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Cronobacter spp. (previously *Enterobacter sakazakii*) invade and translocate across both cultured human intestinal epithelial cells and human brain microvascular endothelial cells

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ABSTRACT

The mechanism of *Cronobacter* pathogenesis in neonatal meningitis and potential virulence factors (aside from host cell invasion ability) remain largely unknown. To ascertain whether *Cronobacter* can invade and transcytose across intestinal epithelial cells, enter into the blood stream and then transcytose across the blood-brain-barrier, we have utilized human intestinal INT407 and Caco-2 cells and brain microvascular endothelial cell (HBMEC) monolayers on Transwell filters as experimental model systems. Our data indicate a wide range of heterogeneity with respect to invasion efficiency among twenty-three *Cronobacter* isolates screened. For selected isolates, we observed significant levels of transcytosis for *Cronobacter sakazakii* across tight monolayers of both Caco-2 and HBMEC, mimicking *in vivo* ability to cross the intestine as well as the blood brain barrier, and at a frequency equivalent to that of a control meningitis-causing *Escherichia coli* K1 strain. Finally, EM analysis demonstrated intracellular *Cronobacter* bacteria within host vacuoles in HBMEC, as well as transcytosed bacteria at the basolateral surface. These data reveal that certain *Cronobacter* isolates can invade and translocate across both cultured human intestinal epithelial cells and HBMEC, thus demonstrating a potential path for neonatal infections of the central nervous system (CNS) following oral ingestion.

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1. Introduction

Enterobacter spp. are motile, rod-shaped, non-spore forming, Gram-negative, facultatively anaerobic members of the family *Enterobacteriaceae*. Although considered normal inhabitants of the gastrointestinal tract, *Enterobacter* are common contaminants of inanimate hospital surfaces and, as such, are responsible for ~10% of nosocomial infections, particularly wound infections, bacteremia, and pneumonia [1]. *Enterobacter sakazakii* is uniquely associated with infections in neonates and can cause necrotizing enterocolitis, bacteremia, and meningitis, with reported mortality rates of 40–80%; even the survivors often develop chronic neurological and developmental disorders [2–6]. There is considerable diversity with respect to both genotypic and phenotypic characteristics among different isolates of *E. sakazakii*. Accordingly, Iversen and coworkers [7–9], recently proposed a taxonomic reclassification of *E. sakazakii*, to *Cronobacter* spp., because *E. sakazakii* isolates are both genetically distinct from other *Enterobacter* spp. and represent at least six different species; recently, Joseph et al. [10] added two new species to this genus.

While this pathogen has been detected in a wide variety of foods [5,11], powdered infant formula remains primarily linked to outbreaks of neonatal meningitis. The mechanism of pathogenicity and expression of potential virulence factors of *Cronobacter* spp. remain largely unknown. To date, the few reports describing putative virulence factors are: by Pagotto et al. [12], who evaluated various *E. sakazakii* isolates for production of an enterotoxin using the suckling mouse assay; by Kothary et al. [13] who described

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a genus-specific zinc-metalloprotease expressed by all isolates tested; a recent report by Raghav and Aggarwal [14] who described the purification and characterization of an *E. sakazakii* enterotoxin; by Franco/Hu et al. [15] who described a major virulence plasmid which contained common and species-specific virulence factors such as iron acquisition systems (i.e. a common factor) and *Cronobacter* plasminogen activator (Cpa) similar to Pla or Pgt of *Yersinia pestis* and salmonellae (i.e. a specific factor expressed by *Cronobacter sakazakii* and *Cronobacter* genomospecies 1 strains) and a filamentous hemagglutinin (possessed by *Cronobacter malonaticus* and *Cronobacter turicensis* strains); and also a recent review of *C. sakazakii* as an opportunistic pathogen [16]. However, at the present time the linkage between enterotoxin or protease production and pathogenesis is unclear. Cpa was shown by Franco et al. [17] to activate plasminogen and to mediate resistance to serum bactericidal activity through the activation of plasminogen and the combined degradation α-2 antiplasmin and several complement proteins.

The abilities of *Cronobacter* to adhere to and invade the intestinal epithelium of the human host are also considered critical to disease pathogenesis. Mange et al. [18] investigated adhesive, but not invasive, characteristics of *E. sakazakii* with human epithelial (HEp-2 and Caco-2) and human brain microvascular endothelial (HBMEC) cells. Townsend et al. [19,20] showed the invasive ability of *E. sakazakii* into rat brain capillary endothelial cells as well as the persistence of this opportunistic pathogen within human macrophages. More recent reports [21–23] of the invasion of *E. sakazakii* into INT407 and Caco-2 cell lines have revealed bacterial outer membrane protein A (OmpA), OmpX, and host actin filaments as requirements for invasion. In addition, *E. sakazakii* invasion of HBMEC has been reported to require microtubules [24]. Recent evidence reported by Jaradat et al. [25] and data accumulated from a yet unpublished analysis of 161 *Cronobacter* isolates (Tall et al., unpublished data) show that not all isolates are *ompA*-positive (131/161 positive, 81.3%) by PCR using primers designed by Nair and Venkitanarayanan [26]. In the current report, we show that *ompA* is present, but heterogeneous among the 23 isolates studied herein (which were included in the above-mentioned unpublished study by Tall et al.). Very recently, the *C. sakazakii* BAA-894 genomic sequence has been reported and employed to analyze related *Cronobacter* genomes [27]. This work has revealed the presence of a copper/silver resistance gene system, known to be linked to invasion of the blood brain barrier (BBB) in meningitis-causing *Escherichia coli*, in all isolates studied of *Cronobacter* linked to neonatal infections.

