RELATEDNESS OF ISOLATES OF A NOVEL GENUS, *CRONOBACTER*, FORMERLY KNOWN AS *ENTEROBACTER SAKAZAKII*

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Relatedness of a novel genus, *Cronobacter*, formally known as

Enterobacter sakazakii

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ABSTRACT

Solseng, Tracy Anne, MS, Food Safety Program, College of Graduate and Interdisciplinary Studies, North Dakota State University, July 2010. Relatedness of Isolates of a Novel Genus, *Cronobacter*, Formerly Known as *Enterobacter sakazakii*. Major Professor: Dr. Penelope Gibbs.

Members of the genus *Cronobacter* were once classified as *Enterobacter sakazakii*. These bacteria are opportunistic pathogens that are associated with necrotizing enteritis, sepsis and meningitis in neonatal or low-birth-weight infants and can result in death, slowed development, or extensive neurological disorders. In adults, they have been documented as a cause of bacteremia, osteomyelitis, and vaginitis. Previously, *E. sakazakii* was found in the midgut of stable flies. Research by Nangoh et al. determined that *Cronobacter* spp. (previously identified as *E. sakazakii)* are present in bison and bovine feces.

In addition to the bison and bovine fecal isolates of *Cronobacter* spp. found by Nangoh et al., other isolates of *Cronobacter* spp. were analyzed phenotypically and genetically for biochemical typing and genotyping. The additional isolates include several American Type Culture Collection isolates, an isolate from a neonatal meningitis case, and multiple isolates of various origins received from Cornell University. These isolates were further categorized using four different biochemical tests. The results of these tests placed the isolates into one of the six different species or subspecies within the genus *Cronobacter*.

For genotyping, the isolates were tested for the gene specifically responsible for the α-glucosidase activity. In addition, Pulsed-Field Gel Electrophoresis using two different enzymes, *Xba*I and *Spe*I, was performed to determine possible genetic similarity of isolates

from the bison and bovine feces to other isolates found in food, clinical and environmental settings. The *Xba*I enzyme showed two Cornell isolates, F6-049 and F6-051, had a high degree of similarity; both of these isolates were from the same clinical source. Isolates from bison and bovine feces, 52 and N72, respectively, have a high degree of dissimilarity to each other, ~ 75%. Isolate 52 showed ~ 35% dissimilarity to an isolate from a food source, and N72 showed ~45% dissimilarity to an isolate from a clinical source. The results using the *Spe*I enzyme showed a wide diversity among the isolates. This study shows that very few of the *Cronobacter* spp. isolates are closely related and that there is a high level of diversity based on pulse-field gel electrophoresis and biochemical analysis.

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OBJECTIVES

The objectives of this study are:

1. To identify the species of *Cronobacter* from the formerly identified *Enterobacter sakazakii* isolates, through biotyping in the newly established typing scheme.
2. To partially determine the genetic relatedness of isolates of *Cronobacter* spp. from many different sources through Pulsed-Field Gel Electrophoresis.

INTRODUCTION

Cronobacter spp., formerly *Enterobacter sakazakii* are Gram negative facultative anaerobes that are part of the Enterobacteriaceae family *(1, 2, 4, 6, 13-15, 24, 29, 31, 33, 36, 41, 47, 56)*. The organisms are motile, peritrichous rods that were once referred to as yellow-pigmented *Enterobacter cloacae (6, 11, 13, 14, 20, 24, 28, 29, 31, 33, 39, 41, 42, 44, 47, 56).* In 1980, the name was changed to distinguish it as different from *E. cloacae (1, 2, 15, 20, 21, 28, 29, 31, 33, 41, 42, 44, 56)*. Currently, these isolates have now been classified as various species of the Genus *Cronobacter* due to genetic and biochemical differences *(22)*.

This bacterium was first known by other names including "Urmenyi and Franklin bacillus, yellow coliform, yellow *Enterobacter*, and pigmented *cloacae* A organism" *(13)*. After it was found to have different characteristics from *E. cloacae* such as DNA relatedness, pigment production, biotyping, and antibiograms, the bacteria was named after a Japanese microbiologist- Dr. Riichi Sakazaki *(8, 13, 31)*.

Cronobacter spp. are foodborne pathogens sometimes found in powdered infant formula and are able to cause severe disease in immunocompromised infants. *Cronobacter* spp. has been reported to be able to outcompete *E. cloacae* even when found in lower numbers *(13)*, causing a variety of diseases that are specific to the patient's age. In adults, *Cronobacter* spp. causes bacteremia, osteomyelitis, and vaginitis *(13, 14, 29, 47, 56)*. In infants, the diseases reported include septicemia, meningitis, and necrotizing enterocolitis *(1, 2, 6, 11-16, 18, 19, 23-29, 31-39, 41, 42, 44, 45, 49, 54-59)*. Septicemia is the severe infection of bacterium in the blood stream. Meningitis is the infection of the meninges, the membrane that encapsulates the brain and spinal cord. Necrotizing enterocolitis is the

infection of the gastrointestinal tract and portions of the bowel become necrotic. The mortality rates for infants has been reported as high as 80%, with the cases that have survived the infection afflicted with severe brain injuries *(6, 19, 31, 32, 35, 38, 41, 42, 44).*

LITERATURE REVIEW

Epidemiology of *Cronobacter* spp.

Cronobacter spp. are Gram-negative bacteria that can survive in a variety of different environments. They are able to survive at temperatures from 4°C to 80°C *(13, 24, 31)*. The optimal temperature for growth is approximately 39.4°C *(24)*. Some strains have been shown to be extremely thermotolerant (*6, 55, 59*) which may be due to the fact that some of the strains are able to produce a heat stable bacterial toxin *(55)*. They have been reported to have a short lag time at the optimal temperature of 39.4°C *(24)*. This genus is able to survive in low water activities such as powdered infant formula for at least four weeks *(24)*. *Cronobacter* spp. was reported to survive in low numbers for a year in powdered infant formula, and to survive the irradiation of smoked sardines *(9)*. There is also a study that reports that *Cronobacter* spp. was isolated in powdered infant formula after two and a half years of storage *(49)*. There have been reports of an increase in antibiotic resistance that in *Cronobacter* spp. *(6, 31, 36)*.

The mode of transmission as well as a reservoir for *Cronobacter* spp. are not yet defined *(2, 6, 36, 38)*. However, these bacteria have been discovered in the midgut of stable flies as well as in a variety of different foods and beverages including grains, fruits and vegetables *(9, 14, 19, 25)*. The severe disease outbreaks associated with *Cronobacter* spp. have been most commonly connected to powdered infant formula (PIF) (*9, 11-16, 18, 19, 23-29, 31-39)*. The mode of transmission has been researched in detail in connection to PIF because of the health risks associated with the immunocompromised infants that consume the PIF. However, despite exhaustive investigation the true reservoir of *Cronobacter* spp. remains undetermined.

