Enterobacter sakazakii invades brain capillary endothelial cells, persists in human macrophages influencing cytokine secretion and induces severe brain pathology in the neonatal rat

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Enterobacter sakazakii is an opportunistic pathogen associated with contaminated powdered infant formula and a rare cause of Gram-negative sepsis that can develop into meningitis and brain abscess formation in neonates. Bacterial pathogenesis remains to be fully elucidated. In this study, the host inflammatory response was evaluated following intracranial inoculation of *Ent. sakazakii* into infant rats. Infiltrating macrophages and neutrophils composed multiple inflammatory foci and contained phagocytosed bacteria. Several genotypically distinct *Ent. sakazakii* strains (16S cluster groups 1–4) were shown to invade rat capillary endothelial brain cells (rBCEC4) *in vitro*. Further, the persistence of *Ent. sakazakii* in macrophages varied between strains. The presence of putative *sod* genes and SOD activity may influence the survival of acidic conditions and macrophage oxidase and contribute to *Ent. sakazakii* intracellular persistence. The influence of macrophage uptake of *Ent. sakazakii* on immunoregulatory cytokine expression was assessed by ELISA. This demonstrated that the IL-10/IL-12 ratio is high after 24 h. This is suggestive of a type 2 immune response which is inefficient in fighting intracellular infections. These findings may help explain how the diversity in virulence traits among *Ent. sakazakii* isolates and an unsuccessful immune response contribute to the opportunistic nature of this infection.

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INTRODUCTION

Enterobacter sakazakii is a rare cause of neonatal Gram-negative sepsis and severe disease manifestations such as meningitis and brain abscess formation. There is also a strong association between some cases of necrotizing enterocolitis and the colonization of *Ent. sakazakii*. The contamination of powdered infant formula with *Ent. sakazakii* has been linked to neonatal outbreaks resulting in devastating morbidity and death (van Acker *et al.*, 2001; Biering *et al.*, 1989; Coignard *et al.*, 2006; Himelright *et al.*, 2002; Jarvis, 2005; Muytjens *et al.*, 1983). This has generated significant concern over the non-sterile condition of

this product and the regulatory standards governing the presence of this ubiquitous, opportunistic pathogen in infant formula powder (FAO-WHO, 2006). While recent studies have focused on the identification and classification of this genetically diverse species, very little is known concerning virulence (Drudy *et al.*, 2006; Iversen *et al.*, 2006; Iversen & Forsythe, 2007). Studies on toxin production by Pagotto *et al.* (2003) showed the production of cytotoxins or enterotoxins by clinical isolates and therefore these may contribute to a possible mechanism of pathogenesis. There is considerable diversity within the *Ent. sakazakii* species, which can be divided into four cluster groups according to 16S rDNA analysis (Iversen *et al.*, 2004). It is of interest whether the virulence of *Ent. sakazakii* varies according to cluster group. The presence of lipopolysaccharide (LPS) in powdered infant formula has

Abbreviations: BBB, blood–brain barrier; CSF, cerebrospinal fluid; H&E, haematoxylin/eosin; i.c., intracranially; i.p., intraperitoneally; PMA, phorbol 12-myristate 13-acetate; SOD, superoxide dismutase.

also been implicated in increasing the permeability of tissue barriers to intestinal bacteria including *Ent. sakazakii* (Townsend *et al.*, 2007). Studies to determine the ability of *Ent. sakazakii* to penetrate the blood brain barrier (BBB) and cause meningitis are hindered due to lack of an animal model. *Citrobacter* spp. and *Escherichia coli* K1 elicit haematogenous meningitis and brain abscess development in the neonatal rat (Kim *et al.*, 1997; Kline *et al.*, 1988); however, similar dissemination is not observed with *Ent. sakazakii* and *in vivo* models of this infection may require the use of immunodeficient animal strains (Pagotto *et al.*, 2007).

This study utilized intracranial inoculation of neonatal rats with *Ent. sakazakii* to facilitate histological characterization of the inflammatory response *in vivo*. *Ent. sakazakii* strains from cluster groups 1 and 2 were shown to significantly invade rat brain capillary endothelial cells (rBCEC4). Gene probing suggests that *Ent. sakazakii* survival in human (U937) macrophages *in vitro* may be influenced by *sodA*. In addition, we suggest that *Ent. sakazakii* may bias early IL-10/IL-12 cytokine expression by macrophages, contributing to the exacerbation of disease.