Based on the pathogenic pathway of the most widely studied meningitis-causing bacterial pathogen, *E. coli* K1, we hypothesize that *Cronobacter* spp. invade and transcytose gut cells, enter into the blood stream, and then traverse across the blood brain barrier (BBB) to enter into the central nervous system (CNS) to cause meningitis and other neurological disorders [28,29]. Kim and coworkers [29,30] have developed an experimental model system to study the translocation of meningitis-causing bacterial pathogens across the BBB. This model is based on using collagen-coated Transwell membrane filters on which an immortalized, differentiated, and polarized HBMEC endothelial cell monolayer can be grown to confluence, forming tight junctions.

To ascertain whether representative isolates of *Cronobacter* can invade into or transcytose across human intestinal epithelial cells, presumably allowing them to enter into the blood stream, and then transcytose across the BBB, we utilized INT407 epithelial cells grown on tissue culture plates plus Caco-2 intestinal and HBMEC endothelial cell monolayers grown on Transwell membrane filters to study invasion and transcytosis ability. Our data verify the previously reported ability of *Cronobacter* to invade host intestinal epithelial cells [19–22], and, for the first time, demonstrate the ability of *C. sakazakii* to transcytose across tight monolayers of both intestinal and HBMEC cells in Transwell-culture. We observed a wide variation in invasion/transcytosis abilities among different isolates of *Cronobacter*. However, these results provide evidence of a molecular mechanism whereby ingested *Cronobacter* cells can transcytose across both the human intestinal and blood–brain barriers, presumably allowing the organism to stimulate meningitis and ensuing neurologic sequelae in neonates. Identification of *Cronobacter* isolates that differ significantly in their invasive and/or translocation efficiencies may facilitate identification of essential virulence functions or regulatory genes controlling these properties, information that will be useful both to understanding pathogenesis and in developing assays for rapid detection of virulent *Cronobacter* spp.

2. Results

2.1. Invasion efficiencies of twenty-three isolates of Cronobacter into INT407 epithelial cells

To ascertain whether representative isolates of *Cronobacter* can invade human intestinal epithelial cells, we utilized INT407 monolayers as an initial experimental model system. We first determined the invasion efficiencies of 20 isolates of *C. sakazakii*, two isolates of *C. malonaticus*, and a *Cronobacter muytjensii* isolate compared to those of noninvasive *E. coli* HB101 and invasive *E. coli* K1 isolates as negative and positive controls, respectively (Fig. 1). There was a wide variation in the invasion efficiencies among the various isolates of *Cronobacter* that were screened. These invasion levels could be placed into three categories: 1) the most invasive was *C. sakazakii* 1588 (Table 1 and Fig. 1, lane 5) at 0.63% invasion efficiency; 2) intermediately invasive *Cronobacter* isolates were 1605, 613, 1431, and 1599 (Table 1 and Fig. 1, lane 4) and averaged

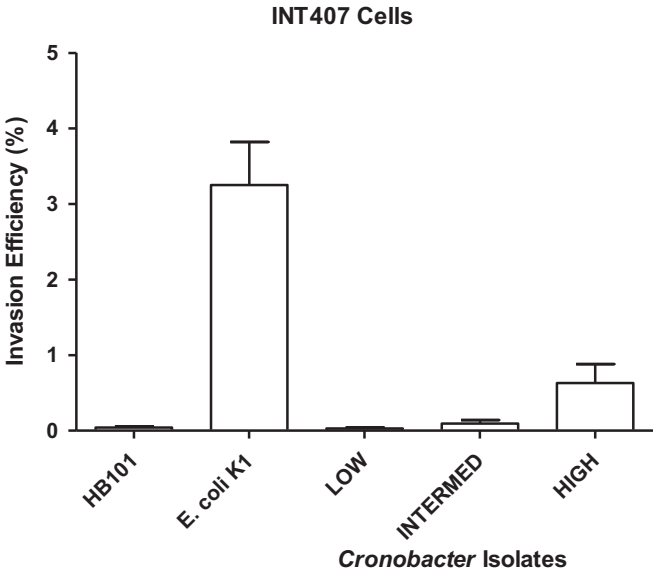

Fig. 1. Percent invasion efficiencies of bacteria into INT407 cells. Invasion assay procedures are as outlined in Methods. Lanes 1 and 2 represents the percent invasion efficiencies of *E. coli* HB101 (i.e. 0.0042%) and *E. coli* K1 strain RS203 (i.e. 3.25%) as negative and positive invasion controls, respectively. Lanes 3–5 represent the averaged percent invasion efficiencies of the three groups of *Cronobacter* isolates showing low (average = 0.028%), intermediate (average = 0.095%), or high (average = 0.63%) invasion levels. The bacterial isolates in each group are identified in the Results section. Each of the three groups were statistically different from each other and the positive control K1 strain at $P < 0.05$.

