# Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *C. helveticus, C. pulveris*, and *C. zurichensis*

E. Jackson, H. Sonbol, N. Masood & S.J. Forsythe

Pathogen Research Centre
School of Science and Technology
Nottingham Trent University
Clifton Lane
Nottingham, UK
NG11 8NS

Tel: 0115 8483529
Fax: 0115 8486636

Corresponding author: Prof SJ Forsythe,stephen.forsythe@ntu.ac.uk

**Abstract**

In 2013, *Enterobacter helveticus, E. pulveris* and *E. turicensis*, were reclassified as *Cronobacter helveticus*, *C. pulveris* and *C. zurichensis*, respectively. Previously these species had been used as negative controls for some *Cronobacter* detection assays. This study examined cultural, biochemical and molecular *Cronobacter* detection and identification assays, with emphasis on the new species. Additionally, 32 *Cronobacter* genomes were examined for the presence of PCR target genes using the BLAST function of the online *Cronobacter* BIGSdb facility. The results of the cultural methods varied and no single medium was able to correctly detect all *Cronobacter* spp. Since the supporting databases have not been updated to include the *Cronobacter* genus, *Enterobacter sakazakii* was returned for four strains of the newly reclassified species with ID32E and none with API 20E. PCR probes targeting *rpoB* and *ompA* could not correctly identify the new *Cronobacter* spp., due to primer specificity or absent target genes. As neonates have been identified as a high-risk group for infection, international standards require the absence of all *Cronobacter* species in powdered infant formula. However, many conventional detection methods cannot correctly identify the newly recognized species. Conversely, DNA sequence-based methods can adapt to taxonomic revisions and will likely become more common.

Keywords: *Cronobacter*, detection methods, identification methods

## 1. Introduction

*Cronobacter* spp. are members of the family *Enterobacteriaceae* that can cause rare but serious infections in humans (FAO-WHO, 2006; Farmer et al., 1980; Iversen and Forsythe, 2003; van Acker et al., 2001). Severe symptoms, including necrotizing enterocolitis and meningitis, have been observed in infected neonates with powdered infant formula (PIF) identified as a route of transmission (FAO-WHO 2004 and 2006; Himelright et al., 2002; Simmons et al., 1989; van Acker et al., 2001). Though only *C. sakazakii, C. malonaticus* and *C. turicensis* have been linked with human illnesses, current international microbiological standards require the absence of all *Cronobacter* species in PIF (test volume 10g), demonstrating the need for specific detection and identification methods (CAC, 2008; Joseph et al., 2012c).

The development and evaluation of methods for the detection and identification of *Cronobacter* from PIF has involved inclusivity and exclusivity strain testing with target and related non-target organisms, respectively. However although the taxonomic definition of *Cronobacter* has changed in recent years, not all methods have adapted to these changes (Brady et al., 2013; Iversen et al., 2008b; Joseph et al., 2012a). Instead there has been a continued reliance on phenotypic identification and biochemical profiling of presumptive *Cronobacter* isolates for their speciation (Cruz et al., 2011; Hochel et al. 2012). The current ISO standard for the detection of *Cronobacter* in PIF relies on cultural and biochemical methods (ISO 2006). Yet, many of these tests have been found to lack sufficient robustness for this diverse genus (Baldwin et al., 2009; Cetinkaya et al., 2013; Joseph and Forsythe, 2012; Joseph et al., 2013). For example, the *C. sakazakii* type strain ATCC 29544 is unable to grow at the raised temperature of 44°C required by some approved isolation methods (Besse et al., 2006; Nazarowec-White & Farber 1997; ISO 2006). Additionally, some commercial phenotyping kits used in the ISO and FDA methods have continued to use the former name *Enterobacter sakazakii* in their identification schemes, which generates an additional source of confusion as this name is no longer taxonomically valid. Additionally an improved knowledge of the diversity of the *Cronobacter* genus, based on multilocus sequence analysis and whole genome sequencing, has shown that speciation by biotyping is also unreliable (Baldwin et al., 2009; Cetinkaya et al., 2013; Iversen et al., 2007a; Joseph et al. 2013).

The most recent taxonomic change in the *Cronobacter* genera is the renaming of *Enterobacter helveticus*, *E. pulveris* and *E. turicensis* as *Cronobacter helveticus*, *C. pulveris* and *C. zurichensis*, respectively (Brady et al., 2013). This is likely to cause significant changes in the efficiency of *Cronobacter* test methods since these three species were previously used as negative control organisms during method evaluation because they are closely related to *Cronobacter* species. Examples include the development of *Cronobacter* screening broth (CSB) (Iversen et al., 2008a) and molecular assays targeting *cgcA*, *rpoB,* the O-antigen locus and iron acquisition genes (Carter et al., 2013; Grim et al. 2012; Jarvis et al., 2011; Mullane et al. 2008; Strydom et al. 2011). Additionally, the cultural and PCR methods described in the FDA Bacteriological Analytical Manual (BAM) included strains of the newly reclassified species as negative controls (Chen et al. 2012).

DNA-based identification methods using DNA probes and PCR amplicon detection are regarded as more reliable than phenotyping; however, they depend upon the accuracy of the initial primer design. Hence, the absence of target genes or sequence variation in primer binding sites in the newly reclassified species may lead to false negative results or misidentification of the species. Target genes for PCR probe based methods include *cgcA*, *gyrB*, *ompA*, *rpoB*, *gluA*, *dnaG*, *zpx*, iron acquisition genes, the macromolecular synthesis operon, the 16S rRNA gene, and the 16S-23S intergenic transcribed spacer (Carter et al., 2013; Grim et al., 2012; Hassan et al., 2007; Huang et al., 2012; Kothary et al., 2007; Lehner et al., 2006b; Lehner et al., 2012; Liu et al., 2006; Mohan-Nair and Venkitanarayanan, 2006; Seo and Brackett, 2005; Stoop et al., 2009). As given already, several of these methods used strains of *E. helveticus*, *E. pulveris* or *E. turicensis* as negative controls in the primer design stage due to their close relationship to the *Cronobacter* genus (Carter et al., 2013; Chen et al. 2012; Jarvis et al., 2011; Mullane et al. 2008). In contrast, phylogenetic and DNA sequencing based methods can be easily updated in response to taxonomic re-evaluations in the *Cronobacter* genus, but these methods are not without their own problems. The 16S rDNA gene has been problematic as a marker in *Cronobacter* as it is present in multiple copies within a single genome and these copies contain microheterogenities (Baldwin et al. 2009). Additionally, the closely related *C. sakazakii* and *C. malonaticus* were indistinguishable based on the 16S rRNA sequences (Iversen et al. 2008a, Strydom et al., 2012b). Hence DNA sequence-based methods for single loci (ie. *fusA*) and multilocus sequence typing (MLST) are becoming more popular methods for species identification of *Cronobacter* isolates (Baldwin et al., 2009; Brady et al., 2013; Huang and Huang, 2013; Kuhnert et al., 2009; Li et al., 2012; Joseph et al., 2012c). In addition, as part of the Bacterial Isolate Genome Sequence Database (BIGSdb), a specific repository for all *Cronobacter* genomes sequenced to date has been established with open access at www.pubMLST.org/*Cronobacter*. The *Cronobacter* BIGSdb enables the scalable analysis of *Cronobacter* genomes, representing all 10 species, for genes of interest (Maiden et al., 2013). Lastly, the *Cronobacter* seven loci multilocus sequence typing (MLST) scheme has recently been extended online to include *ompA* and *rpoB* sequences such that these alleles can add to taxonomic evaluations (Tax-MLST).

The taxonomic revisions within the *Cronobacter* genus challenge the reliability of some detection and identification methods and re-evaluation is needed to ensure compliance with international microbiological safety requirements for the absence of all *Cronobacter* species in PIF (CAC 2008). This study examined the genotypic and phenotypic characteristics of *Cronobacter* spp., with a particular focus on the recently reclassified species of *C. helveticus*, *C. pulveris*, *C. zurichensis*. Isolates were analyzed using a range of *Cronobacter* detection and identification methods to determine which methods and strains produced false negative or false positive results.

## 2. Materials and Methods

### *2.1. Bacterial strains*

A total of twenty-seven bacterial strains were used for the laboratory evaluation of various methods, as given in Table 1. The selected strains included the type strains of each of the seven original *Cronobacter* spp., multiple strains of *C. helveticus*, *C. pulveris* and *C. zurichensis*. These had been previously identified using 7-loci MLST and whole genome sequencing. Further details can be obtained from www.pubmlst.org/cronobacter . The negative control strains of *Escherichia hermanii*, *Pantoea* spp. and *Buttauxiella nokiae* had been previously identified using 16S rDNA sequencing. These latter strains have previously produced false positive results in cultural or molecular *Cronobacter* detection methods. Strains were stored in 20% glycerol at -80°C and were resuscitated on tryptic soy agar (TSA) at 25°C for 72 hours. Single colonies were streaked to TSA for purity and incubated at 37°C for 24 hours before use.

