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BIOLOGICAL CHARACTERIZATION OF *ENTEROBACTER SAKAZAKII*

by

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ABSTRACT

Enterobacter sakazakii, a motile peritrichous gram-negative rod, was designated a unique species in 1980, based on differences from *Enterobacter cloacae* in DNA relatedness, pigment production and biochemical reactions. This organism has been implicated in a severe form of neonatal meningitis. Although studies have failed to find an environmental source for the organism, dried-infant formula has been implicated in both outbreaks and sporadic cases of *E. sakazakii* meningitis. Reported case-fatality rates vary from 40 to 80 %. The severity of the infection in infants plus the lack of information on the ecology and pathogenicity of this organism led to the study of the biological characterization of the organism. Strains of *E. sakazakii* were isolated from dried-infant formula available on the Canadian retail market. The prevalence varied from 0-12 % in 120 samples evaluated from 5 different companies. Nine clinical *E. sakazakii* strains were obtained from Canadian hospital culture collections. Identification of all *E. sakazakii* strains was confirmed using the API 20E identification system. Minimum growth temperatures of 7.0 to 8.5°C were observed by using a temperature-gradient incubator. The potential for growth of *E. sakazakii* was followed using cocktails of food and clinical isolates in three different formula incubated at 4, 10 and 23°C. Average generation times were 40 min at 23°C and 4.98 h at 10°C. *E. sakazakii* strains did not grow at 4°C and appeared to die-off during storage. Heat resistance studies using 10 *E. sakazakii* strains (5 clinical and 5 food isolates) determined the heat resistance of this organism at 52, 54, 56, 58 and 60°C in reconstituted dried-infant formula. D- values of

54.8, 23.7, 10.3, 4.2 and 2.5 min, were obtained at each temperature respectively. The overall calculated z- value was 5.82°C. To date there are no known literature reports describing virulence factors or minimum infectious dose of *E. sakazakii*. Using the standard suckling mouse assay to evaluate enterotoxin production, it was found that four strains (1 food and 3 clinical) produced enterotoxin. Dose response studies using suckling mice indicated that 15 of 18 *E. sakazakii* strains tested caused lethality in mice by intraperitoneal injection, but only two strains through oral feeding. The relatedness of *E. sakazakii* isolates was evaluated using both phenotypic (biotyping, antibiograms) and genotypic (RAPD, PFGE, ribotyping) methods. From the 18 strains examined, 3 biotypes and 4 antibiogram patterns were observed. Ribotyping using the Dupont Riboprinter™ microbial identification system categorized the test strains into 10 clusters. After initially screening 100 decamer primers, two were selected for random amplification of polymorphic DNA (RAPD) typing. Banding patterns were similar for three strains isolated from the same hospital, although each strain was isolated in a different year. All three of the food isolates from company A showed similar profiles, while two food strains isolated from dried-infant formula produced by Company B revealed different profiles. Pulsed-field gel electrophoresis (PFGE) using the restriction enzymes *Xba*1 and *Spe*1 revealed a similar number of bands to that of RAPD (4-13 bands), giving distinguishable patterns that allowed for the comparison of *E. sakazakii* strains. Using the index of diversity, it was found that RAPD and PFGE were the most discriminatory methods for distinguishing clinical and food isolates of *E. sakazakii*. Genotypic typing revealed heterogeneity among Canadian *E. sakazakii* strains. Overall, the abilities of RAPD and

PFGE to differentiate between strains were comparable, followed by ribotyping, biotyping and antibiograms. In conclusion, my studies have found that *E. sakazakii* is prevalent in dried-infant formula available on the Canadian retail market. This organism can grow at a temperature as low as 5.5°C, a temperature found in many home refrigerators. Growth studies in reconstituted dried-infant formula indicate a generation time of only 40 min at ambient temperatures. These data indicate the importance of using aseptic methods and temperature control in preparation, use and storage of dried-infant formula. Heat resistance studies in dried-infant formula over a range of temperatures indicate that *E. sakazakii* appears to be one of the most thermotolerant organisms in comparison to other members of the *Enterobacteriaceae.* The importance of using Hazard Analysis and Critical Control Point (HACCP) during the manufacture of dried-infant formula is evident.

RÉSUMÉ

En 1980, *Enterobacter sakazakii*, bacille ŕ Gram négatif, mobile et péritriche, a été reconnue comme espčce distincte d'*Enterobacter cloacae* d'aprčs le degré d'homologie de l'ADN, la production de pigments et les réactions biochimiques. Une forme sévčre de méningite néonatale lui est imputée. Męme si les études n'ont pas réussi ŕ trouver une source environnementale de l'organisme, des préparations de lait maternisé en poudre ont été mises en cause ŕ la fois dans des poussées épidémiques et des cas sporadiques de la méningite ŕ *E. sakazakii*. D'aprčs les cas signalés, le taux de létalité variait de 40 ŕ 80 %. La gravité de l'infection chez les nourissons, combinée au manque d'information sur l'écologie et la pathogénicité de cet organisme, nous a incités ŕ en étudier les caractéristiques biologiques. Nous avons isolé des souches d'*E. sakazakii* ŕ partir de laits maternisés en poudre vendus au détail au Canada. Dans les échantillons évalués, provenant de cinq sociétés différentes, le taux de prévalence variait de 0 ŕ 12 %. Nous avons également obtenu des souches cliniques des collections d'hôpitaux canadiens. Le systčme d'identification API 20E a été utilisé pour confirmer l'identité de toutes les souches. A l'aide d'un incubateur ŕ gradient de température, nous avons déterminé la température minimale de croissance; celle-ci variait de 5,5 à 8,0 °C. Nous avons examiné le potentiel de croissance de cocktails d'isolats de sources alimentaires et cliniques dans trois préparations différentes incubées ŕ 4, 10 et 23 °C. Le temps de génération moyen était de 40 min ŕ 23 °C et de 4,98 h ŕ 10 °C. A 4 °C, par contre, les souches ne se sont pas développées et ont semblé dépérir lors de l'entreposage. D'aprčs des études de la thermorésistance sur dix souches (cinq isolats cliniques et cinq isolats de

sources alimentaires), *E. sakazakii* résiste f la chaleur jusqu'f des températures de 52, 54, 56, 58 et 60 °C dans les laits maternisés en poudre reconstitués. Des valeurs de D de 54,8, 23,7, 10,3, 4,2 et 2,5 minutes, f chacune de ces températures respectivement, ont été obtenues. La valeur globale calculée pour z est de 5,82 °C. Nous n'avons pas trouvé dans la littérature de rapport décrivant les facteurs de virulence ou la dose infectante minimale d'*E. sakazakii*. Dans l'essai standard sur souriceaux nouveau-nés effectué pour évaluer la production d'entérotoxine, quatre souches (une alimentaire et trois cliniques) ont produit une entérotoxine. Des études de la relation dose-réponse sur des souriceaux nouveau-nés ont indiqué que 15 souches d'*E. sakazakii* sur 18 administré par injection intrapéritonéale s'averaient létales par comparaison aux deux souches létales par voie orale. Pour évaluer le degré d'homologie des isolats, nous avons utilisé des méthodes phénotypiques (biotypage, antibiogrammes) et génotypiques (RAPD, PFGE, ribotypage). Chez les 18 souches examinées, nous avons mis en évidence trois biotypes et quatre profils d'antibiogrammes. Le ribotypage effectué f l'aide du systčme d'identification microbiologique Riboprinter^{MC} de Dupont a réparti les souches étudiées en dix groupes. Pour l'analyse du polymorphisme de l'ADNpar amplification aléatoire (RAPD), nous avons examiné 100 amorces (décamčres) et en avons retenu deux. Les profils de migration obtenus étaient similaires pour trois souches isolées au męme hôpital, quoique au cours d'années différentes. Pour les trois isolats des produits de la société A, les profils étaient similaires; par contre, deux isolats provenant de lait maternisé en poudre de la société B ont donné des profils différents. L'analyse de l'ADN génomique par électrophorčse en champs pulsés (PFGE) digéré avec les enzymes de restriction *Xba*1 et

*Spe*I a révélé un nombre de bandes similaire ŕ celui obtenu par la RAPD, fournissant ainsi des profils distinctifs permettant de comparer les souches. La RAPD et la PFGE se sont révélées les méthodes les plus discriminantes pour les isolats d'*E. sakazakii* de sources alimentaires et cliniques. En conclusion, nos études ont indiqué qu'*E. sakazakii* est présent dans des laits maternisés en poudre vendus au détail au Canada. Cet organisme peut se développer ŕ une température aussi basse que 5,5 °C, température que l'on trouve dans de nombreux réfrigérateurs domestiques. Les études de croissance dans des laits maternisés reconstitués ont indiqué un temps de génération de seulement 40 minutes ŕ température ambiante. Ces données montrent l'importance d'employer des méthodes aseptiques et de contrôler la température durant la préparation, l'utilisation et l'entreposage des laits maternisés. D'aprčs les études de la résistance ŕ différentes températures, *E. sakazakii* semble l'un des organismes les plus thermorésistants lorsqu'on le compare aux autres *Enterobacteriaceae* que l'on peut trouver dans les produits laitiers. L'importance d'appliquer le HACCP (systčme des points critiques pour l'analyse des risques) durant la fabrication des laits maternisés est évidente. Le typage du génotype a révélé que les souches canadiennes d'*E. sakazakii* étaient heterogčnes. De façon globale, les méthodes d'analyse RAPD et PFGE se sont avérées également efficaces pour différencier les souches, suivies du ribotypage, du biotypage et des antibiogrammes.

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ABBREVIATIONS

APC: aerobic plate count

ATCC: American Type Culture Collection

BHI: brain heart infusion

Ca/P: calcium to phosphorus ratio

CCP: critical control point

CFF: cell-free filtrate

CFU: colony forming unit

CNS: central nervous system

CSF: cerebral spinal fluid

d: day

ddH_2O: double distilled water

dNTPs: deoxyribonucleoside 5'-triphosphate

DNA: deoxyribonucleic acid

D value: decimal reduction

EC medium *E. coli* medium

EDTA: ethylenediaminetetra acetic acid

EE Broth: *Enterobacteriaceae* enrichment broth

ESP: EDTA-sarcosine-proteinase K

ET: Electrophoretic type

FAO: Food and Agriculture Organization

g:	gram
h:	hour
HACCP:	hazard analysis and critical control points
HCl:	hydrochloric acid
HTST:	high-temperature short-time
IP:	intraperitoneal injection
LT:	heat labile enterotoxin
Md:	Megadalton
MEE:	multilocus enzyme electrophoresis
MID:	minimum infectious dose
mL:	millilitre
MPN:	most probable number
NaCl:	sodium chloride
NaI:	sodium iodide
NB:	nutrient broth
OD:	optical density
ONPG:	orthonitrophenyl-β-galactosidase
PCR:	polymerase chain reaction
PFGE:	pulsed-field gel electrophoresis
PMP:	Pathogen Modelling Program
PMSF:	phenylmethyl sulfonyl fluoride
RAPD:	random amplification of polymorphic DNA

REA:	restriction enzyme analysis
RNA:	ribonucleic acid
rRNA:	ribosomal ribonucleic acid
s:	second
SDS:	sodium dodecyl sulfate
ST:	heat stable enterotoxin
TBE:	Tris-boric acid-EDTA disodium
TE:	Tris-EDTA disodium
TSA:	trypticase soya agar
TSB-YE:	trypticase soya broth with 6% yeast extract
UHT:	ultra-high temperature
UPGMA:	unweighted pair-group method arithmetic averages
USDA:	United States Department of Agriculture
UV:	ultra-violet
wt/vol:	weight per volume
y:	year
VP:	Voges-Proskaur
VRBG:	violet red bile glucose agar

CHAPTER 1: *ENTEROBACTER SAKAZAKII* – A REVIEW

INTRODUCTION

Enterobacter sakazakii is a member of the family *Enterobacteriaceae*, genus *Enterobacter*. It is a motile, peritrichous, Gram-negative rod. This organism was previously referred to as a "yellow-pigmented *Enterobacter cloacae*". The new name *E. sakazakii* was proposed in 1980 by Farmer and co-workers based on differences between *E. sakazakii* and *E. cloacae* in DNA-DNA hybridization, biochemical reactions and production of yellow-pigmented colonies (Farmer *et al.*, 1980).

In 1961, Urmenyi and Franklin reported the first two known cases of neonatal meningitis caused by *E. sakazakii*, although at the time it was still considered an *E. cloacae* strain. Since 1961, a number of cases of neonatal infections caused by *E. sakazakii* have been reported worldwide. A listing of the reported cases and outbreaks of neonatal infections caused by *E. sakazakii* found in the literature to date can be seen in Table 1. In December 1990, two incidents of neonatal meningitis caused by *E. sakazakii* were reported in two Canadian hospitals (T. Gleeson, personal communication).

Although the reservoir and mode of transmission of this organism has not been clearly identified, dried-infant formula has been implicated in some of the incidents of neonatal meningitis. The manifestation of disease in neonatal infections caused by *E. sakazakii* is severe. Usually newborns with *E. sakazakii* meningitis have a poor prognosis, many of them dying within days of infection. The reported case-fatality rates vary from 40 to 80 % (Urmenyi and Franklin, 1961; Adamson and Rogers, 1981; Kleiman *et al.*, 1981;

Table 1. Worldwide reported sporadic cases or outbreaks of neonatal *E. sakazakii* infection.

Location	Number of cases (deaths)	Source implicated	References
I. Sporadic cases			
Denmark	1(1)	not known	Joker *et al.* (1965)
Georgia	1(0)	not known	Monroe and Tift (1979)
Oklahoma	1(0)	not known	Adamson and Rogers (1981)
Indiana	1(0)	not known	Kleiman *et al.* (1981)
Greece	1(0)	not known	Arseni *et al.* (1984)
Missouri	1(0)	not known	Naqvi *et al.* (1985)
Maryland	1(0)	not known	Noriega *et al.* (1990)
Ohio	1(0)	not known	Gallagher and Ball (1991)
II. Outbreaks			
England	2(2)	incubator	Urmenyi and Franklin (1961)
Netherlands	8(6)	birth canal	Muytjens *et al.* (1983)
Greece	11(4)	not known	Arseni *et al.* (1987)
Massachusetts	2(1)	not known	Willis and Robinson (1988)
Tennessee	4(0)	dried-infant formula	Simmons *et al.* (1989)
Iceland	3(1)	dried-infant formula	Bierling *et al.* (1989)
Ontario	2(?)	not known	Gleeson, T. (personal communication)
Australia	3(1)	not known	Khadilkar *et al.* (1995)

Muytjens *et al.*, 1983; Willis and Robinson, 1988).

GENUS *ENTEROBACTER*

Genera of the family *Enterobacteriaceae,* in some cases, represent natural evolutionary groups as defined by techniques that measure evolutionary distance. These natural groups share phenotypic similarities which differentiate them from other genera (Izard *et al.*, 1983). An example of this type of genus is *Serratia*, which is a unique evolutionary group and which is biochemically distinct from other genera of *Enterobacteriaceae.* Another definition of a genus is "a group of species which are grouped together for convenience rather than because of a close evolutionary relationship" (Brenner, 1981). *Enterobacter* is an example of this kind of genus, which has been found to be heterogeneous by both phenotypic characteristics and molecular techniques such as DNA hybridization. Brenner (1981) considers organisms to be of the same species if they are highly related (> 70 %) by DNA hybridization.

The genus *Enterobacter* was officially accepted by the Judicial Commission in 1963 (Brenner, 1981), with *E. cloacae* designated as the type species. The characteristics of the genus include organisms which exhibit a wide variety of biochemical reactions. *Enterobacter* spp. are straight rods, motile by peritrichous flagella, facultatively anaerobic, grow readily on laboratory media, ferment glucose with production of acid and gas, give positive Voges-Proskauer, citrate assimilation and β-glucosidase (ONPG) reactions and a negative methyl red test (Farmer and Kelly, 1992). Table 2 shows the

Table 2. Biochemical differentiation of opportunistic *Enterobacter* species. [a]

Test	Reaction[b]				
	E.sakazakii	*E.cloacae*	*E.aerogenes*	*E.agglomerans*	*E.gergoviae*
Lysine decarboxylase	–	–	+	–	+
Arginine dihydrolase	+	+	–	–	–
Ornithine decarboxylase	+	+	+	–	+
Tween 80 esterase	+	-	ND	-	ND
KCN growth	+	+	+	v	–
Fermentation:					
sucrose	+	+	+	(+)	+
dulcitol	–	(–)	–	(–)	–
adonitol	–	(–)	+	–	–
D-sorbitol	–	+	+	v	–
raffinose	+	+	+	v	+
α-methyl-D-glucoside	+	(+)	+	–	–
D-arabitol	–	(–)	+	–	+
DNase	+	-	-	-	-
% G+C	57	52-54	53-54	53-58	60
Yellow pigment	+	–	–	(+)	–

[a] Adapted from Farmer and Kelly, 1992 and Aldova *et al.*, 1983.
[b] +=90–100% positive; (+)=75–89% positive; v=25–74%positive; (–)=10–24% positive; –=0–9% positive.

various species in the genus *Enterobacter* and their differentiation based on biochemical reactions

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Over the last several years numerous organisms have been reclassified and renamed, and the number of recognized species has grown tremendously. This rapid expansion of taxonomy is based on the determination of genetic relatedness. By correlating phenotypic characteristics with DNA homology and rRNA sequence analysis, it has been possible to classify organisms more accurately. Nine species of *Enterobacter* are currently recognized, namely, *E. aerogenes, E. amnigenus, E. asburiae, E. cancerogenus, E. cloacae, E. gergoviae, E. hormaechei, E. intermedius,* and *E. sakazakii* (Colonna, 1996).

TAXONOMY AND MICROBIOLOGY

E. sakazakii is a rare, but important cause of life-threatening neonatal meningitis and sepsis. Until 1980, *E. sakazakii* was referred to as a "yellow-pigmented cloacae" (Farmer *et al.*, 1980). The proposed change in classification of this organism is based on differences between *E. cloacae* and *E. sakazakii* in deoxyribonucleic acid (DNA) hybridization, biochemical reactions, pigment production and antibiotic susceptibility. Steigerwalt *et al.* (1976) showed that strains of the species *E. cloacae* formed two separate DNA hybridization groups which correlated with the presence or absence of yellow pigment. At that time, few biochemical characteristics could separate pigmented from non-pigmented strains of *E. cloacae*. Two years later, Brenner *et al.* (1977) showed that the pigmented strains could be separated from non-pigmented strains on the basis of

acid production from D-sorbitol by fermentation and delayed production of DNase. Farmer *et al.* (1980), in the paper proposing reclassification of the yellow-pigmented *E. cloacae* strains, found that in DNA-DNA hybridization studies the type strain of *E. sakazakii* was 83 - 89 % related to the other strains in this species, but only 31 - 49 % related to strains of *E. cloacae*. *E. sakazakii* has a biochemical profile very similar to that of *E. cloacae*, but unlike *E. cloacae* is always D-sorbitol negative, positive for extracellular deoxyribonuclease and produces yellow-pigmented colonies (Table 2). The yellow pigment is stronger at 25 than at 36°C. The only other species of *Enterobacter* that may produce a yellow pigment is *E. agglomerans*. *E. sakazakii* grows on nonselective plating media commonly used in enteric bacteriology. All strains of *E. sakazakii* grow rapidly on trypticase soy agar at 36°C and form colonies 2 to 3 mm in diameter after 24 h; at 25°C, the colonies are 1 to 1.5 mm at 24 h and 2 to 3 mm at 48 h.

Farmer *et al.* (1980) found that freshly isolated strains had two or more morphological colony types when first streaked for purity. One type of colony is dry or mucoid, with scallop edges, and rubbery when touched with a loop. The scallop-edged rubbery colonies reverted to typical smooth colonies on subculturing. It is not known at this time whether differences in virulence or any other phenotypic traits, exist between these two colony types. Farmer *et al.* (1980) also found that after 24 h incubation in trypticase soy broth, all strains of *E. sakazakii* produced a large amount of sediment which appeared to contain clumped cells and amorphous masses.

Muytjens *et al.* (1984) studied the enzymatic profiles of *E. sakazakii* and related species with specific reference to the α-glucosidase reaction. In this study, 226 *Enterobacter* strains (of which 129 were *E. sakazakii*) were evaluated using the API™ ZYM identification system (API System S.A., La Balme Les Grottes, France), which is based on microbial enzyme profiles. These researchers found two major differences between *E. sakazakii* and the other *Enterobacter* species. α-glucosidase activity was demonstrated in all *E. sakazakii* (129), but not in any of the other *Enterobacter* strains, which included *E. aerogenes* (19 strains), *E. cloacae* (60 strains) and *E. agglomerans* (18 strains). The absence of the enzyme phosphoamidase was also unique to *E. sakazakii* isolates. Farmer *et al.* (1985) found 53 of 57 strains of *E. sakazakii* to be positive for α-glucosidase activity. It was concluded that the detection of α-glucosidase permits rapid and reliable differentiation between *E. sakazakii* and other *Enterobacter* species. Aldova *et al.* (1983) evaluated Tween 80 esterase activity in 73 *E. sakazakii* strains isolated in Czeckoslovakia, and found that 97.3 % of them contained the enzyme. Postupa and Aldova (1984) studied six *E. sakazakii* strains isolated from powdered milk and dried-infant formula, and found that all produced Tween 80 esterase after 7 days of incubation at 25 and 37°C. These authors suggested that Tween 80 esterase production could be used to differentiate *E. sakazakii* from *E. cloacae*, along with pigment production, and non-fermentation of sorbitol.

CHARACTERISTICS OF INFECTIONS CAUSED BY *E. SAKAZAKII*

Meningitis is an acute inflammation of the membranes (meninges) of the brain and spinal

cord. Infections of the meninges are usually associated with acute onset and progression, and if untreated, usually progresses to a fatal outcome (Booy and Kroll, 1994).

In meningitis, the risk of serious neurological sequelae or death is great, and clinicians often initiate therapy before results of microbiological analysis are known (Isenberg *et al.*, 1992). Patients with viral infections of the CNS receive supportive care only. This therapy includes specific attention to the metabolic and ventilatory problems that may develop in some cases (Ray, 1994).

E. sakazakii infections have been reported primarily to cause a rare and particularly severe form of neonatal meningitis. Cases of *E.sakazakii* infections are described in the following section. Table 3 summarizes the reported cases of infections associated with *E. sakazakii* and the outcome of the infections.

In 1961, Urmenyi and Franklin in England reported the first two known cases of neonatal meningitis caused by *E. sakazakii* (described as a "yellow-pigmented *E. cloacae*"). Both infants died of a generalized infection (including meningitis) within 2 days of each other. The main pathological abnormalities were seen in the brain. Joker *et al.* (1965) in Denmark reported on a case of a neonate who remained well for the first 4 days of life and then began to show meningeal signs, complicated by brain abscess and hydrocephalus. In consultation with Urmenyi and Franklin, Joker *et al.* (1965) concluded that the bacterium isolated was an "uncommon *Enterobacter*", almost identical to the

Table 3. Symptoms and antibiotic intervention in reported cases of *E. sakazakii* infections.

