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Multiplex PCR Assay Targeting a Diguanylate Cyclase-Encoding Gene, *cgcA*, To Differentiate Species within the Genus *Cronobacter*

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In a comparison to the widely used *Cronobacter rpoB* PCR assay, a highly specific multiplexed PCR assay based on *cgcA*, a diguanylate cyclase gene, that identified all of the targeted six species among 305 *Cronobacter* isolates was designed. This assay will be a valuable tool for identifying suspected *Cronobacter* isolates from food-borne investigations.

Cronobacter spp. are Gram-negative, opportunistic pathogens that cause meningitis, necrotizing enterocolitis, and septicemia in neonates and elderly individuals (1–3). *Cronobacter* spp. are ubiquitous in nature and have been isolated from clinical, environmental, and food sources, most notably powdered infant formula and other dried foods (2, 3), and, more recently, from surfaces and intestinal tracts of wild filth flies (4). The *Cronobacter* genus consists of seven species, *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis*, and *C. universalis* (5, 6). Although all species except *C. condimenti* have been associated with clinical infections, *C. sakazakii* and *C. malonaticus* isolates are responsible for causing the majority of infantile illnesses (7). It is important to identify *Cronobacter* quickly and precisely. Current *Cronobacter* identification and subtyping methods include 16S rRNA gene sequencing, ribotyping, DNA-DNA hybridization, *rpoB* PCR, pulsed-field gel electrophoresis, plasmidotyping, and molecular serogrouping assays (3, 5, 8–12). These methods have detected considerable diversity among *Cronobacter* spp.; however, many of these are not rapid or require multiple PCRs to identify or characterize isolates. Also, 16S rRNA gene sequence analysis has limitations for discriminating between very closely related organisms, such as *C. malonaticus* and *C. sakazakii*, because of minimal sequence diversity or the presence of multiple copies of 16S rRNA gene loci. It is necessary to ensure that reliable and robust identification methods are used so that the control of contamination by these organisms during the food manufacturing process and the reduction of exposure to susceptible high-risk individuals are achieved.

Cyclic diguanylate (c-di-GMP) is a bacterial signal transduction second messenger recognized for its involvement in the regulation of a number of complex physiological processes, including bacterial virulence, biofilm formation, and persistence (long-term survival) (13). Diguanylate cyclase, which synthesizes cyclic diguanylate, possesses a conserved active domain of five amino acids, Gly-Gly-Asp-Glu-Phe, or GGDEF (13). Several food-borne pathogens, such as *Vibrio cholerae*, *Salmonella*, and *Escherichia coli*, encode variable numbers of GGDEF domain proteins (13).

Analysis of 12 *Cronobacter* genomes revealed seven GGDEF domain-encoding genes which were conserved among all *Cronobacter* spp. (15, 16; C. J. Grim, M. L. Kotewicz, K. A. Power, A. A. Franco, G. Gopinath, K. G. Jarvis, Q. Q. Yan, S. A. Jackson, L. Hu, V. Sathyamoorthy, F. Pagotto, C. Iversen, A. Lehner, R. Stephan, S. Fanning, and B. D. Tall, submitted for publication). Phylogenetic analysis of each set of homologous genes from the available genomes revealed that homologs of two of the seven genes (ESA_04212 and ESA_03399 of *C. sakazakii* ATCC BAA-894) were highly similar within the genus and one of these was highly similar throughout the *Enterobacteriaceae* (data not shown). In contrast, the other five GGDEF domain-encoding genes (homologs of ESA_01230, ESA_01822, ESA_03401, ESA_03491, and ESA_ 04315 from *C. sakazakii* ATCC BAA-894) showed species-specific allelic divergence (Fig. 1A), some of which recapitulated the species-specific phylogenetic relationships within the genus (Fig. 1B), previously described by Iversen et al. (5) and as determined through multilocus sequence typing (MLST) (17), *rpoB* sequencing analyses (12), and whole-genome phylogenetic reconstruction (Grim et al., submitted).

