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

76 **1. Introduction**

77

78   *Cronobacter* spp. are members of the family *Enterobacteriaceae* that can cause rare but serious
79   infections in humans (FAO-WHO, 2006; Farmer et al., 1980; Iversen and Forsythe, 2003; van Acker
80   et al., 2001). Severe symptoms, including necrotizing enterocolitis and meningitis, have been
81   observed in infected neonates with powdered infant formula (PIF) identified as a route of
82   transmission (FAO-WHO 2004 and 2006; Himelright et al., 2002; Simmons et al., 1989; van Acker et
83   al., 2001). Though only *C. sakazakii, C. malonaticus* and *C. turicensis* have been linked with human
84   illnesses, current international microbiological standards require the absence of all *Cronobacter*
85   species in PIF (test volume 10g), demonstrating the need for specific detection and identification
86   methods (CAC, 2008; Joseph et al., 2012c).
87   The development and evaluation of methods for the detection and identification of *Cronobacter*
88   from PIF has involved inclusivity and exclusivity strain testing with target and related non-target
89   organisms, respectively. However although the taxonomic definition of *Cronobacter* has changed in
90   recent years, not all methods have adapted to these changes (Brady et al., 2013; Iversen et al.,
91   2008b; Joseph et al., 2012a). Instead there has been a continued reliance on phenotypic
92   identification and biochemical profiling of presumptive *Cronobacter* isolates for their speciation (Cruz
93   et al., 2011; Hochel et al. 2012). The current ISO standard for the detection of *Cronobacter* in PIF
94   relies on cultural and biochemical methods (ISO 2006). Yet, many of these tests have been found to
95   lack sufficient robustness for this diverse genus (Baldwin et al., 2009; Cetinkaya et al., 2013; Joseph
96   and Forsythe, 2012; Joseph et al., 2013). For example, the *C. sakazakii* type strain ATCC 29544 is
97   unable to grow at the raised temperature of 44°C required by some approved isolation methods
98   (Besse et al., 2006; Nazarowec-White & Farber 1997; ISO 2006). Additionally, some commercial
99   phenotyping kits used in the ISO and FDA methods have continued to use the former name
100   *Enterobacter sakazakii* in their identification schemes, which generates an additional source of
101   confusion as this name is no longer taxonomically valid. Additionally an improved knowledge of the
102   diversity of the *Cronobacter* genus, based on multilocus sequence analysis and whole genome
103   sequencing, has shown that speciation by biotyping is also unreliable (Baldwin et al., 2009;
104   Cetinkaya et al., 2013; Iversen et al., 2007a; Joseph et al. 2013).
105   The most recent taxonomic change in the *Cronobacter* genera is the renaming of *Enterobacter*
106   *helveticus*, *E. pulveris* and *E. turicensis* as *Cronobacter helveticus*, *C. pulveris* and *C. zurichensis*,
107   respectively (Brady et al., 2013). This is likely to cause significant changes in the efficiency of
108   *Cronobacter* test methods since these three species were previously used as negative control
109   organisms during method evaluation because they are closely related to *Cronobacter* species.
110   Examples include the development of *Cronobacter* screening broth (CSB) (Iversen et al., 2008a) and
111   molecular assays targeting *cgcA*, *rpoB,* the O-antigen locus and iron acquisition genes (Carter et al.,
112   2013; Grim et al. 2012; Jarvis et al., 2011; Mullane et al. 2008; Strydom et al. 2011). Additionally, the
113   cultural and PCR methods described in the FDA Bacteriological Analytical Manual (BAM) included
114   strains of the newly reclassified species as negative controls (Chen et al. 2012).

