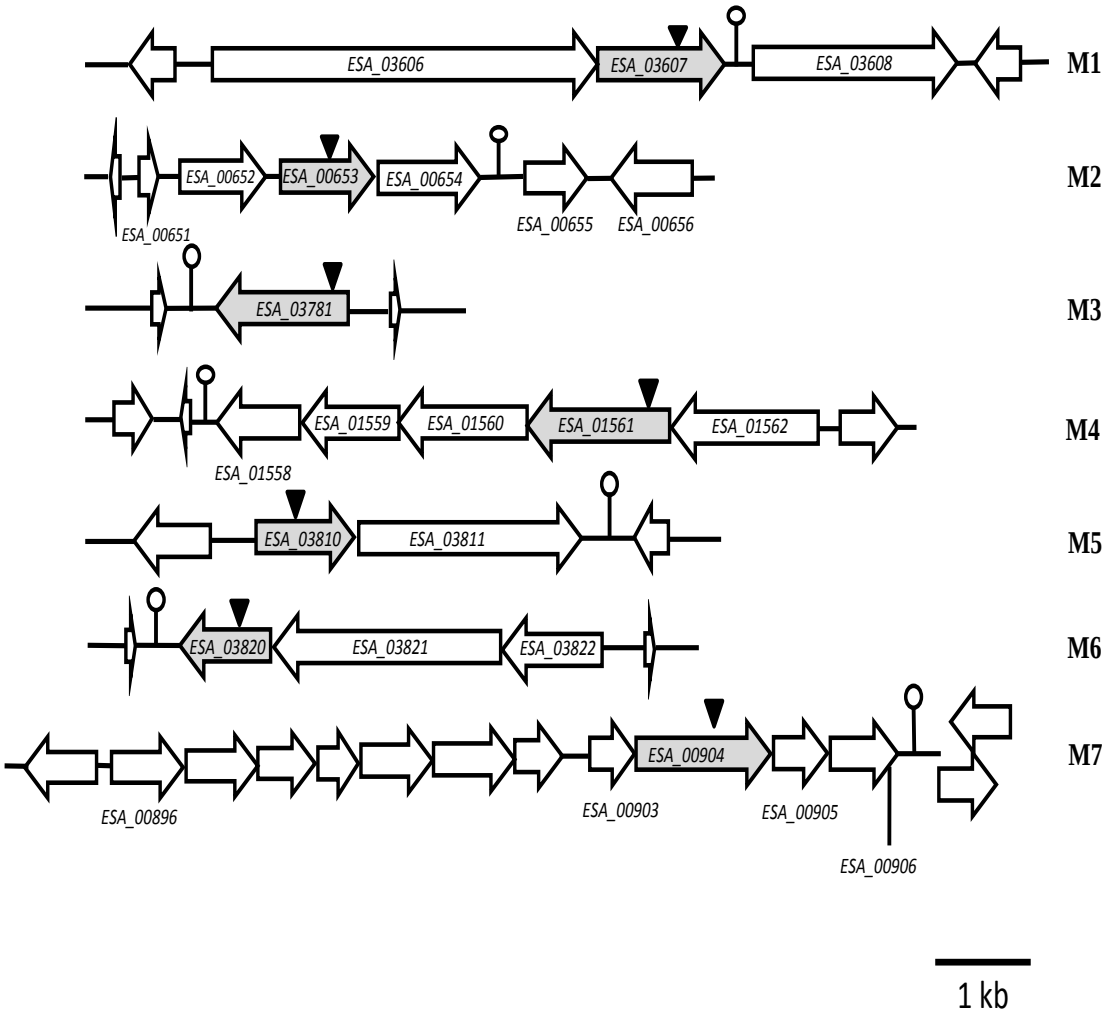
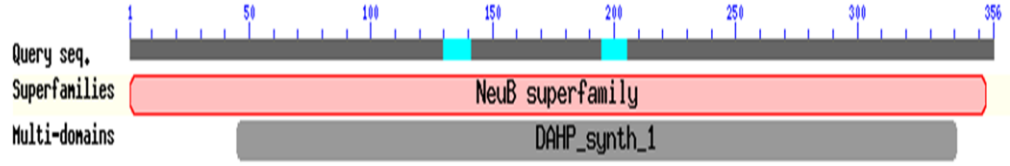


Fig 4. Genomic organisation of insertion sites in transposon mutants with delayed growth in RSM (M1-M7). The diagram was drawn approximately to scale using *C. sakazakii* BAA_894 genome sequence data. Open reading frames (shaded in grey) are genes with transposon insertion. Black arrowheads represent the approximate location of the transposon insertion. White open reading frames are flanking genes. Lollipops indicate predicted terminator locations.

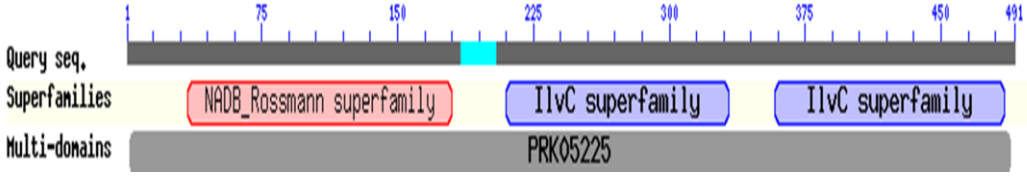
M1
ESA_03607



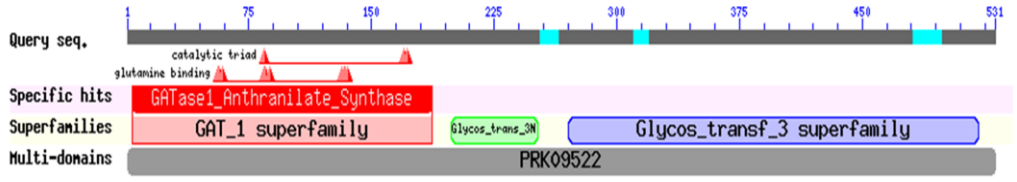
M2
ESA_00635



M3
ESA_03781



M4
ESA_01561



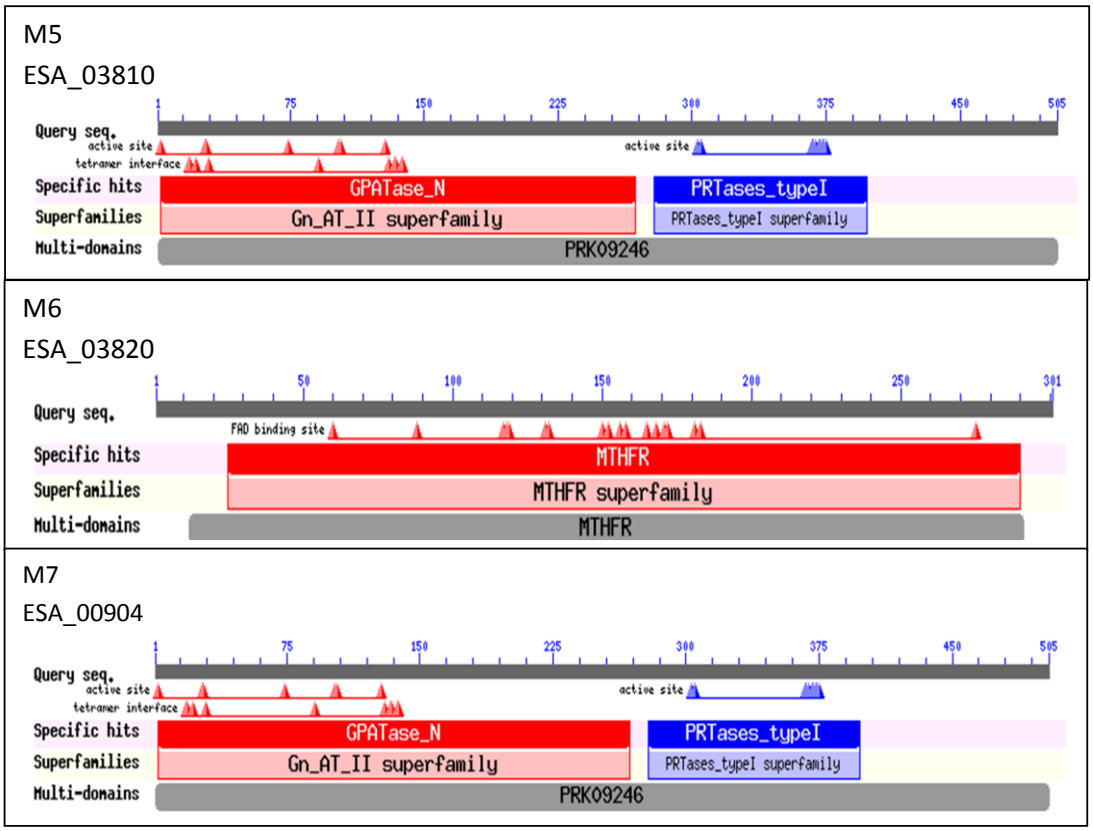


Fig 5. Conserved domains present in the putative proteins encoded by the disrupted genes in M1-M7. Proteins were analysed for the presence of conserved domains using the conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/).

Mutant M1. In M1 the transposon disrupted gene *ESA_03607*. Bioinformatic analysis revealed the product of this gene has 92% maximum identity to the glutamate synthase subunit beta of *E. coli* S88. Glutamate synthase catalyzes the single-step conversion of L-glutamine and α-ketoglutarate into two molecules of L-glutamate. Glutamate synthase is a tetramer of dimers, with each dimer having one large and one small subunit (GltB and GltD, respectively) (Kumar *et al*., 2010). Conserved domain searches illustrate the protein has a GltD domain with an e-value of zero.

Mutant M2. In M2 the transposon inserted into *ESA_00653*. This gene encodes a hypothetical protein. *In silico* analysis suggests that it may be a 3-deoxy-D-arabino-heptulosonate-7-phospahte synthase (DAHP synthase). This protein shows 90% identity to a DAHP synthase in *Klebsiella variicola* At22. DAHP synthase catalyzes the formation of 3-deoxy-D-arabino-heptulosonate-7-phosphate from phosphoenolpyruvate and D-erythrose-4-phosphate and is tyrosine sensitive (Ma *et al*., 2012). A conserved domain search revealed a DAHP synthetase I family (e-value=1.04^{-110}).

Mutant M3. The transposon disrupted gene *ESA_03781* in M3. Bioinformatic analysis indicates this gene encodes a ketol-acid reductoisomerase (IlvC). Conserved domain searches show an IlvC (ketol-acid reductoisomerase) domain (e-value=9.68^{-32}). IlvC catalyzes the formation of (R)-2,3-dihydroxy-3-methylbutanoate from (S)-2,3-hydroxy-2-methyl-3-oxobutanoate and is involved in valine biosynthesis. It also catalyses the formation of (R)-2,3-dihydroxy-3-methylpentoate from (S)-2,3-hydroxy-2-methyl-3-oxopentoate in isoleucine biosynthesis (Tyagi *et al*., 2005). Homology searches reveal 95% identity to ketol-acid reductoisomerase of *Escherichia coli* 536.

Mutnat M4. *ESA_01561* was disrupted in M4. The product of this gene displays homology to a bifunctional glutamine amidotransferase/anthranilate phosphoribosyltransferase (TrpD) in *Shigella flexneri* 5a str (91% maximum identity). It forms a heterodimer with TrpE and this complex catalyzes the formation of anthranilate from chorismate and glutamine (Ramos

et al., 2008). A search of the conserved domains reveal a glutamine aminotransferase (e-value=3.35^{-77}) and an anthranilate phosphoribosyltransferase (e-value=3.85^{-102}).

Mutant M5. In M5 the transposon inserted into *ESA_03810.* Bioinformatic analysis suggests this gene encodes an acetylornithine deacetylase (ArgE). The protein is 91% homologous (maximum identity) to ArgE in *Salmonella enterica* subsp. *enterica* serovar *Typhi* str. P-stx-12. This catalyzes the formation of L-ornithine from (N)-2-acetyl-L-ornithine in arginine biosynthesis (McGregor *et al*., 2007). A search for conserved domains reveal the presence of an acetylornithine deacetylase subfamily domain (e-value=1.29^{-161}).

Mutant M6. In M6 the transposon disrupted gene *ESA_03820. In silico* analysis suggests this gene encodes a 5,10-methylenetetrahydrofolate reductase (MetF). This protein shows 95% identity with MetF in *Salmonella enterica* subsp. *enterica* serovar *Choleraesuis* str. SC-B67. In addition, conserved domain searches illustrate a 5,10-methylenetetrahydrofolate reductase with an e-value of zero. MetF reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. It is a cytoplasmic, NAD(P)-dependent enzyme. MetF is also used in the methylation of homocysteine to form methionine (Sheppard *et al*., 1999).

Mutnat M7. *ESA_00904* was disrupted in M7. Bioinformatic analysis indicates this product of this gene has 95% maximum identity to an amidophosphoribosyltransferase in *Escherichia coli* str. K-12 substr. MG1655. This protein catalyzes the first step of the de novo purine nucleotide biosynthetic pathway (Bera *et al*., 2000). A search of the conserved domain revealed the presence of an amidophosphoribosyltransferase domain (e-value=0).

Growth of mutants in milk supplemented with amino acids

In silico analyses suggested that the disrupted genes in four of the mutants are putatively associated with amino acid metabolism. It was therefore decided to investigate if supplementation with the relevant amino acid would restore growth of these mutants.

For M1, glutamic acid was added to RSM. For M3 isoleucine and valine was added to RSM. For M5 arginine was added to RSM. For M6 methionine was added to RSM. These concentrations fully restored the mutants to wild type levels of growth in RSM (Figure 6 A, B, C and D).

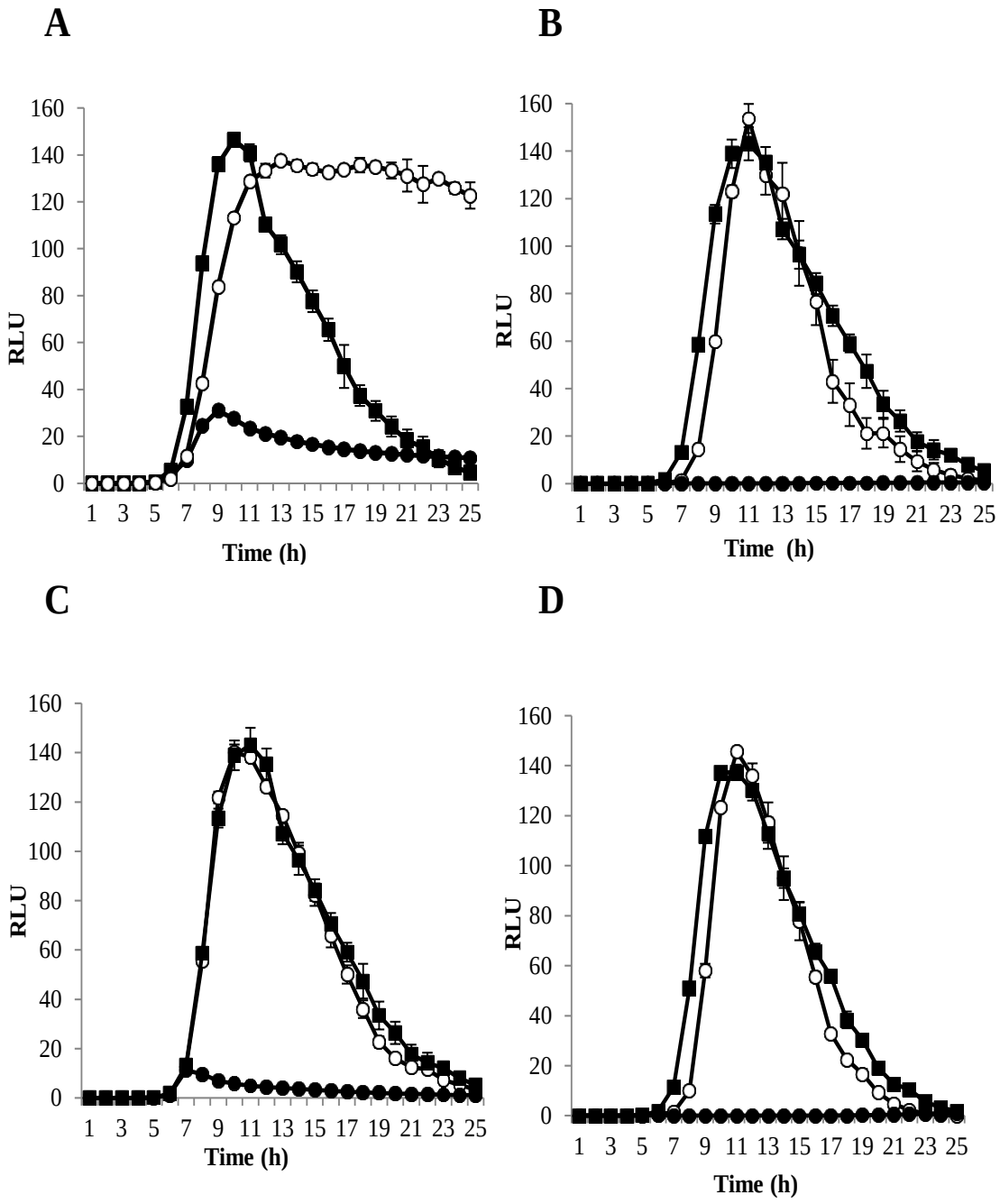


Fig 6. Growth of mutants supplemented with amino acids (A) WT (■), M1 plus 8.76mg/ml glutamic acid (○), M1 (●). **(B)** WT (■), M3 plus 1.6mg/ml isoleucine and 2mg/ml valine (○), M3 (●). **(C)** WT (■), M5 plus 1mg/ml arginine (○), M5 (●). **(D)** WT (■), M6 plus 0.16mg/ml methionine (○) M6 (●). Bioluminescence was measured using a Luminoskan luminometer. Error bars represent the standard deviations of triplicate experiments.