Table 1
Bacterial strains used in this study.

Bacterial strain[a]	Source	Source type
E. coli HB101	LESTD collection, FDA	Negative control, noninvasive
E. coli K1 strain RS203	FDA-CBER[b]	Meningitis strain, invasive
C. sakazakii 1424; 700; ATCC 29544	ATCC	Human, Child's throat
C. sakazakii 1639; ES614	R&F Laboratories[d]	Environ., Dairy Plant
C. sakazakii 1644; ES606	R&F Laboratories	Food, Corn flour, 2004
C. sakazakii 1588; FSM263	R&F Laboratories[c]	Environ., Nestle, Switzerland
C. sakazakii 1604; FSM286	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1605; FSM280	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1642; ES622; LCDC648	R&F Laboratories	Unknown, Canada
C. sakazakii 1593; FSM284	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1426; 702	FDA-CFSAN[c]	Environ.
C. sakazakii 613; ES11	FDA-CFSAN[c]	Food
C. sakazakii 1445; ES30	FDA-CFSAN[e]	Human, Wisconsin
C. sakazakii 1597; FSM296	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1584; RF560; ATCC 29004	ATCC	Human
C. sakazakii 1435; 713	FDA-CFSAN[c]	Dried Infant formula
C. sakazakii 1591; FSM373	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1449; ES34	FDA-CFSAN[e]	Neonate, CSF/Blood, 2002, Israel
C. sakazakii 1590; FSM315	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1428; 704	FDA-CFSAN[c]	Environ., Food manufacturing
C. sakazakii 1606; FSM262	R&F Laboratories	Environ., Nestle, Switzerland
C. sakazakii 1431; 707	FDA-CFSAN[c]	Human
C. muytjensii 1425; 701; ATCC 51329	ATCC	Non-human
C. malonaticus 1600; FSM327	R&F Laboratories	Environ., Nestle, Switzerland
C. malonaticus 1599; FSM275	R&F Laboratories	Environ., Nestle, Switzerland

[a] *Cronobacter* species identity was established by defining species-specific regions of *rpoB* according to the PCR method described by Stoop et al. [43].
[b] Strain was supplied by W. Vann, FDA-CBER.
[c] Strains were part of the Division of Virulence Assessment, OARSA, CFSAN, MOD1 culture collection.
[d] Strains were supplied by Dr. Lawrence Restaino, R& F Laboratories, 2725 Curtiss Street, Downers Grove, Illinois 60515.
[e] Strains were supplied by Dr. Michael Kotewicz, Division of Molecular Biology, OARSA, CFSAN, MOD1.

0.095% invasion efficiency; and 3) the least invasive *Cronobacter* isolates were 1424, 1639, 1644, 1604, 1600, 1605, 1642, 1593, 1426, 1445. 1597, 1584, 1435, 1591, 1449, 1590, 1428, 1606 and 1425 (Fig. 1, lane 3) and averaged 0.028% invasion efficiency, although they were significantly more invasive ($P = 0.0116$) than that of the *E. coli* HB101 negative control (i.e. 0.0042% invasion efficiency).

Because OmpA has been reported to be required for *C. sakazakii* invasion into INT407 cells [22,23], PCR analysis for the detection of *ompA* was performed with all of the isolates. The results show that *C. sakazakii* strain 1588 (FSM263), the most invasive isolate, and *C. sakazakii* strain 1445 (ES30) were repeatedly PCR-negative for the presence of *ompA* as tested according to the protocol and primers described by Nair and Venkitanarayanan [26], whereas the other strains including those isolates that fell into the least invasive category were PCR-positive. However, when a different set of *ompA* primers was employed, as described in the Material and Methods section, all isolates were PCR-positive for *ompA*, indicating some *ompA* heterogeneity among strains.

2.2. Transcytosis of Cronobacter isolates across differentiated Caco-2 intestinal epithelial cell monolayers

The above studies demonstrated that only a limited number of *Cronobacter* isolates show moderate to high levels of host cell invasion. Thus, we next assessed the ability of several selected invasive isolates of *Cronobacter* to invade and translocate across differentiated 7 day-old Caco-2 cells grown on Transwell membrane filters, cultured as described in the Material and Methods section. These confluent Caco-2 monolayers maintained TEER values of 650–800 Ω/cm^2, which correlate with monolayer polarization and tight junction formation [31]. Log phase *Cronobacter* cells, grown in BHI broth, were added at an MOI of ~200 to the upper well of the Transwell apparatus to infect the confluent Caco-2 monolayer. Following a 90 min invasion period and washing, any bacteria remaining in the upper well were killed by gentamicin treatment for 60 min. After washing the monolayer, internalized bacteria were enumerated to assess the efficiency of invasion (Table 2). Next, the internalized bacteria were then assessed for their ability to transcytose through the host cells and pass through the transwell membrane pores into the bottom well. Consistently high TEER values (i.e >650 Ω/cm^2) over the assay period, indicated maintenance of tight junctions. As shown in Table 2, the positive control *E. coli* K1 strain RS230 and the three selected *C. sakazakii* isolates invaded at a level of 2.5–3 logs higher than the noninvasive HB101 control. Transcytosis across the monolayer was measured, following gentamicin treatment and removal, after a further arbitrarily chosen 2 h and 4 h period. Transcytosis of the three selected *Cronobacter* isolates occurred by 2 h and increased substantially by 4 h, probably due to increased transcytosis and bacterial replication (Table 2). These studies show that the selected *C. sakazakii* isolates can invade and transcytose across tight differentiated, cultured intestinal epithelial cell monolayers.

