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Novel PCR-RFLP system based on *rpoB* gene for differentiation of *Cronobacter* species

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ABSTRACT

Bacteria from the genus *Cronobacter* are opportunistic foodborne pathogens that can cause severe infections. More rapid, cost-effective and reliable methods are still required for the species identification of *Cronobacter* spp. In this study, we present a novel PCR-RFLP-based method that uses a newly designed pair of primers for the PCR-amplification of a partial *rpoB* gene sequence (1635 bp). The amplified products of DNA from 80 *Cronobacter* strains were separately digested with three restriction endonucleases (*Csp*6I, *Hin*P1I, *Mbo*I). Using the obtained restriction patterns, a PCR-RFLP identification system was created to enable differentiation between all seven currently-known *Cronobacter* species. The functionality of our method was successfully verified on real food samples. Moreover, the relationships between the *Cronobacter* species were determined via a phylogenetic tree created from the RFLP patterns.

1. Introduction

Bacteria from the genus *Cronobacter* are Gram-negative facultative anaerobic rods belonging to the family *Enterobacteriaceae* (Farmer et al., 1980; Iversen et al., 2008). They are opportunistic pathogens that mostly cause sporadic outbreaks of illness in the adult population, especially in the immunocompromised, the elderly, patients undergoing extended hospital stays, and those with acute, chronic, or serious illnesses (FAO/WHO, 2008; Forsythe et al., 2014; Kucerova et al., 2011). They are also associated with serious neonatal infections (FAO/WHO, 2006; Masood et al., 2015; van Acker et al., 2001), being able to cause neonatal meningitis, necrotising enterocolitis and sepsis (Iversen and Forsythe, 2003; Muytjens et al., 1983).

These bacteria have an interesting and still-evolving taxonomy. Previously known as *Enterobacter sakazakii*, in 2007 they were reclassified into a new genus, *Cronobacter* (Iversen et al., 2007a). Currently, this genus consists of seven species, namely *Cronobacter sakazakii*, *C. malonaticus*, *C. muytjensii*, *C. turicensis*, *C. universalis*, *C. condimenti* (which currently exists as only one strain worldwide) and *C. dublinensis* (which has three subspecies: *C. dublinensis* subsp. *dublinensis*, *C. dublinensis* subsp. *lactaridi* and *C. dublinensis* subsp. *lausannensis*) (Iversen et al., 2008; Joseph et al., 2012a). In 2013, three new species were proposed for inclusion (*C. helveticus*, *C. pulveris* and *C. zurichensis*) (Brady et al., 2013), but in the following year they were excluded from *Cronobacter* spp. and divided into two separate genera (*Siccibacter* and *Franconibacter*) (Stephan et al., 2014).

Owing to their ubiquitous nature, *Cronobacter* strains have been isolated from various environmental samples, as well as from a wide spectrum of foods and food ingredients (Friedemann, 2007; Gičová et al., 2014; Li et al., 2014; Norberg et al., 2012; Turcovsky et al., 2011; Vojkovska et al., 2016). Although fatal infant infections caused by *Cronobacter* spp. have been epidemiologically linked to the consumption of contaminated powdered infant formula, the plant environment is considered to be the natural habitat for these bacteria (Schmid et al., 2009). Indeed, foods of plant origin represent the most common source of *Cronobacter* isolation (Sani and Odeyemi, 2015) and, thus, people using a standard diet can be affected by *Cronobacter*. To date, only three species, *Cronobacter sakazakii*, *C. malonaticus* and *C. turicensis*, have been associated with serious human illnesses (Kucerova et al., 2010). This highlights the need for a simple and rapid method that is able to reliably distinguish between all species of this genus.

Traditional phenotypic discrimination of the *Cronobacter* species is based on biotyping, and genotypic identification is based on

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sequence analysis of the 16S rRNA gene (Iversen et al., 2006; Turcovsky et al., 2011). However, these methods are time-consuming and unreliable (Iversen et al., 2004, 2007b). While the sequencing of the *fusA* gene is a robust technique capable of distinguishing between species as closely related as *C. sakazakii* and *C. malonaticus* (Baldwin et al., 2009; Joseph et al., 2012b) and a complete multilocus sequence analysis has been proposed (Brady et al., 2013; PubMLST, 2015), these sequencing methods require special equipment and are time consuming. Compared with these methods, polymerase chain reaction (PCR) of a suitable part of a chosen gene appears to be a more simple and rapid tool for the purpose.

The classification of many bacterial families is based on PCR of the 16S rRNA gene. Despite this, taxonomic studies of *Enterobacteriaceae*, especially of *Cronobacter* spp., have shown that the *rpoB* gene (coding the β-subunit of DNA-dependent RNA polymerase) is a better alternative (Adekambi et al., 2009; Li et al., 2012; Mollet et al., 1997). The *rpoB* gene is a housekeeping gene, 3400–4200 bp long with a variable part in position 2300–3300 (Adekambi et al., 2009). A species-specific PCR system targeting the *rpoB* gene has been published (Lehner et al., 2012; Stoop et al., 2009). However, the primers used were not specific enough and led to many false positive and negative results (Jackson et al., 2014). In 2011, the PCR-restriction fragment length polymorphism (PCR-RFLP) protocol was introduced (Strydom et al., 2011). It was based on a 600 bp *rpoB* amplicon and the combination of two restriction endonucleases (*Csp*6I and *Hin*P1I), but only included the five *Cronobacter* species known at that time. Furthermore, this protocol is problematic due to simultaneous digestion with two endonucleases. Nevertheless, the PCR-RFLP method is now widely used in the field of food microbiology and food control for the identification of various organisms at the species level (Blažková et al., 2015; Duffy et al., 2015; Mueller et al., 2015; Sumathi et al., 2015; Sun and Liu, 2014; Tofalo et al., 2014).

