Pathogenesis of *Cronobacter* species: Enterotoxin production, adhesion and invasion of the blood brain barrier

Kahina Abdesselam

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the M.Sc. degree in Biochemistry, Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Cronobacter species cause serious infections such as meningitis and enteritis in newborns and neonates, with the major vehicle being contaminated powdered infant formula. The main objectives of this study were i) to identify potential virulence factors, such as enterotoxin production; ii) characterize the gene(s) involved in adhesion and invasion of the human brain microvascular endothelial cells (HBMEC); and iii) determine whether strains from clinical, food, and environmental sources differ in their ability to produce surface-attached bacterial aggregates, known as biofilms. Random transposon mutagenesis was used on strains demonstrating the best adherence and invasion to blood-brain barrier cell lines (BBB). Isogenic mutants were then screened for increased or decreased adherence and invasion. Screening of the transposon library identified one isogenic mutant of a clinical strain which lost the ability to adhere to BBB cells. The transposon rescue revealed the insertion site to be within a diguanylate cyclase (DGC) gene. The major function of DGC in many Gram-negative bacteria is to synthesize cyclic diguanylate (c-di-GMP), a secondary bacterial metabolite known for regulating biofilm formation, motility, and virulence or aspects of microbial pathogenicity. Based on the findings of this study, DGC appears to play an important role in *Cronobacter* species' ability to produce biofilms and may also have a role of the pathogenicity in the microorganism.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LEGEND

ANOVA – analysis of variance

ATCC – American Type Culture Collection

BBB – blood-brain barrier

BLAST – basic local alignment search tool

BPW – buffered peptone water

BSA – bovine serum albumin

CDC – Centers for Disease Control and Prevention

c-di-GMP – cyclic dinucleotide-GMP

CEB – *Cronobacter* enrichment broth

CHO – Chinese hamster ovarian

CNS – Central Nervous System

CSC – Cell system corporation

CSF – cerebrospinal fluid

CV – crystal violet

DFI – Druggan-Forsythe-Iversen

DGC – diguanylate cylcase

EE – *Enterobacter* enrichment

EMEM – Eagle's minimum essential medium

FAO – Food and Agriculture Organization

FBS – fetal bovine serum

FISH – fluorescence *in situ* hybridization

GI – gastrointestinal

h – hour(s)

HBMEC – human brain microvascular endothelial cells

HDMS – hexadimethydisalizone

HPLC – high-performance liquid chromatography

ICMSF – International Commission for Microbiological Specifications for Foods

IDF – International Dairy Federation

ISO – International Standards Organization

LB – Luria Broth

MBEC – Minimum biofilm eradication concentration

MS – Mass spectrometry

NCBI – National Center of Biotechnology Information

NEC – necrotizing enterocolitis

PAGE - polyacrylamide gel electrophoresis

PCR – polymerase chain reaction

PDE – phosphodiesterases

PEPC – phosphoenolypyruvate carboxylase

PEPCK – phosphenolpyruvate carboxykinase

PIF – powdered infant formula

s – second(s)

SEM – Scanning Electron Microscopy

SOC – super optimal broth with catabolite repression

SDS – sodium dodecyl sulfate

TSBYE – tyrptic soy broth with yeast extract

TS – threonine synthase

TSA – tryptic soy agar

WHO – World Health Organization

ACKNOWLEDGEMENT

First and foremost I would like to thank my supervisors Dr. Franco Pagotto and Dr. Jeff Farber for their supervision and providing me with the opportunity to do this research. It would not have been possible without their continuous encouragement and support.

I would also like to thank the members of my thesis advisory committee, Drs. Min Lin and Craig Lee, for their valuable assistance and useful advice throughout the duration of the project.

Many thanks to all members (past and present) of the *Listeria/Cronobacter,* Virology, Botulism and *Campylobacter* laboratories at the Bureau of Microbial Hazards, in particular Karine Hébert, Oksana Mykytczuk, Anu Shukla and Jennifer Harlow, for their kindness, technical assistance, advice and support throughout my work.

Finally, a special thanks to my friends and my family, in particular my dear mother, for her constant support and endless help during all my school years.

Chapter I:
Introduction

The information presented in this introduction was used in the chapter "*Cronobacter* species", to be published the Encyclopedia of Food Safety

BACKGROUND

Cronobacter sakazakii, formerly known as *Enterobacter sakazakii*, is an emerging foodborne pathogen that has been known to the scientific community for the last 50 years. This organism was first characterized in 1929 as a "yellow-pigmented coliform" and was discovered to be the causative agent of septicemia in infants (20). In the 1960s the "yellow pigmented coliform" was suspected to be the cause of two fatal cases of neonatal meningitis. By the 1980s this pathogen was classified as a new species, *Enterobacter sakazakii* and was found to affect infants and neonates by causing sepsis, necrotic enterocolitis and meningitis. *E. sakazakii* is found in a wide range of environmental sources but has been predominantly linked to contaminated powdered infant formula (PIF) (70). Overall, there has been a minimum of 111 reported cases of this severe infection in infants and neonates worldwide, leading to 26 deaths (12).

CHARACTERISTIC OF THE ORGANISM

Cronobacter sakazakii belongs to the genus *Cronobacter* and to the family *Enterobacteriaceae*. Like some members in this family, it is considered to be an opportunistic pathogen. The species *sakazakii* was named after Riichi Sakazakii, a Japanese microbiologist whose research had a major impact in the understanding of enteric bacteriology (20). Currently, there are seven species in the *Cronobacter* genus that are used to classify the biogroups of the formerly known *Enterobacter sakazakii* and they include *sakazakii, malonaticus, turicensis, muytjensii, condimenti, universalis* and *dublinensis* (35, 37).

Cronobacter spp. are Gram-negative organisms that are motile, peritrichously flagellated, oxidase negative, non-sporeforming, non-acid- fast, straight and rod shaped. The

organism has approximate dimensions of 0.3 -1.0 × 1.0-6.0 μm. There are two types of colonies with distinct morphologies when *Cronobacter* is grown on Trypticase Soy Agar (TSA) at 26 to 36 ˚C (27, 70). One type of colony is dry, rubbery and sticky due to the heteropolysaccharide thought to be produced. The second type of colony is smooth and non sticky. Both colony types can produce a yellow pigment, a previously used diagnostic marker that is now not deemed to be useful in identifying *Cronobacter* spp. (70).

Cronobacter is a facultative anaerobe. It can produce ATP in the presence of oxygen through aerobic respiration and can also produce ATP in the absence of oxygen through fermentation pathways (70). *Cronobacter* species have a wide temperature range for growth which varies from 6 to 45 ˚C, with an optimal range of 37 to 43˚C. Certain species have the ability to grow at temperatures of 4 and 47 ˚C (12). In the family *Enterobacteriaceae*, *Cronobacter* spp. are considered to be thermotolerant. Work by Edelson-Mammel *et al.* (18), demonstrated the ability of 12 *Cronobacter* strains to survive heating in rehydrated PIF at 58˚C. The D-values at this specific temperature ranged from 30-35 s to 591-599 s. The D-value for the most heat resistant strain was measured at 71˚C and was found to be 0-7 s (18). Iversen et *al.* (33) predicted a 21-log reduction in the cell count of the pathogen under standard pasteurization processes, indicating that the probable sources of contamination would include non-sterile equipment and/ or additives. A study conducted by Mullane *et al.* (61) demonstrated that air filters in PIF–related processing sites contained *Cronobacter* spp. and that these cells could end up in ingredients found in PIF. Spray drying and milling operations create aerosols and if these aerosols are contaminated with microorganisms, can contaminate the air filters of the processing site (61).

Cronobacter spp. have the ability to survive in acidic environment with pH levels as low as 3 (12). Among the members of the *Enterobacteriacae* family, *Cronobacter* spp. appear to be well adapted to dry stress. Lin and Beuchat (50) conducted a study that determined the effects of water activity (a_w) and temperature on the recovery of *Cronobacter* spp. from infant cereal over a 12-month period. The results showed that increases in a_w or storage temperature increased the death rate of *Cronobacter* spp. in dried infant cereal. Low levels, (2 CFU/g) of *Cronobacter* were able to survive in infant cereals for as long as 12 months at 4°C, 21°C and 30°C when the a_w was low (50).

There are many differences between *Cronobacter* spp. and species belonging to the family of *Enterobacteriacae*. The biochemical characterization of *Cronobacter* spp., according to the second edition of Bergey's manual, are summarized in Table 1 (adapted from references 35 and 70).

Table 1: Biochemical characterization of *Cronobacter species* (35, 70*)*.

Table 1: Biochemical characterization of *Cronobacter* species (35, 70*)*.

Characteristic									
Motility (36°C)	+	Yellow pigment	+	Urea hydrolysis	-	Indole production	d	β-Xylosidase test	+
Methyl red	-	Voges-Proskauer	+	Growth in KCN	+	Gelatin hydrolysis (22°C)	-	Desoxyribonuclease (25°C)	(+)
Lysine decarboxylase	-	Arginine dehydrolase	+	Ornithine decarboxylase	+	Phenylalanine deaminase	d	Glucose dehydrogenase	+
Gluconate dehydrogenase	-	Growth at 41°C	+	Esculin hydrolysis	+	Acetate	-		
Acid from:									
Adonitol	-	L-Arabinose	+	D-Arabitol	-	Cellobiose	+	Sucrose	+
Dulcitol	-	*meso*-erythritol	-	Glycerol	-	*myo*-inositol	(+)		
Maltose	+	D-Mannitol	+	Melibiose	+	α-Methylglucosidase	+		
Raffinose	+	L-Rhamnose	+	Salicin	+	D-Sorbitol	-		
Trehalose	+	D-Xylose	+	Lactose	+	Mucate	+		
Utilization of:									
cis-aconitate	+	*trans*-Aconitate	d	Adonitol	-	4-Aminobutyrate	+	5-Amonivalerate	-
D-Arabitol	-	Benzoate	-	Citrate	+	*m*-Coumarate	-	Dulcitol	d
L-Fucose	-	Gentisate	-	Histamine	-	3-Hydroxybenzoate	-	4-Hydroxybenzoate	-
3-Hydroxybutyrate	-	*myo*-inositol	d	5-Ketogluconate	-	2-Ketoglutarate	-	Lactose	+
Lactulose	+	D-Lyxose	-	D-Malate	(d)	Malonate	(d)	Maltitol	+
D-Melibiose	+	1-*O*-methyl-α-glactoside	+	1-*O*-methyl-D-glucose	-	1-*O*-methyl-α-D-glucoside	+	Mucate	-
Palatinose	+	Phynylacetate	-	L-Proline	+	Protocatechuate	-	Prutesine	+
Quinate	-	D-Raffinose	+	L-Rhamnose	+	D-Sacharate	-	D-Sorbitol	-
Sucrose	+	D-Tagatose	-	*meso*-Tartate	-	Tricarballylate	-	Tryptamine	-
D-Turanose	d	L-Tyrosine	-						

+, 90-100% positive in1-2 days; (+), 90-100% positive in 1-4 days; -, 90-100 % negative in 4 days; d, positive or negative in 1-4 days; (d), positive or negative in 3-4 days

RESERVOIRS

Cronobacter spp. appear to be ubiquitous microorganisms as they have been isolated from a wide variety of foods including milk, cheese, dried foods, meats, water, vegetables, rice, bread, tea and herbs. The most common source epidemiologically associated with human outbreaks appears to be contaminated PIF. Clinically, this pathogen has been isolated from cerebrospinal fluid, bone marrow, blood, intestinal, respiratory tract, urine, ear and eye swabs, and skin wounds (12, 27, 88). Environmentally, they have been isolated from dust, soil, plant materials and even in household vacuum-cleaner bags. It is clear that this bacterium has an ecological niche that is very diverse; however, its primary reservoir has yet to be determined. There are suggestions that plant material, may be a primary source of *Cronobacter* (27, 33).

PIF is the source that is predominantly linked with outbreaks of *Cronobacter* infections in infants and neonates. Possible sources of contamination include plant-derived ingredients that are added to PIF without prior heat treatment and thermally sensitive ingredients that are used in the production of PIF (27, 33). Thus, it appears that raw materials are an important source and possible initial step for the entry of *Cronobacter* species in PIF. Other sources of contamination include utensils, equipment used to handle PIF in hospitals and kitchens and feeding tubes (12, 27, 70).

NEW CLASSIFICATION

Before *Enterobacter sakazakii* was recognized as a new species and named *Cronobacter sakazakii*, it was referred to as yellow pigmented *E. cloacae.* DNA-DNA hybridization results showed that *Cronobacter sakazakii* was 53-54% related two distinct genera: *Enterobacter* and *Citrobacter* (35). However, further biochemical characterization

supported that this organism was more genetically related to *E. cloacae* than *Citrobacter freundii*; thus, the new species was included in the genus *Enterobacter* (20). Fifteen biogroups were then described with suggestions that they represented multiple types within the species (35). Iversen et *al.* (35) investigated several different strains of *E. sakazakii* and based on f-AFLP analysis, ribotyping and full-length 16S rRNA gene analyses, proposed a reclassification of this species. Phenotypic profiling, using 14 biochemical characteristics, permitted better differentiation of the species (Table 2). Joseph *et al.* (37) recently reported a new species, *C. condimenti,* and reclassified *C. genomospecies* as *C. universalis,* while further evaluating *Cronobacter* strains 1330 (formerly *sakazakii*), NCTC 9529, 731 (formerly g*enomospecies*) and 96, 1435 (formerly *turicensis*), isolated from a wide range of food and human sources that included spiced meat to leg infections (37). Currently, *Cronobacter* spp. can be phenotypically differentiated as shown in Table 2.

Table 2: Biochemical differentiation of *Cronobacter* species (adapted from 27 and 37)

Characteristics	*sakazakii*	*malonaticus*	*turicensis*	*muytjensii*	*dublinensis*	*condimenti*	*universalis*
Indole production	-	-	-	++	++*	+	-
Dulcitol	-	-	++	++	-	-	++
Lactulose	++	++	++	++	*	-	++
Malonate	-	++	++	++	*	+	++
Maltitol	++	++	++	-	*	-	++
Palatinose	++	++	++	+	++	-	-
Putrescine	++	+	++	++	++*	-	-
Melezitose	-	-	++	-	-*	-	++
Turanose	++	++	++	+	*	-	-
myo-Inositol	+	+	++	++	++	-	++
cis-Aconitate	++	++	++	+	++	-	-
trans-Aconitate	-	++	-	+	++	-	-
1-0-Methyl α-D-glucopyranoside	++	++	++	-	++	+	++
4-Aminobutyrate	++	++	++	++	++	-	-

++, > 90% positive; +, 20 to 80% positive; -, <10% positive; * variation within the 3 subspecies

CLINICAL MANIFESTATION

Cronobacter is an opportunistic pathogen that has been most often associated with sporadic cases of life-threatening illness. Individuals suffering from a compromised immune system, neonates and infants less than 28 days old or/ and low-birth weight babies are the most susceptible group (88). There are three major clinical manifestation seen in *Cronobacter* infections: (1) necrotic enterocolitis (NEC), (2) septicemia and (3) meningitis and cerebritis.

Analysis of clinical data by Bowen and Braden (7) indicate that the most common symptom associated with *Cronobacter* infection in neonates and infants is meningitis (7). Meningitis caused by *Cronobacter* is established between the fourth and fifth day after birth and can be fatal within hours to a few days if left untreated. Neonatal infections have a mortality rate of 40 to 80% and infants or neonates who survive the infection are often left with severe and often irreversible neurological sequelae (88). These include brain abscess, ventricle compartmentalization due to necrosis of brain tissue and liquefaction of white cerebral matter, hydrocephalus, cyst formation and pulmonary, urinary or blood stream infections (12, 27, 88).

The Centers for Disease Control and Prevention (CDC) have estimated six new cases of *Cronobacter* infection per year, worldwide (27). The lack of awareness of the organism and adequate isolation methods led the World Health Organization (WHO) to declare that *Cronobacter* infections are significantly under-reported in most countries and that the incidence is likely higher (88). Even though the number of reported cases of *Cronobacter* infection are relatively low compared to other pathogens, the consequences of the

outbreaks can be severe. This has led the International Commission for Microbiological Specifications for Foods (ICMSF) to conclude that *Cronobacter* spp. are a severe hazard for restricted populations, causing life-threatening or substantial chronic sequelae (12, 27).

The groups most susceptible to *Cronobacter* spp. are infants and neonates. However, immunocompromised adults, such as the elderly, are also susceptible to *Cronobacter* spp. (22, 27, 88). There have been a total of 20 reported cases of *Cronobacter* infections in adults. The symptoms seen in adults include pneumonia, sepsis, foot ulcers, wound infections, osteomyelitis and splenic abscesses (27). In addition, the mouths of stroke patients can be severely affected by *Cronobacter* (23). Stroke patients who are diagnosed with dysphagia seem to be susceptible to Gram-negative bacilli colonization in the mouth as they are more likely to be fed supplements, such as starch powder, which may also contain *Cronobacter* (23).

Chemotherapeutics are effective against this pathogen as it is sensitive to many classes of antimicrobials including acylureidopenicillins, aminoglycosides, ampicillin, antifolates, aztreonam, carbapenems, cephalosporins, chloramphenicol, nitrofurantoin, quinolones, tetracyclines, ticarcillin, and several β-lactams (27). In contrast, *Cronobacter* spp. are resistant to oxacillin, benzylpenicillin, clindamycin and some macrolides. If the symptoms in infants or adults are observed at an early stage of infection then antibiotics can be used to treat patients more effectively and with minimum complications than at a later stage.

VIRULENCE FACTORS AND PATHOGENICITY

Very little is known of the virulence factors involved in the pathobiology of *Cronobacter* spp., however, in the last decade much progress has been made. A model that demonstrates what is currently known about the host-pathogen relationship is shown in Figure 1.

The main route of infection for *Cronobacter* spp. appears to be through ingestion. As previously mentioned, the vehicle of *Cronobacter* infections in infants and neonates is contaminated PIF. The major factors that contribute to the presence, growth and/or survival of the organism in PIF include temperature, equipment and biofilm formation, defined as bacterial cells able to attach on abiotic and biotic surfaces and form an exopolysaccharide surrounding them . Reconstituted PIF is normally stored at room temperature, which is approximately 23˚C (69). The optimal temperature for *Cronobacter* spp. in reconstituted PIF is 37˚C; however, it has the ability to grow between 6 and 45˚C (35). PIF is not sterile, therefore, the composition of PIF in terms of the background microflora as well as the storage temperature of reconstituted PIF, can affect the growth of the organism (69). Another factor that has been found to be an issue in contaminated reconstituted PIF is the presence of free lipopolysaccharide (LPS) (84). LPS appear to play a role in compromising the intestinal barrier integrity, by allowing the organism to cross the GI tract and become bloodborne (84).

Figure 1: A current model of *Cronobacter* pathogenicity

BIOFILMS

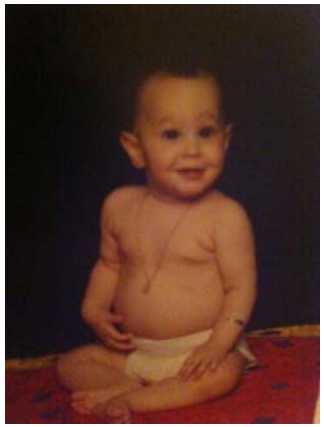
TEMPERATURE

EQUIPMENT

INGREDIENTS

POWDERED INFANT FORMULA (PIF) AND/OR EQUIPMENT

Ingestion



OmpA binds to fibronectin, facilitating invasion of brain endothelial cells

Protease, Zpx, appears to be involved in crossing *BBB*

Can cross the BBB

At-risk population

Can cross GI barrier

When Stomach has a higher pH (>4), this may assist in survival

OmpA can facilitate translocation of *Cronobacter* across the GI tract

Enterotoxin (?)

Other potential sources of contamination of PIF include the equipment used to prepare the reconstitute PIF. Extrinsic contamination from utensils such as blenders, brushes and spoons has been documented (14, 27, 33, 40). Biofilms, defined as a group of bacterial cells that attach to a surface and form an exopolysaccharide that surrounds them (14), are also considered a major concern for contamination of PIF. Abiotic surfaces used to demonstrate *Cronobacter*'s ability to form biofilms include materials commonly associated with PIF-feeding equipment and surfaces such as glass, stainless steel, polyvinyl chloride (PVC), polycarbonate, silicone, and enteral feeding tubes (25, 33, 39, 40, 48).

Kothary *et al*. (45) examined 135 *Cronobacter* strains and identified a cell bound-zinc-containing metalloprotease encoded by a *zpx* gene. The protease was found to be active against azocasein, resulting in the rounding of Chinese hamster ovarian (CHO) cells and may contribute to the organism's ability to cross the blood-brain barrier (BBB) or perhaps help in intestinal cell destruction in neonates with NEC (45).

A virulence mechanism that seems to be common in species belonging to the family Enterobacteriaceae is that of active efflux (27). The latter enables the organism to survive in the presence of xenobiotic compounds, including bile salts, antibiotics, disinfectants and dyes (27). Currently, there is no information on the role of the active efflux transporters in relation to the virulence of *Cronobacter* isolates.

DETECTION METHODS

In 2002, the US Food and Drug Administration (FDA) described a modified method, based on work conducted by Muytjens *et al.* (63) for detecting and isolating *Cronobacter* spp. from PIF (86). The protocol consists of several steps involving enrichments in broth and plating onto selective agar media. The first step requires a primary enrichment of the PIF samples in buffered peptone water (BPW) overnight. Secondary enrichment in Enterobacteriaceae enrichment (EE) broth is followed by plating onto Violet Red Bile Glucose (VRBG) agar for overnight incubation and then purification onto Tripticase Soy Agar (TSA). Yellow-pigmented colonies are selected for biochemical tests using API 20E test strips (10-12, 27). This method is lengthy and not specific to *Cronobacter* spp. Furthermore, this method is not effective at discriminating between *Cronobacter* and *Enterobacter* spp. (10-12, 27).

The International Standards Organization (ISO) and the International Dairy Federation (IDF) published a method in 2006 describing the isolation of *Cronobacter* spp. from milk-based powdered formula (31). The method consists of pre-enriching the PIF samples in BPW at 37˚C overnight (2, 6, 27). The samples are then enriched in modified lauryl sulfate (addition of vancomycin) broth (mLST) at 44˚C overnight. The samples are then plated on to chromogenic agar at 25˚C for a period of 48 to 72 hours (2, 6, 27). For confirmation of *Cronobacter* spp., biochemical identification kits are used. It reduced the analysis time by one day when compared to the FDA approved method. However, not all strains of *Cronobacter* have the ability to grow in mLST (2).

The FDA revised their 2002 method to incorporate both culture steps on chromogenic media and real-time PCR for detection, which proved to be significantly better ($p < 0.05$) than the original method for detection of *Cronobacter* spp. (86). The chromogenic media was developed by Iversen *et al.* (32), which was based on the work of Muytjens *et al.* (64) on the α-glucosidase enzyme. Muytjens *et al.* (64) identified this unique enzyme that was present in all 129 *Cronobacter* isolates tested. This enzyme appears to be unique to only *Cronobacter;* as the closely-related species *Enterobacter cloacae, Enterobacter aerogenes* and *Pantoea agglomerans* were all negative for the enzyme (64). Media which incorporates the chromogenic substrate 5-bromo-4-chloro-3-indolyl-α, α-glucopyranoside (X-αGlc) will result in *Cronobacter* colonies appearing blue-green on a Druggan, Fosythe and Iversen (DFI) agar plate. The α-glucosidase present in *Cronobacter* spp. hydrolyzes X-αGlc to form a bromo-chloro indigo pigment (32).

In 2009, O'Brien *et al.* (68) developed a one-step method for detection, to help address lengthy and multiple enrichments. Their method consisted of a combined pre-enrichment/enrichment broth, *Cronobacter* enrichment broth (CEB formerly known as *Enterobacter sakazakii* enrichment broth) combined with a selective-differential agar (68). The recovery of *Cronobacter* spp. from PIF using this protocol was significantly greater when compared with other enrichment broths. The advantage of this protocol is that it removed the need for separate pre-enrichment and enrichment steps, thus speeding up the process of detecting *Cronobacter* in PIF (68). Al-Holy *et al.* (2) evaluated a new enrichment broth known as Al-Holy-Rasco (AR) medium which consists of a generic brain heart infusion broth with the addition of 1% NaCl, 15% sucrose, and 0.8 g/L sodium deoxycholate as selective ingredients. They used 10 different strains of *Cronobacter* to

compare their enrichment media to EE, CEB, mLST and milk. AR media was superior to the other enrichment broths in both supporting the growth of *Cronobacter* and in suppressing non-*Cronobacter* spp. (2). Further studies in order to validate AR broth for its use as a rapid detection and isolation tool for *Cronobacter* spp. will require a larger strain set of *Cronobacter* spp. in order to address its usefulness in permitting the growth of all *Cronobacter* spp.

