Mining for sensitive and reliable species-specific primers for PCR for detection of *Cronobacter sakazakii* by a bioinformatics approach

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ABSTRACT

Although several studies have reported PCR assays for distinguishing *Cronobacter sakazakii* from other species in the genus, reports regarding assay sensitivity and specificity, as well as applications for food testing, are lacking. Hence, the objective of this study was to develop a sensitive and reliable PCR-based method for detection of *C. sakazakii* by screening for specific target genes. The genome sequence of *C. sakazakii* in the GenBank database was compared with that of other organisms using BLAST. Thirty-eight DNA fragments unique to *C. sakazakii* were identified, and primers targeting these sequences were designed. Finally, 3 primer sets (CS14, CS21, and CS38) were found to be specific for *C. sakazakii* by PCR verification. The detection limit of PCR assays using the 3 pairs of primers was 1.35 pg/μL, 135 fg/μL, and 135 fg/μL, respectively, for genomic DNA, and 5.5×10^5 , 5.5×10^3 , 5.5×10^3 cfu/mL, respectively, using pure cultures of the bacteria, compared with 13.5 pg/μL and 5.5×10^5 cfu/mL for primer set SpeCronsaka, which has been previously described. *Cronobacter sakazakii* were detected in artificially contaminated powdered infant formula (PIF) by PCR using primer sets CS21 and CS38 after 8 h of enrichment. The detection limit was 5.5×10^{-1} cfu/10 g of PIF. Thus, the PCR assay can be used for rapid and sensitive detection of *C. sakazakii* in PIF.

Key words: *Cronobacter sakazakii*, PCR detection, powdered infant formula, species-specific primers

INTRODUCTION

Cronobacter sakazakii is a gram-negative pathogen that can cause necrotizing enterocolitis, sepsis, and meningitis in all age groups (Iversen et al., 2008; Li et al., 2012). The fatality rate of infections caused by *C. sakazakii* can be 50 to 80% (Townsend et al., 2008; Healy et al., 2010). Thus, the International Commis-

sion for Microbiological Specifications for Foods (IC-MSF) ranked *Cronobacter* spp. as a “severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration” (Hoque et al., 2010). *Cronobacter* spp., mostly *C. sakazakii*, can be isolated from a wide range of food sources (Friedemann, 2007; Joseph and Forsythe, 2012); however, many infections caused by this opportunistic pathogen have been epidemiologically linked to contaminated powdered infant formula (PIF; Muytjens et al., 1983; Arseni et al., 1987; Simmons et al., 1989; Ray et al., 2007; Li et al., 2014).

Methods based on PCR have been widely used for detection of pathogens because they are simple and rapid compared with conventional culture-based methods. Several targets have been described for PCR detection of *Cronobacter* genus, including 16S ribosomal (r)DNA (Iversen et al., 2004), 16S–23S rDNA internal transcribed spacer (Liu et al., 2006), 23S rDNA (Derzelle et al., 2007), transfer (t)RNAGlu (Hassan et al., 2007), *dnaG* (Seo and Brackett, 2005), *ompA* (Mohan Nair and Venkitanarayanan, 2006), *gluA* (Iversen et al., 2007), *wehC*, *wehI* (Mullane et al., 2008), *wzx* (Jarvis et al., 2011), *zpx* (Jaradat et al., 2009), and others.

Only 3 species of *Cronobacter* (*C. sakazakii*, *C. malonaticus*, and *C. turicensis*) have been associated with human infection. The virulence of these pathogens differs, and *C. sakazakii* causes the majority of serious neonatal and infant infections (Jackson et al., 2014; Zimmermann et al., 2014). Very few reports have described PCR assays for detection of *C. sakazakii*; thus, it is critical to develop methods targeting this important species of *Cronobacter*. The first gene used as a target for the identification of the 6 *Cronobacter* species was *rpoB* (Stoop et al., 2009). The disadvantage of this target was that 2 PCR amplification reactions were needed to identify *C. sakazakii* and distinguish it from other species. In another report, the sequence of the *cgcA* gene was analyzed to design primers that were specific to *C. sakazakii*, and only one PCR amplification reaction was needed. However, a problem with this assay was that nonspecific bands appeared when the PCR products were analyzed on agarose gels (Carter et al., 2013). The *gyrB* gene has also been used as a

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molecular marker to design primers that are specific to *C. sakazakii* (Huang et al., 2013). All primers could be used for detecting *C. sakazakii* and distinguishing it from other species in the *Cronobacter* genus; however, the sensitivity of the assays, interference due non-*C. sakazakii* bacteria, and PCR assays for testing of food were not reported.

