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Human isolates of *Cronobacter sakazakii* bind efficiently to intestinal epithelial cells *in vitro* to induce monolayer permeability and apoptosis

Quin Liu^{1,*}, Rahul Mittal^{2,*}, Claudia N. Emami³, Carol Iversen⁴, Henri R Ford^{3,5}, and Nemani V. Prasad Rao^{2,3,5}

¹Department of Gastroenterology, Children's Hospital Los Angeles ²Division of Infectious Diseases and Department of Pediatrics, Children's Hospital Los Angeles ³Department of Surgery, Children's Hospital Los Angeles ⁴Microbiological and Molecular Analytics Nestlé Research Center Vers-chez-les-blanc, CH-1000 Lausanne 26, Switzerland. ⁵Keck School of Medicine, University of Southern California, Los Angeles, CA 90027.

Abstract

Background—*Cronobacter sakazakii* (CS) is an emerging opportunistic pathogen that causes life-threatening infections in infants. This pathogen has been implicated in the outbreaks of necrotizing enterocolitis (NEC) with associated rates of high mortality and morbidity. In this study, we compared the abilities of CS strains isolated from human and environmental sources to bind to intestinal epithelial cells and trigger apoptosis.

Materials and Methods—CS strains were isolated from human and environmental sources, and their abilities to bind to intestinal epithelial cells were determined. Monolayer permeability was determined by transepithelial electrical resistance (TEER) and horseradish peroxidase (HRP) leakage. Apoptosis was examined by ApoTag and AnnexinV-7AAD staining. PKC activation was evaluated by non-radioactive PepTag assay.

Results—Human isolates of CS bind to rat and human enterocytes more efficiently than environmental strains. Additionally, these strains induced increased enterocyte monolayer permeability as indicated by a decrease in TEER and an increase in transcellular leakage of exogenously added HRP. Human isolates also caused tight junction disruption and significant apoptosis of enterocytes compared to environmental strains due to increased production of inducible nitric oxide. We also observed that human CS isolates caused two-fold increase in the activation of phosphokinase C (PKC) than environmental strains. Blocking the PKC activity in enterocytes by an inhibitor, Gö 6983, suppressed CS-mediated tight junction disruption, monolayer permeability and apoptosis of the cells.

Conclusion—These results suggest that human isolates of CS more efficiently bind to and cause damage to intestinal epithelial cells compared to environmental strains.

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Corresponding author: Nemani V. Prasad Rao Division of Infectious Diseases, MS #51 Children's Hospital Los Angeles 4650 Sunset Blvd. Los Angeles, CA 90027 Tel: 323-361-5465 Fax: 323-361-2867 pnemani@chla.usc.edu.

*These authors contributed equally to this work.

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Keywords

Cronobacter sakazakii; necrotizing enterocolitis; nitric oxide; phosphokinase C; apoptosis

INTRODUCTION

Necrotizing enterocolitis (NEC) is the most frequent and lethal disease that affects the intestine of premature infants [1]. NEC is the most common gastrointestinal medical/surgical emergency occurring in neonates [2, 3]. The incidence of NEC is reported to be approximately 2-5% [4]. However, it is likely to rise further due to the increased survival of premature infants, which results in a greater number of neonates at risk of developing NEC. Although it is more common in premature infants, it can also be observed in term, and near-term babies [5]. The overall mortality for NEC ranges from 10% to 50% but approaches 100% for infants with the most severe form of the disease characterized by full-thickness destruction of the intestine leading to intestinal perforation, peritonitis, bacterial invasion, and sepsis [6]. The indigenous intestinal microbial flora has been postulated to play a central role in the pathogenesis of NEC [7, 8]. In fact, bacterial colonization may be a prerequisite for the development of NEC. Despite recent advances in the field of neonatal medicine, the incident of NEC has not changed over the course of the past three decades and the morbidity and mortality remain the same [9]. Current trends, therefore, predict that NEC may soon become the leading cause of mortality for infants. While multiple risk factors have been implicated in the pathogenesis of NEC, the exact etiology of the disease remains elusive [10, 11]. The immaturity of the intestinal epithelial barrier exacerbated by intestinal mucosal injury resulting from various perinatal insults, including splanchnic hypoperfusion, hypoxia and formula feeding can lead to bacterial translocation, and exacerbation of epithelial injury.