METHODS

Bacterial strains and culture conditions. *Ent. sakazakii* strains representing each 16S cluster group were used in this study (Table 1). The cluster 1 strain NTU658 (ATCC BAA-894) is currently being sequenced by Washington University in St Louis Genome Sequencing

Center (<http://www.genome.wustl.edu>). *Citrobacter koseri* SMT319 (Townsend *et al.*, 2003), *Citrobacter freundii* NTU340, *E. coli* K1 meningitic cerebrospinal fluid (CSF) isolate RS218 (O18:K1:H7) and *Salmonella enterica* serovar Enteritidis (NCTC 3046) were used as positive controls and non-pathogenic *E. coli* K-12 served as a negative control. For animal studies, gentamicin protection assays and cytokine secretion assays, bacteria were aerobically grown at 37 °C to mid-exponential phase in brain heart infusion (BHI) broth and washed twice with and resuspended in cold PBS. Concentrations of bacterial inoculations were determined by OD₆₀₀ and confirmed by plate count enumeration.

Serum complement resistance and acid tolerance. Serum sensitivity and acid tolerance were assessed via turbidimetric analysis and confirmed by viable plate counts. BHI broth cultures were grown with shaking to exponential phase and resuspended to obtain 10⁷ cells ml⁻¹. *E. coli* K-12 was used as a negative control. For serum studies over 2 h, 50 µl bacterial suspension was added to 50 µl non-immune or heat-inactivated (56 °C for 30 min) human pooled serum (HPS) in triplicate. For acid tolerance studies over 6 h, 100 µl LB broth pH adjusted to 4 and buffered with citrate/phosphate buffer was added to 50 µl of culture in triplicate. The mixtures were incubated at 37 °C and the OD₆₀₀ was determined at 30 min intervals. Percentage survival was determined as (Number of bacteria that survived 2 h treatment/Inoculum size) × 100. Strains able to survive in HPS at levels equal to or greater than that observed initially were considered resistant.

Superoxide dismutase (SOD) activity. SOD activity in *Ent. sakazakii* cell lysates was evaluated using the Superoxide Dismutase Assay kit II (Calbiochem). *Yersinia enterocolitica* strain 8081 served as a positive control (Roggenkamp *et al.*, 1997). Bacterial pellets collected from 2 ml overnight cultures were sonicated in ice-cold 20 mM HEPES buffer containing 1 mM EGTA, 210 mM mannitol

Table 1. Summary of strains used in this study

NTU strain ID	Source†	Origin	16S rDNA cluster group	Organism
1	NCTC 11467 ^T	Child's throat	1	<i>Ent. sakazakii</i>
2	ATCC 12868	Unknown	1	<i>Ent. sakazakii</i>
658	ATCC BAA-894	Infant formula	1	<i>Ent. sakazakii</i>
57	NTU	Milk powder	2	<i>Ent. sakazakii</i>
3	ATCC 51329	Unknown	3	<i>Ent. sakazakii</i>
16*	NTU	Herb	3	<i>Ent. sakazakii</i>
530*	HMMP13	Milk powder	3	<i>Ent. sakazakii</i>
566*	CDC-3523-75	Bone marrow	3	<i>Ent. sakazakii</i>
84	NTU	Herb	4	<i>Ent. sakazakii</i>
564*	CDC-5960-70	Blood	4	<i>Ent. sakazakii</i>
582*	NCTC 9844	Unknown	4	<i>Ent. sakazakii</i>
583*	NCTC 9846	Unknown	4	<i>Ent. sakazakii</i>
NA	NCTC 3046	Unknown	NA	<i>Salmonella</i> Enterica
NA	CHLA	CSF	NA	<i>E. coli</i> K1 RS218
NA	CHLA	Unknown	NA	<i>E. coli</i> K-12 HB101
NA	WU	CSF	NA	<i>C. koseri</i> SMT319
340	NTU	Salad	NA	<i>C. freundii</i>

NA, Not applicable.

*Used in PCR studies only.

†^T, Type strain; NTU, Nottingham Trent University; CHLA, Children's Hospital Los Angeles; WU, Washington University Medical School (Joseph St Geme III).

and 70 mM sucrose (pH 7.2). Centrifugation at 1500 g for 5 min at 4 °C cleared the supernatants used in the assay to detect superoxide radicals according to the manufacturer's instructions. Two independent assays were performed in duplicate.

Tissue culture cell line cultivation and invasion assays. U937 macrophages were obtained from the ATCC (CRL-1593.2) and seeded into 75 ml tissue culture flasks (Sundstrom & Nilsson, 1976). Cells were cultivated, activated and plated as described previously (Townsend *et al.*, 2003). Cells were gently washed with RPMI to remove residual phorbol 12-myristate 13-acetate (PMA) following activation, and fresh medium was added prior to inoculation with unopsonized bacteria. U937 human macrophages were infected at an m.o.i. of 10 for 45 min at 37 °C in 5% CO₂. After a 45 min incubation period, the medium was aspirated and replaced with U937 macrophage medium supplemented with 100 µg gentamicin ml⁻¹ and incubated for an additional 45 min at 37 °C in 5% CO₂. Macrophages were washed twice, lysed with 0.5% Triton X, serially diluted, and plated to determine the number of intracellular bacteria at various time points (Zaidi *et al.*, 1996). The viability of the bacterial strains tested was not affected by 0.5% Triton X treatment. For persistence assays, cells were replenished daily with fresh medium containing 10 µg gentamicin ml⁻¹ (above the MIC). Trypan Blue exclusion staining indicated that macrophage viability ranged from 80 to 95% and was maintained for at least 96 h. For persistence assays, results for each time point are presented as the percentage of inoculum that was intracellular. All assays were performed in triplicate at least twice.