Because of advancements in next-generation sequencing technologies, the genome sequences of a large number of organisms are currently available in the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>). Utilizing the genome sequence data and tools provided by NCBI, molecular targets specific for different pathogens can be obtained easily. For the *Cronobacter* genus, the genome sequences of 2 species (*C. sakazakii* and *C. turicensis*) are available, and sequencing of additional strains is in progress. The objective of this work was to identify molecular targets that are specific to *C. sakazakii* by analyzing the genome sequence of *C. sakazakii* ATCC BAA-894. Primers targeting these DNA fragments were then designed and evaluated. Finally, a new PCR method for the detection of *C. sakazakii* in food was developed.

MATERIALS AND METHODS

Bacterial Strains

A total of 12 *Cronobacter* strains and 26 non-*Cronobacter* strains (Table 1) were used in this study. They were purchased from the China Center of Industrial Culture Collection (CICC, Beijing, China), the National Center for Medical Culture Collections (CMCC, Beijing, China), the American Type Culture Collection (ATCC, Manassas, VA), the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), the National Collection of Type Cultures (NCTC, Salisbury, UK), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The other strains that were used were part of our laboratory culture collection (Table 1). All strains were retrieved from frozen stock cultures and grown in appropriate broth at 37°C, at 180 rpm, and in common air.

Selection of *C. sakazakii*-Specific Targets

Information on the complete genome sequence of *C. sakazakii* ATCC BAA-894 (GenBank accession number: CP000783.1) was obtained from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). The genome of *C. sakazakii* ATCC BAA-894 includes 4,562 genes and 4,422 coding sequences. All of the gene sequences were

compared with those of other bacteria in the nucleotide database (nucleotide collection) using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The coding sequence in the genome of *C. sakazakii* ATCC BAA-894 that matched with those of all 4 strains of *C. sakazakii* (GenBank accession ID: CP000783.1, CP003312.1, CP004091.1 and CP006731.1) with completed genome sequences and that could not be found in other organisms were chosen as candidate targets for detection.

DNA Preparation

All strains of bacteria were cultured in Luria-Bertani (LB) broth at 37°C. The bacterial cultures were then collected by centrifugation at 12,000 × *g* for 5 min. Genomic DNA from these cells was extracted and purified using the E.N.Z.A Bacteria Genome kit (Omega Bio-Tek Inc., Norcross, GA) according to the manufacturer's instructions. The concentration and purity of the DNA were estimated by agarose gel electrophoresis and by using the NanoDrop 2000c UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA). In addition, crude genomic DNA extracts were prepared by boiling cells in sterile water. Cell pellets obtained by centrifugation were resuspended in 200 µL of sterile distilled water, boiled for 10 min, stored at −20°C for 10 min, and then centrifuged at 12,000 × *g* for 5 min. The supernatant with crude DNA was used as template in PCR reactions.

PCR Conditions

The PCR was carried out in a 25-µL reaction mixture, which consisted of 12.5 µL of 2× Taq Master (Vazyme Biotech Co., Jiangsu, China), 9.5 µL of sterile distilled water, 1 µL of each primer at a concentration of 0.25 µM, and 1 µL of genomic DNA. The PCR cycling conditions included an initial incubation at 95°C for 5 min, followed by 30 cycles of 95°C for 40 s, 60°C for 30 s, 72°C for 30 s, and the final extension of 72°C for 10 min. To compare the primers designed in the current study with primers reported previously targeting *gyrB*, primer set SpeCronsaka (SpeCronsaka-F1: AGG TAA AAT CCA CCA GCA AAC; SpeCronsaka-R1: CAG AAT ATC GTA TTC AAA CTC; Huang et al., 2013) was used as a control. The PCR mixture was the same as described above, and the cycling protocol consisted of 1 cycle at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. Purified genomic DNA from *C. sakazakii* CICC21560 extracted with the commercial kit and sterile distilled water were used as the positive and negative controls in the PCR, respec-