*2.2. Cultural and biochemical analyses*

Strains were tested for their ability to grow in *Enterobacteriaceae* enrichment broth (EE; Oxoid ThermoFisher, Basingstoke, UK), *Cronobacter* selective broth with 10 mg/L vancomycin (CSB; Oxoid ThermoFisher, Basingstoke, UK) (Iversen et al. 2008b) and modified lauryl sulphate broth with 0.5 M sodium chloride and 10 mg/L vancomycin (mLSB) (Guillaume-Gentil et al., 2005). Each medium was inoculated with a single colony from the TSA plate. All broths were incubated at 37°C. Additionally, CSB and mLSB were incubated at 42°C and 44°C, respectively. Cultures were observed for growth after 24, 48 and 72 hours, as appropriate. Growth was indicated by turbidity in EE and mLSB, and by turbidity and a colour change from purple to yellow in CSB (Druggan and Iversen, 2009; Guillaume-Gentil et al., 2005; Iversen et al., 2008a; Iversen and Forsythe, 2007; Lehner et al., 2006).

Strains were also assessed for their ability to produce typical colony morphologies on TSA (Oxoid ThermoFisher, Basingstoke, UK), Druggan-Forsythe-Iversen agar (DFI; Oxoid, Basingstoke, UK) and violet red bile glucose agar (VRBGA; Oxoid ThermoFisher, Basingstoke, UK). Each plate was streaked using a single colony from the stock plate. Plates were incubated at 37°C and examined for typical *Cronobacter* colony appearance after 24, 48 and 72 hours. Typical *Cronobacter* colonies are yellow on TSA, blue-green on DFI and red or purple with a halo on VRBGA (Iversen et al., 2004; Iversen and Forsythe, 2007; Lehner et al., 2006; Strydom et al., 2012a). Mucoid colonies may also be observed for some strains on VRBGA (Strydom et al., 2012a). All strains were subject to phenotyping using the API 20E and ID 32E test kits (bioMerieux, France), according to the manufacturer's instructions. The databases at https://apiweb.biomerieux.com were used for species identification. Version 4.1 was used for the API 20E tests and version 3.0 was used for the ID32E tests.

*2.3. Genome searching for PCR target genes*

Using the BLAST function of the online *Cronobacter* BIGSdb facility (www.pubMLST.org/*Cronobacter*), the full genome sequences of 32 *Cronobacter* strains were examined for the presence of target gene sequences used in the original design of PCR primers and

probes for a variety of detection methods. Genes and accession numbers are shown in Table 2a. The presence of genes was reported according to arbitrary divisions. Genes were considered present if ≥90% of the target sequence was detected. Partially present genes were defined by the detection of 50-90% of the target gene. If <50% of the target gene was detected, the gene was considered to be absent. Absent genes were confirmed by genome sequence alignment using WebAct (http://www.webact.org/WebACT/home).

*2.4. PCR detection and identification*

A single colony of each strain was suspended in 100 µl sterile distilled water and boiled at 100°C for 10 minutes. The PCR method targeting *ompA* was performed as described for boiled cell lysate (10 µl) by Mohan-Nair and Venkitanarayanan (2006). The *rpoB* method was performed as described by Stoop et al. (2009) and Lehner et al. (2012) for boiled colony lysate (5 µl). Primer sequences for both assays can be found in Table 2b. Because a different set of primers is used to identify each species, the type strains of *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C. sakazakii*, *C. turicensis* and *C. universalis* were tested only with the appropriate primer sets and therefore served as positive controls. PCR products were visualized on a 1.5% agarose gel stained with SYBR safe.

## 3. Results

*3.1. Cultural detection*

The results of the cultural detection methods are summarized in Table 3. All *Cronobacter* and non-*Cronobacter* strains exhibited growth in EE broth at 37°C. Except for one *C. helveticus* strain (1975), all *Cronobacter* spp. were capable of growth in mLSB at 37°C; however, the growth of many strains, including all of the newly reclassified species, was inhibited at 44°C. *C. sakazakii* exhibited growth at 44°C only after the incubation time was extended from 24 to 48h. In CSB, all *Cronobacter* spp., except *C. helveticus* and *C. zurichensis*, were able to grow and produce the expected colour change (purple to yellow) at both 37°C and 42°C. The only species affected by the difference in temperature were the *Pantoea* spp. Both *Pantoea* strains displayed positive reactions at 37°C, but negative reactions after 24 hours at 42°C. *Pantoea* strain 44 did exhibit a positive reaction at 42°C, but only after incubation for 48 hours.

Almost all strains of the newly reclassified species produced non-pigmented colonies on TSA following incubation at 37°C for 24 hours; however, all but one of these strains (*C. helveticus* 1344) showed some degree of yellow colouration following incubation at 25°C for 72 hours. Some strains did show a slight darkening of the yellow pigment after 72 hours of incubation at 37°C, but five stains of *C. helveticus*, 2 strains of *C. pulveris* and one strain of *C. zurichensis* were not yellow at this temperature, regardless of incubation time. All strains were able to grow on VRBGA, and a variety of colony morphologies were observed. As shown in Table 3, some strains produced large, mucoid

colonies with a very little pink color, while others produced small, pink to purple colonies. Of the newly reclassified species only *C. zurichensis* 2025 produced mucoid colonies. All of the *Cronobacter* and non-*Cronobacter* strains, except the *Pantoea* spp., showed typical blue-green colonies on DFI agar after 24 hours at 37°C.

### *3.2. Biochemical identification*

The results of the API 20E and ID32E assays are shown in Table 4. The API 20E identified six of the seven species type strains as *Enterobacter sakazakii*, but four of these identifications were based on doubtful profiles. This test identified *C. turicensis* as *Enterobacter gergoviae* with 91.2% identification and only a 7.0% identification as *E. sakazakii*. The *C. zurichensis* strains were identified as *Klebsiella pneumoniae* ssp. *ozaenea* or *Buttauxiella agrestis*. *C. helveticus* 1208 was identified as *Yersinia pseudotuberculosis* and *C. pulveris* 1390 was identified as *Citrobacter freundii*. The remaining strains of *C. helveticus* and *C. pulveris* were identified as *Escherichia vulneris* with the API 20E. Three *C. pulveris* strains were identified as *E. sakazakii* with a 0.8% identification and the database report indicated that the identification of these strains was not valid. None of the other strains of the recently reclassified species gave possible identifications as *E. sakazakii*. Of the negative control strains, both *Pantoea* spp. were correctly identified, but the remaining strains were not correctly identified to the species level.

For the ID32E phenotyping method, most identifications with doubtful or unacceptable profiles did not return percentage identifications. The type strains for *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. sakazakii*, *C. turicensis*, and *C. universalis* were identified as *E. sakazakii*. Three of these identifications were the results of 'doubtful' or 'unacceptable profiles'. The species type strain of *C. muytjensii* (ATCC 51329$^T$) was unidentified. The profiles for the type strains for *C. helveticus* and *C. pulveris* returned *E. sakazakii* as the top species identified, but the percent identifications were not given as they were identified with 'unacceptable profiles'. In contrast, the profile for the *C. zurichensis* type strain LMG23730$^T$ returned *Buttiauxella agrestis*, as did the profile for *C. zurichensis* 2025. Only *C. zurichensis* 1383 was identified as *E. sakazakii*, though with an unacceptable profile. None of the *B. noakiae*, *E. hermanii,* or *Pantoea* spp. strains were correctly identified to the species level using the ID32E system, and *E. hermanii* strain 159 was identified as *E. sakazakii* (99.9%). Fifteen of the 22 *Cronobacter* strains (59.1%) gave contradictory identifications when the results from the two kits were compared.

### *3.3. Genome searching for PCR target genes*

Gene sequences previously used to design PCR primers and probes for detection and identification of *Cronobacter* spp. were compared to the full genome sequences of 32 *Cronobacter* strains representing the whole genus (Table 2a). Genes were considered present if 90% or more of the target sequence was aligned. Partial positives were indicated by the presence of 50-90% of the target sequence. Genes were considered absent if less than 50% of the target sequence was

present. The genes for *ompA*, *rpoB*, and *gyrB* were present in all genomes, as expected since these are used in the *Cronobacter* MLST and Tax-MLST schemes. As shown in Table 5, the *cgcA* sequence was absent from all the new *Cronobacter* species, as well as *C. sakazakii* 680, two *C. dublinensis* strains, and it was only partially present in a third *C. dublinensis* strain. Similarly, the zinc metalloprotease gene, *zpx*, was only partially present in some strains.

### *3.4. PCR detection and identification*

The results of the *ompA* and *rpoB* PCR assays are shown in Table 6. The *ompA* PCR assay described by Mohan-Nair and Venkitanarayanan (2006) produced bands of the expected size of 469 bp for the type strains for the seven original *Cronobacter* spp. However, no PCR amplicons were generated with any of the strains for the three newly reclassified species; *C. helveticus*, *C. pulveris* and *C. zurichensis*.