Complementation of Transposon mutants

These four transposon mutants were subsequently complemented with full functional copies of the disrupted genes to determine if this would also restore growth to wild type levels in RSM. Equal initial inoculums of each strain (wild type, transposon mutants and complemented strains) were added to RSM. Fig 7 shows the bioluminescence readings obtained at 12 hours. RLU readings obtained were as follows: WT $1.34x10^9 \pm 0.43$ RLU, M1 $5.5x10^8 \pm 0.6$ RLU, M1Comp $1.33x10^9 \pm 0.43$ RLU, M3 $6.23x10^7 \pm 0.13$ RLU, M3Comp $1.49x10^9 \pm 0.07$ RLU, M5 $3.36x10^8 \pm 0.66$ RLU, M5Comp $1.3x10^9 \pm 0.2$ RLU, M6 $3x10^8 \pm 0.5$ RLU, M6Comp $1.2x10^9 \pm 0.2$ RLU) (Figure 7 A, B, C and D). When compared to the wild-type, all RLU readings for the transposon mutants were significantly lower whereas readings for complemented strains were not significantly different (as determined by Student's t-test).

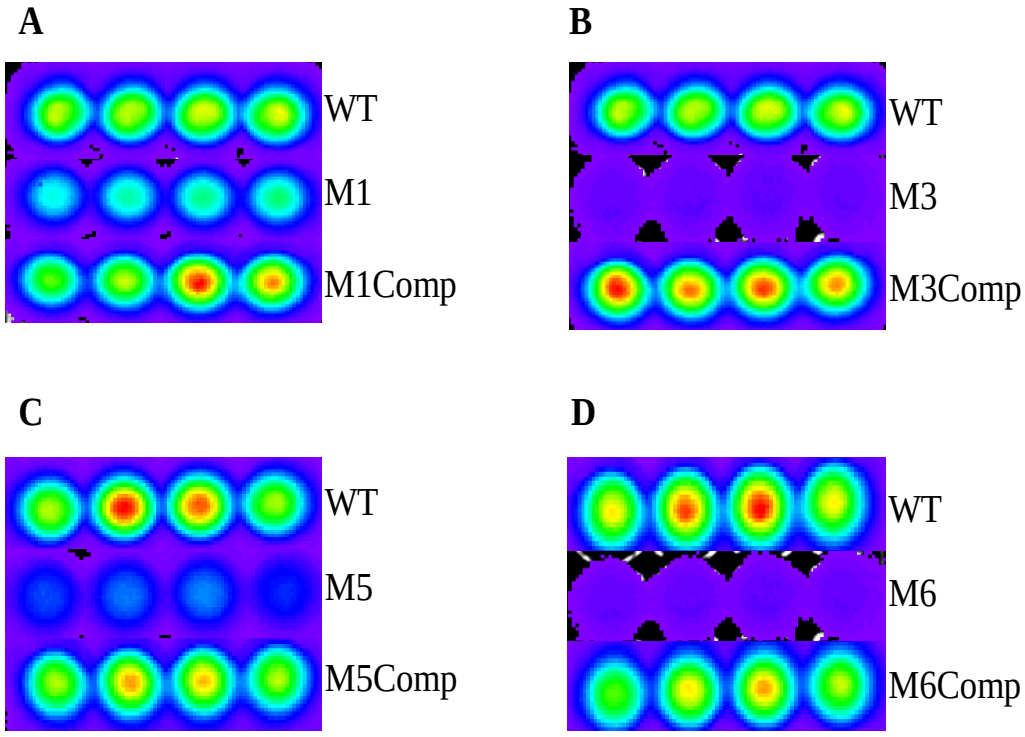


Fig 7. Growth of complemented Tn mutants in RSM using an IVIS 100 imager after 12 hours of growth **(A)** M1 and M1Comp, **(B)** M3 and M3Comp, **(C)** M5 and M5Comp, **(D)** M6 and M6Comp.

Discussion

To date, much of the research into the genes required for growth of bacteria in milk has focused on LAB e.g. *L. lactis* and *S. thermophilus.* Less information is available on genes essential for growth of pathogenic bacteria in milk. Lippolis *et al*., (2008) investigated the proteomic variation in *E. coli* when grown in fresh milk versus LB. This study observed changes in the adaptation of *E. coli* when grown in milk. The strain used was isolated from a clinical case of bovine mastitis. The authors suggest that some of the protein changes required for growth of the pathogenic strain of *E. coli* in milk (compared to a non-mastitis causing strain) could be identified and subsequently be used for therapeutic interventions.

Liu *et al*., (2008) investigated the gene expression profiling of *L. monocytogenes* during growth in ultra high-temperature-processed skim milk at 4°C using microarray technology. The upregulated genes included genes encoding transport and binding proteins, cell division, amino acid biosynthesis, energy metabolism, protein synthesis, transcriptional regulators and hypothetical proteins. This study proposed identification of these genes could ultimately facilitate food processors to develop more effective preservation strategies to control levels of *L. monocytogenes* in milk.

With regards to *C. sakazakii*, information is lacking regarding the genes required for growth of this pathogen in milk. The current study was initiated to address this lack of knowledge. A transposon library was successfully created in a Lux-tagged *C. sakazakii* strain. Transposon mutant libraries have previously been created in naturally light-emitting strains of bacteria. For example, Martin *et al*., (1989) created a bank of 30,000 transposon insertion mutants to identify the loci-encoding functions required for luminescence in *Vibrio harveyi.* Graf *et al*., (1994) screened 2,462 transposon insertion mutations for motility in *V. fischeri.* Ciche *et al*., (2001) screened 2,800 transposon insertion mutants for the ability of

Photorhabdus luminescence to support nematode growth and reproduction. However, to our knowledge our study is the first report of creating a transposon mutant library in a constructed Lux-tagged strain.

Our initial screen identified 35 mutants emitting little or no light in RSM. Some of these Tn mutants were eliminated from further studies by examining their growth in LB broth and by plating them on LB agar and checking the amount of light they emitted compared to the wild type. In mutants with a bioluminescence defect it is possible the transposon either inserted into a gene in p16S*lux* or inserted in a gene in the light emitting reaction. Luciferase catalyzes the reaction of molecular oxygen, reduced flavin mononucleotide ($FMNH_2$) and a long chained aldehyde generating the resulting carboxylic acid, FMN, water and light (490 nm) (Bachmann *et al.,* 2007). Therefore a transposon insertion in this reaction would affect the emission of bioluminescence.

Many of the functions necessary for growth of LAB in milk involve the supply of amino acids (Arioli *et al*., 2007, Monnet *et al*., 2005, Dudley *et al*., 2001, Garault *et al*., 2000). There is a limited availability of free essential amino acids in milk (Tan *et al*., 1993). For this reason LAB have an intricate proteolytic system to convert milk casein to free amino acids and peptides for growth (Law *et al*., 1997). Therefore a mutation in any of the genes that function to metabolise amino acids may cause reduced growth of the bacteria in milk. In our study the majority of the mutants we identified disrupted genes linked to amino acid metabolism: *ESA_03607* (glutamic acid metabolism), *ESA_03781* (isoleucine and valine biosynthesis), *ESA_01561* (tryptophan biosynthesis), *ESA_03810* (arginine metabolism). *ESA_00653* is thought to play a role in the shikimate pathway (Ma *et al.,* 2012) which is used to generate the aromatic amino acids (phenylalanine, tyrosine and tryptophan). *ESA_03820* is believed to be involved in methane metabolism. However, it may also play a role in methionine metabolism as the reaction it catalyzes provides a methyl donor to homocysteine, a precursor to methionine (Sheppard *et al*., 1999). *ESA_00904* is the only gene without a direct link to amino acid metabolism and may be involved in purine

biosynthesis. Derzelle *et al*., (2005) investigated the proteome of *S. thermophilus* in milk and a homolog of *ESA_00904* annotated as *purF* was upregulated when grown in milk. The same gene was upregulated in the proteome profile of *L. lactis* grown in milk (Gitton *et al*., 2005).

Growth of the seven mutants was also investigated in IMF. IMF can be considered to be more nutritious then RSM due to the additional ingredients (e.g. lactose, galactooligosaccharides, vitamins and minerals). This may explain why growth of two of the mutants improved (M4 and M7) in IMF compared to their previous growth in RSM. However, growth of the majority of mutants was affected in IMF.

Growth of four mutants was restored to wild type levels when the relevant amino acid was added (M1, M3, M5 and M6), further highlighting the importance of these genes for progress of the bacteria in milk.

ESA_03607

This gene encodes a putative a glutamate synthase small subunit (GltD). In *C. sakazakii* GltBD may form part of an operon (Figure 4) flanked by an aceltylglutamase kinase. Letort *et al*., (2001) demonstrate omission of both glutamate and glutamine in minimal media prevented growth of *S. thermophilus*. In the proteomic signature of *L. lactis* in milk, Gitton *et al*., (2005) found upregulation of *gltD.* Lippolis *et al*., (2008) investigated the proteomic changes in *E. coli* when grown in fresh milk versus laboratory media and found levels of *gltB* increased when grown in milk compared to laboratory media. Monnet *et al*., (2005) found *glnA* essential for growth of *S. thermophilus* in milk. Glutamine synthetase (encoded by *glnA*) catalyzes the formation of glutamine from glutamate and ammonia. The product of *glnBD* catalyzes the formation of glutamate from glutamine, therefore the two pathways are related and a mutation in *glnB* or *glnD* could reasonably cause reduced growth of the bacteria in milk.

ESA_03781

This gene is predicted to encode a ketol-acid reductoisomerase (IlvC) which may play a role in valine and isoleucine biosynthesis; these are both branched chain amino acids. Garault *et al*., (2000) reported branched-chain amino acid biosynthesis is required for optimum growth of *S. thermophilus* in milk. This paper identified two mutants with reduced growth in milk (*ilvB* and *ilvC*). In *C. sakazakii* BAA-894 the organisation of the *ilvC* gene is similar to *E. coli* as it is flanked by *ilvY*. However, in *C. sakazakii* there is a small (138 base pairs) open reading frame (ORF) between these genes. Gitton *et al*., (2005) and Derzelle *et al*., (2005) found *ilvC* to be upregulated in *L. lactis* and *S. thermophilus* when grown in milk. It was also upregulated in *E. coli* when grown in fresh milk (Lippolis *et al*., 2009).

ESA_03810

In *C. sakazakii* this gene is proposed to function as an acetylornithine deacetylase (ArgE) and may play a role in arginine biosynthesis. ArgE catalyzes the formation of ornithine which is an obligatory element in the 8 enzymatic steps in the biosynthesis of arginine (McGregor *et al*., 2007). Arioli *et al*., (2009) found carbamoylsynthetase (*carB*) activity is essential for growth of *S. thermophilus* in milk. This enzyme has a role in pyrimidine metabolism and is a precursor for the biosynthesis of arginine. Arioli *et al*., (2009) restored growth of their *carB* mutant by addition of arginine and uracil. Growth of M5 also returned to wild type levels by addition of arginine (Figure 6 C).

ESA_03820

In *C. sakazakii* the product of this gene is suggested to function as a methylenetetrahydrofolate reductase (MetF). Similar to *E. coli* the genes are putatively organised in a gene cluster *metJBLF* (Sheppard *et al*., 1999). Although homologs of *metF* have not been directly linked to growth in milk it is believed to play a role in methionine biosynthesis and this amino acid is advantageous for progress of *S. thermophilus* in a minimal chemically defined medium (Letort *et al*., 2001). In addition, Herve-Jimenez *et al*.,

(2008) showed *metF* is expressed during late phase growth of *S. thermophilus* in milk. Lippolis *et al*., (2009) also demonstrated this gene was upregulated during growth of *E. coli* in milk.

The four mutants (M1, M3, M5 and M6) were subsequently complemented. They were chosen as their growth was affected in both RSM and IMF and they were directly linked to amino acid metabolism. As shown in Figure 7 restoration of the complemented mutants was comparable to wild type levels of growth in milk.

In conclusion, a transposon mutagenesis bank was successfully created in a Lux-tagged strain of *C. sakazakii*. This bank was screened for mutants with delayed growth in milk and potential candidates were subsequently identified using single primer PCR and bioinformatic analysis. The disrupted genes compare with previous results obtained in LAB strains, as the majority were found to be involved in amino acid biosynthesis. In these mutants addition of the relevant amino acids fully restored the strain to levels similar to wild type growth in milk. Complemented mutants also displayed growth in milk comparable to the wild type strain. Considering that infant formula is the primary transmission vehicle for *C. sakazakii*, understanding how this pathogen grows in milk is imperative. Identification of these genes may aid in finding targets for more effective control strategies. This could reduce the incidence of infection and decrease the number of high-cost recalls of IMF.

Chapter V

Identification of *Cronobacter sakazakii* genes involved in the tolerance to Lactoferricin B

Abstract

Cronobacter sakazakii is an opportunistic pathogen associated with life-threatening infections, predominantly in neonates. Infection has been linked to the consumption of contaminated infant milk formula (IMF). Lactoferricin B (Lfcin B) is a milk derived antimicrobial peptide obtained from the pepsin cleavage of lactoferrin. It has been shown to be active against a broad range of pathogens including *C. sakazakii*. A transposon mutagenesis library was created in a strain of *C. sakazakii* and screened for mutants with increased sensitivity to Lfcin B. Mutations were identified and the genes involved were characterised by their proposed functions (encoding proteins involved in transport, DNA recombination and repair, central metabolic reactions and stress response). Lfcin B has the potential to be added to IMF as a preservative in order to inhibit *C. sakazakii.* Therefore, identification of the molecular mechanisms of *C. sakazakii* tolerance to Lfcin B is essential to potentiate the activity of this milk derived antimicrobial peptide.

Introduction

Cronobacter sakazakii is an opportunistic pathogen that causes rare but life threatening cases of meningitis, necrotizing enterocolitis and septicaemia in neonates (Nazarowec-White *et al*., 1997a). Powdered infant milk formula (IMF) is often implicated as the primary source of infection. In addition IMF is not sterile therefore contamination with *C. sakazakii* can occur if strict microbiological guidelines are not practiced. This is a problem for IMF manufacturers due to the high mortality rate and the substantial cost of recalls.