In a study by Schmid et al they propose that plants may be a natural habitat for *Cronobacter* spp. *(51).* There are numerous traits that *Cronobacter* spp. express that leads to the suggestion that the natural habitat is of an environmental origin *(51).* These traits include the yellow pigmentation and the extracellular polysaccharide *(51).* In the study by Schmid et al it was found that the *Cronobacter* spp that were studied exhibited the production of indole acetic acid, IAA *(51).* IAA production has been suggested to promote plant development through microbial colonization and root development *(51).* This study by Schmid et al, also documented that *Cronobacter* spp. was able to colonize the root cells of plants and produce factors that are possibly beneficial to the growth of the plants *(51).* This lends credence to the possibility of *Cronobacter* spp. natural habitat being in plants *(51).*

The research has revealed several possible points of contamination of PIF with *Cronobacter* spp. A few studies have shown both intrinsic and extrinsic contamination of the PIF within processing plants *(6, 57).* This means that the contamination could have been introduced during the processing of the PIF or from instruments used to reconstitute the PIF *(6, 24, 31, 57).* The contamination of the PIF may occur after the product has been pasteurized by adding in ingredients that are contaminated such as starches or proteins *(51).* *Cronobacter* spp. is able to form biofilms on numerous different materials including stainless steel, plastic, and glass at a temperature of 25°C *(57).* Biofilms have been reported on spoons and blenders used to reconstitute PIF *(26, 31).*

Cronobacter spp. infects people in many different ways including attachment to the lining of the gut to cause necrotizing enterocolitis; this causes perforations to form in the lining of the stomach and intestines *(35, 40, 55, 57).* *Cronobacter* spp. has also been shown

to live in macrophages and dendritic cells *(6, 37)*. Some strains can also form biofilms, have the ability to make capsules, and express an enterotoxin *(6, 29, 35)*.

PIF has been implicated in most of the infant infections; therefore, new methods to destroy the pathogen in PIF have been researched, including bacteriophages and gamma-radiation with good results *(30)*. A letter has been sent to health professionals to help reduce the risk of *Cronobacter* spp. growing in the PIF reconstituted in hospitals *(56, 58)*. Some of these recommendations include preparing the PIF only when needed and keeping it refrigerated until needed *(56, 58)*. In a case study by Biering, et al. the strains identified from the patients and from the formula were identical through testing (biotyping, plasmid DNA profiles and antibiograms) done at the Centers for Disease Control *(1)*.

There have been few cases worldwide in infants, but the cases that have been documented have been severe with equally severe consequences *(6)*. There have also been cases of meningitis in infants caused by the bacteria traveling into the bloodstream. The movement of *Cronobacter* spp. into the cerebrospinal fluid has not been clearly defined but it has been hypothesized to start with a translocation of the bacteria using endotoxins, OmpA, and other factors to pass through the blood-brain barrier *(13)*. A study done by Mittal, et al. discussed that OmpA is an important factor in the reproduction of the bacterium *(37)*. The study reported that the *Cronobacter* spp that was OmpA positive was taken up by dendritic cells and was able to multiply within these cells without detection, while OmpA negative *Cronobacter* spp are taken up by the dendritic cells and killed *(37)*.

Cases of *Cronobacter* infections in immunocompromised infants may result in meningitis, and brain abscesses. Computerized Axial Tomography and Magnetic Resonance Imaging scans can show these brain abscesses *(*1, 2, *6)*. The infections usually

begin with the children being irritable and having high fevers *(2)*. According to Burdette and Santos, the patients that had *Cronobacter* spp. found in the cerebrospinal fluid then had CT scans to check for brain abscesses followed by a MRI *(2)*. More than 90% of the cases of meningitis caused by *Cronobacter* spp. infections have been found to cause the formation of brain abscesses (2*)*. In a case studied by Burdette and Santos, the fluid in the abscess grew *Cronobacter* spp. *(2)*.

The adult cases are different because they are not usually caused by food. These cases are usually non-life threatening and are found in conjunction with other infections *(6, 8)*. The case of vaginitis was caused by the patient bathing in a warm water pond that contained *Cronobacter* spp. *(47)*. The adults usually are infected with *Cronobacter* spp. when they have an underlying infection *(6)*.

Detection methods for *Cronobacter* spp.

Cronobacter spp. was first reported in 1961 in two cases of neonatal meningitis (*31*). Initially, *Cronobacter* spp. was first identified through checking for yellow-pigmented colonies on blood agar. However, this identification method resulted in false-negative and positives. Since then, detection methods have improved with new methods being discovered on a regular basis in order to develop more rapid methods. The more *Cronobacter* spp. is studied, the more scientists have searched for easier, faster ways to identify *Cronobacter* spp. accurately. The Codex Alimentarius Commission, a worldwide set of codes for food standards and guidelines, has set regulations on how many coliform bacteria can be found in PIF *(6, 36)*. The Codex Alimentarius Commission requirement for the number of coliforms found in PIF is 1-10 bacteria per gram of formula *(6)*. Coliforms consist of any gram-negative, rod-shaped bacteria,

including *Cronobacter* spp., *E.coli, Salmonella* spp., and *Listeria* spp. *(6).* These regulations are forcing manufacturing plants to look into other ways to detect the bacteria as well as making a more sterile product *(6)*. Many manufacturers employ a zero tolerance in regards to *Salmonella* and *Listeria* species *(6).*

The U.S. Food and Drug Administration (FDA) have a protocol in place to test PIF that takes five days to identify *Enterobacteriaceae* (*6, 31, 40)*. This protocol starts with an enrichment process to ensure healthy cells can be isolated *(13, 31)*. After the enrichment process it streaked on violet red bile glucose (VRBG) agar, then from that agar it is transferred to tryptic soy agar (TSA) *(13,31)*. After growing on the TSA agar the colonies are typed using an API 20E® kit *(13, 31)*. This process takes at least 5 days to complete and it still has not accurately identified *Cronobacter* spp. *(13)*, too long for the manufacturer to take appropriate action to prevent the PIF from reaching the consumer. The product can be sold and transported to several different facilities in the time it takes to get the results back from the lab. Therefore a more reliable and faster method of correctly identifying *Cronobacter* spp. is needed to ensure that safe products are being sold and thus reduce the number of recalls of PIF.