Cronobacter (Enterobacter) sakazakii (CS) is an emerging opportunistic pathogen implicated with outbreaks of NEC, and is a common contaminant of powdered milk and infant formulas [12, 13]. The infant mortality rate due to infection by CS has been reported to be 33–100% depending on the severity of the disease [14]. The surviving neonates often develop serious neurological complications and may still require prolonged hospitalization for related complications, such as intestinal obstruction from scarring, liver failure due to a prolonged requirement for total parenteral nutrition, short bowel syndrome with intestinal failure and associated nutritional deficiencies and defects in growth and development [15, 16]. Although the natural reservoir of CS is unknown, however, this pathogen has been isolated from a variety of environmental sources such as factories used to produce milk powder, chocolate, cereal, potato flour, spices, and pasta [17-19]. In humans, it has been isolated from cerebrospinal fluid, blood, skin wounds, breast abscess, urine, respiratory secretions and digestive tract samples [20]. CS has also been isolated from cheese, minced beef, sausage meat, vegetables, and from the guts of the stable fly, *Stomoxys calcitrans*, and the Mexican fruit fly, *Anastrepha ludens* [21, 22]. However, the relationship between these potential environmental sources and infant disease remains unclear.

The binding of CS to intestinal epithelial cells is one of the prerequisites for initiation of infection. Following binding, CS induces disruption of tight junctions (TJ) between enterocytes and ultimately causes apoptosis of the cells [23]. Our previous studies have shown that nitric oxide (NO) plays an important role in TJ disruption of enterocytes by CS [24, 25]. Silencing inducible nitric oxide (iNOS), an enzyme responsible for the production of NO, abrogated CS induced intestinal epithelial cell damage. Since environment can be a potential source for transmittance of CS, we compared the ability of CS strains isolated from human and environmental sources for their binding abilities and to cause apoptosis in intestinal epithelial cells. We observed that human strains of CS have a selective advantage

over environmental strains, which enable these strains to bind to the enterocytes and cause infection.

MATERIALS AND METHODS

Bacterial strains and cell culture

CS strains used in this study are listed in Table 1 and were processed similarly for long-term storage. All the strains were grown in Luria Bertini (LB) broth at 37°C overnight. The strains were identified and characterized as described earlier [26, 27]. IEC-6 and CaCo-2 cells were obtained from American Type Culture collection (ATCC) and maintained as per ATCC instructions. In some experiments, cells were pretreated with PKC inhibitor Gö 6983 (10 µM) or PKC activator PMA (10 µM) before infecting with bacteria.

Binding of CS to intestinal epithelial cells

CS was added to confluent monolayers of IEC-6 or CaCo-2 cells at a multiplicity of infection (MOI) of 100 (cell to bacteria ratio, 1:100) and incubated for 4 h at 37°C under 5% CO₂. After incubation, the cells were washed three times with RPMI-1640 medium and monolayers were solubilized with 0.1% Triton X-100. The samples were serially diluted, plated onto blood agar plates, and incubated at 37°C overnight. The number of bacterial colonies was recorded, and the percentage binding was calculated on the basis of inoculum size.

Non-radioactive detection of PKC activity by PepTag assay

PKC activity was determined using the PepTag (Promega, Madison, WI) assay that utilizes a brightly colored, fluorescent peptide substrate that is highly specific to PKC as per manufacturer's instructions. Phosphorylation of peptide substrate due to increased PKC activity changes the net charge of the peptide from +1 to -1, thereby allowing the phosphorylated and non-phosphorylated peptides to be separated by agarose (0.8%) gel electrophoresis. The phosphorylated peptide migrates toward the positive electrode, whereas the non-phosphorylated peptide migrates toward the negative electrode. Intestinal epithelial cell lysates (10–25 µg in 10 µl) were incubated with the PKC reaction mixture (25 µl) at 30°C for 30 min. The reaction was stopped by exposing the tubes to 100°C in a boiling water bath. After adding 80% glycerol (1 µl), the samples were loaded onto an agarose gel (0.8% agarose in 50 mM Tris-HCl, pH 8.0) and separated at 100 V for 15 min. The peptide bands were visualized under UV light.