2.3. Invasion efficiencies of selected isolates of Cronobacter into human endothelial cells

The HBMEC cell system was developed as a model system to evaluate the ability of meningitis-causing bacteria (e.g. *E. coli* K1 RS203) to invade and transcytose across the BBB [28]. We next determined the invasion efficiencies of selected *Cronobacter* isolates into HBMEC endothelial cells using *E. coli* HB101 and *E. coli* K1 RS203 as negative and positive controls, respectively (Table 3). Similar to the invasion data obtained with INT407 epithelial cells, *Cronobacter* entry into these endothelial cells fell into essentially the same three classes with respect to overall invasion efficiencies, but some *Cronobacter* isolates varied slightly in absolute invasion efficiency between INT407 and HBMEC cells. *C. sakazakii* 1588 was again found to be the most invasive isolate (~3 orders of magnitude more invasive relative to the least invasive one).

2.4. Transcytosis of Cronobacter across Transwell-cultured, confluent HBMEC monolayers

To analyze traversal across a mock BBB, we utilized the experimental model system comprising a tight monolayer of HBMEC cells

Table 2
Efficiency of *C. sakazakii* invasion into and transcytosis across differentiated 7 day-old Caco-2 cell monolayer.

Bacteria	Percent efficiency of		
	Invasion	Transcytosis	
		2h	4h
E. coli HB101	0.00007 ± 0.00003	0.00041 ± 0.0002	0.0007 ± 0.0002
E. coli K1	0.051 ± 0.021	0.006 ± 0.004	0.525 ± 0.09
C. sakazakii isolate			
1590	0.014 ± 0.009	0.007 ± 0.006	1.01 ± 0.29
1588	0.016 ± 0.006	0.014 ± 0.005	2.04 ± 0.59
613	0.048 ± 0.029	0.008 ± 0.003	1.59 ± 0.35

Table 3
Invasion efficiency of bacterial isolates into HBMEC cells

Bacterial strain	Percent invasion efficiency ± SD	
E. coli HB101[a]	0.0065 ± 0.002	
E. coli K1[a]	0.275 ± 0.111	
Cronobacter isolates[b]		
1424	0.039 ± 0.033	
1605	0.016 ± 0.014	
1590	0.001 ± 0.0004	LOW INVASION
1428	0.043 ± 0.010	AVERAGE = 0.021 ± 0.02
1431	0.003 ± 0.002	
613	0.083 ± 0.022	
1449	0.237 ± 0.071	INTERMEDIATE INVASION
1425	0.14 ± 0.017	AVERAGE = 0.14 ± 0.07
1599	0.10 ± 0.044	
1588	4.56 ± 0.94	HIGH INVASION

[a] *E. coli* HB101 served as the noninvasive control, while *E. coli* K1 served as a positive invasion control.
[b] The *Cronobacter* isolates were separated into three groups: low, intermediate, and high invasion levels. The individual isolates in each group and the group average invasion efficiencies are shown.

grown on Transwell membrane filter apparatus [30], as described in the Material and Methods section. Confluent HBMEC displayed typical TEER values of 361 ± 21 Ω/cm^2, which correlate with endothelial cell tight junction formation [30]. For the translocation studies, we chose to include *C. sakazakii* strains 1590, 613, and 1588, representing low, intermediate, and high invasive ability, respectively, both in INT407 and HBMEC cells. *E. coli* HB101 and *E. coli* K1 were utilized as negative and positive transcytosis-controls, respectively. Log-phase *Cronobacter* cells were added at an MOI of ~200 to the upper well of the Transwell apparatus to infect the confluent HBMEC monolayer. Following a 90 min invasion period at 37 °C, the remaining apical extracellular bacteria were killed by gentamicin (100 μg/ml) treatment for 60 min at 37 °C. The results (Table 4) indicate that *C. sakazakii* strain 1590 (which exhibited a low invasion level in both INT407 and HBMEC cells) also displayed the lowest ability to translocate across the HBMEC cell monolayer within the arbitrarily chosen 3 or 6 h period. Although strain 1590 transcytosis was low, note that the negative control *E. coli* HB101 did not transcytose during this 6 h time period. However, the moderately invasive level *C. sakazakii* strain 613 exhibited the highest level of translocation efficiency at both 3 h and 6 h. After 4 cycles of serial passage involving invasion and transcytosis (i.e. using the transcytosed bacteria for subsequent invasion/transcytosis rounds), the translocation efficiency of strain 613 further increased >10-fold. *C. sakazakii* strain 1588, which demonstrated the highest invasion levels in INT407 cells and HBMEC, revealed an intermediate, but significant ability to traverse the HBMEC monolayer, that was comparable to that of the positive control *E. coli* K1 strain RS203. The maintenance, over the assay period, of the high TEER for the infected HBMEC monolayers suggested that bacterial translocation occurred transcellularly.