The rat brain capillary endothelial cell line (rBCEC4) was a kind gift from I. E. Blasig (Forschungsinstitut für Molekulare Pharmakologie, Berlin, Germany). Following the 22nd subculture rBCEC4 cells were seeded at 1×10^5 cells per well into collagen-coated 24-well plates and left to adhere for 48 h. The medium contained DMEM, 4.5 g glucose l⁻¹, 1.2 mM glutamine, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, 2.5 µg amphotericin B ml⁻¹ (Sigma-Aldrich), 100 µg heparin ml⁻¹, 110 µg sodium pyruvate ml⁻¹ (Sigma), 10 µg ECGF ml⁻¹ (endothelial growth factor; Axxora), 10% fetal bovine serum (Blasig *et al.*, 2001). Each bacterial strain was grown in brain heart infusion broth (BHI; CM 10322, Oxoid) to mid-exponential phase from overnight cultures and inoculated in triplicate at an m.o.i. of 1:100. Inoculated cells were incubated with 5% CO₂ at 37 °C for 1.5 h as previously described (Badger *et al.*, 1999). In order to quantify bacterial invasion 100 µg gentamicin ml⁻¹ was added to each well and incubated for 30 min. Then the cells were washed twice with PBS and treated with trypsin to dislodge the adherent cells. The rBCEC4 cells were lysed with 0.5% Triton X and serial dilutions were plated on nutrient agar. Cell integrity following invasion was qualitatively assessed using Trypan Blue staining after 2 h incubation. *E. coli* K-12 and *C. koseri* SMT319 were used as negative and positive controls, respectively. Data are presented as percentage invasion as determined by (Number of bacteria recovered/Number of bacteria inoculated) × 100.

Animal studies. Timed-pregnant (E14) Sprague-Dawley rats (Charles River Laboratories) were obtained and gave birth in our vivarium after a 21 day gestation period. Litters averaged 12 pups and were kept with their mother in an opaque polypropylene cage under a Small Animal Isolator (Forma Scientific). Two- to three-day-old rat pups were anaesthetized with isoflurane and inoculated. For serum cytokine studies (described below), 10⁷ c.f.u. in 0.1 ml PBS were inoculated intraperitoneally (i.p.). For histological studies, 10³ c.f.u. in 0.002 ml PBS were inoculated intracranially (i.c.); i.e. inoculations were administered through a burr hole produced by a 26-gauge needle at coordinates approximately 5 mm caudal to the right eye and 2 mm right of the sagittal suture. A 33-gauge, single internal cannula (Plastics One) was attached to a Hamilton 1801RN 10 µl syringe (Hamilton Company) with a 22-gauge needle via 24-gauge standard

wall tubing, to administer the dose. The needle was inserted perpendicular into the right parietal area approximately 2 mm deep from the external surface; 2 µl was injected over 1 min then the needle was carefully retracted. For the study reported in Table 2, rat pups were anaesthetized as they succumbed to infection up to 9 days post i.c. inoculation. Blood samples were aseptically collected via intracardiac puncture, as previously described (Badger & Kim, 1998). Blood samples (10 µl) were inoculated in LB and plated on agar plates. Rats were euthanized and whole brains were removed for histological analysis. All animal experiments were performed according to protocols approved by CHLA Institutional Animal Care and Use Committee (IACUC).

Histopathology. Whole neonatal rat brains were fixed in 10% buffered formalin, routinely processed, then paraffin embedded. Coronal sections of 4–5 µm were cut and stained with haematoxylin and eosin (H&E). Histological analysis of the midbrain was completed by one blinded pathologist (J.L.).

Electron microscopy. Transmission electron microscopy was used to visualize *Ent. sakazakii* within rat macrophages. *Ent. sakazakii*-infected rat brain samples were fixed with 2.5% glutaraldehyde in 0.1 M PBS. Samples were post-fixed for 1 h with 2% OsO₄, rinsed, dehydrated through graded ethanol solutions, and embedded in polypropylene oxide. Ultrathin sections mounted on collodion grids (single hole) were stained with uranyl acetate and lead citrate and examined with a Philips CM transmission electron microscope.