The methods of detecting *Cronobacter* spp. have changed in the years since the initial cases were reported in literature. After it was found to have a different DNA makeup than *Enterobacter cloacae* there was a change in detection methods that involve looking at the DNA and the 16S rRNA sequences *(31)*. The new methods of detection supplement the other methods that have been used previously. One method studied used *hsp60* sequencing in conjunction with the 16S rDNA to differentiate *Cronobacter* spp.

(13). Another study looked at the internal spacer regions within the 16S and 23S genes to differentiate the isolates at the intraspecies level *(4)*.

There are numerous PCR methods used to detect *Cronobacter* spp. including real-time PCR using a TaqMan probe that targets the macromolecular synthesis operon *(6)*. Another PCR method used is made by DuPont, called the Bax system, and includes many primers in a tablet that are specific for *Cronobacter* spp. *(7)*. One such primer set published by Iversen, et al. is EsAgF, and EsAgR; these primers are specific for the *glu*A gene in *Cronobacter* spp. (otherwise known as *Enterobacter sakazakii)*. Pulse-field gel electrophoresis has also been used to show relatedness between strains *(13, 31, 41)*.

The methods to identify *Cronobacter* spp. include specialized agars that detect different pathogens *(21)*. Many different agars that have been developed are able to target the α-glucosidase enzyme. On Druggan, Forsythe, and Iversen (DFI) agar, *Cronobacter* spp. grows as bright green colonies *(21)*. Formerly, another difference between *Cronobacter* spp. and other *Enterobacter* spp. is that all strains of *Cronobacter* spp. were not able to ferment D-sorbitol. This has since proven untrue *(13)*. Other methods that are used to detect *Cronobacter* spp. include phenotypic methods, comprising of "biochemical traits, serotyping, bacteriophage typing and antibiotic resistance" *(13)*. These typing schemes have not been found to differentiate *Cronobacter* spp. accurately *(13)*.

Risk factors associated with *Cronobacter* spp.

The epidemiologic criteria for causal factors include the strength of the association, consistency of the effect, the timing of the sequence, and the specificity of the effect. These factors are used by epidemiologists to decide whether there is a causal association between

an etiological agent and the disease. The strength of the association shows the correlation between the identified organism and the disease that has then occurred. The consistency of the effect shows that when the *Cronobacter* spp is present in the PIF the infants become ill most of the time. Timing of the sequence shows that the disease occurs after the ingestion of the bacteria laden PIF. The specificity of the effect means that the bacterium does not cause a myriad of diseases or vice versa. This means that in the cases that were reported and researched that the bacterium only caused the one disease (necrotizing enterocolitis, meningitis, or septicemia), and that there were no other causative bacterial agents found in these cases.

Research has shown that *Cronobacter* spp. in infants have a strong association with PIF *(11-16, 18, 23-29, 31-39, 54-59)*. *Cronobacter* spp. does cause different diseases in infants, but they are still connected by PIF. In a case of necrotizing enterocolitis half of the infants were fed a certain brand of PIF which tested positive for *Cronobacter* spp. *(57)*. The infants were also found to have positive cultures *(57)*. This same hospital also tested other infants that were not fed this PIF and found that none of these infants had positive cultures for *Cronobacter* spp. *(57)*. It was also shown that only after the ingestion of the PIF did the infants present with the symptoms of the disease *(57)*. After the symptoms appeared, the infants were tested for bacteria in their sputum, blood, stomach and fecal material *(57)*. The bacteria that was found in most of the patients was *Cronobacter* spp. *(57)*.

Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) is a process that allows for the genetic comparison of bacterial strains that are implicated in disease. In this process the DNA of

the bacterial strains are restriction enzyme digested using the same enzyme and then run on a gel using pulses of electrical current from different directions to ensure that the large DNA fragments are able to move through the gel. The DNA shows bands of differing lengths depending on how many times the enzyme cuts the strands. This allows for the bacterial isolates to be compared to each other. In this comparison the isolates to each other, the bacterial isolates can be shown to be similar in composition if the banding patterns are similar to each other.

The standards of PFGE similarity are not documented other than to be considered similar there must be at least 90% band similarity between isolates *(52, 53)*. Identical isolates must have approximately 98-100% band similarity *(52, 53)*. Any other band similarity would be considered dissimilar.

MATERIALS AND METHODS

Bacterial isolates

The two *Enterobacter sakazakii* isolates found in bison and bovine feces were identified through 16S RNA sequencing by Nangoh et al. Other isolates were obtained from ATCC and Cornell University that had been previously identified as *Enterobacter sakazakii* (see Table 1). All of the isolates used in this study were maintained on agar plates in the refrigerator, and also kept at -80°C with the addition of glycerol. These 36 isolates were then cultured onto tryptic soy agar (Becton, Dickinson and Company, Sparks, MD) plates overnight at 37°C.

Pulsed Field Gel Electrophoresis

A colony was selected and added to 2 ml of cell suspension buffer (10 ml of 1 M Tris, pH 8.0; 20 ml of 0.5 M EDTA (ethylene-diamine-tetra-acetic-acid), pH 8.0. This was further diluted in 100 ml with sterile water to an optical density of between 1.45 and 1.55 at a wavelength of 610 nm. Next, 400 µl of this solution was added to 20 µl of Proteinase K (Amresco, Solon, Ohio). Then 400 µl of 1% SeaKem Gold (SKG) (Cambrex Bioscience Rockland, Inc., Rockland, ME) agarose gel (0.50 g SKG to 49.5 ml of TE (Tris/EDTA) buffer cooled to 58°C) was added to the mixture. This solution was placed in a disposable plug mold (Bio-Rad Laboratories, Hercules, CA) and allowed to set for 10 minutes. After setting, the plugs were placed in 5 ml of cell lysis buffer (25 ml of 1 M Tris, pH 8.0; 50 ml of 0.5 M EDTA, pH 8.0; 5 g of Sarcosyl; and 420 ml sterile water) and 25 µl Proteinase K (Amresco, Solon, Ohio) overnight in a shaking incubator at 54°C at ~90 RPM (New Brunswick Scientific, Edison, NJ). The next day the plugs were washed with 15 ml of 50°C water with shaking at ~150 RPM in the shaking incubator at 50°C for twenty minutes, and

repeated for a total of two washes. The plugs were washed with 10 ml of TE buffer heated to 50°C (10 ml of 1 M Tris, pH 8.0; 2 ml of 0.5 M EDTA, pH 8.0; diluted with sterile water to 1000 ml) with shaking at ~150 RPM at 50°C for twenty minutes, and repeated for a total of four washes. After washing, the plugs were placed in 1.7 ml of TE buffer and stored at 4°C until applied to a gel.