Table 4
Ability of bacterial isolates to transcytose across HBMEC cell tight monolayers.

Bacteria	Percent efficiency of transcytosis	
	3h	6h
E. coli HB101	0	0
E. coli K1	0.010 ± 0.001	0.008 ± 0.006
Cronobacter isolates		
1590	0.002 ± 0.0001	0.004 ± 0.003
1588	0.013 ± 0.002	0.017 ± 0.015
613	0.027 ± 0.004	0.033 ± 0.029
613[a]	0.29 ± 0.14	0.098 ± 0.042

[a] Enriched through four serial transcytosis passages.

Finally, transmission EM analyses of infected HBMEC on Transwell filters were conducted to detect both *Cronobacter* invasion and transcytosis. This study demonstrated both intracellular *C. sakazakii* within tight membrane bound intracellular compartments plus apparent bacterial exocytosis at the basolateral host cell interface within proximity of pores in the Transwell filter, presumably showing *Cronobacter* having undergone transcellular transcytosis, effectively mimicking in cultured cells the crossing of the BBB (Fig. 2).

3. Discussion

Cronobacter spp. are emerging pathogens that were previously referred to as "a yellow-pigmented *Enterobacter cloacae*" until 1980 when they were designated as a new species honoring Japanese bacteriologist Ricchi Sakazakii [32]. Because isolates of *Cronobacter* are now known to represent eight different species, Iversen et al. [7–9] and Joseph et al. [10] proposed reclassifying the 16 biogroups of *E. sakazakii* as *Cronobacter* spp., with *C. sakazakii* as the most common species.

The first reported cases of meningitis caused by "yellow-pigmented *E. cloacae*" occurred in the UK in 1958 [33]. Since then, worldwide reports from Denmark, Iceland, Greece, the Netherlands, Canada, and the U.S. have followed, with most reports coming from hospital nurseries and neonatal ICUs [3,11,34]. Though premature infants and those with underlying medical conditions are at highest risk for developing *Cronobacter* infections, a healthy full-term infant in Iceland became ill prior to hospital discharge and suffered permanent neurological sequelae, apparently as a result of a *Cronobacter* infection [34].

Due to a relative resistance to stress during desiccation, *Cronobacter* can survive the infant formula-drying process [11]. Thus, upon ingestion this occasional contaminant of powdered infant formula can cause rare, but life-threatening forms of neonatal necrotizing enterocolitis (NEC), bacteremia, meningitis, and necrotizing meningoencephalitis following infection [35,36]. Meningitis and NEC caused by *Cronobacter* are associated with mortality rates of 40–80% and 10–55%, respectively [11]. In a retrospective study of 125 neonates with NEC, *Cronobacter* spp. were isolated from 29% of patients [37], demonstrating the prominence of this pathogen in neonatal NEC. Further, Van Acker et al. [38] described 12 NEC cases that occurred in 1998; a total of 11 *Cronobacter* strains were isolated from a cohort of 50 neonates during this outbreak. A recent study of *Cronobacter*-induced NEC in infant rats reported epithelial cell injury due to bacterially-induced apoptosis [39], suggesting that rats may serve as a NEC disease model for *C. sakazakii*. Although *C. sakazakii* are significant pathogens in neonates, it is important to note that adult human infections with these organisms are relatively infrequent and do not typically result in meningitis.

How does this opportunistic pathogen cause infections that lead to intestinal disease and extra-intestinal sequelae? The current study assessed the ability of selected *Cronobacter* isolates to invade and transcytose across cultured human intestinal epithelial cells. Previously, multiple *Cronobacter* isolates have been reported to adhere to human intestinal epithelial and endothelial cells [18]. In a recent study [22], one *Cronobacter* isolate was observed to invade INT407 cells with an efficiency of 0.2%. A separate study reported invasion of 3 *Cronobacter* isolates into Caco-2 cells at efficiencies of ~0.5% [21]. The studies reported here show that *Cronobacter* isolates can be grouped into three categories, relative to invasion ability (Fig. 1, Table 2). Only one of 23 strains displayed high-level invasion efficiency, while 4 strains showed an intermediate level (i.e. averaging 0.1–0.2%). Most isolates exhibited very low invasion efficiency, although significantly higher than the negative control strain *E. coli* HB101.

