# TECHNIQUES FOR THE IDENTIFICATION AND DIFFERENTIATION OF *CRONOBACTER* SPECIES

**EMILY ELIZABETH JACKSON**

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## Dedication

To my parents, Kim and Lee, for their constant love and support,
even when my wild pursuits took me halfway around the world.

To all those who supported me in this academic adventure,
for encouraging me when I needed it most.

I could not have done it without you.

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# ABSTRACT

The *Cronobacter* genus currently consists of seven species: *C. condimenti, C. dublinensis, C. malonaticus, C. muytjensii, C. sakazakii, C. turicensis,* and *C. universalis.* The work presented here was undertaken to examine different methods for detection, identification, and characterization of the members of this genus. First, traditional cultural, biochemical, and molecular detection and identification methods were examined with regard to taxonomic changes that occurred within the genus in 2013. This work showed that these methods may not be sufficient for accurate and specific detection and identification of all *Cronobacter* spp. Next, the DNA sequence-based method of multilocus sequence typing (MLST) was used to identify and characterize a collection of *Cronobacter* strains and these results were used in a variety of applications. These sequences were used to identify a novel species, characterize outbreak strains, and resolve discrepancies in species identification. It was also shown that MLST does not necessarily correlate to observable phenotypes. This led to the final portion of work which used whole genome sequences to guide laboratory experiments and data analysis. First, the *mutS-rpoS* genomic region of *Cronobacter* spp. was examined. Though this region is highly variable in other related species, all seven *Cronobacter* spp. contained the same genes in this region, in the same order, indicating that these genes are not likely to be related differences in virulence observed between the species. Finally, production of cellulose as a component of the bacterial capsule was examined. An attempt was made to link colony morphologies on infant formula agar and Congo red agar to the genotypes as determined from whole genome sequence analysis. While some results could be explained by the differences identified in the cellulose gene cluster, it was not possible to predict the phenotype based only on these gene sequences. This is likely due to the presence of four additional components in the capsule of *Cronobacter* spp. Future work developing a capsular typing scheme and linking these capsular profiles to observable phenotypes is also discussed.

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AFSSA | Agence française de sécurité sanitaire de aliments |
| ANI | Average nucleotide identity |
| AP-PCR | Arbitrarily primed polymerase chain reaction |
| BAM | Bacteriological Analytical Manual |
| BIGSdb | Bacterial Isolate Genome Sequence Database |
| BPW | Buffered peptone water |
| BSAC | British Society for Antimicrobial Chemotherapy |
| CAC | Codex Alimentarius Commission |
| CC | Clonal complex |
| CCI | Chromogenic *Cronobacter* isolation agar |
| CSB | *Cronobacter* screening broth |
| DFI | Druggan-Forsythe-Iversen agar |
| DLV | Double locus variant |
| EE | *Enterobacteriaceae* enrichment broth |
| ESIA | *'Enterobacter sakazakii'* isolation agar |
| ESPM | *'Enterobacter sakazakii'* plating medium |
| FAO | Food and Agriculture Organization of the United Nations |
| FDA | United States Food and Drug Administration |
| HGT | Horizontal gene transfer |
| IF | Infant formula |
| ISO | International Organization for Standardization |
| M9 | M9 minimal medium |
| mLSB | Modified lauryl sulphate broth |
| MLST | Multilocus sequence typing |
| MPN | Most probable number |
| NEC | Necrotizing enterocolitis |
| ORF | Open reading frame |
| pdar | Pink, dry, and rough |
| PFGE | Pulsed field gel electrophoresis |
| PIF | Powdered infant formula |
| PT | Pulsotype |
| rdar | Red, dry, and rough |
| rMLST | Ribosomal multilocus sequence typing |
| R-PIF | Rehydrated powdered infant formula |
| rST | Ribosomal sequence type |
| SLV | Single locus variant |
| spp. | Species |
| ssp. | Subspecies |
| ST | Sequence type |
| TLV | Triple locus variant |
| TSA | Tryptic soy agar |
| TSB | Tryptic soy broth |
| VRBGA | Violet red bile glucose agar |
| WGS | Whole genome sequencing |
| WHO | World Health Organization |

# OBJECTIVES

1. To examine commonly used *Cronobacter* detection and identification methods for their ability to correctly detect and/or identify *Cronobacter* species and strains following a revision of the taxonomy of the *Cronobacter* genus

2. To demonstrate the applications of multilocus sequence typing for identification of a novel species, outbreak investigation, accurate species identification of members of the *Cronobacter* genus, and to explore a possible link between the identified sequence type and the growth rate of *C. sakazakii* strains

3. To use whole genome sequences for the examination of the *mutS-rpoS* genomic region in *Cronobacter* species

4. To show how genome sequence data can be used to guide laboratory experiments and data analysis through examination of the *bcs* gene cluster and to link the identified genotypes to observable phenotypes

# CHAPTER 1

# General introduction & literature review

## 1.1. A brief history of the *Cronobacter* genus

This thesis concerns the bacterial genus now known as *Cronobacter*, the members of which were previously known by several other names. Prior to 1980, the species now known to be members of the *Cronobacter* genus were identified as 'yellow-pigmented *Enterobacter cloacae'.* In 1980, however, these strains were reclassified as a novel species then known as '*Enterobacter sakazakii' [34]*. The *Cronobacter* genus was not defined until 2007. This original classification included four species (*C. sakazakii, C. muytjensii, C. turicensis*, and *C. dublinensis*), two subspecies of *C. sakazakii*, and one genomospecies *[78]*. These classifications were updated in the following year, resulting in the definition of three subspecies of *C. dublinensis* and the reclassification of the two *C. sakazakii* subspecies into two unique species: *C. sakazakii* and *C. malonaticus [77].* The taxonomy was again updated in 2012, with the addition of the novel species *C. condimenti* and the designation of *Cronobacter* genomospecies I as *C. universalis [93].* In 2013, the *Cronobacter* genus gained three additional species when *Enterobacter helveticus, E. pulveris,* and *E. turicensis* were reclassified as *Cronobacter helveticus, C. pulveris,* and *C. zurichensis,* respectively *[15, 144, 145]*. This was very quickly disputed and these three species were removed from the *Cronobacter* genus in 2014. These three species were instead reclassified as members of two novel genera: *Franconibacter* and *Siccibacter [146]*. Figure 1.1 summarizes the taxonomic revisions of the *Cronobacter* genus that occurred between 1980 and 2014.

These frequent taxonomic revisions highlight the fact the *Cronobacter* is an emergent genus. In particular, the use of DNA sequence-based identification methods and whole genome sequencing is leading to a better understanding of the genus and its relationship to other genera *[44]*. It is likely that additional taxonomic revisions will occur in the future as more and more DNA sequences become available. These changes can have significant repercussions as they relate to microbiological criteria applied to food products, particularly powdered infant formula (PIF), which has been identified as a route of transmission for *Cronobacter* spp. *[8, 27, 40, 41, 125, 142].* The implications of the 2013 and 2014 taxonomic revisions will be discussed in detail in Chapter 3 and a further change in the taxonomy will be discussed in Chapter 4.

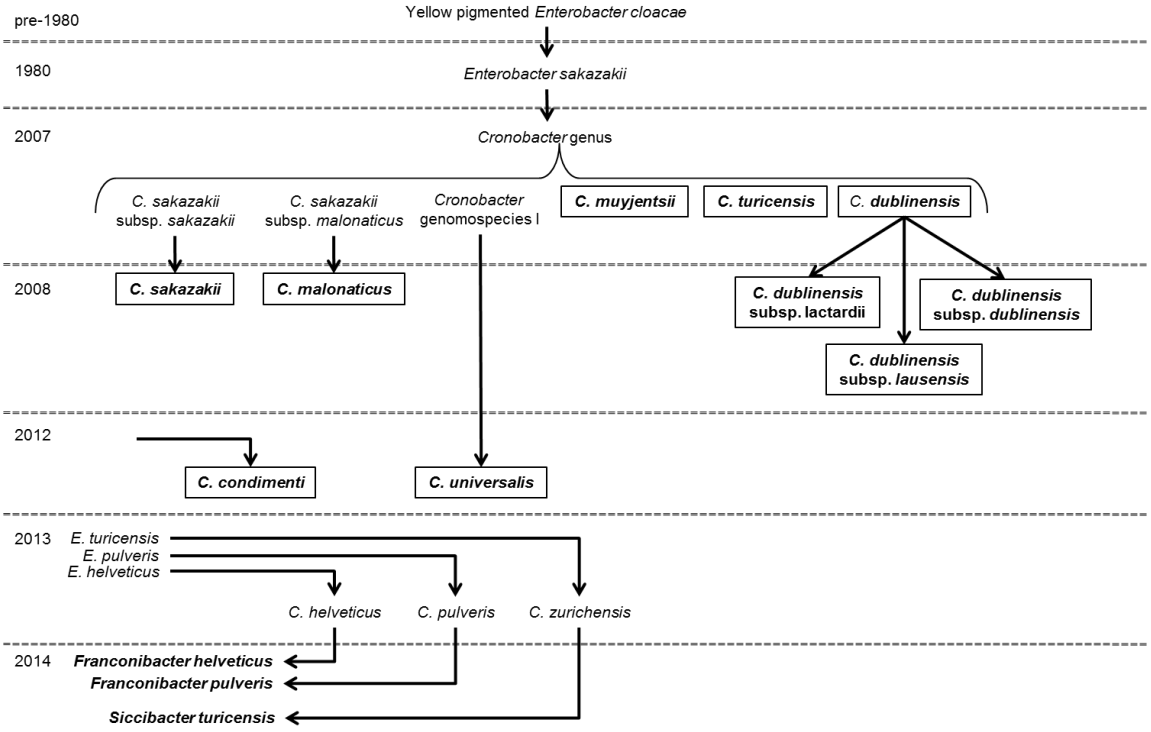


**Figure 1.1. A summary of taxonomic revisions of the *Cronobacter* genus (1980-2014)**

## 1.2. A brief description of the *Cronobacter* genus

As stated above, the *Cronobacter* genus was first described in 2007, with updates to the taxonomy in 2008, 2012, 2013, and 2014 *[15, 77, 78 146]*. The *Cronobacter* genus has been described as consisting of catalase-positive, oxidase-negative, generally motile, facultatively anaerobic Gram negative rods *[77, 78]*. The bacteria are capable of growth between 6° C and 45° C *[77]*. *Cronobacter* spp. are generally Vogues-Proskauer positive and methyl red negative *[77, 78]*. Cells can reduce nitrate, but are negative for hydrogen sulphide production, urease, and lysine decarboxylase *[77, 78]*. Additionally, *Cronobacter* spp. can utilize citrate and are positive for ornithine decarboxylase *[77, 78]*. *Cronobacter* spp. can utilize a variety of compounds as carbon sources, including, but not limited to: D-galactose, D-glucose, D-mannose, D-melibiose, D-ribose, D-xylose, L-arabinose, esculin, glycerol, maltose, raffinose, sucrose, trehalose, and the amino acids D-alanine, L-alanine, L-aspartate, L-glutamate, L-proline, and L-serine *[77, 78]*. Conversely, the following compounds cannot be used as a sole carbon source by members of the *Cronobacter* genus: D-sorbitol, adonitol, arabitol, erythritol, glucose-1-phosphate, glucose-6-phosphate, mucate, sodium pyruvate, tartarte, xylitol, 5-ketogluconate, and the amino acids L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, and L-valine *[77, 78]*.

As of 2014, the *Cronobacter* genus consisted of seven species: *C. condimenti, C. dublinensis, C. malonaticus, C. muytjensii, C. sakazakii, C. turicensis,* and *C. universalis.* The seven species can be differentiated from one another based on the utilization of a variety of carbon sources. In the description of the species *C. condimenti* and *C. universalis*, the following carbon sources were reported to be sufficient for the differentiation of all seven *Cronobacter* spp.: *cis*-aconitate, *trans*-aconitate, dulcitol, indole, inositol, lactulose, malonate, maltitol, melezitose, palatinose, putrescine, turanose, 4-aminobutyrate, and 10-methyl α-D-glucopyranoside *[93]*.