The *rpoB* multiple primer assay utilizes a separate primer set for identification of each of the seven previously recognised *Cronobacter* spp. The primers designed for *C. sakazakii* produced slightly smaller bands for all *C. helveticus* strains. Additionally, both bands of the expected size of 514 bp and the smaller band were detected for *C. pulveris* 1393 and 1978. When tested with the *C. malonaticus* and *C. muytjensii* primers, amplicons of the expected sizes were produced for all strains of the newly reclassified *Cronobacter* spp., except *C. zurichensis* 1383. Both *E. hermanii* strains were also positive with both of these primers sets, and *Pantoea* spp. strain 1318 was positive with the *C. malonaticus* primers. None of the strains for the newly reclassified species were identified with the *C. condimenti* or *Cronobacter* genomospecies (former name for *C. universalis*) primers. No strains, including the positive control strain, produced amplicons with the *C. turicensis* primers.

## 4. Discussion

### *4.1. Cultural and biochemical detection and identification*

Following international concern over the microbiological safety of PIF, the recovery methods initially used for *Cronobacter* detection were EE broth and VRBGA, which are general media for the recovery of *Enterobacteriaceae* prior to phenotypic identification (Chen et al., 2012; Muytjens et al., 1988). As expected, pure cultures of all strains from the ten *Cronobacter* species grew in both media at 37°C, as shown in Table 3. However, since *Cronobacter* can be out-grown by other *Enterobacteriaceae*, mLSB and CSB enrichment broths were developed for the preferential isolation of *Cronobacter* from mixed cultures. Both mLSB and CSB enrichment broths utilize increased incubation temperatures (44 and 42°C respectively) to confer additional selectivity to the cultural detection of *Cronobacter* spp. (Guillaume-Gentil et al., 2005; Iversen et al., 2008a). In this study the type strains of *C. condimenti*, *C. dublinensis*, *C. sakazakii*, *C. turicensis*, C *helveticus*, *C. pulveris*, *C. zurichensis* were unable to grow in mLSB at 44°C as required in ISO/TS 22964|IDF/RM 210:2006

(Besse et al., 2006; ISO, 2006). No strains of *C. helveticus*, *C. pulveris*, or *C. zurichensis* were able to grow in mLSB at 44°C, with the exception of *C. helveticus* 1204. Most of the strains did grow in the medium at the lower temperature of 37°C, but this is not the prescribed temperature in the ISO approved method. Nazarowec-White and Farber (1997) previously reported that the *C. sakazakii* type strain ATCC 29544 was unable to grow above 41°C, and Iversen and Forsythe (2007) reported that 6% of strains then known as *E. sakazakii* were unable to grow in mLSB at 44°C. In our study, extending the incubation period at 44°C to 48h resulted in only the *C. sakazakii* strain displaying slightly greater turbidity. Though this broth was intended for use with selective or differential agars, the absence of turbidity after the prescribed 24 hour incubation indicates that *Cronobacter* spp. may not reach a high enough concentration to result in detection on agar plates (Guillaume-Gentil et al., 2005).

CSB is both a selective and differential medium, containing vancomycin to inhibit the growth of Gram-positive organisms and bromocresol purple to detect the pH change associated with sucrose utilization (Iversen et al., 2008a). This broth was designed to detect presumptive *Cronobacter* positive samples without selective or differential plating to minimize the time required to reach a negative result. Though presumptive positive samples will require further testing, according to the Iversen et al. (2008) negative results can be considered conclusive. Therefore, results for this assay were only considered positive if the expected colour change from purple to yellow was observed after 24 hours. In the current study, CSB enrichment at 42°C supported the growth of only eight of the ten *Cronobacter* species. Though incubation at 42° C was sufficient to exclude all of the negative control strains, *C. helveticus* and *C. zurichensis* were not viable in this broth at any temperature. *E. helveticus*, *E. pulveris*, and *E. turicensis* were all listed as negative control species used for development of this broth and positive results were reported for *E. pulveris* (Iversen et al., 2008a). Prior to the taxonomic reclassification, the recovery of *E. pulveris* from CSB would have been regarded as a false-positive result (color change associated with a non-*Cronobacter* isolate). However, following the taxonomic revisions, the absence of growth for *C. helveticus* and *C. zurichensis* would constitute a false-negative result with CSB (no color change associated with strains identified as *Cronobacter* species).; As current international regulations require the absence of all *Cronobacter* species in PIF (CAC, 2008), such misidentifications can be costly to industry. A batch of infant formula may be rejected due to false-positive identification of *Cronobacter* species, and infant formula containing *Cronobacter* may be mistakenly released due to false-negative identification. Given only three *Cronobacter* species have been epidemiologically- linked to infections the possible revising of international criteria to only those species should be given serious consideration.

Identification of *Cronobacter* spp. based on colony morphology can be unreliable. Yellow pigment production on TSA is often considered to be indicative of *Cronobacter* spp.; however, production of this pigment can be affected by a variety of conditions, including incubation temperature and exposure to light, making it an inconsistent and unreliable test (Druggan and Iversen, 2009; Farmer et al., 1980; Johler et al., 2010). As observed in this study, many strains, including most strains of the newly reclassified species, appeared yellow following incubation at 25°C, but not after incubation at

37°C. Although yellow pigmentation on TSA is stated in the ISO standard protocol, it has been shown that up to 21.4% of *Cronobacter* spp. do not produce yellow pigment after 72 hours of incubation at 25°C (Besse et al., 2006; ISO, 2006; Iversen and Forsythe, 2007). Though all *Cronobacter* strains in the current study produced typical blue-green colonies on DFI, this morphology was also observed for *B. nokiae* and both strains of *E. hermanii.*

Biochemical methods are often used for species identification and confirmation of suspect isolates. Biochemical panels, such as the API 20E and ID32E are popular among testing laboratories, and are used in conjunction with online databases to identify the species of bacteria based on a panel of 20 or 32 biochemical tests. However, these databases are not up to date with the current taxonomy. Though the *Cronobacter* genus was first described in 2007, these databases still report results of "*Enterobacter sakazakii*." Inadequacies in the databases have been noted by other authors, suggesting that these assays are not sufficient for identification of *Cronobacter* spp. (Fanjat et al., 2007; Iversen et al., 2004; Iversen et al., 2007b). Updating the databases will undoubtedly increase the accuracy of identification. Fanjat et al. (2007) examined *E. sakazakii* isolates and found that only 71.4% of these isolates were correctly identified with version 2.0 of the ID32E database. Modification of the database to reflect variability in carbohydrate utilization later resulted in 100% correct identification of these isolates (Fanjat et al., 2007). As demonstrated by the current study, misidentifications of *Cronobacter* spp. are common with these assays. False negative identifications are not the only concern with these methods, as *E. hermanii* 162 was misidentified as '*E. sakazakii*'. This strain could be mistaken for a *Cronobacter* spp. because it also produces blue-green colonies on DFI and yellow colonies on TSA. The possibility of false negative and false positive identifications and the lack of updated databases confirms that these biochemical panels are not sufficient to correctly identify *Cronobacter* spp. (Cetinkaya et al., 2013; Osaili and Forsythe, 2009).

### *4.2. Genome searching for PCR targets and laboratory PCR assays*

The gene *cgcA* encodes a diguanylate cyclase that is involved in signal transduction for the regulation of virulence, formation of biofilms and long-term survival of the organism. As shown in Table 5, the *cgcA* gene sequence was absent from *C. helveticus* genomes. This result was expected since the development of this identification assay used *E. helveticus* as a negative control (Carter et al., 2013). Additionally, the gene was absent from all *C. pulveris* strains and *C. sakazakii* strain 680. Partial sequences were found in *C. dublinensis* 582 and *C. zurichensis* 1974. Absence of the complete gene sequence indicates that the primer binding sites are not present. Absence of a portion of the gene sequence could also indicate the lack of one or both primer binding sites. No amplicon would be produce in either situation. Additionally, if the sequence is only partially present, but the primer biding sites are still intact, a smaller than expected amplicon could be produced. Since multiple strains lack this complete sequence, it is not sufficient for identification of *Cronobacter* spp. and this assay was not used during the laboratory portion of the current study. Similarly, *gluA*, encoding an α- glucosidase, was present in nearly all *Cronobacter* spp. Partial *gluA* sequences were detected in *C. condimenti* 1330 and *C. universalis*. The absence or partial presence of these genes