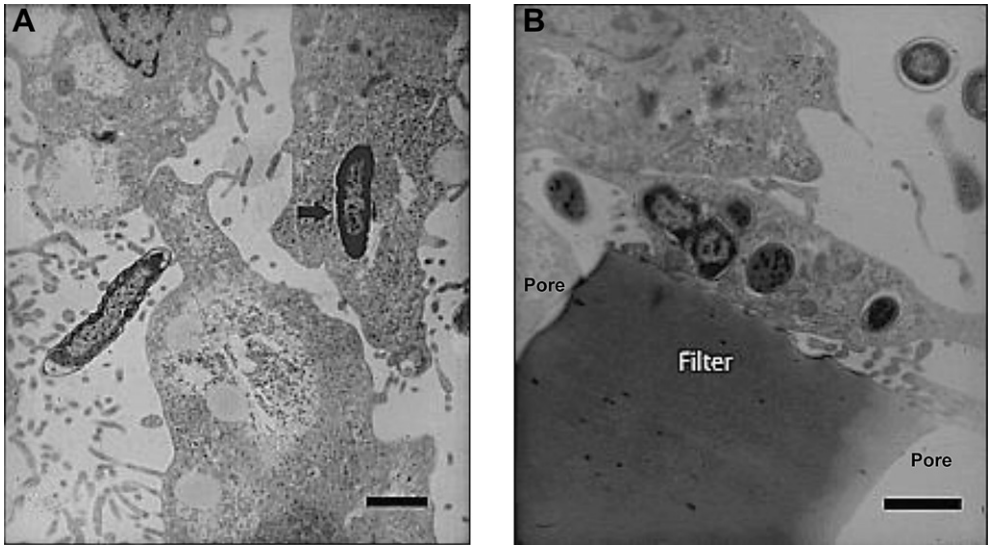

Fig. 2. Transmission electron micrographs of *Cronobacter*-infected HBMEC tight monolayers on Transwell filters. After 90 min infection, micrograph in (A) shows an extracellular *Cronobacter* cell and an internalized *Cronobacter* bacterium (see arrow) contained in a membrane bound intracellular compartment. Micrograph in (B) shows HBMEC monolayer on Transwell filter support with several internalized *Cronobacter* cells in membrane bound intracellular compartments and one bacterium apparently at the host cell basolateral surface-filter support interface in the proximity of a pore within the filter support. Bar markers represent 1 μm.

Earlier reports have suggested that *ompA* is somehow essential for bacterial invasion of host cells [22]. Interestingly, the *Cronobacter* strain (1588) most invasive for Caco-2 and INT407 cells and one other isolate (1445) were reproducibly negative for *ompA* when using the Nair and Venkitanarayanan PCR primers [26]. However, using primers designed to target a nearby region of *ompA* showed that all strains tested do have an *ompA* homolog. Though preliminary, these studies indicate that there is more than one *ompA* homolog among *Cronobacter* strains and support similar observations reported by Jaradat et al. [25]. Speculatively, these results suggest that there may be subgroup of strains containing a variant *ompA* that may somehow affect pathogenic ability.

To cause extra-intestinal infections such as sepsis or meningitis, *Cronobacter* spp. likely cross the intestinal mucosa, gain access to the blood stream and subsequently enter the CNS. We assessed the ability of *Cronobacter* isolates to invade plate-attached HBMEC to determine if there were any notable differences in invasion ability relative to INT407 cells or differentiated Caco-2 cells. No substantial differences were observed in invasion efficiencies across all cultured cell types. *C. sakazakii* strain 1588 had the highest invasion efficiency in all cell lines (Fig. 1, Tables 2 and 3). Subsequent to examination of invasion levels, selected *Cronobacter* isolates were assessed for the ability to translocate across tight monolayers of either Caco-2 cells or HBMEC. Two of three selected isolates were observed to transcytose across both epithelial and endothelial cell monolayers at efficiencies equal to or higher than that of the known meningitis pathogen *E. coli* K1 strain RS203 (Tables 2 and 4). Further, repeated transcytosis of *C. sakazakii* strain 613 for four serial passages through HBMEC increased the monolayer translocation efficiency about 10-fold (Table 4), suggesting that upregulation of these invasion/translocation genes can occur during extended contact with host cells. EM analysis of infected HBMEC monolayers grown on Transwell filters demonstrated the presence of *Cronobacter* cells within membrane-limited intracellular compartments, some of which had apparently migrated to the basolateral host cell surface, prior to exocytic bacterial release at the interface with the support filter (Fig. 2 B). Collectively, these data reveal that some, but not all, *Cronobacter* strains can invade and translocate efficiently across tight monolayers of both human intestinal epithelial cells and HBMEC. Speculatively, these results support a transcellular path for neonatal infection of the human CNS following ingestion of contaminated infant powdered formula. In a related investigation, *Cronobacter* invasion of *in vitro* cultured rat brain microvascular endothelial cells was reported along with inflammatory responses in the infant rat brain following intracranial inoculation with *Cronobacter* cells [19]. More recently, Mittal and colleagues [40] have established an oral infection, newborn rat model of *Cronobacter*-induced meningitis. Interestingly, these investigators have reported that *E sakazakii* strain 51329 (now reclassified as *C. muytjensii*) does not invade cultured rat intestinal epithelial (IEC-6) cells. However, orally administered strain 51329 at a dose of 10^4-10^5 cfu can apparently cause intestinal NEC, which allows the organism to breach the intestinal barrier. OmpA$^+$ strain 51329 then apparently multiplies in the blood stream, enters the CNS, and causes fatal meningitis. An OmpA$^-$ mutant of strain 51329 was found to enter the blood stream following oral infection, but did not multiply, or cause meningitis; however, oral or IV doses $>10^4$ cfu were not attempted to determine if higher doses of blood borne mutant organisms could cause meningitis. This neonatal rat model shows much promise, but may not be an exact mimic for human neonates. For example, rat intestinal epithelial cells are apparently not susceptible to invasion by strain 51329, whereas human intestinal epithelial cells are readily invaded. Thus, the infant rat model may be a good model to study intestinal NEC and meningitis, but may not reflect the potential ability of 51329 to cross the human gut by transcellular transcytosis, as suggested by the studies reported herein and the clinical observation of *C. sakazakii*-induced meningitis in the absence of NEC [2–6]. More recently, Mittal and colleagues [41] have provided new data indicating that *C. sakazakii* invade human dendritic cells via DC-SIGN and induce immunosuppressive responses via OmpA expression. Also, an oral infection model in neonatal CD-1 mice has been reported [42] and together with the rat model should help unfold key mechanisms of pathogenesis.