### 1.3. *Cronobacter* spp. and human illness

*Cronobacter* is most widely known for its association with neonatal infections; however, only three *Cronobacter* spp. have been linked to such infections and illnesses: *C. sakazakii, C. malonaticus,* and *C. turicensis [77, 78]. C. sakazakii* is the most well-known due to its association with neonatal meningitis and necrotizing enterocolitis *[10, 19, 20, 21, 27, 55, 59, 77, 78, 91, 142, 160]*. *C. malonaticus*, on the other hand, has been shown to be more commonly associated with adult infections, with much less serious symptoms *[3, 61, 62, 90, 94]*. Finally, a single case of neonatal meningitis has been linked to *C. turicensis* infection *[77, 78]*. As the effects of *C. sakazakii* infection are most severe, this section will focus on outbreaks linked to this species. In later chapters, analysis of *C. sakazakii* strains is prioritized for the same reason.

#### 1.3.1. *Cronobacter sakazakii* infections in infants and neonates

As stated above, *C. sakazakii* infections primarily affect infants and neonates, particularly those born prematurely or with low birth weights *[14, 59, 124]*. Neonatal infections can result in symptoms including meningitis and necrotizing enterocolitis (NEC) *[14, 19, 20, 21, 27, 59, 124, 142, 160]*. Other symptoms, such as conjunctivitis, have been observed, but these are rare *[27]*. In addition, asymptomatic *C. sakazakii* colonisation of neonates has also been reported *[11, 19, 27]*.

*C. sakazakii* infections are a major concern due to the severe and often long-term effects that result. The mortality rate for neonatal *C. sakazakii* infections has been estimated at 40-80% *[124].* Infants who survive are often left with life-long disability, including severe brain damage in cases of meningitis *[14]*. Due to the high mortality rate and long-term effects of the illness, the cost of *C. sakazakii* infections has been estimated to be greater than $5 million per case *[118]*. Additionally, *Cronobacter* spp. present a unique risk to infants and neonates due to their resistance to desiccation and association with PIF *[8, 27, 40, 41, 125]*. Three neonatal outbreaks

of *C. sakazakii* are described below to illustrate the severity and impact of these infections. Three additional outbreaks will be discussed in detail in Chapter 4.

### 1.3.2. Selected neonatal *Cronobacter sakazakii* outbreaks

*France, 1994.* A large outbreak involving 18 patients occurred in France in 1994, but was not reported in the literature until 2007 *[19]*. To date, this is the largest reported outbreak attributed to *C. sakazakii.* Though originally described as *'E. sakazakii,'* later DNA-sequence based analysis confirmed the identity of the isolates from this outbreak as *C. sakazakii [19, 90]*. During the three month long outbreak, seven cases of NEC, one case of meningitis, one case of septicaemia, and two cases of digestive issues were reported for infants found to be infected with *'E. sakazakii' [19]*. Additionally, four asymptomatic '*E. sakazakii'* colonisations were reported *[19]*. Two of the *C. sakazakii* infections were fatal, including the single case of meningitis *[19]*. A third death from septic shock was also reported at the same time, but the patient was found to be infected with a strain of *E. cloacae,* not '*E. sakazakii' [19]*. Clinical details were not available for the two remaining infants involved in the outbreak *[19].*

Among the '*E. sakazakii'* isolates, four unique pulsotypes (PTs) were identified by pulsed-field gel electrophoresis (PFGE). Three of these PTs (PT1, PT2, and PT3) were identified among patient isolates, while the fourth was associated only with isolates from unopened tins of PIF *[19]*. Strains belonging to PT2 and PT3 were isolated from both patients and batches of prepared infant formula, but not from unopened tins of PIF *[19]*. Thus, the source of these infections could not be determined, though it was suggested that cross-contamination or transmission via hospital workers may have contributed to the illnesses *[19]*. This hypothesis was later supported by genomic analysis of isolates from the outbreak *[112]*.

Identification of the different PTs and later DNA sequence-based analyses also demonstrated that this was not a single outbreak, but rather three or more overlapping outbreaks occurring at the same time *[19, 90, 112]*. This was demonstrated by the isolation of strains belonging to multiple PTs from the same patient. For example, isolates belonging to both PT1 and PT2 were isolated from two infants exhibiting NEC *[19]*. It is not yet known how coinfection with multiple strains of *C. sakazakii* affects the virulence of the organism or patient outcomes following infection. It is also interesting to note that the PT4 strains were only isolated from PIF after the last case had been reported and strains belonging to this PT were never isolated from patients *[19]*. It is not known if any infants in the hospital consumed formula from this batch, but it is possible that

the presence of these organisms in the PIF could have presented an additional hazard to the patients in the hospital at the time had this formula been consumed *[19]*.

Further analyses of the isolates from this outbreak revealed phenotypic differences both between and within the observed PTs. For example, strain 693 (PT3) showed lower attachment to and invasion of Caco-2 cells than strains from PT1 or PT2 *[156]*. As strains from PT3 were not associated with any cases of NEC or meningitis, these results suggest that attachment and invasion to endothelial cells may play a role in the virulence of *C. sakazakii [156]*. Phenotypic differences were also observed within PT2. In particular, two strains within this PT exhibited extended-spectrum β-lactamase activity, but the remaining strains were sensitive to β-lactam antibiotics *[19]*. Additionally, variation in the attachment and invasion of the strains from PT2 was reported by Townsend *et al. [156]*. Together, these results suggest that additional genotypic variation exists within PT2 and that these differences could not be resolved using PFGE. Thus, isolates were further analysed using the DNA sequence-based method of multi-locus sequence typing (MLST) for more detailed genetic characterization.

MLST identified strains from this outbreak as *C. sakazakii,* rather than the former name '*E. sakazakii'.* Additionally, this analysis also revealed three sequence types (STs) that corresponded to the PTs identified in the original outbreak investigation. For example, PT2 was found to consist of strains belonging only to ST4 *[42, 90, 112]*. This is a particularly interesting finding as PT2 was identified as the most virulent PT and ST4 strains have been found to be associated with neonatal infections and neonatal meningitis in particular *[19, 55, 91]*. It should be noted that isolates belonging to PT2 (later identified as ST4) were isolated from both fatal cases in this outbreak *[19, 42, 90]*. Thus, even though only one case of meningitis was reported during the outbreak, the potential hazards of strains belonging to ST4 were demonstrated by the high number of NEC cases and the two deaths that resulted from these infections.

*Israel, 1999.* Five cases of *'E. sakazakii'* infection were reported at a hospital in Jerusalem in 1999 *[11]*. Once case of bacteraemia, one case of meningitis, and three asymptomatic colonisations were observed in this outbreak *[11]*. None of the cases were fatal, but the meningitis case did result in brain damage and unspecified neurological issues *[11]*. Though originally identified as *'E. sakazakii,'* an isolate from the patient with meningitis was later identified as *C. sakazakii* ST4 *[42].*

As with the outbreak in France, isolates were not recovered from PIF, but *'E. sakazakii'* was recovered from prepared formula and the blender used for its preparation *[11]*. Repeated testing of the blender resulted in continued isolation of *'E. sakazakii'* for several months, suggesting that environmental contamination may have also contributed to the illnesses reported during this outbreak *[11]*. Following the onset of symptoms, the affected patients were isolated in the hospital to minimize possible cross-contamination, but there is no way to know if this precaution was effective *[11]*. The hospital also very quickly altered their feeding procedures to prevent infection of other infants in the hospital at the time *[11]*. A damaged blender was removed from use and the hospital staff began using ready-to-feed formula, instead of PIF *[11]*.

In particular, this outbreak highlights the persistence of the organism now known as *C. sakazakii*. The organism was isolated from the blender and faecal samples from the affected infants for several months following the outbreak *[11]*. In this way, *Cronobacter* spp. present a unique hazard for infants being cared for in a neonatal intensive care unit. The infants are in close proximity to one another and members of the hospital staff are responsible for more than one infant. Thus, there is a possibility that cross-contamination within the hospital could lead to further infections. In the Israeli outbreak, the hospital responded promptly to the infections by isolating the affected infants and changing the hospital feeding procedures, possibly reducing the number of resulting infections and the severity of the outbreak.

*United States of America, 2011.* Four cases of *Cronobacter* infection were reported in the United States in 2011 *[20, 21]*. Three cases of meningitis and one case of septicaemia were reported, including two fatal cases *[20, 21]*. No link was found between the patients and the illnesses occurred in four different states, indicating significant geographic distribution. Though all infants were being fed with PIF, the same brand was not used in all cases *[20, 21].* In addition, the genetic fingerprints of isolates from two of the cases differed, suggesting different sources for these infections. MLST of these isolates revealed the presence of strains belonging to two CCs: CC4 or CC8 *[42]*. Thus, though these cases all occurred and were reported in the same time period, they were not necessarily part of the same outbreak event.

In one of the cases, *Cronobacter* was isolated from an opened tin of PIF and an opened bottle of nursery water that had been used to reconstitute the formula *[20, 21]*. Based on DNA fingerprinting, these isolates were found to match the isolates from the patient, but because the tin and bottle had already been opened, it was not possible to determine if either of these materials

were the source of the bacteria *[20, 21]*. Additionally, no *Cronobacter* strains were isolated from unopened tins of PIF, suggesting that the PIF was not the source of the infections *[20, 21]*. It is possible that the opened tin of PIF and the bottle of water had been contaminated by bacteria present in the environment or via a caretaker when preparing the formula.

Following the first death in December 2011, the PIF manufacturer recalled the product from stores as a precaution *[45]*. It was later shown that the PIF in question was not linked to the illnesses; however, the manufacturer saw a 10% drop in their share price *[45]*. This highlights the impact that *Cronobacter* infections can have on the industry and individual PIF manufacturers. Even though their product was not directly linked to any of the illnesses or deaths, the company saw significant losses. While PIF manufacturers do have a responsibility to protect consumers, the manufacturers themselves risk losses, even if they are not directly involved in an outbreak.

### 1.4. Microbiological criteria applied to PIF

It is important to note that PIF is not sterile and, therefore, some microorganisms are expected to be found in the product. Following several serious *Cronobacter* (*'E. sakazakii'*) outbreaks which linked the organism to PIF, it was determined that microbiological criteria needed to be established for the product *[10, 41, 59, 142, 160]*. The Codex Alimentarius Commission (CAC) published their standard in 2008, while the Food and Agriculture Organization of the United Nations (FAO) published reports in 2004 and 2006, in conjunction with the World Health Organization (WHO) *[26, 40, 41]*. An additional FAO-WHO report was published in 2008; however, this report focused on follow-on formula and is, therefore, not relevant to the current discussion of microbiological criteria for PIF *[39]*.

PIF is manufactured using a wet-mix, dry-mix, or combined process *[40, 159].* In the wet-mix process, all ingredients are mixed in liquid form before heat treatment and drying *[40, 159].* Conversely, in the dry-mix process, the individual ingredients are dried before they are combined to make the final product *[40, 159].* A combined process will incorporate elements of both the wet-mix and dry-mix processes *[40].* Dry-mix and combined processes allow for the incorporation of heat labile compounds into the formula; however, this may also present a source of contamination *[40].* The size of a batch of PIF varies between manufacturers. For a wet-mix process, a batch may be 1000-5000 gal, while a batch from a dry-mix process can be between 1000-5000 lbs *[159]*.

