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Profiling of Virulence Determinants in *Cronobacter sakazakii* Isolates from Different Plant and Environmental Commodities

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Abstract *Cronobacter sakazakii* is an emerging pathogen causing meningitis, sepsis and necrotizing enterocolitis in neonates and immune-compromised adults. The present study describes the profiling of different virulence factors associated with *C. sakazakii* isolates derived from plant-based materials and environmental samples (soil, water, and vacuum dust). All the isolates exhibited β-hemolysis and chitinase activity, and were able to utilize inositol. Among the nine virulence-associated genes, *hly* gene coding for hemolysin was detected in all the isolates followed by *ompA* (outer membrane protein); however, plasmid-borne genes were detected at a level of 60% for both *cpa* (cronobacter plasminogen activator) and *eitA* (Ferric ion transporter protein) gene, respectively. Furthermore, the isolate *C. sakazakii* N81 showed cytotoxicity for Caco-2 cells. The presence of the virulence determinants investigated in this study indicates the pathogenic potential of C. *sakazakii* with their plausible connection with clinical manifestations.

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Introduction

Cronobacter is an emerging genus of Gram-negative opportunistic pathogens which have been associated with invasive infections such as meningitis, bacteraemia, and necrotizing enterocolitis mostly in neonates through the consumption of powdered infant formula (PIF) [14, 36]. Among the seven species of *Cronobacter, C. sakazakii, C. malonaticus*, and *C. turicensis* are thought to be the major infantile pathogens with *C. sakazakii* clonal complex 4 (CC4) isolates predominating as the major cause of neonatal infections such as meningitis [23, 29]. Although few putative virulence markers such as production of proteolytic enzymes, outer membrane proteins, and iron acquisition gene systems have been reported in *C. sakazakii*, however, outer membrane proteins (*OmpA* and *OmpX*) are one of the best characterized virulence factors with a role in the adherence and invasion of C. *sakazakii* with animal cell models [8, 27, 30, 36, 41]. Further studies have shown that *C. sakazakii* exploits both microfilaments and microtubules to invade and translocate across human intestinal epithelial cells as well as endothelial cells mimicking the potential path of meningitis [11]. The role of different putative virulence factors such as surface structures (lipopolysaccharides (LPS), exopolysaccharide (EPS), and flagella) in addition to the production of enterotoxin and proteolytic enzymes has been extensively reviewed by Singh et al. [36].

Cronobacter spp. have been portrayed as emergent pathogens; therefore, it is important to examine the genes involved in the pathogenesis of this bacterium. In order to understand the pathogenic process of C. sakazakii infections, this study was designed to identify the prevalence and distribution of putative virulence factors and the genes possessed by this pathogen. So far, the data on the virulence

properties of *C. sakazakii* isolated from Indian commodities are not fully known and the goal of this report is to add such information to the growing body of knowledge on these organisms so that Indian infant food manufacturers can detect and identify this organism quickly so that contaminated products can be removed from commerce.

Materials and Methods

Cultures and Culture Conditions

The rpoB species-specific PCR analyses confirmed the identity of five *C. sakazakii* isolates out of 38 *Cronobacter* spp. which were previously identified by 16 S rRNA [37, 40]. *C. sakazakii* strains ATCC 12,868 and E604 (kindly gifted by Dr. Ben Davies Tall, FDA, USA) and five other isolates were used in this study (Table 1). To determine the cytotoxicity of the strain, Caco-2 cells (ATCC 7,348,406) were obtained from National Centre for Cell Science, Pune, India. The Caco-2 cells were grown in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, USA) supplemented with 10% of fetal bovine serum and 100 U/ml of streptomycin and penicillin [7]. The cells were maintained at 37 °C in a 5% CO_2 /95% air atmosphere.

