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Comparative proteomic analysis of *Cronobacter sakazakii* isolates with different virulences

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ABSTRACT

Cronobacter is a genus of widespread, opportunistic, foodborne pathogens that can result in serious illnesses in at-risk infants because of their immature immunity and high dependence on powdered formula, which is one of the foods most often contaminated by this pathogen. However, limited information is available regarding the pathogenesis and the specific virulence factors of this species. In this study, the virulences of 42 *Cronobacter sakazakii* isolates were analyzed by infecting neonatal SD rats. A comparison of the typing patterns of the isolates enabled groups with close relationships but that exhibited distinct pathogenesis to be identified. Among these groups, 2 strains belonging to the same group but showing distinct virulences were selected, and 2-DE was applied to identify differentially expressed proteins, focusing on virulence-related proteins. A total of 111 protein spots were identified using matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF/TOF-MS), and 89 were successfully identified. Further analysis suggested that at least 11 of these proteins may be involved in the pathogenesis of this pathogen. Real-time PCR was carried out to further confirm the differential expression pattern of the genes, and the results indicated that the mRNA expression levels were consistent with the protein expression levels.

Biological significance: The virulence factors and pathogenesis of *Cronobacter* are largely unknown. In combination with animal toxicological experiments and subtyping results of *C. sakazakii*, comparative proteomics analysis was performed to comprehensively evaluate the differentially expressed proteins of two isolates that exhibited distinct virulence but were closely related. These procedures made it possible to identify the virulence-related of factors of *Cronobacter*. Among the 89 total identified proteins, at least 11 show virulence-related potential. This work provides comprehensive candidates for the further investigation of the pathogenesis of *Cronobacter*.

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1. Introduction

Cronobacter is an emerging genus of Gram-negative opportunistic pathogen that was improperly considered a single species, namely, *Enterobacter sakazakii* [1]. However, with the development of identification techniques, including molecular methods, *Cronobacter* was recognized as a separate genus of the Enterobacteriaceae family in 2008 [2,3]. At least 10 species have been identified in this genus [4].

Cronobacter has been isolated from different food and environments [5,6]. Contamination in infant food, especially in powdered infant formula (PIF), has made this pathogen a great threat to infants due to their immature immunity and intake of PIF as their main food [7,8]. Indeed, commercial PIF has become the most likely source of this pathogen, attracting comprehensive surveillance [9]. However, more than 150 infection cases caused by *Cronobacter* have been recorded, with the typical consequences of meningitis, sepsis and necrotizing enterocolitis (NEC), which are mainly found in neonatal infections [10]. The neonatal infection rates caused by *Cronobacter* remain low (a national FoodNet survey in 2002 in the United States estimated that infection rates caused by invasive *Cronobacter* were 1 per 100,000 infants aged <1 year and 8.7 per 100,000 low-birth-weight infants (<2500 g)), but with a high mortality of 10–42%, which varies depending on the clinical syndrome [11]. Even when infants survive the infection, many of them suffer different sequelae, such as developmental delays, hydrocephaly, and mental retardation [12].

Realizing the great harm associated with *Cronobacter*, many studies explored the mechanism of infection and the virulence factors of this pathogen. Many in vitro studies using human-derived cell lines and in vivo studies using suckling mice or rats have shown that *Cronobacter* can adhere to and invade human intestinal cells, invade the blood/brain barrier, and replicate in macrophages [13–18]. However, little is known about the pathogenesis of *Cronobacter* at the molecular level. As the major virulence marker of microbe pathogenicity, enterotoxin has also attracted active investigation into *Cronobacter*. Pagotto et al. first described putative enterotoxin activity in *Cronobacter* [19], and Raghav and Aggarwal first purified and characterized a putative enterotoxin [20]. Outer membrane protein A (OmpA) is one of the best

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characterized virulence factors and plays an important role in the binding and invasion of endothelial cells [21]. Kim et al. reported that outer membrane protein X (OmpX) was also crucial for *Cronobacter sakazakii* invasion of host cells [22]. After the colonization and invasion of host tissues or cells, *Cronobacter* must exploit some mechanism to avoid the immune system or survive within the host cells. Nevertheless, this mechanism remains incompletely understood. The expression of superoxide dismutase (SODs) and outer membrane protease Cpa (plasminogen activator) was increased after the entry of *Cronobacter* [23,24]. The roles of other factors in the resistance to the immune system are currently known. Type IV secretion systems (T4SSs) and the newly identified VI secretion systems (T6SSs) are considered to be important virulence factors [25,26]. However, their functions remain unclear. Although iron acquisition and sialic acid utilization systems have been proposed to have great clinical relevance [27,28], their extensive existence in *C. sakazakii* (98% for iron acquisition systems and 96% for sialic acid utilization systems) seems to contradict the varied virulences of different isolates of the species. Genome sequencing has provided promising data on putative virulence gene identification through similarity analysis with reported pathogenic factors in other bacteria [29]. However, most of the genes identified require further experimental verification. Although many efforts have been made to explore the pathogenesis of *Cronobacter*, in comparison with other microbial pathogens, knowledge is limited for this pathogen.

To further investigate the virulence factors of *Cronobacter*, especially those determining variations in virulence, proteomic techniques were employed to compare the different protein expression patterns of two *C. sakazakii* isolates that are closely related but have distinct infective abilities.