The CAC criteria state that 30 samples of 10 g each must be analysed for the presence of *Cronobacter* spp. and none of these samples can be positive *[26]*. Additionally, *Salmonella* spp.

must also be excluded from the PIF, based on the analysis of 60 samples of 25 g each. Meanwhile, the FAO-WHO reports do not state specific requirements for *Cronobacter* spp. or *Salmonella* spp. in PIF; however, both *Cronobacter* and *Salmonella enterica* are classified as 'Category A' organisms ("clear evidence of causality") *[40, 41]*. This designation indicates that the organisms in this category are capable of causing illnesses and have been linked to PIF *[40, 41]*. Other possible pathogens are classified as 'Category B' organisms ("causality plausible, but not yet demonstrated") because, while they are known to be capable of causing illness, their transmission via PIF has not yet been demonstrated *[40, 41]*. 'Category B' organisms include *Enterobacter cloacae, Escherichia coli, Esch. vulneris, Hafnia alvei, Klebsiella pneumoniae, Pantoea agglomerans, Serratia* spp., and *Acinetobacter* spp. *[41].* An outbreak involving *Enterobacter* spp., will be discussed in Chapter 4, including the implications this outbreak may have on the designation of species in the *Enterobacter* genus as 'Category B' organisms.

To meet the established microbiological criteria for PIF, accurate and specific detection and identification methods are required. Both the International Organization for Standardization (ISO) and the United States Food and Drug Administration (FDA) have published methods for detection and identification of *Cronobacter* spp. These methods are described below and the application of some techniques utilized by these standard methods will be discussed in Chapter 3.

## 1.5. Recommended detection and identification methods for *Cronobacter* spp.

### 1.5.1. ISO standard methods

The first ISO standard method for the detection and identification of *Cronobacter* spp. was published in conjunction with the International Dairy Federation in 2006 *[9, 71]*. This method was updated in 2015 with the publication of a draft standard titled "Microbiology in the food chain – Horizontal method for the detection of *Cronobacter* spp." *[70]*. Figure 1.2 summarizes and compares these two methods.

Both ISO methods utilize a two-step enrichment process for detection of *Cronobacter* spp. The first, pre-enrichment step is carried out in buffered peptone water (BPW) *[9, 70, 71]*. This allows microorganisms to recover from stresses encountered during processing and storage of the product and multiply to detectable levels. A selective enrichment step is then carried out in either modified lauryl sulphate broth (mLSB) or *Cronobacter* screening broth (CSB) *[9, 70, 71, 76]*. The composition of the broths and increased incubation temperatures inhibit the growth of competing microorganisms in the samples, leading to better recovery of *Cronobacter* spp.

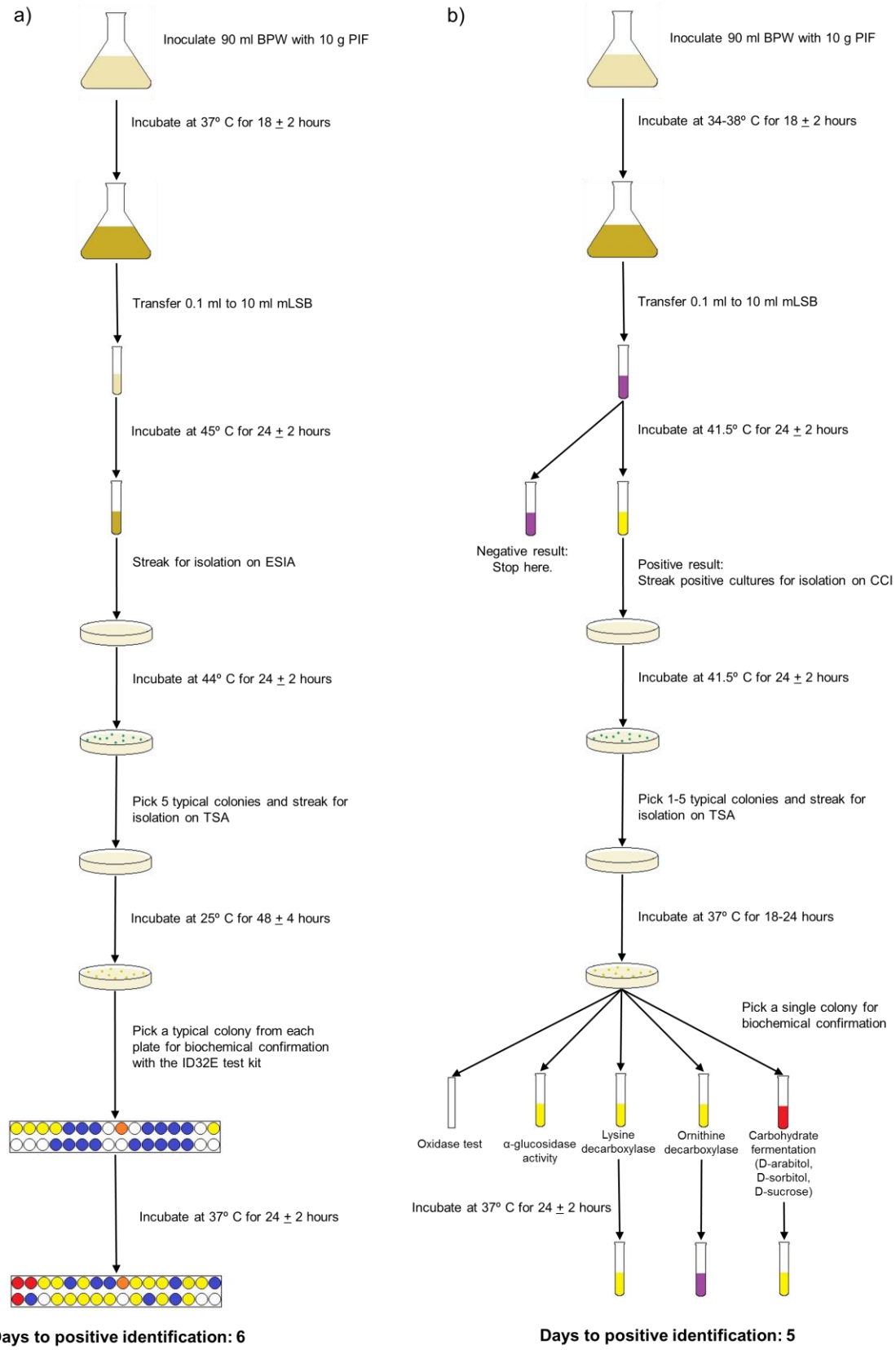


**Figure 1.2. ISO standard methods for detection and identification of *Cronobacter* spp.**
a) ISO-IDF method from 2006 *[9, 71]*; b) Draft ISO standard from 2015 *[70]*. The number of days required to reach a positive identification are indicated beneath each method.

Following the selective enrichment step, the samples are streaked for isolation on either '*E. sakazakii'* isolation agar (ESIA) or Chromogenic *Cronobacter* Isolation agar (CCI) *[9, 70, 71]*. This primary isolation step allows for identification of suspect isolates that must be further analysed to reach a positive identification. Suspect isolates are streaked for isolation on tryptic soy agar (TSA) and the plates are examined for yellow colonies after incubation *[9, 70, 71]*. It should be noted that colony morphology on TSA is not a conclusive test for identification of *Cronobacter* spp. Not all *Cronobacter* strains are capable of producing the yellow pigment and its production can be affected by both incubation temperature and exposure to light, which may result in misidentifications, if further characterization of isolates is not performed *[31, 34, 72, 85, 124]*.

According to the ISO standard methods, the identity of suspect isolates should be confirmed biochemically. While the 2006 method indicated that the ID32E biochemical test panel (bioMérieux, France) should be used for this purpose, the updated method describes more traditional techniques *[9, 70, 71]*. In the 2015 draft ISO method, seven biochemical tests (oxidase, hydrolysis of 4-nitrophenyl $\alpha$-D-glucopyranoside, L-lysine decarboxylase, L-ornithine decarboxylase, and acid production from D-arabitol, D-sorbitol, and D-sucrose) are required for identification of *Cronobacter* species., with three additional optional tests (methyl red, Voges-Proskauer, and acid production from α-methyl-D-glucosidase) *[70]*. The updated method states that 'miniaturized galleries' can be used in place of the more traditional methods for biochemical identification of suspect isolates *[70]*.

Though the ISO standard methods from 2006 and 2015 are similar, the more recent draft standard does have some advantages over the previous version. First, the use of CSB reduces the time required to reach a negative result. As this broth is both selective and differential, only samples giving positive results are analysed further to identify suspect isolates *[76]*. Negative results can be reached in less than 48 hours, allowing PIF manufacturers to release their product sooner *[76]*. Additionally, the 2015 draft standard increased the incubation temperature and decreased the incubation time of the TSA plates, allowing PIF manufacturers or food testing laboratories to reach a positive identification in five days instead of six *[70, 71]*.

The incubation temperatures for the selective enrichment and primary isolation steps in the 2015 ISO draft standard are also better suited to the isolation of *Cronobacter* spp. It has been shown that up to 6% of *Cronobacter* spp. (then known as *'E. sakazakii'*) were not able to grow in mLSB at 44° C, as prescribed in the 2006 ISO-IDF method *[9, 70, 72]*. Thus, the decreased incubation temperature and enrichment in CSB may allow for better recovery of *Cronobacter* spp.

when using the updated method. Yet, the incubation temperatures given in the updated version of the standard may still limit the effectiveness of the method. The secondary enrichment and primary isolation steps of the method use an incubation temperature of 41.5° C, but the type strain of *C. sakazakii* is unable to grow above 41° C *[70, 124]*. Thus, though the temperatures used in the updated method are better for the isolation of *Cronobacter* spp., this standard method may still produce in false negative results.

There are some additional problematic aspects of the method of the updated draft ISO standard from 2015. In particular, out-of-date species names are given for some of the negative control strains used for comparison in the biochemical analyses. The draft standard was published in 2015 and uses the names '*E. helveticus,' 'E. pulveris,'* and *'E. turicensis*,' even though these species names were not valid at the time *[15, 70, 146]*. Furthermore, the method requires only seven biochemical tests for the identification and differentiation of *Cronobacter* spp., with two additional optional tests, if necessary *[70]*. This is a relatively small number of tests to be used for species identification. The method does state that biochemical test panels such as the ID32E can be used in place of the more traditional biochemical assays *[70];* however, such test kits may result in misidentifications *[33, 74, 75]*. The use of biochemical test panels for identification of *Cronobacter* spp. and problems associated with such test kits will be discussed in Chapter 3.

### 1.5.2. FDA Bacteriological Analytical Manual methods

In 2002, the United States FDA added *Cronobacter* detection and identification methods to the Bacteriological Analytical Manual (BAM) *[158]*. These methods were updated in 2012 to include PCR-based identification of suspect isolates *[23-25, 101]*. These two methods are summarized and compared in Figure 1.3. Like the ISO methods, both FDA BAM methods utilize BPW for pre-enrichment to allow any microorganisms to recover from previously encountered stresses *[23-25, 101, 158]*. The method from 2002, however, uses this pre-enrichment as part of a 3-tube, 3-dilution most probable number (MPN) method, which allows for enumeration of *Cronobacter* cells present in the original sample *[158].*

After pre-enrichment, the methods diverge from one another. The method from 2002 continues with cultural detection and identification of *Cronobacter* spp. Subsamples from each MPN tube are transferred to *Enterobacteriaceae* enrichment broth (EE) for selective enrichment *[158]*. Suspect colonies are isolated on violet red bile glucose agar (VRBGA) and are then cultured on TSA and identified with the API20E biochemical test kit *[158]*.