Table 1. Bacterial strains and PCR results (positive, +, or negative, –) obtained in the this study

Bacterial strain	Strain identification ¹	PCR results using 3 primer sets ¹		
		CS14	CS21	CS38
<i>Cronobacter</i> strains				
<i>C. malonaticus</i>	DSM18702	–	–	–
<i>C. turicensis</i>	DSM18703	–	–	–
<i>C. dublinensis</i>	DSM18705	–	–	–
<i>C. sakazakii</i>	CICC21560	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. sakazakii</i>	Food isolate ²	+	+	+
<i>C. muytjensii</i>	CICC21563	–	–	–
<i>C. universalis</i>	NCTC9529	–	–	–
Non- <i>Cronobacter</i> strains				
<i>Salmonella</i> Brenderup	ATCC9812	–	–	–
<i>Salmonella</i> Saintpaul	CICC21486	–	–	–
<i>Salmonella</i> Kentucky	CICC21488	–	–	–
<i>Salmonella</i> Blockley	CICC21489	–	–	–
<i>Salmonella</i> Bonariensis	CICC21527	–	–	–
<i>Salmonella</i> Wandsworth	CICC21504	–	–	–
<i>Salmonella</i> Adelaide	CICC21505	–	–	–
<i>Salmonella</i> Enteritidis	CICC21482	–	–	–
<i>Salmonella</i> Typhimurium	CMCC56041	–	–	–
<i>Listeria monocytogenes</i>	CICC21662	–	–	–
<i>Listeria monocytogenes</i>	CICC21634	–	–	–
<i>Staphylococcus aureus</i>	ATCC29213	–	–	–
<i>Enterobacter cloacae</i>	ATCC13047	–	–	–
<i>Micrococcus luteus</i>	Laboratory strain	–	–	–
<i>Bacillus pumilus</i>	CMCC63202	–	–	–
<i>Escherichia coli</i>	CICC23657	–	–	–
<i>Escherichia coli</i> O157	CICC21530	–	–	–
<i>Escherichia coli</i> DH5α	Laboratory strain	–	–	–
<i>Escherichia coli</i> BL21	Laboratory strain	–	–	–
<i>Pseudomonas fluorescens</i>	AS1.1802	–	–	–
<i>Bacillus cereus</i>	AS1.1846	–	–	–
<i>Bacillus subtilis</i>	Laboratory strain	–	–	–
<i>Vibrio parahaemolyticus</i>	ATCC 33847	–	–	–
<i>Yersinia enterocolitica</i>	CMCC 52225	–	–	–
<i>Bacillus licheniformis</i>	CICC 23584	–	–	–
<i>Serratia marcescens</i>	CICC 10187	–	–	–

¹Strains were purchased from the China Center of Industrial Culture Collection (CICC, Beijing, China), the National Center for Medical Culture Collections (CMCC, Beijing, China), the American Type Culture Collection (ATCC, Manassas, VA), the China General Microbiological Culture Collection Center (CGMCC, Beijing, China), the National Collection of Type Cultures (NCTC, Salisbury, UK), and the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

²Laboratory strains isolated from food samples.

tively. The PCR products were analyzed by agarose gel electrophoresis using the *DS*TM 2000 DNA markers (Dongsheng Biotechnology, Guangdong, China) and visualized using a UV transilluminator.

Primer Design

Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA) was used to design primers targeting the sequences of the *C. sakazakii*-specific DNA fragments described in the section Selection of *C.*

sakazakii-Specific Targets. The primers with the highest rating score were chosen. All primers were synthesized by Shanghai Sangon Company (Shanghai, China).

Primers Selection Based on Their Specificity

Primers described above were tested using genomic DNA from *C. sakazakii* CICC21560 as template. The primers that generated a strong, single band were selected for further testing. To select primers that were specific to *C. sakazakii*, 6 *C. sakazakii* laboratory strains

isolated from food samples, 5 other laboratory strains of *Cronobacter* spp., and 26 strains of non-*Cronobacter* bacteria (Table 1) were used as targets for testing the specificity of the primers. The primers that generated negative results with non-*C. sakazakii* bacteria were considered specific primers, and these were evaluated to develop PCR detection methods.