As compared to biochemical and phenotypic-based tests, real-time PCR (Q-PCR) is becoming more routinely used as a reliable and quick method for accurately detecting food samples containing *Cronobacter* spp. (54). Malorny and Wagner (54) developed a RT-PCR specific for *Cronobacter* spp. which targets the 16s rRNA gene. The assay itself is very quick, shortening the process of detection from two days to 2 hours and can detect *Cronobacter* spp. at an initial population level of 10^3 CFU/g in foods after an 18 hour enrichment process (54). Seo and Brackett (79) developed a more specific Q-PCR in which the target was a sequence within the macromolecular synthesis operon. The advantage of this method is that it can differentiate *Cronobacter* spp. from species belonging to the family Enterobacteriaceae without the need of an additional enrichment step. Its sensitivity of detection is approximately 100 CFU/mL in rehydrated PIF and PBS (79). If a 24h enrichment step is included then the sensitivity of the detection is increased to 0.6 CFU/g of PIF (79). Other target genes for Q-PCR detection method for *Cronobacter* spp. have been described, including the internal transcribed spacer sequence of the 16S-23S rDNA, α-glucosidase gene (*gluA*), outer membrane protein A (*ompA*) and a zinc-containing metalloprotease (27). A recent PCR-based method was developed to differentiate between the various species within the *Cronobacter* genus by targeting the

rpoB gene, a β-subunit of RNA polymerase. This speciation assay depends on single nucleotide polymorphisms in the *rpoB* gene using mismatch PCR (27).

A fluorescence *in situ* hybridization (FISH) method has been described for the detection of low levels of *Cronobacter* spp. in PIF (3). The advantage of this approach is that it is possible to detect low amounts of the organism in 10 grams of PIF, after an 8- hour enrichment, even when mixed in with other bacterial population. FISH requires a specific probe to identify targeted sequences of DNA; the current *Cronobacter* probe, SakPNA971, hybridizes between positions 971 and 985 of the *Cronobacter* strain ATCC29544 16S RNA sequence (3). It was successful in detecting 99 *Cronobacter* strains but also detected 11 non-*Cronobacter* strains; therefore, although the sensitivity of this assay is valid and precise, the specificity is not as accurate (3).

PREVENTIVE MEASURES

PIF is not a sterile product and may contain foodborne pathogens (69). In 2004, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) convened a joint meeting to discuss *Cronobacter* and other important microbial contaminants of PIF, with the aim to revise the 1979 Recommended International Code of Hygienic Infant Formula (15, 21). In 2005, the document was released, and later updated in 2007 (8). The highlights of the guidelines are based on two scenarios: infants more susceptible to infection and infants not at risk. Infants who are in the high-risk group are advised to only consume sterile liquid infant formula, whereas for the feeding of infants who are not at risk, there are several options available for safely preparing PIF (8). Preparing PIF with water at a temperature of 70 ˚C is suggested as it was shown to reduce the potential for disease-causing organisms to survive. The "hang-

time", the amount of time PIF is at room temperature in the feeding bag and accompanying lines during enteral tube feeding, has been recommended to not exceed 4 hours (72, 69). Reducing the time from preparation to consumption and storing the PIF at temperatures no higher than 5 ˚C for a maximum of 24 h have also been shown to be useful in minimizing the risk (69).

Health Canada has provided a PIF guidance document that can be found on their website (www.hc-sc.gc.ca). These general recommendations are meant to assist individuals at home or in a professional setting, such as hospitals and day-care centers. A preliminary summary is shown in Table 3 (69).

Disinfectants used to eliminate many pathogenic microorganisms with the exception of bacterial spores, are effective against planktonic cells. However, they appear to be less effective against the pathogens when they are present as part of a biofilm (4, 12, 27). *Cronobacter* spp. have been shown to produce biofilms on utensils or the machinery used to make PIF (12, 26, 70). In addition to heat, a number of alternative methods have been examined for their ability to inhibit the growth or inactivate *Cronobacter* spp. Bacteriophages, viruses that infect bacteria, have been suggested to inhibit the growth or inactivate pathogenic microorganisms (26, 42). Kim *et al.* (42) investigated the control of *Cronobacter* spp. through the use of bacteriophages. Using sewage and UV irradiation of pure cultures, 6 bacteriophages were isolated. Two bacteriophages specific to the species *sakazakii* were isolated to demonstrate that they could be used to control the growth of *Cronobacter* spp. in both media and reconstituted infant formula at 12, 24 and 37°C. Phages at concentrations of 10^7, 10^8 or 10^9 PFU/ml were used to demonstrate that higher

Table 3: Recommendations on PIF guidance in home or professional settings such as hospitals and day-care centres*

Preparation should take place in a clean environment where counters have been cleaned and sanitized. Hands should be thoroughly washed using soap and lukewarm water.
Bottles, spoons and teats/nipples should be sterilized in boiling water for 2 min and then air-dried before use or storage. Bottles and equipment should be left covered until ready-for-use.
For pre-term and low-birth-weight infants under 2 months of age or immunocompromised infants, water used for preparing PIF should be brought to a rolling boil for 2 min, dispensed into containers of a maximum of 1 L and cooled down to no less than 70 °C before adding the powder. For all other infants, previously boiled water that has been cooled to room or body temperature (37 °C) can be used to prepare PIF, but it should be served immediately to the infant.
Formula that has been mixed or prepared with boiled water that has been cooled to 70 °C should be further cooled down to room or body temperature, before serving, to avoid scalding of the infant's mouth.
It is best to prepare and serve PIF immediately after cooling to body temperature. Reducing the time from preparation to consumption will reduce the risk to infants. Formula in bottles can be cooled quickly by holding the bottle under running tap water or placing in a container of cold water as long as the cooling water is below the teat/nipple.
If it is not possible to serve immediately, prepared formula in bottles or other types of containers should be refrigerated at 4 °C or lower immediately after the powder has been added and dissolved in water. The prepared formula should be used within 24 h.
Due to the possibility of the growth of harmful bacteria at temperatures above 4 °C, stored formula should only be removed from the refrigerator and re-warmed to room or body temperature immediately before feeding. PIF should not be left warming for more than 15 min as re-warming for extended periods means that the PIF will be held at a temperature that is ideal for the growth of bacteria.
Once feeding has started, the individual bottle should be used within the next 2 h. Any leftover formula should be discarded.

*originally published in draft form by Pagotto and Farber (69). Currently available at http://www.hc-sc.gc.ca/fn-an/nutrition/infant-nourisson/pif-ppn-recommandations-eng.php (accessed on April 24, 2012).

temperatures (24 and 37°C) and high titres (10^9) were required to be effective against *Cronobacter* spp. with contaminations levels equivalent to 73, 000 CFU per 100 gram in PIF (42). Zuber *et al.* (90) isolated 67 phages and tested their lytic abilities on a 40-strain set of *C. sakazakii*. They reported that a cocktail of 5 phages were successful in preventing the growth of 35 (of the 40) strains in artificially contaminated formula. This approach may be useful for the elimination of pathogen in reconstituted PIF (27, 42). Another approach put forth by Hayes *et al.* (26) is to use antimicrobial peptides produced by *Lactobacillus acidophilus*, i.e., they identified two peptides derived from milk proteins, caseicins A and B, that may play a role as a bioprotective agent against *Cronobacter* spp. in dairy-based food products such as PIF (26).

Natural organic compounds have also been of interest in their potential use to control *Cronobacter* spp. Water-soluble muscadine seed extracts contain a high phenolic content and phenolic compounds and have been shown to have antimicrobial activity (43). A recent study conducted by Amalaradjou *et al.* (4) demonstrated that the phenolic compound, malic, tartaric and tannic acids have antimicrobial activity against *Cronobacter* spp. (4). Kim et *al.* (43) have also shown that using red muscadine juice in commercial baby juices can cause a 6-log reduction of *C. sakazakii* in approximately two hours. Their results suggest that red muscadine could be used to prevent and/or control *Cronobacter sakazakii* in baby food formula (43). Recently, Yemis *et al.* (89) investigated the antibacterial activity of vanillin, ethyl vanillin and vaillic acids as possible preservatives for the control of *Cronobacter* spp. in tryptic soy broth supplemented with yeast extract (TSBYE). These possible preservatives were used to evaluate the effectiveness of the compounds on the growth and heat resistance (89). Vanillin, ethyl

vanillin and vanillic acid were shown to have bactericidal effects against *Cronobacter* spp., prevent the growth of the organism and reduce the heat resistance in microbiological media (TSBYE) (89).

When possible, mothers should be encouraged to breast feed. Human milk has been shown to prevent the growth of certain pathogens and protects infants and neonates from infections due to protozoans, viruses, and bacteria such as *Staphylococcus,* Group B *Streptococcus,* and *Escherichia coli* (1, 27). Studies suggest that breast-fed infants have a lower chance of getting gastrointestinal (GI) respiratory and meningeal infections (1, 27). Interestingly, *in vitro* studies by Lenati *et al.* (50) have shown that, *in vitro, Cronobacter* growth can occur in human breast milk.

OBJECTIVES AND HYPOTHESIS

Cronobacter spp. are emerging opportunistic pathogens that have the ability to survive in a variety of different environmental conditions such as those encountered in PIF-processing facilities. Although the number of reported cases of *Cronobacter* infections is relatively low when compared to other foodborne diseases, the outcomes can be very severe and infection can lead to death in infants and especially neonates. A better understanding of how *Cronobacter* spp. causes infection in the host is required to improve the detection and characterization tools, and allow for better control of the organism, and thus a reduced incidence and case-fatality rates in high-risk individuals.

The objectives of this study are:

1. To determine if differences in the virulence and pathogenicity amongst clinical, environmental and food isolates existed using a cell culture toxin assay.
2. To characterize the genes responsible for adhesion and/or invasion that help establish *Cronobacter* spp. infections in the central nervous system CNS via the BBB using the following approach:
    a. To develop a transposon mutant library to identify genes involved in adherence and/or invasion
    b. Screen library for hyper or hypo adhesion/invasion mutants
    c. Identify the transposon insertion site of isogenic mutant(s)
3. To determine whether strains from clinical, food, and environmental sources differ in their ability to form biofilm.

The hypothesis of this study is that there is a difference in virulence between clinical, food and environmental isolates. It is proposed that as the organism survives the environment and the journey to the human host to cause illness, it becomes more virulent. Therefore, based on the objectives of this study, it was expected that clinical isolates would be more pathogenic through the study of clinical, environmental and food isolates.

Chapter II:

Enterotoxin Production using Vero, Small and Large Human Intestinal Epithelial Cell Lines

INTRODUCTION

The production of enterotoxin(s) was the first virulence factor described in *Cronobacter sakazakii*. Using a suckling mouse assay, Pagotto et *al.* (71) tested whether *Cronobacter* spp. had the ability to produce enterotoxins. Mice were inoculated with different doses, ranging from 10^5 to 10^8, of the *Cronobacter* spp. either orally or through intraperitoneal (i.p.) injection. The mice were then euthanized at 4 h after oral ingestion and 7 days if i.p. injected and the intestinal tracts of the mice were examined. Using 18 different strains, they demonstrated that certain *Cronobacter* strains, mainly the clinical isolates, were positive for cytopathic effects (71). The possibility of an enterotoxin being produced by *Cronobacter* strains was further supported by *in vitro* assays using CHO, Vero and Y-1 cells where several strains showed cytopathic effects (71). Some of the *in vivo* and *in vitro* results contradicted each other; that is some strains which showed cytopathic effects in the suckling mouse assay but were negative in the cell lines and vice versa, leading to the suggestion that the observed results may be due to differences in receptor-toxin binding, or possibly a complex regulation system. Other possible explanations may be that *Cronobacter* spp. have the ability to produce more than one cytotoxin or that perhaps horizontal gene transfer could occur in some strains (71).

The second study to support the concept that *Cronobacter* spp. have the ability to produce enterotoxin was described by Raghav and Aggarwal (75). They managed to concentrate and purify the putative toxin using ammonium sulphate precipitation, ion exchange chromatography and identified a putative toxin to have a molecular mass of 66 kDa. They also demonstrated that this purified protein was positive for cytopathic effects in the suckling mouse assay.

The objective of this part of the study was to determine if differences in the virulence and pathogenicity amongst clinical, environmental and food isolates existed using a cell culture toxin assay. The cell line that showed the most consistency in the Pagotto et *al.* (71) study was Vero cells. These are derived from African green monkey kidney epithelial cell line and are commonly used to identify whether *E. coli* strains produce verotoxins. Vero cells were also used in this study and the assay was further optimized from the protocol used by Pagotto *et al.* (71) and a larger number of *Cronobacter* strains (10 clinical, 10 environmental and 10 food isolates, plus the putative purified toxin) was also used. Small and large human intestinal epithelial cell lines were also used to address *Cronobacter* spp.'s role within the host.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

The 30 different isolates of *Cronobacter* spp. assessed in the enterotoxin assay were part of the strain collection of the Bureau of Microbial Hazards of Health Canada and comprised of 10 of each clinical, food and environmental isolates that differed in geographic and temporal origin of isolation. Strains were stored at -80°C in TSB containing 50% glycerol. The positive control used for the enterotoxin assay was *E. coli* O157:H7 (VT_1/VT_2), also stored at -80°C. All strains were grown on TSA plates and incubated at 37°C overnight. Individual colonies from each sample were chosen and inoculated in 5 mL of BHI broth and grown at 37 °C overnight. Fifty uL of the overnight growth was transferred to 50 mL of casaminoacids yeast extract broth. This broth was then incubated on a rotary shaker (VWR) set at 120 rpm and 37°C overnight. The composition of the casaminoacids yeast extract was casaminoacids (20 g/L), yeast extract

(6 g/L), NaCl (2.5 g/L), K_2HP0_4 (8.71 g/L) and trace salts (1 mL/L). The composition of the trace salts consisted of 5% $MgSO_4$, 0.5% $MnCl_2$ and 0.5% $FeCl_3$ dissolved in 0.001 mol/L H_2SO_4.

Isolation of Bacterial Culture Filtrates

Bacterial cells were harvested by centrifugation (Sorvall SC5C) at 4000 × g for 20 min. The supernatant, containing the cell filtrate, was filtered through a sterilized 0.22 um low-protein-binding membrane filter (PALL Acrodisk). Amicon Ultra-15 centrifugal filter devices with 10-kD molecular weight cutoffs (Millipore) were used to further concentrate the proteins contained in the *Cronobacter* filtrates. The total amount that can be added to the filter unit was 15 ml and it was centrifuged (Sorvall SC5C) at 4000 × g for 12 min. The ultrafiltrate was discarded and process was repeated until the entire 50 mL sample was filtered. The end of the process left 300 to 500 uL of concentrated protein solution to work with. The Bradford assay (Mandel) was used to measure the total amount of protein in each sample.

Cell Culture Preparations

Vero, small and large intestinal epithelial cells were grown in 100 cm^2 flasks as monolayers at 37 °C in a 5% CO_2 atmosphere. Vero cells were grown in Eagle's minimum essential medium (EMEM), consisting of Eagle's salt andsupplemented with 0.292 g m/L sodium bicarbonate and 10% (v/v) heat-inactivated fetal bovine serum (FBS). The small and large intestinal epithelial cells and media were ordered from Cell System Corporation (CSC) (ACBRI 519 and 510, respectively). Prior to placing ACBRI 519 and 510 cells in the 100 m^2 flask, the flask was coated with the attachment factor (4Z0-210) and grown in CSC complete media.

Cell Culture Toxin Assay

Cells were seeded into Costar plastic dishes containing twenty-four 16-mm diameter wells, with 500 uL of the appropriate media per well. The cells were seeded at approximately 10^5 cells per well and the plates were incubated overnight at 37°C with 5% CO_2. The growth media was then replaced with fresh media and 50 uL of the appropriate *Cronobacter* filtrate, which contained approximately 2 µg of the protein, including the purified putative toxin and *E. coli* O157:H7 filtrate was placed in each well. Two negative controls were used and included the casaminoacids yeast extract broth and the buffer used to maintain the purified putative toxin. The cultures were the incubated for 24 h at 37°C with 5% CO_2.

Crystal Violet (CV) Staining for Cell Culture Assay

After 24 h, the media was aspirated and 500 uL PBS was added to each well to loosen the debris. PBS was aspirated and 500 uL of 4% paraformaldehyde was used to fix the treated cells at room temperature for 30 min. The paraformaldehyde was aspirated and the wells were rinsed gently with water and stained with 300 uL of 0.1% of CV for 30 min. The plates were then rinsed gently with water and left to air-dry overnight. To quantify the results, 800 uL of 1% SDS was added to each well to solubilize the stain and was shaken for 1 h before absorbance reading. The absorbance of the viable cells survival was measured with a spectrophotometer (Thermo Electron Corporation Biomate 3) at 570 nm.

Ammonium Sulphate Precipitation and Dialysis

Cronobacter strains were grown as described under growth conditions. Once the samples were centrifuged and filtered, a stepwise addition of 50%, 60% and 70% ammonium sulphate was used to isolate the protein from the supernatant. At each step of the

ammonium sulphate wash, the sample was centrifuged (Sorvall SC5C) at 4000 × g for 30 min and 5 mL of 0.05 M Tris (hydroxymethyl) aminomethane buffer (pH 8.0) was added. The supernatant was then further concentrated using Amicon-Ultra-15 protein filter. The protein was collected and dialysis, used to purify the protein, was performed against the same buffer using a Spectra/Por Float-A-Lyzer (Spectrum Labs). The amount of protein in each sample was measured using the Bradford assay as described by the manufacturer (Mandel).

Protein Gel and Coomassie Blue Staining

Cronobacter filtrated protein, ranging from 2 μg to 50 μg, was loaded into separate wells of 12 % pre-made sodium dodecyl sulfate (SDS) polyacrylamide gels (Bio-Rad). A 5 μL molecular weight marker, (Precision Plus Protein Dual Color Standard; Bio-Rad), was used in each gel. Subsequently, the gels were inserted into a Mini-Protein Tetra Cell apparatus (Bio-Rad), and the proteins then separated for 19 to 120 min at 100 V.

After electrophoresis, the gels were washed for 30 min with a fixing solution comprised of 50% methanol, 10% acetic acid and 40% water. The gels were then stained for 1 h with a Coomassie blue solution, which includes 50% methanol, 0.05% Coomassie brilliant blue, 10% acetic acid and 40% water. The gels were then rinsed with the fixing solution for 15 min and then washed with a destaining solution (30% methanol, 10% acetic acid, 60% water) for 90 min. After the gels were treated with the destaining solution, they were placed in distilled water overnight and scanned (FluroChem SP).

In-Gel Protein Digestion

Cronobacter filtrate from strain 2878, a strain that showed cytopathic effects in the *in vitro* cell culture toxin assay, was loaded into a 12 % SDS-polyacrylamide gel electrophoresis (PAGE), along with bovine serum albumin (BSA). BSA was used as a positive control for sequencing. The proteins were separated at 100 V for 120 min and stained with the Coomassie blue staining solution. A band, which was present near 66 kDa, was isolated from the gel. The band was sliced into 1-mm square pieces and incubated with 1 mL of destaining solution overnight at room temperature. The band was then dehydrated with 1 mL of acetronitrile for 30 min. The samples were treated with 50 µL of dithiothreitol for 1 h so that the disulfide bond in the proteins could be reduced. The samples were then alkylated with 50 µL of 55 mM iodoacetamide for 30 min, and then washed with 0.5 mL of 100 mM ammonium bicarbonate and 1 mL of acetonitrile, and dried in a vacufuge. The peptide bonds were hydrolyzed with 20 µg of trypsin, which was dissolved in 1 mL of 50 mM ammonium bicarbonate and incubated at 37°C overnight. The extraction of the peptides was conducted in three different solutions and incubated for 15 min; these solutions included: 100 µL of 100 mM ammonium bicarbonate, 100 µL of extraction solution (50% acetonitrile and 5% formic aicd) and 150 µL of extraction solution. The three extractions were then mixed and dried in a vacufuge. The final, dried pepetide mixtures were sent to the mass spectrometry lab for analysis in collaboration with Dr. Terry Cyr (Biologics and Genetic Therapies Directorate, Health Canada).

Statistical Methods

In order to assess if there is a difference in the ability to cause cytopathic effects on Vero, human large and small intestinal epithelial cells amongst clinical, food and environmental isolates, the following one fixed factor ANOVA model was fit to the data:

$$Y_{ij} = \mu + \alpha_i + \varepsilon_i$$

Where,

Y_{ij} represents the absorbance of relative survival cells for the i^{th} type of isolate (where i= 1, 2, 3) population and j^{th} replicate (where j = 1, 2, …, 10).

μ represents the overall mean,

α_i represents the fixed effect of the i^{th} (where i= 1, 2, 3) type of isolate

and ε_{ij} represent the experimental error (81).

The ANOVA results are reliable if the ε_{ij}'s are independent and identically distributed as Normal with mean zero and constant variance. Therefore, prior to the means comparisons, both Levene's and Shapiro-Wilk's tests will be conducted in order to assess respectively the homogeneity of populations variances and the normality of the experimental error (81).

If the results of both Levene's and Wilk-Shapiro's tests are not found to be significant (p-value>0.05), then the ANOVA model will be retained in order to compare all means using the overall F-test. If the result of this test is found to be significant, then all pairwise means comparisons will be conducted using Tukey's t-test.

However, if Levene's and/or Wilk Shapiro's result are found to be significant (p-value ≤ 0.05) then the square root, log, and inverse transformations will be attempted in order to stabilize the variance and/or achieve normality. If data transformation fails to achieve this goal, then a non parametric approach, using Kruskal-Wallis test, will be used to compare the populations. If the result of this test is found to be significant then the pairwise comparisons will be conducted using Wilcoxon Rank Sum Test.

For each pair of individual strains of interest, a two sided 95% confidence interval for the difference between the two corresponding population means were calculated, using a t-distribution. Whenever, the obtained interval does not contain the value zero, we conclude that the two considered population's means were different, otherwise they were considered similar.

All inferential statistical tests were conducted at the 5% significance level and performed using SAS version 9.3.

RESULTS

In vitro Enterotoxin Assay

Initially, the Vero cells were treated with the unconcentrated strain supernatant. Results were inconsistent in independent experiments; however, the Amicon -15 Ultra filter helped to reduce this inconsistency. The relative survival of Vero cells, after being exposed to supernatant filtrates of 30 *Cronobacter* isolates, is summarized in Figure 2. The positive toxin control from *Escherichia coli* O157:H7 and a negative control, casaminoacids yeast extract broth, were included in the assay. The purified protein of strain 2878, a food isolate, and the buffer that the protein was contained as well as an

American type culture collection (ATCC 29544) were also included (Figure 2). The survival of Vero cells was measured by fixing the cells with 4% paraformaldehyde and staining them with 0.1% CV. The absorbance value represents the viable Vero cells and was compared to the negative control. Therefore, the lower the absorbance value, the more cytopathic effect was observed from the strain. The *Cronobacter* strain that demonstrated the greatest ability to kill Vero cells was strain 3267, a food isolate obtained from PIF from Australia. The purified protein showed some cytopathic effect. Most clinical isolates showed slight or no cytopathic effects. The ATCC 29544 strain of *C. sakazakii* demonstrated cytopathic effects only in the Vero cell line.

The relative survival of the human small and large intestinal epithelial cell, after being treated with the *Cronobacter* isolates concentrated supernatant filtrates, is summarized in Figures 3 and 4. There was not much cytopathic activity observed from the *Cronobacter* isolates to the human intestinal epithelial cells. Strain 3267, a food isolate, showed statistically significant cytopathic effects when compared to the 29 other *Cronobacter* strains.

Figure 2: Relative survival of Vero cells in an *in vitro Cronobacter* spp. toxin assay for the 30 strains. Supernatant from strains tested were further concentrated using Amicon Ultra-15 protein filters. The controls consisted of casaminoacids broth (negative control), *E. coli* O157:H7 supernatant (positive control) and the purified protein obtained from isolate 2878. This assay was repeated three independent times.

