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Biochemical and genetic characteristics of *Cronobacter sakazakii* biofilm formation

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Abstract

Cronobacter sakazakii is a wide-spread opportunistic foodborne pathogen that can form biofilms on a number of different substances, creating food safety risk. However, there is little information about biofilm characteristics for this species. In this study, biofilm formation of 14 foodborne *C. sakazakii* strains was examined. Transposon mutants of the strain (IQCC10423), the isolate with the greatest biofilm activity, were prepared. A total of 12 mutants were developed with >40% reduction in biofilm formation ability. Eight of these mutants were successfully sequenced with genes putatively identified for: biofilm formation, fundamental cellular processes, phage tail complete protein and uncertain functional protein. The morphology of the biofilm showed that the wild type strain formed a thick biofilm and mutants formed less extracellular polymeric substances (EPSs). Raman spectroscopy was employed to confirm less biofilm formation by different bacterial mutants and demonstrate a similar chemical composition, but different contents of EPS. Wild type biofilms contained a high level of carotenoids, with the distribution of carotenoids mapped using confocal Raman imaging. We demonstrate that various selective functional genes are responsible for the forming ability of *C. sakazakii* biofilms, which may have the potential to cause risks to food safety.

Keywords: *Cronobacter sakazakii*; Biofilm; Raman spectroscopy

1. Introduction

Cronobacter spp. (formerly *Enterobacter sakazakii*) are opportunistic foodborne pathogens that include 7 species: *Cronobacter sakazakii*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Cronobacter muytjensii*, *Cronobacter dublinensis*, *Cronobacter universalis*, and *Cronobacter condimenti* (Iversen et al., 2008; Joseph et al., 2011). *Cronobacter* spp. can be isolated from the environment (Kandhai et al., 2004) and from a wide variety of foods including milk, cheese, dried foods, meats, vegetables, rice, bread, tea, herbs, spices, powdered infant formula (PIF), and water (Healy et al., 2010). Compared with other Enterobacteriaceae, *Cronobacter* spp. exhibits higher tolerance to dessication and heat stress (Asakura et al., 2007). *Cronobacter* spp. can cause rare but severe diseases, including meningitis, septicemia and necrotizing enterocolitis in neonates and infants (Van Acker et al., 2001). Infant meningitis has an estimated 40–80% mortality (Iversen and Forsythe, 2003). *Cronobacter* spp. can also cause infection in immunocompromised adults (See et al., 2007). A recent comprehensive review by Kucerova et al. (2011) covers the taxonomy, ecology, physiological aspects, virulence and genomics of this microbe.

Cronobacter spp. can form biofilms which are surface-aggregated sessile bacteria embedded in an excreted matrix composed primarily of polysaccharides, proteins and nucleic acids (Costerone et al., 1999; Kolter and Greenberg, 2006). Biofilm formation enhances the resistance of cells to environmental stress and provides protection against drugs and

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disinfectants (Furukawa et al., 2006). In the food industry, biofilms are a major source of bacterial contamination on food contact surfaces (Van Houdt and Michiels, 2010), and removal is difficult. Microbes in biofilms can slough off into foods and can be a source of foodborne infection.

Understanding the mechanisms of biofilm formation and maturation would aid in development of strategies and interventions for their control. The biochemistry of adhesion and EPS development within biofilms and during their formation is not well understood for this the genus. Furthermore, greater understanding of outer membrane proteins and polysaccharides is also required (Grimm et al., 2008; Van Houdt and Michiels, 2010). Recent studies have identified genes associated with cellulose and flagella biosynthesis and virulence in *C. sakazakii* using random transposon mutagenesis (Hartmann et al., 2010). However, in general, there is limited information on *Cronobacter* spp. compared to other biofilm-forming pathogens such as *Pseudomonas aeruginosa*, *Salmonella* spp., *Escherichia coli* O157:H7 and *Staphylococcus aureus*. To further investigate the mechanism of biofilm formation of *Cronobacter* spp., we examined the biofilm-forming abilities and characteristics of 14 foodborne *C. sakazakii* isolates and of mutants derived from the strain with strong biofilm-forming ability.