AUTHORS	COURSE/OUTCOME	ANTIBIOTICS
Uremenyi and Franklin, 1961	neonate: sepsis, meningitis, seizures; death in 48 hours	Oxytetracycline (R)
	neonate: acute collapse and death	None
Joker *et al.*, 1965	neonate: meningitis, seizures, brain cysts, hydrocephalus; survived, severe neurologic deficits	Streptomycin (S) Ampicillin (MIC 2 μg/mL) Chloramphenicol (S) and Sulfa
Monroe and Tift, 1979	neonate: bacteremia, lumbar puncture not done; survived	Ampicillin (MIC 2 μg/mL) Gentamicin
Adamson and Rogers, 1981	neonate: meningitis, seizures; survived, no follow up	Ampicillin (S) Gentamicin (S)
Kleiman *et al.*, 1981	neonate: meningitis, brain cysts, subdural empyema, hydrocephalus; survived with mental retardation	Ampicillin (S) Chloramphenicol (S) Gentamicin (S)
Jimenez and Gimenez, 1982	adult with rectal adenocarcinoma: urosepsis, urinary retention; recovered	Ampicillin Gentamicin
Muytjens *et al.*, 1982 and 1983	neonate: meningitis, seizures; death in 4 days	Ampicillin (MIC 2 mg/L) Gentamicin (MIC 0.125 mg/L)

	neonate: meningitis, seizures; death in 6 days	Ampicillin (MIC 2 mg/L) Gentamicin (MIC 0.125 mg/L)
	neonate: meningitis, seizures, hydrocephalus; survived with severe retardation	Ampicillin (MIC 8 mg/L) Kanamycin Gentamicin
	neonate: meningitis, ventriculitis; death in 4 days	Chloramphenicol Gentamicin
	neonate: meningitis; death in 2 days	Ampicillin (MIC 5 mg/L) Gentamicin
	neonate: meningitis; death in 3 days	Gentamicin Chloramphenicol (MIC 8 mg/L)
	neonate: meningitis; death in 4 days	Ampicillin Gentamicin
	neonate: meningitis; survived, retarded	Ampicillin Gentamicin
Arseni *et al.*, 1984	premature infant: sepsis; death	Ampicillin Netilmicin
Naqvi *et al.*, 1985	neonate: meningitis, seizures, multiple brain abscesses, hydrocephalus; survived, right hemiparesis	Cefotaxime (MIC 1μg/mL) Ampicillin
Pribyl *et al.*, 1985	adult with diabetes: *E. sakazakii* recovered from foot ulcer; recovered	Aztreonam

Arseni *et al.*, 1987	neonate: respiratory distress syndrome; recovered	Cefoxatin Gentamicin
	neonate: respiratory distress syndrome; recovered	Cefoxatin
	neonate: respiratory distress syndrome; died	Penicillin
	neonate: sepsis; died	Cefoxatin Gentamicin
	neonate: anoxia; died	Amikacin Penicillin
	neonate: respiratory distress syndrome; died	Amikacin
	neonate: sepsis; recovered	Amikacin Penicillin
	neonate: sepsis; recovered	Amikacin
	neonate: sepsis; recovered	Amikacin Cefoxatin
	neonate: meningitis; recovered	Cefoxatin Chloramphenicol
	neonate: congenital defects; recovered	Cefoxatin
Willis and Robinson, 1988	neonate: meningitis, seizures, brain cysts; survived with severe deficits	Ampicillin (MIC 1μg/mL) Gentamicin (MIC <0.5 μg/mL) Moxalactam (MIC <1μ/mL)

	neonate: meningitis, seizures, brain cysts, hydrocephalus; survived with deficits	Ampicillin (S) Gentamicin (S) Moxalactam (S) Chloramphenicol (S)
Simmons *et al.*, 1989	bacteremia, bloody diarrhea sepsis, abdominal distention	Not described
Bierling *et al.*, 1989	meningitis, quadraplegia seizures	Ampicillin Gentamicin Chloramphenicol Cefotaxime
Noriega *et al.*, 1990	infant: bacteremia; recovered	Ampicillin Gentamicin
Gleeson, 1991	meningitis	Not described
Hawkins *et al.*, 1991	adult: headaches, blurred vision, neck stiffness, dizziness, nausea (no vomiting), primary bacteremia, no source of infection was identified; recovered	Ceftriaxone Ciprofloxacin
Gallagher and Ball, 1991	neonate: meningitis;	Ampicillin Cefotaxime
Khadilkar *et al.*, 1995	sepsis, shock, altered respiratory status; death	Not described

[a]R=Resistant.
[b]S=Sensitive.
[c] MIC=Minimum inhibitory concentration.

strain isolated from the spinal fluid in the two cases of neonatal meningitis in England. Monroe and Tift (1979) described the first reported case of neonatal bacteremia in the absence of meningitis associated with *E. sakazakii*. The infection occurred 6 days after birth and was treated with ampicillin. At the infant's 2-month check-up, normal growth and development were found.

A case of severe neonatal meningitis in Indiana, USA, was reported that firmly established the pathogenicity of *E. sakazakii* (Kleiman *et al.*, 1981). The report describes a previously healthy 5-week old infant who developed severe meningitis caused by *E. sakazakii*. At admission to hospital the infant was irritable and afebrile, however, there was no clinical or laboratory evidence of congenital malformation of the urinary tract, gastrointestinal tract or central nervous system (CNS). The infant was treated with ampicillin and gentamicin for 21 days and discharged. However, 2 months later, the head circumference rapidly increased, necessitating a ventriculoperitoneal shunt; developmental progress was delayed with severe neurological sequelae.

Adamson and Rogers (1981) also described a case of *E. sakazakii* meningitis with sepsis in a 5 week old male. Blood, cerebral spinal fluid (CSF) and urine cultures were positive for *E. sakazakii*. After a 14 day regimen of ampicillin, the patient was discharged in good condition without apparent sequelae. However, there was no long term follow-up for this patient. This is the second case where infection with *E. sakazakii* occurred at 5 weeks of age, indicating acquisition of the infection in the home environment.

Because *E. sakazakii* was newly named in 1980, and was an unfamiliar organism in the diagnosis of meningitis, Muytjens *et al.* (1983) reanalyzed and reevaluated 38 *Enterobacter* strains from blood and CSF. These researchers found 8 cases of *E. sakazakii* meningitis; 6 of the neonates had died and two recovered, but with severe neurological sequelae. Bacteremia was observed in five of the infants as well as necrotizing enterocolitis in two infants (Muytjens *et al.*, 1983). Lareng *et al.* (1996) also found enterocolitis to be associated with *E. sakazakii* infection. Naqvi *et al.* (1985) in reporting cefotaxime therapy of neonatal gram-negative bacillary meningitis, described a case of *E. sakazakii* meningitis where a 21 day old infant had brain abscesses. The patient, who survived, subsequently developed hydrocephalus which required a shunt.

E. sakazakii has also been reported as the causal agent of infection in a number of cases in Greece. Arseni *et al.* (1984) described a fatal case of *E. sakazakii* bacteremia in a premature newborn who had been treated with ampicillin and netilmicin for the first 3 days of her life and then cefotaxime and amikacin. Within a month of this incident, 11 other neonates nursed in the same neonatal unit were found to be colonized with *E. sakazakii* and had clinical signs of severe sepsis; four of the neonates died. A year later, these same researchers isolated *E. sakazakii* from the blood of both a 3 year old leukemia patient and a 4 year old boy. In both cases, antibiotic therapy (specific antibiotic treatment was not described) was administered and the children survived.

In 1988, Willis and Robinson described a further three cases of infection due to *E.*

sakazakii. In one of the cases, a 5 day old infant developed meningitis, and *E. sakazakii* was isolated from the CSF. Although ampicillin and cefotaxime were administered, the neonate died on the same day the infection was discovered. Powdered infant formula was implicated as the mode of transmission. *E. sakazakii* and *Leuconostoc mesenteroides* were found to be the causal agents of a nosocomial bacteremia in a 6 month old infant (Noriega *et al.*, 1990). Ampicillin and gentamicin were administered after the organisms were identified and the infant survived.

Most cases of *E. sakazakii* meningitis in the literature describe infection in neonates. However, there have been three reports of *E. sakazakii* infection in adults. Pribyl *et al.* (1985) described a case in which *E. sakazakii* was one of three organisms isolated from an ulcer in the foot of a diabetic patient and treated with aztreonem. Jimenez and Gimenez (1982) reported a single case of urosepsis caused by *E. sakazakii* in a 76 year old male. This patient was treated with ampicillin and gentamicin. More recently, Hawkins *et al.* (1991) reported bacteremia in an adult caused by *E. sakazakii*. Antibiotic therapy included ceftriaxone and then cefuroxime. In all three adult cases, appropriate antibiotic therapy resulted in patient recovery. Although optimal therapy of *E. sakazakii* has yet to be determined, in the majority of reported cases, a combination of ampicillin and gentamicin has been used in the treatment of *E. sakazakii* meningitis (Hawkins *et al.*, 1991). Willis and Robinson (1988) call this combination the "gold standard for *E. sakazakii* meningitis". In general, *E. sakazakii* is more susceptible to commonly used antibacterial antimicrobics than other *Enterobacter* species (Hawkins *et al.*, 1991).

According to Hawkins *et al.* (1991), *E. sakazakii* is consistently sensitive to ampicillin, carboxypenicillins, ureidopenicillins and aminoglylosides. Most strains are sensitive to chloramphenicol, and tetracycline, trimethoprim-sulphamethoxazole. However, these reseachers found *E. sakazakii* inconsistently susceptible to second generation cephalosporins.

E. sakazakii is not a reportable disease to Health Canada. Meningitis cases are reported as pneumoncoccol, other bacterial meningitis, viral meningitis and meningicoccal meningitis (E. Todd, personal communication). *E. sakazakii* meningitis would be catagorized under "other bacterial meningitis" and therefore, there is no way of determining the number of cases in Canada.

INCIDENCE IN THE ENVIRONMENT AND FOODS

Little is known about the presence of *E. sakazakii* in the environment. Muytjens and Kollee (1990) could not isolate this organism from surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic animals, cattle and raw cow's milk. Most of the *E. sakazakii* strains reported in the literature have been isolated from clinical sources. However, some literature reports suggest a correlation between *E. sakazakii* meningitis and infant formula (Table 1).

Schwab *et al.* (1982) evaluated the microbiological quality of dry-milk mixes and milk substitute infant formulas in the United States and compared their results to a Canadian

national microbiological survey of dried-infant cereals and powdered infant formula (Collins-Thompson *et al.*, 1980). A comparison of the total aerobic microflora was done using aerobic plate counts, (APCs), with U.S. made dried-infant formula products containing lower APCs than Canadian products. However, the microbiological criteria for this product as recommended by the International Commission for Microbiological Specification (FAO, 1977), was met in both the Canadian and U.S. survey. Muytjens *et al.* (1988) evaluated the quality of 141 powdered substitutes for breast milk from 35 countries, with regard to members of the *Enterobacteriaceae* family. *Enterobacteriaceae* were isolated from 52.3 % of the 141 samples, with *E. agglomerans, E. cloacae, E. sakazakii* and *Klebsiella pneumoniae* being the most frequently isolated organisms. These four bacteria were also found in the Canadian samples, at levels of < 1 colony forming units (CFU)/100 g of dried-infant formula (Muytjens *et al.*, 1988). FAO (1977) recommends bacterial counts for coliforms in powdered formula of less than 3.0 CFU/g; all the formula Muytjens *et al.* (1988) tested met this requirement. Muytjens *et al.* (1983) also isolated *E. sakazakii* from liquid formula, however, the organism could not be isolated from either the powder or the water used for reconstitution of the formula. Two strains of *E. sakazakii* were also isolated from powdered formula in Czeckoslovakia (Postupa and Aldova, 1984). Skladal *et al.* (1993) isolated strains of *E. sakazakii* from cartons of ultra high temperature (UHT) pasteurized milk along with other contaminating bacteria. *E. sakazakii* has also been isolated from ground meat (R. Foster, personal communication), however, to our knowledge, the organism has not been recovered from any other foods. In conclusion, it has been clearly established that *E. sakazakii* can be

found in dried-infant formula.

INFANT FORMULA PROCESSING

Production of dried-infant formula from cow's milk has shown a steady increase since the beginning of this century. Today, dried-infant formula is a highly developed product and its manufacture is an important part of the dry milk industry (Knipschildt, 1986). Health care professionals use the slogan "breast-fed is best-fed" indicating that breast milk is the best source of nutrition for infants up to the age of 6 months. Commercially prepared formulas or breast milk substitutes are the next best thing to breast milk. Breast milk substitutes are formulated to resemble the nutrient composition of breast milk and are a more satisfactory substitute for breast milk than any other product (Health Canada, 1989). Cow's milk, which is used in the production of dried-infant formula, contains a higher amount of fat, protein and minerals than breast milk. Therefore, it is first skimmed and then diluted in order to acheive levels more commonly found in breast milk.

Care must be taken in the manufacture of infant formula to safeguard the microbiological quality of the finished product. The manufacture of dried-infant formula can follow two procedures. The "dry procedure" involves dry mixing of ingredients into powder after spray-drying. This method should be avoided, as it can lead to bacteriological contamination (Lambert-Legace, 1982). Furthermore, by dry-mixing, it is difficult to obtain a uniform distribution and the added ingredients, e.g. lactose, may segregate when the cans are subjected to vibrations. In the second manufacturing method, dried-infant

formula is prepared using a "wet procedure" where the following heat treatments are used: 1) liquid skim milk is heat-treated before processing at 82°C for 20 s; 2) the pre-mix consisting of skimmed milk and fat components is heat-treated at 80°C for 20 s; 3) the total mixture containing all ingredients is heat-treated at 107-110°C for 60 s; 4) the liquid mixture is concentrated using a falling film evaporator and 5) the concentrate is heat-treated again at 80°C and then immediately spray-dried. Often, a combined procedure is used where water souble components are added to the milk before drying and the less soluble components are added to the blend after drying (Caric, 1993). In Canada, some companies use the "dry procedure" while others use the "wet procedure".

INFANT FORMULA RELATED OUTBREAKS

A direct relationship between reconstituted dried-infant formula and *E. sakazakii* infection has been found in some instances (Table 1). Simmons *et al.* (1989) reported an outbreak of *E. sakazakii* infection involving four neonates, where it was suspected that infant formula was contaminated during the manufacturing process. *E. cloacae* was the only other organism isolated. These researchers, in addition to determining total microbial counts in the formula, also extended the incubation period of the original enrichment broth to 7 d at 35°C. After this period of time, *E. sakazakii* grew to large numbers while *E. cloacae* did not grow. The *E. sakazakii* isolated from the formula fed to the infants had the same plasmid and multilocus enzyme electrophoresis (MEE) profile as the strains isolated from the 4 neonates.

In an outbreak in Iceland, strains of *E. sakazakii* isolated from three patients with neonatal meningitis were very closely related (i.e., by plasmid profiles, restriction enzyme analysis (REA), antibiograms) to the strains found in the formula used in the hospital (Bierling *et al.*, 1989). The organism was found in low numbers in the suspect formula. A possible explanation for this outbreak could be that the formula bottles were occasionally kept at 35-37°C for extended periods of time in bottle heaters, therefore possibly allowing for the multiplication of the organism. There is no available data in the literature concerning the growth potential of *E. sakazakii* in infant formula at various temperatures.

In the incident where *E. sakazakii* and *L. mesenteroides* were found to be causal agents of a nosocomial bacteremia in a 6-month old infant (Noriega *et al.*, 1990), stock formula was examined for *E. sakazakii* and *L. mesenteroides*, but none were found. However, the blender used to rehydrate the formula was found to be heavily contaminated with both organisms. Perhaps *E. sakazakii* and *L. mesenteroides* were present in low numbers in the dried-infant formula, but levels of the organisms increased with each preparation in the blender. It is not clear whether *E. sakazakii* or *L. mesenteroides* was the organism responsible for the bacteremia.

Clark *et al.* (1990) investigated two unrelated hospital outbreaks involving meningitis, bacteremia and colonization of neonates. In each of the outbreaks, *E. sakazakii* was isolated from both patients and dried-infant formula. A combination of typing methods (plasmid analysis, antibiograms, chromosomal REA, ribotyping and MEE) were used to

evaluate the relatedness of the strains involved in these outbreaks. Using visual examination of banding patterns, these researchers found that although the typing patterns of isolates between outbreaks were different, within each outbreak patient and formula isolates shared the same typing patterns. Clark *et al.* (1990) found that 26 isolates from the non-U.S. hospital (patient and formula isolates) had the same plasmid profiles containing four bands of 85, 70, 42 and 3.2 megadaltons (Md). All 5 isolates from the U.S. hospital including 3 from patients, 1 from formula and 1 from a blender had a similar plasmid profile which contained three bands, of 75, 29, and 3.2 Md. Results from susceptibility tests showed that the majority of the U.S. isolates were resistant to cephalothin and cefoxitin and were susceptible to all other agents tested. The non-U.S. isolates were resistant to cephalothin, intermediate in susceptibility to cefazolin, cefoxitin and tetracycline and susceptible to the remaining antimicrobial agents tested.

All the U.S. isolates shared the same eletrophoretic type (ET). The 8 non-U.S. isolates tested shared the same ET however, the pattern was different from the U.S. isolates. REA analysis using either *Hind*III or *Bam*HI demonstrated that all U.S. isolates were indistinguishable, but different from the non-U.S. isolates. Ribotype patterns of the non-U.S. and U.S. isolates contained 5 and 3 distinct bands, respectively.

PATHOGENICITY

There has been no published work that we are aware of on the mechanisms of

pathogenicity or associated virulence factors of *E. sakazakii.*

The information presented in this chapter was used in the following publication:

Nazarowec-White, M. and Farber, J.M. 1997. *Enterobacter sakazakii:* a review. Int. J. Food Microbiol. 34:103-113.

RATIONALE AND OBJECTIVES

In 1991, the Hospital for Sick Children in Toronto, Canada reported to Health Canada a case of neonatal meningitis related to the consumption of infant formula. Two months previous to this incident, a case of *E. sakazakii* meningitis associated with dried infant formula had been reported. At the time of these reports, concerns were raised because of the susceptible population (infants), the high mortality rate reported in the literature and the paucity of information with respect to this organism (T. Gleeson, personnal communication). There were also communications from Europe that *E. sakazakii* infections were increasing in number (J. Harwig, personal communication). These factors stressed the importance of initiating investigations into the biology of *E. sakazakii.*

The overall objective of this thesis was to study the basic biology and pathogenicity of clinical and food isolates of *E. sakazakii* from Canadian sources. Strains were characterized using traditional microbiological parameters (growth characteristics, thermotolerance, biochemical reactions and virulence factors). In addition, several molecular techniques (pulsed-field gel electrophoresis, ribotyping and random amplification of polymorphic DNA) were evaluated for their potential as typing methods for typing *E. sakazakii.*

Specific objectives of this study were:

1. To determine the incidence and prevalence of *E. sakazakii* in dried-infant formula available on the Canadian market.
2. To evaluate the growth characteristics of various clinical and food isolates of *E. sakazakii* in reconstituted powdered infant formula.
3. To establish the thermotolerance (D and z values) of *E. sakazakii* strains in reconstituted powdered infant formula.
4. To determine the minimum infectious dose of various *E. sakazakii* strains using suckling mice.
5. To determine if *E. sakazakii* isolates are capable of producing enterotoxin using the suckling mouse model.
6. To develop a highly discriminatory molecular typing method to characterize differences among food and clinical isolates of *E. sakazakii*.

The data resulting from this thesis can be incorporated into a health risk analysis for *E. sakazakii* in infant formula.

The abstracts and introduction sections of the following chapters may be repetitive. However, these chapters are based on scientific peer-reviewed publications.

CHAPTER 2: INCIDENCE, SURVIVAL AND GROWTH OF *ENTEROBACTER SAKAZAKII* IN INFANT FORMULA

ABSTRACT

E. sakazakii has been implicated in a severe form of neonatal meningitis. Although studies have failed to identify an environmental source for the organism, dried-infant formula has been implicated in outbreaks and sporadic cases of *E. sakazakii* meningitis. The high mortality rate (40-80 %), the severity of the infection in infants and the lack of information on the incidence, survival and growth of *E. sakazakii* in foods led to this study. Experiments were undertaken to determine the incidence of *E. sakazakii* in dried-infant formula, the temperature range for growth, and the growth characteristics of *E. sakazakii* in reconsititued dried-infant formula. Strains of *E. sakazakii* were isolated from dried-infant formula available on the Canadian retail market. The incidence varied from 0 to 12 % in 120 samples from five different companies. For both clinical and food isolates, minimum growth temperatures of 5.5-8.0°C were observed by using a temperature-gradient incubator. The potential growth of *E. sakazakii* was followed using a mixture of food and clinical isolates in three different formula incubated at 4, 10 and 23°C. Average generation times were 40 min at 23°C and 4.98 h at 10°C. *E. sakazakii* strains did not grow at 4°C and began to die-off during storage at this temperature. The results of this study stress the importance of using aseptic methods and proper temperature control in the preparation, use and storage of dried-infant formula.

INTRODUCTION

E. sakazakii, designated a unique species in 1980 (Farmer *et al.*, 1980), has been implicated as the causal organism in a rare but severe form of neonatal meningitis (Willis and Robinson, 1988; Simmons *et al.*, 1989; Bierling *et al.*, 1989; Noriega *et al.*, 1990; Gallagher and Ball, 1991). A mortality rate of 40-80 % has been reported in the literature (Willis and Robinson, 1988). In a number of cases, dried-infant formula has been implicated as the mode of transmission. Muytjens *et al.* (1988) evaluated the quality of 141 powdered substitutes for breast milk from 35 countries with regard to members of the *Enterobacteriaceae* family. *Enterobacteriaceae* were isolated from 52.2 % of the 141 samples; in samples from 13 of the 35 countries. Two strains of *E. sakazakii* were also cultured from powdered infant formula in Czeckoslovakia (Postupa and Aldova, 1984). Simmons *et al.* (1989) reported isolation of *E. sakazakii* from infant formula assumed to have been contaminated during the manufacturing process. Three cases of neonatal meningitis caused by *E. sakazakii* found in dried-infant formula were reported by Bierling and co-workers (1989) in Iceland. In the latter two reports, a relationship was established between isolates found in dried-infant formula and clinical isolates from infected neonates (Clark *et al.*, 1990). In 1990, two cases of *E. sakazakii* infection of unknown etiology were reported to Health Canada (T. Gleeson, personal communication). In one case, analysis of two cans of formula obtained from the home of a 1 month old infant showed no microbiological contamination. However, the original can had been discarded and therefore could not be evaluated. Skladal *et al.* (1993) found *E. sakazakii* to be one of the major contaminating bacteria in ultra-high temperature (UHT) milk cartons, implying that

this organism may survive pasteurization treatments. Little information exists with respect to the growth characteristics of this organism. Farmer *et al.* (1980) examined 57 strains of *E. sakazakii* and reported growth of the organism at 25, 36 and 45°C. Fifty of the tested strains grew at 47°C, but not at 4 or 50°C. Growth of microorganisms is measured by change in the number of cells with time. Viable cell counts measure only the living population. The growth of viable cells are determined by placing dilutions of the population on agar plates and counting the number of colonies that occur after incubation. Counts of viable cells are a long and cumbersome process to carry out, but this method is very sensitive (Brock and Madigan, 1991). The growth curve of microorganisms can be divided into distinct phases called the lag phase, exponential phase, stationary phase and death phase (Brock and Madigan, 1991). Growth rate is the change in cell number or mass per unit time. The interval for the formation of two cells from one is called a generation and the time required for this to occur is called the generation time. The generation time is therefore the time required for the cell number to double.