Lactoferricin B (bovine lactoferricin) (Lfcin B) is a 25-residue disulfide cross-linked, cationic peptide corresponding to amino acid residues 17-41 of the N-terminal of bovine lactoferrin (Figure 1). It can be produced by gastric pepsin digestion of bovine lactoferrin and possesses antimicrobial activity against a wide variety of microorganisms including Gram positive and Gram negative bacteria, fungi, protozoa and viruses (Bellamy *et al*., 1992, Orsi 2004, Gifford *et al*., 2005). Shorter derivatives of Lfcin B have also exhibited antimicrobial activity (Liu *et al.,* 2011, Ulvante *et al*., 2001). An 11-mer derivative representing residues 4-14 in Lfcin B, has been recognized as being necessary for the antibacterial effect (Kang *et al*., 1996). The primary sequence of Lfcin B contains mainly hydrophobic and positively charged residues. This may facilitate interaction with the negatively charged elements in the bacterial envelope, such as lipopolysaccharide (LPS) in Gram-negative bacteria and lipotechoic acid in Gram-positive bacteria.

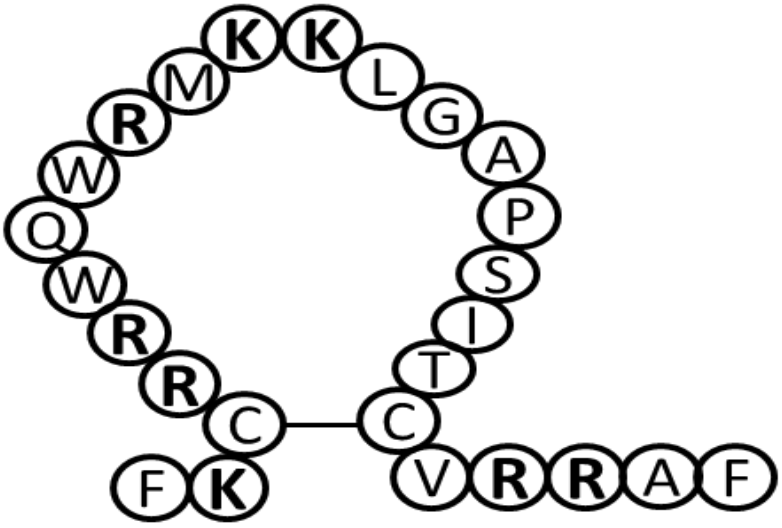


Fig 1. Amino acid sequence of lactoferricin B. Basic amino acids are highlighted in bold.

Due to its broad range of action, it has been suggested that Lfcin B interacts directly with membranes instead of with a specific receptor (Arseneault *et al*., 2010). The antibacterial effect is believed to be accomplished via a pore forming mechanism (Hwang *et al*., 1998). Lfcin B also exerts an intracellular effect, in that Ulvante *et al.*, (2004) observed that sublethal concentrations of Lfcin B inhibited DNA, RNA and protein synthesis of *Escherichia coli* and induced filamentation during an SOS response in bacteria. Tu *et al*., (2011) employed an *E. coli* proteome chip to identify the intracellular targets of Lfcin B. Sixteen proteins were identified and the majority

interact with proteins that affected the tricarboxylic acid (TCA) cycle. Phosphoenolpyruvate carboxylase was identified as a target of Lfcin B, suggesting that one of its mechanisms of action may be connected to pyruvate metabolism. A pyruvate assay was carried out in *E. coli* with and without Lfcin B and it was shown that abnormal accumulation of pyruvate occurred when *E. coli* was incubated with Lfcin B (Tu *et al*., 2011).

Wakabayashi *et al,* (2008) investigated the susceptibility of four *C. sakazakii* strains to lactoferrin derived compounds, including Lfcin B. MIC's were determined and revealed all strains of *C. sakazakii* showed susceptibility to Lfcin B, with MIC's ranging from 5.1µM-9.1µM.

IMF manufacturers are frequently creating new ingredients and formulations that can be added to milk to improve the safety and health benefits of their IMF; therefore an inhibitor of *C. sakazakii* would certainly be of interest. Bovine lactoferrin is added as an ingredient to some infant formulae in Japan, Indonesia and Korea (Tomita *et al*., 2009). The Morinaga milk company was the first IMF manufacturer to add bovine lactoferrin to its infant formulae (Tamura, 2004). The expected effects are anti-infection, improvement of oro-gastrointestinal microflora, immunomodulation, anti-inflammation and anti-oxidation. Tomita *et al*., (2009) also stated that bovine lactoferrin pepsin hydrolysate containing Lfcin B is added to some peptide based infant formulae. As Lfcin B is already added to some IMF, there is potential for further research into the possibility of utilizing Lfcin B as a preservative in IMF.

The aim of the current study was to create a transposon mutagenesis library in *C. sakazakii* and to use it to identify genes involved in the tolerance of *C. sakazakii* to Lfcin B. A better understanding of these mechanisms of bacterial resistance is

essential to progress efforts to exploit milk derived antimicrobials for application as a preservative or for therapeutic interventions. For example developing compounds that target one of the resistance mechanisms could enhance the activity of the milk derived antimicrobial.

Materials and methods

Bacterial strains and culture conditions

Strains used in this study are listed in Table 1. They were routinely grown in Luria-Bertani (LB) broth (Merck, Darmstadt, Germany) statically at 37°C for ~16 h unless otherwise stated. For solid media 1.5% agar was added. For certain experiments Brilliance *Enterobacter sakazakii* (DFI, Druggan, Forsythe and Iversen) agar (Oxoid, UK), TSA (Tryptic Soya Agar) (Oxoid), Mueller Hinton (Oxoid) broth and Mueller Hinton agar were used. If required kanamycin (50µg/ml) was added to cooled autoclaved media. Kanamycin (Sigma-Aldrich) was filter sterilized and made up as a concentrated stock in sterile distilled water.

Test antimicrobials

Lactoferricin B (Sigma-Aldrich L1290) was dissolved in sterile HPLC grade water to give a stock solution of 1mM. Aliquots of stock solution were stored at -80°C until required.

Nisin powder (2.5%, balance sodium chloride and denatured milk solids) (Sigma-Aldrich) was suspended in sterile HPLC grade water to give a stock solution of 10mg/ml.

Ampicillin (Sigma-Aldrich) was filter sterilized and made up as a concentrated stock in sterile HPLC grade water (50mg/ml).

Table 1. Bacterial strains and transposon used in this study

Strains or transposon	Relevant genotype or characteristic(s)	Source or reference
EZ-Tn5<kan-2> (Tn)	Km^r, mini-Tn5 transposon	Epicentre, Madison, WI
6522	*Cronobacter sakazakii*	DPC
6523	*Cronobacter sakazakii*	DPC
6524	*Cronobacter sakazakii*	DPC
6525	*Cronobacter sakazakii*	DPC
6526	*Cronobacter sakazakii*	DPC
6527	*Cronobacter sakazakii*	DPC
6528	*Cronobacter sakazakii*	DPC
6529 (WT)	*Cronobacter sakazakii*	DPC
6530	*Cronobacter sakazakii*	DPC
6531	*Cronobacter malonaticus*	DPC
ATCC51329	*Cronobacter muytjensii*	DPC
NCTC8155	*Cronobacter sakazakii*	DPC
ATCC12868	*Cronobacter sakazakii*	DPC
ATCC29004	*Cronobacter sakazakii*	DPC
ATCC29544	*Cronobacter sakazakii*	DPC
NCTC11434	*Enterobacter gergorviae*	DPC
NCTC11590	*Enterobacter cloacae*	DPC
NCTC11933	*Enterobacter cloacae*	DPC
NCTC10006	*Enterobacter aerogenes*	DPC
DH5-α	*E. coli*	UCC
L1	WT with an insertion in *ESA_04322* Km^r	This study
L2	WT with an insertion in a gene of unknown function Km^r	This study
L3	WT with an insertion in a gene of unknown function Km^r	This study
L4	WT with an insertion in *CTU_32540* Km^r	This study
L5	WT with an insertion in *ESA_03985* Km^r	This study
L6	WT with an insertion in *ESA_00567* Km^r	This study
L7	WT with an insertion in *ESA_01660* Km^r	This study
L8	WT with an insertion in *ESA_00894* Km^r	This study
L9	WT with an insertion in *ESA_00177* Km^r	This study
L10	WT with an insertion in *ESA_02808* Km^r	This study

Km^r = kanamycin resistance

Strains were obtained from the DPC, Dairy Products Research Centre, Moorpark, Fermoy, Co. Cork, Ireland and UCC, University College Cork culture collections.

Limits of tolerance to Lfcin B

DPC6529 (WT) was grown in triplicate and incubated overnight in LB broth for approximately 16 hours. To grow to log phase, a 2% inoculum was added to fresh LB broth for 2.5 hrs until an OD of ~0.3 was reached. 1ml of cells were centrifuged at 8000 x *g* for 7 min, the supernatants were removed and resuspended in 1ml of 10mM sodium phosphate buffer (pH7.4) The washed log phase cells were serially diluted in 10mM sodium phosphate buffer to give a final concentration of ~$5x10^5$ cfu/ml of bacteria. Bactericidal assays were performed according to Ericksen *et al*., (2005). Briefly 50µl of washed cells was transferred to a 96 well plate (Genetix, UK). 50µl of Lfcin B (Sigma) with final concentrations ranging from 125µM to 3.9µM was also transferred to the 96-well plate. The plate was incubated at 37°C over the next 2 hours, after which 100µl of double strength (2X) LB broth was added to each of the wells. Growth was monitored by measuring optical density (OD) at 620nm for the next 24 hours using a temperature controlled automatic plate reader (Thermo Scientific Multiscan FC). Limits of tolerance were also determined for stationary phase cells with the same initial inoculum as the log phase cells.

Transposon mutagenesis

A transposon mutagenesis library was constructed using the EZ-Tn5 <KAN-2>Tnp Transposome kit (Epicentre, Madison, WI) according to protocols provided by the supplier. Briefly, DPC6529 was grown overnight in LB for ~16 h statically. A 1% inoculation was transferred to fresh LB incubated shaking for ~3 hours at 37°C until and OD of ~0.6 was reached. The cells were centrifuged at 900 x *g* for 10 minutes at 4°C, the supernatant was discarded, and cells were resuspended in ice cold sterile distilled water and re-centrifuged. This was repeated 3 times, after which the cells

were resuspended in 10% ice cold glycerol and centrifuged again. The supernatant was removed and cells were resuspended in 500µl of ice cold 10% glycerol. 50µl aliquots were transferred to cold sterile 1.5ml centrifuge tubes and stored at -80°C.

For electroporations, 1µl of transposon DNA was added to 50µl of competent cells on ice in a 2mm cuvette (Molecular BioProducts, San Diego CA). The electroporation conditions were 2.5kV, 200Ω and 25µF with a Harvard Apparatus ECM 630 Electro Cell Manipulator (MA, USA). To aid recovery the cells were resuspended in 1ml super optimal broth with catabolite repression (SOC) and incubated shaking for 1 hour at 37°C. Cells were diluted 1:100 in prewarmed SOC medium. To select for transposon insertion clones, aliquots of 100µl were plated onto LB plates containing 50µg/ml kanamycin and plates were incubated overnight at 37°C. Single colonies were picked and transferred to individual wells of 96-well plates (Genetix, UK), containing 150µl/well of LB, supplemented with 7.5% glycerol and 50µg/ml kanamycin. Plates were incubated overnight at 37°C for approximately 16 hours and subsequently stored at -80°C.

Screening of Transposon mutants

The -80°C stock plates were thawed and replicated into 96-well plates (Genetix) containing LB broth plus 50µg/ml kanamycin using a 96-pin replicator (Genetix). These were incubated at 37°C for ~16 hours. Subsequently, mutants were serially diluted in 10mM sodium phosphate buffer (pH 7.4) to give a final concentration of ~$5x10^5$ cfu/ml of bacteria. 50µl of cells was transferred to a 96-well plate (Genetix, UK) and 50µl of Lfcin B (15.62µM) (Sigma) was added to each well. The plate was incubated at 37°C over the next 2 hours. Following this, 100µl of double strength (2X) LB broth was added to each of the wells. Growth was monitored by measuring

optical density (OD) at 620nm for the next 24 hours using a temperature controlled automatic plate reader (Thermo Scientific Multiscan FC). Viable plate counts were performed at selected time points by serially diluting samples in ¼ strength Ringer's solution and enumeration on LB agar.

DNA extraction, manipulations and sequencing

Strains were grown overnight (~16 hrs) in LB broth. DNA was extracted from 1ml of overnight cultures using the Invitrogen PureLink Genomic DNA Mini Kit following the manufacturer's instructions. PCR amplification was carried out using a G-storm cycler (Essex, UK). The amplified PCR products were separated on 1% (m/v) agarose gels and visualised with the DNR Bio-Imaging System (Jerusalem, Israel). PCR products were purified using Invitrogen PCR purification kit. The concentration of nucleic acids was determined using an Invitrogen Qubit fluorometer (Eugene, Oregon, USA).

Identification of transposon insertion sites

The transposon insertion site for each mutant was identified using a single primer PCR approach. DNA fragments were amplified from chromosomal DNA of the transposon mutants using primer Tn5PCRR or Tn5PCRF (Table 2) with the reaction conditions described by Karlyshev *et al*., (2000). The conditions were 1 min at 94°C, 20 cycles of 94°C for 30s, 50°C for 30s and 72°C for 3 min, 30 cycles of 94°C for 30s, 30°C for 30s and 72°C for 2 min, 30 cycles of 94°C for 30s, 50°C for 30s and 72°C for 2 min, followed by a 7 min extension at 72°C. PCR products were purified and sequenced with primer KAN-2 RP1 or KAN-2 FP1 (Table 2) by MWG Biotechnologies. BLASTn (Basic Local Alignment Search Tool) searches were performed with the obtained sequences (http://blast.ncbi.nlm.nih.gov/) to identify

homologous genes. Homologous regions in the genome of the sequenced *C. sakazakii* BAA-894 strain (accession number NC_009778) were analysed and the information obtained was used to generate Figure 5. Proteins were analysed for the presence of conserved domains using the conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/). The location of each protein in bacterial cells was predicted using PSORT (http://psort.hgc.jp/form.html).

Table 2. Primers used in this study

Primer	Sequence 5'→3'
Kan-2 FP1	ACCTACAACAAAGCTCTCATCAACC
Kan-2 RP1	GCAATGTAACATCAGAGATTTTCAG
Tn5PCRF	GCTGAGTTGAAGGATCAGATC
Tn5PCRR	CGAGCAAGACGTTTCCCGTTG

Survival assays

For survival assays, strains were grown statically at 37°C for approximately 16 hrs. 1ml aliquots of overnight cultures were centrifuged at 8000 x *g* for 7 min, the supernatants were discarded and the cell pellets were resuspended in 1ml 10mM sodium phosphate buffer and diluted in sodium phosphate buffer to give a final concentration of ~$5x10^5$ cfu/ml. 50µl was added to 50µl of Lfcin B incubated statically at 37°C for 1 hour. Viable plate counts were performed by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar in triplicate.

Well assays

The antibacterial activity of Lfcin B and nisin was measured by radial diffusion assay according to the method described by Derache *et al*., (2009). Cultures of DPC6529 (WT) and transposon mutants were incubated at room temperature (~20°C) overnight. The cells were centrifuged at 8,000 x *g* for 7 min, supernatants were removed and cell pellets were resuspended in 1ml sodium phosphate buffer. 100µl of washed cells was added to 15ml 10mM sodium phosphate buffer containing 0.03g of LB, 1% wt/vol ultra-pure agarose (Invitrogen) and 0.02% Tween 20 and poured into a petri dish. Once solid a 4.6mm diameter well was bored into the media. 10µl of the antimicrobial was added to the well. Peptide and control solutions were allowed to diffuse into the gel containing bacteria by incubating the plates for 3 hours at 37°C. The gel was then overlaid with 15ml 10mM sodium phosphate buffer containing double strength (2X) LB, plus 1% wt/vol ultra-pure agarose. After overnight incubation at 37°C, the diameter of the clear zone surrounding each well was measured.

Ampicillin disk diffusion assay

A disk diffusion assay was carried out following the instructions of the Clinical and Laboratory Standards Institute (CLSI, 2005) using ampicillin disks (10µg; Oxoid). 6529 (WT) and transposon mutants were streaked on TSA (Tryptic Soya Agar) and incubated at 37°C for 24 h. Colonies were suspended in 3ml of Mueller Hinton broth. The suspensions were swabbed onto the entire surface of Mueller Hinton agar with sterile swabs after which the antimicrobial disk (ampicillin, 10µg) (Oxoid, UK) was placed onto the surface of the plate with a sterile forceps. The plates were incubated upright at 37°C for 24 hours under aerobic conditions. The diameters of zones of inhibition were measured using a digital Vernier Callipers (Fisherbrand) and interpreted according to the CLSI guidelines for Enterobacteriaceae (Clinical and Laboratory Standards Institute 2005).