394 excluded the corresponding assays from laboratory evaluation the current study. Previously, a PCR
395 assay targeting *zpx* was able to correctly detect all *E. sakazakii* strains tested (Kothary et al. 2007).
396 However, genome searching with the *zpx* gene sequence indicated variation between the species
397 and strains. Though the gene was present in most strains, partial sequences were detected in five of
398 the 28 strains tested. This gene encodes a zinc-containing metalloprotease, and may serve as an
399 indicator of pathogenicity (Kothary et al., 2007). However the presence of only partial sequences in
400 five strains suggests that it is not suitable to detect all *Cronobacter* species or strains. *DnaG* was
401 detected in all *Cronobacter* strains, except *C. zurichensis* 1974, which contained only a fragment of
402 the target sequence. This target sequence was only 319 bp long; therefore, analysis of a larger
403 fragment may allow for the design of PCR primers capable of detection all *Cronobacter* spp.,
404 including all *C. zurichensis* strains.
405 The genes *gyrB*, *ompA*, and *rpoB* were present in the genomes of all 32 strains of *Cronobacter*
406 examined by genome searching. Though the *gyrB* primers used by Huang et al. (2013) were
407 designed for detection of only *C. sakazakii* and *C. dublinensis*, the *gyrB* gene is already part of the
408 seven loci *Cronobacter* MLST scheme (Baldwin et al. 2009). Therefore such a restricted assay is
409 unnecessary and was not included in this study. Although BLAST searching of 32 whole genomes
410 showed that *ompA* gene is present in all species (Table 5), the *ompA* gene PCR primers resulted in
411 amplification products for only the type strains of the initial seven *Cronobacter* species, and not for
412 any strains of the newly reclassified species (Table 6). Jaradat et al. (2009) also reported false
413 negative results for two strains identified as *Cronobacter* spp. when using these primers. Though
414 sequence variability was suggested to explain the lack of detection with the *ompA* primers, the
415 presence of *ompA* in all ten species is of significance as it is proposed as an important trait in the
416 invasion of host brain cells (Jaradat et al., 2009; Kim et al., 2010).
417 The results of the PCR probe assays for *ompA* and *rpoB* showed that neither method was able to
418 detect all *Cronobacter* species. The Stoop et al. (2009) and Lehner et al. (2012) *rpoB* multiple primer
419 assays were not designed for *Cronobacter* spp. detection, but for speciation of *Cronobacter* isolates.
420 The specific primer sets were designed such that amplification should only occur with each of the
421 seven target species. Hence cross-reactivity of the primers with the new species was considered.
422 The *C. sakazakii* primers produced amplicons of a slightly smaller size for all of the *C. helveticus*
423 strains, indicating sequence variation between the two species. Faint bands of both the expected
424 and smaller size were observed for two *C. pulveris* strains. These two strains were also positive with
425 the *C. malonaticus* primers. As the *C. malonaticus* primers are intended for use only with strains
426 producing positive results with the *C. sakazakii* primers, these strains could be misidentified as *C.*
427 *malonaticus*. Additionally, the primers intended to identify *C. malonaticus* and *C. muytjensii* gave
428 positive PCR products for nearly all strains of the newly reclassified species. Except for the two
429 weakly positive *C. pulveris* strains, none of the newly reclassified species would be tested with the *C.*
430 *malonaticus* primers. There is, however, a strong possibility that these species could be
431 misidentified as *C. muytjensii*. As shown in Table 6, some negative control strains also produced
432 amplicons of the expected size when tested with the *C. dublinensis*, *C. malonaticus* and *C.*
433 *muytjensii* primer sets, adding the to the confusion of species identification. The *C. turicensis* primer

set was unable to amplify any of the species in the current study. The remaining primer sets were specific to their target species. Therefore the Stoop et al. (2009) and Lehner et al. (2012) multiple primer *rpoB* assay method is no longer effective for speciating *Cronobacter* isolates. However the generic amplification and sequencing method of *rpoB* described by Stoop et al. (2009) has been incorporated into the Tax-MLST scheme which enables the speciation of *Cronobacter* isolates from a single reaction followed by phylogenetic analysis.

## 5. Conclusions

The reclassification of three *Enterobacter* species into the *Cronobacter* genus limits the utility of some current *Cronobacter* isolation and detection methods. Many published methods utilized the recently reclassified species as negative controls and, thus, these methods will no longer detect all recognized *Cronobacter* species. This is particularly important as current international microbiological standards require an absence of all *Cronobacter* species in PIF (CAC, 2008). It is not possible to ensure compliance with this standard or the safety of PIF if the methods currently in use are not capable of detecting all *Cronobacter* spp. A more practical approach could be to limit the criteria to the three *Cronobacter* species which are epidemiologically-linked to infections; *C. sakazakii, C. malonaticus* and *C. turicensis*. In addition such misidentifications can be costly to industry due to the potential rejection of a batch of infant formula due to false-positive identification, and also the possible release of infant formula containing *Cronobacter* due to false-negative identification.

PCR assays are limited by the presence of the target gene and sequence variation among strains, which may inhibit primer binding and amplification, producing false negative results in both circumstance. Conversely, the recent developments in sequence-based methods, including MLST, allow for highly specific species and strain identification, and are becoming more affordable for routine testing laboratories (Peréz-Losada et al., 2013). These methods are more reliable than subjective biochemical and morphological tests or detection based on amplification of particular gene fragment. Sequence-based methods will detect variations as small as a single base pair and can be used to accurately differentiate between species and strains. A combination of cultural and sequence-based methods offer the most reliable identification and profiling of *Cronobacter* isolates. Currently the reliable alleles for speciation include *fusA*, *rpoB* and *ompA*, with *fusA* having the advantage of over 600 sequence entries in the online MLST database. Sequence-based methods also have the advantage of being able to more easily adapt to expansion or reclassification of the genus. The reliability and adaptability of DNA sequence-based methods, including MLST, provide an advantage over biochemical and PCR probe-based methods for detection and identification of isolates from the emerging genus *Cronobacter*.

## Acknowledgements

The authors thank Nottingham Trent University and Ministry of Higher Education, Saudi and Nottingham Trent University for their financial support of this work. This publication made use of the *Cronobacter* Multi Locus Sequence Typing website (http://pubmlst.org/cronobacter/) developed by Keith Jolley and sited at the University of Oxford (Jolley & Maiden 2010). The development of this site has been funded by the Wellcome Trust.

**References**

Baldwin, A., Loughlin, M., Caubilla-Barron, J., Kucerova, E., Manning, G., Dowson, C., & Forsythe, S. (2009). Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. *BMC Microbiology, 9*, 223. doi:10.1186/1471-2180-9-223; 10.1186/1471-2180-9-223.

Besse, N. G., Leclercq, A., Maladen, V., Tyburski, C., & Lombard, B. (2006). Evaluation of the international organization for Standardization International dairy federation (ISO-IDF) draft standard method for detection of *Enterobacter sakazakii* in powdered infant food formulas. *Journal of AOAC International, 89*(5), 1309-1316.

Brady, C., Cleenwerck, I., Venter, S., Coutinho, T., & De Vos, P. (2013). Taxonomic evaluation of the genus *Enterobacter* based on multilocus sequence analysis (MLSA): Proposal to reclassify *E. nimipressuralis* and *E. amnigenus* into *Lelliottia* gen. nov. as *Lelliottia nimipressuralis* comb. nov. and *Lelliottia amnigena* comb. nov., respectively, *E. gergoviae* and *E. pyrinus* into *Pluralibacter* gen. nov. as *Pluralibacter gergoviae* comb. nov. and *Pluralibacter pyrinus* comb. nov., respectively, *E. cowanii, E. radicincitans, E. oryzae* and *E. arachidis* into *Kosakonia* gen. nov. as *Kosakonia cowanii* comb. nov., *Kosakonia radicincitans* comb. nov., *Kosakonia oryzae* comb. nov. and *Kosakonia arachidis* comb. nov., respectively, and *E. turicensis*, *E. helveticus* and *E. pulveris* into *Cronobacter* as *Cronobacter zurichensis* nom. nov., *Cronobacter helveticus* comb. nov. and *Cronobacter pulveris* comb. nov., respectively, and emended description of the genera *Enterobacter* and *Cronobacter*. *Systematic and Applied Microbiology, 36*(5), 309-319. doi:10.1016/j.syapm.2013.03.005; 10.1016/j.syapm.2013.03.005

Carter, L., Lindsey, L. A., Grim, C. J., Sathyamoorthy, V., Jarvis, K. G., Gopinath, G., Hu, L. (2013). Multiplex PCR assay targeting a diguanylate cyclase-encoding gene, *cgcA,* to differentiate species within the genus *Cronobacter*. *Applied and Environmental Microbiology, 79*(2), 734-737. doi:10.1128/AEM.02898-12; 10.1128/AEM.02898-12

Cetinkaya, E., Joseph, S., Ayhan, K., & Forsythe, S. J. (2013). Comparison of methods for the microbiological identification and profiling of *Cronobacter* species from ingredients used in the preparation of infant formula. *Molecular and Cellular Probes, 27*, 60-64.

Chen, Y., Lampel, K. & and Hammack, T. (2012). Bacteriological analytical manual, chapter 29: *Cronobacter*.
Retrieved 11/06/2013, from
http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm289378.htm.