DNA isolation, gene identification and PCR. Genomic DNA was prepared from 1.5 ml overnight culture grown in LB broth using a GenElute Bacterial Genomic DNA kit (Sigma) according to the manufacturer's instructions. DNA–DNA microarray hybridization of *Ent. sakazakii* DNA to *Salmonella enterica* serotype Typhi and Typhimurium virulence gene arrays (Porwollik *et al.*, 2003) identified homologues to *Ent. sakazakii* genes *sodA* and *ompA* related to macrophage survival and serum resistance. The *Ent. sakazakii* genome project at Washington University School of Medicine Genome Sequencing Center (<http://www.genome.wustl.edu>) provided sequence information of the *Ent. sakazakii* gene homologous to *sodA* and *sodB* from *S. Typhimurium* and PCR primers were designed to probe the chromosomal DNA. The PCR reaction mixture contained 5 × Green GoTaq Flexi Buffer (Promega), 2.5 mM MgCl₂, 0.2 mM each dNTP, 0.5 µM SodA1-F (5'-GTCAAC AACGCTAACG-3') and SodA1-R (5'-CCCATCAGCGGGGAAT-3') primers, 2.5 U GoTaq Flexi DNA polymerase (Promega), 100 ng template DNA, and nuclease-free water to a final volume of 50 µl. The thermocycler (Genius FGEN 05 TD; Techne) was programmed as follows. An initial denaturing step at 94 °C for 2 min was followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, and a final extension step of 72 °C for 5 min; this yielded a 352 bp product. The *ompA* gene has recently been identified and was amplified using primers ESSF and ESSR, which yield a 469 bp product following PCR conditions described previously (Mohan Nair & Venkitanarayanan, 2006). PCR products were visualized on 1% agarose gels stained with 0.5 µg ethidium bromide ml⁻¹.

Quantitative analysis of cytokine secretion. ELISA was used to quantify cytokine secretion (IL-10, IL-12 and TNFα) from U937 macrophages following *Ent. sakazakii* uptake. Human IL-10 (sensitivity <1 pg IL-10 ml⁻¹), IL-12 (sensitivity <0.2 pg IL-12 ml⁻¹), and TNFα (sensitivity <1.7 pg TNFα ml⁻¹) ELISA kits (Biosource International) were used to measure cytokines secreted into the macrophage supernatant at 6 and 24 h post-inoculation with each strain in this study. *E. coli* O111:B4 LPS or PMA (Sigma) was used as a positive control. ELISA was also used to measure rat pup IL-10 (sensitivity <5 pg IL-10 ml⁻¹) concentrations (Biosource

International). Rat serum was isolated 24 h after i.p. injection of *Ent. sakazakii* strain NTU2 or NTU658 and IL-10 concentration was measured. In accordance with the manufacturer's instructions, each sample (serum was diluted 1:10) was run in duplicate wells and the absorbance values were averaged. Standard curves were generated using the cytokine standards supplied by the manufacturer.

RESULTS AND DISCUSSION

Histological characterization of rat brain inflammatory response following intracranial *Ent. sakazakii* infection

Ent. sakazakii causes opportunistic infections and is reported to be associated with the consumption of infant formula in neonates. Traversing the gut and entering the brain requires invasive capabilities and immune evasion techniques that are yet to be fully elucidated. In this study, genetically distinct *Ent. sakazakii* strains were selected and tested *in vivo* and *in vitro* to better characterize mechanisms of virulence and the host immune response to infection. *Ent. sakazakii* strains from cluster 1 are most commonly isolated from food, clinical and environmental sources and have been associated with clinical disease. Cluster groups 2, 3, and 4 are dissimilar and less commonly isolated yet these strains are still recovered from clinical sources. The pathogenic potential of these strains has not been previously described.

For the comparative study summarized in Table 2, strains from clusters 1 and 2 were inoculated into rat pups via intracranial injection. Blood and brains were collected and examined between 6 and 9 days post-inoculation to observe evidence of sepsis and chronic-pattern inflammation. This study showed that NTU1 was attenuated in comparison to other strains such as NTU2, which initiated chronic-pattern inflammation (lymphocytic predominance) in 83 % of rats tested (Table 2). This provides evidence that even within genetically similar strains there are significant differences in the host immune response. These differences may be attributed to distinctions at the level of protein expression, although it cannot be ruled out that they may be due to extensive subculturing resulting in a loss of virulence in NTU1. Strain NTU658, from cluster group 1, is reported as having caused a neonatal outbreak and its genome is currently being sequenced. This strain

caused meningitis and chronic-pattern inflammation in 33 % of infected rat pups (Table 2). Strain NTU57 (cluster group 2) also caused chronic-pattern inflammation in 33 % of infected rat pups, and 50 % of the rat pups developed meningitis as defined by inflammation within the meninges following histological analysis (Table 2). No bacterial growth was detected in blood samples; thus no evidence of sepsis was obtained. This comparative study identified strain NTU2 as having the highest occurrence of chronic-pattern inflammation, and this strain was used for further kinetic studies.