In particular, multiple sequence alignment of homologues of ESA_01230 from *C. sakazakii* ATCC BAA-894 (Fig. 1A), annotated as a putative *Cronobacter* diguanylate cyclase (containing a GGDEF domain) and designated *cgcA* here, yielded a phylogeny in which all six species formed discrete, distinct lineages, with relationships in agreement with those in whole-genome analysis (Fig. 1B). Further, we hypothesized that this gene would be amenable to species-specific primer design based on the length of the coding sequences and the depth of branching exhibited between species, which, taken together, would provide a significant number of variable sites (single nucleotide polymorphisms [SNPs]) for species-specific primer design (see Fig. S1 in the supplemental material).

These observations led us to design a set of species-specific multiplex PCR primers which may be used to identify strains of *Cronobacter* in a single multiplex PCR assay (Table 1 and Fig. 2). Primer sequences were chosen by multiple alignment analysis of the respective *cgcA* sequences using MEGA5 (18). The primer sequences were evaluated for their ability to form homo- and heterodimers as well as hairpins by using OligoAnalyzer 3.1 (Inte-

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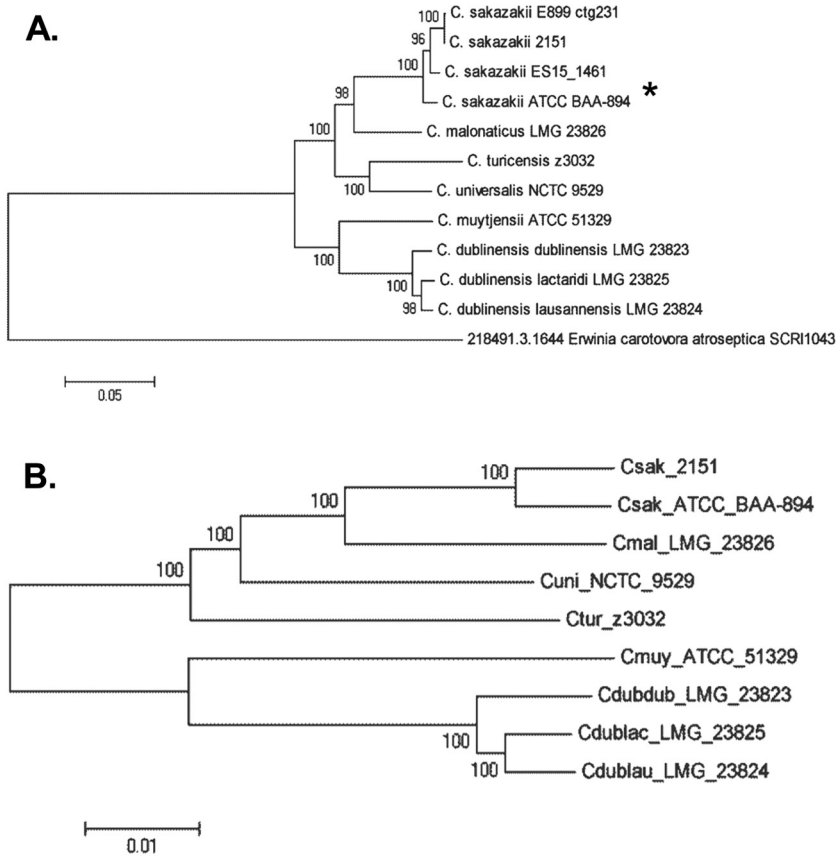

FIG 1 Evolutionary reconstruction of homologues of the GGDEF domain-encoding gene *C. sakazakii* ATCC BAA-894 ESA_01230 (*) among *Cronobacter* spp. (A) and of the genus *Cronobacter* (B) using 0.5 Mb of syntenic whole-genome nucleotide sequences (20). Evolutionary analyses were conducted in MEGA5 (18). Evolutionary history was inferred using the neighbor-joining method. Bootstrap consensus tree inferred from 1,000 replicates. Evolutionary distances were computed using the maximum composite likelihood method, and units are the numbers of base substitutions per site.

grated DNA Technologies, Coralville, IA). All primers were synthesized by Integrated DNA Technologies. Empirical primer concentration and PCR optimization studies were performed by changing primer and template concentrations and PCR assay parameter conditions so that optimal kinetics were achieved.

