Int.J.Curr.Microbiol.App.Sci (2017) *6*(10): 4720-4733

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 6 Number 10 (2017) pp. 4720-4733**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2017.610.438

Characterizing the Rogues and Smooth Pairs of *Cronobacter sakazakii* Involved in Biofilm Formation and Long-Term Survival

Lan Hu* and Sherile K. Curtis

CFSAN, FDA, Laurel, MD 20708, USA
**Corresponding author*

Keywords

Cronobacter, Biofilm, Foodborne pathogens, Rugose phenotype change, Autoaggression, Bacterial resistance.

Article Info

Accepted:
29 September 2017
Available Online:
10 October 2017

A B S T R A C T

Cronobacter species are emerging foodborne pathogens that cause severe sepsis, meningitis, and necrotizing enterocolitis in neonates and infants. Many bacteria use phase variation such as smooth to rugose phenotype change to adapt and survive in stressful environments. In this study we developed two stable smooth and rugose pairs by sequentially streaking a single rugose or smooth colony of *C. sakazakii* on Trypticase Soy Agar at 37°C to understand the roles of the smooth and rugose phase change in *Cronobacter*. The rugose-positive strains produced significantly more biofilms and cell autoaggregation than their rugose-negative counterparts. More bacteria of rugose-positive *C. sakazakii* persisted for five months under desiccated growth environment than their smooth counterparts. Our study showed that *C. sakazakii* did not express curli, but possessed cellulose *bcsABC* genes in both rugose and smooth pairs. The data show that the rugose phenotype of *Cronobacter* allows for more biofilm formation and cell autoaggregation, and the rugose variants could survive much longer than the smooth counterparts in desiccated environment. These results suggest that the smooth to rugose phenotype change plays an important role in *Cronobacter* survival and persistence in harsh environments.

Introduction

Cronobacter species are peritrichous flagella, Gram-negative bacilli, and emerging opportunistic foodborne pathogens (Farmer *et al.*, 1980; Iversen *et al.*, 2008; Jarada *et al.*, 2014). Although the epidemiology and reservoir of *Cronobacter* are still not understood very well, powdered infant formula has been considered as the principal route of transmission among infants (Beuchat *et al.*, 2009; Himelright *et al.*, 2002; Hunter and Bean, 2013; Tall *et al.*, 2015). *Cronobacter* also cause urinary tract infections, septicemia, and catheter-associated wound infections in people of all ages, especially in the elderly and those with other diseases or immune-compromised individuals (Joseph *et al.*, 2012). *Cronobacter* can survive in dried food, fresh produces, and fruit juices (Lin and Beuchat, 2007; Gurtler and Beuchat, 2007; Tall *et al.*, 2015).

In response to environmental stresses, many pathogens undergo phase variation between rugose (also known as wrinkled or rdar) and smooth colony morphology. Although both variants of *Vibrio* and *Salmonella* are pathogenic and are found both in *vivo* and in *vitro*, the rugose phase variants form

corrugated colonies, exhibit increased resistances to acids, low temperatures, osmotic pressure and oxidative stresses, and form more biofilms (Anriany *et al.,* 2001; Beyhan *et al.,* 2007; de Rezende *et al.,* 2005; Hollenback *et al.,* 2014; Morris *et al.,* 1996; Rice *et al.,* 1992; Wai *et al.,* 1998; Yildiz and Schoolnik 1999; Yildiz *et al.,* 2004). In *Vibrio* species, more surface polysaccharide (VPS) are produced in the rugose variants.

The increase is mediated by genes located in the VpsI (VpsA-K) and VpsII (VpsL-Q) loci (Yildiz and Schoolnik 1999). A microarray study of *V. cholerae* shows that smooth and rugose phase variation are directed by complex regulatory genes to raise the resistance of the organisms (Yildiz *et al.,* 2004). A rugose (R) variant of *V. vulnificus* produces a large number of extracellular polysaccharides (EPS) matrix and form biofilms (Grau *et al.,* 2005), while a smooth variant forms less biofilms. The rugose colony morphology and biofilm formation of *S. typhimurium* are associated with extracellular substances including curli, cellulose, LPS, EPS, and capsules, which are important in the bacterial resistances (Anriany *et al.,* 2001; de Rezende *et al.,* 2005).

Biofilm consists of surface attached microbial communities (single species or several species) and extracellular substances. Formation of biofilms on surfaces can be regarded as universal bacterial strategy for bacterial survival and growth (Kaplan, 2010; Hollenback *et al.,* 2014). *C. sakazakii* formed biofilms on stainless steel and enteral feeding tubes have increased their resistances to disinfectants (Beuchat *et al.,* 2009), and the biofilms also facilitated survival and persistence of the bacteria in food and food manufacturing environments (Breeuwer *et al.,* 2003).

Currently, very little information is available on the phase variation involving in *Cronobacter* and their pathogenic mechanisms. Rugosity may play an important role in *Cronobacter* survival and persistence in food and food processing environments. In this study, we tried to determine the possible advantages of rugose state in *Cronobacter*. The rugose and smooth pairs of *C. sakazakii* were compared in biofilm formation, cell autoaggregation, and long-term survival.

Materials and Methods

Bacterial growth conditions

C. sakazakii strain 713 and 632 were identified by using species-specific *rpoB* and *cgcA* assays described by Stoop *et al.,* (2009), Lehner *et al.,* (2012), and Carter *et al.,* (2013). *Cronobacter* strains were stored at -80 C in Trypticase Soy Broth (TSB) (BBL, Cockeysville, MD) supplemented with 1% NaCl (TSBS) and 50% glycerol. Frozen bacterial cultures were streaked onto Trypticase Soy Agar (TSA), Luria-Bertani (LB, Bacto) agar, or LB without salt (LBNS) agar plates or inoculated into tubes contained TSBS or LB broth, and incubated overnight at 37 C or 28 C.