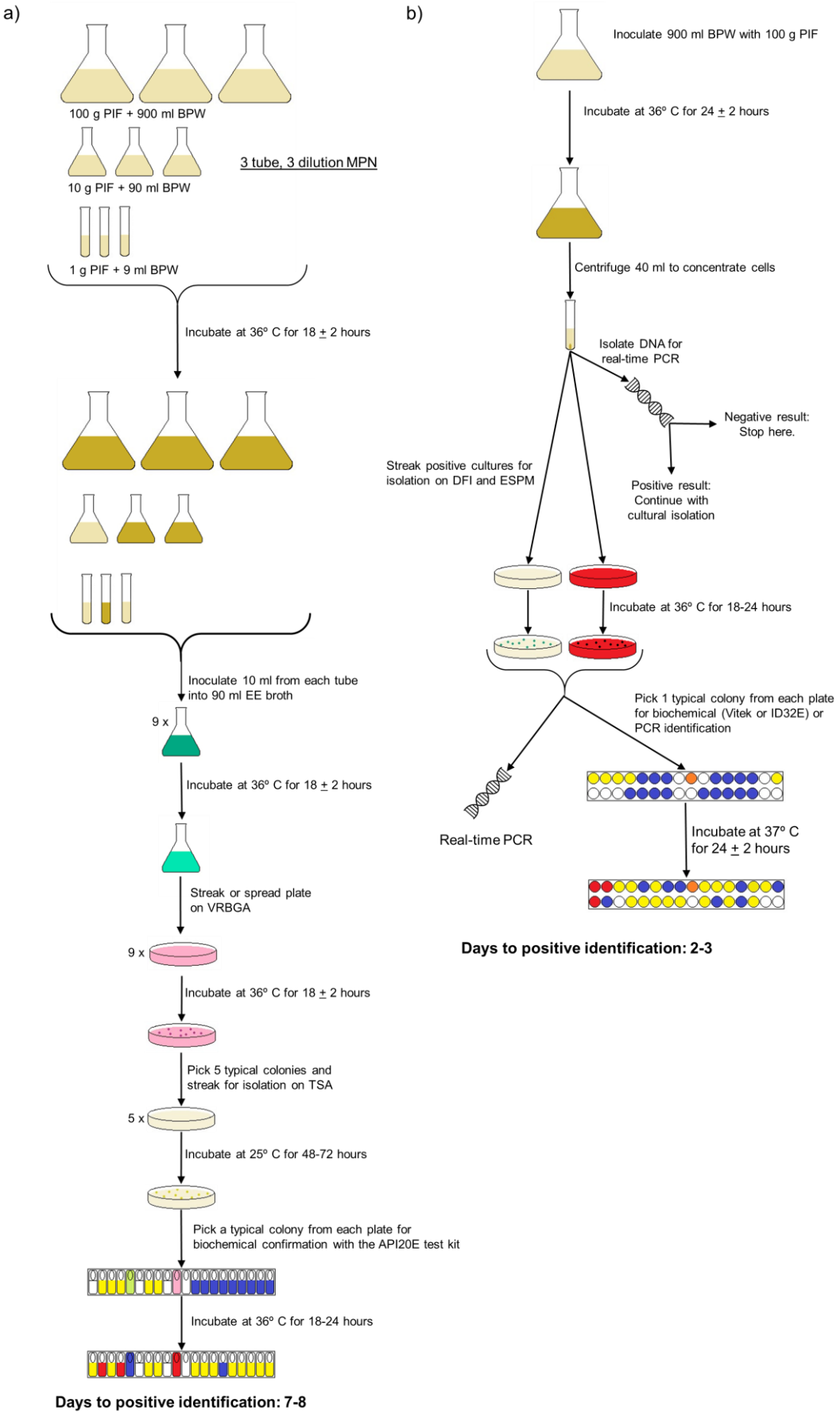


**Figure 1.3. US FDA BAM methods for detection and identification of *Cronobacter* spp.**
a) FDA BAM method from 2002 *[158]*; b) FDA BAM method from 2012 *[23-25, 101]*. The number of days required to reach a positive identification are indicated beneath each method.

Conversely, the updated FDA BAM method from 2012 does not use a selective enrichment step during cultural isolation of *Cronobacter* spp. Instead, the pre-enrichment cultures in BPW are centrifuged to harvest the cells and produce a concentrated cell suspension *[23-25, 101]*. This suspension is then used to perform a real time PCR-based identification method to screen for positive samples *[23-25, 101]*. Samples giving negative results with the PCR assay do not require any further analysis, again allowing for release of tested batches of product in a shorter period of time. Samples testing positive with the real-time PCR method are then confirmed culturally and biochemically; however, it should be noted that the cultural and biochemical methods described in the FDA BAM can be used independently of the PCR-based method *[23]*.

To confirm the results of the PCR assay or to isolate suspect colonies, the concentrated cell suspensions are streaked for isolation on two chromogenic agars: Druggan-Forsythe-Iversen agar (DFI) and R&F '*Enterobacter sakazakii'* chromogenic plating medium (ESPM) *[23-25, 74, 101, 130]*. Suspect colonies are then identified directly from these plates using biochemical or real-time PCR-based identification methods *[23-25, 101]*. This procedure eliminates the step requiring re-isolation of suspect isolates on TSA, reducing the amount of time required to reach a positive identification. When biochemical methods are used for identification of suspect isolates, the FDA BAM method recommends either the ID32E biochemical test panel or the Vitek 2.0 system (bioMérieux, France) *[23-25, 101]*.

While the method from 2002 did allow for enumeration of *Cronobacter* spp. in PIF, it was time consuming, labour intensive, and required large amounts of media, making the procedure highly inefficient. Additionally, as the microbiological criteria applied to PIF require the absence of all *Cronobacter* spp., enumeration is not really necessary to ensure compliance *[26]*. Thus, the updated method from 2012 better fits the needs of PIF manufacturers. In particular, the 2012 FDA BAM method greatly reduces the time required to test samples. As stated above, using the real time PCR method to screen for positive samples will allow manufacturers to release their products faster and will reduce the cost of microbiological testing because not all samples will need to be analysed with the full method. Moreover, the use of real-time PCR for confirmation of suspect isolates reduces the time required to identify these organisms. The biochemical methods both require an overnight incubation step, while the real time PCR method can be completed within a few hours. The updated FDA BAM method is the shortest of those described in this chapter, due to the elimination of the selective enrichment step, the step requiring isolation of suspect colonies on TSA and identification using real-time PCR instead of biochemical methods.

While the updated FDA BAM method shortens the time needed to reach a positive identification and reduces the amount of labour involved, this method may not be accessible to all users. Not all PIF manufacturers or food testing laboratories will have access to real-time PCR technology. These labs will be restricted to use of the cultural methods described in the FDA BAM, which increases the time and labour necessary to reach a positive identification of any suspect isolates. Additional problems may arise from the lack of a selective enrichment step during cultural isolation. This does shorten the time required for detection and identification of *Cronobacter* spp., but may give rise to false negative results. *Cronobacter* spp. are often present at low levels in PIF *[40, 73, 122, 123, 142]*. The use of a non-selective, pre-enrichment broth will allow for the growth of not only *Cronobacter* spp., but also competing microorganisms that may be present. Thus, it is possible that other organisms will outcompete and overgrow the *Cronobacter* spp., leading to lack of isolation from the selective agars.

Like the ISO standard methods, the use of biochemical identification methods in the FDA BAM may lead to misidentifications. Such misidentifications have been reported by other authors and discrepancies between the results of the API20E and ID32E test kits have also been reported *[33, 74, 75]*. As stated above, cultural, biochemical, and molecular identification methods for *Cronobacter* species will be examined in detail in Chapter 3, including a discussion of the use of these biochemical test panels for identification of suspect *Cronobacter* isolates.

# CHAPTER 2

# Materials and Methods

## 2.1. Preparation of culture media and reagents

Unless otherwise specified, culture media were prepared using the following general procedures. Where applicable, all culture media were prepared according to the manufacturer's instructions. Media were prepared and autoclaved at 121° C for 15 minutes. Liquid media were dispensed into tubes (10 ml/tube) and solid media were cooled to approximately 45° C before being dispensed into plates (25 ml/plate). Descriptions of each medium and any deviations from these procedures are given below.

### 2.1.1. Tryptic soy broth (TSB)

TSB contains pancreatic digest of casein (17.0 g/L), enzymatic digest of soya bean (3.0 g/L), sodium chloride (5 g/L), dipotassium hydrogen phosphate (2.5 g/L) and glucose (2.5 g/L). The casein and soya bean digests serve as sources of carbon, nitrogen, amino acids and other nutrients, while glucose serves an additional carbon source. This allows the medium to support the growth of a variety of microorganisms. Dipotassium hydrogen phosphate is a buffering agent to maintain the pH of the broth during growth. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM0129).

### 2.1.2. *Enterobacteriaceae* enrichment (EE) broth

EE broth contains pancreatic digest of gelatine (10.0 g/L), glucose (4.5 g/L), bile salts (sodium desoxycholate, sodium lauryl sulphate and sodium citrate; 3.055 g/L), potassium dihydrogen phosphate (2.0 g/L), disodium hydrogen phosphate (6.4 g/L), and brilliant green (0.015 g/L). The digest of gelatine is a source of amino acids and the glucose provides a carbon source. This medium was designed to allow for the growth of all *Enterobacteriaceae.* The bile salts and brilliant green inhibit the growth of competing Gram positive organisms. The remaining ingredients are buffering agents to maintain a neutral pH during growth. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM1115).

### 2.1.3. *Cronobacter* screening broth (CSB)

CSB contains peptone (10.0 g/L), meat extract (3.0 g/L), sodium chloride (5.0 g/L), bromocresol purple (0.04 g/L), sucrose (10.0 g/L), and vancomycin (10.0 µg/ml) *[76]*. The peptone and meat extract provide nitrogen and amino acids. The high concentration of sucrose selects for organisms that are resistant to osmotic stress and this sugar also serves as a carbon source. The pH change caused by fermentation of the sucrose is indicated by the bromocresol purple, which will cause the broth to change from purple to yellow under acidic conditions. Additional selectivity against Gram positive organisms is provided by the vancomycin. The dehydrated medium containing all ingredients except the vancomycin was obtained from Oxoid Thermofisher, Basingstoke, UK (CM1121). A filter-sterilized stock solution of vancomycin (2.5 mg/mL) was aseptically added to the cooled medium to reach the final concentration of 10 µg/ml. The final pH of the broth was adjusted to 7.4 $\pm$ 0.1, as needed *[76]*.

### 2.1.4. Modified lauryl sulphate broth (mLSB)

Lauryl sulphate broth contains tryptone (20.0 g/L), lactose (5.0 g/L), sodium chloride (5.0 g/L), dipotassium hydrogen phosphate (2.75 g/L), potassium dihydrogen phosphate (2.75 g/L), and sodium lauryl sulphate (0.1 g/L). The modified version of this broth, mLSB, includes an additional 0.5 M sodium chloride (total sodium chloride concentration: 34 g/L) and vancomycin (10.0 µg/ml) *[53]*. The tryptone provides a source of nitrogen and amino acids while the lactose provides a carbon source. The dipotassium hydrogen phosphate and potassium dihydrogen phosphate again serve to buffer the medium against pH changes caused by bacterial growth. The high level of sodium chloride selects for organisms resistant to osmotic stress. Additional selectivity is provided by the sodium lauryl sulphate, which inhibits non-coliforms, and the antibiotic vancomycin, which inhibits the growth of Gram positive organisms. This medium was prepared from its constituent ingredients. All ingredients except the vancomycin were suspended in 1 L of distilled water before autoclaving. A filter-sterilized stock solution of vancomycin (2.5 mg/mL) was aseptically added to the cooled medium to reach the final concentration of 10 µg/ml.

### 2.1.5. Phenol red broth

Phenol red broth contains an enzymatic digest of casein (10.0 g/L), sodium chloride (5.0 g/L), and phenol red (0.018 g/L). The digest of casein acts as a source of amino acids. Phenol

red is a pH indicator which turns from red to yellow in acidic conditions, indicating fermentation of the included carbohydrate(s). This medium was prepared from its constituent components. Filter sterilized solutions of the desired carbohydrates (as indicated where necessary in Chapter 4) were added to the cooled medium to reach a final concentration of 1% (w/v). The final pH of the broth was adjusted to 7.4 $\pm$ 0.1, as needed.

