Development and evaluation of rpoB based PCR systems to differentiate the six proposed species within the genus Cronobacter

Stoop, B ; Lehner, Angelika ; Iversen, C ; Fanning, S ; Stephan, Roger

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Short communication

Development and evaluation of *rpoB* based PCR systems to differentiate the six proposed species within the genus *Cronobacter*

B. Stoop[1], A. Lehner[1], C. Iversen[2], S. Fanning[2], R. Stephan[1]*

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, CH-8057 Zurich, Switzerland

[2]Centre for Food Safety, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland.

* Corresponding author:

R. Stephan, Institute for Food Safety and Hygiene, Vetsuisse Faculty University of Zurich, Winterthurerstr. 272, CH-8057 Zurich, Switzerland, Phone 0041-44-6358651, fax 0041-44-6358908, e-mail stephanr@fsafety.uzh.ch

Abstract

Although there are various PCR based methods described in the literature to detect the genus *Cronobacter*, none of these methods is able to differentiate the six proposed species within the genus *Cronobacter*. Here we report for the first time different *rpoB* based PCR systems which enable the identification to species level of strains previously confirmed to belong to the genus *Cronobacter*.

Different primer pairs based on the *rpoB* sequences of the six *Cronobacter* species type strains were designed. Thereafter, 58 target and non-target strains, previously described in the *Cronobacter* taxonomy paper (Iversen et al. 2007a), were included for the specificity evaluation. *C. turicensis, C. muytjensii, C. dublinensis* and *C. genomospecies* 1 can be reliably identified by the proposed single primer pairs. Only target strains showed a correctly sized amplification product, whereas no amplification product was obtained for all non-target strains used in this study (100% specificity). However, as the *rpoB* gene sequences of *C. sakazakii* and *C. malonaticus* are closely related, a two step procedure is necessary in order to discern *C. malonaticus* from *C. sakazakii*. We therefore recommend a two-step procedure in which the primer pairs Cmalf/Cmalr are used in a follow up PCR on strains that are found to be positive in the amplification with the *C. sakazakii* specific primers Csakf/Csakr.

Keywords: *Cronobacter* spp., PCR, *rpoB* gene, species identification

1. Introduction

E. sakazakii, previously known as yellow-pigmented *Enterobacater cloacae*, was described as a new species in 1980 (Farmer et al., 1980), when the presence of certain biochemical traits, antibiotic susceptibilities and DNA relatedness provided sufficient evidence to distinguish the species from *E. cloacae*. However, members of this species were from the beginning described as relatively heterogeneous on the phenotypic as well as on the molecular level (Farmer et al., 1980, Iversen et al., 2004, Lehner et al., 2004).

Updating the original taxonomy of *E. sakazakii* by using a polyphasic approach has resulted in the definition of six new species based on extensive geno- and phenotypic evaluations. In order to facilitate their continued inclusion in schemata for the diagnosis and the microbiological monitoring of food products, it has been proposed that these species be moved to a novel genus *Cronobacter* (Iversen et al., 2007a, Iversen et al., 2008), with the novel genus being contaxic with *E. sakazakii*. The definition of six species within the genus *Cronobacter* is further supported by a recent study, where a multilocus sequence analysis (MLSA) approach was used (Kuhnert et al. 2009).

In the last few years, different PCR methods have been developed that enable detection of *Cronobacter* spp. (Malorny and Wagner, 2005, Seo and Brackett, 2005, Lehner et al., 2006, Liu et al., 2006, Mohan Nai and Venkitanarayanan, 2006, Mullane et al., 2006). Up to now, a 1,6-α-glucosidase based conventional PCR and a *dnaG* based RT-PCR system are the most extensively evaluated methods (Iversen et al. 2007b). However, none of these methods can differentiate the various species within the genus *Cronobacter*.

Therefore, the aim of the present study was to develop and evaluate conventional PCR systems which enable the differentiation on species level for strains previously identified as *Cronobacter* spp.

2. Materials and methods

Bacterial strains

Overall, 32 *C. sakazakii* strains (E266, E269, E272, E274, E280, E283, E292, E302, E309, E314, E328, E393, E423, E468, E601, E602, E604, E607, E620, E621, E622, E624, E627, E632, E736, E739, E750, E761, E768, E775, E796, E828), three *C. malonaticus* strains (E265, E825, E829), eight *C. turicensis* strains (3032, E609, E625, E626, E676, E681, E688), two *C. genomospecies* strains (E680, E797), six *C. muytjensii* strains (E456, E488, E603, E616, E769, E888) and seven *C. dublinensis* strains (E464, E465, E515, E791, E798, E799, DFS237, DES 187) with isolates originating from human, food and environmental origin were included in this study. The selected strains were part of a taxonomy study performed by Iversen et al. (2007a). This fact guaranteed the correct identification of all strains used for the specificity evaluation.

