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Short communication

Identification of potential virulence factors of *Cronobacter sakazakii* isolates by comparative proteomic analysis

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ABSTRACT

Cronobacter is a group of important foodborne pathogens associated with neonatal meningitis, septicemia, and necrotizing enterocolitis. Among *Cronobacter* species, *Cronobacter sakazakii* is the most common species in terms of isolation frequency. However, the molecular basis involved in virulence differences among *C. sakazakii* isolates is still unknown. In this study, based on the determination of virulence differences of *C. sakazakii* G362 (virulent isolate) and L3101 (attenuated isolate) through intraperitoneal injection, histopathologic analysis (small intestine, kidney, and liver) further confirmed virulence differences. Thereafter, the potential virulence factors were determined using two-dimensional electrophoresis (2-DE) coupled with MALDI/TOP/TOF mass spectrometry. Among a total of 36 protein spots showing differential expression (fold change > 1.2), we identified 31 different proteins, of which the expression abundance of 22 was increased in G362. These up-regulated proteins in G362 mainly contained DNA starvation/stationary phase protection protein Dps, OmpA, LuxS, ATP-dependent Clp protease ClpC, and ABC transporter substrate-binding proteins, which might be involved in virulence of *C. sakazakii*. This is the first report to determine the potential virulence factors of *C. sakazakii* isolates at the proteomic levels.

1. Introduction

Cronobacter species are important foodborne pathogens associated with invasive infections (septicemia, meningitis, and necrotizing enterocolitis) through the consumption of contaminated powdered infant formula (Biering et al., 1989; Gurtler et al., 2005; Nazarowec-White and Farber, 1999; van Acker et al., 2001). The virulence differences of *Cronobacter* isolates were firstly studied by Pagotto et al. (2003). Furthermore, Townsend et al. (2007) demonstrated that *Cronobacter* could invade rat capillary endothelial brain cells (rBCEC4) in vitro and the persistence of *Cronobacter* in macrophages varied between *Cronobacter* strains. *Cronobacter* from an outbreak infection in France attached and invaded Caco-2 human epithelial cells and rat brain capillary endothelial cells (Townsend et al., 2008). The outer membrane proteins such as ompA and ompX were required for adhesion or invasion of *Cronobacter* into Caco-2 and INT-407 (Kim and Loessner, 2008; Mittal et al., 2009; Mohan Nair and Venkitanarayanan, 2007; Singamsetty et al., 2008). In addition, *zpx* gene encoding the cell-bound zinc-containing metalloprotease might be important in dissemination of the pathogen into the systemic circulation (Kothary et al., 2007).

To date, *Cronobacter* consists of seven species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter condiment*, and *Cronobacter universalis* (Iversen et al., 2008; Joseph et al., 2012). Kucerova et al. (2010) reported that only strains from *C. sakazakii*, *C. malonaticus*, and *C. turicensis* were associated with infantile infections, and *C. sakazakii* is the most common species in terms of isolation frequency (Muller et al., 2013). *C. sakazakii* clonal complex 4 (CC4) was principally associated with neonatal meningitis using PubMLST database, but no particular virulence traits have been determined in *C. sakazakii* CC4 compared to other sequence types (Forsythe et al., 2014). As the part of LPS of Gram-negative bacteria, structures of O antigens in *C. sakazakii* have been identified (Arbatsky et al., 2010, 2012; Maclean et al., 2009, 2010). Although several genes such as *zpx*, *ompA*, and *ompX*, have been identified to be implicated in invasion or adherence of *Cronobacter*, little focus has been placed on the molecular basis of virulence differences among *C. sakazakii* isolates.

The global techniques such as comparative proteomic analysis have been widely applied in elucidation of molecular basis involved in virulence differences (Cuervo et al., 2008; Donaldson et al., 2011; Mattow

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et al., 2003). Regulatory factors related to virulence might happen post-translationally, so measurement of mRNA levels might give incomplete information. In contrast, the analysis of global changes by a comparative proteomic assay seems reasonable for revealing differentially expressed proteins between virulent and attenuated isolates.