When a microbial population is inoculated into a fresh medium, growth usually does not begin immediately but only after a period of time called the lag phase, which may be brief or extended depending on conditions. If inoculated cells are in the stationary phase, they are usually depleted of various essential enzymes or other cell constituents and time is required for resynthesis. As well, if cells have been damaged but not inactivated in a previous treatment with heat or chemicals, a lag time is required to repair these cells. Exponential growth can not occur indefinitely. Once exponential growth (doubling or

generation) ceases, the population has reached the stationary phase. In this phase there is no increase or decrease in cell numbers. As the nutrients of the medium are depleted, the cells begin to die off. There are no known reports describing the growth of *E. sakazakii* in reconstituted dried-infant formula.

Although the exact prevalence of neonatal meningitis caused by *E. sakazakii* is not known, the severity of the infection, the high mortality rate and the lack of information on the growth characteristics of this organism prompted the need for this study. The objective was to determine the incidence of *E. sakazakii* in dried-infant formula on the Canadian market and to evaluate the growth characterisics of clinical and food isolates in laboratory medium and reconstituted dried-infant formula.

MATERIALS AND METHODS

Incidence in dried-infant formula

Source of clinical isolates: Clinical strains used in this study were obtained from culture collections of Canadian hospitals (Table 4). Two strains were obtained from St. Jospeh's Hospital, London, ON; 1 strain from Montreal Children's Hospital, Montreal, PQ and 3 strains from the Hospital for Sick Children, Toronto, ON. The Laboratory Centre for Disease Control, Ottawa, ON provided 3 strains of *E. sakazakii* which had been isolated from patients in Canadian hospitals however, the exact location of the hospitals was not known. The *E. sakazakii* type strain, ATCC 29544, obtained from the American Type Culture Collection (ATCC, Rockville, MD) had been isolated (1970) from the throat of a

Table 4. Sources and strain designation of *E. sakazakii* isolates.

Strain Designation		Source		Isolation year
Clinical	Type Strain	ATCC		1979
	LA	St. Joseph's Hospital	London, ON	1990
	LB	St. Jospeh's Hospital	London, ON	1990
	MONT	Montreal Childrens' Hospital	Montreal, PQ	1989
	SK81	Hospital for Sick Children	Toronto, ON	1981
	SK90	Hospital for Sick Children	Toronto, ON	1990
	SK92	Hospital for Sick Children	Toronto, ON	1992
	LCDC1	Laboratory Centre for Disease Control	Ottawa, ON	1979
	LCDC2	Laboratory Centre for Disease Control	Ottawa, ON	1982
	LCDC3	Laboratory Centre for Disease Control	Ottawa, ON	not known
Formula	MNW1	Company A		1991
	MNW2	Company A		1991
	MNW3	Company B		1992
	MNW4	Company B		1992
	MNW5	Company B		1992
	MNW6	Company C		1992
	MNW7	Company C		1992
	MNW8	Company D		1992

person diagnosed with whooping cough in Tennessee (Farmer *et al.*, 1980).

Sampling: The incidence of *E. sakazakii* in dried-infant formula from five different companies that retail on the Canadian market was determined. The protein source in each of the formula was cow's milk (whey protein) and the carbohydrate source, lactose. Samples (cans) from different lots manufactured on different days were obtained at the manufacturing or retail levels, with a total of 120 cans being evaluated, 24 from each manufacturer.

Analysis: The can lid margins and the spoons used for sampling the cans were sterilized by flaming in burning ethanol before samples were withdrawn. Double-distilled water (ddH_2O) was autoclaved in flasks and cooled to 45°C. Dried-infant formula powder (100, 10 and 1 g amounts) was added to the water (1:10), and shaken by hand until the powder was uniformly suspended. After overnight incubation at 36°C, 10 mL of each suspension was withdrawn from each flask and added to 90 mL of *Enterobacteriaceae* Enrichment (EE) broth (buffered glucose, brilliant green, bile broth, Oxoid, Nepean, ON). After overnight incubation (36°C), 1 mL amounts of broth were mixed with 20 mL amounts of fluid violet red bile glucose (VRBG) agar, (Oxoid) in duplicate petri plates. After solidification of the agar, the plates were incubated overnight at 36°C.

Confirmation and Enumeration: All clinical isolates were confirmed using the API 20E biochemical identification system (API System, Laval, PQ). Presumptive colonies of *E.*

sakazakii isolated from dried-infant formula were confirmed using the same biochemical tests (API 20E) and estimates of viable organisms were determined by the most probable number (MPN) technique (Health Canada, 1993).

Determination of Minimum Growth Temperature

Ten clinical and food strains of *E. sakazakii* and the type strain, ATCC 29544 were examined. Each strain was grown overnight (36°C) in 10 mL Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) and then diluted in fresh BHI broth to give a final concentration of 10^3 colony forming units (CFU)/mL. Five mL of the diluted broth was dispensed into L-shaped aerobic culture tubes (L-tubes) and placed into a temperature-gradient incubator (Scientific Industries, Mineola, NY). In preliminary experiments, a constant temperature-gradient was established in the incubator so that the temperature ranged from 4 to 50°C in 30 L-tubes, with the temperature differential between tubes ranging from 1 to 1.5°C. The accuracy of the temperature-gradient incubator was ± 0.2°C. Thirty duplicate tubes were inoculated with *E. sakazakii* and then placed in the temperature-gradient incubator. Growth was assessed by visually examining the turbidity of each culture over a 20 day period.

Growth in Formula

Formula: Infant formula powders from three companies (A, B and C) having the highest market share of Canadian retail sales, were selected to evaluate the growth of *E. sakazakii* in reconstituted infant formula. Formula was prepared according to the manufacturer's

directions. Powder was weighed out (92.0, 82.5 and 93.5 g for formula from companies A, B and C, respectively) and added to 600 mL of sterile ddH_2O. The 600 mL of formula was divided into two flasks, each containing 300 mL. One flask was inoculated with a mixture of 5 clinical strains of *E. sakazakii* and the other with a mixture of 5 food isolates. Each spiked 300 mL of formula was further divided into 50 mL portions. Experiments were done in duplicate.

Inoculation of formula: Each of the 10 test strains was incubated overnight at 36°C in 5 mL of BHI broth, and diluted so as to give a final total concentration of 1.1 x 10^3 CFU/mL of formula. Inoculated formula were incubated at three temperatures, namely 4, 10 and 23°C. The first temperature (4°C) was selected as a "proper refrigeration temperature", 10°C was considered a slightly abusive temperature and 23°C a simulation of room temperature abuse.

Sampling: At 23°C, samples (1 mL) were taken every 2 h over a 24-h period, while at 10°C samples were taken on day 0 and then every day for 10 days. Formula incubated at 4°C was sampled every other day for 20 days. At these timed intervals, samples were diluted in 9 mL 0.1 % (wt/vol) buffered peptone (Difco Laboratories) water, plated onto duplicate plates of VRBG agar using the pour plate technique, and incubated at 36°C for 24 h. Confirmation of presumptive *E. sakazakii* colonies was done using the API 20E test strip.

Statistical analysis: Data were analyzed by the Gompertz equation to give fitted growth curves, using the Inplot™ (Graph Pad, Inplot™ 1992 GraphPad Software Inc. Version 4.04, Sorento, CA) statistical software package to obtain both generation and lag times. Generation time and lag time results were further subjected to an analysis of variance (SAS Institute Inc., 1989) in order to determine significant statistical differences among formula and between clinical and food isolates. The analysis included experiments done on two different days.

RESULTS AND DISCUSSION

Incidence

A total of 120 cans (from 5 different companies) of infant formula were examined for the presence of *E. sakazakii.* The microorganism was cultured from 8 cans of product from 4 of 5 companies (Table 5). The levels of *E. sakazakii* found in the positive samples was 0.36 CFU/100g. These findings were similar to those of Muytjens *et al.* (1988), who reported levels of *E. sakazakii* ranging from 0.36-66.0 CFU/100 g of dried-infant formula in three cans of Canadian formula examined. The MPN is particularly useful for low concentrations of organisms (<10/g) especially in foods where particulate matter may interfere with accurate colony counts.

Minimum Growth Temperature

In a temperature-gradient incubator, a linear temperature gradient is established allowing an organism to be grown simultaneously over a wide temperature range (Matches and

Table 5. Incidence of *E. sakazakii* in infant formula powder.

Company	Positive Samples	Strain Designation
A	2/24 (12%)	MNW1, MNW2
B	3/24 (8%)	MNW3, MNW4, MNW5
C	2/24 (8%)	MNW6, MNW7
D	1/24 (4%)	MNW8
E	0/24 (0%)	
Total	8/120 (6.7%)	

Liston, 1973). The minimum growth temperatures of the clinical (isolates obtained from hospital culture collections) and food (isolated from dried infant formula) isolates of *E. sakazakii* in BHI broth ranged from 5.5°C for strains SK90 and MNW2 to 8.0°C for strain SK92 (Table 6). The minimum growth temperature of the *E. sakazakii* type strain (ATCC 29544), which was the slowest growing isolate, was 7°C. None of the strains grew below 5.5°C. Of the 57 *E. sakazakii* strains tested by Farmer and co-workers (Farmer *et al.*, 1980), none grew at 4°C.

Walker (1990), using a temperature-gradient incubator, investigated the growth of 29 bacterial strains representing 6 recognized pathogenic genera. The organisms were grown in BHI as well as UHT milk, with results being similar for both growth media. It was concluded that the organisms could be divided into either mesophiles or psychrotrophs. The mesophiles (*Salmonella* spp., *Staphylococcus aureus, Escherichia coli*) did not grow below 5°C, while the minimum growth temperature for the psychrotrophic pathogens, *Listeria monocytogenes, Yersinia enterocolitica* and *Aeromonas hydrophila*, ranged from -0.9 to 1.2°C. The results of our study indicate that *E. sakazakii* falls into the mesophile category as defined by Walker (1990). Raghubeer and Matches (1990), also using a temperature-gradient incubator, evaluated the temperature range for growth of *E. coli* O157:H7, *Klebsiella pneumoniae* and *E. aerogenes,* in *E. coli* medium (EC medium). For *K. pneumoniae*, after a 24-h incubation,

the temperature range for growth was 26 to 41°C, while at 36 and 48 h, the minimum temperature for growth decreased to 22.7°C. The minimum growth temperatures for *E.*

Table 6. Minimum and maximum growth temperature of *E. sakazakii* isolates.

Strain Designation	Minimum growth temperature (°C)	Maximum growth temperature (°C)
Type Strain	7.0 (15)[c]	41
LA[a]	6.0 (6)	44
LB[a]	6.0 (7)	43
SK92[a]	8.0 (10)	41
SK90[a]	5.5 (11)	42
SK81[a]	6.5 (8)	41
MNW1[b]	7.5 (11)	43
MNW2[b]	5.5 (10)	45
MNW4[b]	6.0 (12)	45
MNW6[b]	7.0 (13)	41
MNW8[b]	5.5 (11)	42

[a]Clinical strains.
[b]Food strains.
[c]Time (days) at which visual growth was first observed in BHI broth.

aerogenes ranged from 24.3 to 27.6°C after 24 to 48 h of incubation, while the maximum growth temperature was 41°C. After 24 and 48 h, *E. sakazakii* initiated growth at 17 and 13.5°C, respectively. The much lower minimum growth temperature for *E. sakazakii* as compared to those of *E. coli, K. pneumoniae* and *E. aerogenes* reported by Raghubeer and Matches (1990) may be the result of differences in initial cell concentration, i.e., 10 cells in the Raghubeer and Matches (1990) study versus 1000 cells in the present study. The results of our study, as well as those of Farmer *et al.* (1980), indicate that *E. sakazakii* would not grow at the proper refrigeration temperature of 4°C. However, the temperatures of many home refrigerators range from 7 to 10°C (Rhodehamel, 1992). Harris (1989) reported 20% of the home refrigerators surveyed were found to be between 5 and 10°C; however, none were found above 10°C. In contrast, Van Garde and Woodburn (1987) found that refrigerator temperatures in 21% of the households surveyed were ≥10°C, and Daniels (1991) reported that 1 of 4 home refrigerators were above 7.2°C and 1 of 10 above 10°C. These commonly found, but potentially abusive temperatures would allow for the growth of *E. sakazakii*. In terms of maximum growth temperature, Farmer and co-workers (1980) found none of the 57 strains of *E. sakazakii* tested grew at 50°C. In the present study, the maximum temperature at which visible growth of *E. sakazakii* was observed ranged from 41 to 45°C (Table 6).

Growth Study

There were no statistically significant differences found in generation times either among the tested infant formula or between strains at 4, 10 and 23°C ($P>0.05$). At 4°C, in all

three formula and for both clinical and food strains, the concentration of *E. sakazakii* remained at the initial inoculum levels ($1.1x10^3$ CFU/mL) or declined with time. These findings confirm the importance of proper refrigeration temperatures after reconstitution of infant formula powders to ensure that this organism does not grow.

The generation and lag times of *E. sakazakii* grown in the three formula at 10 and 23°C were evaluated (Table 7). There were no statistically significant differences found among the three formula tested for both clinical and food strains. The lag time at 10°C varied from 19 h for Formula A (Food) to 47 h for Formula B (Clinical). Although lag times were generally shorter for the food as compared to the clinical isolates, the differences were not statistically significant ($P>0.05$). Generation times for *E. sakazakii* at 10°C varied form 4.18 to 5.52 h. Again, the generation times for the food isolates were shorter than for the clinical strains, although the differences were not statistically significant ($P>0.05$). Generation and lag times were calculated for *Salmonella* and *E. coli* (Table 8) using the USDA Pathogen Modeling Program (Version 4.0), using the water activity and pH values of the reconstituted dried-infant formula used in the present study. The predicted lag time for *Salmonella* spp. and *E. coli* at 10°C was 74.8 and 47.0 h, respectively (Table 8), while the mean lag time of 32.8 h for *E. sakazakii* (all formula, all strains) was the shortest of all three organisms. At 23°C, both *E. coli* and *Salmonella* spp. had a predicted generation time of 44.4 min (0.74 h), as compared to a mean generation time of 40 min (0.67 h) for *E. sakazakii* (Table 8). A much greater difference was found when comparing lag times at 23°C, where predicted values for *E. coli* and *Salmonella*

Table 7. Generation and lag time of *E. sakazakii* in reconstituted infant formula at 10 and 23°C.

		10°C		23°C	
Strain	Formula	Lag time (h)	Generation time (h)	Lag time (h)	Generation time (min)
Clinical[a]	A	28.08±0.72[b]	5.52±0.54	2.10±0.49	39.09±5.56
	B	46.80±15.60	4.79±0.90	3.02±0.70	39.25±0.07
	C	36.48±17.04	5.06±1.30	3.09±0.83	43.91±8.63
	Mean	37.20±11.12	5.12±0.91	2.75±0.67	40.73±4.75
Food[a]	A	18.96±8.40	4.22±0.54	1.76±0.72	40.11±4.54
	B	24.72±13.44	4.18±0.88	3.40±0.70	36.99±2.07
	C	42.00±0.48	4.20±0.84	3.11±0.12	40.41±6.49
	Mean	28.56±7.44	4.15±0.75	2.76±0.51	39.95±4.36

[a]A mixture of five different strains of *E. sakazakii* was used at an initial cell concentration of 10^3 CFU/mL. (clinical strains included LA, MONT, SK81, SK90, SK92; food strains included MNW1, MNW2, MNW4, MNW6, MNW8).
[b]± Standard deviation.

Table 8. Some reported generation and lag times in BHI broth and milk products.

Organism	Growth medium	T (°C)	GT (h)	LT (h)	Reference
E. sakazakii	Infant formula	23	0.67	2.76	Present study
		10	4.64	32.8	Present study
Salmonella spp.	BHI broth	23	0.74	5.10	PMP[a]
		10	10.3	74.8	PMP
E. coli	BHI broth	23	0.74	5.56	PMP
		10	6.40	47.0	PMP
	UHT milk	19	1.90	6.30	Buchanan *et al.*, 1993
E. aerogenes	Whole milk	23	0.80	NR[b]	Longeveld and Cuperus, 1980
		10	4.20	NR	Longeveld and Cuperus, 1980
E. cloacae	Whole milk	23	0.69	NR	Longeveld and Cuperus, 1980
		10	2.70	NR	Longeveld and Cuperus, 1980
E. hafnia	Whole milk	23	0.85	NR	Longeveld and Cuperus, 1980
		10	4.20	NR	Longeveld and Cuperus, 1980
B. cereus	Whole milk	10	4.00	24.0	Meer *et al.*, 1991
L. monocytogenes	Whole milk	10	7.30	25.0	Hudson, 1994
S. flexneri	Whole milk	28	0.80	1.90	Zaika *et al.*, 1989
		19	4.20	11.1	Zaika *et al.*, 1989
	UHT milk	28	0.70	3.20	Zaika *et al.*, 1992
		19	3.30	14.7	Zaika *et al.*, 1992
Y. enterocolitica	Whole milk	25	1.30	NR	Stern *et al.*, 1980
	UHT milk	8.4	7.18	<24	Walker and Stringer, 1988

[a]USDA Pathogen Modeling Program using pH, a_w and temperature from present study.
[b]Not reported.
T = temperature, GT = generation time, LT = lag time

were 5.7 and 5.1 h, respectively, as compared to 2.7 h for *E. sakazakii*.

Phillips and Griffiths (1987) found generation times of *Pseudomonas fluorescens* at 10°C to be 4.9 and 3.5 h in raw milk and pasteurized cream, respectively; values similar to those of *E. sakazakii*. Longeveld and Cuperus (1980) determined growth rate of different bacteria in pasteurized milk . Apparently, organisms within the genus *Enterobacter*, i.e., *E. sakazakii, E. cloacae, E. hafnia* and *E. aerogenes,* have similar lag and generation times at both 23 and 10°C in dairy products such as milk (Table 8). The generation and lag times reported in the literature for other organisms found in milk or milk products are also shown in Table 8. It appears that both generation and lag times for these organisms are longer than for *E. sakazakii*. Typical growth curves for *E. sakazakii* are shown in Appendix A.

Although low levels of *E. sakazakii* were found in dried-infant formula, the incidence (6.7%) found in this study and the 16% incidence found by Muytjens *et al.* (1988), as well as the relatively short lag time and generation time of *E. sakazakii* may be a cause for concern. Improper storage of reconstituted dried-infant formula at ambient temperatures, e.g., on a bedside table for night feedings, or during shopping, can permit growth of *E. sakazakii*. Growth inhibition appears to be an important control factor for *E. sakazakii*. Our work with suckling mice strongly suggest that large numbers need to be ingested in order to cause illness as discussed in Chapter 5.

We are unaware of any literature reports describing the competitiveness of *E. sakazakii* and other organisms. Simmons *et al.* (1989) demonstrated that when dried-infant formula was mixed with nutrient broth, *E. sakazakii* grew well and survived better than the only other contaminant found, *E. cloacae*. The interaction of *E. sakazakii* with other organisms found in dried-infant formula needs to be examined.

CONCLUSION

Results obtained from this study indicate the importance of proper preparation and storage of reconsitituted dried-infant formula with respect to the survival and growth of *E. sakazakii*. This is the first study to demonstrate that, although present at low levels, *E. sakazakii* can be found in dried-infant formula in the Canadian marketplace. Although *E. sakazakii* does not grow at proper refrigeration temperatures of 4°C, it can grow slowly at slightly abusive temperatures (5.5°C). The short generation time (40 min) of *E. sakazakii* in reconstituted dried-infant formula at room temperature is a cause for concern.

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Nazarowec-White, M. and Farber, J.M. 1997. Incidence, survival and growth of *Enterobacter sakazakii* in infant formula. J. Food Prot. 60:226-230.

CHAPTER 3: THERMAL RESISTANCE OF *ENTEROBACTER SAKAZAKII* IN RECONSTITUTED INFANT FORMULA

ABSTRACT

E. sakazakii, designated a unique species in 1980, has been implicated in a rare but severe form of neonatal meningitis, with dried-infant formula being implicated as the mode of transmission. The high mortality rate (40-80%) and the lack of information about this organism led to a study of the heat resistance of *E. sakazakii* in reconstituted dried-infant formula. Ten Canadian *E. sakazakii* strains (5 clinical and 5 food isolates) were used to determine the heat resistance of this organism at 52, 54, 56, 58 and 60°C in reconstituted dried-infant formula. D-values of 54.8, 23.7, 10.3, 4.2 and 2.5 min were obtained for each temperature, respectively. The overall calculated z-value was 5.82°C. In a comparison of the D-values of several members of the *Enterobacteriaceae* in dairy products, *E. sakazakii* appeared to be one of the most thermotolerant organisms. The importance of process control during manufacture and the use of aseptic procedures during preparation, use and storage of dried-infant formula is discussed.

INTRODUCTION

E. sakazakii was designated as a unique species in 1980 (Farmer *et al.*, 1980) based on differences from *E. cloacae* in DNA relatedness, pigment production and biochemical reactions. This organism has been implicated in a rare, but severe form of neonatal meningitis (Willis and Robinson, 1988; Simmons *et al.*, 1989; Bierling *et al.*, 1989; Noriega *et al.*, 1990; Gallagher and Ball, 1991). Although studies have failed to find an environmental source for the organism, dried-infant formula has been implicated as the mode of transmission in both outbreaks and sporadic cases of *E. sakazakii* meningitis (Muytjens *et al.*, 1983; Postupa and Aldova, 1984; Muytjens *et al.*, 1988; Bierling *et al.*, 1989; Noriega *et al.*, 1990). In survivors of *E. sakazakii* meningitis, severe neurological sequelae such as hydrocephalus, quadraplegia and retarded neural development can occur. A mortality rate of 40-80% has been reported in the literature (Willis and Robinson, 1988), and in many cases neonates die within days of birth.