Intracranial injection of *Ent. sakazakii* was performed in a kinetic study to describe the progression of brain inflammation. The injection site was located in the right cerebral cortex at the approximate junction of the forebrain and midbrain at a depth (2 mm) not penetrating the ventricle. Rat pups were sacrificed at various time points up to 9 days. Histological analysis of H&E-stained coronal sections elucidated the inflammatory response. Fig. 1(a) is an example of normal brain histology observed in rat pups 9 days post-infection with NTU1 (most indistinguishable from controls) to compare to the severe inflammatory reaction observed in NTU2-infected rat pups. Only three rat pups inoculated with NTU1 had extremely small foci of inflammatory cells (mostly neutrophils) in the cingular gyrus, near the base of the brain, or near the meninges. Rat pups inoculated with NTU2 had severe bilateral ventriculitis, meningitis and marginalized neutrophils in local blood vessels 3 days post-inoculation (Fig. 1b, c). Lymphocytes and microglia were activated, reactive astrocytes were observed and inflammation was associated with a micro-haemorrhage (Fig. 1d). The occurrence of oedema and flocculation suggests that increased vascular permeability and fibrin liberated from blood vessels may impair drainage of CSF, causing hydrocephalus. The choroid plexus also harboured inflammatory cells and secreted fibrin, which was not prevalent in the *C. koseri* model (Townsend *et al.*, 2003). Leptomeningitis, ventriculitis and ventriculomegaly, with massive dilation of the ventricles, worsened 6 days post-inoculation (Fig. 1e). Morphological evidence of neuronal or glial cell death via apoptosis was suggested by hyperchromatic, condensed nuclei within the cortex (Fig. 1f) and could be induced by bacterial factors such as LPS or other secreted endotoxins (Pagotto *et al.*, 2003). Further, neuronal death and cell lyses releases acid hydrolase and causes liquefaction and necrosis of brain tissue. It follows that there is also evidence of ischaemic damage (possibly due to oedema and pressure), reduced neuronal density and liquefaction within the cortex; these factors could also influence the observed neuronal cell death. Neutrophils were still margined and infiltrating into the cortex with other inflammatory cells, causing multiple foci of cerebritis and small ischaemic lesions more than a week after inoculation (Fig. 1g, h, i).

The multiple inflammatory foci provide evidence that the bacteria can enter the CSF circulation. The glial limitans, normally a good barrier preventing the extension of

Table 2. Comparative *in vivo* *Ent. sakazakii* study

Strain	Origin	Cluster group	Meningitis	Chronic-pattern inflammation
NTU1	Child's throat	1	0 % (0/7)	0 % (0/7)
NTU2	Unknown	1	83 % (5/6)	83 % (5/6)
NTU658	Milk powder	1	33 % (2/6)	33 % (2/6)
NTU57	Milk powder	2	50 % (3/6)	33 % (2/6)

Fig. 1. Histological analysis of *Ent. sakazakii* in the infant rat brain. *Ent. sakazakii* NTU2 was inoculated into rat pups via i.c. injection. Rat pups were sacrificed at various time points after inoculation. Histological analysis of H&E-stained coronal sections elucidated the inflammatory response. Bars: 2 mm (a, b, e), 500 μ m (k), 200 μ m (c, g), 100 μ m (d, i, l) and 50 μ m (f, h, j). (a) Rat pup inoculated with *Ent. sakazakii* NTU1 with normal brain histology. (b) Rat pup inoculated with *Ent. sakazakii* strain NTU2 showing severe bilateral ventriculitis 3 days post-inoculation. (c) Marginalization of neutrophils (solid arrow) and leptomenigeal inflammation (open arrow) at day 3. (d) Extravagation of red blood cells. Inflammatory cells (i.e. neutrophils) are among the micro-haemorrhage. (e) Ventriculitis is persistent at 6 days post-inoculation and ventriculomegaly ensues with massive dilation of the ventricles. (f) Normal neuron (open arrow) and morphological features of neuronal programmed cell death demonstrated by hyperchromatic, condensed DNA in the cortex (solid arrows) at day 5. (g) Inflammatory foci in close proximity to brain capillaries at day 5. (h) Marginated neutrophils and infiltrating inflammatory cells at day 7. (i) Marginated neutrophils and inflammatory foci (arrow) at day 7. (j) Marginated neutrophils, leptomeningitis dissemination and dilated perivascular space. (k) Micro-abscess (arrow) (l) Marginated neutrophils in an abnormal blood vessel and inflammatory foci at day 9.