115   DNA-based identification methods using DNA probes and PCR amplicon detection are regarded as
116 more reliable than phenotyping; however, they depend upon the accuracy of the initial primer design.
117 Hence, the absence of target genes or sequence variation in primer binding sites in the newly
118 reclassified species may lead to false negative results or misidentification of the species. Target
119 genes for PCR probe based methods include *cgcA*, *gyrB*, *ompA*, *rpoB*, *gluA*, *dnaG*, *zpx*, iron
120 acquisition genes, the macromolecular synthesis operon, the 16S rRNA gene, and the 16S-23S
121 intergenic transcribed spacer (Carter et al., 2013; Grim et al., 2012; Hassan et al., 2007; Huang et
122 al., 2012; Kothary et al., 2007; Lehner et al., 2006b; Lehner et al., 2012; Liu et al., 2006; Mohan-Nair
123 and Venkitanarayanan, 2006; Seo and Brackett, 2005; Stoop et al., 2009). As given already, several
124 of these methods used strains of *E. helveticus*, *E. pulveris* or *E. turicensis* as negative controls in the
125 primer design stage due to their close relationship to the *Cronobacter* genus (Carter et al., 2013;
126 Chen et al. 2012; Jarvis et al., 2011; Mullane et al. 2008). In contrast, phylogenetic and DNA
127 sequencing based methods can be easily updated in response to taxonomic re-evaluations in the
128 *Cronobacter* genus, but these methods are not without their own problems. The 16S rDNA gene has
129 been problematic as a marker in *Cronobacter* as it is present in multiple copies within a single
130 genome and these copies contain microheterogenities (Baldwin et al. 2009). Additionally, the closely
131 related *C. sakazakii* and *C. malonaticus* were indistinguishable based on the 16S rRNA sequences
132 (Iversen et al. 2008a, Strydom et al., 2012b). Hence DNA sequence-based methods for single loci
133 (ie. *fusA*) and multilocus sequence typing (MLST) are becoming more popular methods for species
134 identification of *Cronobacter* isolates (Baldwin et al., 2009; Brady et al., 2013; Huang and Huang,
135 2013; Kuhnert et al., 2009; Li et al., 2012; Joseph et al., 2012c). In addition, as part of the Bacterial
136 Isolate Genome Sequence Database (BIGSdb), a specific repository for all *Cronobacter* genomes
137 sequenced to date has been established with open access at www.pubMLST.org/*Cronobacter*. The
138 *Cronobacter* BIGSdb enables the scalable analysis of *Cronobacter* genomes, representing all 10
139 species, for genes of interest (Maiden et al., 2013). Lastly, the *Cronobacter* seven loci multilocus
140 sequence typing (MLST) scheme has recently been extended online to include *ompA* and *rpoB*
141 sequences such that these alleles can add to taxonomic evaluations (Tax-MLST).