Fluorescence Microscopy

Confluent monolayers of CaCo-2 and IEC-6 cells in eight well chamber slides were infected with CS strains and incubated for 4 h at 37°C. After incubation the cells were washed three times with PBS and incubated with 5% goat serum in PBS to prevent non-specific binding. Cells were then fixed and permeabilized using BD biosciences (San Jose, CA) cytofix and cytoperm, washed and incubated with anti-ZO-1 antibody followed by Alexa Fluor 568 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). The slides were mounted in an antifade Vectashield solution containing 4', 6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were viewed and images were acquired by a confocal microscope LSM710, and arranged using Adobe Photoshop 7.0.

Determination of CaCo-2 cell monolayer integrity

IEC-6 or CaCo-2 cells seeded in transwells in enterocyte differentiation medium (BD Biosciences, San Jose, CA) were cultured for 48 h to reach a transepithelial electrical resistance (TEER) of >300 Ω cm² [25]. At the time of experiment, the medium in the upper

compartment was carefully removed and replaced with 100 μ l of fresh medium containing 0.1 μ g of HRP. CS strains were then added at an MOI of 100 in transwells and incubated for varying periods. 10 μ l of sample was then withdrawn at different post-infection times and HRP concentration was determined by measuring the peroxidase activity by mixing 5 mmol L⁻¹ guaiacol in 50 mmol L⁻¹ Na₂H₂PO₄ and 0.7 mmol L⁻¹ hydrogen peroxide. The color developed was measured at 470 nm using a spectrophotometer. TEER was determined using Millipore ERS apparatus (Temecula, CA) according to the manufacturer's instructions.

Evaluation of apoptosis of intestinal epithelial cells

Confluent monolayers of IEC-6 cells or CaCo-2 cells were infected with CS at an MOI of 1:100 for various time points. The cells were then stained using the ApopTag Red *In Situ* apoptosis detection kit (Chemicon, Temecula, CA) along with 4-, 6-diamidino-2-phenylindole (DAPI) and were visualized using a fluorescent microscope. Apoptosis was also assessed using AnnexinV-7AAD kit as per the manufacturer's instructions (BD biosciences, San Jose, CA).

Determination of iNOS expression and NO production

The cells infected with CS were washed, fixed and permeabilized using BD biosciences (San Jose, CA) cytofix and cytoperm. The cells were incubated with iNOS antibody (BD biosciences, San Diego, CA) followed by Alexa Fluor 568 goat anti-mouse IgG and analyzed by flow cytometry. NO production was determined in the supernatants as nitrite by Griess colorimetric assay as described earlier [25]

Statistical analysis

Statistical significance was determined by Student's *t* test, ANOVA and Fisher's exact test. Values of *P* < 0.05 were considered to be statistically significant.

RESULTS

CS strains isolated from humans adhere to both IEC-6 cells and CaCo-2 cells with a greater frequency compared to environmental sources

Our previous studies showed that CS binds at a frequency of 2.5% when infected at an MOI of 100 to intestinal epithelial cells employing an *in vitro* model of IEC-6 cells [23, 24]. In the present study, we examined the binding capacities of 24 CS strains isolated from environmental and human sources *in vitro* to intestinal epithelial cells (Table 1). Preliminary screening using all these strains revealed that optimal binding of these strains was observed at 4 h post-infection (data not shown). Four CS strains had significantly higher binding to IEC-6 cells at frequencies of 4-5% compared to binding frequencies of 0.01-0.8% for other CS stains (Figure 1A) (*P* < 0.001 human versus environmental strains by Student's *t* test, ANOVA and Fisher's exact test). Four other human isolates also had significantly higher binding at frequencies of ~1%, whereas all of low-binding strains were isolated from environmental samples. The binding experiments were repeated using CaCo-2 cells to determine whether the binding frequencies between human and environmental strains of CS are consistent between rat and human epithelial cells. Although the degree of binding of CS strains to CaCo-2 cells was lower compared with binding to IEC-6 cells, the differences in binding frequency to CaCo-2 cells between CS strains from human (0.6-1.9%) and environmental sources (0.0007- 0.07%) was significant (Figure 1B) (*P* < 0.001 human versus environmental strains by ANOVA and Fisher's exact test). These results suggest that CS strains isolated from humans have greater binding capacities to intestinal epithelial cells compared to CS strains obtained from environmental sources.