512 Codex Alimentarius Commision (CAC). Code of hygienic practice for powdered formulae for infants
513 and young children, (2008).
514 Cruz, A., Xicohtencatl-Cortes, J., González-Pedrajo, B., Bobadilla, M., Eslava, C., & Rosas, I. (2011).
515 Virulence traits in *Cronobacter* species isolated from different sources. *Canadian Journal of*
516 *Microbiology, 57*(9), 735-744.
517 Druggan, P., & Iversen, C. (2009). Culture media for the isolation of *Cronobacter* spp. *International*
518 *Journal of Food Microbiology, 136*(2), 169-178. doi:10.1016/j.ijfoodmicro.2009.09.008;
519 10.1016/j.ijfoodmicro.2009.09.008
520 Fanjat, N., Leclercq, A., Joosten, H., & Robichon, D. (2007). Comparison of the phenotyping
521 methods ID 32E and VITEK 2 compact GN with 16S rRNA gene sequencing for the identification of
522 *Enterobacter sakazakii*. *Journal of Clinical Microbiology, 45*(6), 2048-2050. doi:10.1128/JCM.00961-
523 06
524 Farmer, J. J., Asbury, M. A., Hickman, F. W., & Brenner, D. J. (1980). *Enterobacter sakazakii*: A new
525 species of "*Enterobacteriaceae*" isolated from clinical specimens. *International Journal of Systematic*
526 *Bacteriology, 30*(3), 569-584.
527 Food and Agricultural Organization-World Health Organization (FAO-WHO). (2004). *Enterobacter*
528 *sakazakii* and other organisms in powdered infant formula.
529 Food and Agricultural Organization-World Health Organization (FAO-WHO). (2006). *Enterobacter*
530 *sakazakii and salmonella in powdered infant formula.* (No. MRA series 10). Geneva, Switzerland:
531 World Health Organization.
532 Grim, C. J., Kotewicz, M. L., Power, K. A., Gopinath, G., Franco, A. A., Jarvis, K. G., Tall, B. D.
533 (2013). Pan-genome analysis of the emerging foodborne pathogen *Cronobacter* spp. suggests a
534 species-level bidirectional divergence driven by niche adaptation. *BMC Genomics, 14*, 366-2164-14-
535 366. doi:10.1186/1471-2164-14-366; 10.1186/1471-2164-14-366
536 Guillaume-Gentil, O., Sonnard, V., Kandhai, M. C., Marugg, J. D., & Joosten, H. (2005). A simple
537 and rapid cultural method for detection of *Enterobacter sakazakii* in environmental samples. *Journal*
538 *of Food Protection, 68*(1), 64-69.
539 Hassan, A. A., Akineden, O., Kress, C., Estuningsih, S., Schneider, E., & Usleber, E. (2007).
540 Characterization of the gene encoding the 16S rRNA of *Enterobacter sakazakii* and development of
541 a species-specific PCR method. *International Journal of Food Microbiology, 116*(2), 214-220.
542 doi:10.1016/j.ijfoodmicro.2006.12.011
543 Himelright, I., Harris, E., Lorch, V., & Anderson, M. (2002). *Enterobacter sakazakii* infections
544 associated with the use of powdered infant formula-Tennessee, 2001. *J.Am.Med.Assoc., 287*, 2204-
545 2205.
546 Hochel, I., Růžičková, H., Krásný, L., & Demnerová, K. (2012). Occurrence of *Cronobacter* spp. in
547 retail foods. *Journal of Applied Microbiology, 112*(6), 1257-1265.
548 Huang, C., & Huang, L. (2013). Differentiation of *Cronobacter sakazakii* and related taxa using direct
549 sequencing, species-specific PCR, and mini-sequencing assays. *European Food Research and*
550 *Technology, 236*, 399-403.

Huang, C. H., Chang, M. T., & Huang, L. (2013). Use of novel species-specific PCR primers targeted to DNA gyrase subunit B (*gyrB*) gene for species identification of the *Cronobacter sakazakii* and *Cronobacter dublinensis*. *Molecular and Cellular Probes, 27*(1), 15-18. doi:10.1016/j.mcp.2012.08.004; 10.1016/j.mcp.2012.08.004

International Organization for Standardization (ISO). ISO/TS 22964|IDF/RM 210:2006: Milk and milk products -- Detection of *Enterobacter sakazakii*, (2006).

Iversen, C., Druggan, P., & Forsythe, S. (2004). A selective differential medium for *Enterobacter sakazakii*, a preliminary study. *International Journal of Food Microbiology, 96*(2), 133-139. doi:10.1016/j.ijfoodmicro.2004.01.024

Iversen, C., Druggan, P., Schumacher, S., Lehner, A., Feer, C., Gschwend, K., Stephan, R. (2008a). Development of a novel screening method for the isolation of "*Cronobacter*" spp. (*Enterobacter sakazakii*). *Applied and Environmental Microbiology, 74*(8), 2550-2553. doi:10.1128/AEM.02801-07; 10.1128/AEM.02801-07

Iversen, C., & Forsythe, S. (2003). Risk profile of *Enterobacter sakazakii*, an emergent pathogen associated with infant milk formula. *Trends in Food Science & Technology, 14*(11), 443-454. doi:http://dx.doi.org/10.1016/S0924-2244(03)00155-9

Iversen, C., & Forsythe, S. J. (2007). Comparison of media for the isolation of *Enterobacter sakazakii*. *Applied and Environmental Microbiology, 73*(1), 48-52. doi:10.1128/AEM.01562-06

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Joosten, H. (2007a). The taxonomy of *Enterobacter sakazakii*: Proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. *BMC Evolutionary Biology, 7*, 64. doi:10.1186/1471-2148-7-64

Iversen, C., Lehner, A., Mullane, N., Marugg, J., Fanning, S., Stephan, R., & Joosten, H. (2007b). Identification of "*Cronobacter*" spp. (*Enterobacter sakazakii*). *Journal of Clinical Microbiology, 45*(11), 3814-3816. doi:10.1128/JCM.01026-07

Iversen, C., Mullane, N., McCardell, B., Tall, B. D., Lehner, A., Fanning, S., Joosten, H. (2008b). *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. *International Journal of Systematic and Evolutionary Microbiology, 58*(Pt 6), 1442-1447. doi:10.1099/ijs.0.65577-0; 10.1099/ijs.0.65577-0

Jaradat, Z. W., Ababneh, Q. O., Saadoun, I. M., Samara, N. A., & Rashdan, A. M. (2009). Isolation of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) from infant food, herbs and environmental samples and the subsequent identification and confirmation of the isolates using biochemical, chromogenic assays, PCR and 16S rRNA sequencing. *BMC Microbiology, 9*, 225-2180-9-225. doi:10.1186/1471-2180-9-225; 10.1186/1471-2180-9-225

Jarvis, K. G., Grim, C. J., Franco, A. A., Gopinath, G., Sathyamoorthy, V., Hu, L., Tall, B. D. (2011). Molecular characterization of *Cronobacter* lipopolysaccharide O-antigen gene clusters and development of serotype-specific PCR assays. *Applied and Environmental Microbiology, 77*(12), 4017-4026. doi:10.1128/AEM.00162-11; 10.1128/AEM.00162-11

Johler, S., Stephan, R., Hartmann, I., Kuehner, K. A., & Lehner, A. (2010). Genes involved in yellow pigmentation of *Cronobacter sakazakii* ES5 and influence of pigmentation on persistence and growth under environmental stress. *Applied and Environmental Microbiology, 76*(4), 1053-1061. doi:10.1128/AEM.01420-09; 10.1128/AEM.01420-09

Jolley, K. A., & Maiden, M. C. (2010). BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics, 11*(1), 595.

Joseph, S., Cetinkaya, E., Drahovska, H., Levican, A., Figueras, M. J., & Forsythe, S. J. (2012a). *Cronobacter condimenti* sp. nov., isolated from spiced meat, and *Cronobacter universalis* sp. nov., a species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water and food ingredients. *International Journal of Systematic and Evolutionary Microbiology, 62*(Pt 6), 1277-1283. doi:10.1099/ijs.0.032292-0; 10.1099/ijs.0.032292-0

Joseph, S., Desai, P., Ji, Y., Cummings, C. A., Shih, R., Degoricija, L., Forsythe, S. J. (2012b). Comparative analysis of genome sequences covering the seven *Cronobacter* species. *PLoS One, 7*(11), e49455. doi:10.1371/journal.pone.0049455; 10.1371/journal.pone.0049455

Joseph, S., & Forsythe, S. J. (2012). Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by multilocus sequence typing and analysis. *Frontiers in Microbiology, 3*, 397. doi:10.3389/fmicb.2012.00397; 10.3389/fmicb.2012.00397

Joseph, S., Sonbol, H., Hariri, S., Desai, P., McClelland, M., & Forsythe, S. J. (2012c). Diversity of the *Cronobacter* genus as revealed by multilocus sequence typing. *Journal of Clinical Microbiology, 50*(9), 3031-3039. doi:10.1128/JCM.00905-12; 10.1128/JCM.00905-12