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

149

150 **2. Materials and Methods**

151

152 *2.1. Bacterial strains*

153

154 A total of twenty-seven bacterial strains were used for the laboratory evaluation of various methods,
155 as given in Table 1. The selected strains included the type strains of each of the seven original
156 *Cronobacter* spp., multiple strains of *C. helveticus, C. pulveris* and *C. zurichensis.* These had been
157 previously identified using 7-loci MLST and whole genome sequencing. Further details can be
158 obtained from www.pubmlst.org/cronobacter . The negative control strains of *Escherichia hermanii*,
159 *Pantoea* spp. and *Buttauxiella nokiae* had been previously identified using 16S rDNA sequencing.
160 These latter strains have previously produced false positive results in cultural or molecular
161 *Cronobacter* detection methods. Strains were stored in 20% glycerol at -80°C and were resuscitated
162 on tryptic soy agar (TSA) at 25°C for 72 hours. Single colonies were streaked to TSA for purity and
163 incubated at 37°C for 24 hours before use.
164
165 *2.2. Cultural and biochemical analyses*
166
167   Strains were tested for their ability to grow in *Enterobacteriaceae* enrichment broth (EE; Oxoid
168 ThermoFisher, Basingstoke, UK), *Cronobacter* selective broth with 10 mg/L vancomycin (CSB; Oxoid
169 ThermoFisher, Basingstoke, UK) (Iversen et al. 2008b) and modified lauryl sulphate broth with 0.5 M
170 sodium chloride and 10 mg/L vancomycin (mLSB) (Guillaume-Gentil et al., 2005). Each medium was
171 inoculated with a single colony from the TSA plate. All broths were incubated at 37°C. Additionally,
172 CSB and mLSB were incubated at 42°C and 44°C, respectively. Cultures were observed for growth
173 after 24, 48 and 72 hours, as appropriate. Growth was indicated by turbidity in EE and mLSB, and by
174 turbidity and a colour change from purple to yellow in CSB (Druggan and Iversen, 2009; Guillaume-
175 Gentil et al., 2005; Iversen et al., 2008a; Iversen and Forsythe, 2007; Lehner et al., 2006).
176   Strains were also assessed for their ability to produce typical colony morphologies on TSA (Oxoid
177 ThermoFisher, Basingstoke, UK), Druggan-Forsythe-Iversen agar (DFI; Oxoid, Basingstoke, UK) and
178 violet red bile glucose agar (VRBGA; Oxoid ThermoFisher, Basingstoke, UK). Each plate was
179 streaked using a single colony from the stock plate. Plates were incubated at 37°C and examined for
180 typical *Cronobacter* colony appearance after 24, 48 and 72 hours. Typical *Cronobacter* colonies are
181 yellow on TSA, blue-green on DFI and red or purple with a halo on VRBGA (Iversen et al., 2004;
182 Iversen and Forsythe, 2007; Lehner et al., 2006; Strydom et al., 2012a). Mucoid colonies may also
183 be observed for some strains on VRBGA (Strydom et al., 2012a). All strains were subject to
184 phenotyping using the API 20E and ID 32E test kits (bioMerieux, France), according to the
185 manufacturer's instructions. The databases at https://apiweb.biomerieux.com were used for species
186 identification. Version 4.1 was used for the API 20E tests and version 3.0 was used for the ID32E
187 tests.
188
189 *2.3. Genome searching for PCR target genes*
190
191   Using the BLAST function of the online *Cronobacter* BIGSdb facility
192 (www.pubMLST.org/*Cronobacter*), the full genome sequences of 32 *Cronobacter* strains were
193 examined for the presence of target gene sequences used in the original design of PCR primers and

194   probes for a variety of detection methods. Genes and accession numbers are shown in Table 2a.
195   The presence of genes was reported according to arbitrary divisions. Genes were considered
196   present if ≥90% of the target sequence was detected. Partially present genes were defined by the
197   detection of 50-90% of the target gene. If <50% of the target gene was detected, the gene was
198   considered to be absent. Absent genes were confirmed by genome sequence alignment using
199   WebAct (http://www.webact.org/WebACT/home).
200
201   *2.4. PCR detection and identification*
202
203   A single colony of each strain was suspended in 100 µl sterile distilled water and boiled at 100°C
204   for 10 minutes. The PCR method targeting *ompA* was performed as described for boiled cell lysate
205   (10 µl) by Mohan-Nair and Venkitanarayanan (2006). The *rpoB* method was performed as described
206   by Stoop et al. (2009) and Lehner et al. (2012) for boiled colony lysate (5 µl). Primer sequences for
207   both assays can be found in Table 2b. Because a different set of primers is used to identify each
208   species, the type strains of *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. muytjensii*, *C.*
209   *sakazakii*, *C. turicensis* and *C. universalis* were tested only with the appropriate primer sets and
210   therefore served as positive controls. PCR products were visualized on a 1.5% agarose gel stained
211   with SYBR safe.
212
213   **3. Results**
214
215   *3.1. Cultural detection*
216
217   The results of the cultural detection methods are summarized in Table 3. All *Cronobacter* and non-
218   *Cronobacter* strains exhibited growth in EE broth at 37°C. Except for one *C. helveticus* strain (1975),
219   all *Cronobacter* spp. were capable of growth in mLSB at 37°C; however, the growth of many strains,
220   including all of the newly reclassified species, was inhibited at 44°C. *C. sakazakii* exhibited growth at
221   44°C only after the incubation time was extended from 24 to 48h. In CSB, all *Cronobacter* spp.,
222   except *C. helveticus* and *C. zurichensis*, were able to grow and produce the expected colour change
223   (purple to yellow) at both 37°C and 42°C. The only species affected by the difference in temperature
224   were the *Pantoea* spp. Both *Pantoea* strains displayed positive reactions at 37°C, but negative
225   reactions after 24 hours at 42°C. *Pantoea* strain 44 did exhibit a positive reaction at 42°C, but only
226   after incubation for 48 hours.
227   Almost all strains of the newly reclassified species produced non-pigmented colonies on TSA
228   following incubation at 37°C for 24 hours; however, all but one of these strains (*C. helveticus* 1344)
229   showed some degree of yellow colouration following incubation at 25°C for 72 hours. Some strains
230   did show a slight darkening of the yellow pigment after 72 hours of incubation at 37°C, but five stains
231   of *C. helveticus*, 2 strains of *C. pulveris* and one strain of *C. zurichensis* were not yellow at this
232   temperature, regardless of incubation time. All strains were able to grow on VRBGA, and a variety of
233   colony morphologies were observed. As shown in Table 3, some strains produced large, mucoid