The plugs were cut into 2 mm wide slices and 1 slice per isolate was placed in a tube with 100 µl of an *Xba*I restriction enzyme (Invitrogen, Carlsbad, CA) mixture (86.5 µl sterile water, 10 µl buffer, 2.5 µl *Xba*I enzyme, and 1 µl Bovine Serum Albumin, BSA). The plug slices were then incubated at 37°C for 2 hours. The mixture was removed from the plug slices and incubated for 5 minutes in 200 µl of 0.5X TBE at room temperature. Next, the plug slices were removed and placed on the 15 well comb on teeth 2-7 and 9-14; three lambda marker plug slice were placed on teeth 1, 8, and 15. Any excess moisture was removed and the plugs were allowed to dry for 10 minutes. A 1% SKG (Cambrex) agarose gel (1 g SKG Agarose, 100 ml 0.5X TBE (Tris/ Borate/ EDTA) was heated and allowed to cool to 58°C) was poured into the gel form and allowed to set for 30-45 minutes. The gel was then placed in the Chef Mapper (Bio-Rad Laboratories, Hercules, CA) gel electrophoresis chamber and the program was set for 18 hours with an initial switch time of 2.16 seconds and a final switch time of 63.8 seconds. After 18 hours the gel was placed in an ethidium bromide solution (40 µl into 400 ml of ddH_2O) for 30 minutes, and destained in ddH_2O for 60 min (changing the water every 20 min). Imaging was performed using a fluorescence detection method in the Auto Chemi System (UVP BioImaging Systems, Upland, CA). The images were then placed in the Fingerprinting II Informatix system (Bio-Rad Laboratories, Hercules, CA). This process was then repeated using the *Spe*I enzyme.

Table 1: Bacterial Isolates

Isolate ID	Source
N72	Bovine feces
CT2	Child's throat
52	Bison feces
ATCC 51329	unknown
ATCC 29544	Child's throat
ATCC 29004	unknown
ATCC 12868	unknown
F6-023	cornell clinical
F6-024	cornell food
F6-025	cornell environmental
F6-026	cornell environmental
F6-027	cornell environmental
F6-028	cornell clinical
F6-029	cornell clinical
F6-030	cornell food
F6-031	cornell food
F6-032	cornell food
F6-033	cornell food
F6-034	cornell clinical
F6-035	cornell clinical
F6-036	cornell environmental
F6-037	cornell environmental
F6-038	cornell environmental
F6-039	cornell environment
F6-040	cornell environmental
F6-041	cornell environmental
F6-042	cornell environmental
F6-043	cornell clinical
F6-044	cornell food
F6-045	cornell food
F6-046	cornell food
F6-047	cornell food
F6-048	cornell food
F6-049	cornell clinical
F6-050	cornell clinical
F6-051	cornell clinical
F6-052	cornell clinical

Polymerase Chain Reaction (PCR)

PCR was performed on the isolates looking for the *gluA* gene in the isolates. The isolates were struck onto MacConkey Agar and incubated overnight at 37°C. The isolates, along with ATCC 10798 *Escherichia coli* as a negative control, and water as a reagent control were then placed into a mixture of 1:100, Protease K and TE buffer. This was placed in the thermocycler for 20 minutes, 10 minutes at 55°C and 10 minutes at 80°C. The tubes were then placed in the -20°C freezer. Then PCR was performed using 2 µl of the SCLB, 17.875 µl water, 0.25 µl EsAgF (5' TGA AAG CAA TCG ACA AGA AG 3'), 0.25 µl EsAgR (5' ACT CAT TAC CCC TCC TCC TGA TG 3'), 0.5 µl dNTPs, 5 µl 5x buffer and 0.125 µl taq polymerase. This PCR mixture was placed in the thermocycler for an initial denaturation step of 94°C for 2 minutes, followed by 29 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 60 seconds, and extension at 72°C for 90 seconds, followed by a final elongation step at 72°C for 5 minutes. After the PCR was completed the isolates were then placed in a 2% agarose gel for approximately 1.5 hrs. The gel was then placed in ethidium bromide for approximately 30 minutes to stain, and then water for approximately 5 to 15 minutes. The gel was exposed to fluorescent light for imaging.

Biochemical tests

The four biochemical tests were for dulcitol, indole, malonate and methyl-α-D-glucopyranoside (AMG). Each of the different medias, (phenol red, Methyl Red-Vogues Proskauer, sodium malonate broth, and phenol red broth with Durham tubes, respectively), were prepared as described by Iverson et al. *(22)*. The isolates were cultured on MacConkey Agar and incubated overnight in a 37°C and then a single colony was picked

and added into each of the media. The four different tubes of media were placed in the incubator for 1-2 days at 37°C. The dulcitol, indole, and malonate tests were all performed in triplicate, and the AMG test was performed in duplicate.

Remel RapidID

The isolates that did not show the *glu*A gene were identified using the Remel RapID test. The Remel RapID test was started by growing the isolates overnight in a 37°C incubator on MacConkey Agar. The next day, approximately 3 to 4 colonies were placed in the provided tubes of RapID™ inoculation fluid. The absorbance of a blank tube and the McFarland standard is read at A_{600}. The tubes with the inoculation fluid and isolates are then read to ensure an absorbance of .2, next all of the inoculation fluid in the tubes is pipetted into the trays, and mixed thoroughly. The inoculation fluid was then tipped at a 45° angle into each of the wells equally and the trays are incubated for 4 hours at 37 °C. After the four hours the trays are taken out and placed into the Biomic program (Giles Scientific, Santa Barbara, CA).The computer program is used to identify the results of the tray and identify the isolates.

RESULTS

The PFGE results showed that all of the isolates, when the *Xba*I enzyme was used, had approximately 9-30 DNA fragments. All of the *Cronobacter* spp. isolates were approximately 85% dissimilar. Two out of the ten of the clinical isolates received from Cornell University had a high degree of similarity to each other, approximately 95%. The two isolates provided by Nangoh et al were found to be approximately 75% dissimilar to each other. The bovine fecal isolate was more similar to an isolate from a clinical source, approximately 45% dissimilar. The bison fecal isolate was approximately 35% dissimilar to an isolate from a food source. See Figure 1 for the Pulsed-Field Gel Electrophoresis dendrogram.

The isolates had approximately 10-40 DNA fragments when digested with the *Spe*I enzyme. This enzyme showed again that there was a high degree of diversity among the isolates. The two isolates provided by Nangoh et all were found to be less similar to each other at approximately 35% dissimilar. The bovine fecal isolate was more similar to an isolate from a food source, at approximately 22% dissimilar. The bison fecal isolate was approximately 28% dissimilar to an isolate from a meningitis source. Two different isolates from Cornell University showed a degree of dissimilarity at 15%. See Figure 2 for the Pulsed-Field Gel Electrophoresis dendrogram.