Joseph, S., Hariri, S., & Forsythe, S. J. (2013). Lack of continuity between *Cronobacter* biotypes and species as determined using multilocus sequence typing. *Molecular and Cellular Probes, 27*(3–4), 137-139. doi:http://dx.doi.org/10.1016/j.mcp.2013.02.002

Kim, K., Kim, K. P., Choi, J., Lim, J. A., Lee, J., Hwang, S., & Ryu, S. (2010). Outer membrane proteins A (OmpA) and X (OmpX) are essential for basolateral invasion of *Cronobacter sakazakii*. *Applied and Environmental Microbiology, 76*(15), 5188-5198. doi:10.1128/AEM.02498-09; 10.1128/AEM.02498-09

Kothary, M. H., McCardell, B. A., Frazar, C. D., Deer, D., & Tall, B. D. (2007). Characterization of the zinc-containing metalloprotease encoded by zpx and development of a species-specific detection method for *Enterobacter sakazakii*. *Applied and Environmental Microbiology, 73*(13), 4142-4151. doi:10.1128/AEM.02729-06

Kuhnert, P., Korczak, B. M., Stephan, R., Joosten, H., & Iversen, C. (2009). Phylogeny and prediction of genetic similarity of *Cronobacter* and related taxa by multilocus sequence analysis (MLSA). *International Journal of Food Microbiology, 136*(2), 152-158. doi:10.1016/j.ijfoodmicro.2009.02.022; 10.1016/j.ijfoodmicro.2009.02.022

Lehner, A., Fricker-Feer, C., & Stephan, R. (2012). Identification of the recently described *Cronobacter condimenti* by an rpoB-gene-based PCR system. *Journal of Medical Microbiology, 61*(Pt 7), 1034-1035. doi:10.1099/jmm.0.042903-0; 10.1099/jmm.0.042903-0

Lehner, A., Nitzsche, S., Breeuwer, P., Diep, B., Thelen, K., & Stephan, R. (2006). Comparison of two chromogenic media and evaluation of two molecular based identification systems for *Enterobacter sakazakii* detection. *BMC Microbiology, 6*, 15. doi:10.1186/1471-2180-6-15

Lehner, A., Riedel, K., Rattei, T., Ruepp, A., Frishman, D., Breeuwer, P., Stephan, R. (2006b). Molecular characterization of the α-glucosidase activity in *Enterobacter sakazakii* reveals the presence of a putative gene cluster for palatinose metabolism. *Systematic and Applied Microbiology, 29*(8), 609-625. doi:http://dx.doi.org/10.1016/j.syapm.2006.02.002

Li, Y., Cao, L., Zhao, J., Cheng, Q., Lu, F., Bie, X., & Lu, Z. (2012). Use of *rpoB* gene sequence analysis for phylogenetic identification of *Cronobacter* species. *Journal of Microbiological Methods, 88*(2), 316-318. doi:10.1016/j.mimet.2011.12.002; 10.1016/j.mimet.2011.12.002

Liu, Y., Cai, X., Zhang, X., Gao, Q., Yang, X., Zheng, Z., Huang, X. (2006). Real time PCR using TaqMan and SYBR green for detection of *Enterobacter sakazakii* in infant formula. *Journal of Microbiological Methods, 65*(1), 21-31. doi:10.1016/j.mimet.2005.06.007

Maiden, M. C., van Rensburg, M. J., Bray, J. E., Earle, S. G., Ford, S. A., Jolley, K. A., & McCarthy, N. D. (2013). MLST revisited: The gene-by-gene approach to bacterial genomics. *Nature Reviews.Microbiology, 11*(10), 728-736. doi:10.1038/nrmicro3093; 10.1038/nrmicro3093

Mohan Nair, M. K., & Venkitanarayanan, K. S. (2006). Cloning and sequencing of the *ompA* gene of *Enterobacter sakazakii* and development of an *ompA*-targeted PCR for rapid detection of *Enterobacter sakazakii* in infant formula. *Applied and Environmental Microbiology, 72*(4), 2539-2546. doi:10.1128/AEM.72.4.2539-2546.2006

Mullane, N., O'Gaora, P., Nally, J. E., Iversen, C., Whyte, P., Wall, P. G., & Fanning, S. (2008). Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus. *Applied and Environmental Microbiology, 74*(12), 3783-3794. doi:10.1128/AEM.02302-07; 10.1128/AEM.02302-07

Muytjens, H. L., Roelofs-Willemse, H., & Jaspar, G. H. (1988). Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *Journal of Clinical Microbiology, 26*(4), 743-746.

Nazarowec-White, M., & Farber, J. M. (1997). Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. *Letters in Applied Microbiology, 24*(1), 9-13.

Osaili, T., & Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. *International Journal of Food Microbiology, 136*(2), 214-220. doi:10.1016/j.ijfoodmicro.2009.08.006; 10.1016/j.ijfoodmicro.2009.08.006

Pérez-Losada, M., Cabezas, P., Castro-Nallar, E., & Crandall, K. A. (2013). Pathogen typing in the genomics era: MLST and the future of molecular epidemiology. *Infection, Genetics and Evolution, 16*(0), 38-53. doi:http://dx.doi.org/10.1016/j.meegid.2013.01.009

Seo, K. H., & Brackett, R. E. (2005). Rapid, specific detection of *Enterobacter sakazakii* in infant formula using a real-time PCR assay. *Journal of Food Protection, 68*(1), 59-63.

669 Simmons, B. P., Gelfand, M. S., Haas, M., Metts, L., & Ferguson, J. (1989). *Enterobacter sakazakii*
670 infections in neonates associated with intrinsic contamination of a powdered infant formula. *Infection*
671 *Control and Hospital Epidemiology, 10*(9), 398-401. Retrieved from
672 http://www.jstor.org/stable/30144207
673 Stoop, B., Lehner, A., Iversen, C., Fanning, S., & Stephan, R. (2009). Development and evaluation of
674 *rpoB* based PCR systems to differentiate the six proposed species within the genus *Cronobacter*.
675 *International Journal of Food Microbiology, 136*(2), 165-168. doi:10.1016/j.ijfoodmicro.2009.04.023;
676 10.1016/j.ijfoodmicro.2009.04.023
677 Strydom, A., Cawthorn, D., Cameron, M., & Witthuhn, R. C. (2012a). Species of *Cronobacter* - A
678 review of recent advance in the genus and their significant in infant formula milk. *International Dairy*
679 *Journal, 27*, 3-12.
680 Strydom, A., Cameron, M., & Corli Witthuhn, R. (2011). PCR-RFLP analysis of the *rpoB* gene to
681 distinguish the five species of cronobacter. *Food Microbiology, 28*(8), 1472-1477.
682 doi:10.1016/j.fm.2011.08.015; 10.1016/j.fm.2011.08.015
683 Strydom, A., Cameron, M., & Witthuhn, R. C. (2012b). Phylogenetic analysis of *Cronobacter* isolates
684 based on the *rpoA* and 16S rRNA genes. *Current Microbiology, 64*(3), 251-258. doi:10.1007/s00284-
685 011-0061-8; 10.1007/s00284-011-0061-8
686 van Acker, J., de Smet, F., Muyldermans, G., Bougatef, A., Naessens, A., & Lauwers, S. (2001).
687 Outbreak of necrotizing enterocolitis associated with enterobacter sakazakii in powdered milk
688 formula. *Journal of Clinical Microbiology, 39*(1), 293-297.
689

690 Table 1. Bacterial species and strains used in this study
691

| Species | Strain code | Source | Country of isolation (Year of isolation) |
|---|---|---|---|
| *C. condimenti* | LMG 26250$^T$ | Food | Slovakia (2010) |
| *C. dublinensis* | LMG 23823$^T$ | Environmental | Ireland (2004) |
| *C. malonaticus* | LMG 23826$^T$ | Clinical | United States (1997) |
| *C. muytjensii* | ATCC 51329$^T$ | Unknown | United States |
| *C. sakazakii* | ATCC 29544$^T$ | Clinical | United States (1980) |
| *C. turicensis* | LMG 23827$^T$ | Clinical | Switzerland (2005) |
| *C. universalis* | NCTC 9529$^T$ | Water | United Kingdom (1956) |
| *C. helveticus* | LMG 23732$^T$ | Fruit powder | Switzerland (2007) |
| | 1204 | Follow on formula | Jordan (2009) |
| | 1208 | Follow on formula | Portugal (2009) |
| | 1344 | Spice | United Kingdom (2011) |
| | 1373 | Spice | United Kingdom (2011) |
| | 1374 | Insects | United Kingdom (2011) |
| | 1387 | Spice | UK (2011) |
| | 1392 | Ingredients | UK (2011) |
| *C. pulveris* | LMG 24057$^T$ | Fruit powder | Switzerland (2008) |
| | LMG 24059 | Infant formula | Switzerland (2008) |
| | 1390 | Spice | United Kingdom (2011) |
| | 1393 | Ingredients | United Kingdom (2011) |
| *C. zurichensis* | LMG 23730$^T$ | Fruit powder | Switzerland (2004) |
| | LMG 23731 | Fruit powder | Switzerland (2004) |
| | 1383 | Food ingredient | United Kingdom (2011) |
| Negative control strains | | | |
| *Buttiauxella noakiae* | 53 | Fish | UK (2004) |
| *Escherichia hermanii* | 159 | Dried food | UK (2004) |
| | 162 | Rice | UK (2004) |
| *Pantoea* spp. | 44 | Baby food | Korea (2004) |
| | 1318 | Environment | France (2009) |

