Original Article

Comparison of *Cronobacter sakazakii* from Agra region grown in biofilms, agar surface associated and planktonic mode by proteomic analysis

Abstract

Context: *Cronobacter sakazakii* is an emerging food borne pathogen that causes severe meningitis, meningoencephalitis, sepsis, and necrotizing enterocolitis in neonates and infants, with a high fatality rate. **Aims:** The present paper is for the rapid detection of *C. sakazakii* from milk and milk products of Agra region via PCR method and comparison of *C. sakazakii* in biofilm, on agar surface and planktonic cells by proteomic analysis. **Materials and Methods:** In the present study, 55 samples of milk and milk products of the Agra region were analyzed. 200 isolates were obtained of which 11 were biochemically detected as *C. sakazakii*. The PCR targeting the *ompA* gene was used to amplify a 496 bp DNA segment unique to *C. sakazakii*, in order to confirm *C. sakazakii* isolates. The proteome was investigated to study the differential protein pattern expressed by biofilm, agar surface-associated and planktonic bacteria employing SDS–PAGE. **Statistical Analysis:** UN-SCAN-6.1 gel analysis software. **Results:** The primer pair ESSF and ESSR was successfully used to amplify a 469 bp DNA unique to *C. sakazakii*. Whole cell protein profiles of planktonic, biofilm and agar surface associated were characteristic. **Conclusion:** The cultural procedure for detection of *C. sakazakii* is laborious, taking up to 7 days for completion, whereas PCR combined with enrichment culturing can detect *C. sakazakii* in about 12 hours and thus has the potential to be used as a rapid tool for detecting its presence. Differential protein pattern of *C. sakazakii* cultivated in biofilm versus agar-surface-associated and planktonic cells were observed. Further understanding the role of specific proteins during the biofilm development should permit a better understanding of the mechanisms sustaining the proliferation and the resistance of bacteria on biotic surfaces.

Key words:
Biofilm, Cronobacter sakazakii, planktonic, proteomic analysis

Introduction

Cronobacter sakazakii, a popular contaminant of powder infant formula milk[1] is a reason of causing necrotizing enterocolitis,[2] bacteremia,[3] and a rare form of infant meningitis.[4,5] The gram-negative, rod-shaped bacterium of family Enterobacteriaceae induce signs and symptoms like poor feeding response, jaundice, variation in body temperature, hydrocephalus, developmental delay, and death. Organism is ubiquitous in the water and soil environment[6,7] and is also prevalent in food like cattle and raw cow's milk,[3] infant milk formulae,[6] milk cartons, fermented bread,[8] etc.

Many cases of contaminated powered infant formula (PIF) products by *C. sakazakii* causing severe infections have been reported.[1,2,5] Immunocompromised, premature, and low-birth-weight infants and those aged <28 days are considered to be more at risk than older infants.[4,9,10] Moreover, many cases of infection by *C. sakazakii* have been reported in neonatal intensive care units around the world.[2,10,11] Mortality rates vary from 40% to 80%[2,12] and meningitis survivors suffer severe neurological sequelae

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such as hydrocephalus, quadriplegia, and retarded neural development.

C. sakazakii is found to be resistant to certain antibiotics like vancomycin, penicillin, oxacillin, and lincosamides.[13] Generally, bacterial biofilm are composed of microbial communities attached to surfaces and embedded in an extracellular polymeric matrix. Like other bacteria, biofilm formation of *C. sakazakii* has known to contribute to its persistence on food surfaces.[14] For most bacteria including *C. sakazakii*, limited information is available about the specific properties that are expressed in biofilm. A growing number of works are published on the change in gene expression during the initial phase of biofilm development, but information concerning the properties of mature biofilm is lacking. In the present work, mature biofilm of *C. sakazakii* planktonic culture and agar-surface-associated cells of the same age were studied by a proteomic analysis using SDS–PAGE in order to investigate the induction or repression of individual proteins which could characterize the biofilm, planktonic, and agar-surface-associated phenotypes.