MIC (minimum inhibitory concentration) determination

MICs were determined in LB broth. A two-fold serial dilution of ampicillin was added to the wells of a 96-well plate containing *C. sakazakii* at a concentration of ~$5x10^5$ CFU/ml. Following a two hour incubation at 37°C, 100µl double strength LB broth was added. After 24 h of incubation at 37°C the plates were read, and the MICs were determined as the lowest concentration of ampicillin which resulted in the absence of apparent growth of the bacteria. MIC determinations were carried out in triplicate.

Pigment production

Strains were streaked onto TSA and incubated at 25°C for 72 hours after which the colonies were visually inspected for the production of a yellow pigment.

Results

Growth of *Cronobacter* strains in LB plus Lfcin B

To investigate the variation between *Cronobacter* strains, log phase cells of sixteen *Cronobacter* (fourteen *C. sakazakii*, one *C. malonaticus* and one *C. muytjensii*) were grown in LB plus 7.8µM Lfcin B with an initial inoculum of ~$5x10^5$ cfu/ml. For comparative purposes two *Enterobacter cloacae*, one *Enterobacter gergorviae* and one *Enterobacter aerogenes* were included (Figure 2). There were differences between the susceptibility of individual strains to Lfcin B; e.g. ATCC29544 was the most affected strain. DPC6529 was chosen as the target strain for mutagenesis as it was one of the easiest of the *Cronobacter* strains to genetically manipulate and it was one of the more stress resistant of the *Cronobacter* strains. It was clear all strains were affected by Lfcin B due to their delayed growth compared to the wild type strain.

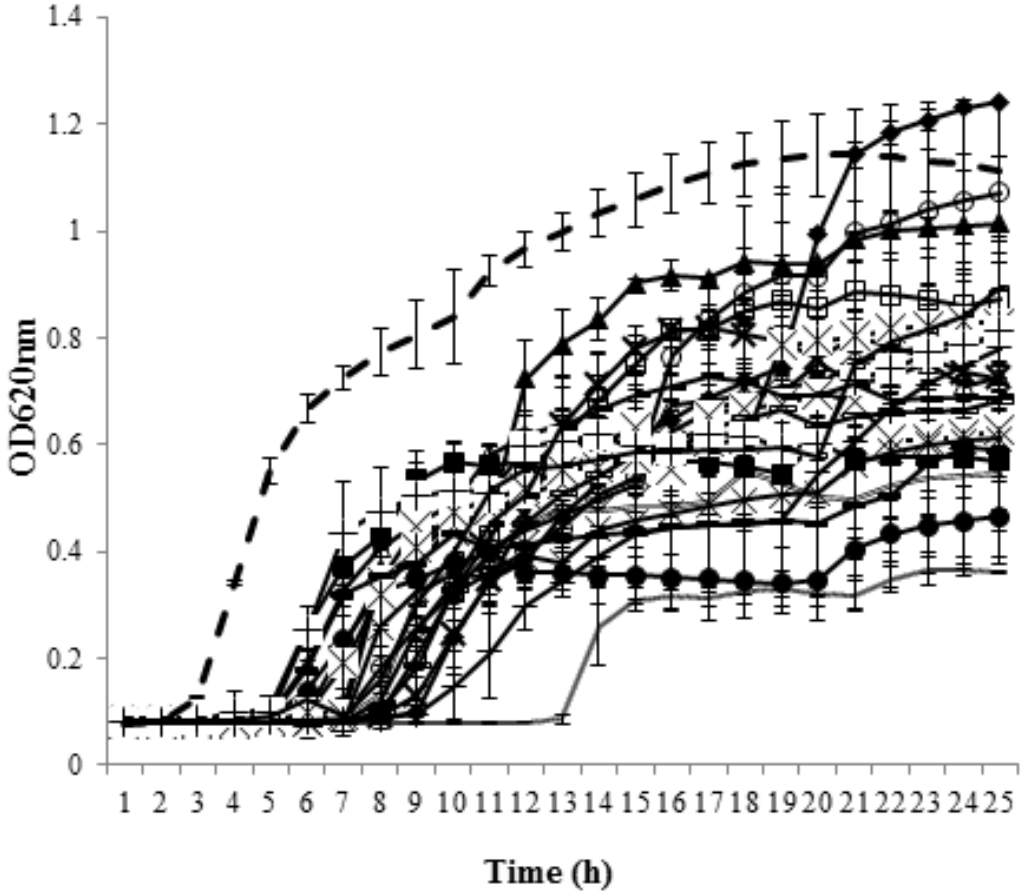


Fig 2. Growth curve of strains in LB containing 7.8 µM Lfcin B. DPC6522 (■), DPC6523 (□), DPC6524 (♦), DPC6525 (◊), DPC6526 (▲), DPC6527 (Δ), DPC 6528 (●), DPC6529 (WT) (○), DPC6530 (X), DPC6531 (⊠), ATCC51329 (▬), ATCC8155 (▭), ATCC12868 (+), ATCC29004 (⊞), ATCC29544 (=) NCTC11434 (✳), NCTC11590 (⊠), NCTC11933 (▭), NCTC10006 (▬), and DPC6529 (WT) containing no Lfcin B (– –).

Limits of tolerance of stationary phase and log phase cells to Lfcin B

The limits of tolerance to Lfcin B of DPC6529 were determined using stationary phase cells (Figure 3A) and log phase cells (Figure 3B) using an initial inoculum of ~$5x10^5$ cfu/ml. This was carried out to establish whether stationary and log phase cells display the same tolerance to Lfcin B. The log phase cells were more sensitive than stationary phase cells to Lfcin B. For example, concentrations of 125µM and 62.5µM prevented growth of log phase cells but stationary phase cells were able to grow at these concentrations.

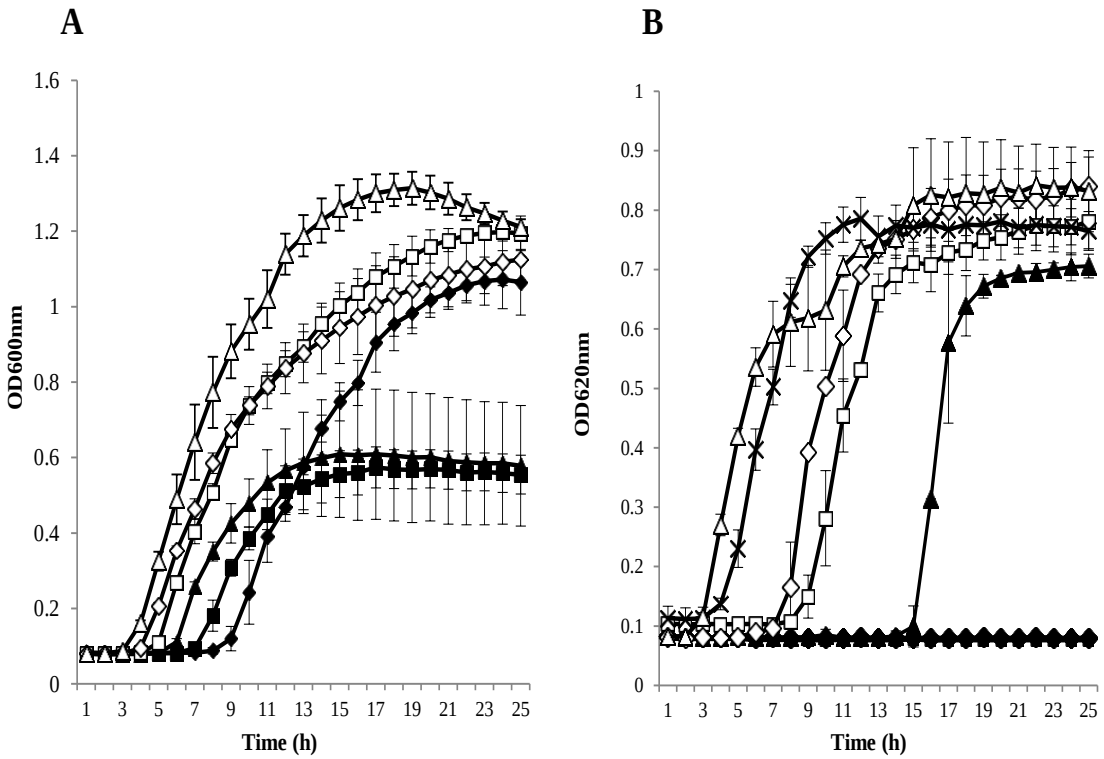


Fig 3. (A) Growth curve of stationary phase inoculum *C. sakazakii* DPC6529 (WT) in LB supplemented with Lfcin B. 125µM (♦). 62.5µM (■), 31.25µM (▲), 15.62µM (□), 7.8µM (◊) and wt without Lfcin B (Δ). **(B) Growth curve of log phase inoculum *C. sakazakii* DPC6529 (WT) in LB supplemented with Lfcin B.** 125µM (♦). 62.5µM (■), 31.25µM (▲), 15.62µM (□), 7.8µM (◊), 3.9 µM (x) and wt without Lfcin B (Δ). Error bars represent the standard deviation of triplicate experiments.

Creation and screening of the transposon mutant library

A *C. sakazakii* DPC6529 random transposon mutant library was created. Mutants with increased sensitivity to Lfcin B were identified using the approach outlined in Figure 4. From the limits of tolerance of stationary phase cells to Lfcin B (Figure 3A) it was decided to use a concentration of 15.62µM Lfcin B for screening of the transposon mutant bank as this was a concentration that had a limiting effect on growth of *C. sakazakii* 6529 but did not prevent growth.

Of 1029 mutants screened ten were chosen for further analysis due to their longer lag phase compared to the wild type strain when grown in LB plus Lfcin B (data not shown). All ten mutants displayed growth similar to the wild type strain when grown in LB at 37°C. Any differences were not statistically significant (Student's t-test $p<0.05$). This indicates that the functions encoded by the genes in question were not essential for growth under normal physiological conditions. The exact location of the transposon insertion site was identified by a single primer PCR approach, followed by sequencing of the insertion site (Table 3). When plated on TSA, all mutants produced a yellow pigment and all mutants produced blue/green colonies on DFI agar.

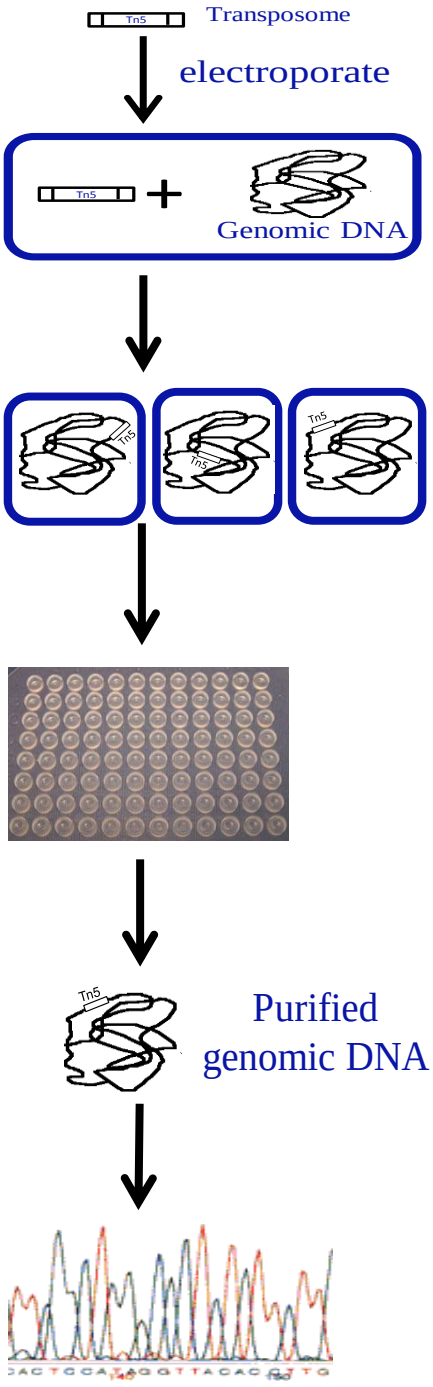


Fig 4. Method used to create and screen the transposon mutagenesis bank.

Table 3. Overview of Lfcin B transposon mutants isolated in this study

Tn mutant	Tn insertion site	Primer used	Protein accession number, Size of protein	Proposed function of encoded protein	PSORT localisation prediction
L1	*ESA_04322*	Tn5PCRR	ABU_79501 702 aa	4-α-glucanotransferase, MalQ, it acts to release glucose from maltodextrins	Bacterial cytoplasm
L2	No sequence similarity	Tn5PCRR	-	Unknown function	-
L3	No sequence similarity	Tn5PCRR	-	Unknown function	-
L4	*CTU_32540*	Tn5PCRR	CBA_33120 339 aa	Integrase gene product involved in DNA integration and recombination	Bacterial cytoplasm
L5	*ESA_03985*	Tn5PCRR	ABU_79171 253 aa	Uncharacterized protein conserved in bacteria, BsmA	Bacterial cytoplasm
L6	*ESA_00567*	Tn5PCRR	ABU_75857 354 aa	RecA (recombinase A), it catalyzes the hydrolysis of ATP in the presence of single-stranded DNA, the ATP-dependent uptake of single-stranded DNA by duplex DNA, and the ATP-dependent hybridization of homologous single-stranded DNAs	Bacterial cytoplasm
L7	*ESA_01660*	Tn5PCRR	ABU_76914 115 aa	Putative anti-adapter protein IraM. It inhibits *rpoS* proteolysis by regulating *rssB* activity, thereby increasing the stability of the sigma stress factor *rpoS* during magnesium starvation. May also be involved in the early steps of isoprenoid biosynthesis, possibly through its role as *rssB* regulator	Bacterial cytoplasm
L8	*ESA_00894*	Tn5PCRR	ABU_76164 924 aa	Subtilisin-like serine protease	Inner or outer membrane protein
L9	*ESA_00177*	Tn5PCRF	ABU_75478 379 aa	Hypothetical protein, putative iron-sulfur cluster binding protein	Bacterial cytoplasm
L10	*ESA_02808*	Tn5PCRR	ABU_78038 1049 aa	Hypothetical protein, multidrug efflux system protein AcrB	Plasma membrane

Bioinformatic analysis

Location of the transposon insertion sites are illustrated in Figure 5. Conserved domains of each of the proteins are illustrated in Figure 6. For two of the transposon mutants (L2 and L3) there was no sequence similarity to any protein in the NCBI datasite, therefore they were assigned as having unknown function.