The PCR results were to show if the isolates carried the *glu*A gene that has been shown by Iverson, et al. to be an indicator of the *Cronobacter* genus. Only three Cornell isolates did not show a band for the *glu*A gene, all of the other isolates did have this gene. The isolates that did not have the *glu*A gene were F6-026, F6-033, and F6-045. These three organisms, bacterial genera were later determined using biochemical analysis.

The biochemical test results were either positive or negative and are shown in Table 2. The Remel RapidID biochemical test results were either positive or negative and the software then identified the three bacterial isolates that did not contain the *glu*A gene as *Enterobacter sakazakii* (F6-026), *Pantoea agglomerans* (F6-033), and *Escherichia hermannii* (F6-045).

Table 2: Biochemical Tests Performed on Isolates, PCR Results, and Identification of Isolates.

Isolate ID	Dulci-tol	Dulci-tol	Dulci-tol	In-dole	In-dole	In-dole	Malo-nate	Malo-nate	Malo-nate	AMG	AMG	PCR	Species
F6-023	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-024	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-025	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-026	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	neg	csak sak
F6-027	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-028	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-029	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-030	neg	neg	K	neg	neg	neg	pos	pos	pos	A	A	pos	csak malon
F6-031	pos	pos	AG	pos	pos	neg	pos	pos	pos	K	K	pos	cmuyt
F6-032	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-033	pos	pos	AG	neg	neg	neg	neg	neg	pos	K	K	neg	pagglom
F6-034	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-035	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-036	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-037	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-038	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-039	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-040	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-041	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-042	neg	neg	K	pos	pos	neg	neg	neg	neg	K	K	pos	cmuyt
F6-043	pos	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-044	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak

Table 2: Continued

Isolate ID	Dulci-tol	Dulci-tol	Dulci-tol	In-dole	In-dole	In-dole	Malo-nate	Malo-nate	Malo-nate	AMG	AMG	PCR	Species
F6-045	pos	pos	A	pos	pos	pos	neg	neg	neg	K	K	neg	eherman
F6-046	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-047	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-048	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-049	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-050	neg	neg	K	pos	pos	neg	neg	neg	neg	K	K	pos	cmuyt
F6-051	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
F6-052	neg	neg	K	neg	neg	neg	pos	pos	pos	AG	A	pos	csak malon
N72	neg	neg	AG	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
CT-2	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	AG	pos	csak sak
52	pos	pos	AG	neg	neg	neg	pos	pos	pos	AG	AG	pos	ctur
ATCC 51329	pos	pos	AG	pos	pos	pos	pos	pos	pos	AG	NG	pos	
ATCC 29544	neg	neg	K	neg	neg	neg	neg	neg	neg	A	A	pos	csak sak
ATCC 29004	neg	neg	K	neg	neg	neg	neg	neg	neg	AG	NG	pos	csak sak
ATCC 12868	neg	neg	K	neg	neg	neg	neg	neg	pos	AG	AG	pos	csak sak
ATCC 10798 E. coli	pos	pos		pos	pos		neg	neg		K	K	neg	ecoli

Neg=Negative, Pos= Positive, AG= Acid with gas production, K= Alkaline, A= Acid, Csak sak= *Cronobacter sakazakii* subspecies *sakazakii*, Csak malon= *Cronobacter sakazakii* subspecies *malonaticus*, Csak muyt= *Cronobacter muytjensii*, Ctur= *Cronobacter turicensis,* Pagglom= *Pantoea agglomerans*, Eherman= *Escherichia hermannii*