234 colonies with a very little pink color, while others produced small, pink to purple colonies. Of the
235 newly reclassified species only *C. zurichensis* 2025 produced mucoid colonies. All of the
236 *Cronobacter* and non-*Cronobacter* strains, except the *Pantoea* spp., showed typical blue-green
237 colonies on DFI agar after 24 hours at 37°C.
238
239 *3.2. Biochemical identification*
240
241 The results of the API 20E and ID32E assays are shown in Table 4. The API 20E identified six of
242 the seven species type strains as *Enterobacter sakazakii*, but four of these identifications were
243 based on doubtful profiles. This test identified *C. turicensis* as *Enterobacter gergoviae* with 91.2%
244 identification and only a 7.0% identification as *E. sakazakii*. The *C. zurichensis* strains were identified
245 as *Klebsiella pneumoniae* ssp. *ozaenea* or *Buttauxiella agrestis*. *C. helveticus* 1208 was identified as
246 *Yersinia pseudotuberculosis* and *C. pulveris* 1390 was identified as *Citrobacter freundii*. The
247 remaining strains of *C. helveticus* and *C. pulveris* were identified as *Escherichia vulneris* with the API
248 20E. Three *C. pulveris* strains were identified as *E. sakazakii* with a 0.8% identification and the
249 database report indicated that the identification of these strains was not valid. None of the other
250 strains of the recently reclassified species gave possible identifications as *E. sakazakii*. Of the
251 negative control strains, both *Pantoea* spp. were correctly identified, but the remaining strains were
252 not correctly identified to the species level.
253 For the ID32E phenotyping method, most identifications with doubtful or unacceptable profiles did
254 not return percentage identifications. The type strains for *C. condimenti*, *C. dublinensis*, *C.*
255 *malonaticus*, *C. sakazakii*, *C. turicensis*, and *C. universalis* were identified as *E. sakazakii*. Three of
256 these identifications were the results of 'doubtful' or 'unacceptable profiles'. The species type strain
257 of *C. muytjensii* (ATCC 51329[T]) was unidentified. The profiles for the type strains for *C. helveticus*
258 and *C. pulveris* returned *E. sakazakii* as the top species identified, but the percent identifications
259 were not given as they were identified with 'unacceptable profiles'. In contrast, the profile for the *C.*
260 *zurichensis* type strain LMG23730[T] returned *Buttiauxella agrestis*, as did the profile for *C. zurichensis*
261 2025. Only *C. zurichensis* 1383 was identified as *E. sakazakii*, though with an unacceptable profile.
262 None of the *B. noakiae*, *E. hermanii,* or *Pantoea* spp. strains were correctly identified to the species
263 level using the ID32E system, and *E. hermanii* strain 159 was identified as *E. sakazakii* (99.9%).
264 Fifteen of the 22 *Cronobacter* strains (59.1%) gave contradictory identifications when the results from
265 the two kits were compared.
266
267 *3.3. Genome searching for PCR target genes*
268
269 Gene sequences previously used to design PCR primers and probes for detection and
270 identification of *Cronobacter* spp. were compared to the full genome sequences of 32 *Cronobacter*
271 strains representing the whole genus (Table 2a). Genes were considered present if 90% or more of
272 the target sequence was aligned. Partial positives were indicated by the presence of 50-90% of the
273 target sequence. Genes were considered absent if less than 50% of the target sequence was