Muytjens *et al.* (1988) evaluated the quality of powdered substitutes for breast milk with regard to members of the *Enterobacteriaceae* family. One hundred and forty-one powdered infant formula samples were obtained from 35 countries. Members of the *Enterobacteriaceae* family were isolated from 52.3% of the 141 different samples, the most frequent species being *E. agglomerans, E. cloacae, E. sakazakii,* and *K. pneumoniae.* Only 6 cans of Canadian formula were tested and *E. sakazakii* was isolated from 1 can of formula (<1 CFU/100g). Postupa and and Aldova (1984) isolated two strains of *E. sakazakii* from powdered infant formula in Czeckoslovakia, while both Simmons *et al.*

(1989) in the US and Bierling *et al.* (1989) in Iceland, also isolated *E. sakazakii* from dried-infant formula. Dried-infant formula is used when mothers are unable or choose not to breast feed their babies. Human and cow's milk differ in the relative content and chemical composition of macronutrients. Therefore, to simulate breast milk, cow's milk must be modified to 1) reduce protein and mineral content, 2) increase the amount of whey protein, 3) increase the carbohydrate content, and 4) increase the Ca/P ratio. In addition, vitamins are added and the fat modified. In the production of powdered infant formula, different processes may be used. One is the "dry procedure", where all ingredients are blended in the dry form; while the other is the "wet procedure" where mixing is done in the wet state prior to drying; frequently these methods are combined (Caric, 1993). In the "dry procedure" the skimmed milk is pasteurized and then evaporated. All other ingredients, i.e., fat, whey, vitamins, emulsifiers and stabilizers, are added and blended. This mix is pasteurized at 110°C for 60 seconds and then spray-dried. However, if the formula is not dry-mixed, both the liquid skimmed milk and the pre-mix of skimmed milk and fat components are treated at 80-82°C for 20 seconds. Then, the total mixture is heated at 107-110°C for 60 seconds, and the liquid mixture is concentrated using a falling-film evaporator. The concentrate is heat-treated again at 80°C and finally spray-dried. However, post-processing contamination in the manufacturing plant is possible and there is no heat treatment in reconstitution of the formula in the home.

The use of high tmeperatures to preserve food is based on their destructive effects on microorganisms. These high temperatures refer to any temperature above ambient. The

D-value is the decimal reduction value or the time required for a microbial population to decrease by 90%, i.e. a 1-$\log_{10}$ decrease at a constant temperature (Whiting and Buchanan, 1994). The value is numerically equal to the number of minutes required for the survivor curve to traverse one $\log_{10}$ cycle. Mathematically it is equal to the reciprical of the slope of the survivor curve and is a measure of the death rate of an organism.

The z-value is the change in temperature needed to decrease the D-value by 90% (or 1-$\log_{10}$), which is often used in thermal processing of foods (Whiting and Buchanan, 1994). Therefore, the z-value refers to the degree Celcius required for the thermal destruction curve to transverse one $\log_{10}$ cycle. While the D-value reflects the thermal resistance of an organism to a specific temperature, z provides information on the relative resistance of an organism to different destructive temperatures. The z-value allows for the calculation of equivalent thermal processes at different temperatures.

Since there are no reports in the literature on the heat resistance of *E. sakazakii*, the objective of this study was to obtain data on the D and z values of this organism in reconstituted dried-infant formula.

MATERIALS AND METHODS

Organism

The ten strains of *E. sakazakii* used in the evaluation of thermotolerance were all Canadian isolates. Five clinical strains were provided from culture collections of various

hospitals across Canada and the five food strains were isolated in Health Protection Branch laboratories from dried-infant formula available on the Canadian market (Table 4, Chapter 2).

Heating Menstruum

The formula from Company A, with the highest market share (38%) on the Canadian retail market, as well as containing the highest amount of fat (3.8 g/100mL), was selected as the heating menstruum. The formula was prepared according to the manufacturer's directions. The reconstituted formula (49.5 mL) was dispensed into two 250 mL stainless steel flat-bottomed centrifuge tubes (Canlab, Mississauga, ON), one for the clinical isolates and the other for food isolates.

Inoculum

Cell suspensions of five clinical and five food isolates were used to inoculate the formula. Each of the 10 strains was incubated in 5 mL BHI broth (Difco Laboratories) at 36°C for 16-17 h, centrifuged at 2800 x g (IEC Centra-8 centrifuge, International Equipment Company, Needham Hts., MA) for 25 min, and then resuspended in 10 mL reconstituted dried-infant formula. A 1 mL aliquot from each of the five clinical isolates was combined so as to obtain a concentration of 10^9 bacterial CFU/mL of inoculum. The five food isolates were similarly combined.

Thermal Resistance Experiments

The stainless steel flat-bottomed centrifuge tubes containing 49.5 mL of reconstituted dried-infant formula and a sterile magnetic stir bar, were placed into a Blue M constant temperature bath (Blue M, Blue Island, IL). This was done so that the heating medium (reconstituted formula) was constantly mixed to minimize temperature gradients. The water level in the circulating water bath was maintained at least 3.0 cm above the level of the medium in the heating vessel. Prior to inoculation, the medium was pre-heated to the appropriate test temperatures of either 52, 54, 56, 58 or 60°C. Water bath temperatures were monitored with a Daye Digistrip 4c monitor/controller (Kaye Instruments, Bedford, MA) attached to copper constantan thermocouples. The latter were calibrated with a platinum resistance temperature detector (model 373A RTD monitor; Kaye Instruments). The formula was inoculated with 0.5 mL of either the clinical or food isolate cocktail to give a final inoculum of 10^7 CFU/mL. At various time intervals 1 mL samples were withdrawn, serially diluted in buffered peptone water (0.1% w/v), surface-plated (0.1 mL) in duplicate onto tryptic soy agar (TSA, Oxoid) plates containing 1% sodium pyruvate (Sigma Chemical Co., St. Louis, MI), and then incubated at 36°C for 48 h. Sodium pyruvate was added to the plating medium to facilitate repair of injured cells (Line *et al.*, 1991).

Typical *E. sakazakii* colonies were selected for confirmation using API 20E test strips (API System, Laval, PQ). Three replicate experiments on three different days were performed for both the clinical and food isolates at each temperature.

D and z value Determinations

Thermotolerance parameters (D- and z-values) were estimated using standard regression analysis based on log linear models. For each treatment (clinical and food), at each temperature, the $\log_{10}$ CFU/mL was plotted as a function of time. A linear model for time versus $\log_{10}$ CFU of the counts was used to estimate D-values. A linear model for temperature versus the log of estimated D-values was used in order to obtain z-values. The test of hypothesis was a standard F-test (SAS Institute, 1989).

RESULTS AND DISCUSSION

In this study, D-values were calculated at five different temperatures. Table 9 shows the D-values at each temperature for both the clinical and food strains. Combining replicates and strains (pooled), the D-values ranged from 54.7 min at 52°C to 2.5 min at 60°C. Although the D-values for the clinical strains were higher at each temperature in comparison to the D-values for the food strains, the differences were not statistically significant ($P>0.05$). Figure 1 shows a typical thermal death time curve at 60°C.

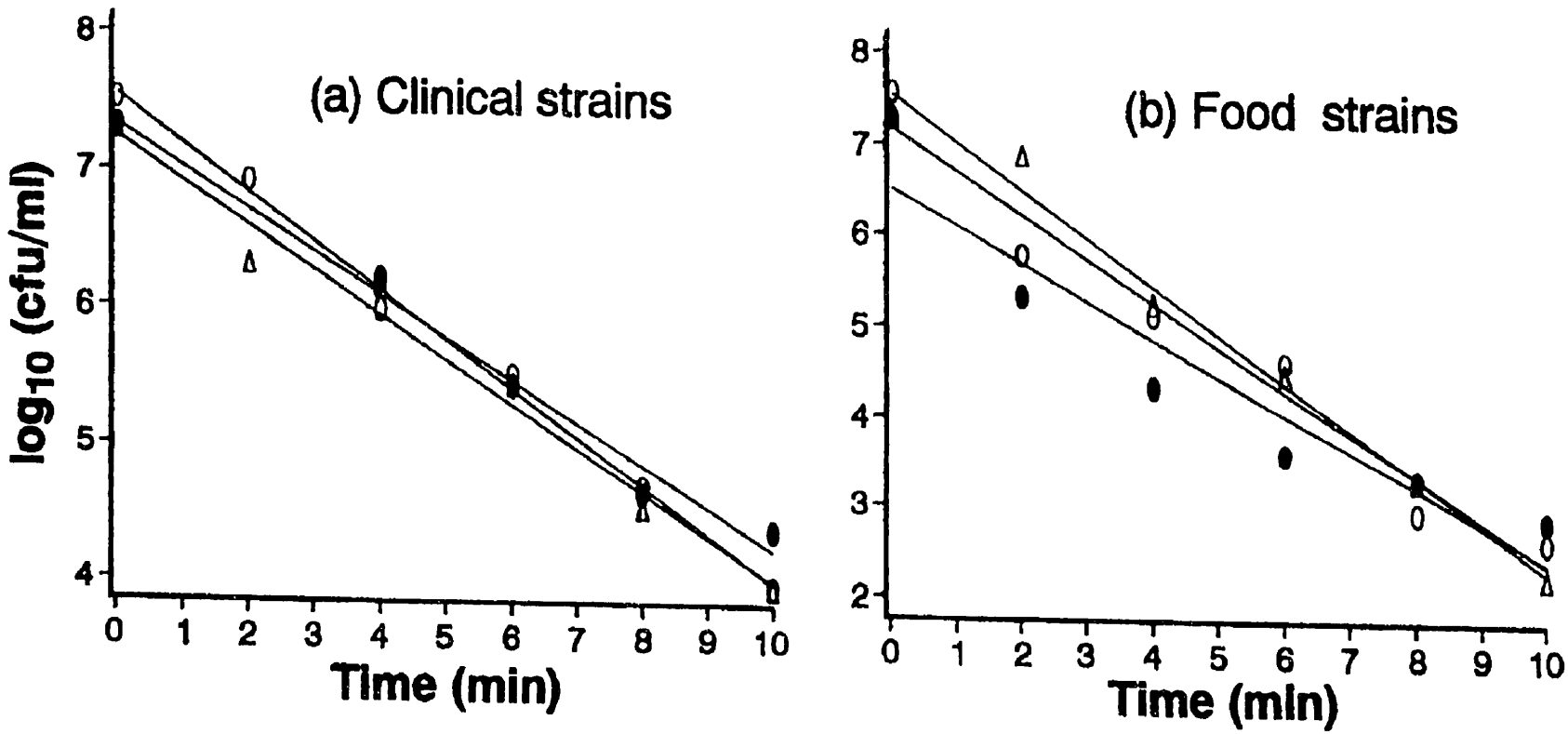
It is well known that many factors can influence the heat resistance of bacteria. Some of these include the physiological state of the organism, growth temperature of the inoculum (Knabel *et al.*, 1990), the heating menstruum (including factors such as fat concentration, amount of solids, and sugar concentration), as well as the methodology used for bacterial recovery; all make it difficult to compare D-values among studies.

Table 9. D-values (min) ± standard deviation (n=3) and z-value for *E. sakazakii* in reconstituted dried-infant formula.

Strains	Temperature (° C)					
	52	54	56	58	60	z-value
Clinical[a]	54.76 ±5.69	36.72 ±6.07	10.91 ±1.52	5.45 ±0.46	3.06 ±0.12	6.02
Food[a]	54.82 ±7.21	18.57 ±1.14	9.75 ±0.47	3.44 ±0.35	2.15 ±0.07	5.60
Pooled[b]	54.79 ±4.71	23.70 ±2.52	10.30 ±0.72	4.20 ±0.57	2.50 ±0.21	5.82

[a]Mixture of five different strains combined to give a final concentration of 10^7 CFU/ml of infant formula (clinical strains included LA, MONT, SK81, SK90, SK92; food strains included MNW1, MNW2, MNW4, MNW6, MNW8).
[b]Includes all 10 isolates.

Figure 1. Thermal inactivation of *E. sakazakii* in reconstituted dried-infant formula at 60°C (Triplicate experiments done on three different days).


(a) Clinical strains
(b) Food strains
log10 (cfu/ml)
Time (min)
Time (min)

Batish *et al.* (1988) reported on the survival of select strains of enterococci in various milks including reconstituted nonfat dried cow's milk, full cream buffalo's milk and reconstituted dried-infant formula. Among the five heating menstrua evaluated, the highest D-values were found for full-cream buffalo's milk and reconstituted dried-infant formula, in comparison to reconstituted non-fat dried-milk. The calculated D-values for reconstituted dried-infant formula were $D_{50°C}$ 87 min, $D_{55°C}$ 58 min, and $D_{60°C}$ 45 min. The corresponding values for non-fat dried milk were 67, 52 and 45 min, at the respective temperatures. It was suggested that the high content of total solids and high amount of fat in the infant formula was responsible for protecting the microorganisms from heat. Singh and Ranganathan (1980) reported $D_{55°C}$ values of 40.10 and 33.28 min for a nonpathogenic strain of *E. coli* in whole milk. However, in the same study, a D-value of 6.56 min at 55°C was also reported for a different strain of *E. coli* in whole milk. In comparison, for *E. sakazakii*, D-values of 23.7 and 10.3 min were obtained at 54 and 56°C, respectively. Morgan *et al.* (1988) reported $D_{62°C}$ values of 0.11 to 0.13 min in human milk for *K. pneumoniae*. D-values reported in the literature for other members of the *Enterobacteriaceae* can be seen in Table 10.

The z-value calculated in our study for the pooled clinical and food strains was 5.82°C (Table 9) which was within the range (4-6°C) reported for most nonsporeforming bacteria (Tomlins and Ordal, 1976). The z-value was slightly higher (6.02°C) for the clinical than for the food strains (5.60°C). However, the difference was not statistically significant ($P>0.05$).

Table 10. A comparison of the heat resistance of various Gram-negative organisms in dairy products.

Organism	Heating menstruum	$D_{72°C}$ (s)[a]	Reference
A. hydrophila	Raw milk	0.01476	Palumbo *et al.*, 1987
C. jejuni	Skim milk	0.07033	Doyle and Roman, 1981
E. coli	Whole milk	0.15669	Singh and Ranganathan, 1980
	Human milk	0.01443	Morgan *et al.*, 1988
E. sakazakii	Infant formula	1.30088	This study
K. pneumoniae	Human milk	0.00008	Morgan *et al.*, 1988
S. muenster	Whole milk	0.07214	D'Aoust *et al.*, 1987
S. senftenberg	Whole milk	0.08417	D'Aoust *et al.*, 1987
S. typhimurium	Whole milk	0.22000	Bradshaw *et al.*, 1987
	Whole milk	0.12125	Bradshaw *et al.*, 1987
S. dysenteriae	Whole milk	0.13045	Stumbo, 1973
Y. enterocolitica	Whole milk	0.46086	Toora *et al.*, 1992
	Whole milk	0.91208	Toora *et al.*, 1992

[a]Calculated in seconds from values given in each reference, as per Shapton and Shapton (1991).

Many thermal processes or pasteurization regimes require a 4 to 7D kill of microorganisms as a prerequisite of process control (USDA, 1990). Using the results of this study ($D_{60°C}$ 2.5 min), in order to obtain a 6 or 7 $\log_{10}$ reduction in numbers of *E. sakazakii*, the length of heat treatment required at 60°C would be 15.0 and 17.5 min, respectively. At minimum high-temperature, short time (HTST) pasteurization schedules (15 seconds at 71.7°C), a greater than 11D kill of *E. sakazakii* in dried-infant formula would be observed (Table 8), and therefore the organism would not survive the pasteurization process. However, post-processing contamination of product is potentially still a problem. It is recognized that the plant environment is of critical importance for all dried products, and that the drying and filling areas are often the principle contamination source for dried products (Caric, 1993). Microbial pathogens can gain access to the powder from the environment or from the addition of ingredients at the powder stage. Pathogens in these products are a major concern since the powdered formula is intended for feeding small babies, and therefore pathogens must either be inactivated or prevented from contaminating the formula post-processing. Pasteurization of milk and all other ingredients (as technically possible) should theoretically destroy all foodborne pathogens. The contamination of infant formula powder can be reduced or eliminated by careful monitoring of the critical control points (CCP) within the manufacturing process. Of particular concern is that this organism can multiply in reconstituted formula, which in certain instances is held for some time before being consumed by infants (see Chapter 2, Table 7).

CONCLUSION

From this data, it appears that *E. sakazakii* is more thermotolerant than many other *Enterobacteriaceae* in dairy products. The importance of using Hazard Analysis Critical Points (HACCP) during the manufacture of dried-infant formula is evident. HACCP is a preventative approach to identify all significant foodborne hazards that could be related to processes at each step of production of a food product. Further work to find the origin of contamination by this particular organism, as well as its possible occurrence in other potentially high-risk foods is needed.

The information presented in this chapter came in part from the following publication:

Nazarowec-White, M. and Farber, J.M. 1997. Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. Letters Appl. Microbiol. 24:9-13.

CHAPTER 4: *ENTEROBACTER SAKAZAKII* CHARACTERIZATION BY PHENOTYPE AND GENOTYPE

ABSTRACT

Enterobacter sakazakii, designated a unique species in 1980, has been implicated as the causative organism in a rare but severe form of neonatal meningitis. Dried-infant formula has been identified as a potential source of the organism. *E. sakazakii* isolated from dried-infant formula available on the Canadian market, and clinical isolates obtained from Canadian hospital culture collections were characterized. The relatedness of the bacterial isolates was evaluated using both phenotypic (biotype and antibiograms) and genotypic (ribotyping, random amplification of polymorphic DNA, and pulsed-field gel electrophoresis) methods. From the 18 clinical and food isolates examined, three biotype and four antibiogram patterns were observed. Ribotyping using the Dupont Riboprinter™ microbial identification system divided the 18 isolates into 10 ribotypes. Three isolates from the same hospital had indistinguishable ribotyping patterns although each was isolated in a different year. In addition, three food isolates from one company had identical robotype patterns. PFGE and RAPD indicated minor differences between isolates indistinguishable by ribotyping. PFGE using the restriction enzymes *Xba*I and *Spe*I and RAPD gave discrete patterns that enabled easy comparison of *E. sakazakii* isolates, with a high degree of discrimination. Using the discriminatory index, RAPD and PFGE were shown to be the most discriminatory typing schemes for *E. sakazakii* followed by ribotyping, biotyping and antibiograms.

INTRODUCTION

Bacterial typing systems are based on the premise that clonally related isolates share characteristics by which they can be differentiated from unrelated isolates (Arbeit, 1995; Maslow *et al.*, 1993). Methods for the identification and discrimination of bacterial isolates can be divided into two broad categories. The traditional microbiological techniques (phenotypic typing) are based on secondary characteristics of bacteria including biochemical reactions, antibiograms, serotyping and bacteriophage typing, (Arbeit, 1995; Eisentein, 1990). In areas such as food safety, epidemiology and ecology it is important to identify species and strains of organisms accurately in order to establish clonality of organisms in outbreaks. The application of molecular techniques to food microbiology has led to the development of new and rapid methods for the detection, identification and characterization of foodborne organisms (Grant and Kroll, 1993). The most common molecular typing methods being used in food microbiology include chromosomal DNA restriction endonuclease analysis (REA), plasmid typing, ribotyping, (PFGE) and polymerase chain reaction (PCR)-based methods such as (RAPD) typing (Farber, 1996). Each of these techniques has their advantages and disadvantages and the objectives of the experiments will influence the technique selected. Table 11 is a composite describing the advantages and disadvantages of the most commonly phenotypic and genotypic molecular techniques used in food research. Advantages and disadvantages of the techniques are described in the following sections.

Biotyping: Biotyping makes use of the pattern of metabolic activities expressed by an

Table 11. Advantages and disadvantages of various typing methods[a]

Typing system	Advantages	Disadvantages
I. Phenotypic methods		
Biotyping	Automated systems available	Inability to differentiate between strains of a different species Random mutations confuse interpretation
Antimicrobial susceptibility testing	Applicability of results to medical intervention	Phenotypes vary Strain genetic history may influence antibiotic susceptibility
Serotyping	Rapid, reliable serological assays available	Difficulty in interpretation
Multilocus enzyme electrophoresis	Powerful tool for population genetics	Requires specialized techniques and equipment
II. Genotypic methods		
Plasmid profile analysis	Relatively quick and easy method Results can be standardized using known markers	Plasmids usually unstable Some organisms contain few or no plasmids Different plasmids can appear to be the same size
Chromosomal DNA Restriction endonuclease analysis	Universally applicable Rapid, inexpensive, and relatively easy to perform	Genomic restriction fragments are usually too numerous and too closely spaced Have to screen a number of restriction endonucleases

Ribotyping	Single probe can be used to subtype all eubacteria Reproducible patterns obtained with reasonable number of fragments after probing Can be automated	A little more tedious and time-consuming (multiple steps) than other molecular methods Not as discriminatory as some newer methods (*rrn* operons cover only about 0.1%) of chromosomal DNA) May not be useful for some bacteria that contain only 1 or 2 *rrn* loci
Pulsed-field gel electrophoresis	A tool for both taxonomic and epidemiological studies Highly reproducible and discriminatory Produces around 10-15 easily visible bands	More tedious and time-consuming than other molecular typing methods Restriction endonucleases tend to be expensive Cost of equipment
Randomly amplified polymorphic DNA	Rapid, easy to perform Does not require prior knowledge of DNA sequence Does not require isotopic labelling nor use of restriction endonucleases Oligo-primers can be made in unlimited amounts anywhere, so can develop standard RAPD typing procedure without need for exchanging research materials	Reproducibility, if not well standardized Only looking at possible difference from a small percentage of total genome when using 1 primer Comparison of different intensity bands of the same size may be problematic

[a] Adapted from Arbeit, 1995 and Farber, 1996.

isolate and may include specific biochemical reactions, colonial morphology and environmental tolerances (Arbeit, 1995). However, biotyping has only limited ability to differentiate among strains within a species and consequently, the technique has relatively poor discriminatory power.

Antibiograms: Antibiotic susceptibility testing is routinely performed in clinical microbiology laboratories. Manual and automated methods are available, easily conducted and relatively inexpensive. Multiple genetic mechanisms are available to a given organism (i.e. point mutation, acquisition of resistance genes), which may impart resistance to the organism against a particular antibiotic. Particularily, in hospitals antibiotic resistance is under selective pressure. The transfer of aminoglycoside modifying enzymes among members of the *Enterobacteriaceae* family is of particular concern (Mayer, 1988).