Sensitivity Evaluation of Specific Primers Using Genomic DNA

Purified DNA of known concentration extracted from *C. sakazakii* CICC21560 was serially diluted 10-fold. One microliter of the DNA from each dilution was used as template in a 25- μ L PCR reaction. The PCR results were analyzed, and the detection limit of the PCR was determined.

Sensitivity Evaluation of Specific Primers Using Whole Cells

Cronobacter sakazakii CICC21560 was cultured in LB broth at 37°C overnight. The cell culture was serially diluted 10-fold with 0.9% normal saline (when the cell concentration reached 10² cfu/mL, the 10-fold dilution turned into doubling dilution) and the cell concentration was determined by plating onto LB agar and counting the colonies. Crude DNA from the different dilutions was extracted by heating 1 mL of the dilutions at 100°C for 10 min. One microliter of crude DNA from each cell concentration was used as template in the PCR assays. The PCR results were analyzed and the detection limit was obtained.

Testing for PCR Interference with Various Concentrations of *Escherichia coli*

Cronobacter sakazakii CICC21560 and *E. coli* CMCC23657 were cultured in LB broth at 37°C overnight. The cell concentrations were determined by plating 10-fold serial dilutions prepared in 0.9% normal saline. Serial dilutions (10-fold) were prepared to obtain *E. coli* CMCC23657 at different cell concentrations, ranging from 10⁸ to 10⁰ cfu/mL (when the cell concentration reached 10² cfu/mL, the 10-fold dilution turned into doubling dilution). *Cronobacter sakazakii* was used at a fixed concentration of 10⁷ cfu/mL. The *E. coli* cells at different concentrations and *C. sakazakii* cells at a fixed concentration were mixed in equal volumes. Then, DNA was extracted from the cell mixtures by heating at 100°C for 10 min and was used as template for the PCR. The PCR products were analyzed by gel electrophoresis and visualized using a UV transilluminator.

Use of the PCR Assay to Detect *C. Sakazakii* in Artificially Inoculated PIF

Cronobacter sakazakii CICC21560 was cultured in LB broth at 37°C for 12 h, and the cell concentration was estimated by plate counting. One milliliter of the cell cultures at concentrations of 10¹, 10⁰, and 10⁻¹ cfu/mL were each mixed with 10 g of PIF, and these were each added to 89 mL of LB medium and incubated at 37°C. One milliliter of sterile distilled water in 10 g of PIF was used as a control check. From 0 to 12 h, 1-mL aliquots of the samples were taken every 2 h and stored at -20°C. Genomic DNA from the samples was extracted by heating at 100°C for 10 min and used as template in PCR assays.

RESULTS AND DISCUSSION

Screening of *C. Sakazakii*-Specific Targets for Detection

Based on previous reports, the primers used in PCR assays to distinguish *C. sakazakii* from other species only targeted the *rpoB*, *gyrB*, and *cgcA* genes (Stoop et al., 2009; Carter et al., 2013; Huang et al., 2013). The sensitivity of these assays and the effect of interference from other bacteria were not reported. Furthermore, the performance of these primers in PCR assays with actual food samples is not known. Hence, there is a need to identify additional specific targets for *C. sakazakii* and to develop sensitive and specific PCR assays for detection of the pathogen in food. In this study, a bioinformatics approach was used to identify as many specific targets as possible for *C. sakazakii*. The complete genome sequence of *C. sakazakii* ATCC BAA-944 was chosen to identify specific DNA fragments. Thirty-eight DNA fragments were identified as described above and found only in the 4 *C. sakazakii* strains as determined using the BLAST program on the NCBI website, and therefore, these were chosen as candidate targets. The 38 DNA fragments and their respective gene IDs are shown in Table 2.