274   present. The genes for *ompA*, *rpoB*, and *gyrB* were present in all genomes, as expected since these

275   are used in the *Cronobacter* MLST and Tax-MLST schemes. As shown in Table 5, the *cgcA*

276   sequence was absent from all the new *Cronobacter* species, as well as *C. sakazakii* 680, two *C.*

277   *dublinensis* strains, and it was only partially present in a third *C. dublinensis* strain. Similarly, the zinc

278   metalloprotease gene, *zpx*, was only partially present in some strains.

279

280   *3.4. PCR detection and identification*

281

282   The results of the *ompA* and *rpoB* PCR assays are shown in Table 6. The *ompA* PCR assay

283   described by Mohan-Nair and Venkitanarayanan (2006) produced bands of the expected size of 469

284   bp for the type strains for the seven original *Cronobacter* spp. However, no PCR amplicons were

285   generated with any of the strains for the three newly reclassified species; *C. helveticus*, *C. pulveris*

286   and *C. zurichensis*.

287   The *rpoB* multiple primer assay utilizes a separate primer set for identification of each of the seven

288   previously recognised *Cronobacter* spp. The primers designed for *C. sakazakii* produced slightly

289   smaller bands for all *C. helveticus* strains. Additionally, both bands of the expected size of 514 bp

290   and the smaller band were detected for *C. pulveris* 1393 and 1978. When tested with the *C.*

291   *malonaticus* and *C. muytjensii* primers, amplicons of the expected sizes were produced for all strains

292   of the newly reclassified *Cronobacter* spp., except *C. zurichensis* 1383. Both *E. hermanii* strains

293   were also positive with both of these primers sets, and *Pantoea* spp. strain 1318 was positive with

294   the *C. malonaticus* primers. None of the strains for the newly reclassified species were identified

295   with the *C. condimenti* or *Cronobacter* genomospecies (former name for *C. universalis*) primers. No

296   strains, including the positive control strain, produced amplicons with the *C. turicensis* primers.

297

298

299   **4. Discussion**

300

301   *4.1. Cultural and biochemical detection and identification*

302

303   Following international concern over the microbiological safety of PIF, the recovery methods

304   initially used for *Cronobacter* detection were EE broth and VRBGA, which are general media for the

305   recovery of *Enterobacteriaceae* prior to phenotypic identification (Chen et al., 2012; Muytjens et al.,

306   1988). As expected, pure cultures of all strains from the ten *Cronobacter* species grew in both media

307   at 37°C, as shown in Table 3. However, since *Cronobacter* can be out-grown by other

308   *Enterobacteriaceae*, mLSB and CSB enrichment broths were developed for the preferential isolation

309   of *Cronobacter* from mixed cultures. Both mLSB and CSB enrichment broths utilize increased

310   incubation temperatures (44 and 42°C respectively) to confer additional selectivity to the cultural

311   detection of *Cronobacter* spp. (Guillaume-Gentil et al., 2005; Iversen et al., 2008a). In this study the

312   type strains of *C. condimenti*, *C. dublinensis*, *C. sakazakii*, *C. turicensis*, C *helveticus*, *C. pulveris*, *C.*

313   *zurichensis* were unable to grow in mLSB at 44°C as required in ISO/TS 22964|IDF/RM 210:2006