2. Materials and methods

2.1. Bacterial strains

There were 42 isolates of *C. sakazakii* in this study, including 3 strains (ATCC29544, ATCC29004 and ATCC12868) from the American Type Culture Collection (ATCC), 2 clinical isolates and 37 food isolates (Table 1). Two clinical isolates were from the Czech Collection of Microorganisms (CCM). The food isolates were collected from different Entry–Exit Inspection and Quarantine Bureaus of China from 2005 to 2010 and were originally identified as isolates of the *Cronobacter* genus using the API 20E, VITEK or Biolog systems. All of the isolates were further identified as *C. sakazakii* through 16S rRNA sequencing and phenotypic tests.

2.2. Animals

Timed-pregnant SD rats were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were placed in clean individual cages (disinfected before using). The rats received drinking water and rodent food and were acclimatized and permitted to give birth naturally. Neonatal rats (48 h old) were separated from their mothers immediately prior to testing (approximately 2–4 h) and were randomly divided into groups. Each group contained at least 3 male and 3 female neonatal rats.

2.3. Preparation of inocula

The *C. sakazakii* strains were stored at −80 °C and then grown on LB agar plates for 24 h at 37 °C. A single colony was inoculated into 5 mL liquid LB broth and cultured overnight at 37 °C with shaking. Overnight cultures were transferred to fresh culture medium (diluted by 1:100) and grown to an OD_{600} of between 0.5–0.8. The bacteria were centrifuged at 5000 ×*g* for 5 min and then washed twice with sterile deionized water. *C. sakazakii* populations were resuspended and serially diluted in sterile powdered infant formula to a concentration of 5×10^6 cells/mL for gavage.

Table 1
C. sakazakii strains involved in this study.

No.	Strain	Origin/source
1	IQCC 10409	Shrimp/China[a]
2	IQCC 10410	Milk powder/China[a]
3	IQCC 10419	Biscuit/China[b]
4	IQCC 10423	Milk power/China[c]
5	IQCC 10449	Soybean/China[d]
6	IQCC 10455	Onion ring/China[e]
7	IQCC 10458	Shrimp crackers/China[e]
8	IQCC 10459	Chocolate/China[e]
9	IQCC10440	Milk powder/China[f]
10	IQCC10442	Unknown/China[g]
11	IQCC10448	Milk powder/China[d]
12	IQCC10451	Taro milk bar/China[f]
13	IQCC10486	Unknown/China[b]
14	IQCC10487	Export starch/China[b]
15	ATCC29544	ATCC[h]
16	90310	Unknown/laboratory
17	90505	Unknown/laboratory
18	6607	Unknown/laboratory
19	ATCC29004	ATCC[h]
20	ENS5329	Milk powder/Ukraine
21	ENS6309-4	Skimmed milk powder/Indian
22	ENS51024-2	Milk powder/Indian
23	ENS51227	Milk powder/New Zealand
24	ENS51229	Milk powder/Australia
25	ENS6106	Milk powder/India
26	ENS6124-2	Milk powder/Holland
27	ENS6607	Milk powder/Ireland
28	ATCC12868	ATCC[h]
29	ENS70101	Milk powder/America
30	ENS70115	Milk powder/France
31	ENS70307-1	Milk powder/China
32	ENS70307-2	Milk powder/China
33	ENS70307-3	Milk powder/China
34	ENS70307-4	Milk powder/Poland
35	ENS6309-1	Skimmed milk powder/India
36	ENS6309-2	Skimmed milk powder/India
37	ENS6309-3	Skimmed milk powder/India
38	ENS6124-1	Whey powder/Holland
39	ENS70216	Milk powder/China[i]
40	ENS07208	Whey powder/India
41	CCM3460	Foot wound/CCM[j]
42	CCM3479	Bronchial secretion[j]

[a] Isolates from food, Liaoning Entry–Exit Inspection and Quarantine Bureau, China.
[b] Isolates from food, Xinjiang Entry–Exit Inspection and Quarantine Bureau, China.
[c] Isolates from food, Tianjin Entry–Exit Inspection and Quarantine Bureau, China.
[d] Isolates from food, Neimenggu Entry–Exit Inspection and Quarantine Bureau, China.
[e] Isolates from food, Shenyang Entry–Exit Inspection and Quarantine Bureau, China.
[f] Isolates from food, Guangdong Entry–Exit Inspection and Quarantine Bureau, China.
[g] Isolates from food, Hunan Entry–Exit Inspection and Quarantine Bureau, China.
[h] American Type Culture Collection (ATCC), USA.
[i] Isolates from food, Jiangxi Entry–Exit Inspection and Quarantine Bureau, China.
[j] Czech Collection of Microorganisms (CCM), Czech Republic.

2.4. Virulence examination of different isolates

Each pup was orally administered with 0.2 mL of reconstituted powdered infant formula containing 1×10^6 bacterial cells using a stainless steel animal feeding needle. The controls received 0.2 mL sterile powdered infant formula only. The pups were observed for symptoms and mortality twice daily during the post-challenge period. After 48 h, all of the pups were euthanized. The abdomens of the mice were opened, and their entire intestinal tracts, excluding the stomachs, were removed with sterile tools; then, the intestinal tract and remaining carcass were weighted, and the weight ratios of the gutted carcass was calculated to evaluate the enterotoxin production of the isolates [30]. Blood and brain samples were excised during necropsy and prepared for

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