Materials and Methods

Collection of samples

Four different region of Agra, i.e. Sadar Bazaar (southern region), Dayalbagh (northern region), Taj Nagri (eastern region), Arjun Nagar (western region), were included in the study. For this, milk samples were collected from cow, goat, and buffalo udder under an aseptic condition. Moreover, food product samples like cheese, butter, and curd were also taken from local vendors. A total of 55 different milk and milk product samples were collected from different areas of Agra which included 15 samples each of buffalo, cow, goat raw milk, and 10 samples of milk products.

Isolation of bacterial strains

About 0.5 g or 0.5 ml of a sample was added to 4.5 ml of Enterobacter Enrichment Broth (EE Broth) in the ratio of 1:9 and incubated overnight at 37°C. Culture from EE broth was streaked on VRBGA media (Violet Red Bile Glucose Agar) and the obtained pink color colonies obtained were examined microscopically. These typical colonies were further streaked on TSA plates (Tryptic Soya Agar) and incubated for 24 h at 37°C. Characteristically yellow color colonies were picked up and tested biochemically for *C. sakazakii*. *C. sakazakii* MTCC-2958 used as positive control was obtained from Institute of Microbial Technology, Chandigarh.

Identification of bacteria using biochemical tests

Viability tests for each organism were carried out by reviving the organism in nutrient agar using serial dilution methods. They were also confirmed by carrying out gram staining procedures. *C. sakazakii* is a Gram-negative rod, glucose fermenter appearing pink to purple colonies on VRBGA media. It is indole, Dnase, oxidase negative and citrate, VP, nitrate, catalase positive, and does not produce H_2S.

Identification of bacteria using molecular methods

Preparation of Genomic DNA—*C. sakazakii* MTCC-2958 and 11 isolates were cultured in 3 ml of EE broth for 24 hours at 370°C. 1.5 ml culture was transferred to a microcentrifuge tube, boiled at 1000°C in a heating block for 10 minutes and centrifuged at 1500 g for 30 seconds. Primers ESSF and ESSR[15] were used to amplify a 469-bp fragment of the *ompA* gene specific to *C. sakazakii*. The PCR mix consisted of 1 × Gene Amp PCR buffer II (50 mM potassium chloride and 10 mM Tris–HCl, pH 8.3). 2.5 mM $MgCl_2$, 200 µM (each) dNTP, 1 µM (each) primer, 1 U of Taq DNA polymerase, 50 ng of template DNA, and sterile deionized water to make the volume up to 50 µl. The samples were subjected to PCR cycles consisting of denaturation at 94°C for 15 seconds, 600 C for 15 seconds, and 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. A 5-µl aliquot of the amplified product was characterized on a 1.5% agarose gel. The amplicons were detected by staining with ethidium bromide (0.5 µg/ml) and were photographed under a UV transilluminator.

Preparation of planktonic, agar-surface-associated and biofilm growth

For planktonic growth, EE broth was inoculated with bacterial suspension standardized to 8 McFarland and cultivated for 24 hours at 37°C. For agar-surface-associated growth, culture were streaked on TSA plates and incubated at 37°C for 24 hours. Plate grown cells were harvested using a wire loop and suspended in EE broth (pH 7). For biofilm growth, broth was inoculated with bacterial suspension and cultivate for 24 hours at 37°C. The supernatant EE broth was decanted. Minor planktonic cells attached were gently washed twice with PBS buffer. The adhering biofilm growth was detached after addition of PBS buffer by vigorous shaking. Equal planktonic, agar-surface-associated and biofilm growth in 1.5 ml EE broth standardized to 2×10^8 CFU/ml was used for extraction of whole cell protein.