Congo red and calcofluor binding assays

These assays were applied to check the expression of rugose morphotypes and cellulose production (Chapman *et al.,* 2002; Hu *et al.,* 2015). In brief, bacteria were cultured on LB agar overnight at 37 C, and inoculated onto LBNS agar plates supplemented with 40 µg/ml Congo red (Sigma, St. Louis, MO) and 20 µg/ml Coomassie brilliant blue (Sigma), or 200 µg/ml fluorescence brightener 28 (Sigma). The inoculated agar plates were incubated at 28 C for 24 h, and then moved to room temperature, and evaluated at 24 h, 48 h, and 72 h. Cellulose production was judged by viewing the florescence of the colonies under a 366 nm UV light. All isolates were screened

in duplicates in each assay and repeated the assay three times. The images of colonies were taken by a Bio-Rad digital camera.

Biofilm formation assay

Detection and quantification of biofilm were performed with glass tubes (11x75 mm) using the procedure of Hu *et al.,* (2015) with slight modification. These strains were incubated in TSB at 37 C or 28 C, shaking at 150 rpm for 48 h. Biofilm formation in the tubes (pellicles at the air-liquid interfaces) was visually tested. The tubes were washed twice with PBS, air-dried for 30 min, stained with 0.1% crystal violet at room temperature for 30 min, and washed twice with distilled water. The crystal violet bound to the biofilm was dissolved with 5 ml of 95% ethanol for 30 min by shaking, 100 µl per well was added to a 96-well plate (three wells for each sample), and absorbance was determined at 570 nm by a UV max microplate reader (Molecular Devices, Corp., Sunnyvale, CA). The biofilm assay was carried out in duplicates, and three independent experiments were performed for each strain. Results are presented as the mean ± SD.

Cell autoaggregation

To determine cell aggregation, the strains grown overnight were inoculated into 5 ml LB broth in a small glass tube with shaking at 37 C or 28 C for 48 h, then left standing at room temperature for 1 h. Cell aggregation was determined by visual estimation of the cell sediment at the bottom of the glass tubes (Gualdi *et al.,* 2008). The assay was carried out at least three independent experiments.

Long-term survival assay

The assay was done following the method of Vestby *et al.,* (2009) with slight modification. Ten microliter overnight cultures of *C. Sakazakii* strain 713 and 632 were inoculated in 50 ml sterile centrifuge tubes. Each tube contained 20 ml LB broth and an autoclaved microscope slide, and all tubes was incubated with shaking at 37°C or 28°C. Two tubes without added bacteria served as negative controls. During incubation, biofilms were allowed to form on both sides of the slides. After 48 h, the slides were washed twice in sterile saline, moved to empty sterile centrifuge tubes, and air-dried in a sterile Class II Biosafety cabinet hood overnight. The lids of the tubes with slides were loosely tightened, and placed at room temperature for 5 months for detecting bacterial long-term survival. After 5 months, the slides were moved to new sterile tubes with 20 ml PBS, the biofilms on the slides were thoroughly scraped using a sterile cell scraper and were disrupted with sterile glass beads (20-30 nm diameter) by vortexing. The number of viable bacteria remained in each sample was determined by serially diluting the cell mixtures and plated in duplicates onto 5% sheep blood TBS agar plates (Remel, Lenexa, KS) and incubated at 37 C. Data were presented as the mean number of recovered colonies ± SD of triplicate wells from three independent experiments.

Molecular typing

Pulsed-field gel electrophoresis (PFGE) analysis of XbaI-digested genomic DNA was used to examine the genetic relatedness of *Cronobacter* rugose and smooth pairs. High molecular mass DNA in agarose plugs was prepared as reported by Herschleb *et al.,* (2007). The DNA samples were digested with the restriction enzyme Xbal, and the plugs were loaded onto a 1.5% agarose gel in 0.5% TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0). The DNA samples were subjected to separation in a CHEF-Mapper (Bio-Rad) using the flowing parameter: pulse-times, 1-14 s for 5 h, 12-25 s for 10 h, 8-50 s

for 4 h, 6 V cm-1; 120 reorientation angle. The gels were stained with 0.1% ethidium bromide solution, and imaged with a Bio-Rad Gel/Chemo Doc System (Bio-Rad, Hercules, CA).

Metabolic typing by VITEK 2

VITEK® 2 compact system (BioMerieux, Hazelwood, MO) was employed to identify metabolic differences between the *C. sakazakii* rugose and smooth phase variants following the manufacturer's instruction. The bacteria were grown overnight in TSA broth at 37 C with shaking, the colonies of a pure culture were suspended to a turbidity reading of ~0.5, and a Gram negative (GN) card was used.

Motility assay

Bacteria were grown on TSA overnight, and a single colony was inoculated into a motility test medium (Remel), and incubated in 37 C for 24 h. The bacterial motility was confirmed and the images of the bacterial motility were taken by a Bio-Rad digital camera.

Transmission electron microscope (TEM)

Cronobacter were grown overnight on TSA at 37 C. A bacteria suspension in 0.25-0.5% phosphotungstic acid (pH 6.8) containing approximately 10^6 CFU/ml was applied to a formavar-carbon-coated 300 mesh copper grids for 2 min. The liquid was removed by blotting. The samples were air-dried and visualized under a JEOL JEM-1011 electron microscope using an accelerating voltage of 80 KV.