PCR mixtures were prepared using the GoTaq green master mix (Promega Corp., Madison, WI) according to the manufacturer's instructions using boiled genomic DNA preparations (approximately 50 ng DNA/25-μl reaction mixture) as the DNA template (19). GoTaq Hot Start green master mix is a premixed, proprietary, ready-to-use solution containing GoTaq Hot Start polymerase, deoxynucleoside triphosphates (dNTPs), $MgCl_2$, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. GoTaq Hot Start polymerase is supplied in 2× Green GoTaq reaction buffer (pH 8.5), 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP, and 4 mM $MgCl_2$. In all PCRs, the polymerase was activated by using a 3-min predenaturation step at 94°C, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and a one-min extension at 72°C, followed by a final extension step at 72°C for

TABLE 1 *Cronobacter* species-specific *cgcA* PCR primers used in this study

Primer[a]	Sequence	Amplicon size (bp)	Species identification
Cdm-469R[b]	CCACATGGCCGATATGCACGCC		
Cdub-40F	GATACCTCTCTGGGCCGCAGC	430	*C. dublinensis*
Cmuy-209F	TTCTTCAGGCGGAGCTGACCT	260	*C. muytjensii*
Cmstu-825F[c]	GGTGGCSGGGTATGACAAAGAC		
Ctur-1036R	TCGCCATCGAGTGCAGCGTAT	211	*C. turicensis*
Cuni-1133R	GAAACAGGCTGTCCGGTCACG	308	*C. universalis*
Csak-1317R	GGCGGACGAAGCCTCAGAGAGT	492	*C. sakazakii*
Cmal-1410R	GGTGACCACACCTTCAGGCAGA	585	*C. malonaticus*

[a] The numbers comprising each primer name indicate the 5′ bp location within the aligned nucleotide sequence of *cgcA* (see Fig. S1 in the supplemental material).
[b] The PCR primer Cdm-469R was used in multiplex reactions, with primers Cdub-40F and Cmuy-209F identifying *C. dublinensis* and *C. muytjensii* strains, respectively.
[c] The PCR primer Cmstu-825F was used in multiplex reactions, with primers Ctur-1036R, Cuni-1133R, Csak-1317R, and Cmal-1410R identifying *C. malonaticus*, *C. sakazakii*, *C. turicensis*, and *C. universalis* strains, respectively.

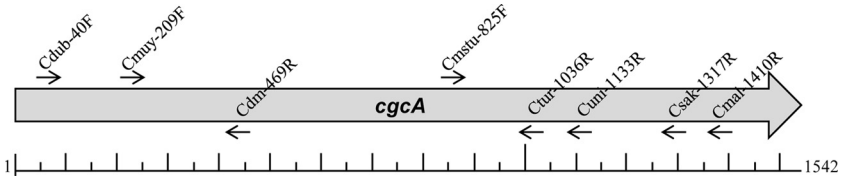

FIG 2 Schematic map of *cgcA* showing locations of PCR primers. The smallest ticks on the scale indicate 50-bp nucleotide positions, with larger ticks indicating 100-bp and 1-kbp nucleotide positions. See Table 1 for an explanation of primer names and nucleotide positions.

five min. PCR amplicons were subjected to agarose gel electrophoresis using 1.5% Tris-borate-EDTA (TBE; Invitrogen, Carlsbad, CA) agarose gels in a RunOne (Embi Tec, San Diego, CA) horizontal electrophoresis unit and were photographed with transilluminated UV light using a Bio-Rad Molecular Imager Gel Chem Doc XR imaging system (Bio-Rad Laboratories, Hercules, CA).