Ribotyping: Ribotyping uses nucleic acid probes to recognize ribosomal genes (Farber, 1996). In this method, which uses Southern blot analysis, strains are characterized for their restriction fragment length polymorphisms (RFLP) associated with ribosomal RNA (rRNA) operon(s), (Stull *et al.*, 1988). The ribosomal operons are comprised of 23S, 16S and 5S rRNA. The genes coding for rRNA are highly conserved among bacteria. In ribotyping, bacterial chromosomal DNA is isolated and a restriction enzyme is used to cleave the DNA into fragments. The DNA is electrophoresed in an agarose gel and then

transferred onto a nitrocellulose or nylon membrane (Farber, 1996). The membrane is then probed with a labelled probe containing, for example, *E. coli* 23S, 16S and 5S rRNA sequences. After probing, each fragment of bacterial DNA containing a ribosomal gene will be highlighted (method used for highlighting willl depend on the type of label used), creating a fingerprint pattern containing approximately 1-15 bands which makes comparison between isolates easy. Only one report (Clark *et al.*, 1990) describes ribotyping of *E. sakazakii*. In that study, *Hind*III and *Bam*HI restriction endonucleases were used to determine the relatedness of *E. sakazakii* isolates from patients as well as dried-infant formula, from two unrelated hospital outbreaks. Ribotyping results differed between the two outbreaks but in each of them, patients' isolates and isolates from the infant formula showed the same ribotype pattern.

Ribotyping has now been successfully used for many bacterial species, especially organisms responsible for nosocomial infections (Bingen *et al.*, 1994). *In vitro* and *in vivo* stability of the studied markers has been shown. Although there are limitations to this technique, such as the tedious multiple steps and the fact that it may not be useful for bacteria which contain only 1 or 2 *rrn* loci. However, in many cases it has been found to be particularly discriminatory for members of the *Enterobacteriaceae* (Bingen *et al.*, 1994).

Random Amplification of Polymorphic DNA: It has been observed that by using a

single arbitrarily chosen primer for PCR amplification, discrete and reproducible sets of products can be generated from bacterial genomes (Farber, 1996). No prior sequence information is necessary for this method to be applicable. The resolution of the patterns is high enough to allow distinguishing different strains of the same species. Compared with other typing methods, PCR-fingerprinting is fast and easy to perform and gives as much or more information than other methods such as ribotyping.

As starting material, cells from a single bacterial colony is sufficient to generate a fingerprint. The cells can be lysed by heat and a small aliquot of this crude preparation added as template DNA for the PCR. A short oligonucleotide of approximately 10 base pairs (bp) of an arbitrarily chosen sequence can be used as a primer. After denaturation of the template DNA, the primer molecules bind to target sequences of full or partial complementarity, depending on the stringency of the conditions used. If two target sites are present on different DNA strands within a distance less than a few kilobases apart and oriented properly, DNA in between the up and downstream sites is amplified during the PCR.

The types of organisms characterized by RAPD analysis is rapidly increasing and includes bacteria belonging to the genera *Staphylococcus, Streptococcus, Escherichia* and *Listeria.*

Using a set of primers alone or in combination, numerous sets of fingerprint patterns can be generated, allowing identification of bacterial strains. However, not all primer sequences are equally efficient in priming DNA synthesis from any template. Several primers should be tested for reproducibility of the results for each bacterial species. In addition, purity of the primers, the source of the *Taq* DNA polymerase and the thermal cycler machine used could impact on the RAPD patterns obtained (Farber, 1996).

The application of PCR for DNA fingerprinting purposes makes automation of the entire process possible. Generation of PCR amplicons is already automated, and staining of the gels can also be automated. In addition, electronic storage and management of the data are directly connected with automated detection. Appropriate software (i.e., Bio-Rad Molecular Analyst™ Software, Bio-Rad Laboratories, Hercules, CA) allows for comparison of newly obtained patterns with patterns in a database, providing the conditions are standardized. This allows for screening a large number of patterns in order to determine the genetic relationship among isolates.

Pulsed-field gel electrophoresis: PFGE is a variation of agarose gel electrophoresis. The orientation of the electric field is pulsed periodically and as a result PFGE can resolve large DNA fragments (>20 kilobase (kb) pairs) which conventional agarose gel electrophoresis, which uses a constant electric field, cannot separate (Tenover *et al.*, 1995). Intact cells of the organisms are embedded in agarose plugs and the DNA is

released *in situ*. This procedure minimizes shearing of the DNA before it is digested with restriction enzymes. Restriction enzymes used in PFGE are enzymes which do not cleave the DNA frequently, thereby allowing for large fragments to be visualized on the agarose gel. This method has been found to be excellent for typing most bacterial pathogens. However, the method is not rapid and requires relatively expensive equipment and enzymes (Farber, 1996).

The discriminatory power of a typing system takes into account the homogeneity of distribution between types and is therefore, an objective measure of the chance that two unrelated strains will belong to two different types (Hunter and Gaston, 1988). A comparison of the discriminatory power using different typing techniques can show the diversity of strains under investigation. Discriminatory power is one criteria for evaluating typing systems. Additional criteria as can be seen in Table 12 are typeability, reproducibility, ease of interpretation of results and ease of performance of the technique.

Typing techniques used in the present study were selected on the basis of discriminatory ability, availablility of equipment as well as the advantages of the different typing methods as described in Tables 11 and 12. Information from both tables was considered in the selection of molecular techniques used in this thesis. In this study, the relatedness

Table 12. Characteristics of bacterial typing systems[a]

Typing system	Proportion of strains typable	Reproducibility	Discriminatory power	Ease of interpretation	Ease of performance
I. Phenotypic methods					
Biotyping	All	Poor to fair	Poor	Excellent	Excellent
Antimicrobial susceptibility testing	All	Fair	Poor	Excellent	Excellent
Serotyping	Most	Good	Fair	Good to excellent	Fair to good
Multilocus enzyme electrophoresis	All	Excellent	Good	Excellent	Fair to good
II. Genotypic methods					
Plasmid profile analysis	Variable	Fair to good	Good	Good	Excellent
Restriction endonuclease analysis	All	Very good	Good	Poor	Excellent
Ribotyping	All	Excellent	Fair to good	Very good to excellent	Fair to good
Pulsed-field gel electrophoresis	All	Excellent	Excellent	Excellent	Very good to excellent
Randomly amplified polymorphic DNA	All	Good	Very good to excellent	Very good	Very good to excellent

[a] Adapted from Farber, 1996; Arbeit, 1995 and Maslow, *et al.*, 1993.

of Canadian strains of *E. sakazakii* was determined using both phenotypic (biotyping and antibiograms) and genotypic (ribotyping, RAPD, PFGE) methods.

MATERIALS AND METHODS

Eighteen strains of *E. sakazakii* were identified using the API 20E system (API System) The source of the isolates is shown in Table 4, Chapter 2. Clinical isolates involved in human illness were obtained from Canadian hospital culture collections. Food strains were isolated from samples of powdered infant formula available on the Canadian retail market (Nazarowec-White and Farber, 1997b). The type strain, ATCC 29544 (ATCC), was also included.

Biotyping

Lyophilized isolates isolated in our laboratory and isolates obtained from Canadian culture collections (Table 4, Chapter 2) were revived in nutrient broth (NB, Oxoid), for 2 h at 36°C. A loopful of the suspended bacteria was added to 9 mL of NB (Oxoid) and incubated at 36°C overnight. The following day, a loopful of the overnight growth was streaked onto TSA (Oxoid) and incubated overnight at 36°C in order to obtain pure, well-isolated colonies. Twenty-one biochemical tests commonly used in the identification of the *Enterobacteriaceae* family were used to determine differences in biotype (API 20E). The directions of the manufacturer of the API20E (API System) test strips were followed.

Antibiograms

Seventeen Canadian strains of *E. sakazakii* plus the type strain (ATCC 29544) were evaluated for antibiotic susceptibility. Strains were grown overnight at 36°C on TSA plates. The following day, 10 colonies of each strain were suspended in sterile ddH_2O to a concentration equivalent to 0.5 McFarland Standard (Oxoid). Within 15 min of preparing the suspension, the isolates were swabbed in three planes onto Mueller-Hinton agar (Difco Laboratories). Antibiotic susceptibility was determined by means of a standard disk diffusion technique (National Committee for Clinical Laboratory Standards: Performance standards for antimicrobial disk susceptibility tests. Approved standard M2-A3. Villanova, PA, 1998). Commercial antibiotic Bactrol Disks™ (Difco Laboratories) were placed onto the agar plates and incubated for 16-18 h. Disk contents were as follows: ampicillin (10 μg), cefotaxime (30 μg), chloramphenicol (30 μg), gentamicin (10 μg), kanamycin (30 μg), polymyxin B (300 units), streptomycin (10 μg), trimethoprim-sulfamethoxazole (25 μg), tetracycline (30 μg), sulfisoxazole (300 μg) and cephalothin (30 μg). To control precision and accuracy of the test procedure, the reference organisms *Staphylococcus aureus* (ATCC 25923), *E. coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853) were used as controls. A standard control strain *Enterococcus faecalis* (ATCC 29212) was tested with trimethoprim-sulfamethoxazole as the media control. The test medium was Mueller-Hinton agar (Difco Laboratories), final pH 7.3. All plates were used within 7 days of preparation.

Strains were categorized as susceptible, intermediate in susceptibility or resistant to the various antimicrobials on the basis of the diameters of the zone of inhibition measured in millimeters (Anon, 1998). All experiments were performed in triplicate.

Ribotyping

E. sakazakii strains were sent to DuPont Central Research and Development (Wilmington, DE) on TSA (Difco Laboratories) slants. The DuPont RiboPrinter Microbial Characterization System™ was used to produce genetic "fingerprints " called RiboPrint patterns. In the RiboPrint System's automated process, bacterial cells were lysed, DNA fragments generated using *Eco*RI restriction endonuclease and patterns were created using a labelled rRNA operon (*rrn*B ribosomal operon of *E. coli*, Brosius *et al.*, 1981). The patterns were imaged using chemoluminescence and a Couple Charged Device (CCD) camera (BioéCan Scientific, Mississauga, ON). Through the use of algorithms for data normalization, patterns were obtained (Bruce, 1996). Visual comparisons among pattern sets for all strains evaluated were made to determine relatedness among strains and strains with similar banding patterns were grouped into clusters.

Randomly Amplified Polymorphic DNA

Primer selection: Thirteen strains of *E. sakazakii*, in addition to the type strain (ATCC 29544) were used in preliminary experiments to screen primers to be used in RAPD.

These thirteen strains were either recently isolated in our laboratory from dried-infant formula or had just been obtained from Canadian hospital culture collections. These strains included: LA, LB, MONT, SK81, SK90, SK92, MNW1, MNW2, MNW4, MNW5, MNW6, and MNW7. Each strain was subcultured onto TSA plates (Difco Laboratories) and grown at 36°C overnight. A single colony was used to inoculate tryptic soy broth containing 0.6% yeast extract (TSB-YE, Difco Laboratories) and incubated at 36°C. After initially screening one hundred decamer primers UBC 201-300 (University of British Columbia, Vancouver, BC), two primers, UBC 245 (5'- CGC GTG CCA G -3') designated as primer 1 and UBC 282 (5'- GGG AAA GCA G -3') designated as primer 2, were selected for subsequent PCR amplification. These primers were selected on the basis of their performance in trial experiments to produce reproducible and manageable RAPD patterns (unpublished data). The decision to select these two primers was based on the number of bands obtained and the wide size range.

DNA isolation: A number of methods were initially explored in order to determine which was the easiest, and most reliable to be used in amplification experiments. A) Crude DNA was isolated from bacterial cells grown overnight in TSB-YE (Difco Laboratories). The cells were pelleted and then ground with a disposable pestle (Mandel Scientific Co. Ltd., Guelph, ON) in the presence of an extraction buffer (0.5% SDS, 25 mM EDTA, 250 mM NaCl and 200 mM Tris HCl pH 8.0, Sigma Chemical Company, St. Louis, MO). The DNA was precipitated with 70% isopropanol, pelleted, and then the

pellet dried and dissolved in sterile ddH_2O. Variations to this method included omitting the SDS from the extraction buffer, and altering the lengths of time spent grinding cells. B) Crude DNA was purified using a Double Gene Clean™ kit (Bio 101 Inc., La Jolla, CA), and the DNA precipitated in the presence of NaI and Si as per the manufacturer's directions. The resultant pellet was washed three times in an ethanol-based Gene Clean buffer and then heated to 55°C for 10 min. The pellet was then dissolved in sterile ddH_2O, centrifuged and the supernatant containing the purified DNA removed to a clean tube before use. C) DNA was extracted from bacterial cells grown in TSB-YE (Difco Laboratories), using a modification of the Birnboim and Doly method (Birnboim and Doly, 1979). Modifications included using 7% cesium chloride (Sigma) and an ultracentrifuge (16,000 x g for 20 h at 4°C, IEC, Needham Hts., MA). For all the above methods the concentration of the extracted DNA was determined on a 0.7% agarose-gel made with 1xTBE buffer (8.9 mM Tris base, 8.9 mM boric acid, 0.2 mM EDTA), Boehringer-Mannheim, Laval, PQ] using known amounts of lambda (λ) DNA (Gibco BRL, Gaithersburg, MD). D) A cell-based RAPD method was also attempted. Confluent overnight growth of *E. sakazakii* was collected from TSA (Difco Laboratories) plates with a swab, using a small amount of sterile ddH_2O. Optical density (OD) readings were taken at 600 nm and the concentration of the cell suspension was adjusted to 0.18, equivalent to approximately 10^7 cells (Woods *et al.*, 1993). E) Each of the *E. sakazakii* isolates were grown overnight at 36°C in a shaking incubator (250 rpm). A portion (500 μL) of a 10-fold dilution of the overnight culture in peptone water (Oxoid) was boiled for

5 min in a 1.5 mL Eppendorf tube in a floating rack in tap water. Immediately after boiling, the cell lysates were frozen at -20°C, and then thawed just prior to use (Trost *et al.*, 1993).

The whole cell method was selected for final testing of *E. sakazakii* isolates as it was the easiest to perform, less expensive than the Double Gene Clean kit and resulted in clear distinguishable banding patterns.

Amplification reactions were performed in a volume of 20 μL mastermix containing a final concentration of 10mM Tris-HCl pH 8.3, 50 mM KCl, 25 mM $MgCl_2$ (Promega Corp, Madison, WI), 200 μM dNTPs (Pharmacia Biotech Inc., Baie d'Urfe, PQ) 0.5 units of *Taq* DNA polymerase (Promega), 1 μM random primer and 1μL cell lysate. Before primer, *Taq* DNA polymerase and cell lysates were added, the reaction mixture was treated with ultra violet (UV) light for 20 min and aliquoted into sterile 0.5 mL Eppendorf tubes (Sarkar and Sommer, 1990). Primer, cell lysates and *Taq* DNA polymerase were then added to each tube. A negative control was included in each trial where cell lysate was replaced with 1 μL sterile ddH_2O. Amplification was performed for 1 cycle of 2 min at 94°C, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 35°C for annealing, 1.5 min at 72°C for extension and then followed by 1 cycle at 72°C for 5 min for further primer extension. The ramp time from 35 to 72°C was 2 min. All PCR reactions were performed in a thermal cycler (Gene Amp® PCR System 9600, Perkin

Elmer, Branchburg, NJ).

The amplified products and a 123 bp marker (Gibco BRL, Gaithersburg, MD) included as a molecular weight standard were resolved by electrophoresis on 1.5% agarose gels in 1 x TBE buffer (Boehringer Mannheim) containing 0.5 μg of ethidium bromide per mL (Dillon *et al.*, 1985). Gels were photographed under UV transillumination.

Pulsed-field Gel Electrophoresis

Preparation of bacterial DNA: Cultures shown in Table 4, Chapter 2 were incubated in 20 mL BHI broth (Difco Laboratories) in a shaking (200 rpm) incubator at 36°C for 3-4 h until growth reached an OD of approximately 0.8 at 610 nm. Cells (1mL) were harvested by centrifugation (16,000 x g for 10 min at 4°C) and washed twice in an equal volume wash buffer (1 M NaCl, 10 mM Tris, pH 8.0; 10 mM EDTA, Sigma). Cells were resuspended in 0.5 mL wash buffer and then warmed for a few minutes at 45°C for easier resuspension. Bacterial suspensions were mixed with an equal volume of 1% high strength analytical grade agarose (BioRad). Agarose was prepared in ddH_2O by boiling in a microwave oven followed by cooling to 55°C in a water bath. Aliquots (100 µL) of the mixture were dispensed into plastic plug molds (BioRad) in order to make multiple plugs. Plugs were allowed to solidify for 30 min at room temperature (~23°C) and then placed into sterile conical tubes containing lysis buffer, (1 M NaCl; 10 mM Tris, pH 8.0; 100 mM EDTA; 0.5% sarkosyl; 0.2% sodium deoxycholate; 1 mg/mL lysozyme, Sigma).

Approximately 1 mL/plug of lysis buffer was used. A stock solution of lysis buffer was made and the lysozyme added immediately prior to use. Bacteria were lysed for 1 to 1.5 h at 37°C. After incubation, the lysis buffer was decanted and several plugs incubated overnight at 50°C in 1 mL/plug of ESP buffer (0.5 M EDTA, 1% sarkosyl; 1 mg/mL proteinase K, Sigma). The following day, plugs were washed twice for 2 h with 1.0 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) prepared in TE buffer (10 mM Tris, pH 8.0; 1 mM EDTA) at room temperature in order to inactivate the proteinase K. Three additional washes for 30 min in TE buffer were performed to remove PMSF. The plugs were then stored in TE buffer at 4°C.

Restriction endonuclease digestion: For restriction endonuclease digestion, three, 1 mm thin rectangular slices were cut from an agarose plugs, and equilibrated for 30 min to 1 h in a microcentrifuge tube containing 100 µL of the appropriate restriction endonuclease reaction buffer. The buffer was then replaced with 100 µL fresh reaction buffer and digestion took place overnight with 0.5 units/mL of enzyme at the appropriate temperature for the enzyme used. Selection of restriction enzymes was based on the G+C content of the recognition site of the enzyme and the 57% G+C content reported for *E. sakazakii* (Farmer *et al.*, 1980). The enzymes investigated were *Apa*I, *Not*I, *Ssp*I, *Spe*I and *Xba*I. *Apa*I and *Not*I produced too many small sized fragments and *Ssp*I digests resulted in unclear banding patterns. Ultimately, enzymes *Xba*I and *Spe*I with recognition sites 5'-TCTAGA-3' and 5'-ACTAGT-3', respectively, were selected because they gave

the clearest and most discriminating bands.

Separation of restriction fragments: Restriction fragments were separated in a 1% ultra-pure DNA grade agarose (BioRad) gel in 0.5 X TBE buffer (4.5 mM Tris base, 4.5 mM Boric acid and 0.1 mM EDTA)(Boehringer Mannheim), by using a CHEF-DR II system (BioRad). Bacteriophage lambda DNA ladders (Boehringer Mannheim) were used as molecular standards. A model 1000 mini-chiller (BioRad) was used to maintain the temperature of the running buffer at 14°C. Following *Xba*I digestion, the pulse time was ramped linearly from 5 to 50 seconds for a running time of 20 h. After *Spe*I digestion, the pulse time was ramped linearly from 5.3 to 38.6 seconds for 16 h. Following electrophoresis, gels were stained for 30 min with ethidium bromide (5 μg/mL in water), destained, and visualized on a UV transilluminator (Fotodyne Incorporated,Hartland, WI).

Analysis of PFGE* and *RAPD patterns: A CCD photography system (Bio/Can Scientific, Mississauga, ON) was used to photograph the stained gels. Images were saved onto diskettes in TIFF file format and each lane analyzed using the Molecular Analyst Software™ (BioRad) in order to determine the banding patterns and their relatedness. The discriminatory power of the genotyping techniques (RAPD, PFGE, ribotyping) was compared using Simpson's index of discrimination (Hunter and Gaston, 1988).

RESULTS AND DISCUSSION

Biotyping

The *E. sakazakii* strains could be grouped into three biotypes (Table 13). The type strain (ATCC 29544) was designated as Biotype 1 and all strains were compared to it. Eighteen strains of *E. sakazakii* exhibited three biotypes, one of which encompassed 9 strains. As can be seen in Table 13, eight strains (44%) belonged to Biotype 1 the same as the type strain. Six strains (33 %) were inositol negative and designated as Biotype 2, which is comparable to the figure of 28% of *E. sakazakii* strains in the API 20E (API System) database which are inositol negative. Three strains (2 clinical: MONT; LCDC3 and 1 powdered infant formula isolate, MNW2) were Voges-Proskaur (VP) positive (Biotype 3).

Postupa and Aldova (1984) describing the biochemical reactions of 6 strains of *E. sakazakii* (4 isolates from skim milk powder and 2 from infant formula), found two to be inositol positive and all to be VP positive. Farmer *et al.* (1980) found 98% of 57 *E. sakazakii* strains to be VP positive and 25% to be inositol negative. These researchers concluded that in the VP negative strains, either acetoin was not expressed under the test conditions or that the organism had lost the genetically encoded ability of expressing acetoin. Farmer *et al.* (1980) found that inositol positive strains decreased the pH of the test medium whereas the inositol negative strains made the medium akaline. acetoin. Farmer *et al.* (1980) found that inositol positive strains decreased the pH of the

Table 13. Biochemical characteristics of *E. sakazakii* evaluated.

Characteristic	Strains evaluated																	
	ATCC	LA	LB	MONT	SK 81	SK 90	SK 92	LCDC 1	LCDC 2	LCDC 3	MNW 1	MNW 2	MNW 3	MNW 4	MNW 5	MNW 6	MNW 7	MNW 8
ONPG	+[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Decarboxylases:	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
arginine																		
lysine	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ornithine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
sulfide																		
Urease	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Tryptophane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
deaminase																		
Indole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Voges-Proskauer	-	-	-	+	-	-	-	-	-	+	-	+	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Acid from:																			
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Inositol	+	+	-	+	+	+	+	+	+	+	+	+	–	–	–	–	+	–	
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Amygdalin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Biotype	1	1	2	3	1	1	1	1	1	3	1	3	2	2	2	2	1	2	

[a]+=positive.
[b]-=negative

test medium whereas the inositol negative strains made the medium akaline. Biochemical evaluations of bacterial isolates are valuable for initial strain characterization and are still in widespread use. In many laboratories biochemical tests are still considered the gold standard for identification. However, the discriminating power of biotyping is poor (Bingen *et al.*, 1994) and this technique has only limited ability to distinguish strains within a given species. Several phenotypic typing systems have been proposed for *Enterobacter* typing: serotyping, phage typing, bacteriocin typing, and biotyping (Poilane *et al.*, 1993). However, none of these schemes could be used alone for epidemiological studies (Gaston, 1988) and cannot be applied to all strains of *Enterobacter* spp. (Grattard *et al.*, 1994).

Antibiograms

The 18 strains of *E. sakazakii* gave four different antibiogram patterns (Table 14). All of the clinical strains of *E. sakazakii* except LCDC3 showed the same antibiotic profiles as the type strain. These clinical strains were susceptible to ampicillin, cefotaxime, chloramphenicol, gentamicin, kanamycin, polymixin B, streptomycin, trimethoprim-sulphamethoxazole and tetracycline but were resistant to sulfisoxazole and cepahalothin (Antibiogram 1). LCDC3 isolates were sensitive to cephalothin and only intermediately resistant to sulfisoxazole. LCDC3 was also found to belong to a different biotype than the other two LCDC strains (LCDC1 and LCDC2). Five of the food isolates of *E. sakazakii* had the same antibiotic profile as the type strain (Antibiogram 1). Food

Table 14. Antibiotic resistance patterns of *E. sakazakii* strains evaluated.