Virulence Determinants Through Biochemical Assays

Various biochemical assays for determination of virulence traits were conducted as per standard protocols. For inositol fermentation test, the overnight grown culture of *C. sakazakii* isolates in Tryptone Soy Broth (TSB) was centrifuged and 100 µl of the cell pellet in phosphate buffer saline (PBS, pH 7.0) with $OD_{600} = 0.5$ was suspended in 5 ml carbohydrate basal medium (peptone 10%, sodium chloride 5%, phenol red 0.018%) with inositol (1 mg/ml) and phenol red as an indicator. The culture was incubated for 24 h at 37 °C. A positive response was shown by a change in color from reddish to yellowish. The DNase test was performed by culturing the isolates on DNase agar for 24 h at 37 °C and pouring 2 ml of 1 N HCl after incubation [39]. Only strong DNase activities displaying a clear zone around growth were taken as positive. The ability of isolates for the production of hemolysin was determined by streaking overnight grown culture on sheep blood agar plates (beef heart peptone 1%, tryptose 1%, NaCl 0.5%, agar 1.5%, and 5% defibrinated blood from HiMedia Laboratories, India) and incubated at 37 °C for 24 h [19]. Complete lysis of erythrocytes around the colonies was considered as positive hemolytic reaction. The siderophore production was determined using the chrome azurol S (CAS) agar (HiMedia Laboratories, India) well diffusion assay [34, 35]. The chitinase activity was determined on colloidal chitin agar

Table 1 Virulence traits identified in *Cronobacter sakazakii* strains

Strains	Source	Plasmids			Virulence determinants through biochemical assays						Detection of virulence-associated genes by PCR								
		pESA3/pCTU1	pESA2/pCTU2	pCTU3	Inositol Fermentation	DNase activity	Hemolytic activity	Siderophore detection	Chitinase activity	Sialic acid utilization	*ompA*	*hly*	*cpa*	*chi*F	*eit*A	BAM (*zpx*)	*iuc*C	*sip*	*fhaB*
E604	Standard	+	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
ATCC 12,868	Standard	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–
N13	Food	+	+	–	+	+	+	–	+	+	+	+	+	–	–	–	+	–	–
N14	Food	+	+	–	+	–	+	–	+	–	+	+	–	–	+	+	+	–	–
N15	Soil	+	+	–	+	–	+	–	+	–	–	+	+	–	–	–	+	–	–
N81	Water	+	+	–	+	+	+	+	+	+	+	+	+	+	–	+	+	–	–
N112	Food	+	+	–	+	+	+	+	+	+	+	+	–	+	+	–	+	–	–

ompA outer membrane protein, *hly* hemolysin, *cpa* cronobacter plasminogen activator, *chiF* chitinaseF, *eitA* iron acquisition gene, BAM zinc-containing metalloprotease, *sip* siderophore-interacting protein, *fhaB* filamentous hemagglutinin, *iucC* from siderophore-mediated *iucABCD/iutA* gene cluster

+ indicates the presence of respective trait, – indicates the absence of respective trait

by inoculating the overnight grown culture of isolates on the colloidal chitin agar medium. The strains considered chitinase positive displayed a zone of hydrolysis surrounding colonies growing on the cream-colored medium after 96 h of incubation at 37 °C [18]. The sialic acid utilization was determined by inoculation of overnight grown *C. sakazakii* cultures in M9 minimal media supplemented with 1 mg/ml of *N*-acetylneuraminic acid (Sialic acid) [17]. The tubes were incubated at 37 °C for 24 h and the growth of each isolate was monitored by measuring the absorbance at 595 nm.

DNA Extraction and Plasmid Isolation

An aliquot of 2 ml of each overnight grown bacterial culture in TSB was used for DNA extraction using phenol:chloroform:iso-amyl alcohol method as described by Sambrook et al. [33]. In parallel, plasmids were isolated from a 3-ml aliquot of overnight grown culture in TSB using QIAprep Spin Miniprep kit as per the manufacturer's instructions. The detection of plasmids was confirmed using the *repA*-targeted PCR assay as described by Franco et al. [11] and finally confirmed by the presence of plasmids using agarose gel electrophoresis.