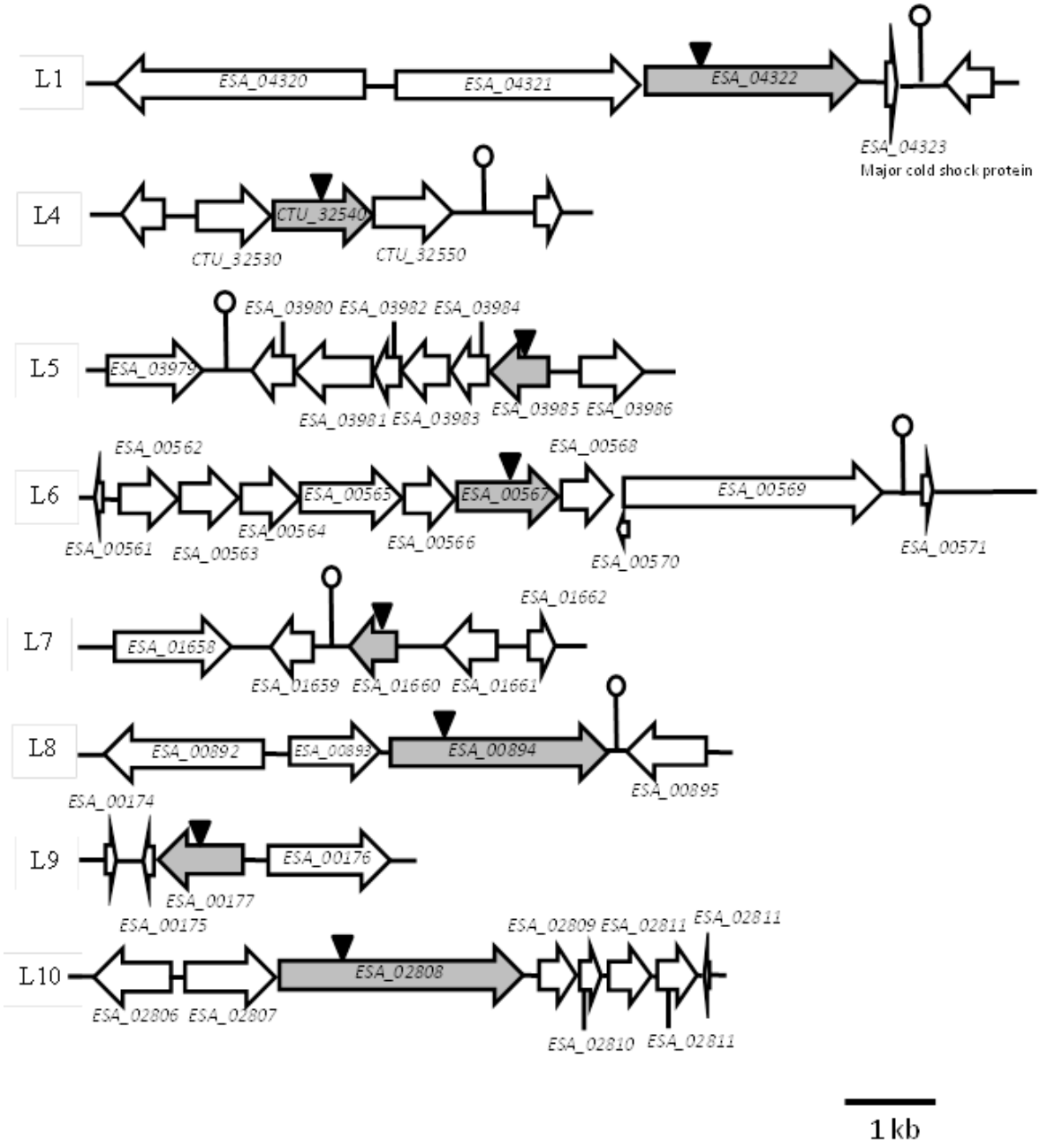
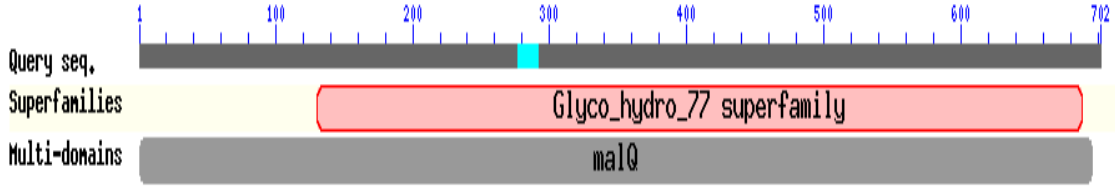
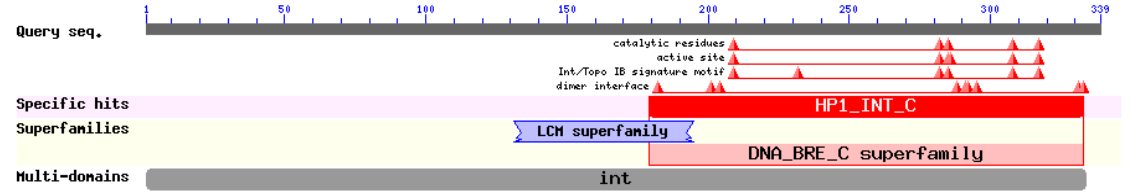


Fig 5. Genomic organisation of the transposon insertion sites (L1-L10) in mutants with delayed growth in Lfcin B. The diagram was drawn approximately to scale using *C. sakazakii* BAA-894 genome sequence data except for *CTU_32540* which was drawn using the *C. turicensis* z3032 genome sequence data. Open reading frames (shaded in grey) are genes with transposon insertion. Black arrowheads represent the approximate location of the transposon insertion. White open reading frames are flanking genes. Lollipops indicate predicted terminator locations.

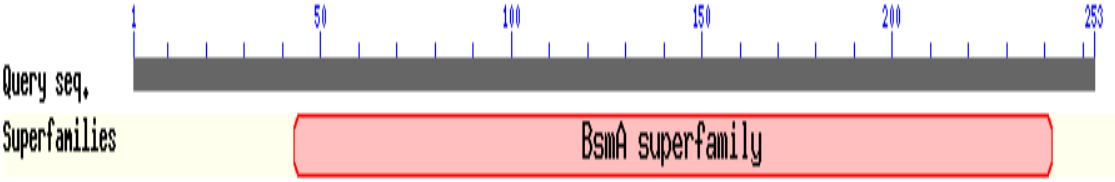
L1
ESA_04322



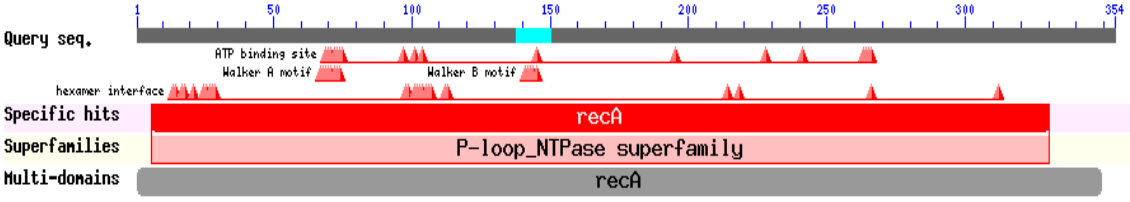
L3
CTU_32540



L4
ESA_03985



L5
ESA_00567



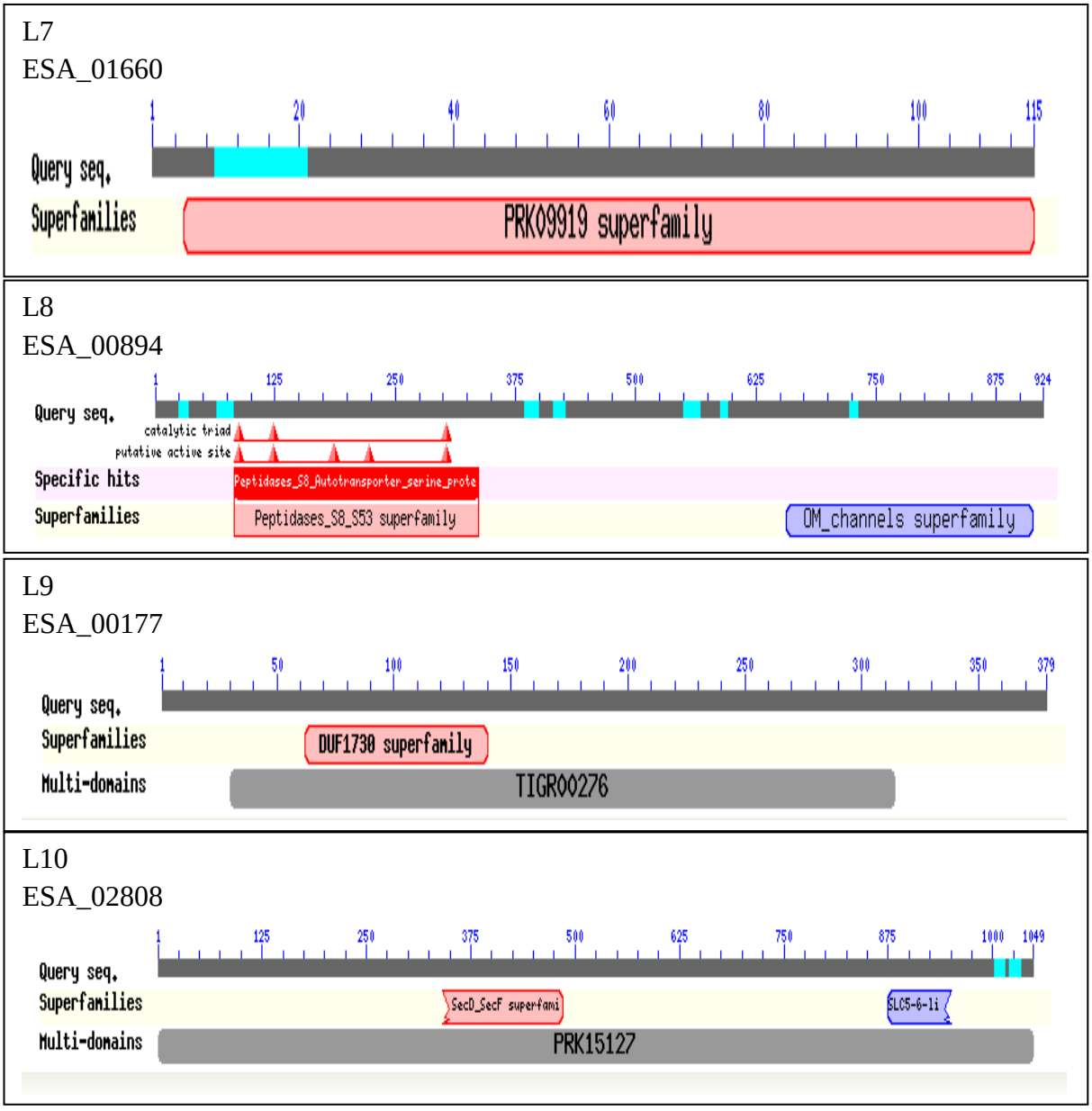


Fig 6. Conserved domains present in the putative proteins encoded by the disrupted genes in L1-L10. Proteins were analysed for the presence of conserved domains using the conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/).

Mutant L1. In L1 the transposon disrupted gene *ESA_04322. In silico* analysis suggests this gene encodes a 4-α-glucanotransferase (MalQ). This protein has a maximum identity of 77% to MalQ in *E. coli* IAI39. It is known that MalQ catalyzes a chemical reaction that transfers a segment of a 1,4 α-glucan to new position in an acceptor carbohydrate which may be glucose or a 1,4 α-glucan (Park *et al*., 2011). A search of the conserved domain showed a 4-α-glucanotransferase superfamily domain with an e-value of zero. PSORT predicted this protein is located in the bacterial cytoplasm.

Mutant L4. In L4 the transposon inserted into *CTU_32540.* A BLASTn of the sequence of this gene revealed no sequence similarity to the *C. sakazakii* BAA-894 genome. A tBLASTx illustrated 91% identity to an integrase gene product of *C. turicensis* z3032. Bioinformatic analysis revealed 92% maximum identity to a phage integrase protein in *Salmonella enterica* subsp. enterica serovar Typhimurium str. ST4/74. A conserved domain search revealed a Phage HP1 integrase with an e-value of 5.82^{-66}. HP1 integrase results in the site-specific recombination of bacteriophage HP1 genome (Hickman *et al.,* 1997). PSORT predicted this gene product is located in the bacterial cytoplasm.

Mutant L5. The transposon disrupted gene *ESA_03985* in L5. This gene encodes an uncharacterised protein conserved in bacteria which has 63% maximum identity to BsmA in *Pseudomonas fluorescens* F113. From the conserved domain search, a BsmA superfamily was identified with an e-value of 3.98^{-70}. This is a biofilm formation and stress response factor (Weber *et al*., 2010). PSORT predicted this protein is located in the bacterial cytoplasm.

Mutant L6. *ESA_00567* was disrupted in L6. *In silico* analysis suggests this gene encodes a recombinase A (RecA). The protein shows high % identity (95%) to recombinase A of *Escherichia coli* str. K-12 substr. MG1655. This bacterial enzyme has roles in homologous recombination, DNA repair and induction of the SOS response (Stohl *et al*., 2003). RecA couples ATP hydrolysis to DNA strand exchange. When a cell's DNA is damaged, RecA undergoes activation, which facilitates the autocleavage of LexA, and this allows the SOS genes to be expressed (Little *et al*., 1980). PSORT predicted this gene product is located in the bacterial cytoplasm.

Mutant L7. In L7 the transposon inserted into *ESA_01660.* This gene encodes a hypothetical protein which shares homology with the anti-adaptor protein IraM in *E. coli* B185 (23% maximum identity). A search of the conserved domains showed an anti-adapter protein family with an e-value of 1.21^{-45}. IraM inhibits *rpo*S proteolysis by regulating *rssB* activity, thereby increasing the stability of the sigma stress factor *rpoS* during magnesium starvation (Bougdour *et al*., 2008). It may also be involved in the early steps of isoprenoid biosynthesis, possibly through its role as *rssB* regulator (Hemmi *et al*., 1998). PSORT predicted this protein is located in the bacterial cytoplasm.

Mutant L8. In L8 the transposon disrupted gene *ESA_00894.* Bioinformatic analysis indicates this gene encodes a hypothetical protein. Conserved domain searches of this protein reveal a peptidase S8 family domain in autotransporter serine proteases (e-value 4.17^{-78}) and an autotransporter beta-domain (e-value=7.71^{-19}). A homology search showed 41% maximum identity to the serine protease of *Xanthomonas axonopodis* pv. citri str. 306. PSORT predicted this gene product has a location in the inner or outer membrane.

Mutant L9. The transposon disrupted gene *ESA_00177* in L9. This gene encodes a hypothetical protein which has 91% maximum identity to a putative iron-sulfur cluster binding protein in *E. coli* MS 16-3. A search of the conserved domains show a domain of unknown function which occurs in iron-sulfur cluster-binding proteins (e-value=3.59^{-31}). PSORT predicted this protein is located in the bacterial cytoplasm.

Mutant L10. *ESA_02808* was disrupted in L10. *In silico* analysis suggests this gene encodes a hypothetical protein with 89% maximum identity to an acriflavine resistance protein (AcrB) in *E. coli* K-12 substr. MG1655. This is part of multidrug efflux pump, where AcrB is the transporter that works in combination with AcrA, a periplasmic accessory protein (Husain *et al*., 2010). The gene product of *ESA_02807* has 85% maximum identity to AcrA in *E. coli* K-12 subst. MG1655, therefore it is possible they form part of the AcrAB multidrug efflux pump. PSORT predicted this gene product is located in the plasma membrane.

Further analysis of mutants

% survival in Lfcin B

A kill curve of the stationary phase mutants in 15.62µM Lfcin B showed variation between strains (Figure 7A). The wild type showed 24.5% survival in the presence of Lfcin B. The highest survival for the Lfcin B mutants was L9 with 21.8% survival. The rest of the mutant strains were significantly more sensitive than the wild type (Student's t-test $p<0.05$). The lowest survival was 4.6% for L7. A kill curve of the log phase mutants in 7.8µM Lfcin B also showed variation between strains (Figure 7B). The wild type strain plus Lfcin B had 10% survival. The highest

survival of the Lfcin B mutants was L5 with 8.3% survival. The rest of the mutant strains were significantly more sensitive than the wild type (Student's t-test $p<0.05$). The lowest survival was 0.13% for L7. This strain had the lowest survival outcome for both stationary and log phase cells.