### 2.1.6. Iso-Sensitest broth

Iso-Sensitest broth was designed for use in antimicrobial susceptibility testing. It allows for the growth of a variety of microorganisms, but is semi-defined to ensure reproducible results when testing antimicrobial resistance. Iso-Sensitest broth contains hydrolysed casein (11 g/L), peptones (3.0 g/L), glucose (2.0 g/L), sodium chloride (3.0 g/L), soluble starch (1.0 g/L), disodium hydrogen phosphate (2.0 g/L), sodium acetate (1.0 g/L), magnesium glycerophosphate (0.2 g/L), calcium gluconate (0.1 g/L), cobaltous sulphate (0.001 g/L), cupric sulphate (0.001 g/L), zinc sulphate (0.001 g/L), manganous chloride (0.002 g/L), menadione (0.001 g/L), cyanocobalamin (0.001 g/L), L-cysteine hydrochloride (0.02 g/L), L-tryptophan (0.2 g/L), pyridoxine (0.003 g/L), pantothenate (0.003 g/L), nicotinamide (0.003 g/L), biotin (0.003 g/L) thiamine (0.00004 g/L), adenine (0.01 g/L), guanine (0.01 g/L), xanthine (0.01 g/L), and uracil (0.01 g/L). The hydrolysed casein and peptones provide nitrogen and amino acids, while glucose and soluble starch serve as carbon sources. The remaining components are buffering agents and trace minerals and nutrients to support the growth of microorganisms. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM471). The pH of the medium was adjusted to 7.4 $\pm$ 0.2, as needed.

### 2.1.7. Tryptic soy agar (TSA)

TSA contains pancreatic digest of casein (15.0 g/L), enzymatic digest of soya bean (5.0 g/L), sodium chloride (5.0 g/L), and agar (15.0 g/L). Casein and soya bean digests provide carbon, nitrogen, amino acids and other nutrients to support growth of a range of microorganisms. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM0131).

### 2.1.8. Violet red bile glucose agar (VRBGA)

VRBGA contains yeast extract (3.0 g/L), peptone (7.0 g/L), sodium chloride (5.0 g/L), Bile Salts No. 3 (1.5 g/L), glucose (10.0 g/L), neutral red (0.3 g/L), crystal violet (0.002 g/L), and agar

(12.0 g/L). The casein and yeast extract provide sources of nitrogen, amino acids and other nutrients. The glucose provides a source of carbon. Again, the bile salts inhibit Gram positive organisms. Neutral red and crystal violet are pH indicators that result in colour changes when glucose is fermented. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM0485). The dehydrated medium was suspended in distilled water and boiled for 2 minutes. This agar is not autoclaved due to the heat sensitive nature of some ingredients.

### 2.1.9. Druggan-Forsythe-Iversen agar (DFI)

DFI contains tryptone (15.0 g/L), soya peptone (5.0 g/L), sodium chloride (5.0 g/L), ferric ammonium citrate (1.0 g/L) sodium deoxycholate (1.0 g/L), sodium thiosulphate (1.0 g/L), chromogen (0.1 g/L) and agar (15.0 g/L) *[74]*. The tryptone and peptone provide sources of carbon, nitrogen, amino acids and other nutrients. Sodium deoxycholate is a bile salt to inhibit Gram positive organisms. Sodium thiosulphate provides a source of sulphur and, in combination with the ferric ammonium citrate, will result in black colonies for organisms that produce hydrogen sulphide. The chromogen results in blue-green colonies when $\alpha$-glucosidase is being produced. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM1055).

### 2.1.10. M9 minimal medium (M9)

M9 is prepared using a 5x salt solution containing disodium phosphate (33.9 g/L), sodium diphosphate (15.0 g/L), sodium chloride (2.5 g/L), and ammonium chloride (5 g/L). M9 salts were obtained from Sigma Aldrich (M6030; Dorset, UK). The 5x M9 salt solution was prepared by dissolving 56.4 g in 1 L of distilled water before autoclaving. To prepare the agar, 200 ml of the 5x salt solution and 15 g of agar were mixed with 800 ml of distilled water before autoclaving.

M9 contains salts and buffering agents, but no nutrients. Filter sterilized solutions of desired carbohydrates and other nutrients (as described below) were added to the cooled agar to reach a final concentration of 1% (w/v). Additionally, 1 M solutions of magnesium sulphate and calcium chloride were prepared and filter sterilized. These salts were added to the cooled agar to reach final concentrations of 2 mM and 0.1 mM, respectively before pouring the agar into plates.

### 2.1.11. Motility agar

Motility agar consists of tryptone (2.0 g/L), sodium chloride (1.0 g/L) and agar (1 g/L). Tryptone provides a source of both carbon and nitrogen. The sodium chloride provides osmotic balance. A reduced concentration of agar results in a semi-solid medium through which motile bacteria can swim. Motility agar was prepared from its constituent components. The cooled agar was dispensed into 5 ml tubes.

### 2.1.12. Iso-Sensitest agar

Iso-Sensitest agar has the same composition as Iso-Sensitest broth described above, but with the addition of agar (8.0 g/L) as a solidifying agent. This medium was obtained from Oxoid Thermofisher, Basingstoke, UK (CM0471).

### 2.1.13. Infant formula (IF) agar

IF agar was prepared as described by Caubilla-Barron and Forsythe *[8]*. Briefly, 0.4 g of ammonium sulphate and 3.0 g of agar were suspended in 40 ml of distilled water and autoclaved. After cooling, 200 ml of pre-sterilized, pre-warmed (55° C) IF (Cow and Gate Infant First Milk) was added to the autoclaved agar mixture. The microorganisms utilize the nutrients in the infant formula and the ammonium sulphate acts as a source of nitrogen.

### 2.1.14. Congo red agar (CRA)

CRA was prepared as described by Monteiro *[120]*. The medium is based on LB agar without salt and contains tryptone (10 g/L), yeast extract (5 g/L), and agar (15 g/L). The tryptone and yeast extract provide carbon, nitrogen, and other nutrients. This medium was prepared from its constituent components. After cooling, the agar was supplemented with filter sterilized solutions of Congo red and Coomassie brilliant blue G-250 to final concentrations of 40 µg/ml and 20 µg/ml, respectively. Congo red binds to cellulose produced by the cells and the Coomassie blue binds to protein. Colony morphologies vary and will be discussed in Chapter 5.

### 2.1.15. Saline solution

A 0.85% (w/v) solution of saline was prepared by dissolving 4.25 g of sodium chloride in 500 ml of distilled water before autoclaving. The solution was aseptically dispensed in appropriate volumes as indicated before use.

## 2.2. Resuscitation of cultures

Unless otherwise specified, cultures were resuscitated from freezer stocks. Cultures were stored in 1 ml of TSB with 20% glycerol at -80° C. Strains were streaked for isolation on TSA and incubated for 72 hours at 30° C. Isolated colonies were then selected from these stock plates for further analysis. Stock plates were stored at 4° C for no longer than one week.

## 2.3. Biochemical phenotyping methods

### 2.3.1. API20E (20 100; bioMérieux, France)

Tests included in the API20E test kit are shown in Table 2.1. A single colony from the stock plate was streaked for isolation on TSA and incubated at 37° C for 18-24 hours. This ensured that a pure, fresh culture was used for biochemical identification. A single colony from this freshly-grown plate was emulsified in 5 ml of sterile 0.85% saline to obtain a homogenous suspension. This solution was used to fill the wells of each test on the strip. In addition, the cupules were also filled for the citrate, Vogues-Proskauer (acetoin production), and gelatinase tests. The arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, hydrogen sulphide production and urease tests were covered with 2 drops of mineral oil to create anaerobic incubation conditions. Test strips were incubated at 37° C for 18-24 hours in a tray with 5 ml distilled water to maintain the humidity.

**Table 2.1 Tests included in the API20E test kit**

| Enzyme activities | Carbohydrate utilization |
|---|---|
| Acetoin production | Amygdalin |
| Arginine dihydrolase | L-arabinose |
| β-galactosidase | D- glucose |
| Citrate utilization | D-inositol |
| Gelatinase | D-mannitol |
| $H_2S$ production | D-melibiose |
| Indole production | L-rhamnose |
| Lysine decarboxylase | D-sorbitol |
| Ornithine decarboxylase | D-sucrose |
| Tryptophan deaminase | |
| Urease | |

After incubation, one drop of TDA reagent (70 402; bioMérieux, France) was added to the tryptophan deaminase test, one drop of JAMES reagent (70 542; bioMérieux, France) to the indole test, and one drop each of VP1 and VP2 reagents (70 422; bioMérieux, France) to the Vogues-Proskauer test. Results were assessed as described by the manufacturer. Species identifications were determined using the APIWeb database (https://apiweb.biomerieux.com) for API20E. Species identifications were determined using version 4.0 or version 5.0 of the APIWeb database, as indicated in Chapter 3.

### 2.3.2. ID32E (32 400; bioMérieux, France)

Tests included in the ID32E test kit are shown in Table 2.2. A single colony from the stock plate was streaked for isolation on TSA and incubated at 37° C for 18-24 hours to ensure that a pure, fresh culture was used for analysis. Isolated colonies were emulsified in 2 ml of sterile 0.85% saline to a turbidity equivalent to 0.5 McFarland. Each well was filled with 55 µl of this suspension. The ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, urease, and L-arabitol, galacturonate, and 5-ketogluconate fermentation tests were covered with mineral oil to create anaerobic incubation conditions. Strips were incubated at 37° C for 18-24 hours.

Before recording results, one drop of JAMES reagent (70 542; bioMérieux, France) was added to the indole test. Results were assessed as described by the manufacturer. Species identifications were determined using the APIWeb database (https://apiweb.biomerieux.com) for ID32E. Species identifications were determined using version 3.0 or version 4.0 of the APIWeb database, as indicated in Chapter 3.

**Table 2.2. Tests included in the ID32E test kit**

| Enzyme activities | Carbohydrate utilization |
|---|---|
| α-galactosidase | Adonitol |
| α-glucosidase | L-arabinose |
| α-maltosidase | D-arabitol |
| Arginine dihydrolase | L-arabitol |
| L-aspartic acid arylamidase | D-cellobiose |
| β-galactosidase | Galacturonate |
| β-glucosidase | D-glucose |
| β-glucuronidase | Inositol |
| Indole production | Malonate |
| Lipase | D-maltose |
| Lysine decarboxylase | D-mannitol |
| N-acetyl-β-glucosaminidase | Palatinose |
| Ornithine decarboxylase | L-rhamnose |
| Phenol red acidification | D-sorbitol |
| Urease | D-sucrose |
| | D-trehalose |
| | 5-ketogluconate |

### 2.3.3. 50CHE (50 300; bioMérieux, France)

The carbohydrates included in the 50CHE test are shown in Table 2.3. A single colony from the stock plate was streaked for isolation on TSA and incubated at 37° C for 18-24 hours, to ensure a pure, fresh culture was used for analysis. Isolated colonies were emulsified in 1 ml of sterile distilled water to a turbidity equivalent to 4 McFarland. The entirety of this suspension was added to one vial of CHBE medium (50 430; bioMérieux, France). The wells of the test strip were filled with the suspension and covered with mineral oil to ensure anaerobic conditions. Results were read after 24 and 48 hours of incubation at 37° C. Positive results were indicated by a colour change from red to yellow due to a decrease in the pH, except for the test for aesculin utilization which turned black due to the hydrolysis of aesculin.