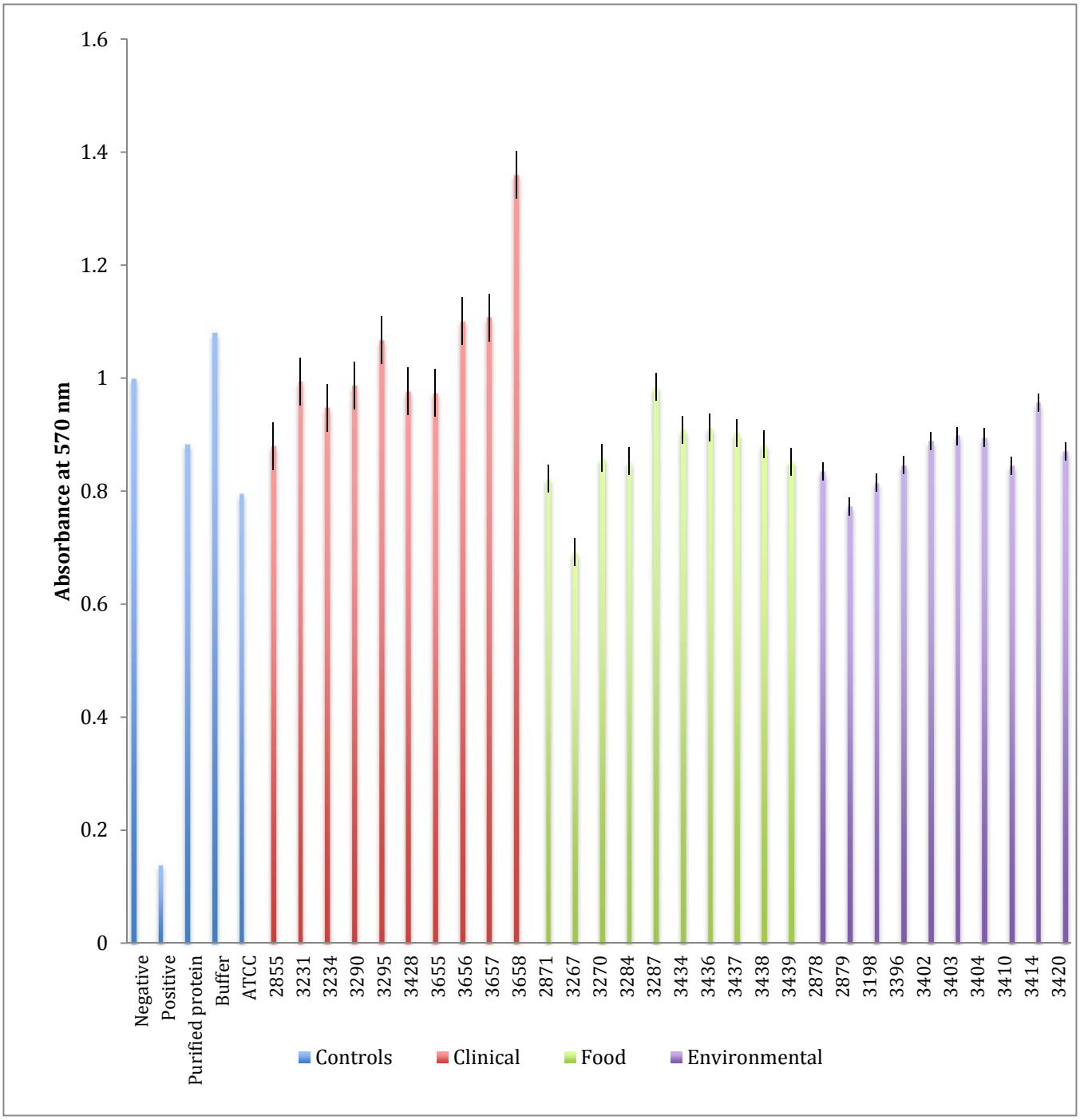

Absorbance at 570 nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Negative
Positive
Purified protein
Buffer
ATCC
2855
3231
3234
3290
3295
3428
3655
3656
3657
3658
2871
3267
3270
3284
3287
3434
3436
3437
3438
3439
2878
2879
3198
3396
3402
3403
3404
3410
3414
3420
Controls
Clinical
Food
Environmental

Figure 3: Relative survival of human small intestinal epithelial cells in an *in vitro Cronobacter* spp. toxin assay for the thirty strains. Supernatant from strains tested were further concentrated using Amicon Ultra-15 protein filters. The controls consisted of casaminoacids broth (negative control) *E. coli* O157:H7 supernatant (positive control) and the purified protein obtained from isolate 2878. This assay was repeated three independent times.

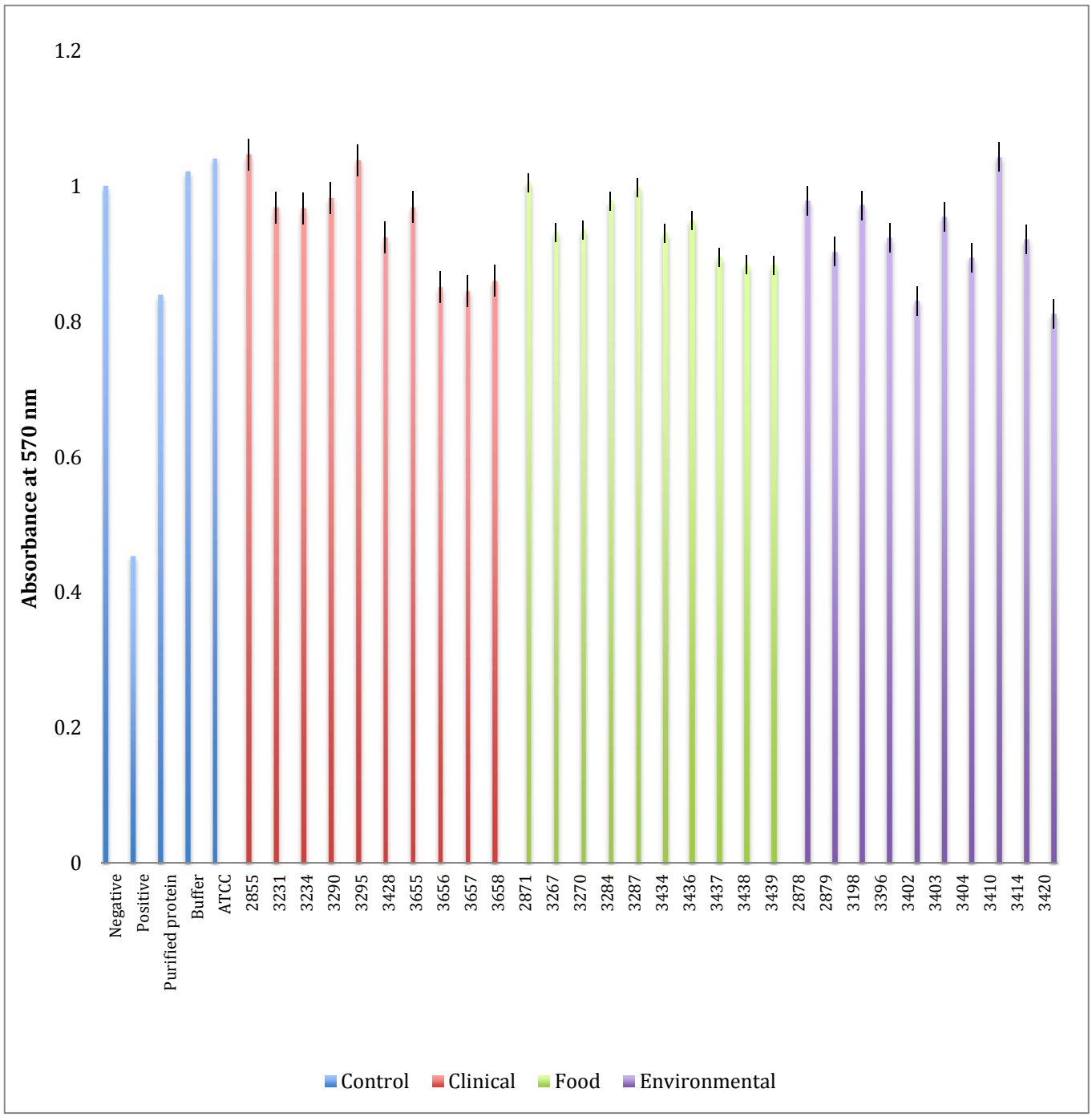
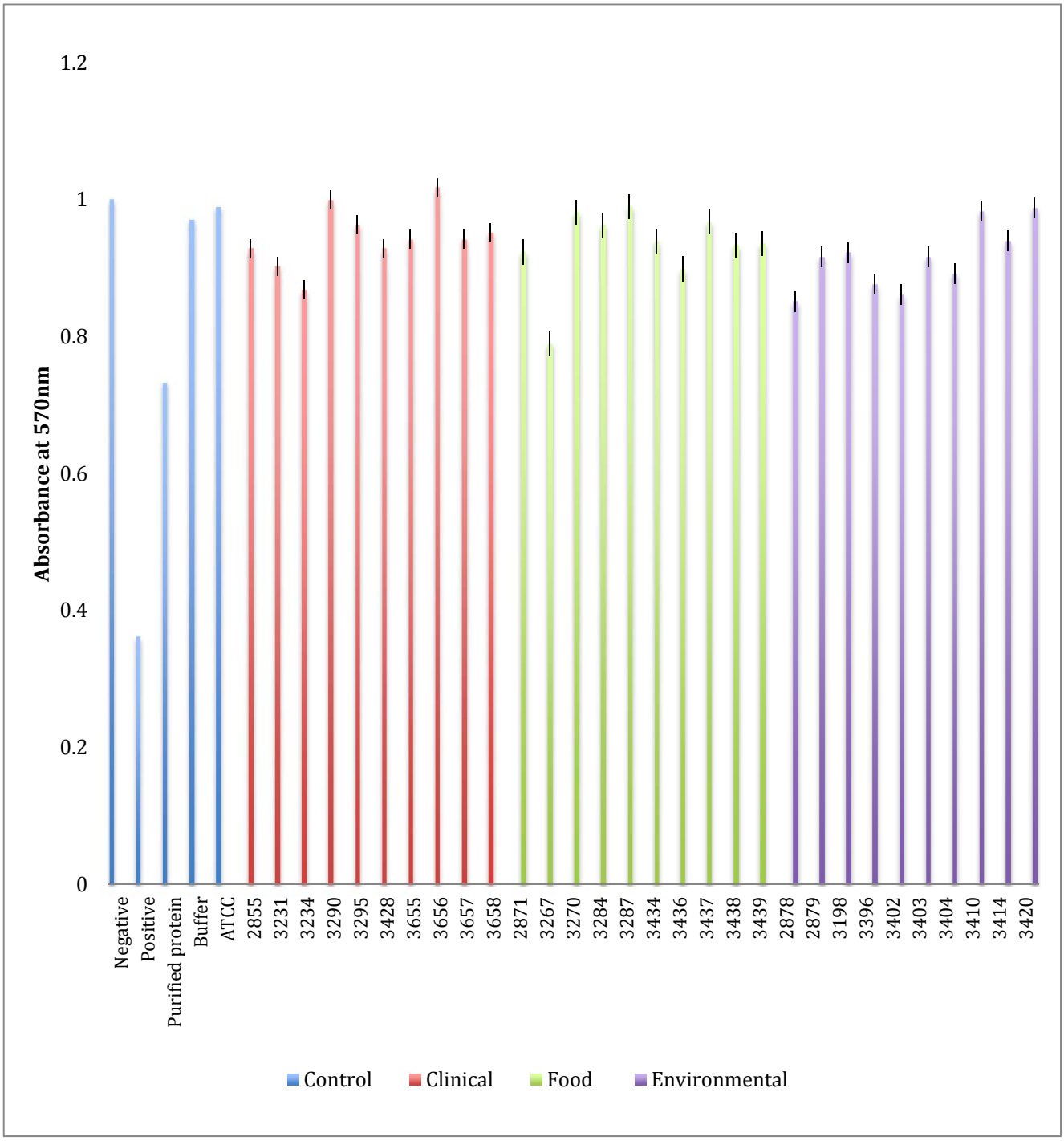

Absorbance at 570 nm
1.2
1
0.8
0.6
0.4
0.2
0
Negative
Positive
Purified protein
Buffer
ATCC
2855
3231
3234
3290
3295
3428
3655
3656
3657
3658
2871
3267
3270
3284
3287
3434
3436
3437
3438
3439
2878
2879
3198
3396
3402
3403
3404
3410
3414
3420
Control
Clinical
Food
Environmental

Figure 4: Relative survival of human large intestinal epithelial cells in an *in vitro* *Cronobacter* spp. toxin assay for the 30 strains. Supernatant from strains tested were further concentrated using Amicon Ultra-15 protein filters. The controls consisted of casaminoacids broth (negative control), *E. coli* O157:H7 supernatant (positive control) and the purified protein obtained from isolate 2878. This assay was repeated three independent times.


1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 570nm
Negative
Positive
Purified protein
Buffer
ATCC
2855
3231
3234
3290
3295
3428
3655
3656
3657
3658
2871
3267
3270
3284
3287
3434
3436
3437
3438
3439
2878
2879
3198
3396
3402
3403
3404
3410
3414
3420
Control
Clinical
Food
Environmental

SDS-PAGE of Concentrated Cronobacter spp. Culture Filtrates

Filtrates of *Cronobacter* spp. were concentrated using Amicon-Ultra 15 protein filters and purified by dialysis. There was approximately 5 μg of total protein for each of the 30 strains. Once the SDS-PAGE and Coomassie blue staining of the *Cronobacter* spp. proteins were conducted, a distinct band was present at 66 kDA for most strains. The 66 kDA protein is the putative molecular weight of the *Cronobacter* spp. enterotoxin (65). Interestingly, there were additional protein bands present in many strains and there were no detectable levels of the 66 kDA protein gel for the negative control, casamino acids yeast extract broth. For strain 2878, there was a band present at 66 kDA and no visible bands present in the negative control (Figure 5).

Figure 5: SDS–PAGE (12%) of proteins stained with Coomassie blue from the culture filtrate of *Cronobacter* food isolate 2878. Casaminoacids growth media was used as a negative control.


Marker
Negative Ctl
Negative Ctl
2878
75 kDa
50 kDa

Protein Sequencing of the Putative 66 kDa Protein

The putative 66 kDa protein in *Cronobacter* strain 2878 and bovine serum albumin (BSA), a positive control known to have a molecular weight of 66 kDa, were analyzed by mass spectrometry (MS). Further details were provided by MS, such as the total amino acid present (539 in total) in the sequence, and the actual molecular weight, which was found to be 59.88 kDA. The actual molecular weight of the putative protein was less than the expected 66 kDa molecular weight of the putative enterotoxin. Sequencing of the BSA positive control confirmed its identity of BSA and the false discovery rates, calculated from algorithms contained in the NCBI database, were 0.00%/9.09% for BSA and 0.00%/0.00% for the putative protein. The sequence of the putative protein of 2878 was compared to the other sequences found in the NCBI database to determine a possible match and to identify the protein of *Cronobacter* strain 2878. There was one protein from the NCBI database that was closely matched to the putative protein sequence of strain 2878. Protein-protein BLAST (BLASTp) was also used to identify conserved domains in the sequences. All the following tools used confirmed that the putative protein from strain 2878 was a phosphenolpyruvate carboxykinase (PEPCK).

Statistical Analysis

The results of the Levene's test revealed no statistical significance ($p>0.05$) for all three cell lines, implying that the variances between the three different groups were not statistically different. However, the results of the Shapiro-Wilk test were found to be significant ($p\leq0.05$) for all three-cell lines. Consequently, in order to achieve normality, the log transformation of the measured relative survival of Vero, human large and small

intestinal epithelial cells was applied. The results of the Shapiro-Wilk test on the log-transformed data were found to be not statistically significant ($p > 0.05$). Therefore, in order to make inference on the three different types of isolates in all the cell lines, a parametric approach, more precisely a homoscedastic one-way ANOVA model was used on the log-transformed data. The results of the overall F-test statistic revealed significance ($p \leq 0.05$) only for the Vero cell assay. Consequently, for the Vero cell assay, the three possible pairwise comparisons between clinical, environmental and food isolates were conducted using Tukey's t–test. The results of this test showed significance ($p \leq 0.05$) for the pairwise comparisons between clinical isolates and food isolates and between clinical isolates and environmental isolates.

DISCUSSION

The mechanisms by which *Cronobacter* spp. infect the human host are still poorly understood. *In vivo* assays, in particular the neonatal suckling mice test, have been used to further progress our understanding of the pathogenicicty of *Cronobacter* spp. The suckling mice assay was used by Pagotto et *al.* (71) to study the putative enterotoxin, the first virulence factor described in *Cronobacter* spp. They were successful in demonstrating that certain *Cronobacter* strains have the ability to produce an enterotoxin. Raghav and Aggarwal (75) provided further information of the putative enterotoxin by purifying it and examining its biochemical properties. However, these are the only studies published related to the potential enterotoxin and still many questions remain about the nature of the toxin and its exact role in the host. As well, to-date, three *Cronobacter* strains have been sequenced in our laboratory, and bioinformatics analyses have not revealed the presence of a toxin (data not shown).

The objectives of this part of the study were to determine whether *Cronobacter* strains were able to produce an enterotoxin in *in* vitro conditions and whether there were any differences amongst clinical, environmental and food isolates. The results of the Vero cell assay showed that enterotoxin production varied amongst the 30 strains (Figure 2). In addition, certain *Cronobacter* strains showed more cytophatic effects than others, whereas others showed little or no effects on the cells. The strain that induced the greatest amount of Vero cell death and was consisted in its activity throughout all three independent experiments was strain 3267 (a food isolate). Interestingly, the food and environmental isolates were shown to exhibit a greater amount of cytopathic effects on Vero cells than clinical isolates. Most clinical isolates showed little to no cytopathic effects. A one-way ANOVA and Tukey's t-test were both conducted in the Vero cell line assay to determine if there were any mean differences between the three types of isolates. In fact, the population mean of the clinical isolates differed statistically from the population mean of the food and environmental isolates, whereas, food and environmental isolates did not statistically differ. From the Vero cell assay results, it was concluded that the food and environmental strains produce more cytopathic effects than the clinical strains.

These results conflict with our hypothesis and the *in vivo* results obtained by Pagotto et *al.* (71), where it was shown that the clinical isolates produced the most enterotoxin. *Cronobacter* strains isolated from clinical specimens would be expected to be the most toxic. It was surprising to see that most clinical isolates had immediate effect on the Vero cells. It is possible that an essential factor was missing in the *in vitro* culture assay or that the conditions were not appropriate for the clinical isolates to produce a toxin. Another

possible explanation could be that the clinical strains may have become host adapted, or that repeated passaging in the lab may have reduced their virulence.

The differences observed in enterotoxin production between *Cronobacter* spp. could also have been due to differences in receptor-toxin binding, or to a complex regulatory system. Another possible explanation could have been that the toxin gene may be present in the strains that showed no cytopathic effects, but it could have been either inactivated or differentially regulated. There is also the possibility that Vero cells may not be an appropriate biological representation of the conditions that the bacteria experience in the host. The two main target areas of this pathogen in the host are the gastrointestinal tract and the BBB, whereas Vero cells are derived from African monkey kidney epithelial cells, and are primarily used to demonstrated the presence of verotoxins in *E. coli.* This could explain why no *Cronobacter* strains were comparable to the positive strain, *E. coli* O157:H7, in inducing major cytopathic effects in Vero cells. This led us to use human intestinal epithelial cell lines, both small and large, as it seemed to be a better representation of the biological phenomenon seen when *Cronobacter* spp. enter the human host.

The results obtained using small and large human intestinal epithelial cell lines were not helpful in confirming enterotoxin production. Most strains showed slight or no impact on the viability of the cell lines. *E. coli* O157:H7 induced the most death in the cell lines. A one-way ANOVA test confirmed that there were no mean differences between the clinical, food and environmental isolates in terms of their effect on the human intestinal epithelial cells. The cell culture assay may not be the best choice for evaluating enterotoxin production and an animal model may be the most appropriate, as it represents

a more suitable biological milieu when attempting to draw comparisons to human disease. Alternatively, intestinal cell assays for *Cronobacter* may need to be optimized further.

Raghav and Aggarwal (75) isolated, purified and characterized the *Cronobacter* enterotoxin and showed that the molecular weight of the toxin was 66 kDa by SDS-PAGE. They also demonstrated that this 66-kDa protein was toxic in the suckling mice assay (75). However, they did not sequence or further characterize the protein. This led us to our next objective in this study, which was to identify the sequence of the potential toxin and determine whether it produces an enterotoxin under *in vitro* conditions. A total of 2 µg of *Cronobacter* protein was analyzed (Figure 5). A distinct protein band was seen at approximately 66-kDa and was present in most of the strains, while it was absent in the negative control. It was demonstrated that the more protein used, the more prevalent the band was (Appendix B). The presence of the putative 66-kDa protein band in the majority of the strains, despite the differences in enterotoxin production using the cell line culture assay, possibly indicates that the protein could be inactivated in certain strains. Interestingly, the strains that produced the most prevalent bands, such as 3270, did not show significant death in the *in vitro* cell culture assay.

The putative enterotoxin from strain 2878, one of the most toxic strains in the Vero cell assay, had a prevalent protein band at 66 kDa. The putative toxin sequence obtained for 2878 was sequence aligned and searched for protein homology by the NCBI database and BLAST. A similar match was seen with the hypothetical protein previously described from *Cronobacter* BA8944 and is reported to have 539 amino acids and a molecular weight of 59.88 kDa. The mass of the isolated protein in our study is slightly less than the 66 kDa putative enterotoxin protein reported by Raggahv and Aggarwal (67). They

determined the weight of the protein using SDS-PAGE, whereas we used the mass spectrometry for determining the molecular weight of the protein. The putative enterotoxin may still be 66 kDa, since there is a possibility that it may contain an extra domain or be bound to another small protein. The false discovery rates for sequencing were 0.00%/ 9.09% for BSA and 0.00%/ 0.00% for the putative toxin, which signifies that the protein band near 66 kDa in the SDS-PAGE is likely the 59.88 kDA hypothetical *Cronobacter* BA8944 protein in the NCBI database. The protein-protein BLAST algorithm (BLASTp) was used to analyze the putative 59.88 kDa, which showed that nearly the whole putative enterotoxin sequence contained the domain of PEPCK and various derivatives of the PEPCK domain.

PEPCK is an enzyme that is involved in a key rate-limiting step in gluconeogenesis, where it is responsible for catalyzing the reversible decarboxylation and phosphorylation of oxaloacetate (OAA) to phosphoenolypyruvate (PEP) and carbon dioxide (9). The identification of the PEPCK as the putative protein in strain 2878 does not support the enterotoxin hypothesis, since the sequence does not resemble a traditional sequence of an enterotoxin. It would also be unusual for a putative enterotoxin to contain a PEPCK domain, since the function of PEPCK is not known to induce cell death and has not been related to toxin functions. However, during the Vero cell and Human (small and large) intestinal epithelial cell assay, the cells were subjected to the purified protein and cell death was observed (Figure 2).

Liu et *al.* (52) have shown that PEPCK activity may be required for successful infection in the host by *Mycobacterium bovis* BCG. They managed to delete phosphenolypyruvate carboxykinase gene (Δ*pckA*) by homologous recombination and tested these mutant

strains both on mice and macrophages (52). They demonstrated that the deletion of *pckA* of *Mycobcterium bovis* BCG reduced the capacity of the bacteria to infect and survive. Mice infected with Δ*pckA* BCG strains were able to reduce the bacterial load much more quickly than mice infected with the wild type.

Utley et *al.* (87) were able to demonstrate that the deficient catabolite repressor/activator (Cra) protein in *Salmonella enterica* serovar Typhimurium, SR-11, is avirulent in mice when administered orally. However, when the Δcra SR-11 was administered intraperitoneally, it remained fully virulent. Cra is a regulator of central carbon metabolism in both *Salmonella* and *E. coli,* and is also a transcription factor that activates genes encoding key enzymes in the glyoxylate bypass, gluconeogenesis and the tricarboxylic acid cycle (TCA) (87). This lead Tchawa Yigma et *al.* (83) to investigate the role of gluconeogenesis and TCA in the virulence of *S.* Typhimurium*,* SR-11, in mice. By using mini transposon mutagenesis, they produced many deficient mutants in key enzymes such as PEPCK and phosphoenolypyruvate synthase (PPS). They concluded that Δ*pckA* and Δ*pps* SR-11 strains were not much different in mice than the SR-11 wildtype when administered both orally and intraperitoneally. However, when a strain of Δ*pckA Δpps*S R-11 was administered orally to mice, it was slightly attenuated (83).

Recently, Schmid et *al.* (78) demonstrated that phosphoenolypyruvate carboxylase (PEPC) is essential for replenishing the oxaloacetate pool in the TCA under virulence conditions. They demonstrated using affinity purification, native gel electrophoresis and small angle x-ray scattering, that YscM1 and YscM2, proteins considered to be regulators in type three secretion systems (T3SS) in *Yersina enterocolitica*, bind to PEPC (78). They were then interested in determining the functional roles of PEPC, YscM1 and YscM2 in

Yop-producing bacteria. They used homologous recombination to generate mutants defective in each of these three proteins and grew them under low calcium conditions in the presence of [U – $^{13}C_6$] glucose (virulence condition) (78). They then analyzed the isotope composition of the secreted Yop proteins by mass spectrometry. Their results demonstrated that a considerable fraction of oxaloacetate was used as precursor for amino acids and was derived from [$^{13}C_3$] PEP by the catalytic action of PEPC in the wild type strain; however, this was not observed in the mutant deficient in PEPC (78).

There have been studies in focusing in on the role of PEPCK and its derivatives in the pathogenesis of bacteria in mice. However, currently, no studies indicate that PEPCK works in a similar manner or has toxin-like functions. PEPCK appeared to induce cell death in the Vero cell assay. However, further tests are necessary to confirm and understand the exact role it may have in *Cronobacter* infections. The results obtained with the *in vitro* techniques and the PEPCK identification at 59.88 kDa does not appear sufficient to support the hypothesis of the enterotoxin in the cell lines. There is a possibility that the protein band isolated for sequencing was not the putative enterotoxin of 66 kDa reported by Ragavh and Aggarwal (75). In Figure 5, there is a faint protein band slightly above the band isolated for sequencing. A different molecular weight ladder which contains a standard 66 kDa protein would facilitate the isolation of the correct band for sequencing. Another alternative method for isolating the putative enterotoxin protein would be high-performance liquid chromatogaphy (HPLC), which was the original method used by Raghav and Aggarwal (75) in identifying the putative enterotoxin.