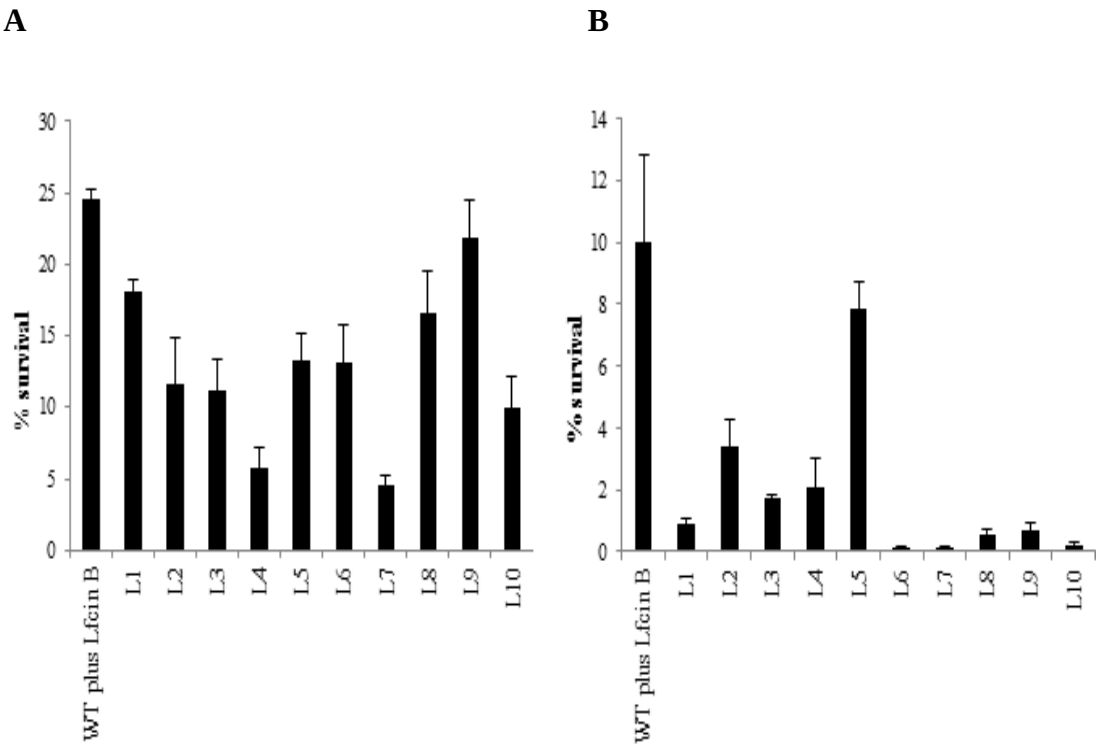


Fig 7. (A) % Survival of stationary phase inoculum WT cells in sodium phosphate buffer containing 15.62µM Lfcin B**. (B) % survival of log phase inoculum WT cells** in sodium phosphate buffer containing 7.8µM Lfcin B. Viable plate counts were performed following one hour incubation by serially diluting in ¼ strength Ringer's solution and enumeration on LB agar. % survival was calculated as a percentage of viable cell counts after challenge expressed as a percentage of viable-cell counts at time zero, i.e. immediately prior to treatment.

Well assays

Well assays with 10mg/ml nisin (Table 4) showed variations in zone size between the Lfcin B mutants. Five strains were significantly more sensitive than the wild type (Student's t-test $p<0.05$) (mutants L2, L3, L6 L7 and L8). L6 had the largest zone of inhibition. Well assays with 10µl of 1mM Lfcin B was done singly. The largest zone of inhibition was L10 and the smallest zone of inhibition was mutant L9.

Ampicillin sensitivity

Ampicillin disk assays (Table 4) revealed the 50% of the Lfcin B mutants were statistically more sensitive than the wild type (mutants L3, L6, L7, L9 and L10) (Student's t-test $p<0.05$). L10 had the largest zone of inhibition. This may be because this mutant is predicted to be a drug efflux pump (AcrB). MICs for all the strains was 10µg/ml except for L10 which had an MIC of 5µg/ml. A bactericidal assay with the Lfcin B mutants plus 8µg/ml ampicillin (Figure 8) also showed variation between the strains with L5 displaying similar growth to the wild type and again L10 was the most sensitive strain.

Table 4. Further characterization of the WT and transposon mutants isolated in this study

Tn mutant	Nisin (well assay) 10mg/ml (mm)	AMP 10µg (mm)	Amp MIC	Pigment production	Growth on DFI
L1	16.00±0.50	20.60±0.60	10 ug/ml	+	Blue green colonies
L2	16.10±0.10	21.40±0.60	10 ug/ml	+	Blue green colonies
L3	16.13±0.23	22.30±0.30	10 ug/ml	+	Blue green colonies
L4	15.70±0.20	20.76±0.80	10 ug/ml	+	Blue green colonies
L5	15.70±0.30	20.60±0.60	10 ug/ml	+	Blue green colonies
L6	16.50±0.50	22.03±0.40	10 ug/ml	+	Blue green colonies
L7	15.93±0.07	22.15±0.05	10 ug/ml	+	Blue green colonies
L8	16.47±0.20	21.9±0.90	10 ug/ml	+	Blue green colonies
L9	15.95±0.45	21.85±0.35	10 ug/ml	+	Blue green colonies
L10	15.25±0.25	24.06±0.26	5 ug/ml	+	Blue green colonies
WT	15.56±0.13	20.65±0.50	10 ug/ml	+	Blue green colonies

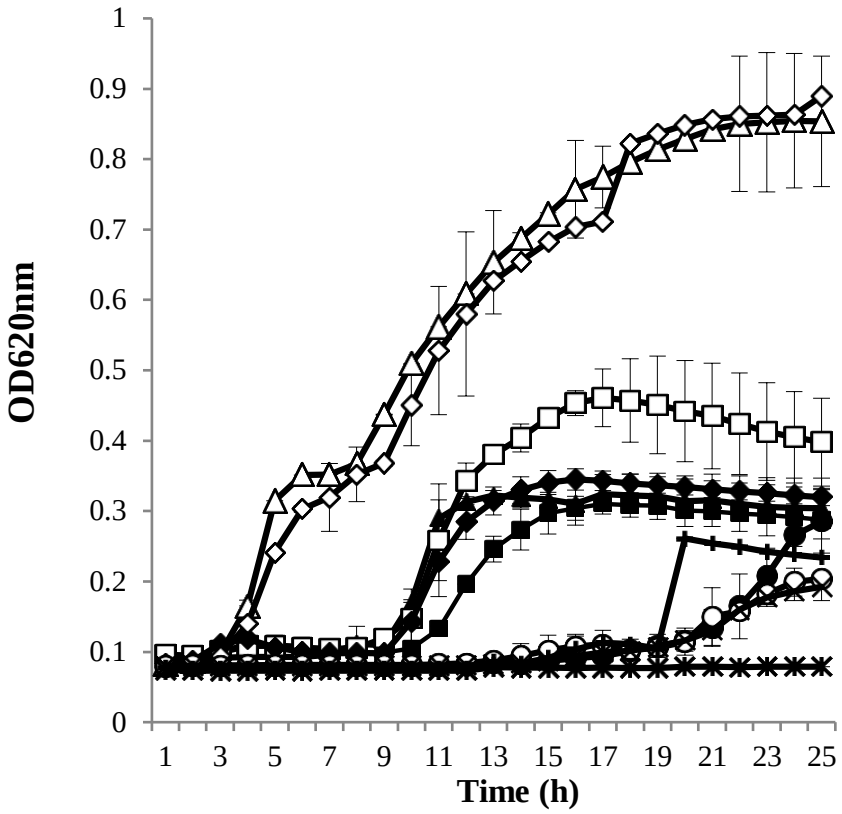


Fig 8. Growth curve of wt and Lfcin B mutants in LB containing 8µg/ml ampicillin. wt (Δ), L1 (▲), L2 (■), L3 (□), L4 (♦), L5 (◊), L6 (○), L7 (●), L8 (x), L9 (+) and L10 (✳).

Discussion

C. sakazakii is an opportunistic foodborne pathogen in neonates and infants. The fatality rate has been reported to vary between 33-80% (Lai, 2001). Although *C. sakazakii* has been isolated from a wide variety of food, contaminated IMF has been epidemiologically linked to cases of *C. sakazakii* infection in infants (Weir, 2002). This is undoubtedly a major concern for IMF manufacturers. In the US, in November and December of 2011 there were four cases of *C. sakazakii* infection in infants in which two infants died (CDC, 2012). A voluntary recall of IMF was initiated. Therefore it is evident *C. sakazakii* is a continuing problem. Antimicrobial peptides (AMPs) such as Lfcin B are of interest to IMF manufacturers due their safety, effectiveness and broad spectrum of activity. As Lfcin B is a milk derived AMP it could act as a preservative by providing protection against *C. sakazakii* infection and increase the safety of the IMF.

It was apparent that log phase cells were more sensitive to Lfcin B than stationary phase cells. This may be due to stationary phase cells expressing more stress response genes (Nair *et al*., 2004). There are also differences in cell membrane composition of stationary phase cells compared to log phase cells (Pagán *et al*., 2000), for example cyclopropane fatty acids can accumulate (Cronan, 1968), which is thought to aid in the bacterial stress response (Chang *et al*., 1999). There was also variation between the collection of *Cronobacter* strains in their sensitivity to Lfcin B, albeit that all strains were affected to some degree. This is significant for IMF manufacturers as it highlights the importance of employing a collection of *Cronobacter* strains instead of a single strain when assessing suitable inhibitory

concentrations for IMF. A selection of strains will give a more realistic readout of the effectiveness of a *Cronobacter* inhibitor that could be added to IMF.

In this study we identified ten mutants with delayed progress when grown in the presence of Lfcin B. Two mutants (mutants L2 and L3) were designated has having unknown function as they had no sequence similarity to any protein in the NCBI datasite. Currently the genome sequences of three *C. sakazakii* strains have been published; however, there are at least fifteen more *C. sakazakii* sequencing projects in progress (http://www.ncbi.nlm.nih.gov/genome/genomes/1170) and publication of this data may aid identification of these unassigned genes.

ESA_04322

The product of this gene is homologous to a 4-α-glucanotransferase (MalQ). In *C. sakazakii* it may form part of an operon with *ESA_04321,* the product of this gene shares homology with MalP, a maltodextrin phosphorylase. Therefore it may be a maltose inducible operon. A gene product downstream from MalQ is homologous to a major cold shock protein (ESA_04323) and may form part of the same operon. In *E. coli* this operon yields glucose and glucose-1-P that enter glycolysis (Park *et al*., 2011). Tu *et al*., (2011) confirmed MalP binds to Lfcin B. Therefore it is plausible that a mutation in this operon has an effect on the sensitivity of the strain to Lfcin B.

CTU_32540

The proposed function of the product of this gene is an integrase protein, which has a role in DNA integration and recombination. This protein may form part of an operon with hypothetical proteins on either side. Integrase proteins have also been shown to form part of pathogenicity islands for integration to the chromosome (Dobrindt *et*

al., 2004). This integrase may be important in the pathogenicity of the strain. Its role in Lfcin B tolerance is not known at this point, nor do we have any reasonable hypothesis to propose.

ESA_03985

This gene product shares homology to a BsmA protein. The gene was previously designated *yjfO*. Weber *et al*., (2010) characterised and proposed renaming of the gene. A mutation in this gene altered the biofilm structure and cell motility and the ability of the bacteria to respond to pH and oxidative stress. Antimicrobial peptides have been shown to induce a stress response in bacteria e.g. upregulation of the PhoPQ two-component regulatory system (McPhee *et al*., 2003). This may explain why a mutation in this stress response gene resulted in increased sensitivity to Lfcin B.

ESA_00567

In *C. sakazakii* the proposed product of this gene is a recombinase A (RecA). In *E. coli*, DNA damaging agents trigger the SOS response; this involves induction of RecA expression (Thi *et al*., 2011). The protein catalyzes an ATP-dependent DNA strand exchange reaction that is the central step in repair of dsDNA breaks in homologous recombination. A mutation in RecA has previously been shown to have increased sensitivity to antibiotics (Liu *et al*., 2010); therefore it is conceivable that a mutation in this gene also resulted in increased sensitivity to antimicrobial peptides such as Lfcin B. In *C. sakazakii* it may form part of an operon with downstream putative *recX* and *alaS* genes.

ESA_01660

In *C. sakazakii* this gene is suggested to encode for a putative antiadapter protein IraM. This protein is believed to be essential for the stabilization of RpoS during magnesium starvation by regulating RssB activity (Bougdour *et al*., 2008). This study also showed IraM is regulated by the two component system PhoPQ. Due to its connection with the general stress response regulator RpoS, it may explain why a mutation in IraM displayed increased sensitivity to Lfcin B. Of all the mutants this was be the most susceptible to Lfcin B, with the lowest % survival in log and stationary phase cells.

ESA_00894

In *C. sakazakii* the proposed function of this gene product is a subtilisin serine protease. It doesn't appear to have a homolog in *E. coli* and the nearest non-*Cronobacter* homolog is in *Rhodanobacter fulvus* with a maximum identity of 45%. Along with a peptidase S8 family domain, a search of the conserved domain revealed an autotransporter β-domain, this family corresponds to the presumed integral membrane β-barrel domain that transports proteins and is also a member of the porin superfamily. PSORT predicted the location of the gene product as an inner or outer membrane protein and Lfcin B is thought to have an effect on the cell membrane of the bacteria (Murdock *et al*., 2010, Ulvante *et al*., 2001). Due to its link to membrane transport, this may explain why a mutation in this gene displayed increased sensitivity to Lfcin B. However, the exact function is not clear at this point.

ESA_00177

In *C. sakazakii* the product of this gene is suggested to be a putative iron-sulfur cluster binding protein (YjeS). Iron sulfur proteins are present in all living organisms and have a variety of functions these include electron transport, redox and non-redox catalysis, stabilization of proteins and sensing for regulatory processes (Ollagnier-de-Choudens *et al*., 2001). This protein has not been fully characterized and its exact function is not known. Of the Lfcin B mutants L9 had the smallest zone of inhibition and the highest % survival of the stationary phase cells. Although it is affected by Lfcin B it is the least susceptible of the mutants.

ESA_02808

The product of this gene is proposed to function as a drug efflux pump, acriflavin resistance protein (AcrB). It may form an operon with AcrA. In *E. coli* this drug efflux pump consists of AcrAB-TolC and displays resistance to a wide variety of compounds. AcrA is an inner membrane lipoprotein, TolC is an outer membrane channel and AcrB captures the drug molecules and pumps them through TolC to the medium (Husain *et al*., 2010). Lfcin B could be one of the compounds this drug-efflux pump can transport. Therefore it is plausible that a mutation in this gene would be more affected by Lfcin B. In addition it had the largest zone of inhibition to Lfcin B compared to the rest of the mutants.

Further analysis of the Lfcin B mutants was undertaken to determine if they are also affected by other antimicrobials. Nisin is a 34 amino acid bacteriocin produced by *Lactococcus lactis*. It forms pores in the cytoplasmic membrane resulting in depolarization in the cell membrane and eventually cell death (Hasper *et al*., 2006). Lfcin B also has an effect on the cell membrane of susceptible bacteria; Lfcin B has

been shown to cause depolarization of the membrane and fusion of negatively charged liposomes (Ulvatne *et al*., 2001). All strains including the wild type were sensitive to nisin, the most sensitive being mutant L6 (the RecA mutant, which is essential for the repair and maintenance of DNA). Gunderson *et al*., (2006) isolated antimicrobial peptides that caused DNA segregation, abnormalities, filamentation and DNA damage, which resulted in induction of the SOS response. As RecA needs to be induced to trigger the SOS response, it is possible a mutation in this gene could render the bacterium more susceptible to other antimicrobial peptides including nisin.

The sensitivity of the mutants to ampicillin was also investigated. Ampicillin is a β-lactam antibiotic that affects cell wall synthesis. Of the disk diffusion assays, the most sensitive of the mutants was mutant L10 which also had the lowest MIC of the strains; this result is understandable as the gene product has homology to a drug efflux pump. Growth of the strains in LB plus 8µg/ml ampicillin showed variation between the strains, with mutant L5 growing similarly to the wild type and with a zone of inhibition similar to the wild type in the ampicillin disk diffusion assay. This gene product has homology to BsmA which is a biofilm formation and stress response factor. However it is the least affected of the Lfcin B mutants to ampicillin.

This study created a transposon mutagenesis library in *C. sakazakii* which were screened for their susceptibility to Lfcin B. The identified genes varied in their proposed function, for some their role in Lfcin B tolerance was not known and will require further investigation. Other genes could be linked to functions with a proposed role in Lfcin B tolerance. Identifying these genes is important to improve the efficacy of Lfcin B and also to identify possible targets for effective therapeutic

delivery. This study could also be of interest to IMF manufacturers due to problems associated with *C. sakazakii* in IMF.

Thesis summary

Cronobacter spp. are opportunistic pathogens associated with the ingestion of contaminated infant milk formula (IMF). This poses a constant challenge to IMF manufacturers, making it essential to understand the factors that affect survival and growth of *Cronobacter* in order to develop more effective control strategies. However, this is problematic due to tolerance of the pathogen to certain environmental stresses, in particular osmotic stress and drying, with some capsulated strains surviving for periods up to 2.5 years in dehydrated powdered infant formula (Edelson-Mammel *et al.*, 2005). This may provide *Cronobacter* with a competitive advantage in powdered IMF.