**Table 2.3. Carbohydrates included in the 50CHE test kit**

| | | |
|---|---|---|
| D-adonitol | D-glucose | Potassium gluconate |
| Amidon (starch) | Glycerol | Potassium-2-ketogluconate |
| Amygdalin | Glycogen | Potassium-5-ketogluconate |
| D-arabinose | Inositol | D-raffinose |
| L-arabinose | Inulin | L-rhamnose |
| D-arabitol | D-lactose | D-ribose |
| L-arabitol | D-lyxose | D-saccharose (sucrose) |
| Arbutin | D-maltose | Salicin |
| D-cellobiose | D-mannitol | D-sorbitol |
| Dulcitol (galactitol) | D-mannose | L-sorbose |
| Erythritol | D-melezitose | D-tagatose |
| Aesculin | Methyl-αD-mannopyranoside | D-trehalose |
| D-fructose | Methyl-αD-glucopyranoside | D-turanose |
| D-fucose | Methyl-ßD-xylopyranoside | Xylitol |
| L-fucose | D-melibiose | D-xylose |
| D-galactose | N-acetyl-glucosamine | L-xylose |
| Gentibiose | | |

### 2.3.4. API ZYM (25 200; bioMérieux, France)

The tests included in the API ZYM kit are shown in Table 2.4. A single colony from the stock plate was streaked for isolation on TSA and incubated at 37° C for 18-24 hours. This ensured that a pure, fresh culture was used for biochemical identification. A single colony from this freshly-grown plate was emulsified in 2 ml of sterile 0.85% saline to obtain a turbidity equivalent to 5-6 McFarland. Each well of the test strip was filled with 65 µl of suspension. Test strips were incubated in a tray with 5 ml distilled water to maintain the humidity at 37° C for 4 hours. One drop each of ZYMA and ZYMB reagents (70 493 and 70 494; bioMérieux, France) was added to each well. Results were assessed as described by the manufacturer, with a score from 0-5 being assigned based on the intensity of the colour in each well.

**Table 2.4. Enzyme activities assayed in the API ZYM test kit**

| | | |
|---|---|---|
| Acid phosphatase | ß-galactosidase | Lipase (C14) |
| Alkaline phosphatase | ß-glucosidase | Leucine arylamidase |
| α-chymotrypsin | ß-glucuronidase | Naphthol-AS-BI-phosphohydrolase |
| α-galactosidase | Cysteine arylamidase | N-acetyl-ß-glucosaminidase |
| α-glucosidase | Esterase (C4) | Trypsin |
| α-fucosidase | Esterase lipase (C8) | Valine arylamidase |
| α-mannosidase | | |

### 2.3.5. Catalase test

Strains were streaked for isolation on TSA and incubated at 37° C for 24 hours. A capillary tube was filled with 3% hydrogen peroxide. The tube was touched to a single isolated colony on the plate. Formation of bubbles within the tube was considered to be a positive reaction.

### 2.3.6. Oxidase test

The oxidase test was performed using Oxidase Detection Strips (MB0266; Oxoid Thermofisher, Baisingstoke, UK). Strains were streaked for isolation on TSA and incubated at 37° C for 24 hours. The test strip was touched to a single isolated colony and observed for 5 seconds. Development of a blue colour on the test strip within this time period was considered to be a positive result.

### 2.3.7. Phenol red test

Phenol red broths were prepared as described in section 2.1.5. A single colony of each strain was inoculated from the stock plate into 10 ml phenol red broth supplemented with each

carbohydrate. The carbohydrates used for analysis are indicated, where necessary in the following chapters. Tubes were incubated at 37° C for 24 hours. Positive results were indicated by a colour change from red to yellow due to acid production from utilization of the supplemented carbohydrate. To test gas production, the broth was dispensed into test tubes each containing an inverted Durham tube before autoclaving. A positive result for gas production was indicated by the formation of air bubbles within the Durham tube.

### 2.3.8. Motility assay

Motility agar was prepared as described in section 2.1.11. A single colony from the stock plate for each strain was picked with a sterile inoculating needle. Cells were inoculated into the motility agar via stab culture. Tubes were inoculated in duplicate and incubated at 25° C or 37° C for 24-72 hours. A positive result was indicated by growth spreading away from the initial point of inoculation.

### 2.3.9. Assessment of sialic acid utilization

M9 agar was prepared as described in section 2.1.10. Agar was supplemented with either glucose, sialic acid, casamino acids, or a combination of sialic acid and casamino acids. All supplements were added to reach a final concentration of 1%. A single colony from each stock plate was streaked for isolation on each type of supplemented M9 agar and on M9 with no supplements. No growth was expected on the unsupplemented medium. The plates containing only glucose or casamino acids were used to ensure that the medium itself was not inhibiting the growth of the organisms. In order to be considered positive for sialic acid utilization, strains must be capable of growth on both types of agar containing sialic acid. The plates containing a combination of both sialic acid and casamino acids were included to ensure that the lack of growth was due to inability to utilize sialic acid and not because the strains were auxotrophic.

### 2.3.10. Antibiotic resistance testing

Antimicrobial resistance was determined according to the methods described by the British Society for Antimicrobial Chemotherapy *[16]*. Strains were streaked for isolation on TSA and incubated overnight (18-24 hours) at 37° C. A sterile loop was used to touch four isolated colonies and inoculate them into 10 ml of Iso-Sensitest broth. These cultures were incubated at

37° C to a McFarland standard of 0.5 or greater. Cultures at a higher density were diluted with sterile distilled water to reach a McFarland standard of 0.5. All cultures were then diluted 1:100 in 10 ml of sterile distilled water, as indicated for *Enterobacteriaceae* in the BSAC method *[16]*. Cotton swabs were used to spread the diluted cell suspensions onto the surface of Iso-Sensitest agar plates. Plates were allowed to dry covered, at room temperature before the addition of the desired antibiotic discs (Mast Diagnostics, Bootle, UK), indicated where appropriate in Chapter 4. Plates were incubated at 37° C for 18-20 hours. Zones of inhibition around each disc were measured. Strains were determined to be resistant, intermediate, or sensitive to each antibiotic based on the size of the zones of inhibition as described in the BSAC method *[16]*.

## 2.4. DNA-based laboratory methods

### 2.4.1. PCR-based identification of *Cronobacter* spp.

*PCR identification of Cronobacter using the gene rpoB.* The PCR assay targeting *rpoB* utilizes a unique set of primers for each of the seven previously defined *Cronobacter* species *[104, 147]*. Only the target species should produce an amplicon of the expected size with each primer set (Table 2.5). Cross-reactivity was reported for *C. malonaticus* strains with the *C. sakazakii* primers; therefore, the original method specified that any isolate testing positive with the *C. sakazakii* primers must also be tested with the *C. malonaticus* primers to distinguish these closely related species *[147]*.

**Table 2.5. PCR primer sequences and amplification conditions for the gene *rpoB***

| Primer name | Primer sequence (5'→3') | Annealing temperature (° C) | Extension time (sec) | Expected amplicon size (bp) |
|---|---|---|---|---|
| Cdublf | GCACAAGCGTCGTATCTC | 62 | 30 | 418 |
| Cdublr | TTGGCGTCATCGTGTTCC | | | |
| Cmalf | CGTCGTATCTCTGCTCTC | 60 | 30 | 251 |
| Cmalr | AGGTTGGTGTTCGCCTGA | | | |
| Cmuyf | TGTCCGTGTATGCGCAGACC | 61 | 30 | 289 |
| Cmuyr | TGTTCGCACCCATCAATGCG | | | |
| Csakf | ACGCCAAGCCTATCTCCGCG | 67 | 60 | 514 |
| Csakr | ACGGTTGGCGTCATCGTG | | | |
| Cturf | CGGTAAAAGAGTTCTTCGGC | 61 | 60 | 628 |
| Cturr | GTACCGCCACGTTTCGCC | | | |
| Cgenomof[a] | ACAAACGTCGTATCTCTGCG | 61 | 30 | 506 |
| Cgenomor | AGCACGTTCCATACCGGTC | | | |
| Ccon-f | AACGCCAAGCCAATCTCG | 55 | 30 | 689 |
| Ccon-r | GTACCGCCACGTTTTGCT | | | |

[a]*Cronobacter* genomospecies I was later renamed *C. universalis* (Joseph *et al.,* 2012)

A single colony from the stock plate was suspended in 100 µl sterile distilled water and held at 100° C for 10 minutes. This boiled cell lysate was used as a source of template DNA. Sterile distilled water was substituted for cell lysate in the negative control reaction. The PCR mixture consisted of: 25 µL GoTaq Green MasterMix (2x GoTaq Green buffer, 400 µM dNTPs, 3 mM magnesium chloride, GoTaq Polymerase; Promega, UK) 10 pM of each primer, 5 µL boiled cell lysate and 19.8 µL sterile distilled water (total volume: 50µl) *[104, 147]*. Primer sequences are shown in Table 2.5. As the primers were designed before *Cronobacter* genomospecies I was renamed *C. universalis*, the primers for this species are Cgenomof and Cgenomor.

PCR amplification was carried out as follows: 94° C for 3 minutes; 30 cycles of 94° C for 1 minute, annealing for 30 seconds and extension at 72° C, followed by a final extension at 72° C for 5 minutes *[104, 147]*. Table 2.5 gives the annealing temperatures and extension times for each primer set. PCR products were separated on a 1.5% agarose gel containing 0.1µl/ml SYBR SAFE (Life Technologies, Paisley, UK) at 90 V for 30 minutes. Results were visualized with UV light. A positive reaction was indicted by a band of the expected size (Table 2.5).

*PCR identification of Cronobacter using the gene ompA*. The PCR assay targeting *ompA* was first described by Mohan-Nair and Venkitanarayanan *[119]*. This assay was designed to detect '*Enterobacter sakazakii.*' As the *Cronobacter* genus encompasses all organisms previously called '*E. sakazakii*', this method should identify all *Cronobacter* species.

Boiled cell lysate was prepared as described above. Sterile distilled water was substituted for cell lysate in the negative control reaction. The PCR mixture consisted of: 5 µL 1x GeneAmp Buffer II (N8080161; Life Technologies, Paisley, UK), 2.5 µL magnesium chloride (2.5 mM), 1 µL dNTP mix (200 µM), 0.5 µL of each primer (1 µM), 2 U AmpliTaq Polymerase (N8080161; Life Technologies, Paisley, UK), 10 µL cell lysate, and 27.6 µL sterile distilled water (total volume: 50 µL) *[119]*. The PCR primers ESSF (5'-GGATTTAACCGTGAACTTTTCC-3') and ESSR (5'-CGCCAGCGATGTTAGAAGA-3') were used for amplification *[119]*.

PCR amplification was carried out as follows: 94° C for 2 minutes; 30 cycles of 94° C for 15 seconds, 60° C for 15 seconds and 72° C for 30 seconds; 72° C for 5 minutes *[119]*. PCR products were separated by gel electrophoresis as described above and were visualized with UV light. The expected amplicon size was 469 bp and results were only considered positive if a band of this size was observed *[119]*.

### 2.4.2. DNA extraction

DNA extraction was carried out using the GenElute Bacterial Genomic DNA kit (NA2110; Sigma-Aldrich, Dorset, UK). A single colony from the stock plate was inoculated into 10 ml TSB and incubated at 37° C for 18-24 hours. Cells were pelleted from 1.5 ml of culture by centrifugation at 14,000 rpm for 2 minutes. The supernatant was discarded.

Cells were then resuspended in 180 µl of Lysis Solution T and 20 µl of Proteinase K Solution were added. Tubes were incubated in a heat block at 55° C for 30 minutes. Following incubation, 200 µl of Lysis Solution C were added. Tubes were incubated at 55° C for 10 minutes. This process lyses the cells and releases the DNA into solution. The DNA was then precipitated from this lysate by the addition of 200 µl of ethanol (95-100%).