Detection of Virulence-Associated Genes by PCR

The PCR was conducted in individual reactions using primers for the following nine genes: *ompA* (outer membrane protein), *hly* (hemolysin), *cpa* (cronobacter plasminogen activator), *chiF* (chitinaseF), *eitA* (iron acquisition gene), BAM (zinc-containing metalloprotease), *sip* (siderophore-interacting protein), *fhaB* (filamentous hemagglutinin), and *iucC* (from siderophore-mediated *iucABCD/iutA* gene cluster) as per the conditions described in literature [5, 9, 15, 42]. The PCR was performed with 1 µl of DNA, 2.5 µl of 10×PCR buffer, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP (Promega) mix, 1 µl each of 10 pM primers, and 0.25 µl of 5 U Taq DNA polymerase (Intron), and the final volume was made up to 25 µl using nuclease-free water. The amplified products were analyzed in 1.5% agarose gel along with 1 kb DNA marker (Gene Ruler–Fermentas) by staining with ethidium bromide.

Cell Infection Assay

Among all the *C. sakazakii* isolates, isolate N81 demonstrated maximum association with virulence genes and was used for cell infection assay. The differentiated Caco-2 cells at late post-confluence were used for infection assays (15 days post seeding). The adhered cells were trypsinized, transferred to 24-well plates containing round glass slides, and re-incubated. After 24 h, the DMEM with antibiotics was replaced by DMEM supplemented with 2% of fetal bovine serum. The overnight grown culture of *C. sakazakii* N81 in TSB was diluted in DMEM containing 2% of fetal bovine serum and added to each well (ca. 10^6 cfu/ml) containing the Caco-2 cells. After 3 h of incubation at 37 °C, the infected monolayers were washed with PBS and incubated with 1 mL DMEM [5]. The infected monolayers were fixed with methanol and stained with Hoechst dye 33,258. The immunofluorescent staining and cell morphology were examined by fluorescence with filters for UV excitation and phase-contrast microscopy using an Olympus microscope BX53.

Results and Discussion

The investigation of bacterial virulence factors and their interactions with the host is essential to understand the pathogenesis of any bacterial species. To detect the essential virulence traits and associated genes, the present investigation reports both phenotypic and genotypic data on the presence of virulence traits among *C. sakazakii* isolates of Indian origin which were obtained from a variety of foods and environmental sources. Our results revealed that, among the tested isolates, all the isolates were capable of utilizing inositol; however, sialic acid utilization and DNase activity were observed in only three isolates, respectively (Table 1). The presence of genes for inositol fermentation has been reported in two other meningitic strains of C. sakazakii (strains 701, 767) belonging to ST4, and the fact that all seven strains could utilize inositol supports the recent results by Hamby et al. [13], suggesting that this phenotypic trait may represent a marker of pathogenicity for *C. sakazakii*. DNase activity has also been used as the fundamental virulence criterion to identify factors which interferes with the antimicrobial activity of neutrophil-produced extracellular traps (NETs) [26]. However, DNase is not entirely reliable as an indicator of pathogenicity. Sialic acid, which is a usual constituent of breast milk, infant formula, mucin lining of intestinal tract and brain ganglioside comples, and is activated by nanAKT gene cluster may too serve as a marker of pathogenicity [38]. Interestingly, among the different species of *Cronobacter, C. sakazakii* is the only species that possesses this gene cluster and subsequently it is the only species to date that can utilize sialic acid as a carbon source [16, 17]. Grim et al. revealed the presence of two genomic regions (GR127 and GR129) that are involved in the utilization of sialic acid in the genome of *C. sakazakii* BAA-894 [12]. In our study, *C. sakazakii* isolates from plant-based products were found to utilize sialic acid, emphasizing that the PIF manufacturers need to maintain microbiological standards before incorporating

plant-based materials in food products. The importance of hemolysins in virulence of bacterial pathogens has been extensively characterized; however, the reports on the production of hemolytic toxins by C. sakazakii are very scarce. All the isolates were hemolytic on blood agar plates and possessed chitinase activity. Expression of siderophores using the CAS assay was observed in two of the five isolated strains. However, iron depletion studies were not performed with the two CAS-negative strains to determine if siderophore activity was inducible in these strains.