Figure 1: PFGE Dendrogram Using *Xba*I Enzyme

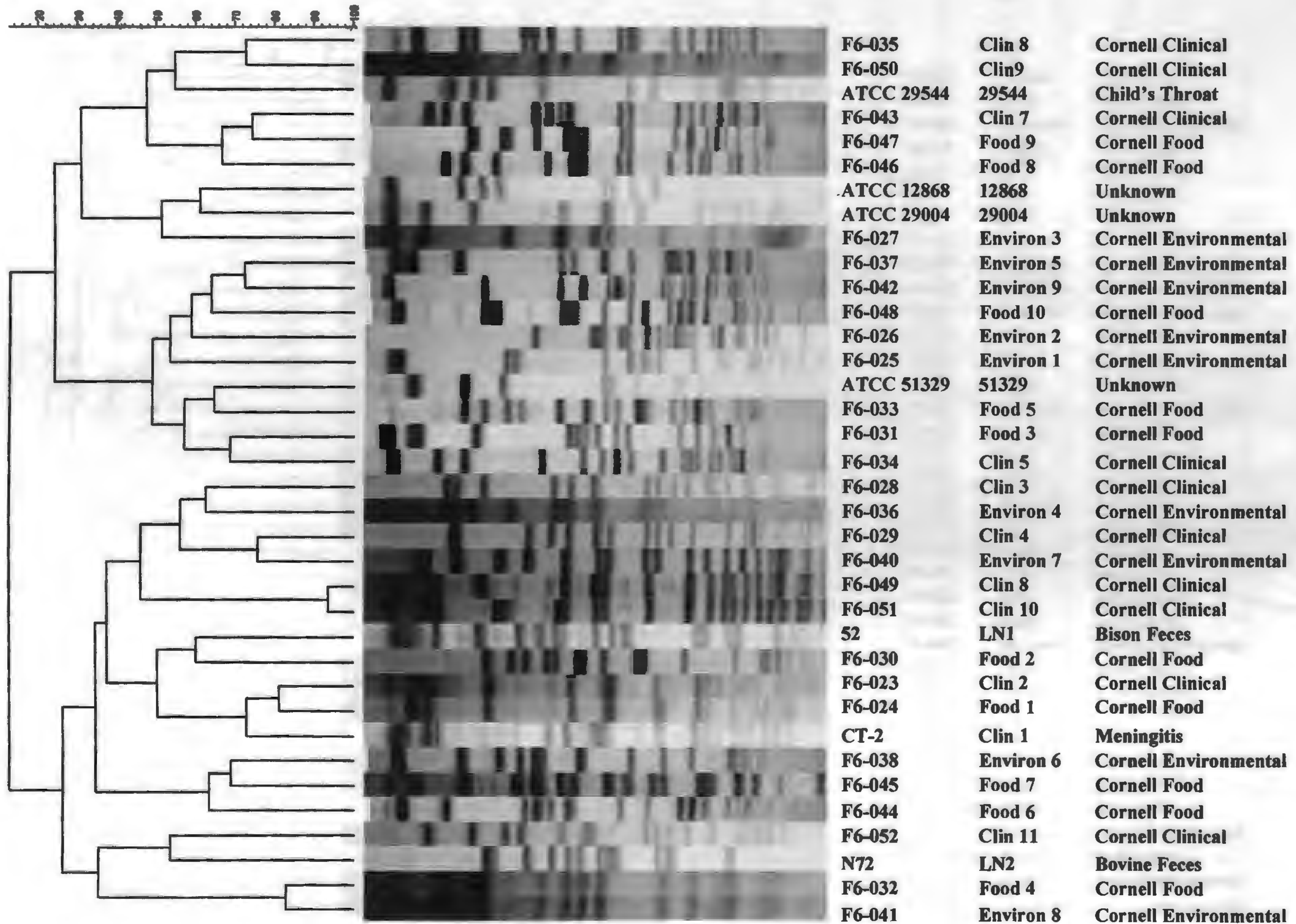
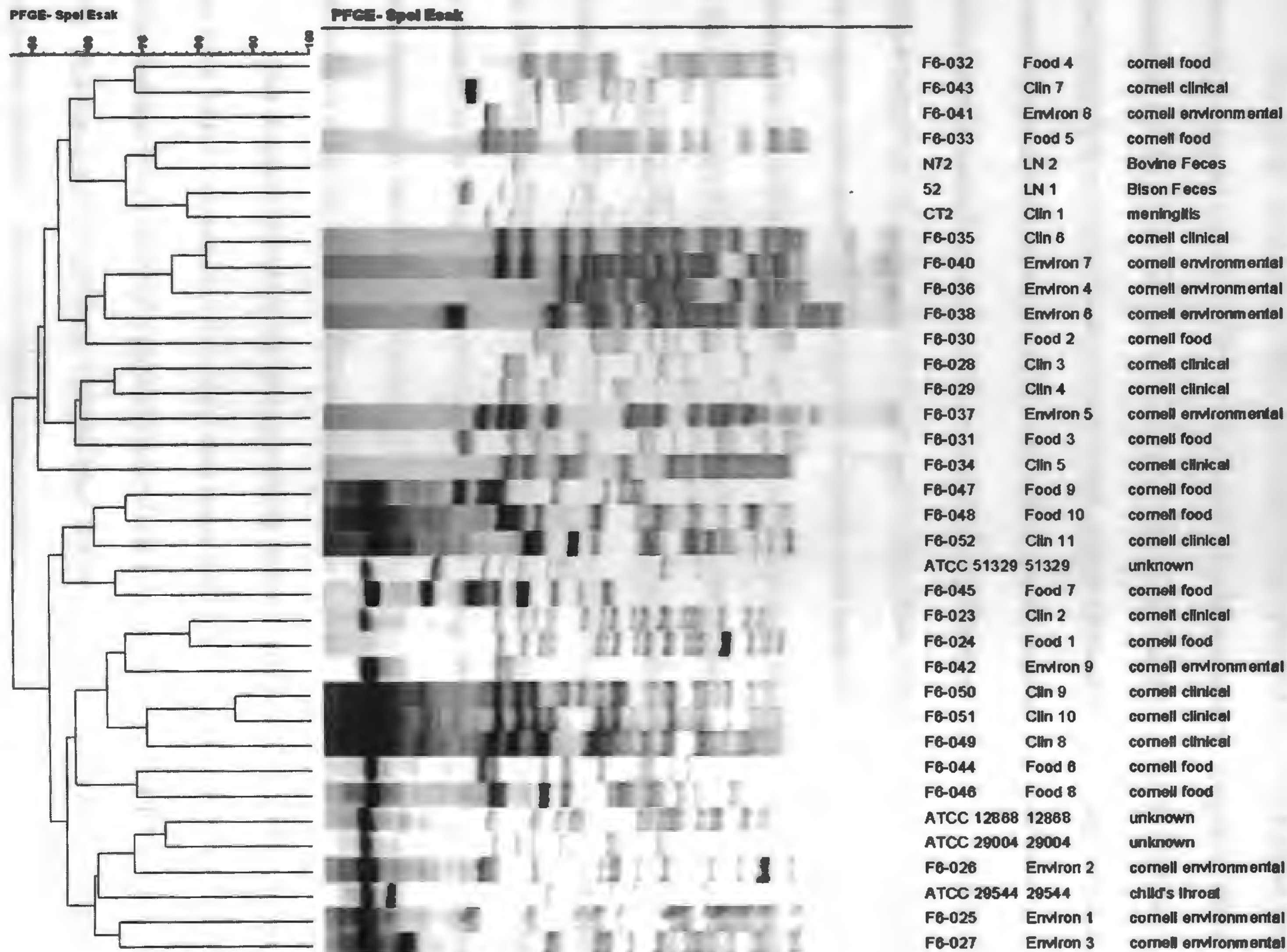


Figure 2: PFGE Dendrogram Using *Spe*I Enzyme



DISCUSSION

The overall results of the PFGE lead to the conclusion that all of the isolates tested have a high degree of diversity. This lends credence to the proposal by Iversen, et. al. to change the genus to *Cronobacter* with multiple species *(22)* as the isolates in this study were highly diverse. All the isolates show a high degree of dissimilarity to each other, as the standards that have been previously set are that isolates must have at least 90% similarity to be considered truly similar. The pulsed-field analysis showed that each of the isolates tested was uniquely different from each of the others, resulting in the fact that only two isolates were similar according to the standards of at least 90% band similarity, between the two different enzymes used. Therefore this study shows the genetic diversity of the isolates tested as well as the similarity of the types of species.

The highest degree of similarity was found between two isolates from Cornell University, F6- 049 and F6- 050, which were found to be 95% similar to each other using the *Xba*I enzyme. These two isolates were both from clinical sources. Therefore, it should be checked to see if they are from the same patient or from two separate patients within the same outbreak to see if this finding is significant. The degree of dissimilarity between these two isolates using the *Spe*I enzyme was 30%.

The degree of dissimilarity amongst all the isolates tested using the *Xba*I enzyme was 87%, which demonstrates that there were more differences than similarities amongst all the isolates. When the *Spe*I enzyme was used, all the isolates showed a dissimilarity of 55%. This was a better result than with the *Xba*I enzyme but still demonstrates that there are greater differences than similarities amongst the isolates tested.