The confirmation that *Cronobacter* spp. produce enterotoxin was not obtained using *in vitro* techniques such as cell culture assay, but the discovery of PEPCK and its role as a

potential virulence factor may shed some light in understanding the mechanism(s) by which this organisms causes infection in humans. The purified PECPK was subjected to all three cell lines and did demonstrate cytopathic effects. It would be interesting to see the effects of this purified protein in the suckling mice assay or to ascertain the role that bacterial PEPCK has on eukaryotic cells. Additional studies on both PEPCK and the enterotoxin are required to fully understand their role in the pathogenesis of *Cronobacter* spp. It would be also interesting to test all strains used in this study on other cell lines such as Caco-2, Hep-2 and HBMEC, as these cell lines are known targeted areas of *Cronobacter* infections in the host. In this study, we focused primarily on the effects of enterotoxin on the GI tract. Further testing on blood brain barrier cells may provide new insights into the pathogenicity of *Cronobacter* meningitis.

Chapter III:

Adhesion and Invasion of the Blood Brain Barrier

INTRODUCTION

Adhesion and invasion of the blood-brain barrier (BBB) and the gastrointestinal (GI) tract are important for *Cronobacter* spp. to be able to infect the human host and a better understanding of these processes would help in unraveling the mechanism(s) of pathogenesis. The use of mammalian tissue culture assays is a common approach to investigate bacterial adhesive and invasive properties. The most common cell lines used to study adherence and invasion are HEp-2, Caco-2 and human brain microvascular endothelial (HBMEC) (30, 55, 58, 59, 65). Mange *et al.* (55) screened 50 *Cronobacter* strains and found two distinctive adherence patterns in all three cell lines tested. They were described to be diffuse adhesion and/ or with the formation of localized clusters of bacteria on the cell surface. Some strains demonstrated both patterns (55). Mange *et al.* (55) also demonstrated that *Cronobacter* spp. adherence was optimal during the late exponential phase of bacterial growth with a 10-fold increase in adherence cells, and also concluded that adhesion to epithelial and endothelial cells is predominantly non-fimbrial based (55). Townsend *et al.* (85) studied the attachment and invasion properties of seven different *Cronobacter* strains associated with symptoms such as NEC, bacteremia, and meningitis using Caco-2 cells and rat-brain capillary endothelial cells. All seven strains attached and invaded Caco-2 cells after 3 h. These particular isolates also have the ability to replicate and adapt in U937 macrophage cells (85).

Bacterial translocation from the gastrointestinal barrier is critical in the pathogenesis of *Cronobacter* meningitis (36, 42, 50, 52, 53, 58, 72). Translocation from the GI tract allows the bacteria to have access to the blood stream, leading to bacteremia in the host and giving access to the central nervous system. *Cronobacter* species are able to

overcome host defensive mechanisms, penetrate the BBB and survive in the cerebrospinal fluid (41, 47, 55, 58, 59, 65, 80). One virulence factor that has been shown to be essential in *Cronobacter* pathogenesis is the outer membrane protein A (OmpA). Interestingly, OmpA plays a role in suppressing the maturation of dendritic cells (19). OmpA expression is also required for invasion of mammalian host cells and is necessary for *Cronobacter* resistance to blood and serum killing in newborn rats (41, 55, 59, 80). Mittal *et al.* (58) have shown that *Cronobacter* isolates that are positive for OmpA can successfully cross the intestinal barrier in newborn rats, multiply in the blood and transverse the BBB, whereas isolates with *ompA* deletion mutant, fail to bind to intestinal epithelial cells both *in vivo* and *in vitro* (58). Recent studies from Kim *et al.* (41) demonstrated that the outer membrane protein X (OmpX) is also required for translocation. They were the first to conduct in-frame deletion mutants for OmpA and OmpX and showed that translocation does not occur in the host when both proteins are not expressed (41). While comparing *Cronobacter* BAA-894 genome *and Enterobacter cloacae* using BLAST search, *ompX* was found to be similar, i.e., 81% identity (41). OmpX in other bacteria, including *E. cloacae*, plays an important role in virulence since it assists in the invasion of host cells and helps to overcome the host's defenses (41). Kim *et al.* (41) have demonstrated (using Caco-2 and INT-407 cells and a rat pup model) that for translocation and basolateral invasion and adhesion of mammalian cells to occur in *Cronobacter*, both OmpA and OmpX need to be expressed.

Singamsetty *et al.* (80) noticed that meningitis caused by Gram-negative bacteria also require the expression of OmpA to invade HBMEC. They demonstrated using HBMEC that OmpA is required for *Cronobacter* spp. to invade the BBB, and that it does this by

inducing microtubule condensation and PI3-kinase and PKC-α activation (80). Both Nair *et al.* (65) and Mittal *et al.* (58) showed that OmpA binds to fibronectin, thereby facilitating the invasion of brain endothelial cells. Mittal *et al*. (58) were the first to demonstrate that OmpA expression affects the onset of meningitis in newborn rats. Mortality rate is 100% when newborn rats are infected with *Cronobacter* OmpA positive strains and no pathological symptoms were observed when using *ompA* deletion mutant strains (58).

In this study, the adhesive and invasive abilities of 30 different *Cronobacter* spp. isolates to the HBMEC line were investigated. A transposon mutant library was developed for certain isolates and screened through the standard adhesion and invasion assay to help identify gene(s) involved in adherence and/or invasion. This study was conducted to help shed further light on the mechanism(s) of pathogenesis of *Cronobacter* spp.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Thirty *Cronobacter* isolates were thawed out from the frozen stock cultures and plated onto Tryptic Soy Agar (TSA) plates. The isolates were grown overnight at 37°C and the following day a single colony for each isolate was inoculated into 10 ml of Tryptic Soy Broth (TSB) and were incubated with shaking for 4 h at 37°C. The cultures were then harvested by centrifugation (3,200 × *g* for 10 min) and washed once with phosphate-buffered saline (PBS). The cells were then re-suspended in minimal Cell System Corporation (CSC) complete medium (Cell System Coporation, Kirkland, WA), which was pre-equilibrated at tissue culture conditions (5% CO_2, 95% relative humidity, 37°C).

Cell concentrations were determined by plating serial dilutions on TSA. This was referred to as infectious media.

Tissue Culture

HBMEC (ACBRI 376) was obtained from Cell System Corporation, Kirkland, WA. Tissue culture flasks (75 cm^2) were treated with attachment factor (4Z0-210) to support the growth of the HBMEC monolayers by evenly coating the bottom of the culture flasks. The remaining liquid was removed, and 12 mL of pre-warmed CSC medium was added to the flask. The thawed HBMEC (passage 3) were added to the prepared flasks and incubated at 37°C in a humid atmosphere of 5% CO_2, and the old CSC media was replaced with fresh CSC complete media the following day. The monolayers were split in a ratio of 1:4 once every two weeks with the passage reagent kit (PRG-1 dPBS-EDTA solution, PRG-2 Trypsin-EDTA Solution and PRG-3 Trypsin Inhibitor Solution) according to standard procedures provided by Cell System Corporation. Monolayers of passages 8 to 16 were used for all experiments described.

Adhesion and Invasion Assay

Approximately 1×10^5 of HBMEC were seeded into 24-well plates which were treated with attachment factor and contained 500 µL of warm CSC complete medium. The cell lines were then incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The following day the media was aspirated and replaced with 200 µl of infectious media at a multiplicity of infection (MOI) of 100:1 and incubated for 1.5 h. The media was aspirated and the 24-well plates were washed twice with PBS and treated with 250 µl of 1% Triton-X 100 and incubated for 30 min. After the addition of 750 µl double distilled water in each well, the

released bacteria were enumerated on TSA plates. Control wells without HBMEC cells were prepared in a similar manner in order to quantify non-specific bacterial adherence to the plastic wells. Adherence to HBMEC for each strain was determined as the total number of CFU minus the number of CFU adherent to wells without HBMEC cells. Invasion assays were conducted the same way with an additional 30 min incubation time after the 1.5 h with CSC media containing 100 μg/ml of gentamicin. *E.* coli K12 (non invasive) was used as a negative control. Data representing the invasive abilities of the *Cronobacter* strains was determined as [100 × (number of bacteria recovered/number of bacteria inoculated)]. The adhesion and invasion assays for each strain were done in duplicates and replicated 3 times.

Construction of the Transposon Mutant Library

A library of random transposon mutants of *Cronobacter* strains 2855, 3231, 3267, 3287 3414 and 3658 were constructed using an EZ-Tn5 <Kan-2> Tnp Transposome kit (Interscience, Markham, Canada). *Cronobacter* electrocompetent cells were prepared by growing strains in 50 mL of LB and incubated overnight at 37°C. The following day, 25 mL of the broth was transferred to 1L of LB. The 1L bottle was shaken and incubated overnight. Each bottle was split into four 250 mL centrifuge bottles and centrifuged at 11, 000 × g at 4°C for 10 min. The supernatant was discarded and the pellets were combined and washed with 200 mL of ice-cold 10% glycerol. They were then once again centrifuged at 11, 000 × g for 10 min at 4°C; these centrifuging conditions were used throughout the procedure. After the supernatant was discarded, another 150 mL of ice-cold 10% glycerol was added to wash the pellets and the mixed content was centrifuged and supernatant discarded. The last wash of 100 mL of cold 10% glycerol was mixed

with the pellets and centrifuged. The pellet was re-suspended with a pipette containing 2 mL of cold 10% glycerol and was transferred, in aliquots of 150 µL, to sterile 1.5 mL Eppendorf tubes. The tubes were stored at -80 ºC until ready for use. Insertion of the transposon in the genome of *Cronobacter* strains was done by electroporation. One hundred µL of the electrocomptent cell stock was inserted in to an electroporation cuvette and placed in a bucket of ice. Two µl (40 ng) of the EZ-Tn5 <Kan-2> Tnp Transposome, as well as 2 µl (10 µg) of Type I inhibitor was added to the electroporation cuvette and was well mixed by pipetting up and down. For the negative control, only 100 µl of electrocompetent cells were used, whereas, for the positive control, 2 µl (100 ng) of green fluorescent protein (GFP) and 2 µl of type I inhibitor were also included. Following electroporation, 1 mL of super optimal broth with catabolite repression (SOC) was added to the cuvette, the cell content was transferred to a 1.5 ml Eppendorf tube and incubated with shaking at 120 rpm at 37°C for 1h. Once the cells were incubated, 200 µl of the cell suspension was plated on to TSA + Kanamycin (50 µg/ml) plate and incubated at 37°C overnight. A stock was made for each mutant grown and the mutants were screened through the standard adhesion and invasion assay.

Confirmation of the Insertion of the Transposon

PCR was used to amplify the integrated transposon using the primers FWKAN and RVKAN. The PCR conditions for the insertion of the transposon were the following: 2 min at 94ºC and 35 cycles of 1 min at 94ºC, 1 min at 55 ºC, 1 min and 30 s at 72ºC and 5 min at 4ºC. Visualization of the insertion of the transposon was done using QIAxcel Biocalculator (Version 3).

Identification of Transposon Insertion Site

The extraction of the genomic DNA of Δ3231 was conducted using Wizard Genomic DNA purification kit (Promega, Madison, WI). The extracted DNA was further purified by the microcon centrifugal filter Ultracel YM-30 (Millipore) kit and the amount of DNA was measured using the NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific). The extracted DNA was digested by first adding 1 µg/µl of the DNA in a PCR tube with 16 µl of nuclease-free water, 2 µl of 10× Buffer EcoRI and 2 µl of EcoRI (Fermentas Life Science). The second step involved mixing the solution and centrifuging for a few seconds. The last step was the incubation of the PCR tube at 37ºC for 16 h. The following day, the digested DNA was ligated in the following reaction: 120 ng of digested DNA and 2 µl of Ligase 10x buffer, 1 µl of T4 DNA ligase and the remainder was nuclease-free water, with the total volume being 10 µl. The ligation reaction was incubated at 16 ºC for 18 h. In separate experiments, the direct ligation of the PCR product for Δ3231 was sequenced by Genome Quebec Innovation Centre (Montreal, Quebec).

Growth Curves

Strain 3231 was grown in Luria Broth (LB), whereas, Δ3231 was grown in LB + KAN overnight. The inoculated broths were diluted in peptone water (Oxoid) to approximately 10 – 100 CFU/ml and incubated at 37°C. A non-inoculated negative control was included throughout the 24 h. For each sampling time, 1 ml samples were diluted in peptone water and plated in triplicate for bacterial plate counts. Inoculation for enumeration was done

manually and using a spiral plater (WASP2, Don Whitley Scientific Limited) on TSA plates and TSA + KAN plates, for strains 3231 and Δ3231, respectively.

Statistical Methods

To assess if there was a difference in adhering and invading HBMEC between the clinical, food and environmental isolates, a one-way ANOVA was conducted. All inferential statistical tests were performed using SAS version 9.3. Prior to the means comparisons, the Levene and Shapiro-Wilk tests were both conducted in order to assess respectively the homogeneity of variances and the normality of the experimental error and therefore, to validate the use of ANOVA (81). Whenever either the listed ANOVA assumptions are violated, a non -parametric approach, more precisely a Kruskal-Walis test, was used to assess the overall difference between the 3 types of isolates in HBMEC. Furthermore, the three possible pairwise comparisons between the three different types of isolates were performed using Wilcoxon Rank Sum test (81).

RESULTS

Adherence and Invasive Abilities of Different Cronobacter strains to HBMEC

To evaluate the adherence ability of different *Cronobacter* strains to HBMEC, a standard adhesion assay was conducted (Figure 6). All environmental and food isolates were successful in adhering to HBMEC; whereas, 9 out of 10 clinical isolates demonstrated the ability to adhere to HBMEC. The majority of the strains adhered greater than the positive controls used in this quantitative assay. The strain that demonstrated the greatest ability to adhere was strain 3404, an environmental isolate.

The different *Cronobacter* isolates were further examined for their ability to invade HBMEC. Relative frequency was used to quantify the invasive abilities of *Cronobacter* spp. by counting the number of viable bacteria in the presence of gentamicin and dividing the total number of bacteria before gentamicin was added. Once again, the majority of the environmental isolates (9/10) demonstrated the ability to invade, whereas 8/10 food and 6/10 clinical isolates were able to invade HBMEC. The strain that demonstrated the greatest ability to invade HBMEC was 3267, a food isolate. Only two strains (3404 and 3267) demonstrated the ability to both adhere to and invade HBMEC.

Screening for Isogenic Mutants

A transposon mutant library was developed for strains 2855, 3267, 3231, 3404 and 3414 to further assist in characterizing genes involved in adhesion and/or invasion of HBMEC. The mutant library was successful for all five strains, and there were around 300 to 1000 mutants for each strain. PCR was used to amplify the transposon and Qiaexcel was used to visualize the insertion site of all the potential mutants. Once the transposon was confirmed to be present in the bacterial genome, mutants were screened using the standard adhesion and invasion assay. Only one mutant of strain 3231 (clinical isolate), Δ3231, demonstrated the inability to adhere to HBMEC. The genome sequence approach was used to identify the transposon insertion site. Blast homology analysis of genomic sequence data generated from the Δ3231 transposition clone compared to the complete genome of *C. sakazakii* BAA-894, identified with 98% identity the insertion site to be diguanylate cyclase (DGC).

To further confirm that the transposon did not interfere with a gene essential to growth, a 24-h growth curve was conducted three independent times. As seen in Figure 8, there was

no statistically significant ($p \leq 0.05$) difference in growth rate between 3231 and Δ3231. Therefore, differences observed between the two strains in adhering to HBMEC were not due to growth rates.

Statistical Analysis

The results of the Levene's test revealed no statistical significance ($p > 0.05$), implying that the variances between the three different groups were not statistically different. However, the results of the Shapiro-Wilk test were found to be significant ($p \leq 0.05$). Consequently, in order to achieve normality, the log and root transformation of the measured adhesive and invasive abilities to HBMEC was applied. However, the results of the Shapiro-Wilk test on the transformed data were still found to be statistically significant ($p > 0.05$). Therefore, in order to make inference on the three different types of isolates a Kruskal-Wallis test was conducted (81). This test revealed no significance at the 5% level. Furthermore, the three possible pairwise comparisons between clinical, environmental and food isolates were conducted using Wilcoxon Rank Sum test (81). The results of this test showed no statistical significance ($p > 0.05$), implying that there were not significant differences in the ability to adhere to and invade HBMEC between the clinical, food and environmental isolates.

Figure 6: Quantitative analysis of the adherent ability of different *Cronobacter* isolates to HBMEC. Adhesion is reported as the number of bacterial CFU adhered to the surface of endothelial cells. Positive controls include strains 5039 (*E.coli* K12) and 385 (*Bacillus cereus*). Strain 5040 (*Klebsiella pneumonia*) was used as the negative control. Results are based on mean of three independent experiments.

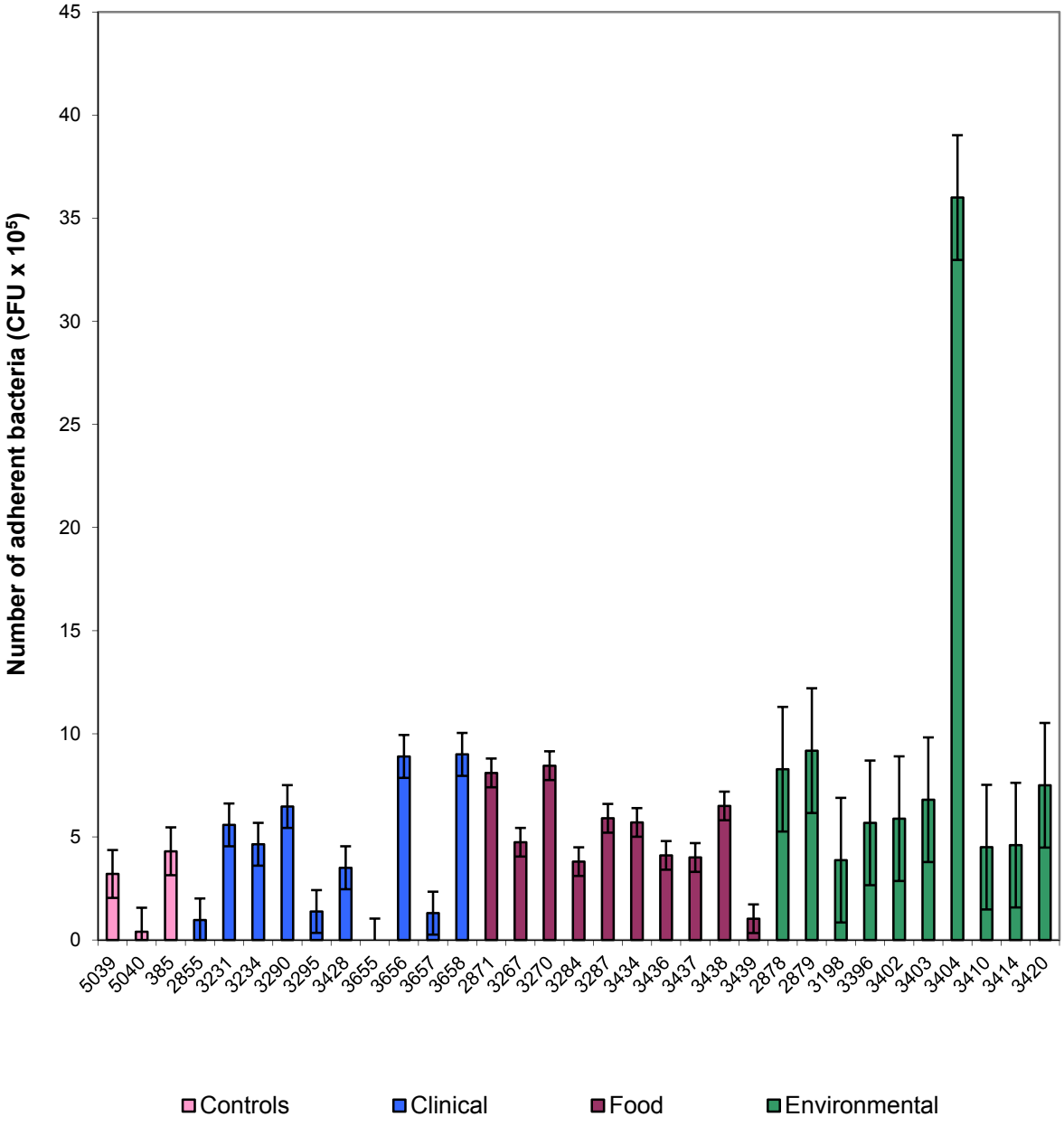

Number of adherent bacteria (CFU x 10⁵)
45
40
35
30
25
20
15
10
5
0
5039
5040
385
2855
3231
3234
3290
3295
3428
3655
3656
3657
3658
2871
3267
3270
3284
3287
3434
3436
3437
3438
3439
2878
2879
3198
3396
3402
3403
3404
3410
3414
3420
Controls
Clinical
Food
Environmental

Figure 7: Quantitative analysis of the invasive ability of different *Cronobacter* isolates to HBMEC. Negative controls include strains 5039 (*E. coli* K-12), 5040 (*Klebsiella pneumonia*) and 385 (*Bacillus cereus*). The results are based on the mean of three independent experiments.