In this study, we present a reliable new method for the differentiation of all seven *Cronobacter* species based on PCR amplification of the *rpoB* gene using specific new primers followed by restriction fragment length polymorphism analysis. The schema for species differentiation is created from a set of patterns obtained by the separate digestion of the PCR amplicon with three restriction endonucleases. We show that our method can be easily adapted to the differentiation of *Cronobacter* strains isolated from food.

2. Materials and methods

2.1. Bacterial strains

A total of 80 *Cronobacter* strains of all seven species were used, together with 10 *Franconibacter*, 2 *Siccibacter* and 3 *Enterobacter* strains, all from our bacterial collection (Table 1). All strains were characterised by biochemical tests, genus-specific PCRs (Hassan et al., 2007; Keyser et al., 2003; Lehner et al., 2004), species-specific PCRs (Lehner et al., 2012; Stoop et al., 2009) (data not shown) and *fusA* sequencing (Baldwin et al., 2009; Joseph and Forsythe, 2012). The strains were stored at −80 °C with 50% glycerol in a ratio of 1:1.

The strains were cultivated on Brain Heart Infusion Agar (BHA, Oxoid, United Kingdom) at 37 °C for 24 h. One separate colony from the BHA plate was transferred onto and further cultivated on *Enterobacter sakazakii* Isolation Agar (ESIA, AES Laboratoire, France) at 42 °C for 24 h. A blue-green ESIA colony was inoculated into Brain Heart Infusion Broth (BHI, Oxoid, United Kingdom) and cultivated at 37 °C for 18 h.

2.2. Isolation of Cronobacter spp. From spice

Bacterial strains were isolated from commercial spice samples of dried basil and ground chilli. Separately, each spice (10 g) was mixed with 90 mL of Lauryl Tryptose Broth (LSB, Oxoid, UK) and incubated in a sterile Whirl-Pak Filter Bag (Nasco, USA) at 37 °C for 24 h. Subsequently, and in parallel, 15 μL of bacterial suspension were inoculated on ESIA (cultivation at 42 °C for 24 h) and BHA (cultivation at 25 °C for 24 h). After 24 h, all of the suspected colonies, blue-green on ESIA and richly yellow on BHA, were inoculated on ESIA and cultivated at 42 °C for 24 h. The single ESIA colony was inoculated into BHI and cultivated at 37 °C for 18 h. The isolated bacterial strains were stored at −80 °C with 50% glycerol in a ratio of 1:1.

2.3. DNA isolation

DNA was isolated from the bacteria cultivated in BHI using GenElute Bacterial Genomic DNA Kit (Sigma, USA). The DNA concentrations were determined photometrically (NanoDrop, 2000 Spectrophotometer, Thermo Fisher Scientific, USA) and the samples stored at −20 °C.

2.4. fusA sequencing

The amplification and sequencing of the *fusA* gene was performed according to a previously published procedure (PubMLST, 2015). The FastStart *Taq* DNA Polymerase dNTPack kit (Roche, Germany) was used for the amplification of a suitable part of the *fusA* gene; the QIAquick PCR Purification Kit (QIAGEN, USA) was used for amplicon purification prior to sequencing. Sequencing was carried out by GATC Biotech Company (Germany).

2.5. PCR of rpoB gene

Using FastPCR 2.6 software (PrimerDigital), new primers were designed on the basis of the whole genome sequence of *C. sakazakii* ATCC BAA-894. The same software was used for *in silico* PCR. The genome sequence was obtained from GenBank (GenBank, 2015). Genetyx 5.2 software (Genetyx Corporation) was used for *in silico* restriction analysis.

The amplification of the variable region of the *rpoB* gene was performed using our newly designed primers, rpoB-1939F (5′-TTGGTCACTTGCCGCAGCAAAGG-3′) and rpoB-3553R (5′-ACG-CAGGTTTTCAGCCAGACG-3′). The FastStart *Taq* DNA Polymerase dNTPack kit (Roche, Germany) was used for gene amplification. The PCR reaction mixture contained 0.4 μL of polymerase (2 U), 5 μL of 10× buffer, 3 μL of $MgCl_2$ (1.5 mM), 1 μL of dNTPs (0.2 mM), 1.5 μL of each primer (0.3 μM), 4 μL of DNA template (300–600 ng genomic DNA per reaction) and 33.6 μL of nuclease-free water (Thermo Fisher Scientific, USA) in a total volume of 50 μL; the parentheses show the final concentrations of the reaction mixture components. The thermal cycling parameters (T-professional Basic Gradient thermocycler, Biometra, Germany) were as follows: initial denaturation at 94 °C for 4 min; 35 cycles of denaturation at 92 °C for 30 s; annealing at 67 °C for 30 s; and elongation at 68 °C for 3 min. Thermal cycling was completed by a final elongation step at 72 °C for 7 min.

The correct molecular size of the *rpoB* amplicons was proved by electrophoresis (TAE electrophoresis buffer, 30 min, 90 V) on a 1% (w/v) agarose gel. The amplicons in the gel were stained with 0.02 μL/mL of DNA Stain G (Serva, Germany) and visualised under UV light. OGeneRuler™ 1 kb Plus DNA Ladder (Thermo Fisher Scientific, USA) was used as a molecular size standard.