Collection columns were prepared by adding 500 µl of Column Preparation Solution. Columns were centrifuged at 13,500 rpm for 1 minute. The eluate was discarded and the columns were ready to bind DNA. Using a wide-bore pipettor tip, the entire lysate, including the precipitated DNA, was transferred to the DNA binding column. Columns were centrifuged at 10,000 rpm for 1 minute. After centrifugation, columns were transferred to new collection tubes. The DNA bound to the columns was washed with 500 µl of Wash Solution containing 80.0% ethanol. Columns were centrifuged at 14,000 rpm for 3 minutes. After ensuring columns were dry and contained no residual ethanol, the columns were transferred to new collection tubes. DNA was eluted from the columns using 200 µl of Elution Solution. To increase DNA concentration, the columns were incubated at room temperature for up to 15 minutes before centrifugation. Columns were centrifuged at 10,000 rpm for 1 minute to collect the DNA. DNA concentration in the eluate was measured with the NanoDrop 2000 (Thermo Scientific, UK). DNA was stored at -20° C until use.

### 2.4.3. Multilocus sequence typing (MLST)

MLST was carried out as described by Baldwin *et al*. *[6]*. This method utilizes the sequences of seven housekeeping genes to assign strains to sequence types (STs). The genes used in this scheme were selected based on their function and necessity for survival *[6]*. The genes are also distributed around the genome to ensure that they are not affected by the same selective pressures *[6].* The genes, their functions, primer sequences, and expected allele sizes are shown in Table 2.6.

**Table 2.6. Genes, functions, primers and allele sizes for MLST of *Cronobacter* spp.**

| Gene | Function | Direction | Amplification primers (5'→3') | Sequencing primers (5'→3') | Allele size (bp) |
|---|---|---|---|---|---|
| *atpD* | ATP synthase (ß-chain) | F | CGACATGAAAGGCGACAT | CGAAATGACCGACTCCAA | 390 |
| | | R | TTAAAGCCACGGATGGTG | GGATGGCGATGATGTCTT | |
| *fusA* | Elongation factor G | F | GAAACCGTATGGCGTCAG | GCTGGATGCGGTAATTGA | 438 |
| | | R | AGAACCGAAGTGCAGACG | CCCATACCAGCGATGATG | |
| *glnS* | Glutaminyl t-RNA synthetase | F | GCATCTACCCGATGTACG | GGGTGCTGGATAACATCA | 363 |
| | | R | TTGGCACGCTGAACAGAC | CTTGTTGGCTTCTTCACG | |
| *gltB* | Glutamate synthase (large subunit) | F | CATCTCGACCATCGCTTC | GCGAATACCACGCCTACA | 507 |
| | | R | CAGCACTTCCACCAGCTC | GCGTATTTCACGGAGGAG | |
| *gyrB* | DNA gyrase (subunit B) | F | TGCACCACATGGTATTCG | CTCGCGGGTCACTGTAAA | 402 |
| | | R | CACCGGTCACAAACTCGT | ACGCCGATACCGTCTTTT | |
| *infB* | Translation initiation factor IF-2 | F | GAAGAAGCGGTAATGAGC | TGACCACGGTAAAACCTC | 441 |
| | | R | CGATACCACATTCCATGC | GGACCACGACCTTTATCC | |
| *ppsA* | Phosphoenolpyruvate synthase | F | GTCCAACAATGGCTCGTC | ACCCTGACGAATTCTACG | 495 |
| | | R | CAGACTCAGCCAGGTTTG | CAGATCCGGCATGGTATC | |

*Amplification.* The first stage of MLST is amplification of the target genes. Separate reactions were run for each gene. Each 25 µl amplification reaction mixture contained: 5 µl of 5x Green GoTaq Flexi buffer, 2 mM $MgCl_2$, 0.4 mM of each dNTP, 1 pmol/µl forward and reverse amplification primers, 1 µl genomic DNA (40-50 ng) and 1.25 U GoTaq Polymerase (Promega, South Hampton, UK) *[6]*. Sterile distilled water was used in place of the DNA for negative control reactions. Amplification conditions were as follows: initial denaturation at 94° C for 2 min; 30 cycles of denaturation at 94° C for 1 min, annealing at 58° C for 1 min and extension at 72° C for 2 min; and a final extension at 72° C for 5 min *[6]*.

Amplification was confirmed by gel electrophoresis. A 5 µl aliquot of each sample was loaded into a 1.5% agarose gel containing 0.1 µl/ml SYBR Safe (S33102; Life Technologies, Paisley, UK). The gel was run at 90 V for 30 min and was visualized with UV light.

*Purification of PCR amplicons*. Following confirmation of PCR amplification by gel electrophoresis, the amplicons were purified using the QIAquick PCR Purification Kit (28106; Qiagen, Manchester, UK). The PCR reaction mixture was combined with 5x volumes of Buffer PB and transferred to a QIAquick column. Columns were centrifuged for 1 min at 13,000 rpm and the flow-through was discarded. The columns were then washed with 750 µl of Buffer PE containing 70% ethanol. Columns were again centrifuged for 1 min at 13,000 rpm and the flow-through was discarded. The column was centrifuged a second time to remove any residual ethanol. The columns were then placed in clean tubes to collect the purified PCR product. The DNA was eluted in 50 µl of sterile distilled water by centrifugation at 13,000 rpm for 1 minute. The concentration of the DNA was measured with the Nanodrop 2000 (Thermo Scientific, UK).

*Sequencing of PCR amplicons*. The concentration of the DNA was diluted to approximately 10 ng/µl in 40 µl of sterile distilled water. Samples were loaded in duplicate into MicroAmp Optical 96-well Reaction Plates (N8010560; Life Technologies, Paisley, UK) and plates were covered with MicroAmp Optical Adhesive Film (4360954; Life Technologies, Paisley, UK). Duplicate samples were necessary as forward and reverse sequences were determined separately. Sequencing primers were diluted to 3.2 pmol/µl and 12 µl of each sequencing primer (forward and reverse) were prepared for each sample. Samples were sequenced using the Sanger sequencing service at Source BioScience (Nottingham, UK).

*Sequence analysis.* Chromatograms for each sequence were examined. Analysis was continued for those with clear base calls. Forward and reverse sequences were combined into a single contig using CAP3 Sequence Assembly Program at http://doua.prabi.fr/software/cap3 *[66]*. Contigs were then aligned with the available allele sequences from the *Cronobacter* PubMLST database using CLUSTALW (http://www.ebi.ac.uk/Tools/msa/clustalw2) *[42, 153]*. The sequences were then trimmed to the appropriate length and compared to the sequences in the *Cronobacter* PubMLST database *[42]*. Allele numbers and STs were assigned based on matches to the sequences in the database. New allele numbers and STs were assigned sequentially by the database curator (S. J. Forsythe), as necessary. Concatenated sequences (3,036 bp) were used for phylogenetic analyses as described below.

### 2.4.4. Whole genome sequencing (WGS)

For WGS, DNA was extracted as described above. DNA samples were sent to either Exeter University or Swansea University for sequencing. The resulting sequences were assembled by P. Ogrodzki (NTU) using SPAdes and QUAST for quality assessment *[7, 54]*. Genomes were also annotated by P. Ogrodzki (NTU) using Prokka *[140]*. All whole genome sequences are available via the *Cronobacter* PubMLST database *[42]*.

## 2.5. *In silico* DNA sequence-based analyses

### 2.5.1. Ribosomal multilocus sequence typing (rMLST)

Concatenated sequences were extracted using the Genome Comparator tool at www.pubmlst.org/Cronobacter *[42]*. In total, 53 loci were used in these analyses with a concatenated length of 22,511 bp. Phylogenetic analyses were performed as described below.

### 2.5.2. Phylogenetic analyses

*DNA sequence alignment.* For analysis, DNA sequences were aligned using the CLUSTALW method in MEGA6 *[153]*. Where indicated, the number of polymorphic sites were determined by MEGA6, based on these alignments *[153]*.

*Construction of phylogenetic trees.* Phylogenetic trees were constructed using the neighbour joining or maximum likelihood method in MEGA6, as indicated *[153]*. Bootstrapping was used to test the phylogenies and the number of replicates is indicated where necessary.

*Construction of phylogenetic networks.* Phylogenetic networks were constructed with SplitsTree4 *[69]*. These networks do not necessarily show the evolutionary history of the included strains, but indicate conflicting phylogenies that result when different fragments of the sequences are analysed. Thus, splits within the network may represent evidence of horizontal gene transfer.

### 2.5.3 Average nucleotide identity (ANI)

Average nucleotide identity (ANI) analyses were carried out using the ANI tool at http://enve-omics.ce.gatech.edu/ani/ *[48]*. Reported values are the result of the two-way analysis. Values of ≥95% indicate that two strains belong to the same species *[48]*. ANI values below this threshold indicate that strains belong to separate species.

### 2.5.4. Other *in silico* analyses

*Calculation of variable sites and percentage of polymorphic nucleotides.* After the DNA sequences were aligned, MEGA6 was used to identify and count the number of variable sites in each gene *[153]*. The total number of polymorphisms was calculated by determining the consensus sequence for each gene and counting the number of polymorphisms in all strains being

analysed. The total number of nucleotides included in the analysis was calculated by multiplying the total number of strains by the length of the gene being analysed. The total number of polymorphisms was then divided by the total number of nucleotides and multiplied by 100 to calculate the percentage of polymorphic nucleotides in each gene.

*Z-test for selection.* The Z-test for selection was used to determine if sequences were undergoing purifying selection, based on the number of synonymous ($d_S$) or nonsynonymous ($d_N$) substitutions per site *[5].* Individual gene sequences were extracted using the BLAST function of the *Cronobacter* PubMLST database *[42].* To test for purifying selection, codons were aligned and a one-tailed Z-test for was performed in MEGA6 with 500 bootstrap replicates *[5, 153].* A p-value of >0.05 indicates neutrality ($d_S = d_N$) and a p-value of <0.05 indicates that the sequence is under purifying selection ($d_N < d_S$) *[5]*. Thus, the DNA sequence is changing when strains are undergoing purifying selection, but the translated protein sequence is not.

*Analysis of recombination by the difference sum-of-squares method.* The difference sum-of-squares method was used to analyse sequences for possible recombination using Topali2 *[114, 117].* A window size of 500 bp and a step size of 10 bp were used for this analysis. Starting at the beginning of the sequence, two windows of 500 bp are identified. The distance matrix is calculated for the first window and the phylogeny of the region is determined by minimizing the sum-of-squares value *[114].* A distance matrix is also calculated for the second window and it is fitted to the topology determined for the first window. This results in a sum-of-squares value for the second window. The difference between these two sum-of-squares values indicates whether a possible recombination event has occurred. The windows are then shifted by 10 bp and the process is repeated to the end of the sequence.

Topali2 plots the difference in the sum-of-squares values against the position in the sequence *[114, 117]*. A significance value of 0.95 ($p < 0.05$) was used for all analyses. This automatically generates a threshold marker on the graphical output. Any peaks which appear above the marked threshold indicate possible sites of recombination *[114, 117]*.

# CHAPTER 3

# Re-evaluation of *Cronobacter* detection and identification methods

*The results presented in this chapter have been published as peer-reviewed journal articles.*
***Jackson, E. E.**, et al. (2014)* Food Microbiology *44: 226-235*.
***Jackson, E. E.** & Forsythe, S. J. (2016)* BMC Microbiology *16.1*

*See Appendix B for the full text versions of these publications.*

## 3.1. Introduction

As discussed in Chapter 1, the taxonomy of the *Cronobacter* genus was revised multiple times between 2007 and 2013 *[15, 77, 78, 93, 146]*. A summary of these changes is shown in Figure 3.1. Of importance for this chapter is the reclassification of *Enterobacter helveticus*, *E. pulveris* and *E. turicensis* as *Cronobacter helveticus*, *C. pulveris* and *C. zurichensis*, respectively *[15, 144, 145]*. It should be noted that the majority of the experiments described in this chapter were carried out in 2013, when these species were considered to be members of the *Cronobacter* genus. As such, the results will be presented using the correct taxonomy for that time. In 2014, however, a further taxonomic revision occurred, which removed these species from the *Cronobacter* genus *[146].* This later revision and its implications will be discussed in more detail in Section 3.5.