Human CS isolates induce greater apoptosis of intestinal epithelial cells compared to environmental isolates

To determine if the binding capacity of CS strains correlates with CS-induced apoptosis, CaCo-2 cells were infected with the bacteria at an MOI of 100 for 4 h and apoptosis was then determined by staining the cells using ApoTag. High-binding CS strains induced more apoptosis of the cells, whereas low-binding CS strains had less effect (Figure 2A). The degree of apoptosis was also quantified by counting the apoptotic cells per high power field. As shown in Fig. 2B, high-binding CS strains induced apoptosis in significantly greater percentage of cells compared to low-binding CS strains ($26\% \pm 4.5\%$ for human isolates versus $6.5\% \pm 2.5\%$ for environmental strains) ($P < 0.001$ by ANOVA and Fisher's exact test). AnnexinV-7AAD staining also revealed higher apoptosis of cells infected with human isolates compared to cells infected with environmental isolates (Figure 2C). Similar results were also observed with IEC-6 cells. These data indicate that high-binding CS strains that are isolated from humans induce significantly more apoptosis compared to low-binding CS strains that are isolated from environmental sources.

High-binding CS strains induce greater permeability of intestinal epithelial cells compared to low-binding CS strains

Loss of gut barrier integrity is an important factor in the development of NEC. To determine if CS binding capacity influences the intestinal epithelium permeability, CaCo-2 cells grown to confluence in 0.4 μm transwells were utilized as a model for the intestinal barrier. Two high-binding strains (1123-79 and E-784) from human-sources and two low-binding strains (BAA-896 and 3523-75) from environmental sources were used to infect CaCo-2 cells up to 4 h. High-binding CS strains from human sources induced a greater decrease in the TEER of CaCo-2 cell monolayer to approximately 45% from baseline by 4 h post-infection. However, low-binding CS strains decreased TEER to 80% from baseline (Figure 3A) ($P < 0.001$ human versus environmental strains by ANOVA and Fisher's exact test). The high-binding CS strains also induced greater HRP leakage across the CaCo-2 monolayer over the 4 h period compared to low-binding CS strains (Figure 3B) ($P < 0.001$ human versus environmental isolates by ANOVA and Fisher's exact test). Similar increase in monolayer permeability was also obtained with IEC-6 cells (Figure 3C and D). These results suggest that CS binding capacity translates to an increase in intestinal permeability.

CS strains obtained from humans trigger greater production of NO and activate PKC in enterocytes

Our earlier studies have demonstrated that NO plays an important role in CS-induced tight junction disruption and apoptosis of intestinal epithelial cells [23]. Therefore, we examined iNOS expression and NO production in CaCo-2 and IEC-6 cells by flow cytometry and Griess assay, respectively after infection with human and environmental strains of CS. iNOS expression was significantly higher in CaCo-2 and IEC-6 cells infected with human isolates of CS than enterocytes infected with environmental strains of CS (Figure 4A and B). Quantitation of these results in terms of mean fluorescence intensity (MFI) revealed that iNOS expression was four fold higher in epithelial cells infected with high-binding CS strains compared to low-binding CS strains (Figure 4C and D). On par with these findings, human CS isolates also induced significantly higher NO production than environmental strains of CS (Figure 4E and F) ($P < 0.001$ human versus environmental strains by ANOVA and Fisher's exact test). These results suggest that high binding CS strains induces greater tight junction disruption and more apoptosis of enterocytes than low binding CS strains that may be attributed to higher iNOS expression and greater NO production. NO has been demonstrated to activate PKC pathway that can play an important role in the disruption of tight junctions and apoptosis of intestinal epithelial cells. Therefore, we examined the activation of PKC in CaCo-2 and IEC-6 cells infected with human and environmental strains

of CS by non-radioactive PepTag assay. The human isolates of CS were more efficient in inducing phosphorylation of PKC compared to environmental strains of CS in CaCo-2 cells (Figure 5A, data for only one strain from each category is shown). Quantitation of phosphorylated protein (lower band) by fluorometric assay revealed that high-binding CS induced a 3-fold increase in PKC activity compared to low-binding CS strains (Figure 5B). Similar results were also obtained with IEC-6 cells (data not shown). These data suggest that CS-induced tight junction disruption may be induced by activation of PKC pathway initiated by NO production.