314 (Besse et al., 2006; ISO, 2006). No strains of *C. helveticus*, *C. pulveris*, or *C. zurichensis* were able
315 to grow in mLSB at 44°C, with the exception of *C. helveticus* 1204. Most of the strains did grow in the
316 medium at the lower temperature of 37°C, but this is not the prescribed temperature in the ISO
317 approved method. Nazarowec-White and Farber (1997) previously reported that the *C. sakazakii*
318 type strain ATCC 29544 was unable to grow above 41°C, and Iversen and Forsythe (2007) reported
319 that 6% of strains then known as *E. sakazakii* were unable to grow in mLSB at 44°C. In our study,
320 extending the incubation period at 44°C to 48h resulted in only the *C. sakazakii* strain displaying
321 slightly greater turbidity. Though this broth was intended for use with selective or differential agars,
322 the absence of turbidity after the prescribed 24 hour incubation indicates that *Cronobacter* spp. may
323 not reach a high enough concentration to result in detection on agar plates (Guillaume-Gentil et al.,
324 2005).
325 CSB is both a selective and differential medium, containing vancomycin to inhibit the growth of
326 Gram-positive organisms and bromocresol purple to detect the pH change associated with sucrose
327 utilization (Iversen et al., 2008a). This broth was designed to detect presumptive *Cronobacter*
328 positive samples without selective or differential plating to minimize the time required to reach a
329 negative result. Though presumptive positive samples will require further testing, according to the
330 Iversen et al. (2008) negative results can be considered conclusive. Therefore, results for this assay
331 were only considered positive if the expected colour change from purple to yellow was observed
332 after 24 hours. In the current study, CSB enrichment at 42°C supported the growth of only eight of
333 the ten *Cronobacter* species. Though incubation at 42° C was sufficient to exclude all of the negative
334 control strains, *C. helveticus* and *C. zurichensis* were not viable in this broth at any temperature. *E.*
335 *helveticus*, *E. pulveris*, and *E. turicensis* were all listed as negative control species used for
336 development of this broth and positive results were reported for *E. pulveris* (Iversen et al., 2008a).
337 Prior to the taxonomic reclassification, the recovery of *E. pulveris* from CSB would have been
338 regarded as a false-positive result (color change associated with a non-*Cronobacter* isolate).
339 However, following the taxonomic revisions, the absence of growth for *C. helveticus* and *C.*
340 *zurichensis* would constitute a false-negative result with CSB (no color change associated with
341 strains identified as *Cronobacter* species).; As current international regulations require the absence
342 of all *Cronobacter* species in PIF (CAC, 2008), such misidentifications can be costly to industry. A
343 batch of infant formula may be rejected due to false-positive identification of *Cronobacter* species,
344 and infant formula containing *Cronobacter* may be mistakenly released due to false-negative
345 identification. Given only three *Cronobacter* species have been epidemiologically- linked to infections
346 the possible revising of international criteria to only those species should be given serious
347 consideration.
348 Identification of *Cronobacter* spp. based on colony morphology can be unreliable. Yellow pigment
349 production on TSA is often considered to be indicative of *Cronobacter* spp.; however, production of
350 this pigment can be affected by a variety of conditions, including incubation temperature and
351 exposure to light, making it an inconsistent and unreliable test (Druggan and Iversen, 2009; Farmer
352 et al., 1980; Johler et al., 2010). As observed in this study, many strains, including most strains of the
353 newly reclassified species, appeared yellow following incubation at 25°C, but not after incubation at

354   37°C. Although yellow pigmentation on TSA is stated in the ISO standard protocol, it has been

355   shown that up to 21.4% of *Cronobacter* spp. do not produce yellow pigment after 72 hours of

356   incubation at 25°C (Besse et al., 2006; ISO, 2006; Iversen and Forsythe, 2007). Though all

357   *Cronobacter* strains in the current study produced typical blue-green colonies on DFI, this

358   morphology was also observed for *B. nokiae* and both strains of *E. hermanii.*

359   Biochemical methods are often used for species identification and confirmation of suspect isolates.

360   Biochemical panels, such as the API 20E and ID32E are popular among testing laboratories, and are

361   used in conjunction with online databases to identify the species of bacteria based on a panel of 20

362   or 32 biochemical tests. However, these databases are not up to date with the current taxonomy.