PCR assay

Cronobacter strains were cultured on TSA overnight at 37 C, and single colony was transferred to TSB, and incubated with shaking overnight. The DNA of the *Cronobacter* strains was prepared by boiling cell culture in 100 µl distilled water for 10 min served as DNA templates. Primers for detecting cellulose and flagella genes of *Cronobacte*r were designed from the conservative sequences of *bcsABC, fliC, flgE, and flgK* of *C. sakazakii* strains (Table 1). GoTaq Green Master Mix (Promega Corp., Madison, WI) was used as manufacture's instruction. One µM of each primer and 1 µl bacterial cell lysate (approximately 50 ng DNA) were missed with PCR buffer. Distilled water served as a negative control. All PCR reactions were run in an Applied Biosystem's models 2720 thermal cycler (Applied Biosystems, Foster City, CL). The PCR assay started by 5 min incubation at 94 C, followed by 30 cycles of 30 sec at 94 C, and 30 sec at 55-60 C, and 1-2 min at 72 C, and terminated with a final extension period of 7 min at 72 C. PCR products were separated with 1.5% agarose gels and stained with 0.1% ethidium bromide solution, and imaged with a Bio-Rad Gel/Chemo Doc System.

Statistical analyses

Each experiment was performed in triplicate. The means of the biofilm formation were compared using Student's t-test.

Results and Discussion

Isolation of rugose and smooth phase variants

C. sakazakii 713 and 632 were grown on TSA plates at 37 C. To develop stable, phase variants, serial passages (>20) of colonies from rugose or smooth phase variants were performed by sequentially streaking a single rugose or smooth colony every 3 days onto a fresh TSA plate at 37°C. The stable rugose (rough and dry) colonies were designated as "rugose" (R) while the smooth and wet colonies were named "smooth" (S). The *Cronobacter* colonies were grown on LBNS

supplemented with Congo red and Coommassie blue at 28 C. Figure 1 shows the colony morphotypes of *C. Sakazakii* strain 713 and 632 on LBNS agar supplemented with Congo red and Coomassie blue. Strain 713R and 632R were presented as brown, dry, and rough (Bdar) colonies while the 713S was presented as smooth and light yellow colony (YAS) (Fig. 1). 632S showed smooth and light brown colonies.

Comparing rugose and smooth pairs on biofilm formation and cell aggregation

After incubating in TSB in glass tubes for 48 h at 37 C or 28 C with shaking, the formed biofilms were characterized by the presence of a pronounced ring at the air-liquid interface, and the production of the biofilm was assayed by crystal violet staining method. The quantity of biofilm formation of the strains was slightly different when the strains were grown at varying temperatures (Fig. 2). The biofilm formation of 713R at OD570 was ~1.80 at 37 C (Fig. 2A) and ~1.90 at 28 C (Fig. 2B). The rugose strains produced significantly more biofilms than their smooth counterparts at 37 C and 28 C (all $\rho<0.01$). These results indicated that *Cronobacter* rugose strains expressed greater biofilm formation at both temperatures than the smooth counterparts.

Cell aggregation is the auto-clumping of bacterial cells. *C. sakazakii* 713R and 632R produced large clumps in TSB, leading to rapid bacterial aggregation. Lesser cell aggregates were observed in the 713S and 632S cultures compared to their rugose counterparts (Table 2).

Long-term survival of rugose and smooth pairs in biofilms

After bacterial biofilms formed on the glass slides, the slides were washed, air-dried, and kept in sterile tubes for 5 months at room temperature. Then the biofilm on the slides were thoroughly scraped and the number of viable bacteria remained in each slide was determined by plate counts. Survival of 732R and 632R were significant higher than 713S and 632S ($\rho<0.001$) (Table 3). The results clearly indicated that *Cronobacter* could survive in desiccated biofilm communities for at least 5 months, and the ability of producing large quantities of biofilms by rugose variants provided greater survival and persistence advantages than that of their smooth phase variant counterparts.

PFGE and VITEK typing of rugose and smooth pairs

The genetic typing of strain 713 and 632 was assessed by XbaI-digested genomic DNA using PFGE. The PFGE analysis showed the DNA patterns of the rugose and smooth pairs. There were two distinctly different bands between 713R and 713S in Figure 3. No obvious different patterns were identified between 632R and 632S. The patterns suggested that no remarkable genetic differences were found between the rugose and smooth variants. The difference in strain 713R and 713S may not result from the phase change from rugose to smooth due to no similar common pattern changes between 632R and 632S. The VITEK 2 compact system is a fully automated system that performs bacterial identification by biochemical analysis using colorimetry. All strains were identified as *C. sakazakii* in this typing method. There are three different reactions between strain 713 (GGT+, BXYL-, and GlyA+) and strain 632 (GGT-, BXYL+, and GlyA-), and one reaction differed between 713R (ILATK+) and 713S (ILATK). No common differences were found between two rugose and smooth pairs. To understand if flagella, curli and cellulose cause the phase changes between strain 713R and 713S,

further experiments were carried out.

Detection of motility of *C. sakazakii* 713 and 632

The motility test agar and transmission EM were used to check the bacterial motility and presence of flagella. The tubes with 0.6% TSA agar were incubated at 37 C, and were observed at 24 h, 48 h, and 72 h. Figure 4 I shows the images of motility tubes incubated for 24 h at 37 C. All strains except 713S showed positive motility in the assay. EM observation of negative stained *Cronobacter* strains grown on TSA plates at 37 C revealed flagella in 713R, 632R and 632S, but not in 713S (Fig. 4 II). The result confirmed that the lacking of motility in strain 713S was resulted from no expression of its flagella.