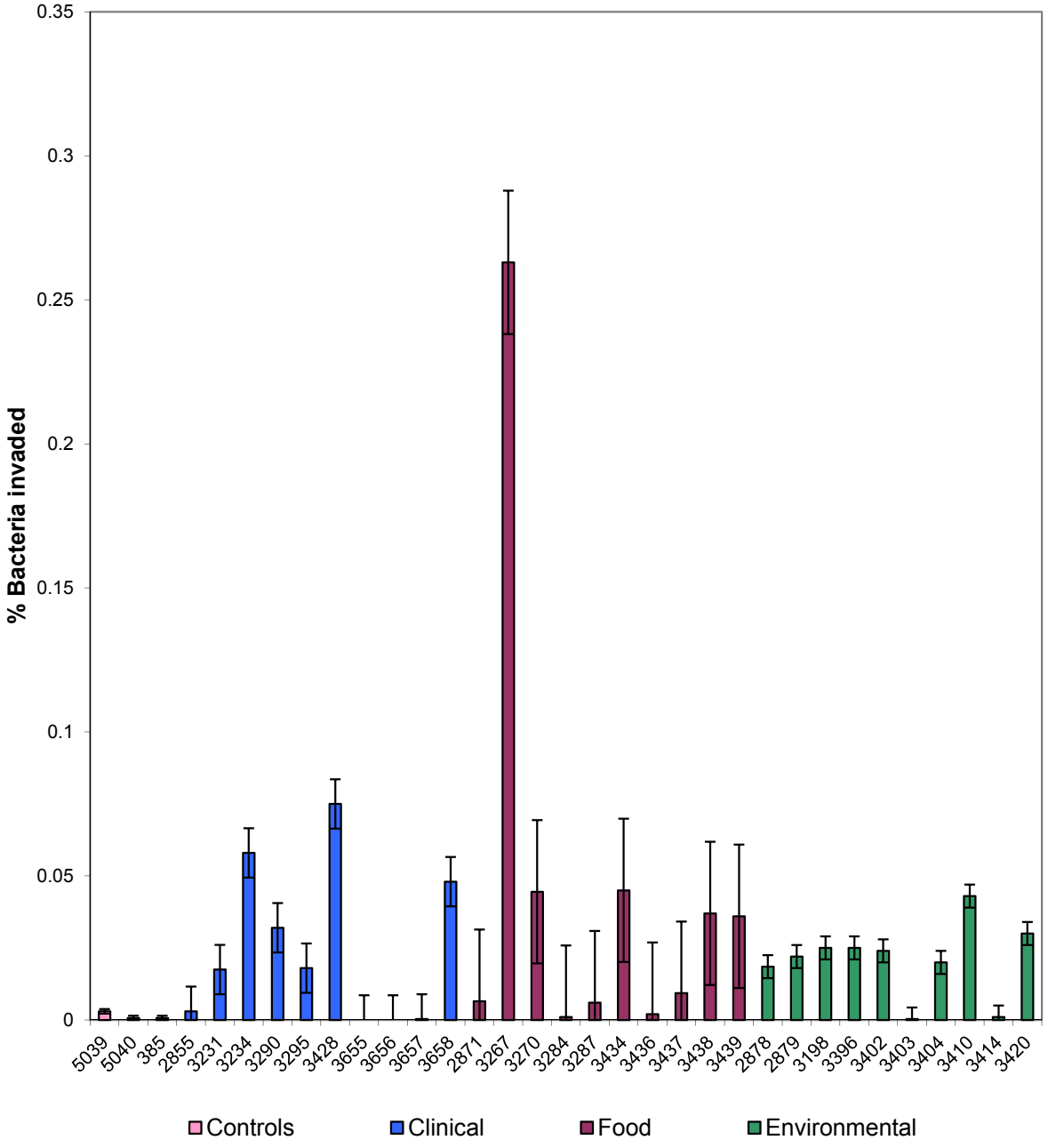
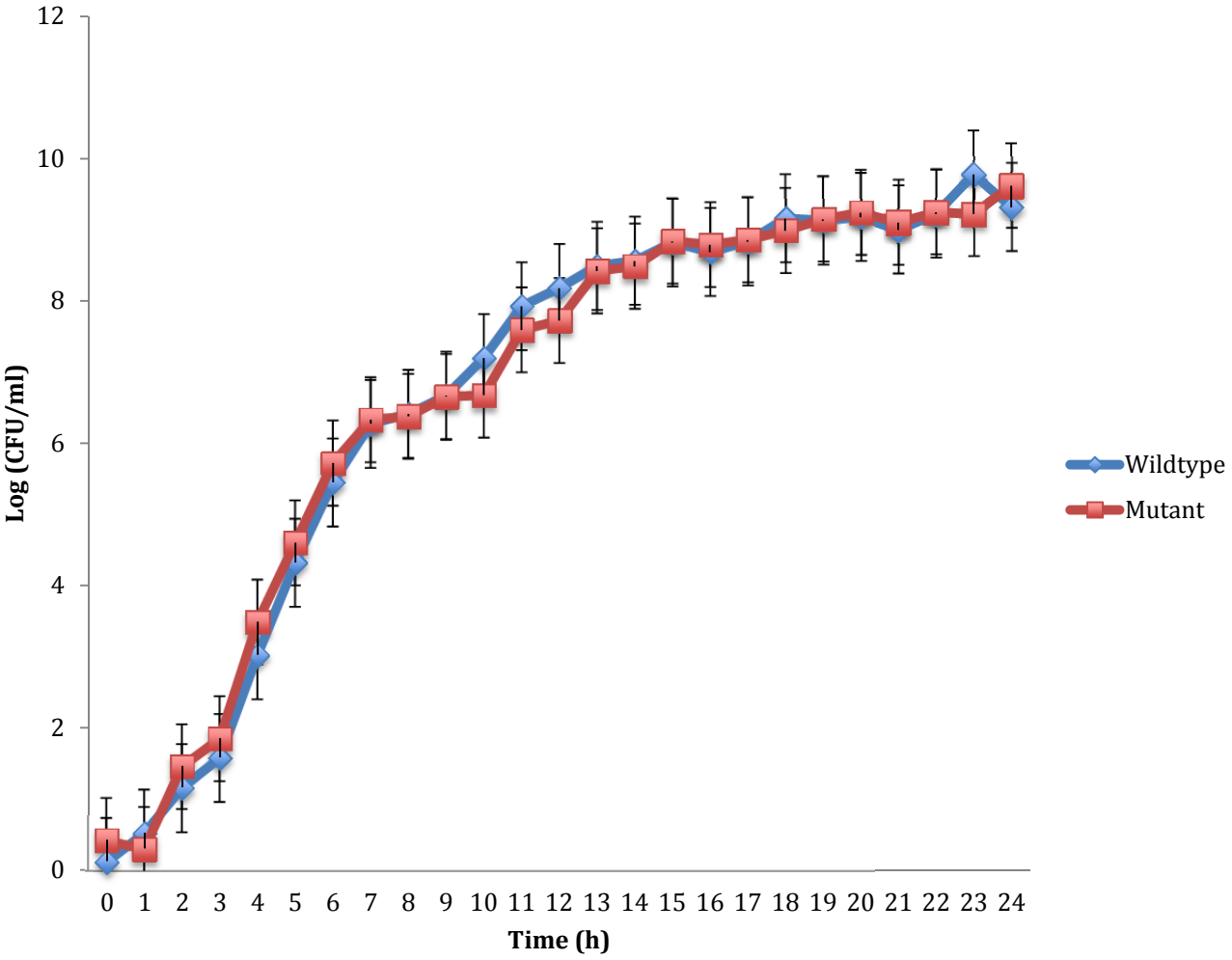

% Bacteria invaded
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
5039
5040
385
2855
3231
3234
3290
3295
3428
3655
3656
3657
3658
2871
3267
3270
3284
3287
3434
3436
3437
3438
3439
2878
2879
3198
3396
3402
3403
3404
3410
3414
3420
Controls
Clinical
Food
Environmental

Figure 8: Growth curve of *Cronobacter* strains of 3231, a clinical isolate, and Δ3231 at 37°C in LB for a period of 24 h


12
10
8
6
4
2
0
Log (CFU/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (h)
Wildtype
Mutant

DISCUSSION

The major clinical features associated with *Cronobacter* infection in neonates are meningitis, septicaemia and NEC. In general, *Cronobacter* infections are rare but quite severe, there is limited information available on the mechanisms that *Cronobacter* spp. use to adhere and/or invade host cells resulting in a variety of infections. In this study, the primary focus was to identify virulence factors associated with the pathogenesis of *Cronobacter* meningitis by characterizing genes required in adhesion and/or invasion of the BBB. The 30 different *Cronobacter* isolates were first quantified for their adhesive and invasive abilities to HBMEC. The next step was to develop a transposon mutant library for strains that demonstrated strong adhesive and/or invasive characteristics to HBMEC. The isogenic mutants were then screened through the standard adhesion and invasion assays to identify hypo or hyper adhesion/invasion mutants.

Attachment to host cells is an important first step in the pathogenesis of *Cronobacter* spp. Using a standard adhesion assay, the ability of 30 *Cronobacter* strains to adhere to HBMEC were quantified and compared to the non-invasive controls *E. coli* K-12 (5039), *Klebsiella pneumonia* (5040) and *Bacillus cereus* 385. The majority of the *Cronobacter* spp. tested adhered to HBMEC in greater amounts than the controls. One strain appeared to attach in much greater numbers than the controls. This strain, an environmental isolate 3404 isolated in Switzerland, was therefore one of the primary targets for developing a transposon mutant library. Using different statistical tests, overall there was no significant difference in the mean adherence to HBMEC between the clinical, food and environmental isolates ($p > 0.05$).

One of the fundamental prerequisites needed for bacterial meningitis is the ability to cross the BBB (47, 58, 65). One of the main hypotheses for the cause of *Cronobacter* meningitis is that once the organism is ingested, it will either adhere/or invade and transcytose across human intestinal epithelial cells. This will result in *Cronobacter* spp. having access to the blood stream and the central nervous system (CNS) (47, 58, 65). The standard invasion assay provided insight on the invasive difference in HBMEC among the clinical, environmental and food isolates.

Based on the exploratory statistical analysis of the data, descriptive statistics and graphs generated (Figures 6 and 7), one might conclude that the there appears to be a difference in invading HBMEC amongst the three different isolates in a descending order, with respective proportions of 10/10, environmental, 8/10, food, and 6/10, clinical isolates. However, based on the inferential statistic, we can conclude that there was no statistically significant difference ($p > 0.05$) in the ability to adhere and invade HBMEC between the three different sources of isolates. Strains 3404 and 3267 were significantly ($p \leq 0.05$) different from the remainder *Cronobacter* isolates in the adhesive and invasive assay, respectively. Clinical isolates are considered to be more virulent since they have successfully demonstrated that they can cause the disease in a host and yet certain strains lacked the ability to invade BBB cells. A possible explanation for the decreased invasive ability seen in clinical strains may be that these strains have become host adapted and require a key nutrient and/or co-factor, or that repeated passaging in the lab may have affected their ability to invade.

Strain 3267 and 3404 were the main strains used to develop a transposon mutant library but other strains included were 2855, 3231 and 3414. Transposon mutagenesis, using Ez

Tn5 <Kan-2>, done using electroporation, was successful for developing mutants for all strains. The isogenic mutants were screened using both the standard adhesion and invasion assay. Only one mutant from the hundreds screened lost the ability to adhere to HBMEC, which was referred to as Δ3231. The original strain, 3231, was obtained from the cerebrospinal fluid (CSF) of a patient in Netherlands. Genomic sequencing, conducted by the Genome Quebec Innovation Center (Montreal), identified the transposon insertion site. Using BLAST, the insertion site of the transposon was identified as diguanylate cyclase (*DGC*).

Diguanylate cyclase (DGC) and phosphodiesterases (PDEs) are enzymes known to control the levels of cyclic dinucleotide-GMP (c-di-GMP) in many organisms (5, 13, 15, 28, 77, 82). DGC contains an amino acid motif GGDEF domain and PDEs contain an EAL domain. DGC is responsible for synthesizing c-di-GMP, whereas PDEs role is to degrade it (5, 13, 15, 28, 77, 82). There are many other derivatives of DGC and PDE's and all are considered to be part of the GGDEF and EAL family, and play an important role in the synthesis and degradation of c-di-GMP. An organism can have multiple GGDEF and EAL domain proteins. C-di-GMP is well known for being a second messenger for many Gram-negative bacteria stimulating many cellular functions such as motility, virulence, cell cycle progression and biofilm formation (5, 13, 15, 28, 77, 82). The levels of c-di-GMP can be influenced by environmental and cellular signals that are not quite fully understood. At present, it is known that high levels of c-di-GMP can promote the biosynthesis of adhesins and expolysaccharide matrix substances in biofilms, which has been shown in many Gram-negative bacteria such as *Pseudomonas* spp.,

Salmonella spp., and *Vibrio cholerae.* Low concentrations of c-di-GMP can favour planktonic lifestyles such as motility and virulence (5, 13, 15, 28, 77, 82).

The *dgc* gene was disrupted by the transposon, allowing for questions regarding its role in *Cronobacter* spp. to be put forth. Based on previous work on the family of DGCs, Kim *et al.* (44) demonstrated that *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* depend on these GGDEF and EAL domain proteins for biofilm formation. They managed to mutate and/or overexpress many of these genes, which resulted in either the inhibition or promotion of biofilm formation (44). *Pseudomonas aeruginosa* and *V. cholerae* genome sequences contained a total of 34 and 61 GGDEF and EAL domain proteins, respectively, that function as DCGs and PDEs (15, 28). An overexpression of one of the GGDEF domain proteins in *V. cholerae* increased the level of c-di-GMP, which resulted in the increased expression of *vpsA-Q* genes, which encodes for *V. cholerae* expolysaccharide. Microarray analysis of *V. cholerae* identified two genes within the tcpPH operon known as *acgA* and *acgB*, which encode for proteins containing EAL and GGDEF domains, respectively (15, 28). An overexpression of AcgA reduced biofilm formation in *V. cholerae* and promoted motility. When *acgB* was overexpressed, an increase in biofilm formation was observed and motility was inhibited. However, when either of these genes was deleted, motility and biofilm formation appeared not to be affected (15, 28).

In this study, the adhesive and invasive abilities of the collective clinical, environmental and food isolates tested were not significantly different from each other. However, based on the data presented in Figure 6 and 7, differences were observed and individual strains did stand out. Environmental isolates demonstrated both the ability to adhere and invade HBMEC and, interestingly, 4 of the clinical isolates could not invade HBMEC. Strains

3404 and 3267 were either dominant in the adhesion or invasion assay and transposon mutant library generation was successful for both strains. Unfortunately, to-date, no isogenic mutant has shown a significant increase or decrease in adhesion to and invasion of HBMEC from the original strains. However, one isogenic mutant of strain 3231 lost the ability to adhere to HBMEC, and the insertion site of the transposon mapped to the *dgc* gene was disrupted. The role DGC in strain Δ3231 raises a lot of questions regarding c-di-GMP and its role in the pathogenesis of *Cronobacter* spp. Currently, DGC has been shown in many Gram-negative bacteria to synthesize c-di-GMP, considered to be a central regulator of biofilm formation and virulence depending on its expression levels within the cell. Further work is required to understand what role, if any, c-di-GMP, plays in the pathogenesis of *Cronobacter* spp.

Chapter IV:

Biofilm Study

INTRODUCTION

The identification of the transposon site, diguanylate cylcase (DGC), led us to investigate biofilm phenomenon in *Cronobacter* spp. Biofilms are a group of bacterial cells that attach on abiotic and biotic surface and form an exopolysaccharide that surrounds the group of bacteria (14). Once a biofilm is formed, bacteria may be released in the environment in their planktonic form, where it may form further biofilms. Most bacteria are found naturally in the environment in biofilms, because this is a survival strategy that protects them from harsh conditions such as heat, desiccation, detergents, acidic conditions and antibiotics (4, 14, 15, 25, 36, 39, 40, 48, 49). However, when pathogenic bacteria produce biofilms in medical devices, industrial water systems, and food-processing facilities, then it can facilitate transmission to the host and becomes a major concern to the public (14, 15, 25, 36, 39, 40, 48, 49). Many foodborne pathogens have the ability to form biofilms in equipment used to handle and/or prepare food and these biofilms have proven to be a major source of food contamination.

Cronobacter spp. have been shown to attach and form biofilms on abiotic surfaces (4, 14, 15, 25, 36, 39, 40, 48, 49). Abiotic surfaces have been used to demonstrate the ability of *Cronobacter* to form biofilms on materials commonly associated with PIF-feeding equipment and surfaces and include glass, stainless steel, polyvinyl chloride (PVC), polycarbonate, silicone, and enteral feeding tubes (4, 14, 15, 25, 36, 39, 40, 48, 49). The characterization of biofilm formation in *Cronobacter* is a major interest and new findings could provide a better understanding of the mechanism and conditions required for biofilm formation in food facilities.

There are three main areas considered important in addressing the role of biofilms in the pathogenesis of *Cronobacter* spp.: (1) understanding the physiology and genetic basis of biofilms (i.e., identifying key genes); (2) determining whether biofilm formation is a virulence factor for *Cronobacter*; and (3) evaluating and determining treatments that are effective in reducing biofilm formation (4, 15, 25, 36, 39, 40, 48). Amalaradjou & Venkitanaravanan (4) have recently shown that trans-cinnamaldehyde, an ingredient in cinnamon oil, had the ability to inhibit and inactivate *C. sakazakii* biofilm formation in the presence or absence of PIF on polystyrene plates, stainless steel coupons, feeding bottle coupons and enteral feeding tube coupons at both 12 and 24 ˚C. Hartmann *et al.* (25) screened a library of mutants of strain ES5 (a clinical isolate) using the crystal violet (CV) microtiter assay, and found four mutants defective in the cellulose biosynthesis, three in flagellar structure, three in basic functions (such as cell division, energy metabolism and acid fermentation), one virulence and four unknown functions (25). Of the 14 mutants, two hypothetical proteins (ESA_00281 and ESA_00282) were found to be important in biofilm formation structure using confocal laser scanning microscopy (25).

The aim of this part of the study was to first demonstrate whether DGC plays a role in the ability of *Cronobacter* spp. to produce biofilms by showing that there is a difference in biofilm production between strain 3231 and one of its isogenic mutants, Δ3231. The next objective was to determine whether there are difference in biofilm production between the clinical, food and environmental isolates. The last object was to further characterize genes that are necessary for biofilm formation by developing a transposon mutant library and screening them through the microtiter plate biofilm assay.

The microtiter plate biofilm assay, a 96-well plate assay, is a simple, low cost commonly used method to study and quantify biofilms using the crystal violet (CV) dye (56). Even though this assay does not allow for the formation of mature biofilms, typically seen in continuous flow methods, the high throughput nature of the assay allows for useful genetic screens and testing biofilm formation for multiple strains at a time under various growth conditions. This method has been established as a standard in investigating important factors involved in biofilm development, such as flagella, pili, adhesins, and various enzymes (56). This protocol assisted us in determining the optimal growth conditions for *Cronobacter* biofilms and provided insights in to biofilm formation of the clinical, food and environmental isolates. The strains that showed a greater ability to form biofilms were used to develop a transposon mutant library, which was screened through the microtiter plate biofilm assay, and assisted us in characterizing genes required in the early stages of biofilm formation.

The 96-well plate assay is a good semi quantitative method assessing biofilm formation but it is not sufficient to confirm biofilm production and structure. This can be done using a scanning electron microscope (SEM) (38). The *Cronobacter* strains that showed good biofilm production were grown on a minimum biofilm eradicated concentration (MBEC) device, which contains a flat bottom 96-well plate with a lid that contains polystyrene pegs. The MBEC device, formerly known as the Calgary Biofilm device, was designed to provide optimal conditions for biofilm production, where, under the appropriate growth conditions, the biofilm is formed on the bottom of the peg. The pegs are then broken off the lid and can be fixed, sputter-coated and viewed under SEM (24).

MATERIALS AND METHODS

Media and Growth Conditions

The 33 different isolates of *Cronobacter* spp. assessed in the biofilm assay were part of the strain collection of the Bureau of Microbial Hazards of Health Canada and comprised clinical, food and environmental isolates. Strains were stored at - 80°C in TSB containing 50% of glycerol. The isolates were streaked onto TSA plates and incubated at 37°C overnight. A colony from each plate was inoculated in 10 mL of LB and incubated at 37°C overnight.

CV Microtiter Biofilm Assay

Quantification of biofilms grown in 96-well microtiter dishes (Corning, Canada) was performed by first diluting the overnight culture (concentration of 10^8) in a 1:10 ratio in peptone water and vortexing. The samples were further diluted in a ratio of 1:10 dilution in AB minimal media (2 g/L $(NH_4)_2SO_4$, 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3 μM $FeCl_2 \bullet 6H_2O$) supplemented with 0.4% maltose, and then vortexed. One hundred μL of diluted culture was pipetted into 6 wells and incubated at 37°C for 45 h. The plates were then rinsed twice in water to remove planktonic bacteria. The wells were then treated with 125 μL of 0.1% CV solution for 30 min at room temperature. The CV solution was then removed and rinsed in water twice to remove excessive dye and plates were left to air-dry overnight. Once the plates were dried, 200 μL of DMSO was added to each well and incubated for 15 min at room temperature. The DMSO/CV solution in each well was mixed and 125 μL of the solution was transferred into another separate well in an optically clear flat-bottom 96-well plate for absorbance reading. The

absorbance at 570 nm was measured for each well using a Tecan microplate reader (model Sunrise Basic, Austria).

Construction of the Transposon Mutant Library

A library of random transposon mutants of *Cronobacter* strains 2855, 3231, 3267, 3414 and 3658 was constructed using an EZ-Tn5 <Kan-2> Tnp Transposome kit (Interscience, Markham, Canada) as described in Chapter II. Confirmation and visualization of the insertion of the transposon was done using PCR and QIAxcel Biocalculator (Version 3), respectively. A growth curve of the mutant of interest and its corresponding strain was conducted to show that the growth rate did not account for any observed differences in biofilm production.

The Minimum Biofilm Eradication Concentration (MBEC) Assay

A colony from the strain of interest was streaked onto a TSA plate, and incubated at 37°C overnight. A second sub-culture was plated onto another TSA plate and incubated at 37°C overnight. Each colony was inoculated in 10 mL of AB minimal media supplemented with 0.4% maltose and further diluted in a 1:30 dilution of the same media. They were then poured into a reagent reservoir and 150 µL of each sample was placed in each row of the MBEC device (Innovtec Inc., Edmonton). The peg lid was placed onto the microtiter plate. The MBEC device was sealed with parafilm and placed in an incubator at 37°C and shaken at 120 rpm for 24 and 45 h. Once the incubation period was completed, a 96 well plate was set up with 100 µl of 0.9% saline and the pegs were removed from the lid with a pair of flamed pliers and placed into a well for 1 min. The pegs were then fixed in 2.5% gluaraldehyde for 1 h at room temperature.

Scanning Electron Microscopy (SEM)

Pegs were fixed with 2.5% glutaraldehyde and placed in a glass vial, under a fume hood. They were rinsed with 0.2 M cacodylate buffer. The buffer was then aspirated and enough osmium was placed in to the vials to cover the pegs and was left then under the hood at room temperature for 1 h. The osmium was removed and the pegs were once again rinsed with 0.2 M cacodylate buffer and then replaced with different concentration of ethanol in the order 70, 90 and 2 times 100 % ethanol in order; 15, 20 and 30 min of incubation, respectively, was used for each step. After the last 100% ethanol wash, hexadimethydisalizone (HDMS) was added and incubated for 10 min in a fume hood. The HDMS was poured in to a petri dish to evaporate and the samples were placed on a plate with filter paper and lid. The samples were then placed in a vacuum oven set at maximum and incubated overnight. The following day the pegs were sputter-coated with 30 nm platinum and viewed under a scanning electron microscope (LE2100; Vickers Nanolab, Ottawa, ON, Canada) operating at an accelerating voltage of 15 kV.

DNA Extraction and Direct Genomic Sequencing

The extraction of the genomic DNA of M17 was conducted using the commercial Wizard Genomic DNA purification kit (Promega, Madison, WI). The extracted DNA was further purified by the microcon centrifugal filter Ultracel YM-30 (Millipore) kit and the amount of DNA was measured using the NanoDrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific). The extracted DNA was further concentrated by the Vacufuge (Eppendorf, Germany) and re-suspended in 20 μL of water. The chromosomal DNA was

then subjected to direct sequencing using the BigDye Terminator (BDT) cycle sequencing kit (PE Biosystems, Foster City CA).

Sequencing primers were Kan-2 FP-1 (5' ACCTACAACAAAGCTCTCATCAACC3') and Kan-2 RP-1 (5' GCAATGTAACATCAGAGATTTTGAG 3') and diluted to 3 uM. Five microliters of DNA was added to 15 µl (2x) reactions containing 2 µl of dye terminator premix and were centrifuged. Sequencing reactions were cycled according to the following program: 1 min at 96°C and 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C. The reaction products were then washed with 70% ethanol and re-suspended in 20 µl of Template Supression Reagent (PE Biosystems, Foster City, CA). The samples were denatured at 95°C for 5 min and electrophoresed in a Genetic Analyser (Version 3130) with version 5.4 Sequence Analysis software. The transposon insertion site was identified using the Bio-edit Sequence alignment Editor version 7.0.9.0 and the BLAST program maintained at the NCBI web site of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical Methods

After testing for normality and equality of variances, a one-way ANOVA overall F-test was used to determine if the three biofilm measurement means between the clinical, environmental and food isolates were significantly different.

RESULTS

Biofilm formation of Cronobacter spp.

Optimization of the growth conditions of *Cronobacter* spp. for biofilm production was most successful when the organism was grown in AB minimum media supplemented with 0.4% maltose at 37°C for 45 h. Incubation periods exceeding 45 h were inconsistent within trials and could not be reproducible. Figure 9 summarizes the 30 different *Cronobacter* spp. as well as strains Δ3231, 5159 (ATCC 29554) and 5723 (clinical isolate). The strains that showed the greatest biofilm production were 3287 (food isolate), 3414 (environmental isolate) and 3658 (clinical isolate). A significant difference in biofilm growth was observed between 3231 (environmental isolate) and its isogenic mutant, Δ3231.

Screening for Cronobacter Biofilm Defective Mutants

Strains 3287 (F), 3414 (E) and 3658 (C) showed the greatest potential for biofilm formation and were the strains targeted for transposon mutagenesis (Figure 9). The transposome <Kan-2> was introduced into *Cronobacter* by electroporation and was screened by growth on TSA + Kan plates. Strain 3287 produced a total of 167 mutants, all screened using the CV microtiter assay. A number of mutants showed a non-significant decrease in biofilm formation, with, strain M17 showing a significant decrease (approximately 85% reduction) in biofilm formation (Figure 10). A 24 h growth was conducted for both strains 3287 and M17 in LB at 37°C to demonstrate that the difference in biofilm formation observed between the two strains was not due to growth rate. There was no significant difference between the two growth curves (Figure 11).

Isogenic mutants for strains 3414 and 3658 were also successfully developed and screened using the CV microtiter assay. Preliminary work on strain 3414, led to potential mutants (M10 and M15) from a total of 93 that showed a two-fold increase in biofilm production, as compared to the wild type strain (Appendix C). The current screening of 3658 mutants has not been successful in identifying an isogenic mutant that differs in biofilm production from the parent strain.

The SEM images obtained for the fixed and sputter-coated pegs of 3231, Δ 3231, 3287 and 3414 were visualized at 5000 × magnification (Figure 12). Strains 3231, Δ 3231 and 3414 were grown for 45 h, whereas, strain 3287 was grown for 24 h. The biofilms formed for strain 3231 were at the mature stage since the bacterial cells were all clustered and enveloped in extracellular polymeric substances (EPS). The "pocket holes" observed were probably due to a loss of water retention and this may have been due to the ethanol washes which were omitted. This mature biofilm formation was also observed for strain 3231 in other independent experiments. The isogenic mutant of 3231 appeared to have lost the ability to produce biofilms. In Figure 12 (b), single cells are seen to adhere to the pegs and in other replicate experiments, no bacterial cells were observed. Strain 3287 was analyzed after 24 h and showed the first few stages of biofilm formation. The bacterial cells were aggregated and the EPS structure was being produced. Preliminary work with strain 3414 showed a very different type of crystal-like structure that was seen all over the peg and it is unclear whether these crystal-like structures represent a biofilm.

Figure 9: Quantification of biofilm formation of 30 different *Cronobacter* isolates, as well as Δ 3231, 5159 (ATCC 29554) and 5723 (clinical isolate), based on CV staining of cells adherent to 96-well plates after 45 h of growth in AB minimum media supplemented with 0.4% maltose . The values are means from at least three independent experiments with 6 replicates in each experiment. The error bars indicate standard error.