Antibiotic	Strains evaluated																	
	ATCC	LA	LB	MONT	SK 81	SK 90	SK 92	LCDC 1	LCDC 2	LCDC 3	MNW 1	MNW 2	MNW 3	MNW 4	MNW 5	MNW 6	MNW 7	MNW 8
Ampicillin	S[a]	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R[b]	S
Cefotaxime	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S	S	S	S	S	I[c]	S	S	S	I	R	S
Gentamicin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Kanamycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Polymyxin B	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Streptomycin	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Sulfamethoxazole	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S	S	S	S	S	I	S	S	S	I	S	S
Sulfisoxazole	R	R	R	R	R	R	R	R	R	I	R	R	R	R	R	R	I	R
Cephaolothin	R	R	R	R	R	R	R	R	R	S	R	R	R	R	R	R	R	R
Antibiogram pattern	1	1	1	1	1	1	1	1	1	2	1	3	1	1	1	3	4	1

[a]S=Sensitive.
[b]R=Resistant.
[c]I=Intermediate resistance.

isolates MNW2 and MNW6, were intermediately resistant to chloramphenicol and tetracycline in addition to the two antibiotics in Antibiogram 1 and were assigned to the Antibiogram 3 category. MNW7 was the isolate demonstrating resistance to the greatest number of antibiotics (Antibiogram 4). MNW7 was resistant to ampicillin, chloramphenicol, cephalothin and showed intermediate resistance to sulfisoxazole.

In general, *E. sakazakii* is more susceptible to commonly used antimicrobials such as ampicillin, gentamicin, tetracycline and cefotaxime, than other *Enterobacter* spp. (Hawkins *et al.*, 1991). Most *E. sakazakii* isolates have been found to be consistently sensitive to ampicillin, the carboxypenicillins and ureidopenicillins and aminoglycosides (Farmer *et al.*, 1980; Willis and Robinson, 1988; Muytjens and van der Ros-van der Repe, 1986; Jimenez and Gimenez, 1982; Adamson and Rogers, 1981; Monroe and Tift, 1979).

Bacterial meningitis is a rapidly fatal disease and host defences in the cerebrospinal fluid (CSF) of neonates are limited. To a large extent, the patient is dependent on the bactericidal activities of antibiotics to eradicate the causal organisms (Eng *et al.*, 1987). Although the outcome with standard therapies has been very poor, optimal antibiotic therapy of *E. sakazakii* meningitis has yet to be determined. In the majority of reported cases (Table 3, Chapter 1), a combination of ampicillin and gentamicin has been used in the treatment of *E. sakazakii* meningitis (Willis and Robinson, 1988), frequently before culture results are available and identification of the causal organism is made.

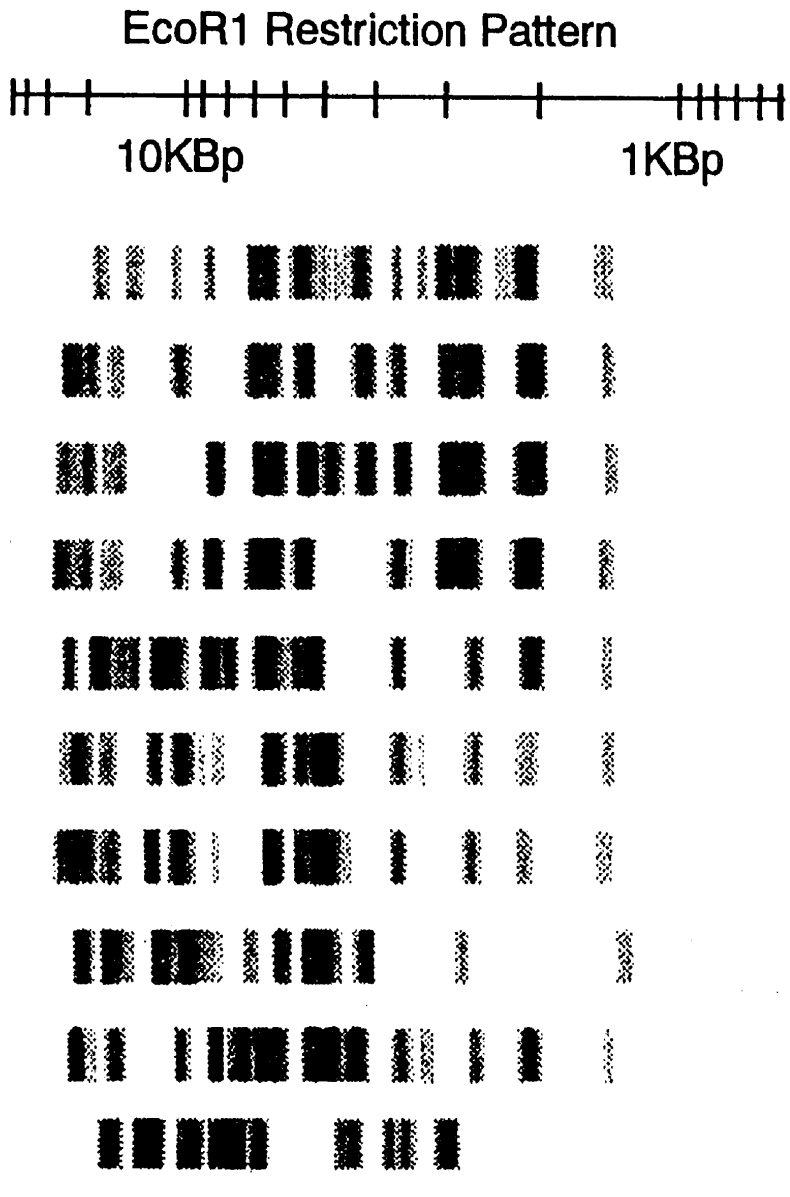
Gentamicin provides a broad spectrum coverage of gram-negative enteric bacilli, is rapidly bactericidal and absorbed readily after administration (Farmer and Kelly, 1992). However, Wolff *et al.* (1993) stated that one of the concerns of using gentamicin alone in the treatment of meningitis due to gram-negative bacilli, is the inability to achieve adequate concentrations of this antibiotic in the CSF. Willis and Robinson (1988) referred to the combination of gentamicin and ampicillin as "the gold standard" for the treatment of *E. sakazakii* meningitis.

The present study found antibiograms to be of limited use in discriminating strains of *E. sakazakii* as the strains fell into only four patterns. Similarly, Clark *et al.* (1990) found antibiograms to be the least useful epidemiological tool for typing *E. sakazakii* strains. Not only were antibiograms not as discriminating as ribotyping and multilocus enzyme electrophoresis (MEE), but variability in antibiotic resistance was found among different colonies (clones) selected from each isolate upon retesting.

Ribotyping

E. sakazakii isolates could be grouped into 10 ribotypes profiles using the restriction enzyme *Eco*RI (Figure 2). Four isolates fell into ribotype pattern 1. This pattern consisted of two clinical isolates provided by LCDC (LCDC2, LCDC3) and strain LA which had the same ribotype profile as the Type Strain (ATCC 29544), also a human clinical isolate. Three isolates (MNW3, MNW4 and MNW5) from powdered formula manufactured by the same company, obtained from three different lots, had the same

Figure 2. Ribotype patterns of *E. sakazakii* isolates.



Strain Designation	Pattern no.
MNW2	**4**
LB	**5**
MONT	**6**
SK81, SK90, SK92	**3**
MNW3, MNW4, MNW5, MNW8	**2**
LCDC1	**7**
LCDC2, LCDC3, Type strain, **LA**	**1**
MNW1	**8**
MNW7	**9**
MNW6	**10**

ribotype profile (ribotype 2). MNW8 also belonged to ribotype 2. Ribotypes 1 and 2 appeared to differ by four banding pattern areas as can be seen in Figure 2. Three clinical strains, SK81, SK90 and SK92 isolated at the same hospital in different years (1981, 1990 and 1992, respectively) gave the same profile (ribotype 3). Ribotypes 1 and 3 varied by six band differences when examined visually. All other isolates had unique ribotypes. These results suggested heterogeneity among Canadian isolates of *E. sakazakii*, results which were later confirmed by PFGE and RAPD typing.

Clark *et al.* (1990) found ribotyping to be superior to REA in the discrimination of specific *E. sakazakii* strains. The RiboPrinter Microbial Characterization System (DuPont) was also used to type *E. sakazakii* strains isolated from the environment, raw milk and powdered infant formula from three manufacturing plants in Europe (Anon, 1996). Thirty strains of *E. sakazakii* found in one factory producing dried-infant formula clustered into 8 RiboGroup pattern sets, suggesting multiple sources of the organism. Identification by RiboGroup enabled company officials to determine which strains of *E. sakazakii* were isolated from only a single site and which strains were common to all three factories producing dried-infant formula (Anon, 1996).

Ribotyping was previously found to be suitable for the study of all major bacterial species responsible for nosocomial infections, and to be particularly discriminating for members of the *Enterobacteriaceae* (Bingen *et al.*, 1994). In a comparison of biotyping, antibiogram typing and ribotyping, the latter was found to be the most discriminatory

method permitting detection of cross-contamination of *E. cloacae* in a pediatric ward (Poilane *et al.*, 1993). Regarding the choice of restriction enzymes for ribotyping, Clark *et al.* (1990) found that *Hind*III restriction digests, as compared to *Bam*HI, gave more discriminating ribotyping patterns for the *E. sakazakii* strains tested. *Eco*RI is the standard enzyme used in the Dupont RiboPrinter Microbial Characterization System and therefore was the only restriction enzyme used in this study.

Pulsed-Field Gel Electrophoresis

PFGE analysis of genomic DNA from 18 Canadian *E. sakazakii* strains digested with *Xba*I resulted in 18 different restriction enzyme digest profiles with fragments of 28-690 kb (Figure 3), indicating the genomic diversity of these strains (Tables 15 and 16). Any difference in banding patterns were considered to indicate a different pattern type. The three isolates found in dried-infant formula produced by one company which had clustered into one ribotype (ribotype 2), fell into three different pulsovars (Figure 3, lanes L, M and N). However, when using restriction enzyme *Spe*I, two of those same isolates, MNW3 and MNW4, had an identical profile (Figure 4, lanes M and N). However, the third isolate (MNW5) had an additional band at approximately 200 kb, as can be seen in Figure 4, lane O.

The three clinical strains isolated from one hospital in three different years had similar ribotype profiles (ribotype3), but different PFGE profiles using both *Xba*I and *Spe*I (Figure 3, lanes D, E and F; Figure 4, lanes D, E and F). Both enzymes used in this study

Table 15. Phenotypic and genotypic profiles of *E. sakazakii* isolates.

Source	Strain	Biotype	Antibiogram	Ribotype	PFGE		RAPD	
					*Xba*1	*Spe*1	Primer 1	Primer 2
Clinical	Type strain	1	1	1	1	1	1	1
	LA	1	1	1	2	2	2	2
	LB	2[a]	1	5	3	3	3	3
	MONT	3[b]	1	6	4	4	4	4
	SK81	1	1	3	5	5	5	5
	SK90	1	1	3	6	6	6	6
	SK92	1	1	3	7	7	7	7
	LCDC1	1	1	7	8	8	8	8
	LCDC2	1	1	1	9	9	9	9
	LCDC3	3	2	1	10	10	10	10
Food	MNW1	1	1	8	11	11	11	11
	MNW2	3	3	4	12	12	12	12
	MNW3	2	1	2	13	13	13	13
	MNW4	2	1	2	14	13	13	14
	MNW5	2	1	2	15	14	14	15
	MNW6	2	3	10	16	15	15	16
	MNW7	1	4	9	17	16	16	17
	MNW8	2	1	2	18	17	17	18

[a] All biotype 2 strains were inositol negative.
[b] All biotype 3 strains were VP positive.

Table 16. Discriminatory indices for phenotypic and genotypic methods.

Method		No. of types	Size (%) of largest group	Discriminatory index
Biotyping		3	50.0	0.647
Antibiogram		4	77.7	0.399
Ribotyping		10	22.2	0.922
PFGE	*Xba*I	18	5.5	1.000
	*Spe*I	17	11.1	0.994
RAPD	primer 1	18	11.1	0.994
	primer 2	17	5.5	1.000

Figure 3. Pulsovars of *E. sakazkaii* isolates using restriction enzyme *Xba*I. Pulsovar A= λ marker; B= MONT; C= LA; D=SK81; E=SK90; F=SK92; G=LCDC1; H=LCDC2; I=LCDC3; J=MNW1; K=MNW2; L=MNW3; M=MNW4; N=MNW5; O=MNW8; P=MNW6; Q=MNW7; R=Type Strain.

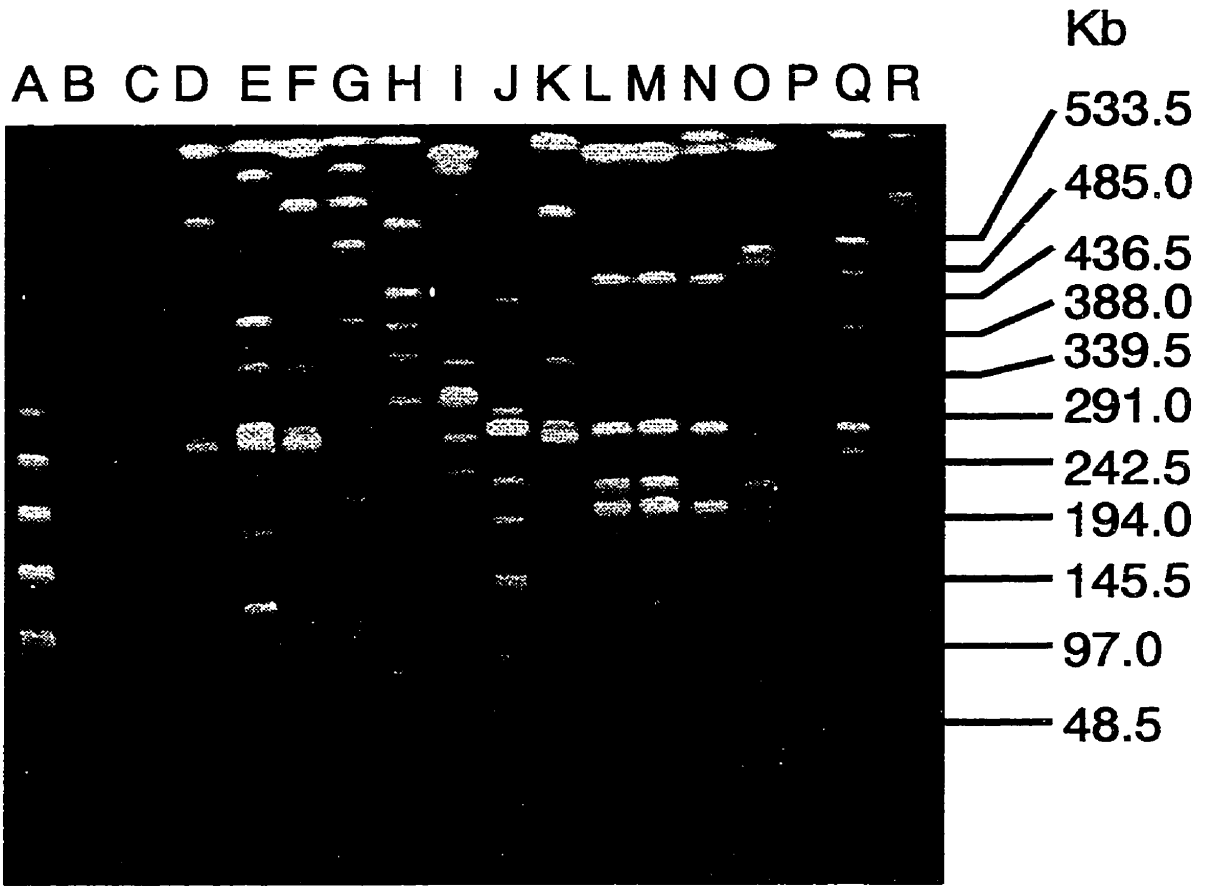
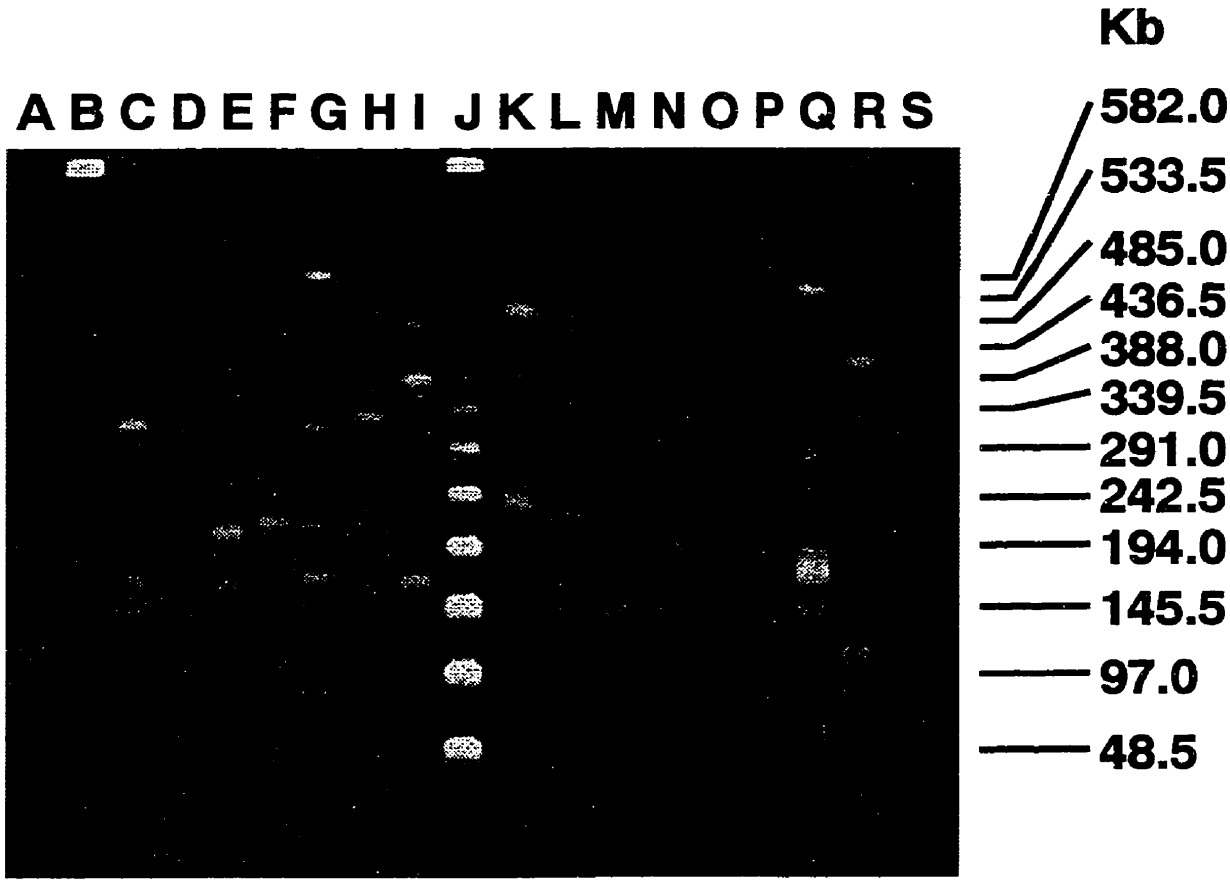

A B C D E F G H I J K L M N O P Q R
Kb
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

Figure 4. Pulsovars of *E. sakazakii* isolates using restriction enzyme SpeI. Pulsovar A=MONT; B=LA; C=LB; D=SK81; E=SK90; F=SK92; G=LCDC1; H=LCDC2; I=LCDC3; J=λ MARKER; K=MNW1; L=MNW2; M=MNW3; N=MNW4; O=MNW5; P=MNW8; Q=MNW6; R=MNW7; S=Type Strain.


A B C D E F G H I J K L M N O P Q R S
Kb
582.0
533.5
485.0
436.5
388.0
339.5
291.0
242.5
194.0
145.5
97.0
48.5

produced highly discriminatory patterns, allowing visual interpretation of banding profiles to be performed with relative ease.

Depending on the bacterial species analyzed, PFGE may be the most discriminating genotyping method available (Arbeit, 1995). Cameron *et al.* (1994) found that within one ribotype, 14 different PFGE patterns were identified among 70 isolates of *Vibrio cholerae*. In the present study, as well as in others (Cameron *et al.*, 1994), PFGE was found to be more discriminatory than ribotyping and as discriminatory as RAPD typing (Tables 15 and 16).

Tenover *et al.* (1994) feel that the lack of standard interpretation of results is a major disadvantage of electrophoretic typing techniques. While Prevost and coworkers (1991) attempted to address this issue by describing criteria for interpretation of pulsed-field gels, few investigators have followed these rules. More recently, Tenover *et al.* (1995) described guidelines to aid microbiologists in interpreting the fragment patterns resolved by PFGE to make use of molecular techniques to identify and differentiate bacterial strains. These guidelines were intended for analyzing discrete sets of isolates during epidemiological studies. Differences among isolates from outbreaks can usually be explained by the occurrence of genetic event(s). However, the interpretation of PFGE results in long term studies may be more problematic (Boerlin *et al.*, 1996). Cookson *et al.* (1996) suggested that more importance be placed on inter-laboratory comparisons in the development of proposed typing techniques. However, the lack of standardization of

methods and interpretation of results using molecular techniques precludes any valuable comparisons obtained between laboratories (Bille and Rocourt, 1996).

Randomly Amplified Polymorphic DNA

Random amplification of polymorphic DNA using primer 1 and primer 2 gave 17 and 18 independent banding patterns, respectively. Both primers yielded from 4 to 13 amplified products ranging in size from 0.3 to 2.5 kb (Figures 5 and 6). In Figure 5, lanes J and L are identical and lane K is different, containing a band at 861 bp. These lanes were the food isolates, MNW3, MNW5 and MNW4, respectively, which also showed the same pulsed-field patterns (Figure 4). Figure 6 shows a composite gel of all *E. sakazakii* strains evaluated using primer 2. This figure clearly shows the banding differences among the three clinical isolates from one hospital, SK81, SK90 and SK92 (Figure 6, Lanes D, E and F), and among the three food isolates strains MNW3, MNW4, and MNW5 (Figure 6, Lanes J, K and L). The reproducibility of the RAPD method was examined by repeating the RAPD analysis on three separate occasions. No change in the DNA fingerprints was observed in any of the replicate experiments (data not shown). The negative controls used on each gel were consistently negative.