Feeding of *C. sakazakii*-contaminated, reconstituted powdered infant formulas has been established as a primary link to *Cronobacter* infection in neonates. *C. sakazakii* colonization most likely occurs in the neonatal intestinal tract. Our findings, together with previous studies of human epithelial cell adherence and invasion

[18–22,24,28] demonstrate through the use of *in vitro* cultured human cell systems that *Cronobacter* isolates can colonize and invade human intestinal epithelial cells. This study of 23 *Cronobacter* isolates shows that there is a wide variation in their abilities to invade host cells, which does not seem to correlate with source of isolation (e.g. environment vs. human infection). Following intestinal colonization (which may lead to NEC in some neonates), *Cronobacter* have the ability to invade and transcytose transcellularly across human host intestinal epithelial cells and gain access to the circulatory system. Low level bacteremias may be cleared by effective innate host defenses, but increasing bacterial loads due to bacterial multiplication will lead to threshold bacterial levels where *Cronobacter,* like *E. coli* K1, can cross the BBB and enter the CNS to cause meningitis and other neurological sequelae. There are yet very limited data on specific *Cronobacter* virulence factors [11,16]. However, bacterial genes controlling their ability to survive during the milk-drying process, to adhere to host cells, to invade and transcytose host intestinal cells, and to cross the BBB may serve as good candidates in future investigations of *Cronobacter* pathogenesis.

4. Materials and Methods

4.1. Bacterial strains and growth conditions

Table 1 lists the bacterial strains used in this study. A total of 23 isolates of *Cronobacter* spp., including a wide range of clinical, environmental and food isolates, were chosen because they represent a broad spectrum of pulse field gel electrophoretic (PFGE) patterns (C. Keys, unpublished data). Unless otherwise indicated, all bacterial strains were routinely grown without shaking at 37 °C in Brain-Heart-Infusion (BHI) broth (Becton–Dickinson, Franklin Lakes, NJ), supplemented with 0.3 M NaCl, prior to invasion studies.

4.2. Cell culture

Human intestinal epithelial INT407 cell monolayers were routinely cultured in minimal essential medium (MEM), supplemented with 2 mM glutamine, nonessential amino acids, penicillin/streptomycin, and heat-inactivated 10% fetal bovine serum (Life Technologies, Grand Island, N.Y.) in a 5% CO_2 atmosphere at 37 °C. SV40 T-Ag-immortalized, HBMEC cells [28,30,31] were grown as monolayers on rat tail collagen-coated tissue culture plates in media containing 1:1 Ham's F12 : Medium 199 with glutaMAX-1 (Invitrogen, Inc., Carlsbad, CA), supplemented with 20% heat-inactivated fetal bovine serum, nonessential amino acids, sodium pyruvate, MEM vitamins, heparin, 0.003% endothelial cell growth supplement from bovine neural tissue (Sigma–Aldrich, Corp., St. Louis, MO), and penicillin/streptomycin. Human intestinal Caco-2 cells were maintained in liquid nitrogen and cultured in MEM containing 20% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 0.1 mM nonessential amino acids, as recommended by the American Type Culture Collection.

4.3. Cellular invasion

One-day-old INT407 or 2-day old HBMEC monolayer cells ($\sim 3 \times 10^5$ cells/well) in a 24-well plate were infected with mid-log phase bacteria at a multiplicity of infection (MOI) of ~100. *E. coli* HB101 and K1 strain RS203 were utilized as negative and positive invasion controls, respectively. Following an invasion period of 90 min at 37 °C, the infected cells were washed 3 times with Hanks' balanced salt solution containing $CaCl_2$ and $MgCl_2$ (HBBS; GIBCO/Invitrogen, Inc.). Any remaining extracellular bacteria were killed by addition of 100 μg/ml gentamicin and incubation for 60 min at 37 °C. In control experiments, treatment with 100 μg/ml gentamicin at 37 °C for 1 h killed ~99.99% of the *Cronobacter* isolates and any remaining bacteria had no appreciable effect on calculation of invasion efficiency. Infected monolayers were then lysed with 0.1% Triton X-100 in DPBS (GIBCO/Invitrogen, Inc.) for ~10 min at 37 °C to release intracellular bacteria, which were enumerated after appropriate dilution and plating on LB agar plates. Each invasion assay was conducted in two separate wells and repeated in 3 separate experiments. The results are expressed as mean ± standard deviation of the mean for all experiments. Invasion efficiency represents the percent of the inoculum recovered as internalized at the end of the assay.