Determination of Primers Specific for *C. sakazakii*

The primers for the PCR assays were designed according to the sequences of the 38 DNA fragments. Three primer sets, numbers 14, 21, and 38, produced strong, single bands for *C. sakazakii* CICC21560 and 6 other *C. sakazakii* isolates, but no bands with 5 strains belonging to other *Cronobacter* species and 22 non-*Cronobacter* strains (Tables 1 and 2). Only 3 out of 38 (7.89%) primer sets showed perfect specificity in PCR assays and the others generated nonspecific products,

Table 2. Primer sets used in PCR assays for *Cronobacter sakazakii* and their target genes

Gene ID	Location	Primer	Forward primer (5'→3')	Reverse primer (5'→3')	<i>C. sakazakii</i> specific ¹	Product (bp)
ESA_01316	1273202–1273804	1	GAAGAACTCAAAGCACGCATAA	CCACGCCGTAGATTTTCATAC	–	386
ESA_01404-ESA_01406	1357289–1357618	2	AAGAGTTTGGCTGAAGCG	TCTGCCTACCTGATGGAGTT	–	218
ESA_01600	1553679–1554035	3	AGGGCGTTTGTGCGGGTAT	CGGCAAGTTCGGCATCCA	–	219
ESA_01690	1631811–1632131	4	CCTGCTGGTCCGTGATGCTG	GCTTCTGCCCTGCCATTTT	–	247
ESA_01813-ESA_01814	1751551–1752024	5	TGCTGAATGCTGACGGCTCT	GGCTGATAAGGCGCAAACG	–	223
ESA_01842-ESA_01844	1785425–1785757	6	TTCGGCAAACCTGAACTG	CCACGCTAAATGTCGTAAAA	–	109
ESA_01846-ESA_01847	1787361–1788356	7	ACGTTACGCTGCATGGTCC	GCGGCGGTATCAACAAGAG	–	329
ESA_01854-ESA_01855	1793571–1793861	8	CGTGCGGCAGGCTTTCTT	CTTCGGCTGGCTGATGGA	–	141
ESA_01864	1799525–1799917	9	CGGGTGGTGACATAGGTAAA	GCTCGGCTGGTAGAAAAC	–	171
ESA_01917-ESA_01918	1851961–1852797	10	TTCGCCAAGAAGAATCAACG	CTATTTAGCGCAAACGGTAGTG	–	196
ESA_01953	1886711–1887226	11	GTTATCGCTGATGAACAGTCGC	GATGGCATGGCACCACAAT	–	370
ESA_02004-ESA_02005	1942675–1943193	12	CCAGCGGTTTTCGGATGAT	TGGTGACTGACAAAGGCGTAG	–	399
ESA_02129	2077070–2077579	13	AGGGTCTCGTCCGCTTTC	GCTGCTGGTTGCATTTCC	–	452
ESA_02130	2077786–2078235	14	GCCCAGACTTTTGATCTCC	TCATTCCCCTGACTATTT	+	148
ESA_02197	2133810–2134192	15	CGGTGTAATAACGGGTGTC	GGCGGATAGGGTGATAAA	–	152
ESA_02199	2136757–2137560	16	GGTGGGAGCCGTTGAATA	ACAGGAAGTGCCAGGAGG	–	282
ESA_02372	2305278–2306234	17	TGTCGGATAGTTGCGGTTGT	CGTCGGCTAAAGAGGATTTTCG	–	247
ESA_02487	2435050–2435367	18	CAGAGCGAGCCGATAGAGC	AGACGGGGCCGTGAACTT	–	158
ESA_02538	2490652–2491191	19	GCTCCTCCTCGCTCACAT	AGGCTTTGGTCAACGGTTT	–	225
ESA_02544	2498505–2499350	20	CGCGGCCAGGAAGTGATT	TCGGCGAGCGTGACATAA	–	245
ESA_02797	2745128–2747617	21	GGCAGCATGTCATTATCGG	CATCAGTGGCATTGCGTCTA	+	152
ESA_02799	2748430–2748966	22	ATCCGTATTACCGTCGTTT	CCATCACCCCTTTCTTCCTG	–	185
ESA_03005-ESA_03006	2958749–2959192	23	GCAGCGAGTGCTGGAAG	CGCACCGAGAAAGTGAGTA	–	239
ESA_03007	2959236–2959697	24	GAACTCGCTCCCTGCTTT	CTCATCGTCACGCCTCCA	–	188
ESA_03008	2959764–2960207	25	CGAAGCCGAACTCAAACA	CGGTAGGTCAGCCAGTCA	–	147
ESA_03009	2960351–2960794	26	ATGGCTTAAATATCGTGATG	CCAGGCTCGTTATGTGAA	–	149
ESA_03010	2960874–2961338	27	CCCAGTCTTCATAGCCCTCC	CGCAGATGCTGAGCCACTA	–	174
ESA_03321	3256913–3257251	28	CATGTTGCGGTCAATAGC	TGATGAAGCGTGAAAGAAA	–	116
ESA_03358	3298780–3299445	29	CATCAATGGCATCATCAACA	GTGCGACAATCTCCACTCAC	–	104
ESA_03490-ESA_03491	3436595–3437245	30	CGGTCAATATCAATTATCAGCA	ACAGCAGCAGTTCGTGGC	–	177
ESA_03743	3683541–3683936	31	TTAAGCGACAAGTGGAAG	GACAGGCGAAATAGAACG	–	292
ESA_03887	3838625–3839092	32	CGACCGTAATCGCTTGCT	CGCTTCCCATTGAAACAC	–	154
ESA_03999-ESA_04000	3946069–3946689	33	GCTGAAGGGCTTTGGTATG	CTATCTGGCTGGTATTGCTGA	–	291
ESA_04072	4024815–4025576	34	TGGAGTATGTTTGAGCGTGTA	AAGATTTGCCCTTTGTGG	–	431
ESA_04112	4068989–4069789	35	TGGCATTACGCCGCATTT	AGCGACGGACGCACTTTT	–	450
ESA_04152	4104173–4104634	36	AACCTATGGTGGTGGACT	GATCTGACTTTCAATTTTCG	–	256
ESA_04314-ESA_04315	4279292–4279753	37	TCTGGCGGGTGCTTGCT	GGCGGCTCCGTAAATAAA	–	219
ESA_04347-ESA_04349	4318202–4318678	38	CGGGTTACGCAGGGTTGA	GCGGTTGCCAGTGAATAAGAT	+	222