For RAPD typing, the selection of primers for producing reproducible and easily interpretable DNA fingerprints is essential. RAPD is more rapid and easier to perform than PFGE, with same day results being obtained if boiled cells are used for template DNA in amplification reactions (Louie *et al.*, 1996). The use of at least two independent primers

Figure 5. RAPD profiles using primer 1. Lane A=MONT; B=LA; C=LB; D=SK81; E=SK90; F=SK92; G=123 bp marker; H=MNW1; I=MNW2; J=MNW3; K=MNW5; L=MNW4; M=MNW6; N=MNW7.

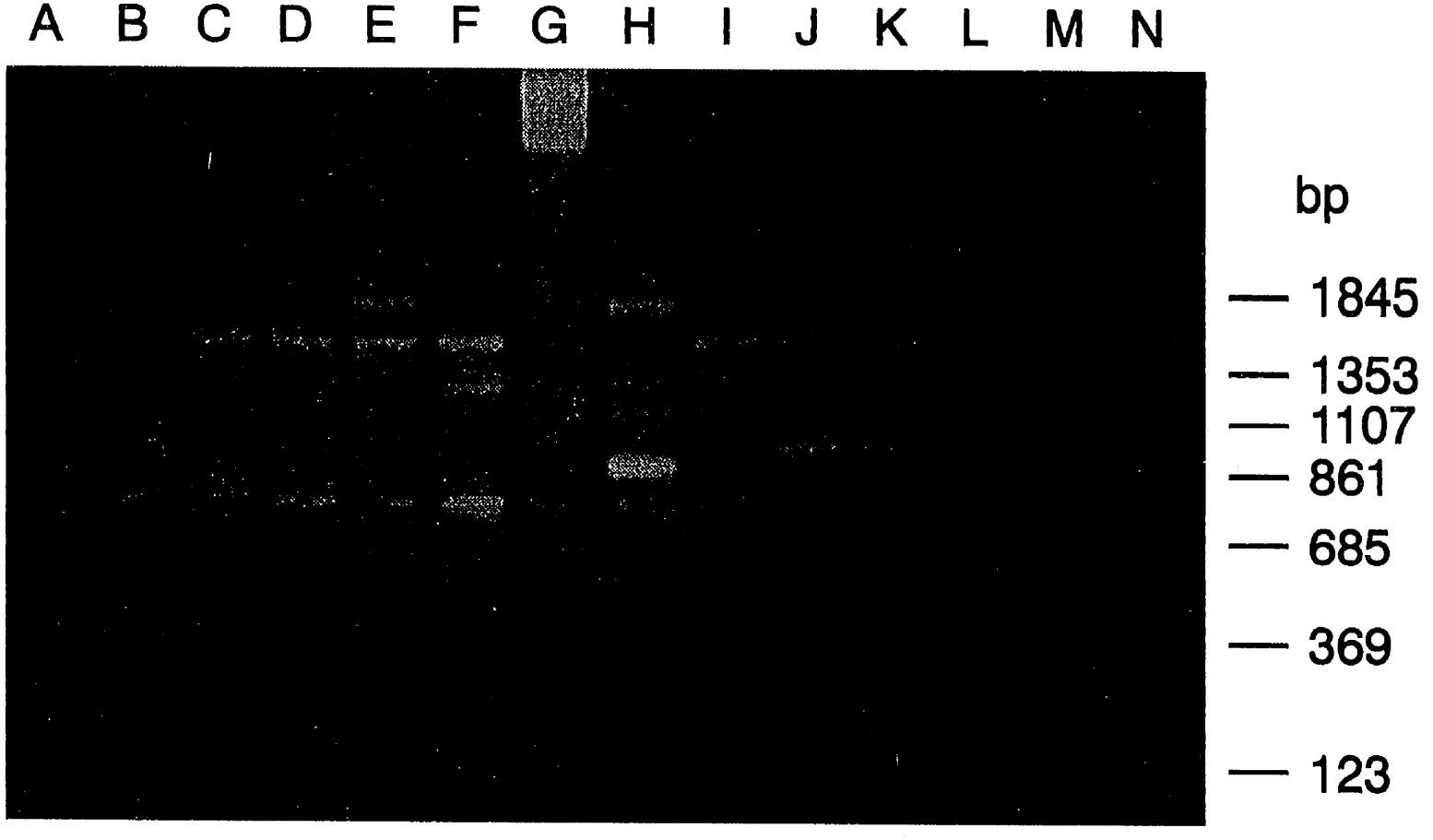
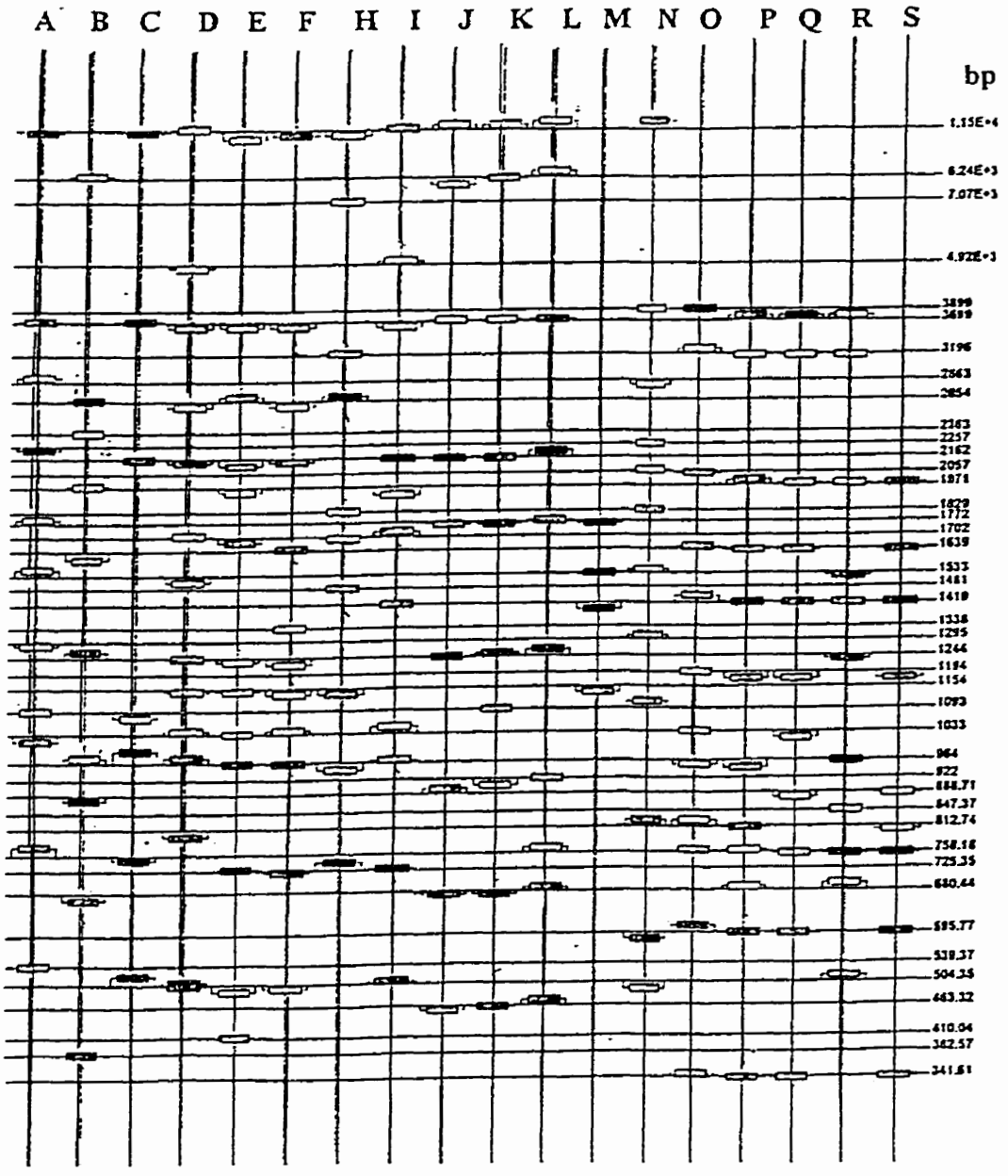

A B C D E F G H I J K L M N
bp
1845
1353
1107
861
685
369
123

Figure 6. Graphic representation of a composite RAPD gel using Primer 2. Lane A=MONT; B=LA; C=LB; D=SK81; E=SK90; F=SK92; H=MNW1; I=MNW2; J=MNW3; K=MNW4; L=MNW5; M=MNW6; N=MNW7; O=LCDC1; P=LCDC2; Q=LCDC3; R=MNW8; S=Type Strain.


A B C D E F H I J K L M N O P Q R S
bp
1.15E+4
6.24E+3
7.07E+3
4.92E+3
3899
3699
3196
2863
2854
2383
2257
2162
2057
1871
1829
1772
1702
1639
1533
1481
1419
1338
1295
1244
1194
1154
1093
1033
964
922
888.71
847.37
812.74
758.16
725.35
680.44
595.77
539.37
504.35
463.32
410.04
382.57
341.61

is recommended in order to improve the discrimination among isolates (Wagner *et al.*, 1996). The ability of a typing scheme to distinguish between unrelated bacterial isolates is a measure of its discriminatory power. Hunter and Gaston (1988) suggest using a single numerical index of discrimination (DI) based on Simpson's index of diversity to compare the discriminatory power of a typing scheme. Table 16 shows a comparison of the indices of discrimination for the five typing methods used in this study. As expected, the genotypic typing schemes were more discriminatory than the phenotypic typing schemes. Hunter and Gaston (1988) suggest that an index greater than 0.90 is desirable in order to interpret typing results with statistical confidence. As shown in Table 16, the DI for RAPD, PFGE and ribotyping was greater than 0.90. However, one should be cautioned, that although typing schemes should not be validated with small numbers of samples, there are no recommendations as to the minimum number of strains needed to validate a typing scheme.

Although DNA fingerprinting methods have not replaced conventional techniques as yet, they have contributed to a more rapid and discriminatory typing of bacterial strains. This could provide great advantages in many fields, especially for bacterial starter cultures used in food processing, the determination of causality in foodborne infections, HACCP, *etc.*

CONCLUSION

In conclusion, both clinical and food isolates of *E. sakazakii* were shown to be genetically heterogeneous. RAPD and PFGE were found to be the most discriminating typing

methods for *E.sakazakii*, followed in order by ribotyping, biotyping and antibiograms. In the present study, it was found that once appropriate primers were selected, RAPD was more rapid and easier to perform than PFGE. Although the discriminatory power of PFGE was comparable to RAPD, the specialized equipment required, the length of time for the protocol and the expertise required make it a more difficult and complex method than RAPD. It is recommended that in outbreak situations, biotyping be used as a screening tool but that in addition, for the best discrimination, either RAPD or PFGE be used.

The information presented in this chapter came in part from the following publication:

Nazarowec-White, M. and Farber, J.M. 1998. Phenotypic and genotypic typing of clinical and food isolates of *Enterobacter sakazakii.* J. Med. Microbiol. (Accepted).

CHAPTER 5: *ENTEROBACTER SAKAZAKII* VIRULENCE

ABSTRACT

Enterobacter sakazakii has been implicated as the causal organism in a severe form of neonatal meningitis with reported mortality rates of 40-80%. Dried-infant formula has been identified as a potential source of the organism in both outbreaks and sporadic cases. There are no published data describing any virulence factors or pathogenicity of *E. sakazakii*. In this study, strains of *E. sakazakii* were evaluated for enterotoxin production using the suckling mouse assay. In addition, suckling mice were challenged both orally and by intraperitoneal injection with clinical and food isolates of *E. sakazakii*. Of 18 *E. sakazakii* strains evaluated, four were found to be positive for enterotoxin production. All strains of *E. sakazakii* were lethal to suckling mice at 10^8 CFU/mouse by intraperitoneal injection, while two strains caused death using the peroral route. Further studies on the putative virulence factors of *E. sakazakii* are warranted.

INTRODUCTION

Enterobacter sakazakii, previously referred to as a "yellow-pigmented *Enterobacter cloacae*" was designated a unique species in 1980 (Farmer *et al.*, 1980). This reclassification was based on differences from *E. cloacae* in DNA relatedness, pigment production and biochemical reactions. *E. sakazakii* has been implicated in a severe form of neonatal meningitis. Dried-infant formula has been suggested as the source of transmission in both outbreaks and sporadic cases of *E. sakazakii* meningitis (Nazarowec-White and Farber, 1997a). Bacterial meningitis, even when treated with highly active antibiotics, is fatal in 5 to 40 % of the patients and causes neurologic sequelae in up to 30 % of survivors (Tauber and Zwahlen, 1994). In comparison, mortality rates for *E. sakazakii* reported in the literature range from 40 to 80 % (Willis and Robinson, 1988).

The bacterial organisms which are most frequently reported to cause meningitis in humans include *Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae*, Group B streptococci, *E. coli* and other *Enterobacteriacae*. Although *N. meningitidis, H. influenzae* and *E. coli* are gram negative organisms and *S. pneumoniae* and Group B streptococci are gram positive organisms, all ot these organisms are reported to possess a capsular polysaccaride antigen. It is possible that the presence of a capsule in these organisms may allow for their survival and subsequent migration to the human CNS..

The biological requirements for pathogenicity of an organism include its ability to multiply in the host's environment, to interfere with the host defence mechanisms and to damage

the host. Efforts in pathogenicity research focus on virulence determinants, recognizing and identifying the chemical structure of their toxin(s), and relating these to biological action (Smith, 1992). A logical first step is to establish a method for comparing virulence of strains in a relevant animal model (Smith, 1992). In bacterial meningitis, there are a number of important questions which remain to be investigated. The circumstances under which bacteria gain access to the central nervous system (CNS) need to be elucidated, as well as the microbiological and host-dependent factors which determine the extent of the resulting injury. A better understanding of these determinants can then be translated into regimens of treatment which will limit or prevent morbidity and mortality due to bacterial meningitis (Moxon *et al.*, 1977).

Species of *Enterobacter* are considered opportunistic pathogens and rarely cause disease in otherwise healthy individuals (Sanders and Sanders, 1997). Very little is known about the factors impacting on the pathogenicity and virulence of the organisms in the genus *Enterobacter*. As gram-negative pathogens, they possess endotoxin and therefore have all the pathogenic properties imparted to an organism by this virulence factor (Bone, 1993). Most epidemiologic aspects of *Enterobacter* spp. infections reflect the opportunity for infection rather than the intrinsic virulence of the organism involved (Sanders and Sanders, 1997). *E. sakazakii* infections are usually reported in neonates (Table 3, Chapter 1). However, at this time it is not known whether this predilection for neonates reflects intrinsic virulence or the fact that the organism has the opportunity to be an early colonizer of infants (Sanders and Sanders, 1997).

At present, there are no published data pertaining to the virulence factors or pathogenicity of *E. sakazakii.* However, it is known that *E. sakazakii*, unlike other enteric organisms, may cause a highly lethal syndrome of bacteremia and meningitis with CNS involvement in neonates (Gallagher, 1990). Weischer and Kolmos (1992) in a retrospective study of *Enterobacter* bacteremia found that one of the most frequently mentioned portals of entry among patients with bacteremia due to *Enterobacter* spp. was gastrointestinal. In a study by Keller *et al.*, (1998) it is stated that there are few reports on the ability of *E. cloacae* or other *Enterobacter* spp. to produce recognized virulence factors such as lipases, DNases, adhesions, *etc.* However, in the same study, they found that several strains of *E. cloacae* (genetically related to *E. sakazakaii)* were able to produce exotoxins, aerobactin and hemagglutinin.

Since it was known that i) a portal of entry for *Enterobacter* spp. could be gastrointestinal; ii) *E. sakazakii* is found in infant formula and iii) *E. cloacae* is an organism very closely related genetically to *E. sakazakii* which produces exotoxins, it was decided to explore the enterotoxin production potential of *E. sakazakii.* In this report, the suckling mouse assay (Dean *et al.*, 1972) was used to test strains of *E. sakazakii* for enterotoxin production. In addition, suckling mice were challenged with clinical and food isolates of *E. sakazakii* by both the oral and intraperitoneal (IP) routes.

MATERIALS AND METHODS

Enterotoxin Production

Bacterial strains for suckling mouse assay: Eight food isolates of *E. sakazakii* isolated in our laboratory from various dried-infant formula available on the Canadian retail market were evaluated for enterotoxin production (Table 4, Chapter 2). Nine clinical isolates implicated in human illness were obtained from Canadian hospital culture collections (Table 4, Chapter 2) and also tested for enterotoxin production. The positive control, *E. coli* strain 339, is a known producer of both heat labile (LT) and heat stable (ST) enterotoxin. The negative control was *E. coli* 711, a strain which does not produce either ST or LT (Stavric *et al.*, 1992). The control *E. coli* strains were provided by S. Stavric, Bureau of Microbial Hazards. Culture medium was used as an additional negative control.

Preparation of culture filtrates: Strains at time of isolation or receipt into the laboratory were grown on TSA (Difco Laboratories) plates for 24 h at 36°C, and then transferred to Microbank™ tubes (Pro-Lab Diagnostics, Richmond Hill, ON), according to the manufacturer's directions, and then stored at -70°C. Prior to each experiment, individual strains (one Microbank bead per strain) were grown in 10 mL BHI broth (Difco Laboratories). After strains were cultured with shaking overnight at 36°C in BHI broth, cells were harvested by centrifugation (2500 x g; 30 min at 4°C), and the supernatant passed through a 0.22 μm low protein binding membrane filter, (Acrodisc™, Gelman Sciences, Ann Arbour, MI) to obtain a cell-free filtrate (CFF).

Assay for enterotoxin activity: Swiss Webster, 16-18 day timed-pregnant mice (Charles River Canada Inc., St. Constant, PQ) were placed in individual cages, and fed food and

water *ad libitum*. Provisions were made to allow pregnant mice to prepare birthing nests. Newborn suckling mice (3-4 d old) were separated from their mothers immediately prior to testing and randomly divided into groups of four. Each mouse was dosed orally with 0.1 mL of the CFF or controls containing 2 drops/mL of 2% Evans blue dye (Sigma), using a 1 mL Tuberculin™ syringe (Canlab) attached to a blunt end 25-gauge needle (Canlab) cut down to 1 cm in length. Mice were discarded if the inoculum went into the lungs rather than the stomach as indicated by the Evan's blue dye . Approximately 4 h after dosing, mice were euthanized with 0.1 mL sodium pentobarbitol (MTC Pharmaceuticals, Cambridge, ON). The abdomens were opened, and the entire intestinal tracts excluding the stomach, were removed with sterile forceps. Upon opening of the peritoneal cavity, intestines were visually examined for distention and fluid accumulation. Intestines from each group of four mice were pooled, weighed, and the ratio of gut weight to remaining carcass weight was calculated. Results were considered positive when the ratio was equal to or greater than 0.083 (Matte *et al.*, 1994).

Infectivity Studies

***Bacterial Strains*:** Clinical and food isolates of *E. sakazakii* (Table 4, Chapter 2) were used in experiments to approximate the minimum infectious dose of *E. sakazakii* using IP injections as well as oral dosing (Dean *et al.*, 1972). Stock cultures of the organism were maintained at -70°C on beads in Microbank tubes (Pro-Lab Diagnostics), as previously described. Mice were housed as described above.

Experiments: Strains of *E. sakazakii* were grown overnight at 36°C in 5 mL BHI broth (Difco Laboratories). Overnight cultures were harvested (2500 x g, 30 min), supernatant discarded and the cells washed twice with 0.85% saline (Difco Laboratories). Serial 10-fold dilutions were made in saline, and 0.1 mL of each dilution was injected IP into each of three or four (3-4 d old) suckling mice. Initial cell concentrations used were 10^5, 10^7 and 10^8 CFU/mouse. The exact number of mice born determined the number of bacterial dilutions tested in each experiment.

The same procedures as in the IP infectivity portion of the study were followed in peroral infectivity testing. However, suckling mice were dosed as described in the assay for enterotoxin activity. Strains of *E. sakazakii* where death occurred in all mice within a group, were retested using a lower concentration of cells, i.e., 10^2, 10^3, and 10^4 CFU/mouse. Control mice were given 0.85% saline plus Evan's blue dye; a medium control was also included. All mice were observed over a 7 d period.

RESULTS AND DISCUSSION

The present study is the first to examine the virulence factors of *E. sakazakii*. Using the suckling mouse assay model, it was found that of 18 strains of *E. sakazakii* evaluated, four (22%) were positive for enterotoxin production (Table 17). Of these four positive strains, three were clinical strains (MONT, LA, SK92) isolated in three different hospitals in three different geographic locations in Canada (Montreal, PQ; London, ON; and Toronto, ON, respectively). Only one (MNW5) of the 8 *E. sakazakii* food isolates produced enterotoxin.

Table 17. Screening of *E. sakazakii* isolates for enterotoxin production.

	Strain	Ratio[a]	Response[b]
	+ Control *E.coli 339*	0.128	+
	- Control *E. coli 711*	0.055	-
	Media control (BHI)	0.045	-
	Type strain (ATCC 29544)	0.065	-
Clinical	MONT	0.095	+
	LA	0.099	+
	LB	0.069	-
	SK81	0.056	-
	SK90	0.059	-
	SK92	0.085	+
	LCDC1	0.065	-
	LCDC2	0.052	-
	LCDC3	0.053	-
Food	MNW1	0.068	-
	MNW2	0.049	-
	MNW3	0.061	-
	MNW4	0.058	-
	MNW5	0.083	+
	MNW6	0.065	-
	MNW7	0.062	-
	MNW8	0.057	-

[a]Ratio of intestinal weight to carcass weight (4 mice).
[b]A positive test for enterotoxin production is when the ratio is ≥ 0.083.

Two other food strains isolated from different lots of the same dried-infant formula were negative in the enterotoxin assay.

Bacteria produce a variety of extracellular toxins and enzymes (i.e., exotoxins, enterotoxins, proteases, lipases, DNase, hemolysins, etc.) which make them potentially virulent. Enterotoxins are exotoxins which primarily affect the intestinal tract and act by inducing fluid accumulation in the lining of the intestine (Grover *et al.*, 1990). Although enterotoxins are produced by a wide variety of bacteria (*Staphylococcus, Clostridium,* and *Bacillus* spp.), the most important enterotoxins causing infection are those produced by the Gram-negative bacilli and vibrios (Berkowitz, 1991). A bacterial exotoxin reaches its target by a sequence of events, beginning with its attachment to the surface of the target cell. This is followed by internalization of the toxin, its attachment to its target molecule and execution of its biological effect (Berkowitz, 1991). Many factors influence each of these events.