692
693

694 Table 2a. Target genes and sequence accession numbers used for genome searching.
695
696

| Gene | Reference | Genbank accession number[a] |
|---|---|---|
| *cgcA* | Carter et al. 2013 | ESA_01230 |
| *gluA* | Lehner et al. 2006b | AM075208[b] |
| *gyrB* | Huang et al. 2013 | JX088572 |
| *dnaG* | Seo and Brackett 2005 | L01755 |
| *ompA* | Mohan-Nair and Venkitanarayanan 2006 | DQ000206 |
| *rpoB* | Stoop et al. 2009 | FJ717638 |
| | | FJ717652 |
| | | FJ717656 |
| | | FJ717657 |
| | | FJ717658 |
| | | FJ717659 |
| | Lehner et al. 2012 | JQ316670 |
| *zpx* | Kothary et al. 2007 | EF061082 |

697
698
699
700 [a] These sequences were used for *Cronobacter*-BIGSdb BLAST searches
701 [b] Sequences for *gluA* and *gluB* were extracted from the partial genome sequence available with this accession
702 number.

703 Table 2b. Table 2b. Primer sequences used in PCR assays.
704
705

| Gene | Reference | Primer name | Primer sequence | Genbank accession number[a] |
|---|---|---|---|---|
| *ompA* | Mohan-Nair and Venkitanarayanan 2006 | ESSF | GGATTTAACCGTGAACTTTTCC | DQ000206 |
| | | ESSR | CGCCAGCGATGTTAGAAGA | |
| *rpoB* | Stoop et al. 2009 | Cdublf | GCACAAGCGTCGTATCTCC | FJ717638 |
| | | Cdublr | TTGGCGTCATCGTGTTCC | FJ717652 |
| | | Cmalf | CGTCGTATCTCTGCTCTC | FJ717656 |
| | | Cmalr | AGGTTGGTGTTCGCCTGA | FJ717657 |
| | | Cmuyf | TGTCCGTGTATGCGCAGACC | FJ717658 |
| | | Cmuyr | TGTTCGCACCCATCAATGCG | FJ717659 |
| | | Csakf | ACGCCAAGCCTATCTCCGCG | |
| | | Csakr | ACGGTTGGCGTCATCGTG | |
| | | Cturf | CGGTAAAAGAGTTCTTCGGC | |
| | | Cturr | GTACCGCCACGTTTCGCC | |
| | | Cgenomof | ACAAACGTCGTATCTCTGCG | |
| | | Cgenomor | AGCACGTTCCATACCGGTC | |
| | Lehner et al. 2012 | Ccon-f | AACGCCAAGCCAATCTCG | JQ316670 |
| | | Ccon-r | GTACCGCCACGTTTTGCT | |

706

707 Table 3. Comparison results for *Cronobacter* spp. cultural detection methods.
708

| | | Growth in broth culture | | | | | Colony appearance | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | EE | mLSB | | CSB[a] | | TSA | TSA | VRBGA | DFI |
| Bacterial species | Strain | (37°C) | (37°C) | (44°C) | (37°C) | (42°C) | (25°C, 72 h) | (37°C, 24 h) | (37°C, 24 h) | (37°C, 24 h) |
| *C. condimenti* | LMG 26250$^T$ | + | + | - | + | + | Dark yellow, glossy | Yellow, glossy | Pink with beige centres, mucoid | Blue-green |
| *C. dublinensis* | LMG 23823$^T$ | + | + | - | + | + | Yellow, glossy | Pale yellow, glossy | Pink with beige centres, mucoid | Blue-green |
| *C. malonaticus* | LMG 23826$^T$ | + | + | + | + | + | Yellow/pale yellow, glossy | Pale yellow, glossy | Pink/purple with small halo | Blue-green |
| *C. muytjensii* | ATCC 51329$^T$ | + | + | + | + | + | Yellow, glossy | Pale yellow, glossy | Pink with beige centres, mucoid | Blue-green |
| *C. sakazakii* | ATCC 29544$^T$ | + | + | - | + | + | Yellow, glossy | Pale yellow, glossy | Pink/purple with pale halo | Pale blue-green |
| *C. turicensis* | LMG 23827$^T$ | + | + | - | + | + | Yellow, glossy | Pale yellow, glossy | Pink with beige centres, mucoid | Blue-green |
| *C. universalis* | NCTC 9529$^T$ | + | + | + | + | + | Yellow, glossy | Pale yellow, glossy | Pink/purple with small halo | Blue-green |
| *C. helveticus* | LMG 23732$^T$ | + | - | - | - | - | Yellow/pale yellow, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| | 1204 | + | + | + | - | - | Pale yellow, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| | 1208 | + | + | - | - | - | Pale yellow, glossy | Pale yellow, glossy | Pink/purple with halo | Blue-green |
| | 1344 | + | + | - | - | - | Pale yellow/cream, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| | 1373 | + | + | - | - | - | White/cream, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| | 1374 | + | + | - | - | - | White/cream, glossy | Cream, glossy | Pink/purple with large halo | Blue-green |
| | 1387 | + | + | - | - | - | Yellow, smooth, dry | Cream, glossy | Pink/purple with large halo | Blue-green |
| | 1392 | + | + | - | - | - | Yellow, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| *C. pulveris* | LMG 24057$^T$ | + | + | - | + | + | Dark yellow, glossy | Pale yellow, glossy | Pink/purple with halo | Blue-green |
| | LMG 24059 | + | + | - | + | + | Cream/colourless, glossy | Cream/colourless, glossy | Pink/purple with halo | Blue-green |
| | 1390 | + | + | - | + | + | Dark yellow, dry, rough | Cream, glossy | Pink/purple with large halo | Blue-green |
| | 1393 | + | + | - | + | + | Yellow, glossy | Cream, glossy | Pink/purple with halo | Blue-green |
| *C. zurichensis* | LMG 23730$^T$ | + | + | - | - | - | Pale yellow/cream, glossy | Pale yellow, glossy | Pink/purple with halo | Blue-green |
| | LMG 23731 | + | + | - | - | - | Pale yellow, glossy | Pale yellow/cream, glossy | Pink/purple mucoid | Blue-green |
| | 1383 | + | + | - | - | - | Pale yellow, glossy | Cream/colourless, glossy | Pink/purple with large halo | Blue-green |
| Negative control strains | | | | | | | | | | |
| *B. noakiae* | 53 | + | + | - | - | - | White/cream, glossy | Colourless, glossy | Pink/purple with halo | Pale blue-green |
| *E. hermanii* | 159 | + | + | - | - | - | Pale yellow/cream, glossy | Pale yellow, glossy | Pink/purple with large halo | Blue-green |
| | 162 | + | + | - | - | - | Yellow, glossy | Pale yellow/cream, glossy | Pink/purple with halo | Blue-green |
| *Pantoea* spp. | 44 | + | + | - | + | - | Yellow, glossy | Pale yellow, glossy | Pink/purple mucoid with halo | Pale yellow |
| | 1318 | + | + | - | + | - | Yellow, glossy | Yellow, glossy | Pink/purple mucoid with halo | Yellow |

709 [a] Positive reaction in CSB was indicated by turbidity and a colour change from purple to yellow, as prescribed by the original method. Strains exhibiting
710 turbidity but no colour change were considered negative.