bacteria and inflammatory cells into the brain, is compromised. During meningitic infections lesions frequently occur where the Virchow–Robin (perivascular) space fuses with the cortex. In this study the Virchow–Robin space was dilated and contained inflammatory cells such as macrophages, sensing lymphocytes and local inflammatory cells that can recirculate as antigen-presenting cells (Fig. 1j). Multiple inflammatory foci within the brain closely associated with blood vessels suggest that in this model bacteria travel down the perivascular spaces (Fig. 1). This suggests a way that the bacteria may enter the CSF, causing a massive influx of inflammatory cells into the ventricles and meninges that may break down adhesion junctions and give access to the brain parenchyma. Micro-abscesses, inflammatory foci and marginated neutrophils were visible at day 9 (Fig. 1k, l). Electron microscopy was used to further discern the host cell interactions and intracellular location of internalized bacteria. Electron microscopic analysis of infant rat brains 7 days post-inoculation found *Ent. sakazakii* in spacious membrane-bound phagosomes within infiltrating neutrophils and macrophages associated with inflammation (Fig. 2).

Rat brain capillary endothelial cell invasion by *Ent. sakazakii*

The pathogenesis of *Ent. sakazakii* meningitis involves haematogenous spread to the CNS and penetration of the BBB. The pathogenesis of most infant meningitis involves invasion of the BBB. This has long been assumed of *Ent. sakazakii* meningitis due to a lack of any other biologically plausible explanation. In order to demonstrate BBB invasion the gentamicin protection assay was performed using the rat rBCEC4 cell line. *C. koseri*, *C. freundii* and *E. coli* K1 are reported to invade human microvascular endothelial cells and were used as positive controls. *Ent. sakazakii* strains NTU2, NTU658 and NTU57 had average percentage invasion values ranging from 0.43 to 1 %, which were not significantly different ($P=0.095$, 0.552 and 0.672 , respectively) from positive control *E. coli* K1

(0.57 ± 0.08 %) or *C. freundii* (0.54 ± 0.09 %; Fig. 3). The *C. koseri* positive control was extremely invasive (2.95 %) while *E. coli* K-12 did not invade this cell line (Fig. 3). Strains NTU1, NTU3 and NTU84 were at least twofold less invasive than *E. coli* K1 ($P \leq 0.0002$) *in vitro* (Fig. 3) and this has been shown to be biologically relevant since 0.1 % invasion is associated with enhanced CNS entry *in vivo* (Badger & Kim, 1998; Wang *et al.*, 1999; Badger *et al.*, 2000). Strains NTU1, NTU3 and NTU84 were also significantly less invasive than NTU2 ($P=0.001$, 0.041 and 0.002 , respectively) *in vitro* (Fig. 3), further illustrating that invasiveness was dependent on strain. Our study appears to be the first to show that *Ent. sakazakii* invades capillary endothelial cells, suggesting that some strains from clusters 1 and 2 may be more likely to invade the BBB and cause CNS infection.

Ent. sakazakii persists within human macrophages

Macrophage uptake of invading microbes is an innate process that employs antimicrobial defence mechanisms such as oxidative burst, acidic compartmentalization and nutrient deprivation to eliminate harmful pathogens. The ability of some bacteria to persist or replicate within these long-lived immune cells offers protection from the immune response. Human U937 macrophages were utilized to determine if *Ent. sakazakii* was able to persist within human macrophages following phagocytosis. All strains of *Ent. sakazakii* tested were able to persist in macrophages up to 96 h. However, this ability differed between strains. Strain NTU2 was able to replicate significantly over this time period while strains NTU1, NTU57 and NTU84 showed modest levels of replication (Fig. 4). Strain NTU3 (16S rDNA cluster group 3) showed the least resistance to macrophage killing among *Ent. sakazakii* strains (Fig. 4). *E. coli* K1 (persists), *E. coli* K-12 (killed), and *C. koseri* strain 319 (replicates) were used as controls.

Fig. 2. Transmission electron micrographs of *Ent. sakazakii* in the infant rat brain. (a) *Ent. sakazakii* NTU57 (arrowed) was found in neutrophils (scale bar, 2 μ m) and (b) membrane-bound vacuoles of macrophages (arrowed) associated with chronic-pattern inflammation within the brain at 6 days post-inoculation (scale bar, 1 μ m).

Fig. 3. *Ent. sakazakii* invasion of rat brain capillary endothelial cells. The gentamicin protection assay was performed using rat rBCEC4 cells to model the BBB invasion. Mid-exponential-phase bacteria were inoculated and incubated for 1.5 h then treated with gentamicin for 30 min. No bacteria were recovered at t_0 . Results for t_2 are presented as the percentage of the initial inoculum that was intracellular after 2 h. *E. coli* K-12 (non-invasive), *C. koseri* strain SMT319, *C. freundii* strain 340 and *E. coli* K1 (invasive) were used as controls. An asterisk marks the means with P values ($P \leq 0.041$) significantly less invasive than *Ent. sakazakii* NTU2, *C. freundii* strain 340 and *E. coli* K1. Data are means $\pm$ SEM from two independent assays performed in triplicate.