Detection of cellulose *bcsABC* genes and expression in the rugose and smooth pairs

The rugosity of *E. coli* and *Salmonella* species were reported to relate to cellulose and curli of the organisms. PCR assay was employed to detect cellulose *bcsABC* genes in the rugose and smooth pairs**,** and all strains presented *bcsABC* genes (Table 4). The PCR results indicated that cellulose *bcsABC* genes were present in both rugose and smooth strains.

Fig.1 Morphology of *Cronobacter* colonies grown on LB agar supplemented with Congo red and Coomassie brilliant blue for 48 h at 28 C. The image was taken by a digital camera

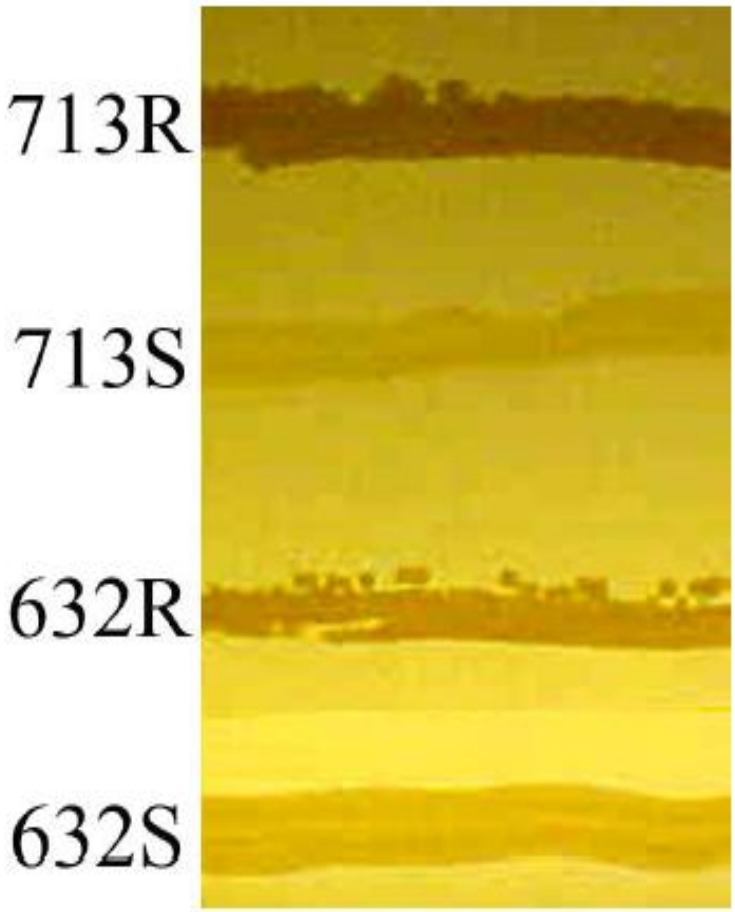


Fig.2 Measuring the biofilm formation of *Cronobacter* strain 713 and 632. Bacteria were cultured in LBNS broth with shaking at 37 C (A) or 28 C (B) for 48 h. The biofilm formation was quantified by crystal violet staining as described in Methods. The values are the means of three independent experiments ± SD

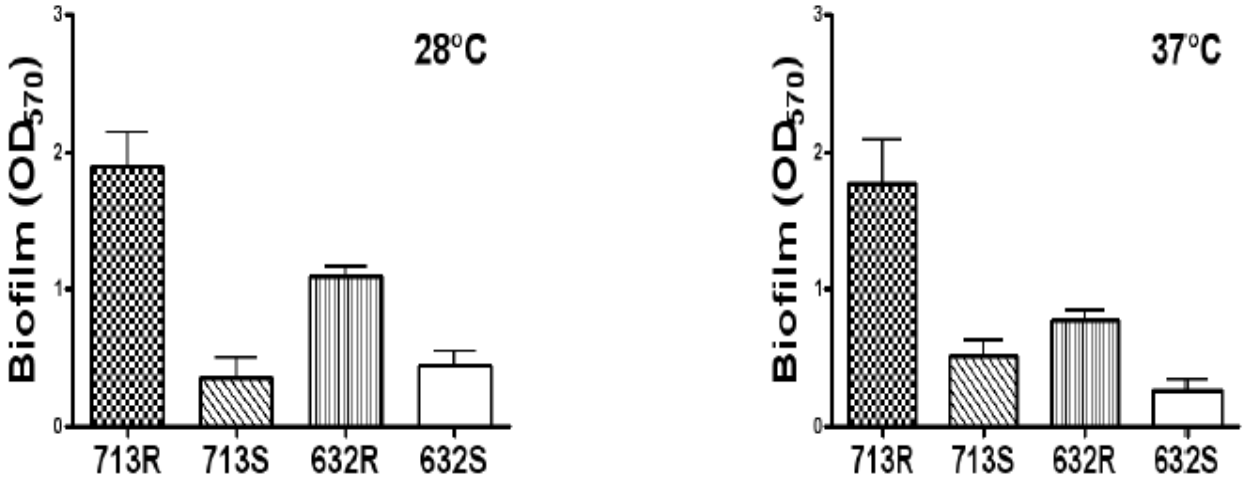
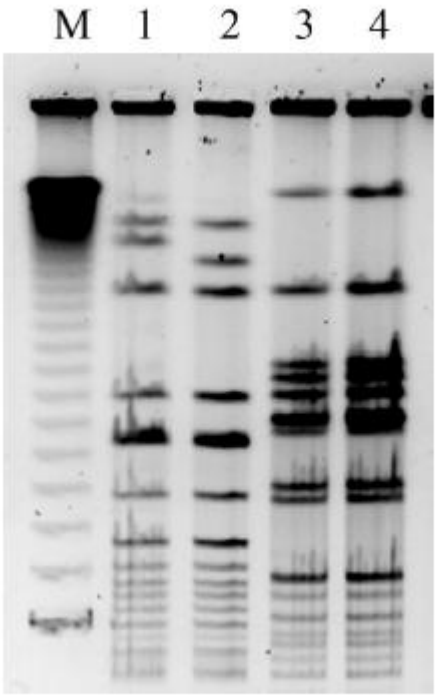