4.4. Bacterial translocation across Caco-2 or HBMEC monolayer cells grown on Transwell filters

Caco-2 or HBMEC cells (~ 2 X 10^5) were seeded on collagen-coated Transwell filters (6.5 mm insert, 24 well plate, Transwell-COL, 3μ pore size PTFE membrane; Corning Life Sciences, Inc.) and then incubated in a 5% CO_2 atmosphere at 37 °C. For some experiments, 6-well Transwell plates were employed. The culture media was changed at 2-day intervals. The Caco-2 monolayers generated polarization and tight junction formation by 7 days, as measured by trans-epithelial electrical resistance (TEER) using a Millicell-ERS meter (Millipore, Inc., Billerica, MA), when the TEER was 650–800 Ω/cm^2, after correction for that of filter blanks without cells [28,31]. The HBMEC monolayer cells generated tight junction formation within 5 days, as evidenced by TEER measurement; after 5 days of incubation, HBMEC monolayers routinely displayed a TEER of 361 ± 21 Ω/cm^2. The confluent monolayers of Caco-2 or HBMEC cells were infected with log phase-grown bacteria at MOI = ~200 for 90 min at 37 °C. Both lower and upper wells were washed 3 times with HBSS. Any remaining extracellular bacteria were killed by incubation for 60 min at 37 °C in culture medium containing 100 μg/ml gentamicin. Both upper and lower wells were then washed three times with HBSS followed by replenishment with fresh culture medium without antibiotics. Transcytosis of the *Cronobacter* isolates across Caco-2 cell monolayers was followed arbitrarily for 2 h and 4 h. after infection. Translocation of bacteria across HBMEC cell monolayers was allowed arbitrarily to occur for 3 or 6 h at 37 °C. The translocated bacteria released into the lower compartment were then enumerated by plating on LB agar plates. Monolayer integrity was monitored throughout these studies using TEER measurements, which did not vary over the course of the studies. Transcytosis assays for each strain studied were conducted in duplicate on each occasion and were repeated in at least 3 separate assays. The results are expressed as mean ± standard deviation of the mean for all wells.

4.5. Transmission electron microscopy (TEM)

For TEM, infected HBMEC monolayers were grown on Transwell filters, as described above. The monolayers infected with *Cronobacter* cells for 90 min were then washed three times with PBS, and fixed for 2 h at 4 °C in a mixture of 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2). The samples were rinsed twice in 0.1 M sodium cacodylate buffer, post-fixed for 2 h at 4 °C in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, rinsed twice in 0.1 M sodium cacodylate buffer, dehydrated in an ascending graded series of ethanol solutions, cleared in propylene oxide and embedded using Spurrs' low viscosity medium. Ultrathin sections were prepared, doubly stained using 2% uranyl acetate followed by Reynolds' lead citrate, and examined using a Zeiss CM 10 electron microscope (Carl Zeiss SMT Inc., Peabody, MA) operating at an accelerating voltage of 80 kV.

4.6. ompA PCR

PCR analysis for the presence of *ompA* was initially performed with primers, parameters and conditions described by Nair and Venkitanarayanan [26]. Primers ESSF and ESSR (Integrated DNA Technologies; Coralville, IA) were used to amplify a 469 bp fragment of the *ompA* gene by using a HotStarTaq kit (Qiagen, Inc.) which supplied each of the 50 ul reactions with 1 unit of Taq DNA polymerase, 1.5 mM MgCl, and 200 uM of each dNTP. Primers were added at 3 mM each and 5 ul of bacterial cell lysate served as DNA template [12]. The HotStarTaq polymerase was activated by incubation for 15 min at 95 °C, and the PCR amplification of the amplicon was performed with 30 cycles of 15 s at 94 °C, 15 s at 60 °C, and 30 s at 72 °C, followed by a final extension period of 5 min at 72 °C. The assay was run using an Applied Biosystems 2400 thermal cycler. DNA templates were prepared from the strains according to a procedure described by Kothary et al. [13]. When strains 1588 and 1445 were found to be negative for *ompA* using primers ESSF and ESSR, a second primer pair was designed to detect a region of *ompA* adjacent to the 469 bp fragment amplified by the primer pair of Nair and Venkitanarayanan [26]. Primer pair DKF4, *ggcgtgctgattcctcttctaac*, and DKB4, *ctccaaaggtatcccgtccaa*, targets a 514 bp region that includes the 3′ prime end of the 469 bp fragment of the Nair and Venkitanarayanan primer reaction. This product was amplified using the same parameters and conditions as described above except that the annealing temperature was changed to 58 °C for 15 s.

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