¹+ = a strong, single band was obtained using the primers in the PCR assay; – = no band was visible when the primers were used in the PCR assay.

Figure 1. Similarity of DNA sequence within 7 species of *Cronobacter*. (A) Similarity of target gene sequences within 7 species of *Cronobacter*; (B) similarity of the sequence of DNA fragments amplified by PCR using 5 primer sets within 7 species of *Cronobacter*.

which could be attributed to limitations in the sequence information in the NCBI GenBank database. Thus, it is necessary to verify the results of BLAST by performing PCR assays. The 3 primer sets were designated CS14, CS21, and CS38. The identities in GenBank of the genes they targeted were ESA_02130, ESA_02797, and ESA_04347-ESA_04349, respectively. The protein ESA_02797 encodes a membrane porin protein known as PapC. In another report, comparative genomic hybridization (CGH) analysis showed that PapC was unique to *C. sakazakii* and absent in *C. malonaticus*, *C. muytjensii*, *C. turicensis*, and *C. dublinensis* (Kucerova et al., 2010). However, the proteins ESA_02130 and ESA_04347-ESA_04349 encode hypothetical proteins, and thus their function is still unknown.

The genes *RpoB* (Stoop et al., 2009), *gyrB* (Huang et al., 2013), and *cgcA* (Carter et al., 2013), and the 3 genes (ESA_02130, ESA_02797 and ESA_04347-ESA_04349) we screened were used to identify *C. sakazakii*. The sequences of the 6 genes of *C. sakazakii* ATCC BAA-894 were used as target and they were compared with 6 other species of *Cronobacter* using the tool provided by *Cronobacter* MLST Databases (<http://pubmlst.org/cronobacter/>). One hundred thirty-five strains of *C. sakazakii*, 24 strains of *C. malonaticus*, 10 strains of *C. turicensis*, 2 strains of *C. universalis*, 13 strains of *C. dublinensis*, and 1 strain of *C. condimentii* were used in this alignment. The results showed that the 3 genes (ESA_02130, ESA_02797 and ESA_04347-ESA_04349) we screened showed better specificity than genes in previous reports (Figure 1A). The specificity of *rpoB* was lower and therefore, 2 PCR reactions were

needed to identify *C. sakazakii*. The primer sets (CS14, CS21, CS38, *Csak*, and *SpeCronsaka*) targeting the other 5 genes could identify *C. sakazakii* in one PCR reaction, and the DNA fragments amplified by PCR