Fig.3 Molecular typing of *C. sakazakii* strains by PFGE. The strain 713 and 632 were digested by XbaI restriction enzyme and separated by a 1.5% agarose gel



(i) 713R, (ii) 713S, (iii) 632R, (iv) 632S

Fig.4 Images of the motility assay and EM. All strains grown overnight were inoculated into TSA motility test tubes or TSA plates, and incubated at 37 C for 24 h. (I) The imagines of motility of *C. sakazakii* 713R, 713R, 632R, and 632S; (II) EM observation of negative-stained *C. sakazakii* strains: A. 713R, B. 713S, C. 632R, D. 632S. All bars are 1.0 um

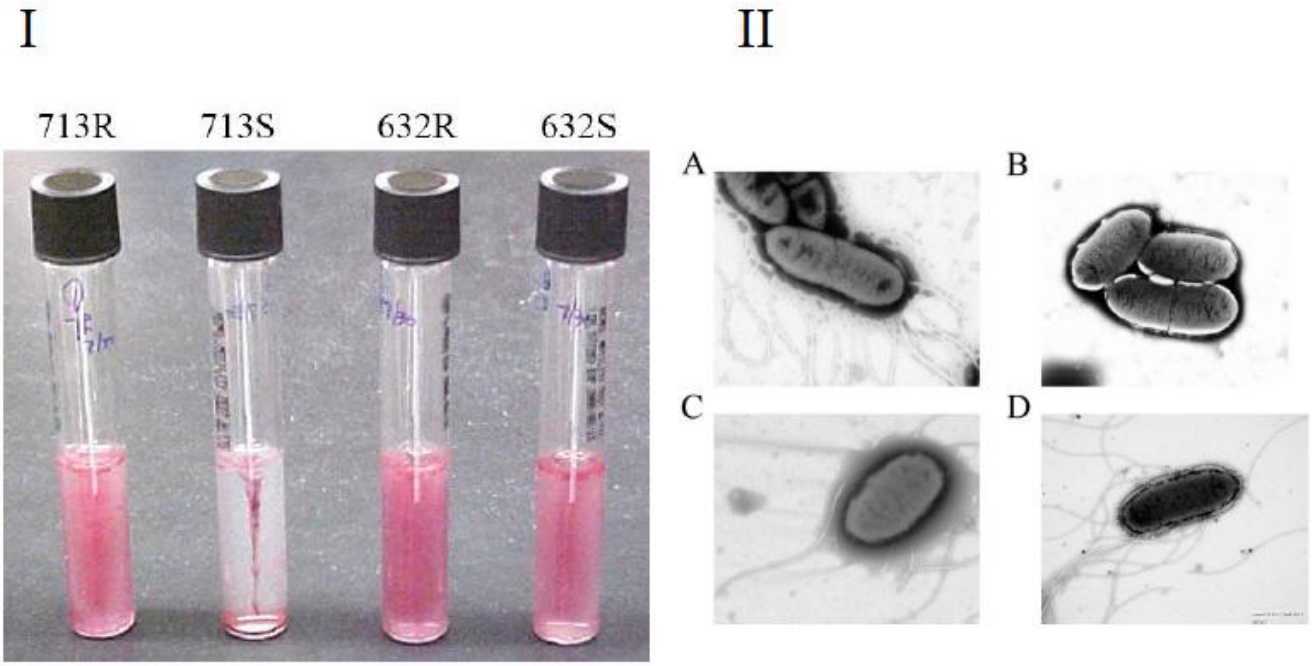


Fig.5 Morphology of *Cronobacter* colonies grown on LB agar supplemented with Calcofluor for 48 h at 28 C. The picture was taken by a digital camera.