Extraction of whole cell protein

Whole cell protein was extracted following the procedure of Du Toit *et al*.[16] 1.5 ml broth from the 24 hours culture was withdrawn and centrifuged at 5600 rpm for 7 minutes. Pellet was washed two times with PBS buffer (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4, 0.8% (w/v) NaCl, pH 7.3) and resuspended in 300 µl of TEGL buffer (25 mM Tris, 10 mM EDTA pH 8, 0.9% glucose (w/v), 10 mg/ml lysozyme) and incubated at 370°C 3 hours. Subsequently, the pellet was obtained by centrifugation at 5600 rpm for 7 minutes and supernatant was discarded. 200 µl of sample reducing buffer (1 M Tris HCl, pH = 6.8, 10% SDS, 20% glycerol, and 0.005% Bromophenol blue) and 0.5 ml of β-mercaptoethanol were added to the preparations and boiled immediately in water bath at 100°C for 5 minutes. After boiling, the samples were immediately cooled and stored at –20°C for further use.

Preparation of SDS–PAGE

For electrophoresis 12% separating gel and 6.5% resolving gel were prepared. A volume of 30 µl of each sample was loaded on gel and was run on mini gel electrophoresis at 100 V for 2 h and stained in a solution containing 0.1% (by mass per volume) Coomassie blue, 10% (by volume) acetic acid and 40% (by volume) methanol. Destaining was performed in a 7% acetic acid solution. Gel was analyzed by using UN-SCAN-6.1 gel analysis software. The protein molecular mass marker (14.3 to 100 kDa; SIGMA, USA) was used as standard.

Results

Out of 55 samples of milk and milk products, 200 bacterial isolates were obtained. Eleven out of 200 isolates were found to be *C. sakazakii* after biochemical characterization. The primer pair ESSF and ESSR was successfully used to amplify a 469 bp DNA unique to *C. sakazakii* to confirm these 11 isolates. PCR results of representative five isolates are shown in Figure 1. Thus, all biochemically confirmed isolates correlated to the PCR amplification of *C. sakazakii* and these 11 (5.5%) out of 200 isolates were confirmed to be *C. sakazakii* [Figure 2].

Although whole cell protein profile of planktonic, biofilm, and agar-surface-associated cells displayed an overall similarity for each isolate, distinct differences in the presence/absence of protein bands were observed [Figure 3]. Table 1 shows the whole cell protein profile of planktonic (P), biofilm (B), and agar-surface (AS) associated cells of *C. sakazakii* isolates Jal 1 and Jal 2. Whole cell protein profiles of planktonic, biofilm, and agar-surface-associated cells were characteristic [Table 1]. Distinct protein bands expressed in biofilm phase include bands of corresponding to 78.65 kDa, 58.18 kDa, 44.34 kDa molecular weights. Prominent proteins uniquely expressed in agar-surface-associated phase include bands of 65 kDa and 57.36 kDa. Distinct proteins expressed in planktonic phase include bands corresponding to 59.21 kDa and to 43.62 kDa molecular weights.

Discussion

The cultural procedure for detection of *C. sakazakii* is laborious, taking up to 7 days for completion, whereas PCR combined with enrichment culturing can detect *C. sakazakii* in about 12 hours and thus has the potential to be used as a rapid tool for detecting its presence.

Differential protein expression between planktonic and sessile cells is well documented.[17-20] However, there is much debate about biofilm-specific phenotypes and whether the biofilm phenotype can be defined by a specific trend in the proteome of biofilm bacterial cells.[21-23] In the present study, differences

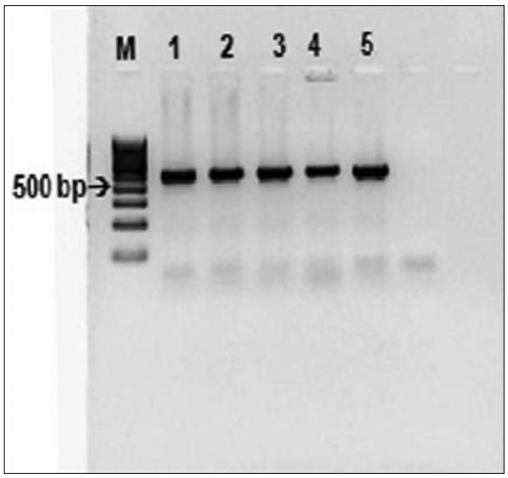

Figure 1: Detection of *C. sakazakii* by PCR amplification of the ompA gene. Lanes M: 100 bp ladder, 1: PCR *C. sakazakii* MTCC -2958 DNA, Lane 2 to 5: *C. sakazakii* isolates