Comparative resistance to serum, acid and reactive oxygen

The innate immune system facilitates a quick, non-specific immune response. Neonate susceptibility may be increased due to reduced gastric acid secretion up to 5 h immediately following birth (Euler *et al.*, 1977). Bacteria that gain access to the bloodstream will be exposed to complement factors that cause cell lysis. Polymorphonuclear leukocytes are capable of releasing reactive oxygen species that damage cell membranes, proteins and essential macromolecules such as DNA. *Ent. sakazakii* strains were tested to determine resistance to acid, complement and reactive oxygen species. Cluster 3 and 4 strains and NTU1 were not resistant to serum complement (data not shown). All strains were resistant to acid at pH 4 except NTU1. Superoxide dismutases (SODs) are metalloenzymes which are found in all aerobic organisms and help protect bacteria from oxidative stress. All bacterial strains tested showed some

level of SOD activity between 0.23 and 2.0 units ml^{-1} as assessed by the SOD assay (Fig. 5). *Ent. sakazakii* strains NTU1, NTU2, NTU3 and NTU84 demonstrated the highest SOD activity. Among *Ent. sakazakii* strains, NTU1 and NTU84 had significantly higher activity than NTU658 ($P \leq 0.02$) and NTU57 ($P \leq 0.03$). All the *Ent. sakazakii* strains exhibited SOD activity significantly lower than the positive control value ($P \leq 0.05$) that has a demonstrable affect on *Y. enterocolitica* virulence (Roggenkamp *et al.*, 1997); however, these studies demonstrate that SOD is functioning in *Ent. sakazakii*. While SOD is generally stationary-phase induced, and Fe-SOD is constitutively expressed, further *sodA* (Mn-SOD) activity may rely on specific induction such as acidic conditions that lead to oxidative stress (Kim *et al.*, 2005). *Deinococcus radiodurans* is an example of a bacterium that can survive long term under harsh conditions, a property attributed to its strong SOD and DNA-repair activity (Tian *et al.*, 2004). *Ent. sakazakii* is also known to

Fig. 4. Long-term survival of *Ent. sakazakii* within macrophages. The gentamicin protection assay was performed on human U937 macrophages using mid-exponential-phase bacteria at 24 h time points up to 96 h (t_{96}). Results are presented as the percentage of the initial inoculum that was intracellular. Data are means $\pm$ SEM from a representative assay performed in triplicate. *E. coli* K1 (persists), *E. coli* K-12 (killed), and *C. koseri* strain SMT319 (replicates) were used as controls.

Fig. 5. *Ent. sakazakii* SOD activity. Supernatants from sonicated bacterial pellets were assessed for SOD activity. The range of SOD detection was between 0.025 and 0.25 units SOD ml⁻¹. One unit of SOD is the amount of enzyme resulting in 50% dismutation of the superoxide radical. *Y. enterocolitica* was used as a positive control. Data are means $\pm$ SEM from two independent assays performed in duplicate.

survive long term in powdered infant formula (Caubilla-Barron & Forsythe, 2007). It is possible that SOD activity may contribute to this ability; this is currently under investigation.

Gene probes for *ompA* and *sodA*

The *ompA* and *sodA* genes were identified in a DNA–DNA microarray analysis showing hybridization of *Ent. sakazakii* genes to *Salmonella ompA* and *sodA* DNA probes. This was confirmed by recent studies (Mohan Nair & Venkitanarayanan, 2006) and the finding of a partial gene sequence for *sodA* and *sodB* in the ongoing genome sequencing project (<http://www.genome.wustl.edu>) using the BLASTX program with homologous *Salmonella* Typhimurium LT2 sequences. The *sodA* and *sodB* genes are known to protect bacteria from oxidative stress (McCord & Fridovich, 1969) by catalysing the conversion of oxygen radicals to hydrogen peroxide (Farr & Kogoma, 1991). Sensitivity to oxidative stress may contribute to reduced virulence so we considered the capability of *Ent. sakazakii* to survive early contact with the macrophage oxidative burst (Beaman & Beaman, 1984) in addition to long-term persistence. While *sodA* was amplified from *Ent. sakazakii* cluster groups 1, 2 and 4 and *C. koseri*, it was not from cluster group 3 *Ent. sakazakii* strain NTU3 (data not shown). NTU3 was also the only strain found to be less numerous in macrophages at t_{24} compared to t_0 (Fig. 4). However, the SOD assay results do not correspond to these data, in that NTU3 showed one of the highest SOD activity values (Fig. 5); this suggests that the poor ability of this strain both to survive early macrophage interactions and to replicate in macrophages may not be a result of a diminished SOD activity or lack of the *sodA* gene. The lack of amplification cannot rule out the presence of *sodA* in this strain, as it is a gene that exhibits a degree of variability. Considering the concern for rapid accurate

identification of *Ent. sakazakii* it is of interest that the variability of *sodA* has recently been utilized in multiplex PCR reactions to identify enterococci at the genus and species level (Jackson *et al.*, 2004). With respect to infection, inactivation of *sodA* in *Y. enterocolitica* resulted in a marked reduction in virulence of the organism in a mouse infection model (Roggenkamp *et al.*, 1997). However, inactivation of *sodA* in *Salmonella* Typhimurium did not significantly attenuate infection in mice, which suggests that SodA is only required for initial resistance to macrophage oxidative burst. Studies utilizing deletion mutants are ongoing to clearly define the importance of *sodA* during *Ent. sakazakii* infection.