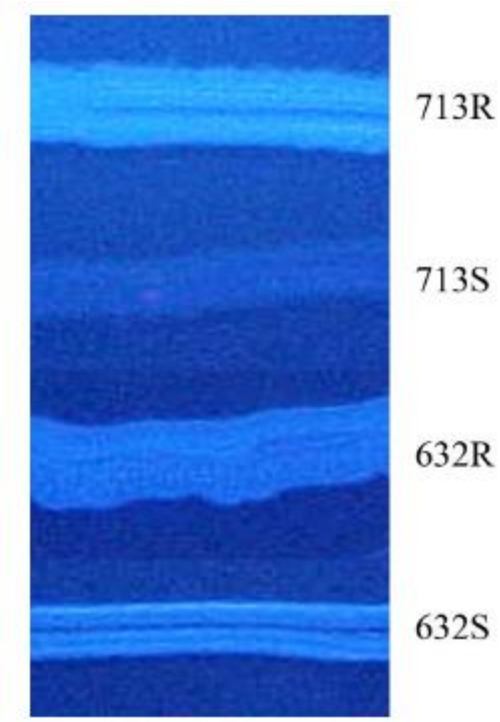


Table.1 PCR primers used in this study

Targeted gene	Primer	Primer sequence	Amplicon size (bp)	Source
bcsA	*bcsA*-12sF	gatggtaacgacatgtgggatg	1296	This study
	bcsA-12sR	gataaacgacactatccaggaaacc		
bcsB	*bcsB*-2sF	gtgaaactgctggtgattgaag	1195	This study
	bcsB-2sR	gaataatcgagaccgagccata		
bcsC	*bcsC*-2sF	ctggtatcagcagattcagggtat	1085	This study
	bcsC-2sR	gattagccgtctccatcagttc		
fliC	*fliC*-1sF	atgacctctcaggttaaaggtatgac	636	This study
	fliC-1sR	agacagtacagaagtaccagcctgt		
flgE	*flgE*-1sR	atgttgaccagttctttaatcaggtc	559	This study
	flgE-1sF	acctgaagctgtactacgtgaaaac		
flgK	*flgK*-1sF	atgtagtactgctgatagcgttgc	662	This study
	flgK-1sR	gtaaggctttctttaagctcggttct		

Table.2 Comparison of cell aggregation properties in *C. sakazakii* rugose and smooth pairs

Strain	Cell aggregation	
	37 C	28 C
713R	+++	+++
713S	+	-
632R	++	++
632S	+	+

Note: "+" and "-" represent: undetectable (-), weak (+), strong reaction (++), Stronger reaction (+++) in the assay.

Table.3 Long-term survival of *C. sakazakii* in biofilms on the surface of glasses

Strain	Bacteria survival for 5 smooth on glass	
	37 C*	28 C*
713R	$2.00 \times 10^{7} \pm 5.17 \times 10^{6}$	$8.1 \times 10^{6} \pm 1.16 \times 10^{6}$
713S	$5.50 \times 10^{2} \pm 2.06 \times 10^{2}$	3.25 ± 5.63
632R	$3.88 \times 10^{5} \pm 1.10 \times 10^{5}$	$1.58 \times 10^{6} \pm 1.19 \times 10^{5}$
632S	$2.00 \times 10 \pm 2.89 \times 10$	$6.57 \times 10 \pm 4.39 \times 10$

*Bacteria were cultured at 37 C or 28 C for 2 days.

Table.4 PCR analysis with primers targeting *bcsABC* genes

strain	*bcsA*	*bcsB*	*bcsC*	*fliC*	*flgE*	*flgK*
713R	+	+	+	+	+	+
713S	+	+	+	-	+	+
632R	+	+	+	+	+	+
632S	+	+	+	+	+	+

Expression of cellulose in the strains was tested by Calcofluor binding assay. The bacteria were inoculated in LBNS agar supplemented with Calcofluor, and cultivated at 28 C for 48 h. The colonies of strain 713R, 632R, and 632S emitted strong fluorescence, however, 713S showed no fluorescence under a 366 nm UV light (Fig. 5). Because of the PCR results, the deficient expression of cellulose in strain 713S was clearly not caused by lacking *bcsABC* genes.

Detection of flagella genes in the rugose and smooth pairs

Bacterial motility is mostly driven by flagella (Ridgway *et al.*, 1977). A set of primers were designed to check flagella genes in the two pairs (Table 1). *fliC* encodes flagellin (FliC), which is a flagellin monomer while *flgE* encodes a flagellar hook protein and *flgK* encodes a hook-associated protein. The PCR results are shown in Table 4. *flgE* and *flgK* were positive in all of the tested strains. *fliC* presented in all of the strains except strain 713S. The negative PCR result of flagellin gene *fliC* in 713S may be the reason why motility or flagella were absent in 713S in the motility test or the EM analysis.

Once excreted from the host, enteric pathogens face to harsh environments characterized by limited nutrients, high or low temperatures, acid, osmotic stress, and dryness (Winfield and Froisman, 2003). The rugose morphology is considered to help *Vibrio* and *Salmonella* survival in the stressful environments (White and Surette, 2006). In this study, we identified and defined rugose and smooth phenotypes in *C. sakazakii*. Based on these results, we showed the advantages of rugose phenotype in survival and persistence of *C. sakazakii*. Rugose variants produced more biofilms and cell aggregation than their smooth phase variant counterparts. The capacity of *Cronobacter* to switch between smooth to rugose phase phenotypes increase bacterial resistances to temperature and desiccative stresses; thus contributes to their survival in harsh environments. In contrast with *Vibrio*, the rugose morphology of *Salmonella* and other bacteria do not produce Vps. The rugosity of *Salmonella* species and pathogenic *E. coli* are reported to related to the expression of cellulose, curli, lipopolysaccharide (LPS), capsules or other polysaccharides (Kim and Wei, 2009). We try to find if the rugosity of *Cronobacter* related to curli and cellulose.

C. sakazakii do not possess curli *csgBAC* and *csgDEFG* genes (Hu, unpublished data). Both rugose and smooth pairs tested here possessed cellulose *bcsABC* genes using the PCR assay (Table 4). Furthermore, the rugose variants could form biofilms as much at 37 C as at 28 C, and the biofilms could form when the bacteria were cultured in rich culture media

with 1% sodium chloride. In contrast to that, the biofilms formation associated with curli and cellulose usually occurred at lower temperature and low osmotic media (no salt or low salt); however, the biofilm formation and cell aggregation of the rugose strains could occur at 37 C and high osmotic condition. Thus, the difference between the rugose and smooth pairs in biofilm formation, cell aggregation, and long-term survival indicated that some unknown factors might mediate the smooth to rugose phase change except curli and cellulose (Hu *et al.,* 2015; Hu, unpublished data*).*

To understand what differences exist between rugose and smooth strains, a molecular typing (PFGE) and a colorimetric typing (VITEK) were used to detect potential differences. After genomic DNA of the organisms was separated by PFGE, no remarkable differences between the rugose and smooth pairs were found in PFGE typing except for two different bands between 713R and 713S. Furthermore, no differences in VITEK typing were shown between the rugose and smooth pairs. These results suggest that no obvious genomic and biochemical differences are found between the rugose and smooth variants using the assays.