High-binding CS strains induce greater ZO-1 disruption

To determine if CS-induced intestinal epithelium permeability is due to tight junction (TJ) disruption, the distribution of the tight junction protein, zonula occludens-1 (ZO-1), was investigated in cells infected with human and environmental isolates of CS. Infection with high-binding CS leads to increased ZO-1 disruption at the periphery of the cells as compared to low-binding CS infected CaCo-2 monolayers and the control (Figure 6). Similar results were obtained with IEC-6 cells (data not shown). To examine the role of PKC in CS induced tight junction disruption, we pretreated CaCo-2 cells with a PKC activator, phorbol 12-myristate 13-acetate (PMA), and a PKC inhibitor, Gö 6983. The cells were then infected with high-binding CS strains; TEER and HRP leakage were determined. CaCo-2 monolayers pretreated with the Gö 6983 showed significantly higher TEER (Figure 7A) and lower HRP leakage (Figure 7B) when compared to untreated cells ($P < 0.01$ by ANOVA and Fisher's exact test). In contrast, CaCo-2 monolayers pretreated with PMA demonstrate lower TEER and higher HRP leakage, indicating greater intestinal barrier permeability compared to high-binding CS strain infected cells. On par with these findings pretreatment of CaCo-2 cells treated with Gö 6983 significantly reduced CS induced apoptosis, whereas cell pretreated with PMA showed marked increase in apoptosis upon infection with high-binding CS as examined by AnnexinV-7AAD staining (Figure 7C). Immunocytochemistry staining of ZO-1 in CaCo-2 cells treated with Gö 6983 and infected with high-binding CS also showed less ZO-1 disruption (Figure 7D). On the contrary, greater disruption of ZO-1 was observed in PMA treated and infected cells than untreated infected cells. Similar results were also obtained with IEC-6 cells (data not shown). Therefore, high-binding CS-induced tight junction disruption is due to activation of PKC pathway.

DISCUSSION

Cronobacter sakazakii is an opportunistic pathogen implicated in the pathogenesis of meningitis, NEC, and sepsis in neonates [13-15]. It is assumed that farm environments and cattle could play a role in the transmission of *Cronobacter* spp although the exact source of the pathogen remains unclear [17, 18]. Colonization of CS requires that the organisms adhere to host cell surfaces [23, 24]. Agents that prevent attachment of the pathogen to epithelial cells could be useful in reducing infections [28]. In this study, we observed that human strains of CS were more able to bind to intestinal epithelial cells than environmental strains in culture. It has earlier been demonstrated that CS strains obtained from environmental sources were more persistent than the clinical strains in inulin and lecithin, indicating that patho-adaptation may have contributed to a reduction in the desiccation tolerance phenotype [29]. Therefore, the increased efficiency of human isolates of CS to bind to enterocytes may also represent an adaptation to cause NEC. These findings corroborates with the findings of Pirone et al who also observed that clinical strains of *Burkholderia cenocepacia* have the better ability to invade human respiratory epithelial cells compared to environmental strains [30]. *Campylobacter jejuni* and *Campylobacter coli* strains isolated from environment were found to be less invasive and less cytotoxic than clinical strains to HeLa cells determined by immunofluorescence and electron microscopy

[31]. It was suggested that virulence of environmental strains was enhanced by passage. Studies from our lab have demonstrated that outer membrane protein A (OmpA) plays a decisive role in the adhesion of CS to intestinal epithelial cells [25, 32]. In agreement, subsequent studies demonstrated that *ompA* and *ompX* deletion mutants exhibited significantly reduced invasion of human enterocyte-like epithelial CaCo-2 and human intestinal epithelial INT-407 cells [33]. Therefore, it remains to be determined whether structural differences in the OmpA sequences between human and environmental strains of CS contribute to the pathogenesis. Alternatively, OmpA mediates initial binding of CS to enterocytes, and the pathogenic strains express or secrete some other virulence factors for the onset of NEC. It has been shown that human intestinal colonization creates a hyperinfectious bacterial state in *Vibrio cholerae* that is maintained after dissemination [34].