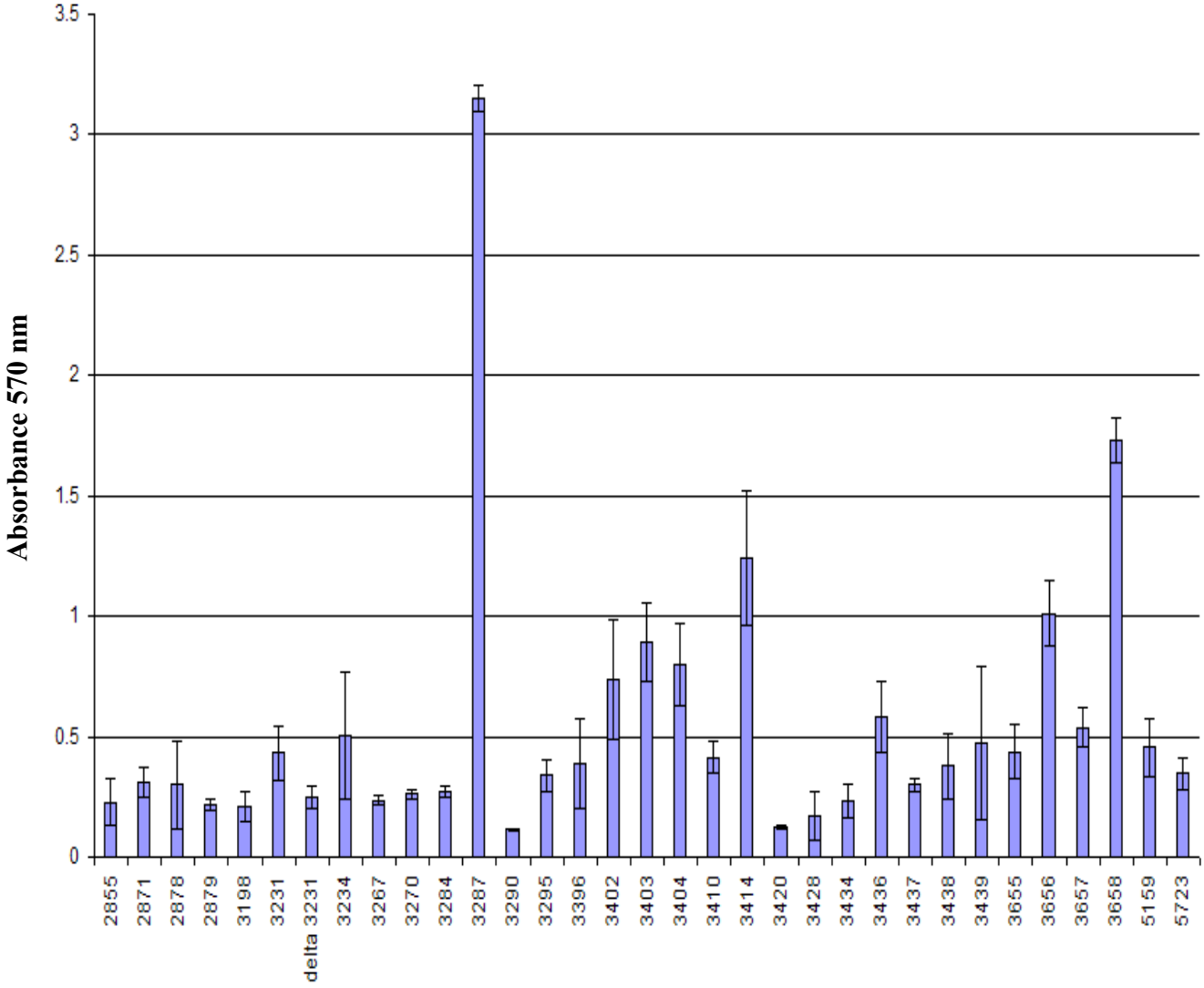

Absorbance 570 nm
3.5
3
2.5
2
1.5
1
0.5
0
2855
2871
2878
2879
3198
3231
delta 3231
3234
3267
3270
3284
3287
3290
3295
3396
3402
3403
3404
3410
3414
3420
3428
3434
3436
3437
3438
3439
3655
3656
3657
3658
5159
5723

Figure 10: Quantification of biofilm of 3287 and its corresponding mutants based on CV staining of cells adherent to 96-well plates after 45 h of growth in AB minimum media supplemented with 0.4% maltose. The values are means from at least three independent experiments with 6 replicates in each experiment. The error bars indicate standard error.

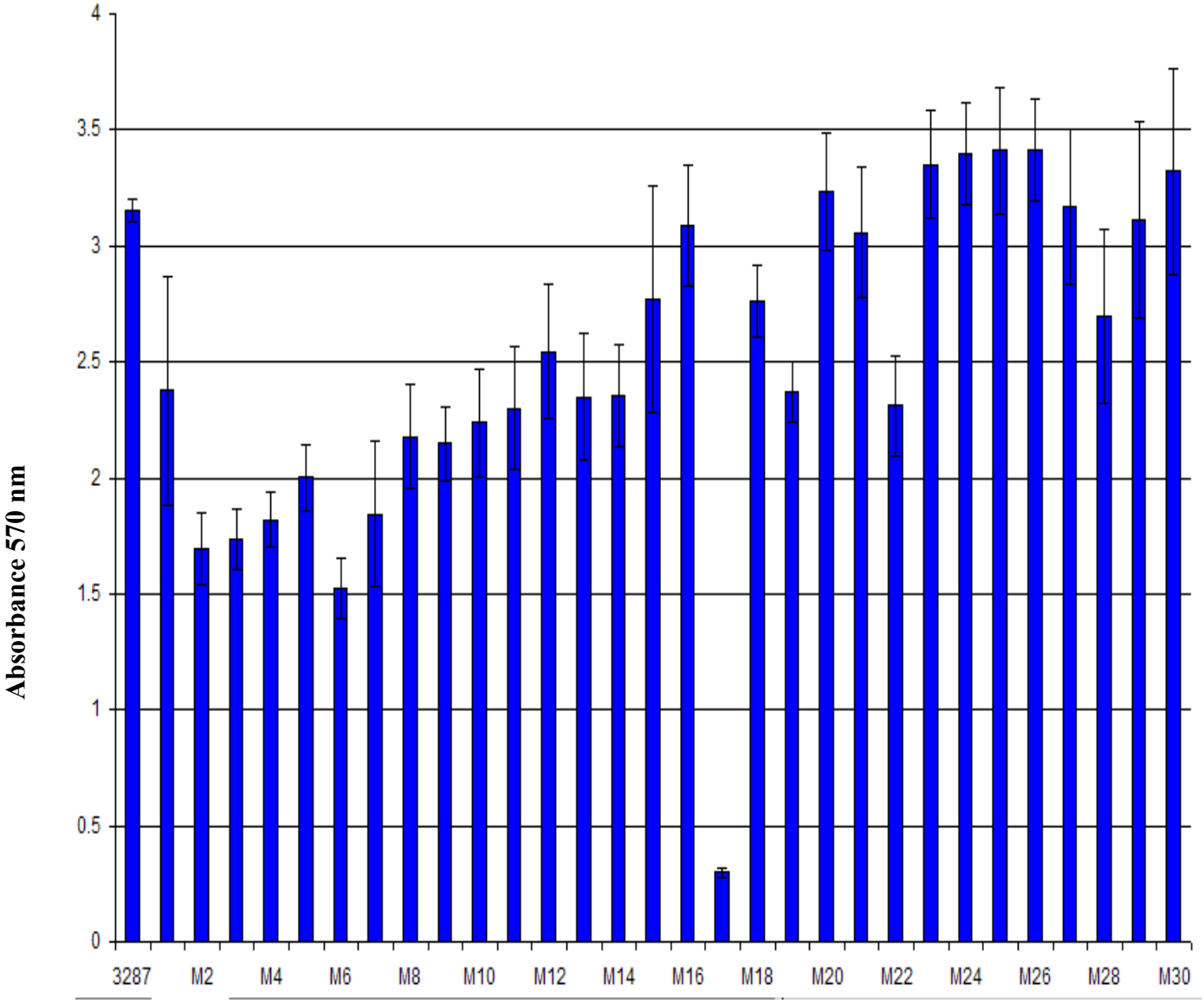

Absorbance 570 nm
4
3.5
3
2.5
2
1.5
1
0.5
0
3287
M2
M4
M6
M8
M10
M12
M14
M16
M18
M20
M22
M24
M26
M28
M30

Figure 11: Growth curve of *Cronobacter* strains of 3287 and M17 at 37°C in LB for a period of 24 h

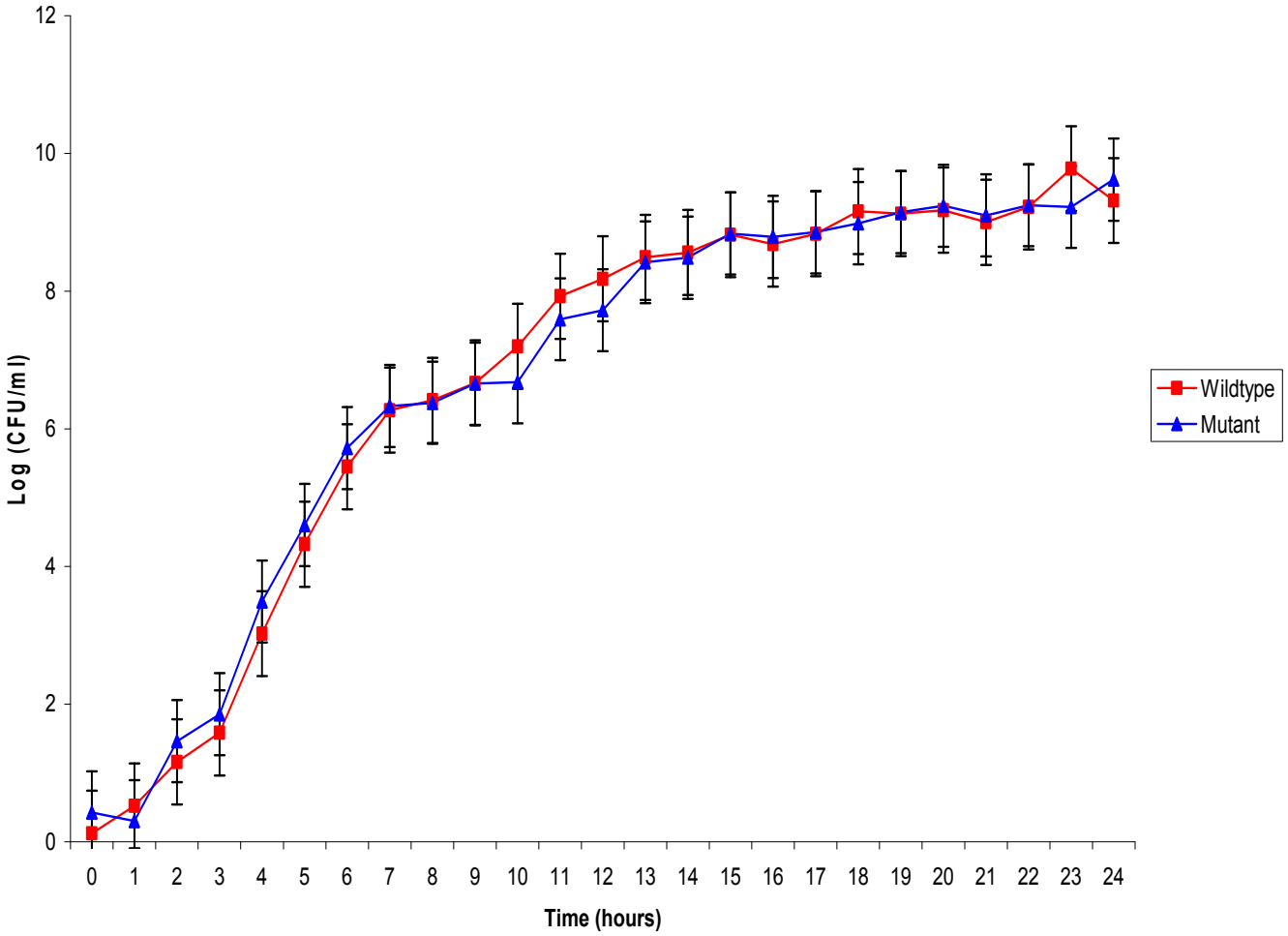
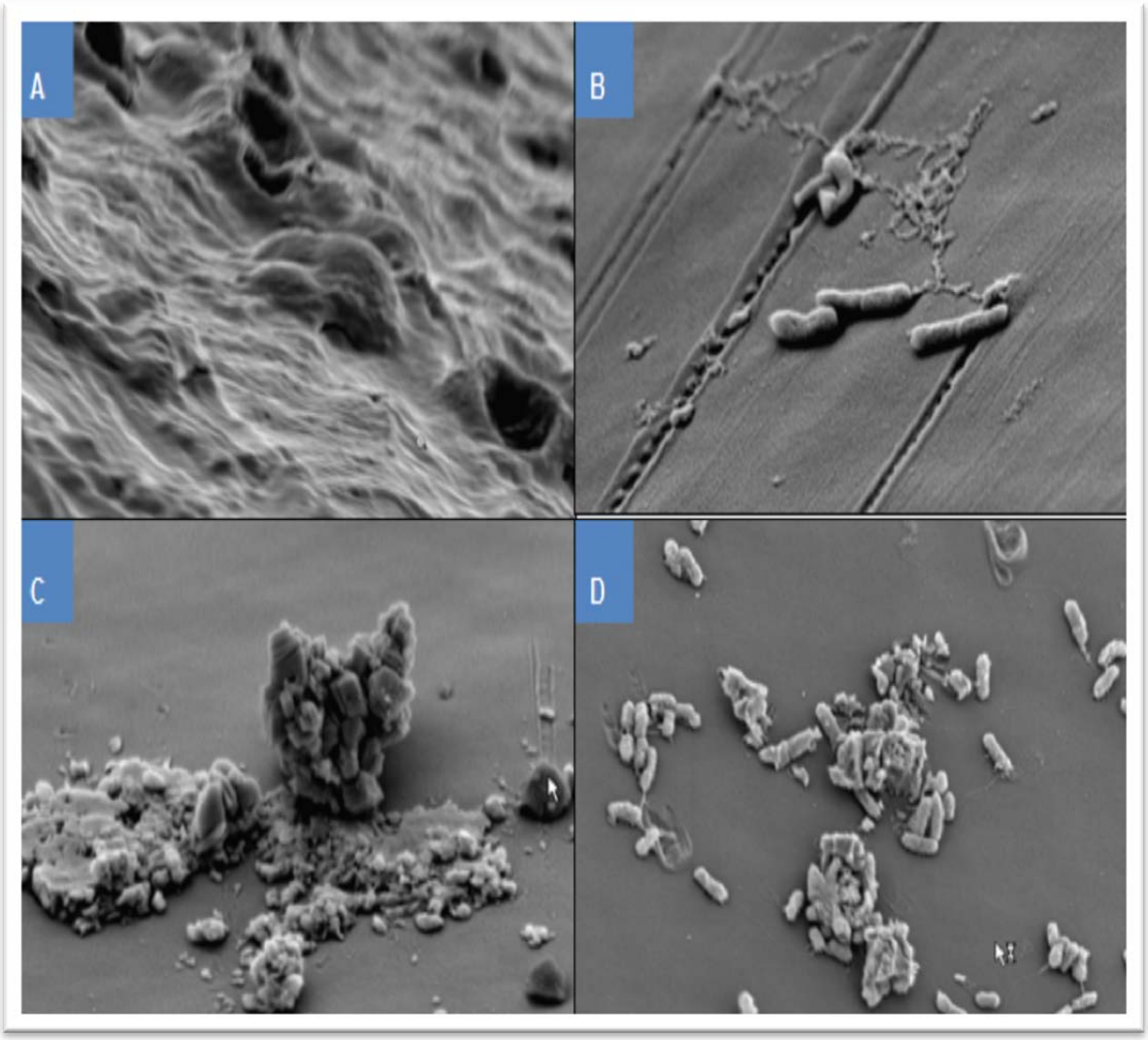

12
10
8
6
4
2
0
Log (CFU/ml)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Time (hours)
Wildtype
Mutant

Figure 12: Biofilm formation on MBEC pegs among the various strains of *Cronobacter* spp. at 37°C in AB minimum media supplemented with 0.4% maltose; (A) 3231 (environmental isolate) at 45 h, (B) Δ3231 at 45 h, (C) 3414 (environmental isolate) at 45 h and (D) 3287 (food isolate) at 24 h. Images were captured at 5000 × magnification .


A
B
C
D

Identification of the Transposon Insertion Site

The confirmation for seeing if the transposon was successfully inserted in the genome was done by running a PCR and visualizing the transposon band using a Qiaxcel. The direct genome sequence approach that was used to identify the transposon insertion site was successful. Blast homology analysis of the genomic sequence data generated from the M17 transposition clone was compared to the complete genome of *C. sakazakii* BAA-894 and identified the insertion site to be threonine synthase with 82% identity.

Statistical Analysis

The result of the overall F statistic revealed no significance between the biofilm measurement means between the clinical, environmental and food isolates, with a p value > 0.05.

DISCUSSION

Biofilms continue to be a major issue for the medical and food industries as they can be resistant to disinfection and/or sanitisation and are an ideal way for transmission of diseases to the public. Understanding the factors that influence biofilm formation could assist in preventing and controlling the spread of infection. In the second part of our study (Chapter III), the identification of the DGC gene in Δ3231, an isogenic mutant that lost the ability to adhere to HBMEC, led to the investigation of the ability of *Cronobacter* spp. ability to produce biofilms. This study was divided into three parts: (i) to determine whether the isogenic mutant Δ3231 had reduce or lost the ability to produce biofilms, (ii) whether there is a difference in the amount of biofilm produced between clinical,

environmental and food isolates, and (iii) to identify gene or genes that are required for biofilm formation in *Cronobacter* spp.

The first approach used to investigate the ability of *Cronobacter* spp. to form biofilms was the CV microtiter assay. Although the CV microtiter assay on its own may not provide sufficient evidence of the occurrence of biofilm formation, it is inexpensive, relatively quick, and currently the standard assay for testing multiple strains. It is also well complemented with SEM analysis. The required conditions for successful biofilm formation in *Cronobacter* spp. were to grow the strains in AB minimum media supplemented with 0.4% maltose in 96-well bottom plates at 37°C for 45 h. The results showed that the 3 strains with the greatest ability to form biofilm were strains 3287, 3414 and 3658. These three strains were thus chosen for developing transposon mutant libraries and were screened using a CV microtiter assay. Although strain 3231 was not one of the stronger formers of biofilms a significant decrease in biofilm production was observed in Δ3231. A one-way ANOVA test was conducted to assess whether there were differences in the mean of biofilm formation in the clinical, environmental and food isolates, and no significant difference were at the 5% significance level. However, on an individual strain level, strain 3287 was statistically higher compared to all strains.

The MBEC assay was used to grow biofilms on the pegs attached to the lid of the MBEC plates. The pegs were fixed and sputter-coated before being analyzed by SEM. Due to the lengthy process of SEM analysis and the availability of the facilities, only a few pegs could be analyzed. SEM images visualized at 5000 × magnification for strain 3231 and Δ3231 demonstrated significant biofilm difference, which was not observed with the CV microtiter assay. Strain 3231, an environmental isolate, showed mature biofilm formation

after 45 h, whereas with Δ3231 we observed either no signs of bacteria or a couple of planktonic cells attached to the peg. This result provides good evidence (and strengthens others studies on Gram-negative bacteria) that DGC plays some role in biofilm formation. The primary role of DGC is to produce c-di-GMP, a secondary messenger responsible for regulating motility, virulence and biofilm in bacteria (5, 13, 15, 25). Many Gram-negative bacteria, such as *Pseudomonas* spp., *Salmonella* Typhimurium and *Vibrio* spp. rely on c-di-GMP for activating biofilm formation (5, 13, 15, 25). Further experiments such as genetic complementation may further assist in confirming the role of DGC in biofilm formation in *Cronobacter* spp. The use of SEM as a supplemental test to the CV microtiter assay was found to be useful because of the visualization of the biofilm structure.

The food isolate 3287 was significantly higher than all other *Cronobacter* isolates tested in biofilm formation when using the CV microtiter assay; SEM images showed the early stages of biofilm formation. Isolate 3287, (Figure 12), which was only incubated at 37°C for 24 h showed a remarkable difference in stages of biofilm when compared to strain 3231. Strain HPB3287 was also examined at 45 h but due to time restrictions and SEM availabilities, we were unable to capture a SEM picture. This strain was used for developing a transposon mutant library. A total of 167 mutants were obtained and screened using the CV microtiter assay. One mutant showed significant reduction in the ability to produce biofilm formation and using direct genomic sequence, the insertion site of the transposon was identified. The conventional method involving DNA extraction, cloning, transformation and then sequencing, was not successful in identifying the insertion site. This may have been due to the ligation products being too big to

electroporate into *E. coli* cells. Only one study to-date has been able successful in producing mutants for *Cronobacter* isolate ES5 (25). Discussions with Dr. Roger Stephen confirmed the difficult nature in the use and recovery of transposon mutagenesis in this organism (R. Stephen, personal communication). Direct genomic sequencing, consisting of isolating the transposition clones from genomic DNA and directly sequencing with Kan-2 FP-1 and Kan-2 RP-1 primers that are specific for the ends of the transposon was used (28, 74). The insertion site of the transposon was the identified by homology to known genes or sequences using BLAST. The insertion site of the gene disrupted by the transposon in M17 was identified as threonine synthase (TS).

Threonine synthase is an enzyme that catalyzes the final step in the biosynthesis pathway in which $_{L}$-homoserine phosphate and water are converted into inorganic phosphate and threonine (16, 62). Not much is known about the role of threonine synthase in bacteria, other than it does produce threonine when required. No work has been done on demonstrating the potential involvement of threonine synthase in biofilm formation. However, there have been several studies showing the importance of the concentration of inorganic phosphate, one of the products of threonine synthase, in biofilm formation (60, 67). In *Pseudomonas aeurofaciens* and *fluorescens,* the limitation of inorganic phosphate has an impact of the level of c-di-GMP. It appears that inorganic phosphate is an important extracellular signal to Pho regulon, phosphodiesterase, known as RapA, is activated and breaks down c-di-GMP (60, 67). RapA appears to be an inhibitor of biofilm formation as it lowers the level of c-di-GMP, which in turns inhibits the secretion of LapA, a large adhesion protein that plays a role in keeping bacteria attached to a surface or to other cells (60, 67). A possible role for threonine synthase is that it may be an

important source of inorganic phosphate for the cell. Disruption of the *thrS* gene demonstrated that the level of inorganic phosphate was limited, causing activation of the RapA protein, and resulting in the inhibition of biofilm formation (60, 67). This later hypothesis is solely based on the results obtained for strain M17, and would require further experiments to demonstrate the association between threonine synthase and c-di-GMP.

HPB strains 3414 and 3658 were also good biofilm producers based on the CV microtiter assay. Transposon mutagenesis was successful for both strains. Preliminary work on strain 3414 identified two potential mutants that have a significantly increased ability to produce biofilms. Preliminary SEM analysis of strain 3414 showed some type of crystal like structure that may or may not be related to biofilms. It will be interesting to compare the genes disrupted to further progress in the understanding of the physiology of *Cronobacter* biofilms.

In conclusion, this study demonstrated that both DGC and TS appear to be important factors that may contribute to the ability of *Cronobacter* spp. to produce biofilms. The interruption of the *dgc* gene demonstrated that strain Δ3231 could not produce biofilms under optimal biofilm conditions, whereas strain 3231 produced mature biofilms under the same condition (Figure 12 a). If high levels of c-di-GMP inhibit a planktonic lifestyle and promote biofilm production, then TS may influence indirectly the level of c-di-GMP in *Cronobacter* spp. Further investigations are required to determine the relation between TS and DGC and investigate their roles in both biofilm formation and virulence, which could possibly shed light on the pathogenicity of *Cronobacter* spp.

Chapter V:

Conclusion

Progress continues to be made on the biology, ecology and pathogenicity of *Cronobacter* spp., formerly known as *Enterobacter sakazakii*. This Gram-negative organism has been recently reclassified into its own genus and currently encompasses a total of 7 species. To-date, the major vehicle associated with *Cronobacter* outbreaks or sporadic cases has been contaminated PIF. The main clinical features are NEC, sepsis and meningitis. The lethality of these clinical manifestations from the period of 2000 to 2007, which included a total of 150 neonatal cases of *Cronobacter* infections, for necrotizing enterocolitis, sepsis, and meningitis, were 19%, < 10% and 41.9%, respectively (22). Although *Cronobacter* infections are quite rare, during the period between 2000 and 2008, in the US, the incidences of *Cronobacter* infections were the following: 1 per 100 000 in infants, 8.7 per 100 000 in low-birth weights and 1 per 10 660 in very low-birth weight neonates (22). Though much of the focus *Cronobacter* infections has been on neonates, these organisms also have the ability to infect individuals of all ages. *Cronobacter* spp. seem to have the biggest impact on immunocompromised individuals. The mechanism(s) by which *Cronobacter* species cause infection still remains a mystery. The objectives of the thesis were to identify and characterize potential virulent factors associated with pathogenicity of this organism. The summary of the findings from this work is presented in Table 4.