In *E. coli* K1 the *ompA* gene has been linked with serum resistance and CNS invasion in the neonatal rat model (Weiser & Gotschlich, 1991). The *ompA* gene was not amplified from cluster 4 strain NTU84 (data not shown). Our studies also show that NTU84 is serum sensitive. Since OmpA is reported to contribute to serum resistance we sought to determine if the presence of *ompA* among cluster 4 strains was associated with serum sensitivity. However, all other cluster 4 strains had the *ompA* gene and these strains were serum sensitive (data not shown), suggesting that OmpA may not play an essential role in *Ent. sakazakii* serum resistance. OmpA has been associated with increased HBMEC invasion *in vitro* (Shin *et al.*, 2005). NTU84 had relatively low invasive abilities in our endothelial cell line, suggesting that OmpA may influence capillary endothelial cell invasion. Alternatively, analogous mechanisms for resistance and invasion may not be present in NTU84. The development and specificity of the *ompA* PCR as a potential tool for the rapid detection of *Ent. sakazakii* in infant formula is impressive (Mohan Nair & Venkitanarayanan, 2006). However, the finding of an *Ent. sakazakii* strain (NTU84) that is not amplified with this method suggests that *ompA* PCR would not detect *Ent. sakazakii* in every case.

Cytokine secretion from U937 macrophages containing *Ent. sakazakii*

Since macrophages are thought to be early regulators of the innate immune response that further dictates the adaptive immune response, cytokine secretion from macrophages was assessed via ELISA following *Ent. sakazakii* inoculation. TNF α levels were not significantly different from 6 to 24 h and strain NTU84 was most robust, secreting 500 pg TNF α ml⁻¹ after 24 h (Fig. 6a). IL-6 levels more than doubled and again strain NTU84 had the most robust response, secreting nearly 1200 pg IL-6 ml⁻¹, indicative of a strong inflammatory response elicited from macrophages in response to NTU84 infection (Fig. 6b). Both patterns of cytokine expression match those expected: TNF α expression occurs rapidly and levels out while IL-6 gradually increases over time. IL-10 secretion was induced (Fig. 6c); however, IL-12 was not recovered in significant amounts (data not shown). Cytokine levels in serum collected from

Fig. 6. Cytokine secretion from *Ent. sakazakii*-exposed U937 macrophages. Human U937 macrophages were inoculated with *Ent. sakazakii* strains as in the gentamicin assay. Supernatant samples were collected after 6 and 24 h incubation (t_6 and t_{24}). ELISA was used to determine the concentration of (a) TNF α , (b) IL-6, (pro-inflammatory cytokines), and (c) IL-10 (immunosuppressive cytokine). IL-12 was not recovered in significant amounts. Data are means $\pm$ SEM from duplicate assays.

neonatal rats 24 h following i.p. inoculation with strain 2 were also tested using ELISA. The level of IL-10 elicited by strain 2 was $562 \pm 133 \text{ pg ml}^{-1}$. The level of IL-6 was $2199 \pm 497 \text{ pg ml}^{-1}$. The type 2 immune response is induced when the IL-10/IL-12 ratio is high, because anti-inflammatory IL-10 can suppress pro-inflammatory IL-12. This response is insufficient at clearing intracellular infections. An early bias towards a type 2 immune response may contribute to an inability to successfully eliminate this intracellular pathogen.

Concluding remarks

Ent. sakazakii is a heterogeneous bacterial species, containing strains with disparate phenotypic and genotypic characteristics that have yet to be clearly characterized. In

this study we comparatively analysed *Ent. sakazakii* for a number of known virulence traits and studied the host immune response in an attempt to gain a broader understanding of the phenotypic and infective differences that occur at the strain level. During meningitis additional damage is almost certainly caused by the host response to the pathogen. While the host response serves a protective purpose, it can be indiscriminate and cause irreversible damage to host brain tissues. More studies are needed; however, modulation of the host response may become an important way to mitigate the long-term sequelae of *Ent. sakazakii* meningitis. In the short term, reducing the bacterial load via antibiotics helps lessen the morbidity and mortality of meningitis but it is best to avoid exposing neonates to these organisms altogether.

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