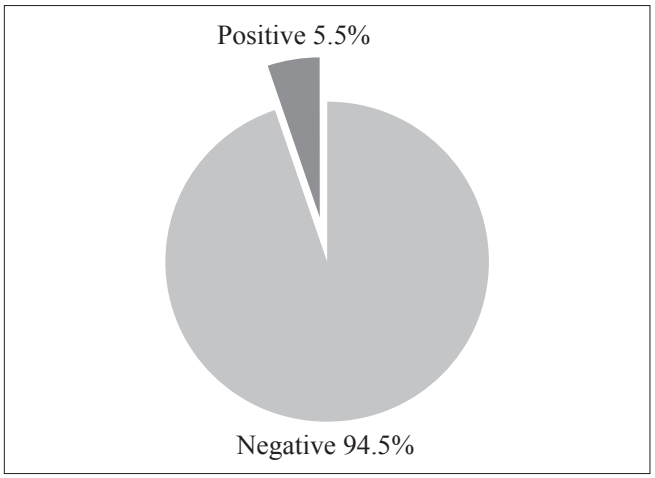

Figure 2: Percentage of isolates identified as *C. sakazakii*

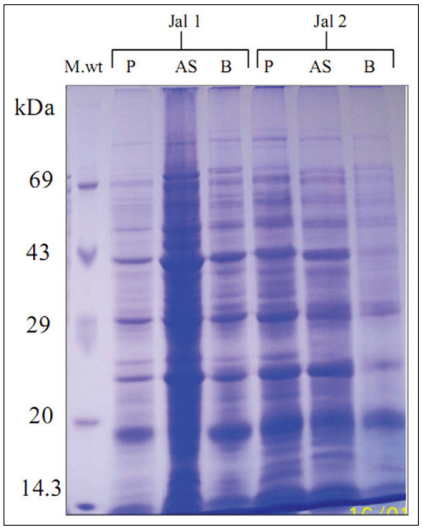

Figure 3: Electrophoregram displaying whole cell protein profiles of planktonic (P), agar surface-associated (AS) and biofilm (B) of *Cronobactersakazakii* isolates

Table 1: Whole cell protein profile of planktonic, biofilm, and agar-surface-associated cells of *C. sakazakii* isolates Jal 1 and Jal 2

Isolate growth phase	Jal 1			Jal 2		
	P	AS	B	*P*	AS	B
No. of major bands	13	14	14	13	14	14
Sizes (kDa)	82	82	82	82	82	82
			78.65			78.65
	80.5	80.5	80.5	80.5	80.5	80.5
	78	78	78	78	78	78
	74	74	74	74	74	74
		65.57			65.57	
	59.21			59.21		
			58.18			58.18
		57.36			57.36	
	51.07	50.29	51.8	51.07	50.29	51.8
	43.62		44.34	43.62		44.34
		39.69			39.69	
	27.11	27.11	27.11	27.11	27.11	27.11
	28.62	28.62	28.62	28.62	28.62	28.62
	24.73	24.73	24.73	24.73	24.73	24.73
	22.28	22.28	22.28	22.28	22.28	22.28
	19.43	19.43	19.43	19.43	19.43	19.43
	13.66	13.66	13.66	13.66	13.66	13.66

AS – Agar surface-associated

in protein expression between planktonic, biofilm, and agar surface-associated growth of *C. sakazakii* isolates of Agra region were investigated and proteins expressed differentially between the three growth phases and/or uniquely-expressed in each of the three phases were identified. One-dimensional SDS–PAGE whole cell protein profiles obtained for the *C. sakazakii* isolates exhibit specific phenotypes for planktonic, biofilm, and agar surface-associated cells. Variation with respect to protein molecular masses was also observed between planktonic, biofilm, and agar surface-associated whole cell protein profiles of respective isolates following visual analysis [Table 1].

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