*rpo*B amplification and direct sequencing

For *rpoB* gene amplification, reaction mixtures (total volume 50 µl) containing primers RpoB-F (5'-AAC CAG TTC CGC GTT GGC CTG G-3') and RpoB-R (5'-CCT GAA CAA CAC GCT CGG A-3') (Mollet et al. 1997) at 20 pmol each were prepared by using the GoTaq Green Master Mix (Promega, Madison, WI). As PCR conditions, 90 s at 94°C followed by 40 cycles at 94 °C for 10 s, 55°C for 20 s and 72°C for 50 s were applied. Cycling was completed by a final elongation step at 72°C for 5 min (Mollet et al., 1997). The amplification was performed in a T3 thermocycler (Biometra, Germany).

After PCR the reaction products were separated on a 1% agarose gel, stained with ethidium bromide and visualized under UV light. The correct size products were excised from the gel and purified using the MinElute™ gel extraction kit (Qiagen, Switzerland). The products were thereafter sequenced. Sequencing of the *rpoB* gene products was outsourced (Microsynth, Balgach, Switzerland).

98

99 **Design of species specific primers**

100 Primer sequences were designed by using the *rpoB* sequences of the following strains: *C.*
101 *sakazakii* (ATCC 29544^T), *C. malonaticus* (LMG 23826^T, E265, E829), *C. turicensis* (LMG
102 23827^T), *C. genomospecies 1* (NCTC 9529^T), *C. muytjensii* (ATCC 51329^T), *C. dublinensis*
103 (LMG 23825^T, LMG 23823^T, LMG 23824^T).
104 Primer sequences were chosen by multiple alignment analysis of the respective *rpoB*
105 sequences using MultAlign (Corpet F., 1988) and ClustalW (Thompson et al., 1994). The
106 primer sequences were evaluated for their ability to form homo- and heterodimers as well as
107 hairpins by using oligo-analyzer 3.1 (Integrated DNA technologies, U.S.). All primers were
108 synthesized by Microsynth (Balgach, Switzerland).
109

110 **PCR reaction conditions**

111 For amplification, reaction mixtures (total volume 50 µl) containing GoTaq Green Master
112 Mix (Promega, Madison, WI) with a final concentration of 1.5 mM $MgCl_2$, 200 µM dNTPs
113 each and primers (Table 1) at 10 pmol each were prepared. Thermal cycling was carried out
114 using an initial denaturation step of 94°C for 3 min, followed by 30 cycles of denaturation at
115 94°C for 1 min and 30 sec of annealing. Annealing temperature as well as elongation time
116 were chosen dependent on expected fragment size and primer constitution (Table 1).
117 Elongation was performed at 72°C.
118 Amplification conditions were optimized by gradually increasing the annealing temperature in
119 the assay. The reaction products were resolved on a 1.5% agarose gel followed by ethidium
120 bromide staining and examination under UV light. The PCR systems were tested on extracted
121 DNA (DNeasy® Blood & Tissue Kit, Qiagen, Switzerland) and on boiled colony material.
122

123 **Accession number of *rpoB* sequences**

C. sakazakii (ATCC 29544^T, FJ717657), *C. malonaticus* (LMG 23826^T, FJ717659), *C. turicensis* (LMG 23827^T, FJ717652), *C. genomospecies 1* (NCTC 9529, FJ717656), *C. muytjensii* (ATCC 51329^T, FJ717658); *C. dublinensis* (LMG 23825^T, FJ717655; LMG 23823^T, FJ717653; LMG 23824 ^{T}, FJ717654).

Results and Discussion

The *rpoB* gene was described as a useful target for bacterial identification and phylogenetic studies (Mollet et al., 1997). We therefore decided to use this target gene to develop different conventional PCR systems which enable strains previously confirmed as belonging to the genus *Cronobacter* to be further discriminated to the species level. The evaluation of the specificity of all primer pairs with 32 *C. sakazakii,* three *C. malonaticus*, eight *C. turicensis,* seven *C. dublinensis,* six *C. muytjensii* and two *C. genomospecies* strains are summarized in Tables 2 and 3. With the primer pairs Cturf/Cturr, Cdubf/Cdubr, Cmuyf/Cmuyr and Cgenomof/Cgenomor all target strains showed a correctly sized amplification product, whereas no amplification product was obtained for all non-target strains (100% specificity). As the *rpoB* gene sequences for *C. sakazakii* and *C. malonaticus* are very closely related, a two step procedure in which the primer pairs Cmalf/Cmalr are used in a follow up PCR on strains that have shown to be positive in the amplification with the *C. sakazakii* primers Csakf/Csakr is necessary in order to reliably discern *C. malonaticus* from *C. sakazakii*. Originally, the malonate positive strains were thought to be a subspecies within the species *C. sakazakii* (Iversen et al. 2007a). However, more recent data provided evidence that *C. sakazakii* and *C. malonaticus* are indeed separate species (Iversen et al. 2008).

In conclusion, we propose these *rpoB* based PCR assays, in combination with a *Cronobacter* genus specific PCR, as a reliable and time saving metod to discriminate *Cronobacter* spp. strains to the species level.

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