711 Table 4. Species identification as according to API 20E and ID32E biochemical profiles.
712

| Bacterial species | Strain | API 20E Profile | API 20E species identification (% identification; t-value)[a] | API 20E Report | ID32E Profile | ID32E species identification (% identification; t-value)[a] | ID32E Report |
|---|---|---|---|---|---|---|---|
| *C. condimenti* | LMG 26250$^T$ | 3367373 | *Enterobacter sakazakii* (99.9; 0.26) | Doubtful profile | 34217360051 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| *C. dublinensis* | LMG 23823$^T$ | 7347373 | *Enterobacter sakazakii* (61.3; 0.26) | Doubtful profile | 34256166211 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| *C. malonaticus* | LMG 23826$^T$ | 3305173 | *Enterobacter sakazakii* (51.1; 0.92) | Excellent identification to the genus | 34276763251 | *Enterobacter sakazakii* (99.9; 0.83) | Excellent identification |
| *C. muytjensii* | ATCC 51329$^T$ | 3365373 | *Enterobacter sakazakii* (99.9; 0.42) | Doubtful profile | 34217041041 | Unidentified | Unacceptable profile |
| *C. sakazakii* | ATCC 29544$^T$ | 3305373 | *Enterbacter sakazakii* (98.4; 1.0) | Good identification | 32276767051 | *Enterobacter sakazakii* (99.9; 0.12) | Doubtful profile |
| *C. turicensis* | LMG 23827$^T$ | 7315373 | *Enterobacter gergoviae* (91.2; 0.36) | Doubtful profile | 34276767211 | *Enterobacter sakazakii* (99.9; 0.62) | Very good identification |
| *C. universalis* | NCTC 9529$^T$ | 3205373 | *Enterobacter sakazakii* (98.0; 0.84) | Good identification | 24276777051 | *Enterobacter sakazakii* (99.9; 0.35) | Good identification |
| *C. helveticus* | LMG 23732$^T$ | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 30675567010 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| | 1204 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 4675561001 | *Aeromonas hydrophila/caviae/sobria* (NS; NS) | Unacceptable profile |
| | 1208 | 1014153 | *Yersina pseudotuberculosis* (97.5; 0.92) | Good identification | 34215461041 | Unidentified | Unacceptable profile |
| | 1344 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 00674563011 | *Buttiauxella agrestis* (86.2; 0.32) | Doubtful profile |
| | 1373 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 6635771041 | Unidentified | Unacceptable profile |
| | 1374 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 4677563011 | *Leclercia adecarboxylata* (NS; NS) | Unacceptable profile |
| | 1387 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 35275663311 | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| | 1392 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | 6675563011 | *Leclercia adecarboxylata* (NS; NS) | Unacceptable profile |
| *C. pulveris* | LMG 24057$^T$ | 3004173 | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | 4275773310 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| | LMG 24059 | 3004173 | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | 4075773310 | *K. pneumoniae* ssp. *ozaenae* (NS; NS) | Unacceptable profile |
| | 1390 | 3004573 | *Citrobacter freundii* (48.8; 0.73) | Low discrimination | 4075763310 | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| | 1393 | 3004173 | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | 4275763310 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| *C. zurichensis* | LMG 23730$^T$ | 3204153 | *K. pneumoniae* ssp. *ozaenae* (66.7; 0.71) | Identification not valid | 14475563310 | *Buttiauxella agrestis* (98.3; 0.28) | Doubtful profile |
| | LMG 23731 | 1224153 | *Buttiauxella agrestis* (63.0; 0.30) | Doubtful profile | 1407461041 | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile |
| | 1383 | 3204153 | *K. pneumoniae* ssp. *ozaenae* (66.7; 0.71) | Identification not valid | 4077563310 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile |
| Negative control strains | | | | | | | |
| *B. noakiae* | 53 | 0004153 | *Pantoea* spp. 4 (53.2; 0.78) | Doubtful profile | 4134563410 | *Buttiauxella agrestis* (NS) | Unacceptable profile |
| *E. hermanii* | 159 | 1204153 | *Buttiauxella agrestis* (63.0; 0.80) | Low discrimination | 34074703051 | *Enterobacter cancerogenus* (96.9; 0.51) | Good identification |
| | 162 | 1004153 | *Escherichia vulneris* (61.5; 1.0) | Low discrimination | 34676767050 | *Enterobacter sakazakii* (99.9; 0.67) | Doubtful profile |
| *Pantoea* spp. | 44 | 1005333 | *Pantoea* spp. 3 (99.8; 0.95) | Very good identification | 04476563051 | *Buttiauxella agrestis* (89.7; 0.46) | Acceptable identification |
| | 1318 | 0221133 | *Pantoea* spp. 3 (NS[b]; NS) | Unacceptable profile | 30014601001 | *Aeromonas sobria* (NS; NS) | Unacceptable profile |

713

714 [a] Only the first species identified by the assay is listed for each strain. [b] NS: Not specified

Table 5. Presence and absence of PCR probe target genes as indicated by BLAST searching *Cronobacter* BIGSdb (www.pubmlst.org/*Cronobacter*) for genes that were found to vary between species or strains.

| Bacterial species | Strain | Target gene *cgcA*[a] (ESA_01230) | Target gene *zpx* (ESA_00752) |
|---|---|---|---|
| *C. condimenti* | LMG 26250$^T$ | Present | Present |
| *C. dublinensis* | LMG 23823$^T$ | Present | Present |
| | LMG 23824 | Absent | Absent |
| | LMG 23825 | Absent | Absent |
| | NCTC 9844 | Partial | Present |
| *C. malonaticus* | LMG23826$^T$ | Present | Present |
| | 507 | Present | Present |
| *C. muytjensii* | ATCC 51329$^T$ | Present | Present |
| *C. sakazakii* | ATCC-894 | Present | Present |
| | 377 | Present | Present |
| | 680 | Absent | Present |
| | 696 | Present | Absent |
| | 701 | Present | Partial |
| | E764 | Present | Present |
| | ES15 | Present | Present |
| | ES35 | Present | Present |
| | ES713 | Present | Present |
| | G-2151 | Present | Present |
| | SP291 | Present | Present |
| *C. turicensis* | LMG 23827$^T$ | Present | Present |
| | 564 | Present | Present |
| *C. universalis* | NCTC 9529$^T$ | Present | Absent |
| *C. helveticus* | LMG 23732$^T$ | Absent | Absent |
| | LMG 23733 | Absent | Absent |
| | 1392 | Absent | Absent |
| | 1204 | Absent | Present |
| *C. pulveris* | LMG 24057$^T$ | Absent | Absent |
| | LMG 24058 | Absent | Absent |
| | LMG 24059 | Absent | Present |
| | 1978 | Absent | Present |
| | 1390 | Absent | Absent |
| | 1393 | Absent | Absent |
| *C. zurichensis* | LMG 23730$^T$ | Absent | Present |
| | 2025 | Absent | Absent |
| | z610 | Absent | Present |
| | 1383 | Absent | Absent |

[a]Present: ≥90% of the target sequence detected. Partial: 50-90% of the target sequence detected. Absent: <50% of the target sequence detected.

Table 6. Detection and identification of strains with PCR assays targeting the *ompA*, and *rpoB* genes

| | | *ompA*[a] | *rpoB*[b] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Bacterial species | Strain | ESSF/ ESSR | CconF/ CconR | CdubF/ CdubR | CgenomF/ CgenomR | CmalF/ CmalR | CmuyF/ CmuyR | CsakF/ CsakR | CturF/ CturR |
| *C. condimenti* | LMG 26250$^T$ | + | + | NT | NT | NT | NT | NT | NT |
| *C. dublinensis* | LMG 23823$^T$ | + | NT[c] | + | NT | NT | NT | NT | NT |
| *C. malonaticus* | LMG 23826$^T$ | + | NT | NT | NT | + | NT | NT | NT |
| *C. muytjensii* | ATCC 51329$^T$ | + | NT | NT | NT | NT | + | NT | NT |
| *C. sakazakii* | ATCC 29544$^T$ | + | NT | NT | NT | NT | NT | + | NT |
| *C. turicensis* | LMG 23827$^T$ | + | NT | NT | NT | NT | NT | NT | + |
| *C. universalis* | NCTC 9529$^T$ | + | NT | NT | + | NT | NT | NT | NT |
| *C. helveticus* | LMG 23732$^T$ | - | - | + | - | + | + | -[d] | - |
| | 1204 | - | - | + | - | + | + | -[d] | - |
| | 1208 | - | - | + | - | + | + | -[d] | - |
| | 1344 | - | - | + | - | + | + | -[d] | - |
| | 1373 | - | - | + | - | - | - | -[d] | - |
| | 1374 | - | - | + | - | + | + | -[d] | - |
| | 1387 | - | - | + | - | + | + | -[d] | - |
| | 1392 | - | - | + | - | + | + | -[d] | - |
| *C. pulveris* | LMG 24057$^T$ | - | - | + | - | + | + | - | - |
| | LMG 24059 | - | - | + | - | + | + | +[e] | - |
| | 1390 | - | - | + | - | + | + | - | - |
| | 1393 | - | - | + | - | + | + | +[e] | - |
| *C. zurichensis* | LMG 23730$^T$ | - | - | + | - | + | + | - | - |
| | LMG 23731 | - | - | + | - | + | + | - | - |
| | 1383 | - | - | - | - | + | + | - | - |
| Negative control strains | | | | | | | | | |
| *B. noakiae* | 53 | - | - | + | - | - | + | - | - |
| *E. hermanii* | 159 | - | - | + | - | + | + | - | - |
| | 162 | - | - | + | - | + | + | - | - |
| *Pantoea* spp. | 44 | - | - | - | - | - | - | - | - |
| | 1318 | - | - | - | - | + | - | - | - |

[a]Method described by Mohan-Nair and Venkitanarayanan 2006. [b]Methods described by Stoop et al. 2009 and Lehner et al. 2012. [c]NT: Not tested since *rpoB* PCR primer sets are for specific species. [d]Expected amplicon size is 514 bp, however an amplicon of approximately 490 bp was detected. [e]Faint bands at both 514 and approximately 490 bp were detected.