In this study, on the basis of pathological and histologic analyses of virulent isolate (G362) and attenuated isolate (L3101), we used 2-DE technology coupled with MAIDI/TOF/TOF mass spectrometry for comprehensive proteomic analysis and identification of differential expression proteins between G362 and L3101. As a result, we found proteins that might play important roles in virulence differences among *C. sakazakii* isolates, and such information might contribute to revealing molecular basis of its pathogenicity.

2. Materials and methods

2.1. C. sakazakii isolates used in this study

In Table 1, virulence differences among 31 *C. sakazakii* isolates from food samples (dry fungus and frozen food) obtained by the ISO method combined with PCR assay targeting *rpoB* (Stoop et al., 2009) were determined through intraperitoneal injection (1.2×10^6 cfu/mouse) into one week-old mice from the animal experimental center of Anhui Provincial Hospital.

Table 1
The animal toxicity test in vivo of 31 *C. sakazakii* isolates.[a][b]

C. sakazakii isolates	Time of death after injected infection (h)														
	6	10	14	18	22	26	30	34	38	42	46	50	54	60	64
L[b]2101	2	1	1	0	0	0	1	0	0	0	0	0	0	0	0
G[b]121	1	1	0	2	0	1	0	0	0	0	0	0	0	0	0
G132	0	0	4	0	1	0	0	0	0	0	0	0	0	0	0
G371	2	0	1	1	0	0	0	1	0	0	0	0	0	0	0
G581	1	2	2	0	0	0	0	0	0	0	0	0	0	0	0
G441	0	0	1	1	0	0	1	1	0	0	0	1	0	0	0
G362	2	1	2	0	0	0	0	0	0	0	0	0	0	0	0
L073	0	1	2	0	2	0	0	0	0	0	0	0	0	0	0
L381	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0
L481	0	0		3	0	0	1	0	0	0	1	0	0	0	0
L3101	0	0	0	0	0	0	1	0	0	0	0	1	1	0	2
G051	0	0	2	0	0	0	1	0	1	0	1	0	0	0	0
G142	1	3	0	1	0	0	0	0	0	0	0	0	0	0	0
G271	0	0	1	0	0	1	3	0	0	0	0	0	0	0	0
G351	2	1	1	0	1	0	0	0	0	0	0	0	0	0	0
G282	1	1	2	1	0	0	0	0	0	0	0	0	0	0	0
G312	1	1	1	0	1	1	0	0	0	0	0	0	0	0	0
G151	0	1	0	2	0	1	0	0	0	0	0	0	0	0	0
L982	2	1	1	0	0	0	0	0	0	0	0	0	0	0	0
L114	0	0	0	1	0	2	2	0	0	0	0	0	0	0	0
G481	1	0	2	1	0	0	1	0	0	0	0	0	0	0	0
G381	2	0	2	0	0	0	1	0	0	0	0	0	0	0	0
L781	0	2	1	1	0	0	0	1	0	0	0	0	0	0	0
G071	0	2	1	1	0	0	0	0	0	0	0	0	0	0	0
L681	0	1	2	2	0	0	0	0	0	0	0	0	0	0	0
G032	1	0	2	2	0	0	0	0	0	0	0	0	0	0	0
L541	0	0	1	2	0	0	0	0	1	0	0	0	0	1	0
L492	0	1	0	0	0	1	0	0	0	1	0	0	0	1	1
G084	0	1	2	1	0	0	0	0	1	0	0	0	0	0	0
L5101	0	0	0	2	0	0	0	1	0	0	1	0	0	0	1
L173	1	3	0	0	0	0	1	0	0	0	0	0	0	0	0

[a] The experiments in vivo were carried out by intraperitoneal injection. *C. sakazakii* isolates were inoculated into LB and incubated at 37 °C for 14 h. Then, bacterial liquid with different concentrations was made by ten-fold and each mouse was injected into 1.2×10^6 cfu/mouse by intraperitoneal injection. We observed the death of mice and take record. The speed to death was considered as the index to judge the virulence of isolates. The biggest speed means the strongest virulence.