The prevalence and distribution of nine virulence-associated genes, some of which are on the common virulence plasmid pESA3/pCTU1, were studied in these *C. sakazakii* isolates (Table 1). All the seven isolates possessed the pESA3 virulence plasmid; however, only the five Indian isolates possessed pESA2. Interestingly, pCTU3 was detected in only *C. sakazakii* ATCC 12,868. The ability of the opportunistic pathogen *Cronobacter* spp. to cause meningitis is dependent upon its capacity to survive in blood. All the *C. sakazakii* strains in this research were confirmed positive for *hly* gene, with one isolate being observed negative for *ompA*. Mittal et al. reported that o*mpA*$^{+}$*C. sakazakii* isolates breach blood–brain barrier and invade central nervous system (CNS), and therefore it is imperative for the commencement of meningitis in a neonatal mice model [27]. In addition to *ompA*, Kim and Loessner [21] reported that the expression of *ompX* of *C. sakazakii* also plays vital roles in both the apical and basolateral invasion of the host cells and these proteins are also involved in systemic circulation (i.e., involvement in spleen and liver abscesses) [20]. It was expected that *ompA* would be detected in all of the isolates due to its importance in cell invasion but one isolate was negative for *ompA* gene. Nevertheless, not all *C. sakazakii* were reported to encode *ompA* using the primers described by Nair & Venkitanarayanan [30], as observed by Jaradat et al. [15] and Giri et al. [11]. Nowadays, proteomics approaches are also garnering attention to screen virulence factors and to recognize new diagnostic markers and therapeutic targets in pathogenic bacteria. To evaluate *Cronobacter*, Alzahrani et al. identified 18 outer membrane vesicle (OMV)-associated proteins by mass spectrometry in *C. sakazakii* strain 767 which may function in the activation of cytopathogenic and host cell responses on human intestinal epithelial cells [1].

The type III hemolysin, an integral outer membrane protein with hemolytic activity, is reported to be a potential virulence factor in several pathogens [2, 3]. In our study, all the isolates from plant-based products carried hemolysin genes, and therefore it is suggested that individuals in the food production and food service industries should therefore be adequately informed on the risks of *Cronobacter* contamination to enable the provision of products that are safe for consumption. Earlier, Cruz et al. [5] reported the prevalence of *hly* gene in only *Cronobacter* spp. isolates mainly from clinical sources.

Chitinase is a putative virulence factor, and its expression has proven to be vital not only for nutrient acquisition and environmental survival but also for infection in humans and animals [43]. The *chiF* and BAM (*zpx*) genes were less frequently detected in the isolates. The role of chitinase has been already defined in infections caused by Legionella pneumophila [6], Listeria monocytogenes [24], and Salmonella enterica serovar *Typhimurium* [25]. A bacterial metalloprotease, encoded by *zpx*, is thought to be a cell-bound zinc-containing metalloprotease which may play an important role in enhancing the dissemination of *Cronobacter* sp. into systemic circulation [22, 28]. The fact that only four of the seven isolates were PCR positive for *zpx* indicates that there must be some other mechanism(s) in our isolates which might play a role in the evasion of the host's immune response to promote systemic circulation. The fact that two of these zpx-negative isolates also possess *cpa* suggests that both *zpx* and *cpa* may complement each other's role in systemic circulation.

A plasmid-borne putative gene for the plasminogen activator (*cpa*) was identified in the isolates from plant-based products and environmental samples. Plasminogen activators are serine proteases, proposing that this gene could be engaged in exhibiting the maximum invasiveness of *Cronobacter* spp. Franco et al. showed that *cpa*, expressed by *C. sakazakii* BAA-894, slowly cleaves plasminogen and increases survival in serum in comparison with a *cpa* deletion mutant [10]. The low proteolytic activity of BAA-894 may be due to *cpa* inhibition by BAA-894 smooth lipopolysaccharides. In our study, pESA3/pCTU1 was detected in all the isolates with the presence of *cpa* in 70% of the isolates only. The capacity of iron acquisition is mostly believed to be a requirement for a pathogen to establish infections when entering a host [4]. Franco et al. demonstrated that *C. sakazakii* contains two iron acquisition systems (*eitCBAD* and *iucABCD*/*iutA*) [9]. The gene *eitA* was mostly present in isolates from plant-based products. It was observed that all the *C. sakazakii* strains lack genes namely *sip, fhaB*. The isolate N81 was negative for *eitA*, whereas it was capable of producing iron siderophores with CAS agar demonstrating orange halo around the wells which might be ascribed to the presence of *iucABCD* gene cluster. However, iron depletion assays were not performed with CAS-negative strains.