Cronobacter could form biofilm, however, the quantity of biofilm formation varied among strains. Rugose variant 713R produced more biofilms and auto aggregation than rugose variant 632R. It was not clear why some rugose variant produced more biofilms than the others. The VITEK typing showed three biochemical reactions differed between strain 713 and 632. Whether the different reactions affect the quantity of biofilm formation still needs further investigation. A bacterial flagellum is comprised of a basal body, a hook and a filament (Apel and Surette, 2008). PCR primers targeted flagella hook and filament structure genes showed only filament gene *fliC* was absent in strain 713S. The failed expression of flagella in 713S may be due to the absent of *fliC* gene. It is still unknown if the failed expression of the filament or other factors in 713S caused no significant fluorescence in strain 713S in Calcofluor binding assay. However, it is clear that the absent of *fliC* in strain 713S did not relate to the rugose and smooth phase changes. From our results, the rugosity of *C. sakazakii* strain 713R and 632R were not involved in curli or cellulose. Other possible contributing factors may be LPS, extracellular polysaccharides, and capsules, or regulatory factors (Anriany *et al.,* 2001; de Rezende *et al.,* 2005).

Taking together, our data revealed that the rugose phenotype of *Cronobacter* formed more biofilms and cell autoaggregation, and enhanced the resistance to desiccated stress. The rugose variants of *Cronobacter* could survive longer than their smooth counterparts in the desiccated environment. Furthermore, *Cronobacter* rugosity did not associate with expression of curli and cellulose. PFGE and VITEK typing analyses of the pairs showed no significant differences between the rugose and smooth pairs in the assays. These results suggest that the smooth to rugose phenotype change may play an important role in *Cronobacter* survival and persistence in environments.

Acknowledgements

The authors thank Dr. Ben D. Tall for his guidance and critical review of the manuscript, and thank Dr. Junia J. Gilles-Beauburn, Dr. Mahendra H. Kothary, and Dr. Barbara A. McCardell for their technical assistances and valuable comments.

References

Anriany, Y.A., Weiner, R.M., Johnson, J.A., De Rezende, C.E., Joseph, S.W. 2001. *Salmonella* enterica serovar

Typhimurium DT104 displays a rugose phenotype. *Appl. Environ. Microbiol*. 67: 4048-56.

Apel. D., Surette. M.G. 2008. Bringing order to a complex molecular machine: the assembly of the bacterial flagella. *Biochim. Biophys. Acta.* 1778: 1851-8.

Beuchat, L.R., Kim, H., Gurtler, J.B., Lin, L.C., Ryu, J.H., Richards, G.M. 2009. *Cronobacter sakazakii* in foods and factors affecting its survival, growth, and inactivation. *Int. J. Food. Microbiol*. 136: 204-13.

Beyhan, S., Bilecen, K., Salama, S.R., Casper-Lindley, C., Yildiz, F.H. 2007. Regulation of rugosity and biofilm formation in *Vibrio cholerae*: comparison of VpsT and VpsR regulons and epistasis analysis of vpsT, vpsR, and hapR. *J. Bacteriol*. 189: 388-402.

Breeuwer, P., Lardeau, A., Peterz, M., Joosten, H.M. 2003. Desiccation and heat tolerance of Enterobacter sakazakii. *J. Appl. Microbiol.* 95: 967-73.

Carter, L., Lindsey, L.A., Grim, C.J., Sathyamoorthy, V., Jarvis, K.G., Gopinath, G., Lee, C., Sadowski, J.A., Trach, L., Pava-Ripoll, M., McCardell, B.A., Tall, B.D., Hu, L. 2013. Multiplex PCR assay targeting a diguanylate cyclase-encoding gene, *cgcA*, to differentiate species within the genus *Cronobacter*. *Appl. Environ. Microbiol*. 79: 734-7.

Chapman, M.R., Robinson, L.S., Pinkner, J.S., Roth, R., Heuser, J., Hammar, M., Normark, S., Hultgren, S.J. 2002. Role of *Escherichia coli* curli operons in directing amyloid fiber formation. *Science*. 295: 851-5.

De Rezende, C.E., Anriany, Y., Carr, L.E., Joseph, S.W., Weiner, R.M. 2005. Capsular polysaccharide surrounds smooth and rugose types of *Salmonella enterica* serovar Typhimurium DT104. *Appl. Environ. Microbiol*. 71: 7345-51.

Farmer, J.J., Asbury, M.A., Hickman, F.W. Brenner D.J. and The *Enterobacteriaceae* Study Group. 1980. *Enterobacter sakazakii*: A new species of "*Enterobacteriaceae*" isolated from clinical specimens. *Int. J. Syst. Bacteriol*. 30: 569-84.

Grau, B.L., Henk, M.C., Pettis, G.S. 2005. High-frequency phase variation of *Vibrio vulnificus* 1003: isolation and characterization of a rugose phenotypic variant. *J. Bacteriol.* 187: 2519-25.

Gualdi, L., Tagliabue, L., Bertagnoli, S., Ieranò, T., De Castro, C., Landini, P. 2008. Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. *Microbiology*. 154: 2017-24.

Gurtler, J.B., Beuchat, L.R. 2007. Survival of *Enterobacter sakazakii* in powdered infant formula as affected by composition, water activity, and temperature. *J. Food Prot.* 70: 1579-8.