In the present study, factors such as pH, aeration, phase of growth, *etc.*, which might influence the events of exotoxin action were not evaluated to determine their effects on *E. sakazakii* enterotoxin production. Grover *et al.* (1990), in studying the production and properties of the crude enterotoxin of *P. aeruginosa*, concentrated the supernatant 8 to10-fold prior to evaluation for enterotoxin activity. Yuan *et al.* (1994) found that there was a need to concentrate (by at least 20-fold) the culture supernatant of *Vibrio mimicus* and *Vibrio cholerae* non-O1 to obtain consistent results with the suckling mouse assay.

In order to ascertain if a quicker and more simple method to detect enterotoxin production than the suckling mouse assay could be developed, initially a search using "BLAST" (http://www.ncbi.nlm.nih.gov:80/cgi-bin/BLAST/nph-blast) was used. The homology of protein sequences among enterotoxins from different microorganisms was examined. Using "BLAST" it was found that the purified ST expressed by *V. cholerae, V. mimicus, E. coli and Y. enterocolitica* had 10 of 17 amino acid residues in common (Altschul *et al.*, 1990). A further literature search of GeneBank (http://www2.ncbi.nlm.nih.gov/cgi-bin/birx-doc?genbank) DNA sequences for DNA homology among different enterotoxins showed that enterotoxins expressed by *Y. kristensenii* and *Y. enterocolitica* have 150 bp in common in a 200 bp sequence (Moseley *et al.*, 1983). Other enterotoxin genes had less than 40 bp in common (Altschul *et al.*, 1990). Thus, from our limited search, we could not find any common nucleotides from which one could prepare genetic primers for the detection of enterotoxin in *E. sakazakii*.

Challenge studies showed that all of the tested strains of *E. sakazakii* were lethal to suckling mice at 10^8 CFU/mouse using IP injections. All deaths occurred within 3 days post dosing, typically within 24-48 h. Strains SK92 (enterotoxin +) and MNW6 (enterotoxin -) had the lowest minimum infectious dose (MID) by the IP route, but were non-lethal at high oral doses (Table 18). These strains, therefore, may be missing some accessory virulence factors which would allow them to survive passage through the stomach and/or translocate across the intestinal wall. In comparison, only two strains of *E. sakazakii*, one clinical (SK81) and one food isolate (MNW2) caused death by the

peroral route, i.e., the clinical and food isolates were lethal at 10^7 CFU to 1 out of 4 mice after 48 and 72 h, respectively. Thus, it appears that these two strains may be more virulent than the others tested. Interestingly, however, under the conditions tested, both of these strains did not produce enterotoxin.

Enterobacteria involved in extraintestinal infections are known to possess virulence associated characteristics which distinguish them from random fecal isolates (Keller *et al.*, 1998). In addition, penetration of the epithelial layer of the intestinal mucosa is a key virulence mechanism of several enteric pathogens such as *Vibrio* spp., *Salmonella* spp. and *E. coli.* (Keller *et al.*, 1998). The most frequently cited risk factors for acquisition of an *Enterobacter* infection is the prior use of antimicrobial agents as well as severe debilitating underlying illness. These factors provide an excellent opportunity for *Enterobacter* spp. (Fuchs, 1998) to colonize the human intestinal tract. The immune system in neonates is not fully developed, thereby predisposing them to *E. sakazakii* infection. As previously mentioned it is not known whether the predilection of *E. sakazakii* to cause infection in neonates, reflects intrinsic virulence or the fact that the organism is an early colonizer. From the present study, it was observed that the high doses of *E. sakazakii* administered to mice did not cause death in the majority of cases and therefore some host factor(s) required for infection may not have been present. Clinical strains used in this study may have lost some of their virulence factors typically carried on plasmids or at least the ability to express those virulence factors. e.g. some of the clinical strains used in the present study were at least twelve years old and may have been

Table 18. Minimum infectious dose of *E. sakazakii* for suckling mice.

	Strain	Infectious dose administered	
		Oral	IP
	Type strain	$>10^{8}$[a]	$10^{8}(3/3)$[b]; $10^{7}(0/4)$
Clinical	MONT	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(0/4)$
	LA	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(0/4)$
	LB	$>10^{8}$	10^{8} (4/4); 10^{7} (0/4)
	SK81	$10^{8}(1/4)$; $10^{7}(1/4)$;$10^{5}(0/4)$	$10^{8}(4/4)$; $10^{7}(4/4)$; $10^{5}(0/4)$
	SK90	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(4/4)$; $10^{5}(0/4)$
	SK92	$>10^{8}$	$10^{8}(2/4)$; $10^{7}(1/4)$; $10^{5}(2/4)$; $10^{4}(0/4)$
	LCDC1	$>10^{8}$	$10^{8}(3/4)$; $10^{7}(0/4)$
	LCDC2	$>10^{8}$	$10^{8}(3/4)$; $10^{7}(0/4)$
	LCDC3	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(0/3)$
Food	MNW1	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(0/4)$
	MNW2	10^{8} (3/4); $10^{7}(1/4)$; $10^{5}(0/4)$	$10^{8}(3/3)$; $10^{7}(0/4)$
	MNW3	$>10^{8}$	$10^{8}(1/4)$; $10^{7}(3/4)$; $10^{5}(0/4)$
	MNW4	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(0/4)$
	MNW5	$>10^{8}$	$10^{8}(3/4)$; $10^{7}(0/4)$
	MNW6	$>10^{8}$	$10^{8}(4/4)$; $10^{7}(4/4)$; $10^{5}(3/4)$; $10^{4}(0/4)$
	MNW7	$>10^{8}$	$10^{8}(3/3)$; $10^{7}(0/4)$
	MNW8	$>10^{8}$	$10^{8}(1/4)$; $10^{7}(0/4)$

[a]Total number of CFU.
[b]Total number of mice dead/ total number of mice inoculated after observation for 7 d.

frequently subcultured The availability of genomic information is currently used as a powerful tool for further study of the molecular mechanisms of pathogenicity. As data is acquired, microbiologists will be able to identify specific factors involved in microbial pathogenicity and the genetic differences between pathogenic and nonpathogenic strains of bacteria will be more quickly identified (Fuchs, 1998).

CONCLUSION

Only two strains of *E. sakazakii* were capable of causing death by the peroral route. This finding suggests that there are obvious differences in virulence among the various strains, which may in part be related to the organisms ability to survive the acidic conditions present in the stomach. If one can extrapolate from the suckling mouse model, then it is evident that the MID for infants would be fairly high, and therefore, some temperature abuse of reconstituted dried-infant formula would have to occur. Previous work in this study showed that when starting at an initial concentration of 1 CFU/mL, reconstituted formula stored at room temperature, would take approximately 10 h to reach 10^7 cells in one infant feeding of 100 mL (Nazarowec-White and Farber, 1997b). Potentially hazardous levels of *E. sakazakii* would be reached even sooner in formula held at 35 to 37°C. Thus, strict adherence to temperature control at critical control points (CCP) during the manufacturing process and aseptic procedures in home and hospital preparation, storage and use of dried-infant formula are essential to ensure a safe product.

CHAPTER 6: GENERAL DISCUSSION AND FUTURE WORK

On December 11, 1990, Health Protection of Health Canada was notified of a case of neonatal meningitis where *E. sakazakii* was isolated from an infant in London, Ontario. One week later a second incident of *E. sakazakii* neonatal meningitis was reported to Health Canada in Toronto. Both infants had been fed infant formula prior to their illness. Although attempts were made to isolate *E. sakazakii* from remaining formula, no microbiological contamination was found.

As described in Chapter I of this thesis, very little information exists with respect to *E. sakazakii*. There are no Canadian nor American researchers actively working on this organism. The severe impact on neonates and the devastating outcome of *E. sakazakii* meningitis warranted this investigation. The wide scope of the objectives in this thesis was established in an attempt to gain some initial insight into the biological characterization of *E. sakazakii.*

The research data presented in this thesis are the most extensive examination of *E. sakazakii* from a single laboratory and describe a number of findings. The market survey of infant formula on the Canadian retail market showed for the first time the extent that *E. sakazakii* is present in Canadian formula. The prevalence of 6.7% in the Canadian dried-infant formula is slightly lower in comparison to the 16% found by Muytjens *et al.* (1988). However, one must consider that Muytjens *et al.* (1988) evaluated only six cans of Canadian formula colleagues had gathered in their travels and was not a market survey. Further investigations could include determining the incidence and prevalance of *E.*

sakazakii in formula which contains pre-digested proteins used in feeding immunocompromised neonates, who may be even more susceptible to *E. sakazakii* infection. This kind of formula is currently used in many hospitals in Canada.

The minimum growth temperature of strains of *E. sakazakii* ranged from 5.5 to 8.0°C. Although this temperature is above the "proper" refrigeration temperature, it is well documented that many refrigerators operate at temperatures ranging from 7 to 10°C, or higher. *E. sakazakii* can therefore survive and grow slowly at these temperatures. The importance of proper storage temperature is emphasized.

There are no known literature reports on the growth of *E. sakazakii* in reconstituted dried-infant formula. The findings from growth experiments showed that *E. sakazakii* has a generation time of 40 min at 23°C and 4.12 h at 10°C. Considering the length of time from reconstitution of formula to the time of feeding infants, *E. sakazakii* could grow to substantial numbers. For example, starting with an inoculum of one CFU/mL, it would take approximately 10 h at room temperature (23°C) to reach a potentially lethal dose of 10^7 organisms/feeding of formula. Formula stored at higher temperatures (35-37°C) would be even more potentially hazardous. This is a cause for concern which needs to be communicated not only to manufacturers of infant formula, but also to health care professionals and consumers. Educational pamphlets could be developed and distributed to pediatricians, neonatal nurses, dieticians and other caregivers responsible for neonatal feeding.

Novel thermotolerance studies indicated that *E. sakazakii* is more thermotolerant than some other members of the *Enterobacteriaceae* family. Using the data from this thesis, it appears that *E. sakazakii* would not survive the pasteurization process. The D and z values obtained in this thesis could be used by manufacturers to re-work contaminated dried-infant formula. However, if manufacturers use the dry blending process, where the final product is not subjected to any heat treatment, there is the potential for individual ingredients to contribute to contamination of final product. It is very difficult to clean manufacturing areas where powdered infant forumla is produced. Personnel and equipment movement from one area of the factory to another can contribute to product contamination. The manufacturing plant environment is of critical importance for all dried products and the drying and filling areas are often the source of contamination of dried foods. The findings in this thesis emphasize the importance of aseptic methods in the manufacture, reconstitution and storage of dried-infant formula.

The mortality rates reported in the literature for *E. sakazakii* infection range from 40 to 80% (Nazarowec-White and Farber, 1997b). In this thesis, it was found that of 18 *E. sakazakii* strains evaluated, four were capable of producing an enterotoxin. In some of the incidents of *E. sakazakii* infections, bloody diarrhea has been reported as a symptom. Further investigations are warranted to more fully characterize this enterotoxin in terms of heat stability, composition, mode of action, etc. As well, other toxins may be produced by this organism and should be considered in future investigations.

Although PCR methods are currently available for *E. coli, Y. enterocolitica* and *V. cholera* enterotoxins, initial gene bank searches indicate that there is great diversity in gene sequences among these enterotoxins. Therefore, it is recommended that initially traditional methods such as column chromatography for fractionation and purification of the toxin, electrofocussing to determine isoelectric point and an amino acid analyzer to determine the amino acid sequence, be used in *E. sakazakii* enterotoxin characterization.

Minimum infectious dose experiments using intraperitoneal injection of suckling mice indicate that 15 of 18 isolates tested caused lethality to suckling mice at $\leq 10^8$ cells per mouse. Two *E. sakazakii* strains caused death in suckling mice using the oral dosing at 10^5 cells per mouse. This finding shows that the MID for humans could be as low as 10^5 cells. Further investigations to elucidate the pathogenic mechanisms of *E. sakazakii* should include an initial evaluation of the attachment of this organism to tissue culture cell lines, including an intestinal cell line. Experiments using the meningitis suckling mouse model in order to observe the action of *E. sakazakii* in causing meningitis, should also be considered.

Phenotypic and genotypic characterization of *E. sakazakii* showed that there was a great diversity among the strains evaluated. This is the first report of the development of molecular typing schemes (RAPD, PFGE) which could be used in epidemiological investigations of *E. sakazakii* infections. In the present study, the genetic heterogeneity among the strains evaluated did not show the existence of any "hot" strains. As molecular

methods become automated and the ability to evaluate a large number of samples becomes more common, molecular typing schemes will provide more accurate and sensitive information. These methodologies will not only be used in clinical or food research laboratories for epidemiological studies but also in the food processing industry. Source of contamination of final product could be determined, whether from the environment, food handlers or raw ingredients.

E. sakazakii causes a rare but very severe form of neonatal meningitis and dried-infant formula has been implicated as the mode of transmission in some of the incidents. *E. sakazakii* is found in dried-infant formula on the Canadian retail market which is used for feeding infants. Oral dosing of suckling mice with *E. sakazakii* showed that 10^5 cells can cause death and therefore under the right circumstances can cause illness in Canadian infants.

Information gleaned from this study can be used in a qualitative risk assessment to dtermine the probability of *E. sakazakii* causing illness in Canadian infants. Factors that would be considered in a risk assessment include the incidence of *E. sakazakii* in dried-infant formula, the severity of the disease caused by *E. sakazakii,* the dose response of the suckling mice, the growth characteristics as well as the thermotolerance of the organism. All these experiments have been carried out in the present thesis work, and will be very important when doing the risk assessment. Findings in this thesis are only initial findings and more work remains in order to elucidate some of the important biological

characteristics of *E. sakazakii*.

CONCLUSIONS

1. The incidence of *E. sakazakii* in dried-infant formula on the Canadian retail market is 6.7%.
2. Eight strains of *E. sakazakii* were isolated from dried-infant formula available on the Canadian retail market.
3. Minimum growth temperatures of *E. sakazakii* ranged from 5.5 to 8.0°C. The fact that many refrigerators operate at temperatures above the "proper" refrigeration temperature of 4°C, is cause for concern to those feeding infants, as *E. sakazakii* can survive and grow slowly at these temperatures.
4. The generation time of *E. sakazakii* at room temperature (25°C) is only 40 min. The importance of proper storage of reconstituted formula is emphasized.
5. Thermotolerance studies showed that *E. sakazakii* is more thermotolerant than some of the other members of the *Enterobacteriaceae* family.
6. Four out of 18 strains of *E. sakazakii* tested are capable of producing an enterotoxin. Enterotoxins may be one of the virulence factors involved in the initial stages of *E. sakazakii* meningitis.
7. Minimum infectious doses for suckling mice as observed by the oral and IP routes were 10^7 and 10^5 total cells, respectively.
8. PFGE and RAPD are highly discriminatory typing methods for *E. sakazakii* strains.

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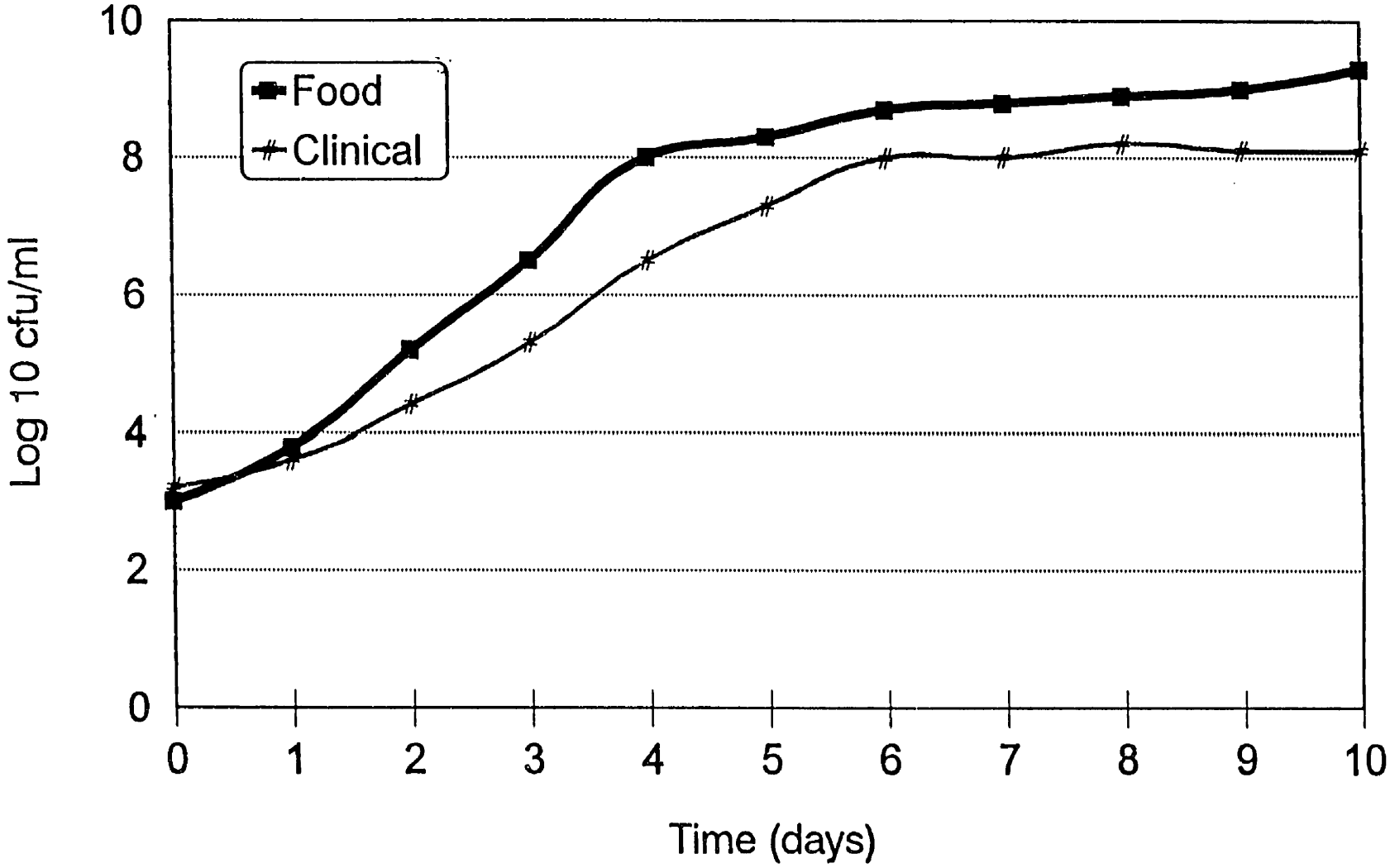
Growth of *Enterobacter sakazakii* in Infant Formula A at 10°C



Appendix A

IMAGE EVALUATION
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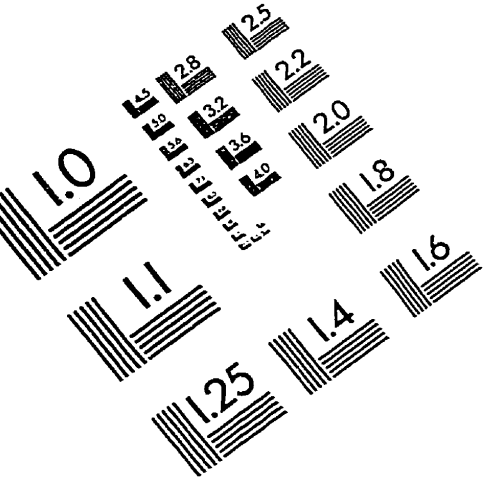

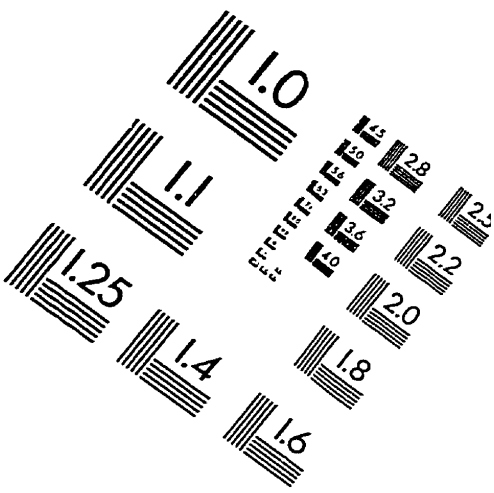

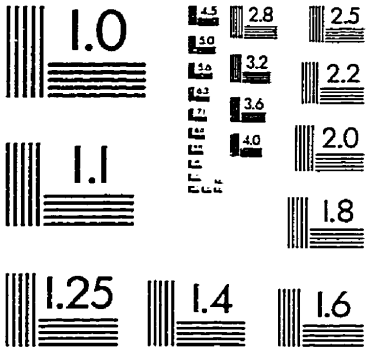

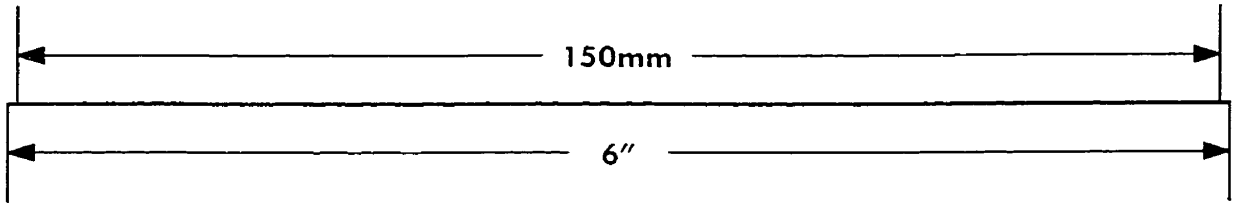

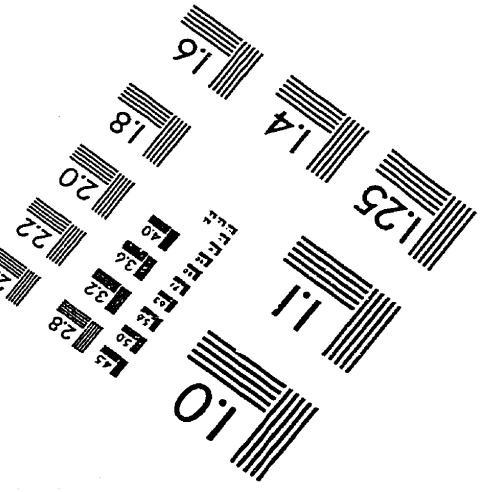

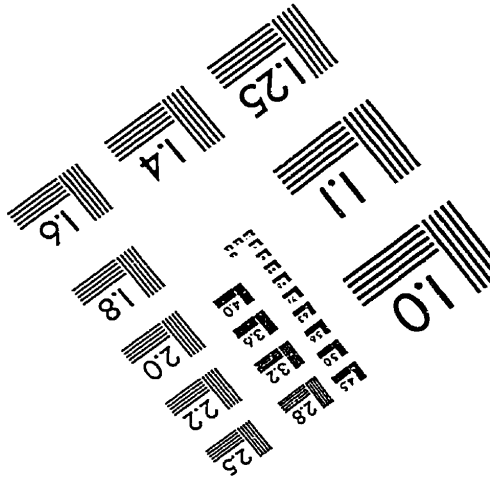

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