Chapter 2 of this thesis examines the ability of a collection of sixteen *Cronobacter* strains to cope with sub-optimal conditions of the gastrointestinal tract (low pH, salt and bile). The majority of studies on the capability of *Cronobacter* to tolerate environmental stresses focus on a single stress using a collection of strains, or a single strain is tested against a variety of stresses. In our study, while all strains were able to endure the stress conditions tested, noteworthy differences in tolerance were observed. It is therefore essential that future research on survival of *Cronobacter* to gastrointestinal stress conditions should include a bank of strains rather than just a single strain.

Chapter 3 of this thesis investigated whether bioluminescence could be used to monitor growth of *Cronobacter* in IMF. As milk is opaque it does not lend itself to optical density measurements. Nine *Cronobacter* strains (from the collection of strains in chapter 2) were Lux-tagged, which enabled their growth to be easily monitored in real time by means of light emission (bioluminescence). Differences in

RLU readings were observed between strains, but these differences did not always directly correlate with differences in cell counts. Experiments with mixes of strains demonstrated that a cocktail of two Lux-tagged *Cronobacter* strains provides a good representation of the average growth of all ten strains in IMF. This means that rather than performing future growth analyses in IMF with all ten strains, the use of two strains would reduce the amount of work and samples required. This bank of Lux-tagged strains could be employed to examine the effects of various infant formula compositions on the growth of *Cronobacter*. The bank of Lux-tagged strains may have other potential applications, for example, examining biofilm formation or biocide efficiency.

Chapter 4 describes the creation of a transposon mutagenesis library in a Lux-tagged strain of *C. sakazakii*. Although transposon mutagenesis libraries have been created in naturally light emitting strains of bacteria this is the first time to our knowledge a transposon mutagenesis library has been created in a constructed Lux-tagged strain. This library was screened for mutants with growth affected in milk. These mutants were identified and the majority were found to be involved in amino acid metabolism. This information could contribute further to our understanding of how *C. sakazakii* grows in milk. This knowledge is essential to aid identification of potential inhibitors of the pathogen in IMF.

Chapter 5 of this thesis describes the creation of a transposon mutagenesis library in *C. sakazakii*. This library was screened for mutants with increased sensitivity to the milk derived antimicrobial peptide, Lactoferricin B (Lfcin B). This antimicrobial peptide could be added to IMF as a preservative to inhibit *C. sakazakii*. A variety of genes were identified that varied in their proposed function (encoding proteins involved in transport, DNA recombination and repair, central metabolic reactions

and stress response). Identification of these genes is important to potentiate the affectivity of Lfcin B.

In conclusion, this body of work found that *Cronobacter* strains vary in their ability to tolerate sub-optimal stress conditions. From Lux-tagging a collection of strains it was established that RLU readings may differ from strain to strain despite cell number counts. Therefore, it is essential that more than one *Cronobacter* strain is employed for investigating the efficiency of a potential anti-*Cronobacter* product. Amino acid metabolism genes were determined to be needed for the growth of *C. sakazakii* in milk. An array of genes including an antibiotic efflux pump were found to be significant in the tolerance of *C. sakazakii* to Lfcin B. Identification of such genes could facilitate approaches to help improve the efficacy of Lfcin B in turn, may aid control of this life-threatening opportunistic pathogen.

Appendix

Identification of genes involved in the antibiotic tolerance of *Cronobacter sakazakii*

This project was initiated by an undergraduate student as her final year lab project. I continued the work with the aim of generating enough data for a peer-reviewed publication.

Introduction

The spread of antibiotic resistant bacteria is a significant public health concern. *C. sakazakii* infections have traditionally been treated with a combination of ampicillin and gentamycin or ampicillin and chloramphenicol (Drudy *et al*., 2006). However, ampicillin, chloramphenicol and gentamycin resistant *Cronobacter* spp. have arisen due to acquisition transposable elements, production of β-lactamases and the presence of several antibiotic resistance operons (Girlich e*t al*., 2001). Even though the antibiotic resistance of *Cronobacter* spp. is less of a problem than for other food borne pathogens, antibiotic resistant strains may become increasingly common due to drug overuse or external and internal stresses linked to environmental conditions (Lee *et al*., 2012b).

Molloy et *al*., (2009) isolated 33 *Cronobacter* spp. from farming and domestic environments, food production animals and retail foods. Antibiotic resistant profiling indicated that all the *Cronobacter* strains were sensitive to seven of the eight antibiotics tested, but 51% of the isolates were resistant to cephalothin. This is a first

generation cephalosporin traditionally used for treatment of neonatal infections, but increased resistance has resulted in the recommended use of third generation cephalosporins (Gurtler *et al*., 2005).

Kilonzo-Nthenge *et al*., (2012) isolated *Cronobacter* spp. from domestic kitchens. Antibiotic resistant profiling on these strains revealed 33% were resistant to ampicillin, 19% resistant to chloramphenicol and none resistant to gentamycin. Overall, *C. sakazakii* isolates were resistant to two or more of the ten antibiotics tested. Lee *et al*., (2012b) investigated the antibiotic susceptibility of 66 *Cronobacter* spp. isolated from various foods in Korea. The majority of *C. sakazakii* strains were susceptible to ampicillin (98.2%), tetracycline (98.2%) and chloramphenicol (96.6%) but 12% were resistant and 75.8% displayed intermediate sensitivity to streptomycin. In addition, a clinical case has been documented in which multiple antibiotics were ineffective in the treatment of a *Cronobacter* infection and required prolonged broad spectrum treatment (Dennison *et al*., 2002). These studies highlight an increase in antimicrobial resistance, with third generation cephalosporins now required to treat some infections. It is crucial antibiotic resistant strains of *C. sakazakii* do not proliferate. This is especially significant as stronger or different combinations of antibiotics may cause serious damage to a neonate.

Currently, the genes used by *C. sakazakii* to tolerate antibiotics are unknown. This study was initiated to address this lack of knowledge.

Materials and methods

Creation and screening of a transposon mutagenesis bank

A *C. sakazakii* transposon mutagenesis library was created as per Chapter V of this thesis. The library was screened for mutants that were impaired in growth on LB agar supplemented with ampicillin, chloramphenicol or gentamycin. Ampicillin (Sigma-Aldrich) was filter sterilized and made up as a concentrated stock in sterile HPLC grade water (32mg/ml). Chloramphenicol was made up as a concentrated stock in ethanol (32mg/ml) and gentamycin was purchased as a concentrated liquid stock (50mg/ml) (Gibco). The transposon library was replicated from 96-well plates onto Q-trays (Genetix, UK) of LB agar, and LB agar supplemented with antibiotics; 2µg/ml ampicillin, 2µg/ml chloramphenicol or 0.09375µg/ml gentamycin. Plates were examined after 24 and 48 hours and mutants which didn't grow or grew poorly were selected for DNA extraction, PCR and sequencing.

MICs determination

MICs were determined as per Chapter V of this thesis. Briefly, a two-fold serial dilution of the antibiotic was added to the wells of a 96-well plate containing *C. sakazakii* at a concentration of ~$5x10^5$ cfu/ml. Following a two hour incubation at 37°C, 100µl double strength LB broth was added. After 24 h of incubation at 37°C the plates were read, and the MICs were determined as the lowest concentration of antibiotic which resulted in the absence of apparent growth of the bacteria. MIC determinations were carried out in triplicate.

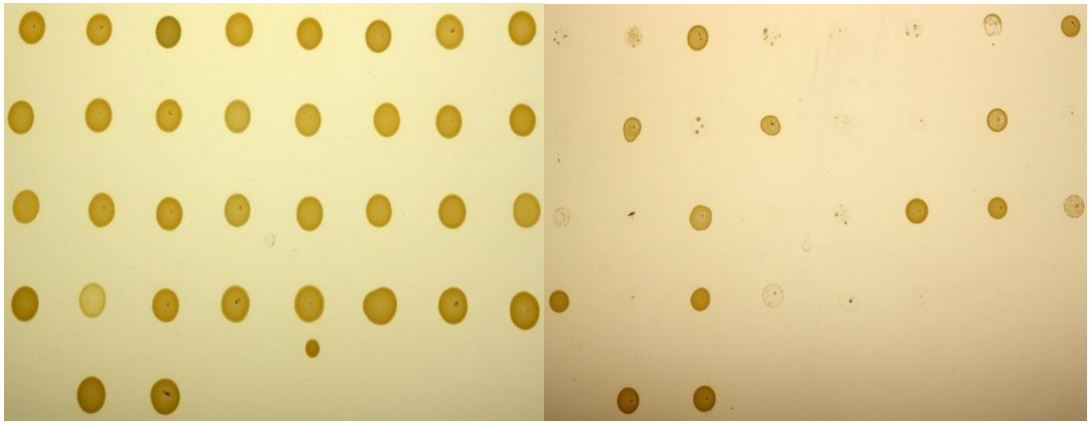
DNA extraction, manipulations and sequencing

DNA extraction and single primer PCRs were set up as per Chapter IV of this thesis using Tn5 PCRR (CGAGCAAGACGTTTCCCGTTG) and sequencing was performed with KAN-2 RP-1 (GCAATGTAACATCAGAGATTTTCAG). Bioinformatic analysis was performed as per chapter three of this thesis.

Results and Discussion

Approximately 1,920 mutants were screened for antibiotic tolerance. Thirty antibiotic sensitive mutants were identified. Some of these mutants were sensitive to more than one antibiotic. In total 23 were sensitive to ampicillin, six were sensitive to chloramphenicol and six were sensitive to gentamycin (Table 1). In order to confirm the initial findings, mutants were grown overnight in 10ml LB, and 10µl aliquots were spotted onto LB and LB supplemented with antibiotics (Figure 1). The transposon insertion sites were identified using a single primer approach (Table 1). MICs for each of the antibiotic sensitive mutants are also shown in Table 1. The wild type strain had an MIC of 20µg/ml against chloramphenicol, 10µg/ml against ampicillin and 0.0625µg/ml against gentamycin.

A **B**



C **D**

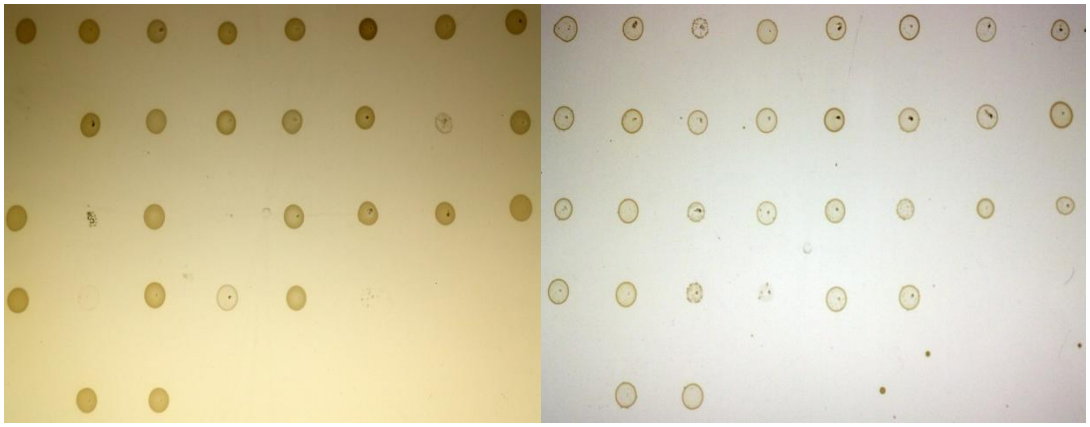


Figure 1. 10µl of overnight cultures of thirty antibiotic sensitive mutants and wild type strain were spotted onto LB agar (A), LB agar plus 2.5µg/ml ampicillin (B), LB agar plus 2µg/ml chloramphenicol (C) and LB agar plus 0.09375µg/ml gentamycin (D). The two spots at the bottom of each plate represent the wild type strain.

Table 1. Overview of the antibiotic sensitive transposon mutants.

Mutant	Antibiotic sensitivity	MIC	Tn insertion site	Proposed function of encoded protein
Ampicillin sensitive mutants				
A-1	Ampicillin	10µg/ml	*ESA_03633*	Hypothetical protein, predicted membrane protein
A-2	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-4	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-5	Ampicillin	5µg/ml	*ESA_03191*	Membrane carboxypeptidase penicillin binding protein 1b MrcB
A-7	Ampicillin	10µg/ml	*ESA_03828*	ATP-dependent protease peptidase subunit, HslU
A-8	Ampicillin	10µg/ml	Intergenic region between two genes 44 bp before *ESA_00124*, may form part of an operon	Hypothetical protein
A-10	Ampicillin	5µg/ml	*ESA_03188*	Iron-hydroxamate transporter substrate-binding subunit FhuD
A-11	Ampicillin	10µg/ml	No sequence similarity	Unknown function
A-12	Ampicillin	5µg/ml	*ESA_02437*	Phosphoserine aminotransferase
A-13	Ampicillin	5µg/ml	*ESA_03325*	Hypothetical protein
A-14	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-16	Ampicillin	5µg/ml	*ESA_04118*	Preprotein translocase subunit, SecA
A-17	Ampicillin	10µg/ml	Intergenic region between two genes 44 bp before *ESA_00124* may form part of an operon	Hypothetical protein
A-21	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-23	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-25	Ampicillin	10µg/ml	*ESA_03741*	Putative chloramphenical resistance permease RarD
A-26	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase
A-29	Ampicillin	5µg/ml	*ESA_03346*	Hypothetical protein, lytic murein transglycosylase

Chloramphenicol sensitive mutants				
A-15	Chloramphenicol	10µg/ml	Intergenic region 96 bp before *tolC*, may form part of an operon	
Gentamycin sensitive mutants				
A-3	Gentamycin	0.03125µg/ml	*ESA_00036*	Potassium transporter peripheral membrane component, TrkA
A-19	Gentamycin	0.0625µg/ml	*ESA_04003/ESA_04005*	Hypothetical protein F0F1 ATP synthase subunit epsilon, AtpC
A-22	Gentamycin	0.03125µg/ml	*ESA_01596*	Hypothetical protein, antimicrobial peptide ABC transporter substrate-binding protein, SapA
A-24	Gentamycin	0.03125µg/ml	*ESA_03779*	Hypothetical protein. ATP-dependent DNA helicase Rep, UvrD
A-28	Gentamycin	0.03125µg/ml	*ESA_04048*	Glutamine synthetase, GlnA
Mutants sensitive to more than one antibiotic				
A-6	Ampicillin and Chloramphenicol	2.5µg/ml 10µg/ml	*ESA_02859*	DNA binding ATP dependent protease La, Lon
A-9	Ampicillin and Chloramphenicol	5µg/ml 5µg/ml	*ESA_02808*	Multi-drug efflux pump AcrB
A-18	Ampicillin and Chloramphenicol	5µg/ml 5µg/ml	*ESA_02808*	Multi-drug efflux pump AcrB
A-20	Ampicillin and Chloramphenicol	5µg/ml 2.5µg/ml	*ESA_00373*	Outer membrane protein TolC
A-30	Ampicillin and Chloramphenicol	5µg/ml 5µg/ml	*ESA_02808*	Multi-drug efflux pump AcrB
A-27	Ampicillin and Gentamycin	10µg/ml 0.0625µg/ml	*ESA_01178*	dTDP-glucose 4,6 dehydratase

Two mutants (A-5 and A-20) were chosen for supplementary bioinformatic analysis. Location of transposon insertion sites these mutants are illustrated in Figure 2. Conserved domains of the proteins are shown in Figure 3.

Mutant A-5

In A-5 the transposon disrupted gene *ESA_03191*. In *C. sakazakii* the proposed product of this gene is a penicillin-binding protein (PBP) 1b (MrcB). Bioinformatic analysis revealed this protein has 85% maximum identity to the penicillin-binding protein in *Salmonella enterica* subsp. enterica serovar Paratyphi C. PBPs catalyze the final stages of murein biogenesis and are targets of β-lactam antibiotics (Ghosh *et al*., 2008). This may explain why a mutation in this gene displayed increased sensitivity to ampicillin. A search of the conserved domain revealed an MrcB multi-domain with an e-value of zero.