Since bison is used mainly for its meat, it makes sense that the bison fecal isolate is more similar to a food source. Bison milk is also used to make certain specialty cheeses, but this would also lend itself to being more similar to the food source isolate. The bovine fecal sample was similar to a clinical isolate, and this is logical as the source of milk for powdered infant formula is from bovine sources. It is tempting to see this as evidence that the bovine was infected with a *Cronobacter* spp. isolate that may be able to cause disease. This supports unpublished data from Dr. Gibbs' Lab performed by Lilian Nangoh *(46)*. Ms. Nangoh carried out an embryo lethality assay (ELA) on the two fecal isolates and the four ATCC strains as well as the clinical isolate from a meningitis case *(46)*. The ELA showed that 75% of the embryos died when infected with the isolate from the bovine feces *(46)*. Conversely, the ELA determined that the isolate from bison feces showed a low to moderate lethality rate of 19.4% *(46)*. The bovine isolate had a lethality rate more commensurate with the isolates that were associated with clinical disease, and the bovine isolate in fact had the highest lethality rate in the study by Ms. Nangoh *(46)*. The CT-2 isolate had a lethality rate of 66.7%, and the ATCC 29544 had a lethality rate of 72.2% *(46)*. These findings are interesting because the true source of *Cronobacter* spp. in powdered infant formula (PIF) has yet to be determined. Further studies determining whether *Cronobacter* spp. are present in the milk provided for PIF would be essential to determine if the milk used in the process of making PIF is a potential source of contamination. This possibility is feasible because previous studies have shown that the *Cronobacter* spp. is extremely resistant to desiccation.

The DNA of the isolates were also cut with the *Spe*I enzyme and had different results than with when they were cut with the *Xba*I enzyme. This actually switched the

bison and bovine results around. The bison, with this enzyme was more closely related to a meningitis case. While the bovine isolate was more closely related to a food source isolate. An important note to make at this point is that this food source isolate did turn out to not have the *glu*A gene that was shown in the PCR, and through a Remel Id was actually shown to be *Pantoea agglomerans*. *P. agglomerans* is an opportunistic bacterium within the Enterobacteriaceae family. It is a gram-negative bacillus that is associated with infections in humans that are in contact with penetrating vegetation wounds *(5)*.

The second enzyme, *Spe*I, was utilized to determine if there was more or less similarity among the isolates. The results were different than the *Xba*I enzyme; lending more credence to the suggestion that these isolates within the *Cronobacter* spp. are very different from each other. The isolates that were identified as *C. muytjensii* were distributed amongst the other isolates and were found to be approximately 75% dissimilar, when the *Xba*I enzyme was used. The isolates that were identified as *C. sakazakii* subspecies *malonaticus* were also separated by other isolates when the *Xba*I enzyme was used and were found to be only approximately 75% dissimilar as well. This is interesting due to the wide differences even among the species.

When the *Spe*I enzyme was used the two isolates that were identified as *C. muytjensii* were distributed amongst the other isolates and were found to be approximately 38% dissimilar, when the third isolate was added then the dissimilarity was 55%. The isolates that were identified as *C. sakazakii* subspecies *malonaticus* were also separated by other isolates and were found to be only approximately 55% dissimilar as well.

Another interesting note is that the two isolates that have been identified as *C. sakazakii* subspecies *malonaticus*, F6-030 and F6-052, were from an environmental source

and a clinical source, respectively. The isolates that were identified as *C. muytjensii*; F6-031, F6-042, and F6-050; were from a food source, an environmental and a clinical source, respectively. The isolate identified at *C. turicensis* was the isolate from the bison feces that was isolated by Nangoh et al.

These results lend confidence to the proposal by Iverson, et al. that the name of the *Enterobacter sakazakii* should be changed to a new genus name of *Cronobacter* with several different species and subspecies; *Cronobacter sakazakii* subspecies *sakazakii*, *Cronobacter sakazakii* subspecies *malonaticus*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter turicensis*, and *Cronobacter* genomespecies 1.

This study also showed that the isolates were different among the different species and similar to other species within the *Enterobacteriaceae* family. This was shown by the biochemical tests that identified each of the isolates, the four biochemical tests differentiated among the *Cronobacter* spp. and the Remel RapidID identified the other bacteria. The isolates that were identified as a *Cronobacter* spp. were mostly identified as *C. sakazakii* subspecies *sakazakii* but the PFGE showed that they were not closely related. This means that there was a great diversity even among the species. The results also showed that although they were in the same species the most closely related isolate may not have even been a *Cronobacter* spp. This was seen in the bovine feces isolate that was typed as a *C. sakazakii* subspecies *sakazakii* was more closely related to a *P. agglomerans* through the PFGE.

There was one isolate that was not able to be speciated by the biochemical tests. The isolate was ATTC 51329. This isolate had test results that did not match up with any of the standard results as listed in the paper by Lehner, et al. If the Indole test was negative

instead of positive the isolate could be *Cronobacter turicensis*, or if the AMG test was K (alkaline) instead of AG (acid with gas production) then the isolate could be *Cronobacter muytjensii*. Therefore all tests for this isolate should be repeated to see if the tests come back differently. The other isolates that did not fall into one of the species were F6-033 and F6-045. These isolates were identified by Remel ID to not be *Cronobacter* spp. but were instead identified as *Pantoea agglomerans*, and *Escherichia hermannii*. There was another isolate, F6-026, that was identified as *Cronobacter sakazakii* subspecies *sakazakii* through biochemical tests, but was not in possession of the *glu*A gene as indicated by PCR results.

The PCR was performed to ensure the isolates were in fact part of the genus *Cronobacter*. The PCR was specific for the *glu*A gene that Lehner et al. showed was particular for *Cronobacter* species. The *glu*A gene is responsible for α-glucosidase activity by the *Cronobacter* spp. After the PCR results were shown to either have the *glu*A gene or not have the gene, the biochemical tests were performed to speciate each of the isolates into the 5 different *Cronobacter* species. Approximately 75% of the isolates were shown to be *Cronobacter sakazakii* subspecies *sakazakii*, including the isolate from the bovine feces. The other isolates were *Cronobacter sakazakii* subspecies *malonaticus*, *Cronobacter muytjensii*, *Cronobacter* genomespecies 1 or *Cronobacter turicensis*. These biochemical tests were to show whether the isolates were utilizing dulcitol, methyl-α-D-glucopyranoside, malonate and tryptophane (for indole production). Iverson, et al. determined these tests to be the four most important tests to define each of the six different species and subspecies. The only problem tends to be in the differentiation of *C. turicensis* and *C.* genomespecies 1. At this time there is no phenotypic way to differentiate *C.* genomespecies 1 from *C. turicensis* but the paper by Iverson et al. explains that there were

only two isolates tested in their lab that fell within the category of *C.* genomespecies 1. Therefore, the one isolate tested in our lab, 52 (bison feces), is most likely part of the *C. turicensis* group rather than the *C.* genomespecies 1 since *C.* genomespecies 1 isolates are relatively uncommon. Another reason to think that this isolate is part of *C. turicensis* is that the proposed type strain isolate for *C. turicensis* is from a fatal case of neonatal meningitis as published by Iversen et al. and the ELA results for isolate 52 were a low to moderate lethality. Isolate 52 was also relatively dissimilar, 23%, to the CT-2 meningitis case when the *Spe*I enzyme was used in the PFGE. The paper published by Iversen et al. also proposes that the type strain isolate for *C.* genomespecies 1 is from a water source.