2. Materials and methods

2.1. Bacterial strains

Fourteen isolates of *C. sakazakii* used in this study were selected from strains collected by entry–exit inspection and quarantine bureaus in China from 2005 to 2010 and archived in the China Center of Industrial Culture Collection (CICC) or Inspection and Quarantine Culture Collection (IQCC) of the Chinese Academy of Inspection and Quarantine culture libraries. These isolates were classified as the *Cronobacter* genus using the API 20E, VITEK or Biolog system. 16S rDNA sequencing and 10 phenotypic tests (Voges-Prosauer, methyl red, nitrate reduction, ornithine utilization, motility at 37 °C, acid production from inositol, acid production from dulcitol, indole production, malonate utilization and gas production from glucose) were carried out to identify the strains (Hangzhou Tianhe Microorganism Reagent Co. Ltd, Hangzhou, China) (Table 1).

Table 1
C. sakazakii isolates used in this study.

No.	Strain[a]	Origin/source
1	IQCC 10409	Shrimp/China[b]
2	IQCC 10410	Milk powder/China[b]
3	IQCC 10419	Biscuits/China[c]
4	IQCC 10423	Milk power/China[d]
5	IQCC 10449	Soybean/China[e]
6	IQCC 10455	Onion ring/China[f]
7	IQCC 10458	Shrimp crackers/China[f]
8	IQCC 10459	Chocolate/China[f]
9	IQCC10440	Milk powder/China[g]
10	IQCC10442	Milk powder/China[h]
11	IQCC10448	Milk powder/China[e]
12	IQCC10451	Taro milk bar/China[g]
13	IQCC10486	Chocolate/China[c]
14	IQCC10487	Export starch/China[c]

[a] The isolates are stored at the China Center of Industrial Culture Collection (CICC) or the Inspection and Quarantine Culture Collection (IQCC) of Chinese Academy of Inspection and Quarantine.
[b] Isolates from food, Liaoning Entry–Exit Inspection and Quarantine Bureau, China.
[c] Isolates from food, Xinjiang Entry–Exit Inspection and Quarantine Bureau, China.
[d] Isolates from food, Tianjin Entry–Exit Inspection and Quarantine Bureau, China.
[e] Isolates from food, Neimenggu Entry–Exit Inspection and Quarantine Bureau, China.
[f] Isolates from food, Shenyang Entry–Exit Inspection and Quarantine Bureau, China.
[g] Isolates from food, Guangdong Entry–Exit Inspection and Quarantine Bureau, China.
[h] Isolates from food, Hunan Entry–Exit Inspection and Quarantine Bureau, China.

2.2. Analysis of biofilm formation capacity

A crystal violet staining method was employed to examine biofilm-forming abilities of the 14 *C. sakazakii* isolates (O'Toole and Kolter, 1998) with modifications. The isolates were inoculated into 5 mL LB broth and grown overnight at 37 °C with constant shaking. Overnight cultures were transferred to new culture medium (diluted by 1:100) and grown to OD_{600} between 0.6 and 0.8. Thirty microliters of bacteria in log phase growth were inoculated into 96-well polystyrene plates containing 100 μL fresh LB broth and incubated at 37 °C for 24 h. The plates were rinsed 3 times with deionized water and the adherent bacteria cells were stained with 1% crystal violet for 30 min. After being rinsed 3 times with deionized water, the crystal violet was liberated by ethanol and acetone (3:1) following a 10 min incubation. The OD values of each well were measured at 570 nm. A minimum of triplicate experiments were conducted for each mutant in triplicate.

2.3. Generation of transposon mutants

Isolate IQCC 10423 had the highest biofilm-forming ability and was selected to generate transposon mutants. Overnight cultures of isolate IQCC 10423 were inoculated into 50 mL fresh LB broth and cultivated to exponential phase. Lysozyme was added (10 μg/mL) and culture incubated at 37 °C for 30 min to improve the efficiency of transformation. The bacterial culture was cooled on ice for 30 min and then centrifuged at 1500 × g for 10 min at 4 °C. The pellet was washed with ice-cold sterile water and glycerol (10% V/V), and resuspended in 500 μL glycerol. The Tn5 transposome was electrically transformed to competent cells to construct a mutant library following the protocol for the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicenter, Madison, WI, USA). The mutants were picked from plates and cultured in LB broth and then tested for biofilm-forming ability as described above. Triplicate experiments were conducted for each mutant in triplicate.