[b] G: isolates from dry fungus samples; L: isolates from frozen food samples.

2.2. Analysis of slices of tissues (small intestine, kidney, and liver)

C. sakazakii isolate (100 μl) G362 and L3101 (with 3.5×10^8 cfu/ml) was injected into one week-old mice via oral route of infection for every two days. After fifteen days, the mice were killed after ether treatment for collection of small intestine, kidney, and liver samples. Then, the tissues were fixed in 10% buffered formalin (Sangon, Shanghai), then routinely dehydrated by ethanol (Sangon, Shanghai) with different concentrations (*v*/*v*), and thereafter embedded by paraffin (Sangon, Shanghai). Tissues sections of 4–5 μm were cut and stained with hematoxylin and eosin (HE, Sangon, Shanghai). Histological analysis was performed by one pathologist who was blind to the treatment. The corresponding samples from the mouse treated with normal saline were analyzed as negative controls.

2.3. Preparation of whole cell proteins of C. sakazakii isolates

Two *C. sakazakii* isolates were inoculated into Luria-Bertani (LB, Haibo, Qingdao) at 37 °C for 16 h. The whole cell proteins were extracted from 1.0 g wet cells using protein extraction kit 786–258 according to the manufacturer's instructions (G-Biosciences, St. Louis, MO, USA) and the extracted proteins were treated using Perfect-FOCUS™ (G-Biosciences, St. Louis, MO, USA). The concentration of proteins was determined using non-interference protein concentration determination kit SK3071 (Sangon, Shanghai).

2.4. 2-DE conditions

For IEF, the protein samples (800 μg) were mixed with rehydration solution (with IPG buffer pH 3–10). The Immobiline™ DryStrip IPG strips (13 cm, pH 3–10) were rehydrated at 30 V for 12 h and continuously focused for 1 h at 500 V, 1 h at 1000 V, 8 h at 8000 V, 4 h at 500 V at 20 °C under mineral oil. Then, the IPG strips were incubated for 20 min in 2D Equilibration Buffer (SD6030 with 1% DTT, Sangon, Shanghai) followed by 2D Equilibration Buffer (SD6030 with 2.5% IAA, Sangon, Shanghai)for 20 min. After the equilibration steps, the strips were transferred to 13 cm × 13 cm 12.5% SDS-PAGE gels for the second dimension. Electrophoresis was performed at 30 A at 10 °C for 30 min. Proteins spots were stained with low background Silver Stain Kit (Sangon, Shanghai) according to the manufacturer's instruction.

2.5. Image analysis and statistics

The gels for triplicates were scanned with a densitometric Image Scanner (GE Healthcare) and the raw images were analyzed using the Image Master™ platinum version 7.0 (GE Healthcare). For each comparison (G362 and L3101), the 2-D gels in triplicate were analyzed. To sufficiently screen for virulence factors, proteins with intensities of >1.2 fold and p values <0.05 are considered significant differences between G362 and L3101 and were identified with MAIDI/TOF/TOF mass spectrometry.

2.6. Identification of proteins by MS

To identify differentially expressed proteins, the respective spots were excised from the 2-DE gel and subjected to in-gel digestion as described by Riedel and Lehner (2007). The excised proteins were analyzed on a 4700 proteomics analyzer MALDI-TOF/TOF (Applied Biosystem) as described by Riedel and Lehner (2007).

Protein identities were based on a combination of peptide fingerprint and MS/MS spectra. MS and MS/MS data were searched using MASCOT version 1.9.05 (Matrix Science) as search engine against the non-redundant NCBI database. Global Proteomics Server (GPS) explorer software (Applied Biosystem) was used for submitting data acquired with MALDI-TOF/TOF mass spectrometer for database searching.