The tight junction constitutes the principle barrier in intestinal epithelium cells to passive movement of fluid electrolytes, macromolecules and cells [35, 36]. Protein kinases have been demonstrated to play a role in the regulation of tight junction assembly and in paracellular permeability changes [37-39]. In the present study, we observed that human isolates of CS caused increased intestinal epithelial permeability *in vitro* leading to disruption of tight junctions and more apoptosis of enterocytes, an important hallmark of CS induced NEC, than environmental strains. Interestingly, the inhibition of PKC- α intracellular signaling pathway abrogated these effects indicating that PKC- α is involved in CS induced intestinal epithelial cell permeability. The activation of PKC- α by phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) has been correlated with tight junction permeability in renal epithelial cells corroborating with the findings of the present study [40]. Furthermore, the virulence of three strains of CS, MNW2 (a food isolate), SK81 (a clinical isolate), and 3290 (a clinical isolate), were tested in neonatal CD-1 mice [35]. CS strain 3290 was significantly more invasive in brains (42.1% of mice) than were strains MNW2 (6.7%) and SK81 (15.9%). Mortality was observed for all strains of CS tested, with SK81 being significantly more lethal (5.6%) than MNW2 (1.2%) or 3290 (0.6%) [41]. The data in these studies also suggest that invasiveness does not necessarily correlate with mortality among different strains of CS, however, the clinical isolates are more virulent than the food isolates. Although infant formula has been implicated to NEC outbreaks, our studies showed that CS strains isolated from milk powder do not efficiently bind intestinal epithelial cells. Therefore, it is possible that CS strains found infant formula could be of human origin due to contamination during formula preparations. Further studies in rat or mouse models of NEC using these strains are necessary to clearly identify the pathogenic potential of environmental strains versus human isolates.

In summary, our studies have demonstrated that CS isolates from humans exhibit a greater binding capacity to enterocytes *in vitro* compared to environmental strains. The binding efficiency has been correlated to increased permeability of enterocyte monolayers and apoptosis of the cells, indicating that adherence is a prerequisite for subsequent damage to enterocytes. Although the roles of virulence-associated factors in the human and environmental CS strains is not clear, it is possible that environmental strains may be precursors of pathogenic strains or may participate in gene transfer events leading to the origination of pathogenic strains. Alternatively the adaptation of environmental strains to the human intestine possibly contributed to the enhanced expression of virulence genes allowing the bacteria to evade potent host immune responses and colonize the gut. Further comparative analysis studies between human and environmental CS strains using genomic microarrays are warranted which are likely to provide a better understanding of the events that led to the evolution of pathogenic CS clones from environmental progenitor strains. The complete understanding of the molecular mechanisms that underlie the pathogenesis of NEC by CS would provide novel avenues to develop preventative strategies against CS-induced NEC.

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Figure 1. Human CS isolates bind more efficiently to intestinal epithelial cells than environmental strains

Confluent monolayers of IEC-6 cells were infected with various human and environmental CS strains and percentage binding was calculated (A). Binding of selected CS strains to CaCo-2 cells was also examined (B). Data represent mean $\pm$ SD. Results are representative of three independent experiments carried out in triplicate. The binding of human isolates to enterocytes was significantly greater than environmental strains, $*p < 0.001$ by Student's *t* test, ANOVA and Fisher's exact test.

Figure 2. The high binding CS strains cause more apoptosis of intestinal epithelial cells
 The high binding strain (1123-79) and the low binding strain (3523-75) of CS was incubated with the IEC-6 or CaCo-2 cells for 4 h. The cells were then stained with ApopTag Red *in situ* Apoptosis Detection kit and DAPI (blue), and viewed under a fluorescence microscope for apoptotic cells (red) (A). The number of apoptotic cells per high power field (HPF) was measured in 10 different areas of each slide, and the results were plotted (B). Apoptosis was also determined in CS infected IEC-6 or CaCo-2 cells by AnnexinV-7AAD kit (C). Data represent mean values $\pm$ SD. The experiments were repeated three times in triplicate with similar results. High binding CS strains induced apoptosis in greater number of cells compared to low binding strains, * $p < 0.001$ by ANOVA and Fisher's exact test.