Table 4: A summary of selected *Cronobacter* isolates ability to produce enterotoxin, adhered to and invade HBMEC and form biofilms

Strains	Enterotoxin production (Vero cell assay)	Adhesive properties (HBMEC)	Invasive properties (HBMEC)	Biofilm Formation	
				MBEC	CV
2855 (C)	+	+/-	-	n/a	-
3231 (C)	-	+	+/-	yes	+
3267 (F)	++	+	+++	n/a	+/-
3287 (F)	+/-	+	-	yes	+++
3404 (E)	+	+++	-	n/a	+
3414 (E)	+	+	+	yes	++
3658 (C)	-	++	+	n/a	++
M17 (Δ 3287)	n/a	n/a	n/a	n/a	+/-
5159 (ATCC 29544)	+	n/a	n/a	n/a	+
5723 (C)	+/-	n/a	n/a	n/a	+

(C) clinical, (F) food, and (E) environmental isolates
+ compared to the negative control
+/- very close to the negative control
+, ++, +++ positive
- negative
n/a, yet to be conducted

Pagotto et *al*. (71) were the first to investigate virulence factors of this organism. They succeeded in demonstrating enterotoxin production in certain *Cronobacter* isolates using the suckling mice assay. The first part of the thesis was to expand on that study by investigating the ability of clinical, food and environmental isolates to produce an enterotoxin in *in vitro* conditions using three different types of cell lines. The standard *in vitro* enterotoxin assay using the Vero cell line was used to investigate the cytopathic effects of 30 *Cronobacter* strains. However, Vero cells may not be an appropriate representation of the targeted area in the host, which for *Cronobacter* spp. are the GI tract and the BBB. Therefore, due to time constraints, only small and large human intestinal epithelial cells were included in this study to attempt to make inference about *Cronobacter* NEC infection in the human host and correlate results obtained in the Vero cell assay.

The quantification of cell death from the *Cronobacter* isolates was statistically analyzed using SAS 9.3 and showed that there was significant difference in the mean of cytopathic effect in Vero cells amongst the clinical and food isolates, as well as clinical and environmental isolates. Interestingly, clinical isolates appeared to be less virulent in the enterotoxin assay, which possibly suggests that clinical strains may become less pathogenic once lab adapted or grown outside the host. Another possibility is that the *in vitro* conditions were not providing a good representation of the GI environment and thus, the virulence potential may have been suppressed.

Isolation of the putative protein was successful for strain 2878, a food isolate, and the sequence of the protein was identified using mass spectrometry. The protein isolated was identified as phosphoenolypyruvate carboxykinase (PEPCK). Also it is important to

note that the actual molecular weight of the purified protein obtained from strain 2878 was 59.88 kDA and not 66 kDA, which was the putative toxin molecular weight described by Raggahv and Aggarwal (75). PEPCK is an enzyme known to play a critical role in gluconeogenesis, in which it catalyzes the reversible decarboxylation and phosphorylation of oxaloacetate (OAA) to phosphoenolypyruvate (PEP) and carbon dioxide. This purified PEPCK did demonstrate some cytopathic effects on the cell lines but based on the results obtained in this study and available information in the literature, there is not enough evidence to unequivocally state that PEPCK is a virulence factor in *Cronobacter* spp.

The standard adhesion and invasion assays, using the HBMEC cell line, were used to measure the invasive and adhesive ability of the 30 *Cronobacter* strains in order to identify the strains of interest that would be used to develop a transposon mutant library. This transposon mutant library was screened for hypo- hyper adhesion and/or invasion mutants and would help identify virulence genes that could be associated with the ability of *Cronobacter* spp. to cause meningitis. The quantification of the adhesive and invasive abilities of 30 *Cronobacter* strains identified two strains in particular that were dominant in either assay, strain 3404, an environmental isolate that adhered the greatest to HBMEC, and strain 3267, a food isolate that was highly invasive to HBMEC. Transposon mutant library generation for strains 2855, 3231, 3267, 3404 and 3414 was successful. The screening of the isogenic mutants using the standard adhesion and invasion assay is a lengthy process and unfortunately to-date, only one isogeneic mutant of strain 3231 (clinical isolate) lost the ability to adhere to HBMEC. The insertion site of the transposon revealed that the *dgc* gene was disrupted. The finding of DGC in strain 3231 raises many

questions regarding c-di-GMP and its role in *Cronobacter* spp. To-date, DGC has been shown in many gram-negative bacteria to synthesize c-di-GMP, which is the main trigger for switching planktonic cells to biofilms. The levels of c-di-GMP not only have an impact on biofilm formation but also on virulence (5, 13, 15, 28, 77, 82). Consequently, as *Cronobacter* spp. are Gram-negative, bacteria DGC and c-di-GMP may have an important role in both biofilm formation and virulence. Further work is required to understand c-di-GMP regulation of pathogenicity in *Cronobacter* spp.

The results obtained from the adhesion and invasion assay led to the investigation of biofilm formation in *Cronobacter* spp. The *Cronobacter* strains were tested for their ability to produce biofilms under different conditions. Strain 3231 and its isogenic mutant, Δ3231, were also compared. It was hypothesized that if the *dgc* gene was disrupted, then there should be a reduction in biofilm formation or no biofilm formation. A CV microtiter assay was used to quantify the biofilm formation and for certain strains further confirmation of biofilm production was done by SEM analysis. Strain 3231 did form biofilms, whereas Δ3231 cells could not aggregate. From the CV microtiter assay, strain 3287 demonstrated the greatest ability to produce biofilms and was the primary target for developing a transposon library. Isogenic mutants for 3287 were successfully developed and screened for defective mutants. Among these, strain M17 showed a decrease of approximately 85% in biofilm formation as compared to its parental wildtype. The insertion site of the transposon was identified using direct genomic sequencing and it was shown that the *thrS* gene was disrupted. Threonine synthase is an enzyme known to convert $_{L}$-homoserine phosphate and water to inorganic phosphate and threonine. There have not been any studies investigating the role of threonine synthase in biofilm

formation. However, researchers have investigated the role of inorganic phosphate and how it regulates c-di-GMP (54, 60). This could imply that TS is indirectly influencing the levels of c-di-GMP in the organism.

Biofilms are important in *Cronobacter* physiology because it is believed that food processing equipment may allow for the formation of *Cronobacter* biofilms. It has been shown that *Cronobacter* spp. can produce biofilms on many abiotic surfaces, with suggestions that they can produce biofilms in PIF (17, 33). Therefore, the results obtained in part III and IV of this study may help to shed some light in the process of *Cronobacter* spp. infection.

The overall hypothesis of the thesis was that there is a difference in virulence potential amongst clinical, food and environmental isolates with clinical isolates being the most virulent. Based on the exploratory statistical analysis of the data, there appears to be differences in the enterotoxin, adhesion and invasion of the BBB and biofilm formation between the clinical, environmental and food isolates. The clinical isolates appear to be the least dominant source in all the assays. However, inferential statistics does not provide us with sufficient evidence to conclude that there are differences among the three different sources in all assays, except in the case of the Vero cell assay. In Table 4, it can be seen that there are individual differences among of the highlighted isolates in the three assays. The food and environmental isolates are almost positive for all assays; where the clinical isolates are negative or not as dominant. Therefore, a larger sample size of each of the three different sources of strains would be necessary to further address differences observed. This is especially important as the clinical strains did not show the greates effects in the assays used.

In summary, two isogenic mutants, Δ3231 and M17, showed a significant decrease in biofilm production compared to their corresponding parental wildtype. The transposon rescue of strain Δ3231 revealed the insertion site to be within a *dgc gene*. DGC synthesizes c-di-GMP, which is a well-known second messenger for many Gram-negative bacteria and is known to influence many cellular functions such as motility, virulence, and biofilm formation. Disrupting the *dgc* gene also inhibited the adherence to BBB cell line. These findings correspond to published literature based on other Gram-negative bacteria and further supports the hypothesis that DGC plays an important role in the production of *Cronobacter* biofilms. Strain M17 transposon insertion site was revealed within a thrS gene. No previous research has been conducted on TS and its role in biofilm formation. However, TS is responsible for producing threonine and inorganic phosphate. Inorganic phosphate in certain *Pseudomonas* spp. promotes the production of c-di-GMP. Based on our findings, it could be hypothesized there appears to be an association between TS and DGC in biofilm formation of *Cronobacter* spp. The hypothesized relationship between TC and DGC is shown in Figure 13. Levels of inorganic phosphate promote DGC, which in return increases the level of c-di-GMP and potentially enhances the formation of biofilm. If inorganic phosphate is limited, PDE is favored, resulting in a breakdown of c-di-GMP, which in return potentially inhibits biofilm formation.

The findings of this work adds to the growing body of scientific knowledge that is starting to accumulate on *Cronobacter* spp. Figure 14 illustrates the integration of our results with previous published work and also demonstrates the importance of understanding the physiology of *Cronobacter* spp. in food industries in order to prevent and control sporadic outbreaks. Further work is required to reveal the role of TS and DGC in *Cronobacter* spp.

and to determine whether TS and DGC have a collaborative role in biofilm production, especially in certain situations such as hospital kitchens where PIF is reconstituted.

Figure 13**:** Proposed hypothetical schematic of a potential role for threonine synthase (TS) and c-diMP in biofilm formation in *Cronobacter* isolates 3287 and M17.

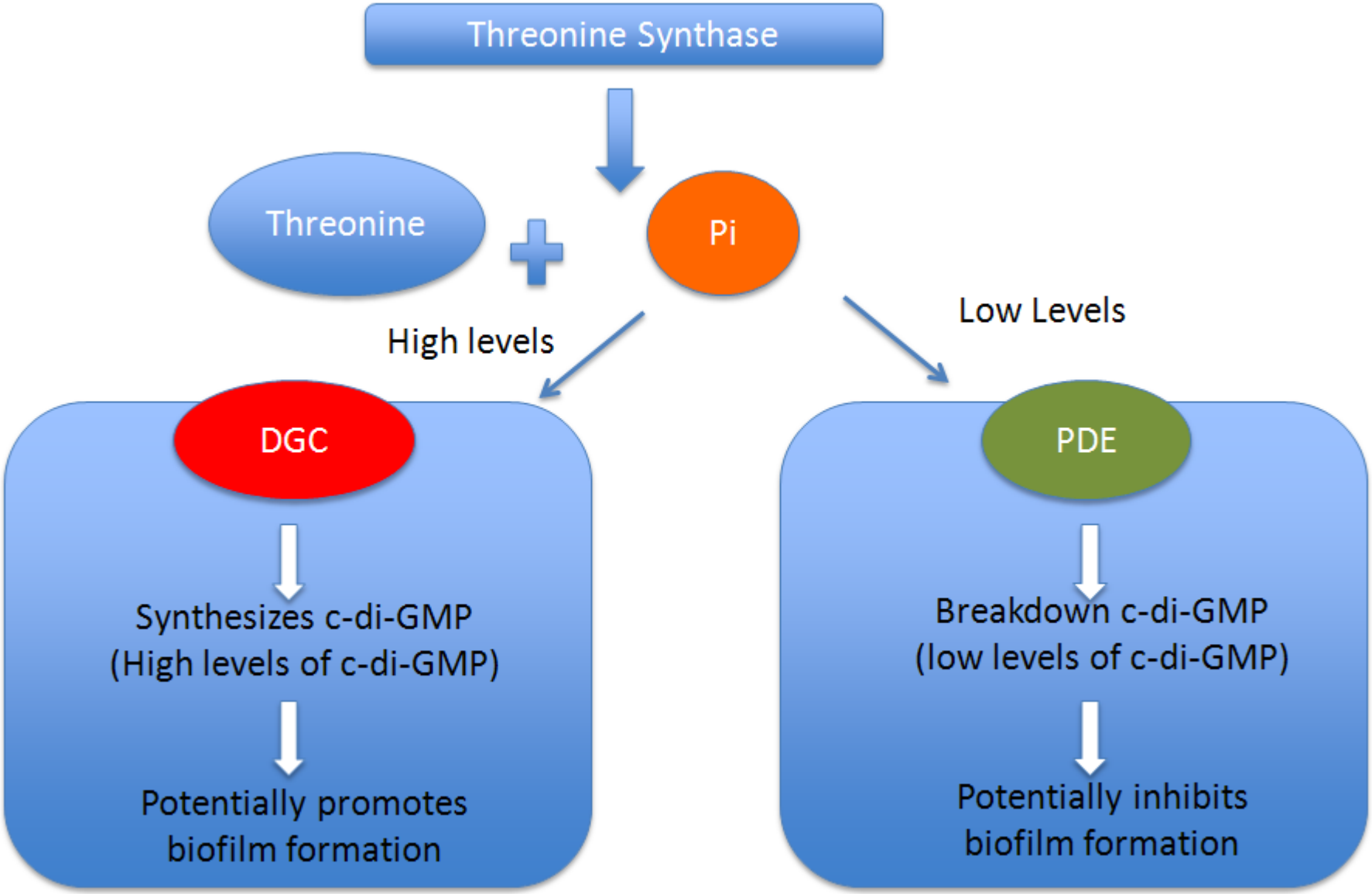
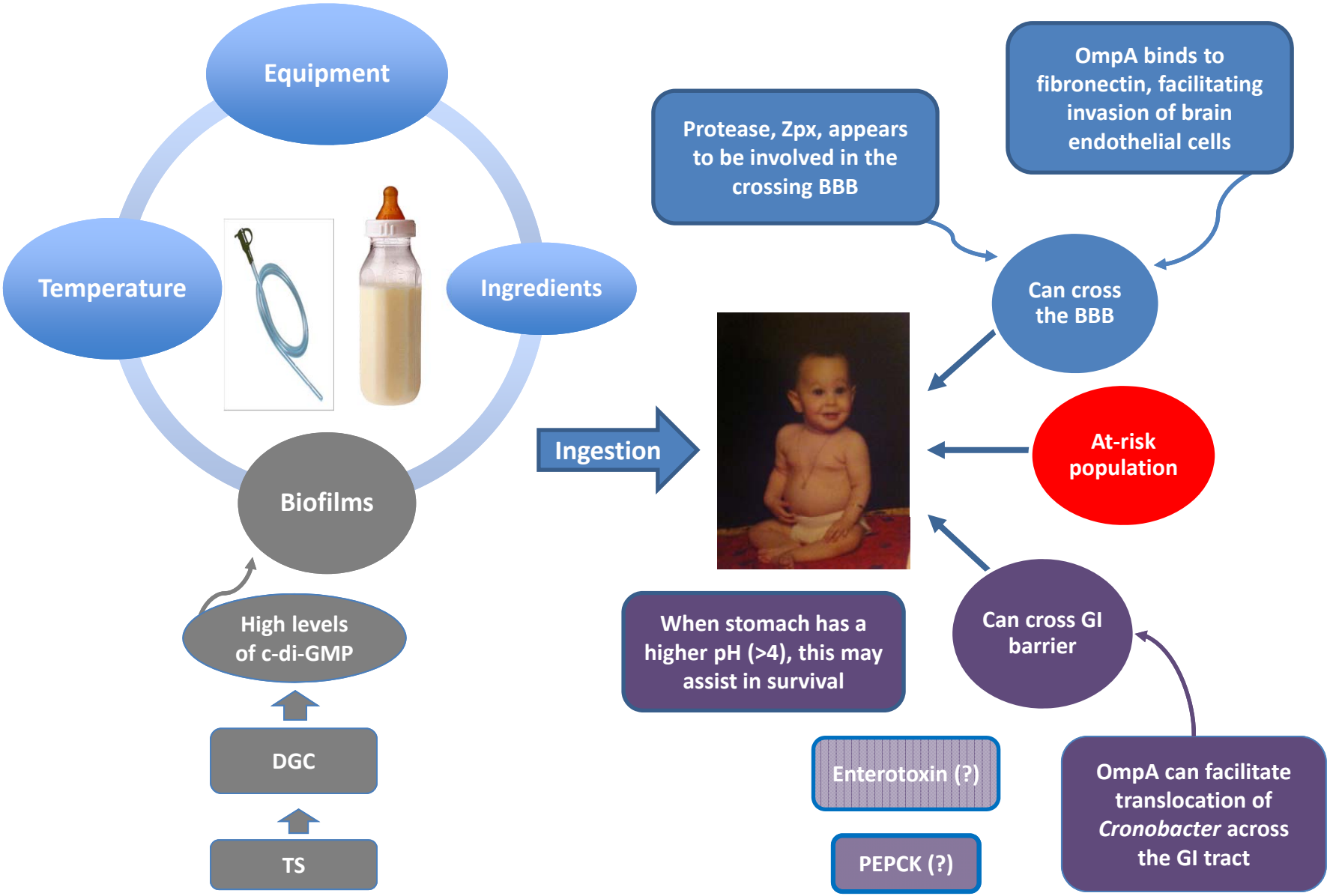

Threonine Synthase
Threonine
Pi
High levels
Low Levels
DGC
PDE
Synthesizes c-di-GMP
(High levels of c-di-GMP)
Potentially promotes
biofilm formation
Breakdown c-di-GMP
(low levels of c-di-GMP)
Potentially inhibits
biofilm formation

Figure 14: An updated model for the pathogenesis of *Cronobacter* infection based on this study


Equipment
Temperature
Ingredients
Biofilms
High levels of c-di-GMP
DGC
TS
Ingestion
Protease, Zpx, appears to be involved in the crossing BBB
OmpA binds to fibronectin, facilitating invasion of brain endothelial cells
Can cross the BBB
At-risk population
When stomach has a higher pH (>4), this may assist in survival
Can cross GI barrier
Enterotoxin (?)
PEPCK (?)
OmpA can facilitate translocation of *Cronobacter* across the GI tract

REFERENCES

1. **Abrahams, S. W., and M. H. Labbok.** 2011. Breastfeeding and otitis media: a review of recent evidence. Curr. Allergy Asthma Rep. **11:**508-512.

2. **Al-Holy, M. A., J. H. Shin, T. M. Osaili, and B. A. Rasco.** 2011. Evaluation of a new enrichment broth for detection of *Cronobacter* spp. in powdered infant formula. J. Food Prot. **74:**387-393.

3. **Almeida, C., N. F. Azevedo, C. Iversen, S. Fanning, C. W. Keevil, and M. J. Vieira.** 2009. Development and application of a novel peptide nucleic acid probe for the specific detection of *Cronobacter genomospecies* (*Enterobacter sakazakii*) in powdered infant formula. Appl. Environ. Microbiol. **75:**2925-2930.

4. **Amalaradjou, M. A., and K. Venkitanarayanan.** 2011. Effect of trans-cinnamaldehyde on inhibition and inactivation of *Cronobacter sakazakii* biofilm on abiotic surfaces. J. Food Prot. **74:**200-208.

5. **Antoniani, D., P. Bocci, A. Maciag, N. Raffaelli, and P. Landini.** 2010. Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. Appl. Microbiol. Biotechnol. **85:**1095-1104.

6. **Baumgartner, A., M. Grand, M. Liniger, and C. Iversen.** 2009. Detection and frequency of *Cronobacter* spp. (*Enterobacter sakazakii)* in different categories of ready-to-eat foods other than infant formula. Int. J. Food Microbiol. **136:**189-192.

7. **Bowen, A.B. and Braden, C.R**. 2006. Invasive *Enterobacter sakazakii* disease in infants. Emerg Infect Dis. 12:1185-1189.

8. **CAC (Codex Alimentarius Commission).** 2008. "Code of Hygienic Practice for Powdered Formulae for Infants and Young Children," CAC/RCP 66-2008.

9. **Cadoudal, T., F. Fouque, C. Benelli, and C. Forest.** 2008. Glyceroneogenesis and PEPCK-C: pharmacological targets in type 2 diabetes. Med. Sci. (Paris) **24:**407-413.

10. **Cawthorn, D. M., S. Botha, and R. C. Witthuhn.** 2008. Evaluation of different methods for the detection and identification of *Enterobacter sakazaki*i isolated from South African infant formula milks and the processing environment. Int. J. Food Microbiol. **127:**129-138.

11. **Chen, Y., T. S. Hammack, K. Y. Song, and K. A. Lampel.** 2009. Evaluation of a revised U.S. Food and Drug Administration method for the detection and isolation of *Enterobacter sakazakii* in powdered infant formula: precollaborative study. J. AOAC Int. **92:**862-872.

12. **Chenu, J. W., and J. M. Cox.** 2009. *Cronobacter* (*'Enterobacter sakazakii'*): current status and future prospects. Lett. Appl. Microbiol. **49:**153-159.

13. **Ching, S. M., W. J. Tan, K. L. Chua, and Y. Lam.** 2010. Synthesis of cyclic di-nucleotidic acids as potential inhibitors targeting diguanylate cyclase. Bioorg. Med. Chem. **18:**6657-6665.

14. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711-745.

15. **Cotter, P. A., and S. Stibitz.** 2007. c-di-GMP-mediated regulation of virulence and biofilm formation. Curr. Opin. Microbiol. **10:**17-23.

16. **Covarrubias, A. S., M. Hogbom, T. Bergfors, P. Carroll, K. Mannerstedt, S. Oscarson, T. Parish, T. A. Jones, and S. L. Mowbray.** 2008. Structural, biochemical, and *in vivo* investigations of the threonine synthase from *Mycobacterium tuberculosis*. J. Mol. Biol. **381:**622-633.

17. **Dancer, G. I., J. H. Mah, and D. H. Kang.** 2009. Influences of milk components on biofilm formation of *Cronobacter* spp. (*Enterobacter sakazakii*). Lett. Appl. Microbiol. **48:**718-725.

18. **Edelson-Mammel, S. G., M. K. Porteous, and R. L. Buchanan.** 2005. Survival of *Enterobacter sakazakii* in a dehydrated powdered infant formula. J. Food Prot. **68:**1900-1902.

19. **Emami, C. N., R. Mittal, L. Wang, H. R. Ford, and N. V. Prasadarao.** 2011. Recruitment of dendritic cells is responsible for intestinal epithelial damage in the pathogenesis of necrotizing enterocolitis by *Cronobacter sakazakii*. J. Immunol. **186:**7067-7079.

20. **Farmer, J.J.III., Hickmann, A.M., and Brenner, DJ**. 1980. *Enterobacter sakazakii*: a new species of "Enterobacteriaceae" isolated from a clinical specimens. Int J Syst Bacteriol. **30**:569-584.

21. **Food and Agriculture Organization / World Health Organization.** 2004. Food and Agriculture Organization/World Health Organization. Joint FAO/WHO workshop on *Enterobacter sakazakii* and other microorganisms in powdered infant formula. Geneva, 2-5 February, 2004.

22. **Friedemann, M.** 2009. Epidemiology of invasive neonatal *Cronobacter* (*Enterobacter sakazakii*) infections. Eur. J. Clin. Microbiol. Infect. Dis. **28:**1297-1304.

23. **Gosney, M. A., M. V. Martin, A. E. Wright, and M. Gallagher.** 2006. *Enterobacter sakazakii* in the mouths of stroke patients and its association with aspiration pneumonia. Eur. J. Intern. Med. **17:**185-188.

24. **Harrison, J. J., C. A. Stremick, R. J. Turner, N. D. Allan, M. E. Olson, and H. Ceri.** 2010. Microtiter susceptibility testing of microbes growing on peg lids: a miniaturized biofilm model for high-throughput screening. Nat. Protoc. **5:**1236-1254.

25. **Hartmann, I., P. Carranza, A. Lehner, R. Stephan, L. Eberl, and K. Riedel.** 2010. Genes involved in *Cronobacter sakazakii* biofilm formation. Appl. Environ. Microbiol. **76:**2251-2261.

26. **Hayes, M., E. Barrett, R. P. Ross, G. F. Fitzgerald, C. Hill, and C. Stanton.** 2009. Evaluation of an antimicrobial ingredient prepared from a *Lactobacillus acidophilus* casein fermentate against *Enterobacter sakazaki*i. J. Food Prot. **72:**340-346.

27. **Healy, B., S. Cooney, S. O'Brien, C. Iversen, P. Whyte, J. Nally, J. J. Callanan, and S. Fanning.** 2009. *Cronobacter* (*Enterobacter sakazakii)*: An Opportunistic Foodborne Pathogen. Foodborne Pathog. Dis. **7:**339-350.

28. **Hengge, R.** 2009. Principles of c-di-GMP signalling in bacteria. Nat. Rev. Microbiol. **7:**263-273.

29. **Hoffman, L. M., J. J. Jendrisak, R. J. Meis, I. Y. Goryshin, and S. W. Reznikof.** 2000. Transposome insertional mutagenesis and direct sequencing of microbial genomes. Genetica **108:**19-24.