363   Though the *Cronobacter* genus was first described in 2007, these databases still report results of

364   "*Enterobacter sakazakii*." Inadequacies in the databases have been noted by other authors,

365   suggesting that these assays are not sufficient for identification of *Cronobacter* spp. (Fanjat et al.,

366   2007; Iversen et al., 2004; Iversen et al., 2007b). Updating the databases will undoubtedly increase

367   the accuracy of identification. Fanjat et al. (2007) examined *E. sakazakii* isolates and found that only

368   71.4% of these isolates were correctly identified with version 2.0 of the ID32E database. Modification

369   of the database to reflect variability in carbohydrate utilization later resulted in 100% correct

370   identification of these isolates (Fanjat et al., 2007). As demonstrated by the current study,

371   misidentifications of *Cronobacter* spp. are common with these assays. False negative identifications

372   are not the only concern with these methods, as *E. hermanii* 162 was misidentified as '*E. sakazakii*'.

373   This strain could be mistaken for a *Cronobacter* spp. because it also produces blue-green colonies

374   on DFI and yellow colonies on TSA. The possibility of false negative and false positive identifications

375   and the lack of updated databases confirms that these biochemical panels are not sufficient to

376   correctly identify *Cronobacter* spp. (Cetinkaya et al., 2013; Osaili and Forsythe, 2009).

377

378   *4.2. Genome searching for PCR targets and laboratory PCR assays*

379

380   The gene *cgcA* encodes a diguanylate cyclase that is involved in signal transduction for the

381   regulation of virulence, formation of biofilms and long-term survival of the organism. As shown in

382   Table 5, the *cgcA* gene sequence was absent from *C. helveticus* genomes. This result was expected

383   since the development of this identification assay used *E. helveticus* as a negative control (Carter et

384   al., 2013). Additionally, the gene was absent from all *C. pulveris* strains and *C. sakazakii* strain 680.

385   Partial sequences were found in *C. dublinensis* 582 and *C. zurichensis* 1974. Absence of the

386   complete gene sequence indicates that the primer binding sites are not present. Absence of a

387   portion of the gene sequence could also indicate the lack of one or both primer binding sites. No

388   amplicon would be produce in either situation. Additionally, if the sequence is only partially present,

389   but the primer biding sites are still intact, a smaller than expected amplicon could be produced. Since

390   multiple strains lack this complete sequence, it is not sufficient for identification of *Cronobacter* spp.

391   and this assay was not used during the laboratory portion of the current study. Similarly, *gluA*,

392   encoding an α- glucosidase, was present in nearly all *Cronobacter* spp. Partial *gluA* sequences were

393   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*.

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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.
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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

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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

Bacterial species	Strain	Growth in broth culture					Colony appearance			
		EE (37°C)	mLSB (37°C)	(44°C)	CSB[a] (37°C)	(42°C)	TSA (25°C, 72 h)	TSA (37°C, 24 h)	VRBGA (37°C, 24 h)	DFI (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

715 Table 5. Presence and absence of PCR probe target genes as indicated by BLAST searching *Cronobacter* BIGSdb
716 (www.pubmlst.org/*Cronobacter*) for genes that were found to vary between species or strains.
717
718

Bacterial species	Strain	Target gene	
		cgcA[a] (ESA_01230)	*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

719
720 [a]Present: ≥90% of the target sequence detected. Partial: 50-90% of the target sequence detected. Absent: <50% of
721 the target sequence detected.

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

Bacterial species	Strain	*ompA*[a] ESSF/ ESSR	*rpoB*[b] CconF/ CconR	CdubF/ CdubR	CgenomF/ CgenomR	CmalF/ CmalR	CmuyF/ CmuyR	CsakF/ CsakR	CtufF/ CtufR
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.