Adhesion and invasion to the host cell is a property that is associated with bacterial pathogenesis, especially of intracellular pathogens (e.g., *Salmonella* spp., *S. flexneri*, and *Y. enterocolitica*) and non-intracellular pathogens (e.g., uropathogenic E. *coli*) [31, 32, 42]. Our results indicated that the isolate N81 resulted in a massive disruption of the

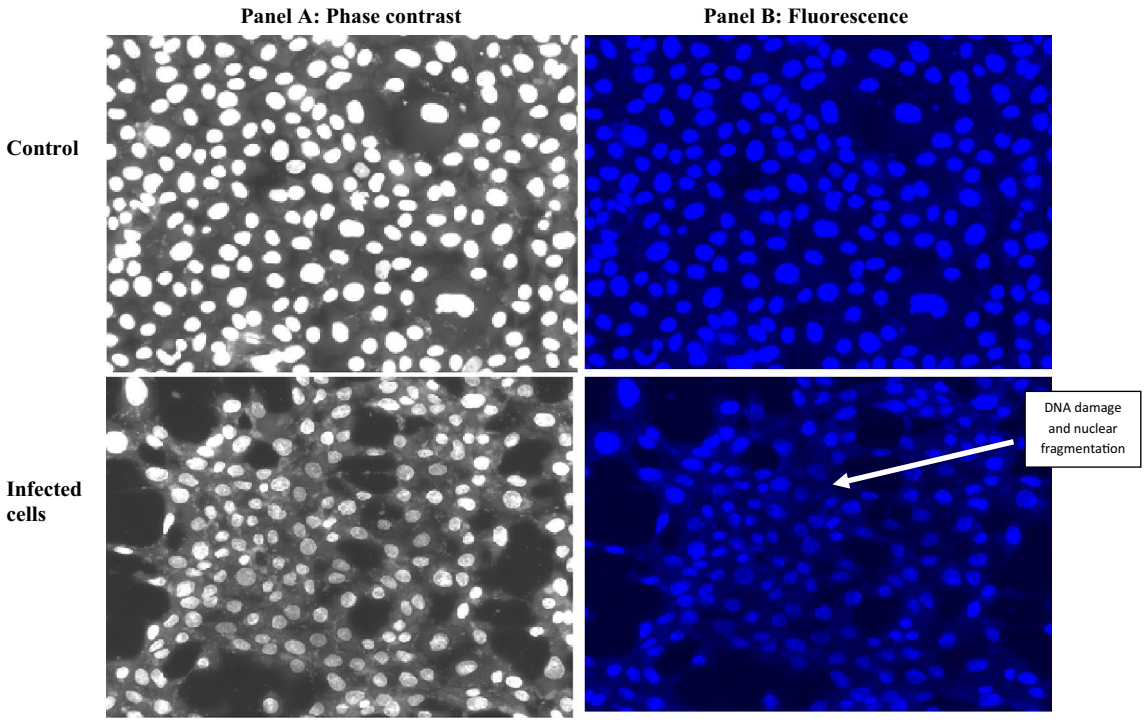

Fig. 1 Cytotoxicity of *C. sakazakii* N81 in Caco-2 cells stained with Hoechst 33,258 at 20X. **A** Phase contrast image and **B** Fluorescent image

host cell with the release of cell contents (Fig. 1). The Hoechst dye 33,258 staining indicated nuclear condensation and fragmentation leading to cell lysis in Caco-2 cells with rounding off of the cells due to the presence of *zpx*.

The present work reports the virulence determinants in C. *sakazakii* strains isolated from different plant-based and environmental sources. Our results revealed the maximum presence of *hly* followed by *ompA* and *eitA* which could be critical in the pathogenesis of enteric infections and in its systemic dissemination. A larger sample size of each source would be necessary to further address the observed differences. These findings may help in explaining the diversity of the virulence traits in *C. sakazakii* which might contribute to the opportunistic nature of this pathogen.

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