Mutant A-20

In A-20 the transposon inserted into *ESA_00373. In silico* analysis suggests this gene encodes an outer membrane channel protein, TolC. Homology searches reveal 84% maximum identity with TolC in *E. coli* O157:H7 str. EC4024. TolC interacts with inner membrane efflux pumps in order to export materials (e.g. antibiotics) outside of the cell. TolC mutants in *E. coli* have previously been shown to be sensitive to antibiotics (Augustus *et al*., 2004). Conserved domain searches reveal a TolC multi-domain with an e-value of zero. In A-15, the transposon inserted 96 base pairs before *ESA_00373*. This insertion has less of an effect on antibiotic susceptibility compared to A-20. A-15 is sensitive to chloramphenicol whereas A-20 is sensitive to ampicillin

and chloramphenicol. In addition A-15 has a higher MIC against chloramphenicol than A-20. However TolC may form part of an operon (Figure 2) which may explain the increased antibiotic sensitivity of A-15 compared to the wild type.

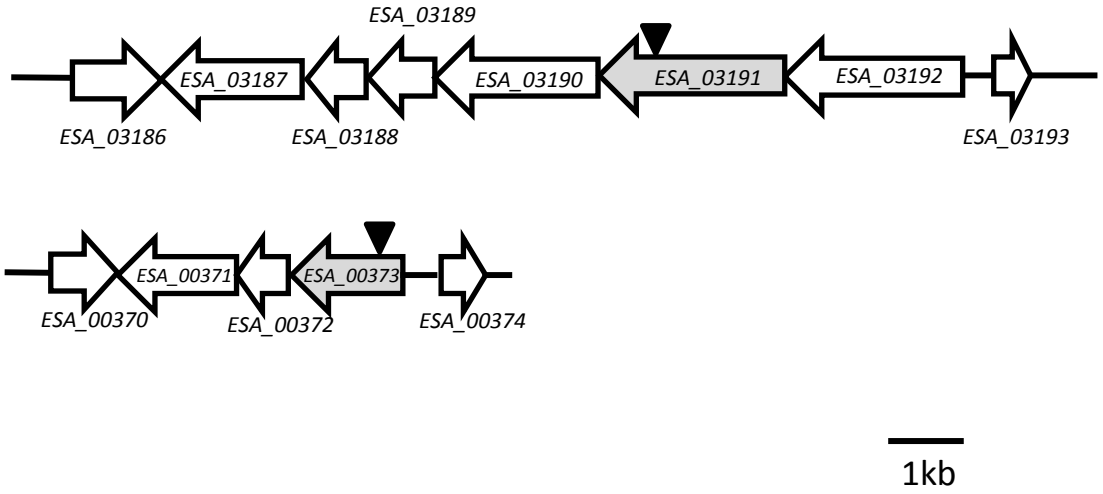


Fig 2. Genomic organisation of insertion sites in transposon mutants (A-5 and A-20) with increased antibiotic sensitivity. The diagram was drawn approximately to scale using *C. sakazakii* BAA-894 genome sequence data. Open reading frames (shaded in grey) are genes with transposon insertion. Black arrowheads represent the approximate location of the transposon insertion. White open reading frames are flanking genes.

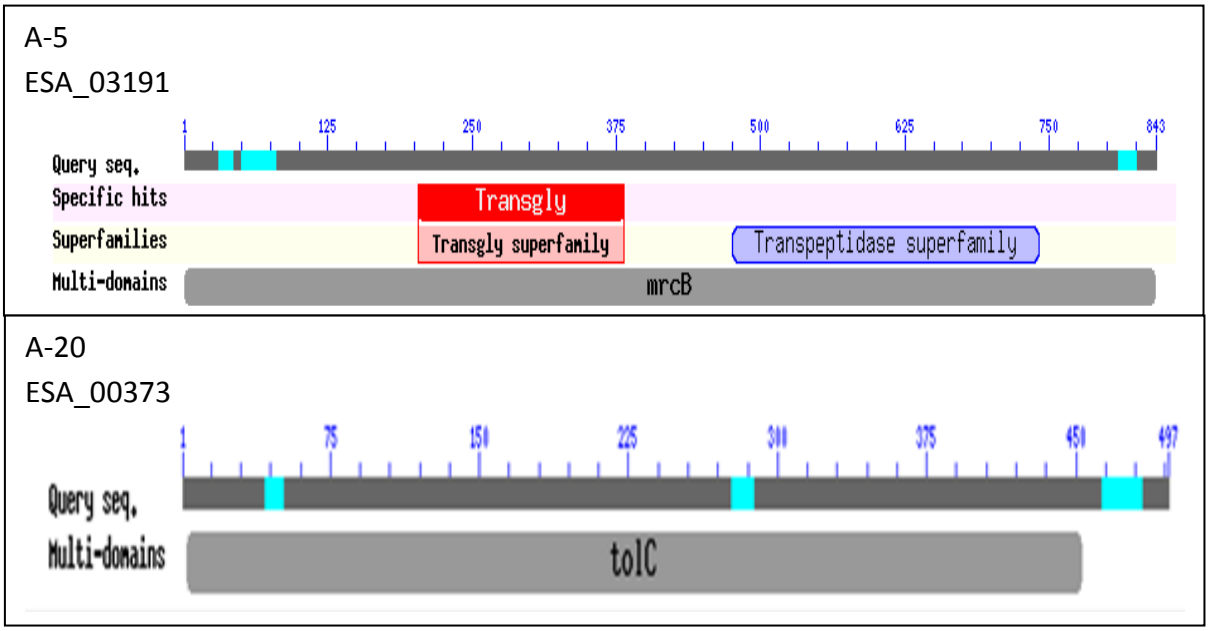


Fig 3. Conserved domains present in the putative proteins encoded by the disrupted genes in A-5 and A-20. Proteins were analysed for the presence of conserved domains using the conserved domain database (http://www.ncbi.nlm.nih.gov/cdd/).

Ampicillin sensitive mutants

For one of the transposon mutants (A-11) there was no sequence similarity to any protein in the NCBI datasite, therefore it were assigned as having unknown function.

ESA_03633

In A-1 the transposon disrupted *ESA_03633*. In *C. sakazakii* BAA_894 the proposed product of this gene is a hypothetical protein and it is suggested to be a predicted

membrane protein. A mutation in a membrane protein may affect membrane permeability therefore increasing the susceptibility of the bacteria to antimicrobials.

ESA_03346

In A-2, A-4, A-14, A-21, A-23, A-26 and A-29 the transposon inserted in the same location in *ESA_03646.* Bioinformatic analysis indicates this gene encodes a lytic murein transglycosylase protein. This is a general peptidoglycan binding molecule. Many antibiotics block the biosynthesis of peptidoglycan (Bateman *et al*., 2000) indicating this protein may function in tolerance of *C. sakazakii* to antibiotics. As the transposon inserted in seven of the mutants identified in the screen, this implies it may be a hotspot for the transposon. In addition, the results are consistent with all the mutants having an MIC of 5µg/ml against ampicillin.

ESA_03828

In A-7 the transposon inserted 49 base pairs before *ESA_03828.* The proposed product of this gene is an ATP-dependent protease peptidase subunit, HslV. It may form part of an operon with HslU therefore the transposon insertion may have a negative effect on the transcription of the operon. HslVU form a novel heat shock locus (Rohrwild *et al*., 1996). Heat shock proteins have been shown to increase antimicrobial tolerance (Tran *et al*., 2011) therefore this may explain why a mutation in this heat shock locus resulted in increased antibiotic sensitivity.

ESA_00124

In A-8 and A-17 the transposon inserted 44 base pairs before *ESA_00124.* Bioinformatic analysis indicates this gene encodes a hypothetical protein. It may form an operon with *ESA_00125* therefore the transposon insertion may have

affected the functioning of the operon. Both these mutants have an MIC of 10µg/ml against ampicillin, therefore the results are consistent. The role of this protein in antibiotic tolerance is not known but it is more susceptible than the wild type strain.

ESA_03188

In A-10 the transposon disrupted gene *ESA_03188*. In *C. sakazakii* the proposed product of this gene is an iron hydroxamate transporter substrate binding subunit (FhuD). In *E. coli* it forms part of the ABC transporter operon FhuACDB involved in $iron^{3+}$-hydroxamate import (Mademidis *et al*., 1997). Currently its role in antibiotic tolerance is not known.

ESA_02437

ESA_02437 was disrupted in A-12. *In silico* analysis suggests this gene encodes a phosphoserine aminotransferase. It catalyses the conversion of 3-phosphohydroxypyruvate to L-phosphoserine in serine biosynthesis (Hester *et al*., 1999). However, its function in antibiotic tolerance is not currently known.

ESA_03325

The transposon disrupted gene *ESA_03325* in A-13. Bioinformatic analysis indicates this gene encodes a hypothetical protein. It has 72% maximum identity to a glutamate dehydrogenase (GDH) in *E. coli* UTI89. GDH catalyses a reversible reaction for the reductive amination of α-ketoglutarate to produce glutamate in the presence of the cofactor NAD(P)H (Kumar *et al*., 2010). Lee *et al*., (2012a) observed that a *Bacillus subtilis* glutamate dehydrogenase mutant was more sensitive to β-lactams (e.g. ampicillin). This may explain why a mutation in *ESA_03325* resulted in increased antibiotic sensitivity.

ESA_04118

In A-16 the transposon inserted into *ESA_04118. In silico* analysis suggests this gene encodes a preprotein translocase subunit, SecB. It is a molecular chaperone that functions in the post translational protein translocation pathway of some bacteria (Zhou *et al*., 2005) and forms part of the general secretory Sec system. There is interest in exploiting the Sec system as a potential target of novel antibiotics, as it functions in assembly and maintenance of the cell envelope and pathogenicity through the delivery of toxins (Economou, 2002).

ESA_03741

ESA_03741 was disrupted in A-25. The proposed product of this gene is a putative chloramphenicol resistance permease RarD. Jack *et al*., (2001) classifies RarD as a member of the drug/metabolite transporter superfamily.

Gentamycin sensitive mutants

ESA_03829

The transposon disrupted *ESA_00036* in A-3. *In silico* analysis suggests this gene encodes a potassium transporter peripheral membrane component, TrkA. Parra-Lopez *et al*., (1994) describe a *Salmonella* protein SapG, which is 99% identical to TrkA. A mutation in SapG displayed increased sensitivity to the antimicrobial peptides protamine and melittin. A mutation in TrkA could also have increased susceptibility. This may explain the deceased tolerance of this mutant to antibiotics.

ESA_04003/ESA_04005

In A-19 the transposon inserted into *ESA_04003*/*ESA_04005*. These genes are overlapping and in reverse orientation to each other. In *C. sakazakii* the proposed

product of *ESA_04003* is a hypothetical protein. Bioinformatic analysis suggests *ESA_04005* encodes an F0F1 ATP synthase subunit epsilon (ε). It forms part of catalytic core of ATP synthase in addition to α, β, γ and δ subunits and is involved in producing ATP from ADP in the presence of the proton motive force across the membrane (Stock *et al*., 2000). McCoy *et al*., (2001) identified a mutant in the transmembrane domain of ATP synthase in *Proteus mirabilis* which had increased sensitivity to antimicrobial peptides. A deficiency in the ability to produce ATP may cause the strain to have less energy to repair damage caused by antimicrobial peptide or antibiotics

ESA_01596

In A-22 the transposon disrupted gene *ESA_01596. In silico* analysis suggests this gene encodes an antimicrobial peptide ABC transporter substrate-binding protein, SapA. Parra Lopez *et al*., (1993) investigated a locus required for resistance to antimicrobial peptides in *Salmonella Typhimurium*, *sapABCDF* (sap, sensitivity to antimicrobial peptides). Therefore it is reasonable that a mutation in this gene also has increased sensitivity to antibiotics.

ESA_03779

In A-24 the transposon disrupted gene *ESA_03779*. Bioinformatic analysis indicates this gene encodes an ATP-dependent DNA helicase Rep. Liu *et al*., (2010) previously showed a mutation in an ATP-dependent DNA helicase in *E. coli* K-12 to have increased antibiotic sensitivity.

ESA_04048

ESA_04048 was disrupted in A-28. *In silico* analysis suggests this gene encodes a glutamine synthetase GlnA. It catalyzes the formation of glutamine from glutamate and ammonia (Kumar *et al*., 2010). However its role in antibiotic tolerance is not yet known.

Mutants sensitive to more than one antibiotic

ESA_02859

The transposon disrupted gene *ESA_02859* in A-6. Bioinformatic analysis indicates this gene encodes a DNA binding ATP dependent protease La (Lon). This protein plays a significant role in regulating many biological processes in bacteria such as cell differentiation, sporulation, pathogenicity and survival under starvation conditions (Tsilibaris *et al*., 2006). Liu *et al*., (2010) created an antibiotic profile with an *E. coli* gene knockout collection and Lon was one of the antibiotic sensitive mutants they identified. This highlights the importance of Lon in antibiotic tolerance.

ESA_02808

In A-9, A-18 and A-30 the transposon inserted in different locations in *ESA_02808*. *In silico* analysis suggests this gene encodes a hypothetical protein with 89% maximum identity to an acriflavine resistance protein (AcrB) in *E. coli* K-12 substr. MG1655. This is part of multidrug efflux pump, where AcrB is the transporter that works in combination with AcrA, a periplasmic accessory protein (Husain *et al*., 2010). The gene product of *ESA_02807* has 85% maximum identity to AcrA in *E. coli* K-12 subst. MG1655, therefore it is possible they form part of the AcrAB multidrug efflux pump.

ESA_01178

In A-27 the transposon disrupted gene *ESA_01178.* Bioinformatic analysis indicates this gene encodes a dTDP-glucose 4,6 dehydratase. This catalyses the transformation of dTDP-glucose into dTDP-4-keto-doeoxyglucose (Okazaki *et al*., 1962). It is further metabolized to produce components of the lipopolysaccharide (LPS) (Marolda *et al*., 1995). LPS mutants have previously been shown to have increased sensitivity to antibiotics (Kropinski *et al*., 1978).

Conclusions and suggestions for future work

In conclusion, this is a novel study that identified thirty antibiotic sensitive mutants of *C. sakazakii*. The majority of the genes that were disrupted could be associated with antibiotic tolerance in other bacteria. It could be postulated from this study that *C. sakazakii* utilizes many of the same mechanisms of antibiotic resistance as other *Enterobacteriaceae*. This knowledge is imperative due to the increase in antibiotic resistance occurring worldwide. It could also aid in the development of novel and more enhanced antibiotics.

Future work in this study could include further bioinformatic analysis of each of the mutants, creation of clean deletion mutants and observing the resulting affects and complementation of selected transposon mutants to restore function to wild type levels.

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Acknowledgements

First I want to thank my supervisors Prof. Colin Hill and Dr. Máire Begley for all their help and advice over the last few years. Thank you also for your contribution to the writing of this thesis, Máire in particular for your continuous support and motivation throughout.

I want to thank the Microbiology Department, especially Margaret, Julianna, Paddy, Máire and Dan. Thanks to everyone in the FHI lab, Martha, Harriët, Avelino, Keira, Cristina, Tríona, Sandra, Brenda, Amy, Wessel and Wesley and all the students who have passed through our lab. Thanks especially to Tanya and Thérèse (baby James gets a mention too) for listening to my rants, making me smile, constantly encouraging me and making the lab a more enjoyable place to be. Thanks also for all the stuff I "borrowed" off both of you throughout the last four years.

I want to thank everyone in lab 4.23, Aurilie, James, Eamonn and most importantly Ruth. Thanks for all your encouragement and motivating chats while running around The Lough, you kept me going. I also want to thank everyone in lab 335 and 337, Karen, Des, Harsh, Brian, Alicia, and my housie Lorraine for listening and always making me feel better at any time for the day.

A few other people I need to thank Evelyn Molloy, Aoife Thompson, Karen O'Donovan, Netty Collins and my other housie Nicola Curtis. I want to thank Pat for being so understanding and learning off the name *Cronobacter sakazakii*! Thanks for all your encouragement and for the constant teasing. Lastly I want to thank my family, David and Niamh, thanks for everything and especially my parents for their continuing emotional and financial support.

Claire