Figure 2. Results of PCR of genome DNA of the strains within the *Cronobacter* genus using primer sets CS14, CS21, and CS38. Lane M = *DS*TM 2000 marker (Dongsheng Biotechnology, Guangdong, China); lane N = negative control (double-distilled H₂O); lanes 1–6: *C. malonaticus* DSM18702, *C. turicensis* DSM18703, *C. dublinensis* DSM18705, *C. sakazakii* CICC21560, *C. muytjensii* CICC21563, and *C. universalis* NCTC9529, respectively. (A) Primer set CS14; (B) primer set CS21; (C) primer set CS38; (D) primer set *SpeCronsaka*. All concentrations of genomic DNA were diluted to about 20 ng/ μ L.

Figure 3. (A) PCR detection sensitivity using dilutions of genomic DNA from *Cronobacter sakazakii* CICC21560. Lane M = *DS*TM 2000 marker (Dongsheng Biotechnology, Guangdong, China); lane N = negative control (double-distilled H₂O); lanes 1–7 = 13.5 ng/ μ L, 1.35 ng/ μ L, 135 pg/ μ L, 13.5 pg/ μ L, 1.35 pg/ μ L, 135 fg/ μ L, and 13.5 fg/ μ L, respectively. (a) Primer set CS14; (b) primer set CS21; (c) primer set CS38; (d) primer set SpeCronsaka. (B) Results of Figure 1A showing detection limits (fg/ μ L) of genomic DNA.

Figure 4. (A) PCR detection sensitivity using dilutions of a pure culture of *Cronobacter sakazakii* CICC21560. Lane M = *DS*TM 2000 marker (Dongsheng Biotechnology, Guangdong, China); lane N = negative control (double-distilled H₂O); lanes 1–9 = 4.1×10^8 cfu/mL, 4.1×10^7 cfu/mL, 4.1×10^6 cfu/mL, 4.1×10^5 cfu/mL, 4.1×10^4 cfu/mL, 4.1×10^3 cfu/mL, 4.1×10^2 cfu/mL, 5.1×10^1 cfu/mL, 6.4×10^0 cfu/mL, respectively. (a) Primer set CS14; (b) primer set CS21; (c) primer set CS38; (d) primer set SpeCronsaka. (B) Results of Figure 3A using whole cells showing detection limits (cfu/mL).

using these primers were analyzed as well (Figure 1B). Our primer sets CS21 and CS38 showed better specificity than previous primers and primer set CS14.

Primer set SpeCronsaka target *cgcA* (Huang et al., 2013) was selected for comparison with the 3 primer sets, CS14, CS21 and CS38, designed in the current study. The PCR results of the 4 primer sets using DNA from 6 species within the *Cronobacter* genus showed that they were specific for *C. sakazakii* (Figure 2), showing one clear band, and thus indicating that the 4 primer sets were specific for *C. sakazakii*.

Evaluation of Sensitivity of PCR Assays Using the Specific Primer Sets

We determined the detection limit of the PCR assays using the 4 primer sets and different cell or DNA concentrations of *C. sakazakii* CICC2156. The detection limits using genomic DNA for the SpeCronsaka, CS14, CS21, and CS38 primer sets were 13.5 pg/ μ L, 1.35 pg/ μ L, 135 fg/ μ L, and 135 fg/ μ L, respectively. Clearly, PCR assays using CS21 and CS38 primer sets were more sensitive than those using CS14 and SpeCronsaka primer sets (Figure 3), because the detection limit of PCR assays using these 2 primer sets was about 100 times greater compared with SpeCronsaka primers. Moreover, when using cells, the detection limit for SpeCronsaka, CS14, CS21, and CS38 primer sets were 4.1×10^6 , 4.1×10^5 , 4.1×10^3 , and 4.1×10^3 cfu/mL, respectively (Figure 4). The results were in agreement with the detection limits using different DNA concentrations, showing again that PCR assays using primer sets CS21 and CS38 were more sensitive than those using SpeCronsaka for the detection of *C. sakazakii*.