There was a proposal to change the classification of *E. sakazakii* to *Cronobacter* spp. by Carol Iversen, et al. *(22)*. This name change was proposed due to the high diversity among the *E. sakazakii* isolates. This not only changed the name but introduced new species. The new classification is *Cronobacter sakazakii* subspecies *sakazakii, Cronobacter sakazakii* subspecies *malonaticus*, *Cronobacter muytjensii*, *Cronobacter* genomespecies 1 and *Cronobacter turicensis (22)*.

The results of this study lend significant support to the name change. The isolates showed a wide amount of diversity when digested with the enzyme. The different species and subspecies did not clump together for either enzyme used. They showed dissimilarities to different species or subspecies. As previously mentioned a few that were thought to be *Cronobacter* spp., and later turned out to not be, were found to be more closely related to isolates that were *C.sakazakii* subspecies *sakazakii*, or *C.muytjensii.* Using the *Xba*I enzyme the ATCC 51329 was found to be about 38% dissimilar to the *P. agglomerans*

isolate, and it was also found to be more closely related using the *Spe*I enzyme to the *E. hermanii* isolate at about 34% dissimilar.

This study has a few limitations associated with it. The main limitation was that there were only a few isolates tested. The lack of isolates comes from the fact that it is difficult to identify *Cronobacter* spp. Another limitation was that the isolates that were tested gave different results in the tests that were performed. Isolates that were previously identified as *Cronobacter* spp. (*Enterobacter sakazakii)* were now identified as other genus or species by one test and by another test were confirmed as being a part of the *Cronobacter* spp. This was the case with the Cornell isolate F-026. Through PCR the isolate was shown to not have the *glu*A gene. The isolate was then tested through the Remel ID program and was reported as being *Enterobacter sakazakii (Cronobacter* spp.). The tests that were used do not agree if this isolate is indeed a member of the *Cronobacter* spp. or if it is merely a member of the *Enterobacteriaceae* group that is related to the *Cronobacter* spp. Although the different results lend credence to the paper from Lehner, et al. it also shows the difficulty in ensuring that the isolates that are available are in fact *Cronobacter* spp. These problems show that there is an immediate need to be able to find a test or a series of tests to show definitively that an isolate is a part of the *Cronobacter* spp.

The next step in this study would be to check the isolates with more tests to ensure the identity of the isolates. More isolates should be procured for testing as well. These isolates should be from known sources and have known species identification, to ensure the tests done are corroborating the identity. A wider variety of isolates within the *Cronobacter* spp. would be of importance to test as well. An isolate from each of the different subspecies would be of interest to test. Testing that was analyzed by a computer would be

an excellent idea so that there would be no inaccuracy in reporting of the results. The test results for the biochemical tests can sometimes be read as either positive or negative (a blue/green color).

Following this, the study should do a more comprehensive testing to discover the lethality of all of the isolates available. This could be done using the embryo lethality assay that was used previously in Dr. Gibbs' lab. The isolates should also be digested with other enzymes to show any similarities or differences amongst the isolates using PFGE. A select few isolates could also be tested with a variety of other isolates within the *Enterobacteriaceae* family to see if the isolates show any similarity to other species. Isolates from each of the subspecies of *Cronobacter* spp. would be important to compare using PFGE to see if there are any similarities, or if there are just more differences.

CONCLUSION

A future step in this study that could be taken is to test the isolates for biofilm formation to see if isolates that are similar, according to the PFGE, are able to cause infection and also form biofilms. This could also be evidence of biofilm formation as a pathogenic mechanism if the isolates that have a higher lethality rate are able to form biofilms. Bacterial virulence factors should be studied as well, such as siderophore production and indole acetic acid production to form a more comprehensive view of the pathogenesis of *Cronobacter* spp.

An additional area that needs to be studied is how the bacterium is found in PIF. There are several hypotheses as to how this is happening. The hypothesis within this lab is that cows are possible carriers of the bacteria and it is then passed from the cow to the milk and this milk is then dried to make PIF. This hypothesis should be tested by testing both cows and their milk to see if *Cronobacter* spp. are discovered. In a study by Chaves-Lopez, et al. they discuss that Enterobacteria are a part of the normal flora of many dairy products, and that contamination seems unavoidable *(3)*. This lends credence to the hypothesis that it is already in the milk before processing.

Plants should also be studied to see if they are the natural habitat of *Cronobacter* spp. as described in Schmid et al *(51)*. A wide variety of plants should be checked to find if *Cronobacter* spp. is found to grow in them naturally. Another area to check would be if the ingredients that are added post sterilization are plant based. If it is found that plants are a natural habitat of *Cronobacter* spp. and these plants are used to produce ingredients used in the production of PIF, then this would lend credence to the hypothesis of *Cronobacter* spp. being added during the production of PIF.

It would also be interesting to discover if the isolates that have a lower lethality rate are in possession of a working *Omp*A gene as discussed in the paper by Mittal, et al. *(37)*. This could be done easily with a PCR test. This would show that the *Omp*A gene is important not only in the pathogenicity but in the virulence of *C.*spp. If there was found to be a connection then it would also be a starting point to search for ways to possibly prevent the bacterium from causing disease in infants and immune compromised individuals.

Cronobacter spp. should continue to be studied in the areas of pathogenesis, and habitat. In continuing to study the bacterium a deeper understanding of how it is infecting infants, and how it is contaminating the PIF will be found. This will lead to ways to ensure *Cronobacter* spp. does not contaminate PIF in the future and if an infection does occur, the best way to treat the patients.

This study highlighted the fact that there is a high diversity among all of the isolates tested. The study also demonstrated the wide diversity among the isolates making it difficult to positively identify the isolates as belonging within the genus *Cronobacter* spp. as shown with the contradictory identifications of the F6-026 and the inability to speciate the ATCC 51239. This study reports there are some similarities among the isolates and most of them were identified as *C. sakazakii* subspecies *sakazakii*, but there are differences in their profiles that make them unique. It also highlighted the need for more observation of *Cronobacter* spp. both in transmission and in identification.

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