Examples of the *cgcA* PCR assay results for type strains of *Cronobacter* are shown in Fig. 3. Because *C. condimenti* is recently described and the taxonomic description is based on a single isolate, this species was not included in these studies. The multiplex PCR assay was evaluated by interrogating a collection of 305 well-characterized *Cronobacter* strains (5, 9, 10, 19, 20; Grim et al., submitted), which included 15 strains of *C. dublinensis*, two strains of *C. universalis*, 12 strains of *C. muytjensii*, 11 strains of *C. turicensis*, 231 strains of *C. sakazakii*, and 34 strains of *C. malonaticus*. The strain collection included isolates from clinical (69 strains), food (144 strains), environmental (63 strains), fly (18 strains), and unknown (11 strains) sources which were obtained from diverse geographic locations worldwide. These isolates were initially characterized biochemically according to Iversen et al. (5) and later confirmed using the species-specific *rpoB* PCR assays as described by Stoop et al. (12). Additionally, this collection of isolates was subjected to RepF1B plasmidotyping (9) and molecular serogrouping (10, 11), the results of which further corroborated the *rpoB*-based PCR species identification for each strain. The multiplex *cgcA* PCR assay correctly identified (100%) the species identity of the 305 *Cronobacter* isolates. These results confirm the species specificity of the *cgcA* multiplex PCR assay. To test for exclusivity, 20 non-*Cronobacter* strains, which included *Enterobacter amnigenus*, *Enterobacter aerogenes*, *Enterobacter gergoviae*, *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter cloacae*, *Enterobacter cloacae* subsp. *dissolvans*, *Enterobacter helveticus*, *Enterobacter pulveris*, *Enterobacter turicencis*, *Citrobacter koseri*, *Pantoea agglomerans*, *Erwinia carotovora*, *Kluyvera intermedia*, *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* subsp. *enterica* serovar Enteritidis, were assayed, and all were negative using these PCR primers.

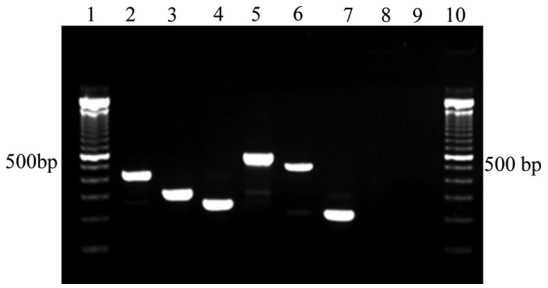

FIG 3 Representative gel image of *Cronobacter* species-specific GGDEF multiplex PCR. Lanes 1 and 10, TrackIt 100-bp DNA ladder (Invitrogen); lane 2, *C. dublinensis* strain LMG 23823T; lane 3, *C. universalis* strain NCTC 9529; lane 4, *C. muytjensii* strain ATCC 51329; lane 5, *C. malonaticus* strain LMG 23826T; lane 6, *C. sakazakii* strain BAA-894; lane 7, *C. turicensis* strain z3032; lane 8, *Enterobacter helveticus* strain z513; lane 9, no-DNA-template control. Five microliters of each PCR (amplicons) was subjected to gel electrophoresis using 1.5% agarose gels and visualized with ethidium bromide (at a final concentration of 0.5 μg/ml).

In conclusion, this study demonstrates that the *Cronobacter* multiplex *cgcA* PCR assay can be used to identify *Cronobacter* strains in a single reaction. The PCR assay described in this report was found to be 100% specific (305/305 correctly identified as *Cronobacter* sp. strains) and 100% sensitive (did not identify 20/20 non-*Cronobacter* species). Its main advantage over the currently used *rpoB* PCR method is that species identity of *C. sakazakii* and *C. malonaticus* can be accomplished in a single reaction as opposed to two separate PCRs. The assay reported here will be a valuable tool for identifying suspected *Cronobacter* isolates from clinical, environmental, and food-borne outbreak and surveillance investigations, quickly and precisely.

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