Testing for PCR Interference and Specificity Using Various Concentrations of *E. coli*

Food samples generally contain background microflora in addition to the contaminating pathogen, and DNA from these non-*Cronobacter* bacteria may affect PCR detection of *C. sakazakii*, leading to false-positive or false-negative results. Therefore, PCR interference using CS14, CS21, CS38, and SpeCronsaka primers was evaluated with a mixture of DNA from *C. sakazakii* and *E. coli* cells. As shown in Figure 5, only one target band appeared even with a cell concentration of *E. coli* up to 10^8 cfu/mL, indicating that the 4 primer sets were not affected by DNA from other bacteria (*E. coli* in this case).

Application of the New Primer Sets in PCR Assays for Detection *C. Sakazakii* in Artificially Contaminated PIF

Artificially contaminated PIF was used to evaluate the sensitivity, specificity, and reliability of the primer sets CS14, CS21, CS38, and SpeCronsaka. The cell concentrations of *C. sakazakii* added to PIF were 5.5×10^1 , 5.5×10^0 , and 5.5×10^{-1} cfu/10 g of PIF. As shown in Table 3, PCR assays using primer sets CS14 and SpeCronsaka could not detect *C. sakazakii* because of their lower sensitivity, even after 12 h of enrichment. However, *C. sakazakii* was detected using PCR assays with primer sets CS21 and CS38 after 8 h of enrichment. The detection limit was 5.5×10^{-1} cfu/10 g of PIF after 8 h of enrichment. Therefore, detection of *C. sakazakii* by PCR in PIF can be accomplished within 11 h.

Table 3. Results of PCR (positive, +, or negative, -) of powdered infant formula (PIF) samples artificially contaminated with *Cronobacter sakazakii*

Primer	<i>C. sakazakii</i> concentration (cfu/10 g)	Enrichment time						
		0 h	2 h	4 h	6 h	8 h	10 h	12 h
CS14	5.5×10^{-1}	-	-	-	-	-	-	-
	5.5×10^0	-	-	-	-	-	-	-
	5.5×10^1	-	-	-	-	-	-	-
	Control check	-	-	-	-	-	-	-
CS21	5.5×10^{-1}	-	-	-	-	+	+	+
	5.5×10^0	-	-	-	-	+	+	+
	5.5×10^1	-	-	-	-	+	+	+
	Control check	-	-	-	-	-	-	-
CS38	5.5×10^{-1}	-	-	-	-	+	+	+
	5.5×10^0	-	-	-	-	+	+	+
	5.5×10^1	-	-	-	-	+	+	+
	Control check	-	-	-	-	-	-	-
SpeCronsaka	5.5×10^{-1}	-	-	-	-	-	-	-
	5.5×10^0	-	-	-	-	-	-	-
	5.5×10^1	-	-	-	-	-	-	-
	Control check	-	-	-	-	-	-	-

Figure 5. Results of PCR assays using primers targeting *Cronobacter sakazakii* testing for interference with different concentrations of *E. coli* DNA. Lane M = *DS*TM 2000 marker (Dongsheng Biotechnology, Guangdong, China); lane N = negative control (double-distilled H₂O); lanes 1–9 = mixtures of *C. sakazakii* CICC21560 (concentration fixed at 10⁷ cfu/mL) and *Escherichia coli* CMCC23657 at 10⁸, 10⁷, 10⁶, 10⁵, 10⁴, 10³, 10², 10¹, and 10⁰ cfu/mL, respectively. (A) Primer set 14; (B) primer set CS21; (C) primer set CS38; (D) primer set SpeCronsaka.

CONCLUSIONS

In this study, 38 DNA fragments that were specific for *C. sakazakii* were identified from the complete genome sequence of *C. sakazakii* ATCC BAA-944, and PCR primers were designed for these fragments. After screening all of the primers, 2 primer sets, CS21 and CS38, targeting the *ESA_02797* and *ESA_04347-ESA_04349* genes, were found to be more specific, more sensitive, and more reliable than the other designed primers and a primer set reported previously in PCR assays. Use of the 2 primer sets in PCR assays to detect *C. sakazakii* in artificially contaminated PIF showed that the detection limit reached 5.5×10^{-1} cfu/10 g of PIF after 8 h of enrichment. To our knowledge, this is the first report using these 2 gene targets for PCR detection of *C. sakazakii* in food.

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