Herschleb, J., Ananiev, G., Schwartz, D.C. 2007. Pulsed-field gel electrophoresis. *Nat. Protoc.* 2: 677-84.

Himelright, I., Harris, E., Lorch, V., Anderson, M., Jones, T., Craig, A., *et al.*, 2002. *Enterobacter sakazakii* infections associated with the use of powdered infant formula—Tennessee, 2001. *JAMA.* 287: 2204–5.

Hollenbeck, E.C., Fong, J.C., Lim, J.Y., Yildiz, F.H., Fuller, G.G., Cegelski, L. 2014. Molecular determinants of mechanical properties of *V. cholerae* biofilms at the air-liquid interface. *Biophys. J.* 107: 2245-52.

Hu, L., Grim, C.J., Franco, A.A., Jarvis, K.G., Sathyamoorthy, V., Kothary, M.H., McCardell, B.A., Tall, B.D., 2015. Analysis of the cellulose synthase operon genes, *bcsA*, *bcsB*, and *bcsC* in *Cronobacter* species: Prevalence among

species and their roles in biofilm formation and cell-cell aggregation. *Food Microbiol*. 52: 97-105.

Hunter, C.J., Bean, J.F. 2013. *Cronobacter*: an emerging opportunistic pathogen associated with neonatal meningitis, sepsis and necrotizing enterocolitis. *J. Perinatol.* 33: 581–585.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R., Joosten, H. 2008. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. Nov. *Int. J. Syst. Evol. Microbiol.* 58: 1442-7.

Jaradat, Z.W., Al Mousa, W., Elbetieha, A., Al Nabulsi, A., Tall, B.D. 2014. *Cronobacter* spp. – opportunistic food-borne pathogens. A review of their virulence and environmental-adaptive traits. *J. Med. Microbiol.* 63: 1023–37.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M.J., Forsythe, S.J., 2012. *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter unversalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *Int. J. Syst. Evol. Microbiol*. 62: 1277-83.

Kim, S.H., Wei, C.I. 2009. Molecular characterization of biofilm formation and attachment of *Salmonella enterica* serovar *typhimurium* DT104 on food contact surfaces. *J. Food Prot.* 72: 1841-7.

Lin, L.C., Beuchat, L.R. 2007. Survival of *Enterobacter sakazakii* in infant cereal as affected by composition, water activity, and temperature. *Food Microbiol.* 24: 767-77.

Morris, J.G. Jr., Sztein, M.B., Rice, E.W., Nataro, J.P., Losonsky, G.A., Panigrahi, P., Tacket, C.O., Johnson, J.A. 1996. *Vibrio cholerae* O1 can assume a chlorine-resistant rugose survival form that is virulent for humans. *J. Infect. Dis.* 174: 1364-8.

Rice E.W., Johnson C.J., Clark R.M., Fox K.R., Reasoner D.J., Dunnigan M.E., Panigrahi P, Johnson J.A., Morris J.G. Jr. 1992. Chlorine and survival of "rugose" *Vibrio cholerae*. *Lancet.* 340: 7403.

Ridgway, H.G, Silverman, M., Simon, M.I. 1977. Localization of proteins controlling motility and chemotaxis in *Escherichia coli*. *J. Bacteriol*. 132: 657-65.

Stoop, B., Lehner, A., Iversen, C., Fanning, S., Stephan, R. 2009. Development and evaluation of *rpoB* based PCR systems to differentiate the six proposed species within the genus *Cronobacter*. *Int. J. Food Microbiol.* 136: 165-8.

Tall, B.D., Franco, A.A., Jarvis, K.G., Grim, C.J., Kopecko, D.J., Hu, L., Kothary, M.H., Gopinath, G., Sathyamoorthy, V., Carter, L., Curtis, S.K., Restaino, L., 2015. Chapter 38: *Cronobacter* species (Formerly *Enterobacter sakazakii*). In: Tortorello M.L. (Ed.), Compendium of Methods for the Microbiological Examination of Foods. American. Public. Health Association, Washington, DC.

Vestby, L.K., Møretrø, T., Balance, S., Langsrud, S., Nesse, L.L. 2009. Survival potential of wild type cellulose

deficient *Salmonella* from the feed industry. *BMC Vet. Res.* 5: 43.

Wai, S.N., Mizunoe, Y., Takade, A., Kawabata, S.I., Yoshida, S.I. 1998. *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. *Appl. Environ. Microbiol.* 64: 3648-55.

White, A.P., Surette, M.G. 2006. Comparative genetics of the rdar morphotype in *Salmonella*. *J. Bacteriol.* 188: 8395-406.

Winfield, M.D., Groisman, E.A. 2003. Role of nonhost environments in the lifestyles of *Salmonella* and *Escherichia coli*. *Appl. Environ. Microbiol*. 69: 3687-94. Review.

Yildiz, F.H., Liu, X.S., Heydorn, A., Schoolnik, G.K. 2004. Molecular analysis of rugosity in a Vibrio cholerae O1 El Tor phase variant. *Mol. Microbiol.* 53: 497-515.

Yildiz, F.H., Schoolnik, G.K. 1999. *Vibrio cholerae* O1 El Tor: of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proc. Natl. Acad. Sci. U.S.A.* 96: 4028-33.

How to cite this article:

Lan Hu and Sherile K. Curtis. 2017. Characterizing the Rogues and Smooth Pairs of *Cronobacter sakazakii* Involved in Biofilm Formation and Long-Term Survival. *Int.J.Curr.Microbiol.App.Sci.* 6(10): 4720-4733. doi: https://doi.org/10.20546/ijcmas.2017.610.438