Figure 3. Infection with high binding strains of CS increases tight junction dysfunction and barrier permeability in intestinal epithelial cells
IEC-6 (A and B) or CaCo-2 (C and D) cells were infected with CS strains for varying time periods. Tight junction disruption and monolayer permeability was then measured using transepithelial electrical resistance (TEER) (A and C) and HRP leakage (B and D). Infection with high binding strains of CS (1123-79 & E784) results in faster decline in TEER of the epithelial monolayers compared to the low binding CS strains (3523-75 & BAA-896). Also, there was more HRP leakage across intestinal epithelial cells indicating monolayer permeability infected with high binding strains of CS compared to the ones infected with the low binding strains. The error bars represent SDs of triplicate samples. The results are representative of four individual experiments performed in triplicate. The decrease in TEER or increase in HRP leakage was significant in cells infected with human CS isolates compared to environmental CS strains, * $p < 0.001$ by ANOVA and Fisher's exact test.

Figure 4. iNOS expression and NO production are significantly higher in enterocytes infected with human CS isolates

iNOS expression was measured in CaCo-2 (A) and IEC-6 (B) cells infected with human and environmental CS strains for 4 h using flow cytometry. For quantitative measurements, mean fluorescence intensity (MFI) was calculated subtracted from isotype-matched controls (C and D). Levels of soluble nitrite were also measured in the cell culture supernatants using Griess colorimetric assay in both CaCo-2 (E) as well as IEC-6 (F) cells infected with CS. Data represent mean $\pm$ SD from five individual experiments carried out in triplicate. The increase in iNOS expression and NO production was statistically significant in cells infected with high-binding CS strains compared to low-binding CS strains, * $p < 0.001$ by Student's *t* test, ANOVA and Fisher's exact test.

Figure 5. Human CS isolates trigger activation of PKC in intestinal epithelial cells
 PKC phosphorylation was examined in CS infected CaCo-2 cells at different post-infection time periods using PepTag assay (A). Quantitation of results in terms of kinase units/ml revealed that human CS isolate induce more phosphorylation of PKC than environmental strain, * $p < 0.001$ by Student's *t* test (B). Data represent mean $\pm$ SD. Results are representative of three independent experiments carried out in triplicate.

Figure 6. Tight junction disruption in intestinal epithelial cells infected with CS
 Confluent monolayers of CaCo-2 cells grown in 8-well chamber slides were infected with CS strains for 4 h and stained for tight junction molecule ZO-1 and for nuclei with DAPI. Human isolates of CS caused more disruption of ZO-1 than environmental strains. The results are representative of four individual experiments. Arrows indicate either bacterial binding or disruption of ZO1 staining.

Figure 7. Inhibition of PKC phosphorylation reduces CS mediated tight junction disruption and monolayer permeability

CaCo-2 cells were pretreated with PKC inhibitor Gö 6983 or PKC activator PMA and then infected with CS (1123-79). TEER (A) and HRP leakage (B) was then determined. The decrease in TEER or increase in HRP leakage was significantly reduced in cells pretreated with PKC inhibitor Gö 6983, $*P < 0.001$ by ANOVA and Fisher's exact test. Apoptosis of CaCo-2 cells induced by high-binding strain of CS was significantly decreased in the presence of Gö 6983 compared to untreated and infected cells ($*p < 0.001$ by Student's *t* test, ANOVA and Fisher's exact test), whereas it was significantly increased in PMA pretreated cells compared to untreated cells as measured by AnnexinV-7AAD staining ($**p < 0.001$ by Student's *t* test, ANOVA and Fisher's exact test) (C). Tight junction disruption was also abrogated in the presence of Gö 6983 compared to PMA pretreated CaCo-2 cells (D). Data represent mean $\pm$ SD and is representative of five individual experiments carried out in triplicate. The arrows indicated the bacterial binding or disruption at the tight junctions.

Table 1*C. sakazakii* strains used in this study

Strain	Origin	Serotype
E784	Neonate	1
CDC1123-79	Clinical	2
CDC 1058-77	Breast Abcess (Human)	ND
E755	Human	2
3523-75	Environmental	1
BAA896	Environmental	2
SK-81	Environmental	1
LPS-LAC	Milk Powder	2
CDC 0996-77	Human spinal fluid	1
CDC0289-81	Clinical	2
E786	Milk Powder	2
2484	Human	2
1895-73	Faeces (Human)	1
9369-75	Unknown	ND
322-78	Clinical	2
SK-90	Human	2
3128-77	Sputum (Human)	1
4963-71	Environmental	1
1/10/2001	Environmental	1
9363-75	Environmental	1
2874	Milk Powder	2
BAA-893	Milk Powder	2
11467	Clinical	1
5960-70	Blood (Human)	2

ND, not determined