30. **Hunter, C. J., V. K. Singamsetty, N. K. Chokshi, P. Boyle, V. Camerini, A. V. Grishin, J. S. Upperman, H. R. Ford, and N. V. Prasadarao.** 2008. *Enterobacter sakazakii* enhances epithelial cell injury by inducing apoptosis in a rat model of necrotizing enterocolitis. J. Infect. Dis. **198:**586-593.

31. **ISO/TS 22964.** ISO/TS 22964 Milk and Milk Products - Detection of *Enterobacter sakazakii* (TC 34/SC; ISO Standards).

32. **Iversen, C., P. Druggan, S. Schumacher, A. Lehner, C. Feer, K. Gschwend, H. Joosten, and R. Stephan.** 2008. Development of a novel screening method for the isolation of "*Cronobacter*" spp. (*Enterobacter sakazakii*). Appl. Environ. Microbiol. **74:**2550-2553.

33. **Iversen, C., M. Lane, and S. J. Forsythe.** 2004. The growth profile, thermotolerance and biofilm formation of *Enterobacter sakazakii* grown in infant formula milk. Lett. Appl. Microbiol. **38:**378-382.

34. **Iversen, C., A. Lehner, N. Mullane, E. Bidlas, I. Cleenwerck, J. Marugg, S. Fanning, R. Stephan, and H. Joosten.** 2007. The taxonomy of *Enterobacter sakazaki*i: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter*

muytjensii sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter genomospecies 1*. BMC Evol. Biol. **7:**64.

35. **Iversen, C., N. Mullane, B. McCardell, B. D. Tall, A. Lehner, S. Fanning, R. Stephan, and H. Joosten.** 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter genomospecies 1*, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and C*ronobacter dublinensis* subsp. *lactaridi* subsp. nov. Int. J. Syst. Evol. Microbiol. **58:**1442-1447.

36. **Jo, S. H., S. B. Baek, J. H. Ha, and S. D. Ha.** 2010. Maturation and survival of *Cronobacter* biofilms on silicone, polycarbonate, and stainless steel after UV light and ethanol immersion treatments. J. Food Prot. **73:**952-956.

37. **Joseph, S., E. Cetinkaya, H. Drahovska, A. Levican, M. J. Figueras, and S. J. Forsythe.** 2011. *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. *genomospecies 1*, recovered from a leg infection, water, and food ingredients. Int. J. Syst. Evol. Microbiol. in press, Doi: 10.1099/ijs.0.032292-0.

38. **Kalmokoff, M. L., J. W. Austin, X. D. Wan, G. Sanders, S. Banerjee, and J. M. Farber.** 2001. Adsorption, attachment and biofilm formation among isolates of *Listeria monocytogenes* using model conditions. J. Appl. Microbiol. **91:**725-734.

39. **Kim, H., J. Bang, L. R. Beuchat, and J. H. Ryu.** 2008. Fate of *Enterobacter sakazakii* attached to or in biofilms on stainless steel upon exposure to various temperatures or relative humidities. J. Food Prot. **71:**940-945.

40. **Kim, H., J. H. Ryu, and L. R. Beuchat.** 2006. Attachment of and biofilm formation by *Enterobacter sakazakii* on stainless steel and enteral feeding tubes. Appl. Environ. Microbiol. **72:**5846-5856.

41. **Kim, K., K. P. Kim, J. Choi, J. A. Lim, J. Lee, S. Hwang, and S. Ryu.** 2010. Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. Appl. Environ. Microbiol. **76:**5188-5198.

42. **Kim, K. P., J. Klumpp, and M. J. Loessner.** 2007. *Enterobacter sakazakii* bacteriophages can prevent bacterial growth in reconstituted infant formula. Int. J. Food Microbiol. **115:**195-203.

43. **Kim, T. J., W. L. Weng, J. L. Silva, Y. S. Jung, and D. Marshall.** 2010. Identification of natural antimicrobial substances in red muscadine juice against *Cronobacter sakazakii*. J. Food Sci. **75:**M150-4.

44. **Kim, Y. K., and L. L. McCarter.** 2007. ScrG, a GGDEF-EAL protein, participates in regulating swarming and sticking in *Vibrio parahaemolyticus*. J. Bacteriol. **189:**4094-4107.

45. **Kothary, M. H., B. A. McCardell, C. D. Frazar, D. Deer, and B. D. Tall.** 2007. Characterization of the zinc-containing metalloprotease encoded by *zpx* and development of a species-specific detection method for *Enterobacter sakazakii*. Appl. Environ. Microbiol. **73:**4142-4151.

46. **Lampel, K. A., and Y. Chen.** 2009. Method for the isolation and detection of *Enterobacter sakazakii* (*Cronobacte*r) from powdered infant formula. Int. J. Food Microbiol. **136:**179-184.

47. **Lee, H. A., S. Hong, H. Park, H. Kim, and O. Kim.** 2011. *Cronobacter sakazakii* infection induced fatal clinical sequels including meningitis in neonatal ICR mice. Lab. Anim. Res. **27:**59-62.

48. **Lehner, A., K. Riedel, L. Eberl, P. Breeuwer, B. Diep, and R. Stephan.** 2005. Biofilm formation, extracellular polysaccharide production, and cell-to-cell signaling in various *Enterobacter sakazakii* strains: aspects promoting environmental persistence. J. Food Prot. **68:**2287-2294.

49. **Lehner, A., and R. Stephan.** 2004. Microbiological, epidemiological, and food safety aspects of *Enterobacter sakazakii*. J. Food Prot. **67:**2850-2857.

50. **Lenati, R. F., D. L. O'Connor, K. C. Hebert, J. M. Farber, and F. J. Pagotto.** 2008. Growth and survival of *Enterobacter sakazakii* in human breast milk with and without fortifiers as compared to powdered infant formula. Int. J. Food Microbiol. **122:**171-179.

51. **Lin, L. C., and L. R. Beuchat.** 2007. Survival of *Enterobacter sakazakii* in infant cereal as affected by composition, water activity, and temperature. Food Microbiol. **24:**767-777.

52. **Liu, K., J. Yu, and D. G. Russell.** 2003. *pckA*-deficient *Mycobacterium bovis* BCG shows attenuated virulence in mice and in macrophages. Microbiology **149:**1829-1835.

53. **Loo, C. Y., D. A. Corliss, and N. Ganeshkumar.** 2000. *Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes. J. Bacteriol. **182:**1374-1382.

54. **Malorny, B., and M. Wagner.** 2005. Detection of *Enterobacter sakazakii* strains by real-time PCR. J. Food Prot. **68:**1623-1627.

55. **Mange, J. P., R. Stephan, N. Borel, P. Wild, K. S. Kim, A. Pospischil, and A. Lehner.** 2006. Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. BMC Microbiol. **6:**58.

56. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. **Chapter 1:**Unit 1B.1.

57**. Mittal, R., S. Bulgheresi, C. Emami, and N. V. Prasadarao.** 2009. *Enterobacter sakazakii* targets DC-SIGN to induce immunosuppressive responses in dendritic cells by modulating MAPKs. J. Immunol. **183:**6588-6599.

58. **Mittal, R., Y. Wang, C. J. Hunter, I. Gonzalez-Gomez, and N. V. Prasadarao.** 2009. Brain damage in newborn rat model of meningitis by *Enterobacter sakazakii*: a role for outer membrane protein A. Lab. Invest. **89:**263-277.

59. **Mohan Nair, M. K., and K. Venkitanarayanan.** 2007. Role of bacterial OmpA and host cytoskeleton in the invasion of human intestinal epithelial cells by *Enterobacter sakazakii*. Pediatr. Res. **62:**664-669.

60. **Monds, R. D., P. D. Newell, R. H. Gross, and G. A. O'Toole.** 2007. Phosphate-dependent modulation of c-di-GMP levels regulates *Pseudomonas fluorescens* Pf0-1 biofilm formation by controlling secretion of the adhesin LapA. Mol. Microbiol. **63:**656-679.

61. **Mullane, N., B. Healy, J. Meade, P. Whyte, P. G. Wall, and S. Fanning.** 2008. Dissemination of *Cronobacter* spp. (*Enterobacter sakazakii*) in a powdered milk protein manufacturing facility. Appl. Environ. Microbiol. **74:**5913-5917.

62. **Murakawa, T., Y. Machida, and H. Hayashi.** 2011. Product-assisted catalysis as the basis of the reaction specificity of threonine synthase. J. Biol. Chem. **286:**2774-2784.

63. **Muytjens, H. L., H. Roelofs-Willemse, and G. H. Jaspar.** 1988. Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacter*iaceae. J. Clin. Microbiol. **26:**743-746.

64. **Muytjens, H. L., van der Ros-van de Repe,J., and H. A. van Druten.** 1984. Enzymatic profiles of *Enterobacter sakazakii* and related species with special reference to the alpha-glucosidase reaction and reproducibility of the test system. J. Clin. Microbiol. **20:**684-686.

65. **Nair, M. K., K. Venkitanarayanan, L. K. Silbart, and K. S. Kim.** 2009. Outer membrane protein A (OmpA) of *Cronobacter sakazakii* binds fibronectin and contributes to invasion of human brain microvascular endothelial cells. Foodborne Pathog. Dis. **6:**495-501.

66. **Newell, P. D., C. D. Boyd, H. Sondermann, and G. A. O'Toole.** 2011. A c-di-GMP effector system controls cell adhesion by inside-out signaling and surface protein cleavage. PLoS Biol. **9:**e1000587.

67. **Newell, P. D., R. D. Monds, and G. A. O'Toole.** 2009. LapD is a bis-(3',5')-cyclic dimeric GMP-binding protein that regulates surface attachment by *Pseudomonas fluorescens* Pf0-1. Proc. Natl. Acad. Sci. U. S. A. **106:**3461-3466.

68. **O'Brien, S., B. Healy, C. Negredo, S. Fanning, and C. Iversen.** 2009. Evaluation of a new one-step enrichment in conjunction with a chromogenic medium for the detection of *Cronobacter* spp. (*Enterobacter sakazakii*) in powdered infant formula. J. Food Prot. **72:**1472-1475.

69. **Pagotto, F. J., and J. M. Farber.** 2009. *Cronobacter* spp. (*Enterobacter sakazakii*): advice, policy and research in Canada. Int. J. Food Microbiol. **136:**238-245.

70. **Pagotto, F. J., R. F. Lenati, and J. M. Farber.** 2007. *Enterobacter sakazakii*, p. 271-287. *In* M. P. Doyle and L. R. Beuchat (ed.), Food Microbiology Fundamentals and frontiers, Third ed. ASM Press.

71. **Pagotto, F. J., M. Nazarowec-White, S. Bidawid, and J. M. Farber.** 2003. *Enterobacter sakazakii*: infectivity and enterotoxin production in vitro and in vivo. J. Food Prot. **66:**370-375.

72. **Pediatric Nutrition Practice Group of the American Dietic Association.** Infant Feedings: Guidelines for Preparation of Formula and Breastmilk in Health Care Facilities.

http://www.infantformula.org/for-health-professionals. Accessed on January 6, 2012.

73. **Proudy, I.** 2009. *Enterobacter sakazakii* in powdered infant food formulas. Can. J. Microbiol. **55:**473-500.

74. **Qimron, U., N. Madar, R. Ascarelli-Goell, M. Elgrably-Weiss, S. Altuvia, and A. Porgador.** 2003. Reliable determination of transposon insertion site in prokaryotes by direct sequencing. J. Microbiol. Methods **54:**137-140.

75. **Raghav, M., and P. K. Aggarwal.** 2007. Purification and characterization of *Enterobacter sakazakii* enterotoxin. Can. J. Microbiol. **53:**750-755.

76. **Richardson, A. N., S. Lambert, and M. A. Smith.** 2009. Neonatal mice as models for *Cronobacter sakazakii* infection in infants. J. Food Prot. **72:**2363-2367.

77. **Romling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol. **9:**218-228.

78. **Schmid, A., W. Neumayer, K. Trulzsch, L. Israel, A. Imhof, M. Roessle, G. Sauer, S. Richter, S. Lauw, E. Eylert, W. Eisenreich, J. Heesemann, and G. Wilharm.** 2009. Cross-talk between type three secretion system and metabolism in *Yersinia*. J. Biol. Chem. **284:**12165-12177.

79. **Seo, K. H., and R. E. Brackett.** 2005. Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. J. Food Prot. **68:**59-63.

80. **Singamsetty, V. K., Y. Wang, H. Shimada, and N. V. Prasadarao.** 2008. Outer membrane protein A expression in *Enterobacter sakazakii* is required to induce microtubule condensation in human brain microvascular endothelial cells for invasion. Microb. Pathog. **45:**181-191.

81.**Steel, G.D., R. and Torrie, J.** 1996. Principles and Procedures of Statistics: A Biometrical approach. McGraw Hill, Third edition

82. **Tamayo, R., J. T. Pratt, and A. Camilli.** 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. Annu. Rev. Microbiol. **61:**131-148.

83. **Tchawa Yimga, M., M. P. Leatham, J. H. Allen, D. C. Laux, T. Conway, and P. S. Cohen.** 2006. Role of gluconeogenesis and the tricarboxylic acid cycle in the virulence of *Salmonella enterica* serovar Typhimurium in BALB/c mice. Infect. Immun. **74:**1130-1140.

84. **Townsend, S., J. Caubilla Barron, C. Loc-Carrillo, and S. Forsythe.** 2007. The presence of endotoxin in powdered infant formula milk and the influence of endotoxin and *Enterobacter sakazakii* on bacterial translocation in the infant rat. Food Microbiol. **24:**67-74.

85. **Townsend, S., E. Hurrell, and S. Forsythe.** 2008. Virulence studies of *Enterobacter sakazakii* isolates associated with a neonatal intensive care unit outbreak. BMC Microbiol. **8:**64.

86. **United States Food and Drug Administration (Center for Food Safety and Applied Nutrition Office of Nutritional Products, Labeling and Dietary Supplements).** 2002. April 11, 2002; Revised October 10, 2002. Health professionals letter on *Enterobacter sakazakii* infections associated with the use of powdered (dry) infant formulas in neonatal intensive care units.

87. **Utley, M., D. P. Franklin, K. A. Krogfelt, D. C. Laux, and P. S. Cohen.** 1998. A *Salmonella typhimurium* mutant unable to utilize fatty acids and citrate is avirulent and immunogenic in mice. FEMS Microbiol. Lett. **163:**129-134.

88. **WHO** (World Health Organization). *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: Meeting report. MRA Series 10, 2006. Available from

http://www.who.int/foodsafety/publications/micro/mra6/en/index.html. Accessed on April 15, 2012.

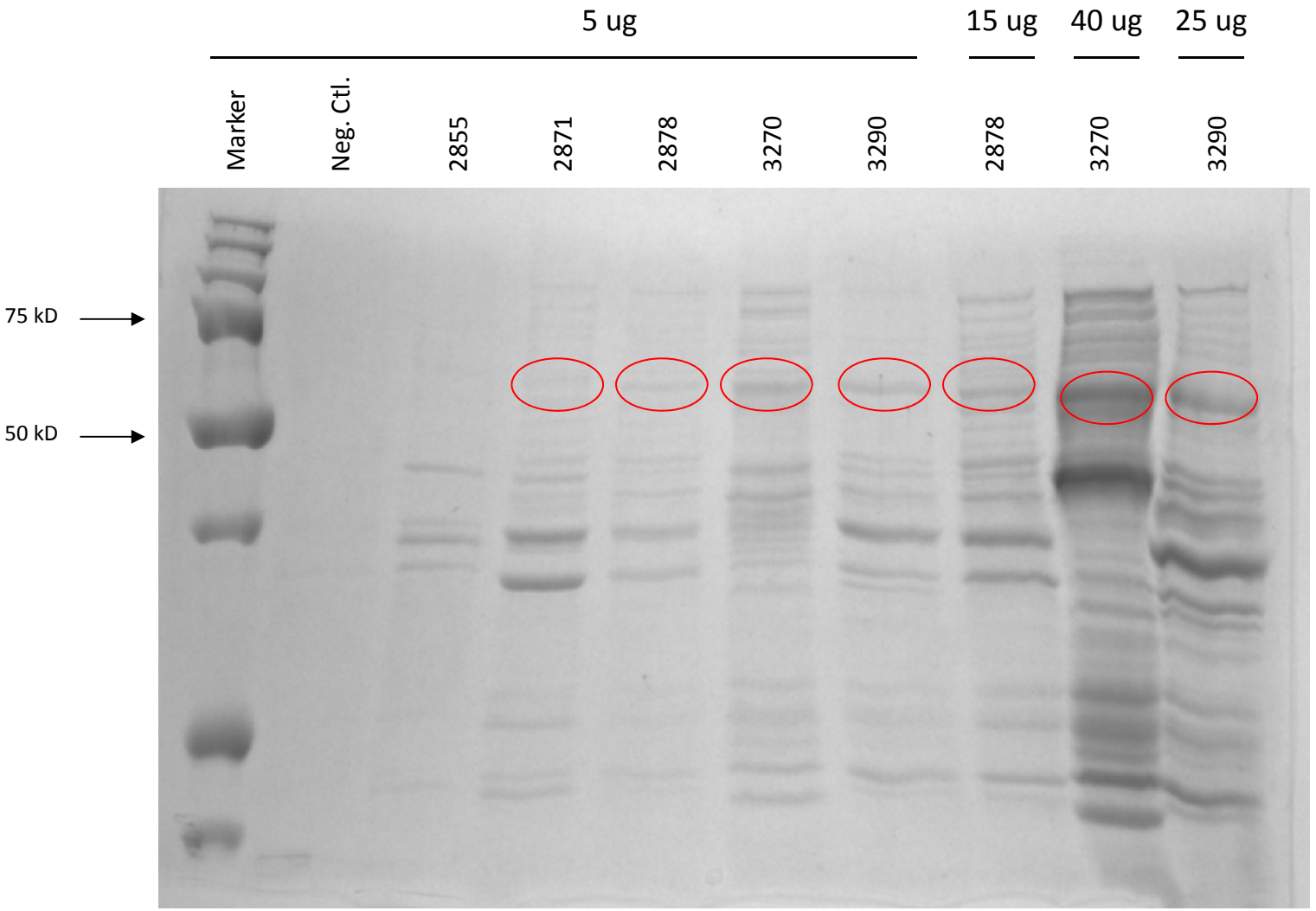
89. **Yemis, G. P., F. Pagotto, S. Bach, and P. Delaquis.** 2011. Effect of vanillin, ethyl vanillin, and vanillic acid on the growth and heat resistance of *Cronobacter* species. J. Food Prot. **74:**2062-2069.

90. **Zuber, S., C. Boissin-Delaporte, L. Michot, C. Iversen, B. Diep, H. Brussow, and P. Breeuwer.** 2008. Decreasing *Enterobacter sakazakii* (*Cronobacter* spp.) food contamination level with bacteriophages: prospects and problems. Microb. Biotechnol. **1:**532-543.

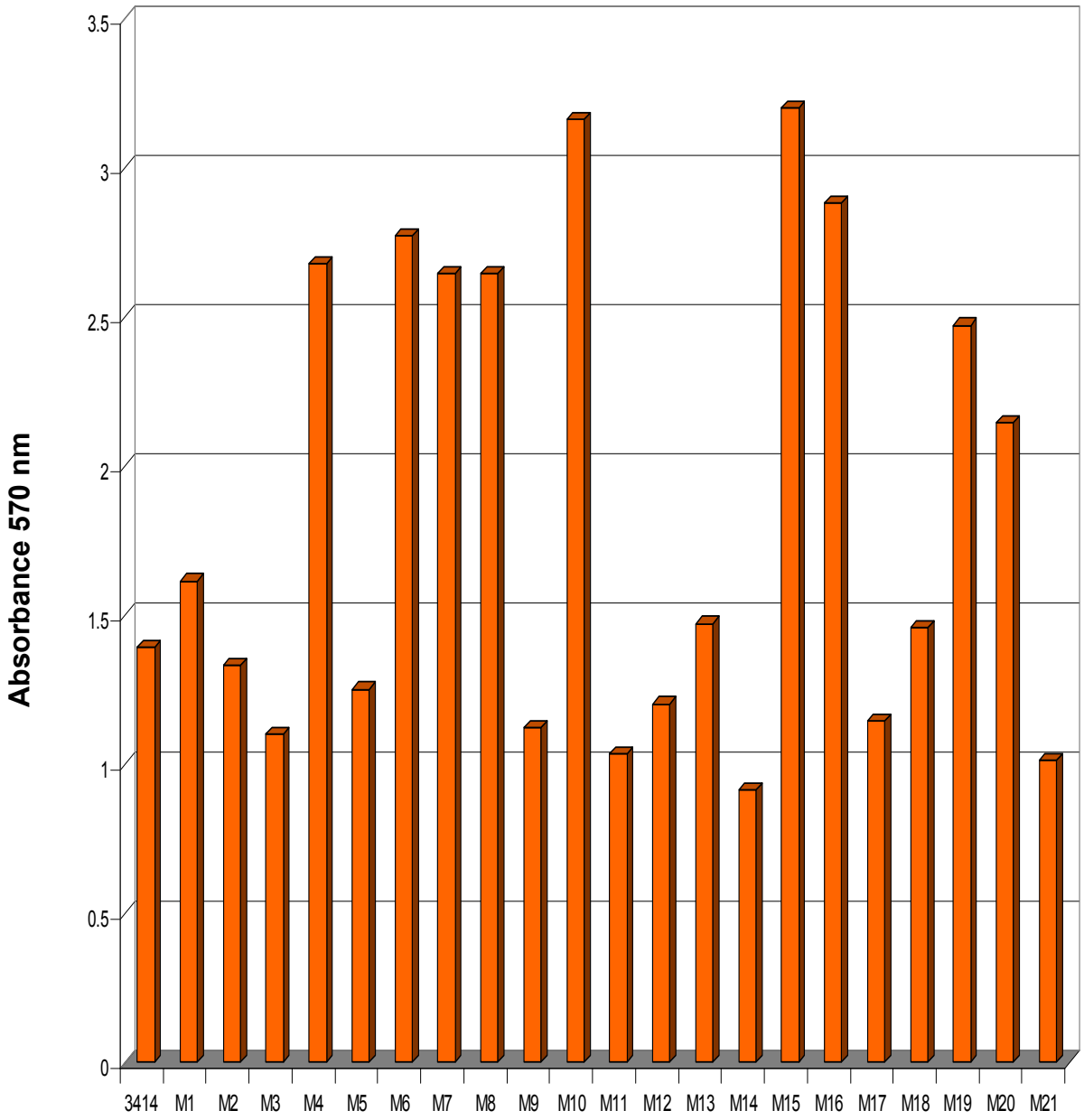
Appendix A: The *Cronobacter* strains used in this study

HPB #	Source	Species	Country of Isolation
2855	Clinical	*sakazakii*	Canada
2871	Food	*sakazakii*	Canada
2878	Environmental	*sakazakii*	Canada
2879	Environmental	*sakazakii*	Canada
3198	Environmental	*sakazakii*	USA
3234	Clinical	*sakazakii*	Netherlands
3267	Food	*malonaticus*	Australia
3270	Food	*muytjensii*	Denmark
3284	Food	*sakazakii*	Uruguay
3287	Food	*turicensis*	India
3290	Clinical	*sakazakii*	USA
3295	Clinical	*sakazakii*	USA
3396	Environmental	*sakazakii*	Malaysia
3402	Environmental	*sakazakii*	France
3403	Environmental	*sakazakii*	Netherlands
3404	Environmental	*sakazakii*	Switzerland
3410	Environmental	*sakazakii*	Netherlands
3414	Environmental	*sakazakii*	USA
3420	Environmental	*sakazakii*	Germany
3428	Clinical	*sakazakii*	Israel
3434	Food	*sakazakii*	Not available
3436	Food	*sakazakii*	Not available
3437	Food	*sakazakii*	Not available
3438	Food	*sakazakii*	Not available
3439	Food	*sakazakii*	Not available
3655	Clinical	*malonaticus*	USA
3656	Clinical	*sakazakii*	USA
3657	Clinical	*sakazakii*	USA
3658	Clinical	*malonaticus*	USA
5159	Clinical	*sakazakii*	Same as ATCC29544
5723	Clinical	*sakazakii*	France

Appendix B: Supplemental information for Figure 5. In preliminary work, the gels were clearer and the excised band from Figure 5 was eventually sequenced. Once the PEPCK was identified, the gene was amplified and sent to GenScript (New Jersey, USA) for production of the intact protein. The company was chosen as they were able to produce the protein without any markers such as the traditional HIS-tag, etc. They sent us the purified protein in a lyophilized form. The red circles indicate the protein what was excised from strain 2878 (Figure 5).


5 ug
15 ug
40 ug
25 ug
Marker
Neg. Ctl.
2855
2871
2878
3270
3290
2878
3270
3290
75 kD
50 kD

Appendix C: Preliminary work for biofilm formation of strain 3414 and its corresponding mutants based on CV staining of cell adhered to 96-well plates after 45 h of growth in AB minimum media supplemented with 0.4% maltose. Experiment was done only one time.


Absorbance 570 nm
3.5
3
2.5
2
1.5
1
0.5
0
3414
M1
M2
M3
M4
M5
M6
M7
M8
M9
M10
M11
M12
M13
M14
M15
M16
M17
M18
M19
M20
M21