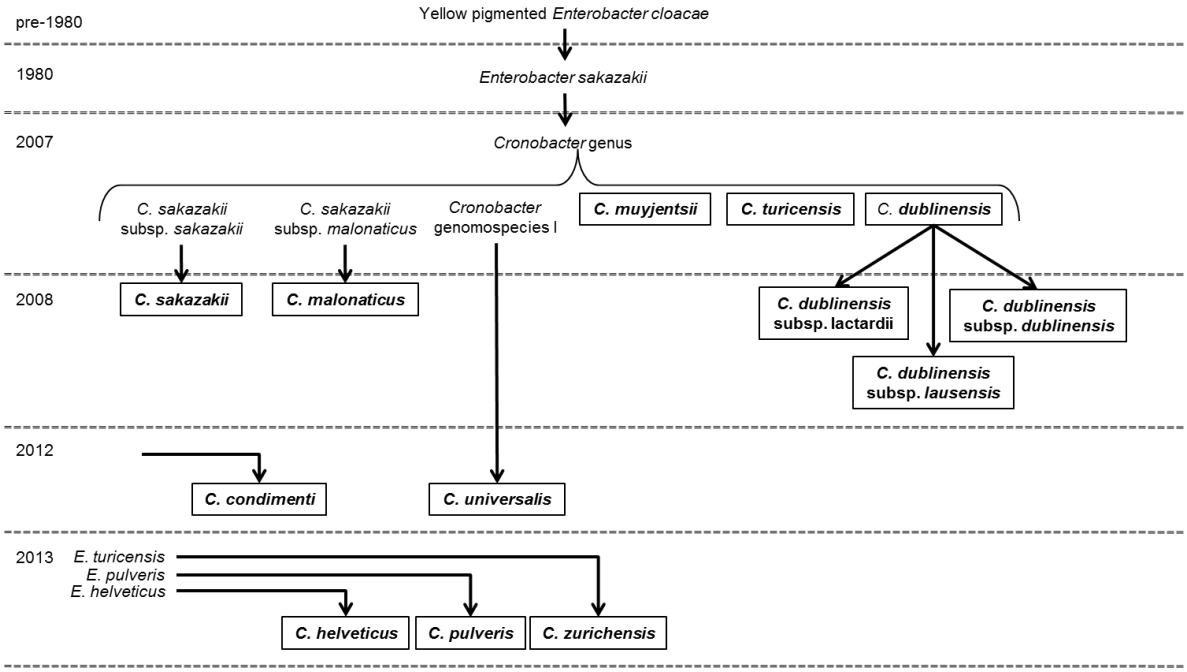


**Figure 3.1. A summary of taxonomic revisions of the *Cronobacter* genus (1980-2013)**

The 2013 reclassification of three *Enterobacter* spp. as *C. helveticus, C. pulveris,* and *C. zurichensis* had serious implications for infant formula manufacturers because international microbiological criteria require the absence of all *Cronobacter* species in powdered infant formula (PIF) *[26, 40, 41]*. In order to meet these criteria, specific and accurate detection and identification methods must be utilized to ensure detection of all *Cronobacter* spp., including the reclassified species of *C. helveticus, C. pulveris,* and *C. zurichensis.* Detection and identification of *Cronobacter* species is typically performed using a combination of cultural, biochemical, and/or molecular methods, as described in Chapter 1 *[9, 23-25, 70, 71, 101, 158]*. Recommended procedures for the isolation of *Cronobacter* species from food products utilize one or more enrichment steps, followed by isolation on selective and/or differential agars. The nonselective, pre-enrichment stage allows the organisms to multiply and recover from stresses (heat, desiccation, etc.) encountered during food processing. Buffered peptone water (BPW) is commonly used for pre-enrichment of *Cronobacter* spp. from PIF, but this broth also allows for the growth of other microorganisms that may be present *[9, 23-25, 70, 71, 101, 158]*.

The subsequent enrichment step is more selective and permits the growth of only a limited range of species. The inclusion of components such as bile salts and antibiotics will inhibit the growth of other microorganisms present in the PIF while still allowing the target organisms to multiply. *Enterobacteriaceae* enrichment broth (EE), modified lauryl sulphate broth (mLSB) or *Cronobacter* screening broth (CSB) may be used for this step during the isolation and identification of *Cronobacter* spp. *[9, 53, 70, 71, 76 158]*. While EE broth will allow for the growth of all *Enterobacteriaceae*, the composition of mLSB and CSB combined with increased incubation temperatures (45° C or 41.5° C, respectively) limits the growth of many non-*Cronobacter* species *[9, 53, 70, 71, 76]*. In particular, CSB is recommended in the draft standard for detection of *Cronobacter* spp. published by the International Organization for Standardization (ISO) in 2015, while mLSB was recommended in the previous version of the standard *[9, 70, 71, 76]*. Conversely, the US Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) formerly recommended the use of EE broth, but now proposes concentration of the bacteria from pre-enrichment cultures by centrifugation in place of a selective enrichment step *[23-25, 101, 158]*. Regardless of the method, microorganisms are isolated from the culture or concentrated cell suspension by plating onto selective, differential or chromogenic agars, such as violet red bile glucose agar (VRBGA), Druggan-Forsythe-Iversen agar (DFI), Chromogenic *Cronobacter* isolation

agar, R&F '*Enterobacter sakazakii*' chromogenic plating medium (ESPM), or tryptic soy agar (TSA) *[9, 23-25, 70, 71, 74, 101, 129, 158]*.

Despite their wide use, cultural methods can be limited by many factors. For example, increased incubation temperatures may inhibit the growth of both target and non-target organisms. Iversen *et al. [76]* found that four *Cronobacter* strains were not able to grow in mLSB at 44º C, even though incubation at 45º C was specified in the previous version of the ISO standard method *[9, 71]*. Similarly, colony morphologies can be misleading as well. Yellow pigment production on TSA is often considered to be indicative of *Cronobacter* spp., but production of this pigment can be affected by a variety of conditions, including incubation temperature and exposure to light *[31, 34, 85]*. Further confusion may arise as members of the *Cronobacter* genus are not the only organisms capable of producing the expected colony morphologies on selective and differential agars. For example, in addition to *Cronobacter* species, strains of *Escherichia hermanii, Citrobacter koseri,* and *Pantoea* spp. also produce $\alpha$-glucosidase, resulting in typical blue-green colonies on DFI *[74]*. Thus, further characterization of suspect isolates is required in order to obtain a positive species identification of presumed *Cronobacter* isolates.

Species identification of suspect isolates can be achieved using biochemical or molecular methods. Commercial biochemical identification kits are frequently employed for this purpose as they are convenient and easy to use. The substrates are already included in the test strips and the user needs only to add a suspension of the bacteria. Following incubation, the results are interpreted using an online database. The API20E and ID32E biochemical test panels use 20 or 32 biochemical tests, respectively, to generate a specific code, linked to a species identification database. Though the API20E kit was specified for identification of *Cronobacter* in a previous version of the FDA BAM, the updated method recommends either the ID32E test panel or the Vitek 2.0 system for biochemical identification of suspect *Cronobacter* isolates *[23-25, 101, 158]*. Similarly, a previously published ISO-IDF method recommended use of the ID32E test kit; however, the draft method for *Cronobacter* detection published in 2015 requires the use of seven biochemical tests (oxidase, hydrolysis of 4-nitrophenyl $\alpha$-D-glucopyranoside, L-lysine decarboxylase, L-ornithine decarboxylase, and acid production from D-arabitol, D-sorbitol, and D-sucrose) for identification and differentiation of *Cronobacter* spp. (see Box 3.1) *[9, 70, 71]*. Though traditional biochemical methods are described for each of these assays, six of the seven required tests are included in the ID32E biochemical panel and the draft ISO standard method indicates that 'miniaturized galleries' are an acceptable alternative for biochemical identification *[70]*.

**Box 3.1. Percentage of strains giving positive results for biochemical tests specified in the 2015 draft ISO standard for detection and identification of *Cronobacter* spp. *[70]***
Required test are shown in bold. Tests included in the ID32E biochemical test panel are shaded grey.

| | *C. dublinensis* | *C. malonaticus* | *C. condimenti* *C. muytjensii* | *C. sakazakii* | *C. turicensis* *C. universalis* |
|---|---|---|---|---|---|
| **Oxidase** | **0** | **0** | **0** | **0** | **0** |
| **α-glucosidase** | **100** | **100** | **100** | **100** | **100** |
| **Lysine decarboxylase** | **0** | **0** | **0** | **0** | **0** |
| **Ornithine decarboxylase** | **100** | **95** | **100** | **94** | **90** |
| Methyl red | 0 | 0 | 0 | 1 | 0 |
| Voges-Proskauer | 100 | 100 | 100 | 99 | 100 |
| Acid production from: | | | | | |
| **D-arabitiol** | **17** | **0** | **0** | **0** | **0** |
| α-methyl-D-glucoside | 100 | 100 | 0 | 100 | 100 |
| **D-sorbitol** | **0** | **0** | **0** | **0** | **0** |
| **D-sucrose** | **100** | **100** | **100** | **100** | **100** |

Though frequently used, commercial phenotyping kits do have some significant drawbacks. Their reliance on subjective, visual detection of colour changes can introduce uncertainty in the species identifications. They are also limited by the relatively small number of tests included in each test panel, which may not be able to accurately represent the variation that exists between and within bacterial species. Finally, the databases associated with the biochemical test kits contribute to the confusion, as they are not frequently updated. Despite the definition of the *Cronobacter* genus in 2007, the API20E and ID32E databases continued to use the former name '*E. sakazakii'* until 2015. The consequences and implications of the 2015 update to these databases will be discussed in more detail later in this chapter.

Molecular, DNA-based methods are considered to be more reliable than biochemical phenotyping for species identification of suspect *Cronobacter* isolates. PCR probe-based methods for *Cronobacter* spp. target a variety of genes, including *cgcA*, *gyrB*, *ompA*, *rpoB*, *gluA*, *dnaG*, *tuf, zpx*, iron acquisition genes, the macromolecular synthesis operon, the O-antigen locus, the 16S rRNA gene, and the 16S-23S intergenic transcribed spacer *[17, 50, 57, 64, 65, 83, 99, 104-106, 109, 119, 141, 147, 150, 151].* These methods, however, are limited by the PCR primer sequences. False negative results may occur due to the absence of target genes or sequence variation in the primer binding sites. Meanwhile, false positives could result from the presence of the primer binding sites in species that are closely related to the target organisms.

In addition to the specific issues discussed for each test above, the efficacy of all detection and identification assays can be affected by taxonomic revisions. Development and evaluation of detection and identification methods requires inclusivity and exclusivity strain testing. Inclusivity testing with a variety of *Cronobacter* species and strains ensures that most or all *Cronobacter* isolates will be correctly identified. Conversely, exclusivity testing using closely-related, non-target organisms ensures that most or all non-target organisms are excluded.

As stated previously, the reclassification of three *Enterobacter* species as members of the *Cronobacter* genus in 2013 is particularly important for this chapter. Prior to this reclassification, strains of '*E. helveticus,' 'E. pulveris,'* and '*E. turicensis'* had been used for exclusivity testing during development of some *Cronobacter* detection and identification assays. In particular, these species were used as negative control strains during the development of CSB and to test PCR-based assays targeting *cgcA*, *rpoB,* the O-antigen locus, and iron acquisition genes *[17, 50, 76, 83, 121, 151]*. Additionally, the methods described for cultural detection and real-time PCR-based identification of *Cronobacter* species described in the FDA BAM included strains of the reclassified species as negative controls *[24, 101, 158]*. The biochemical profiles of these species are also provided for comparison in the 2015 ISO draft method for *Cronobacter* spp. *[70]*.

Thus, the taxonomic revisions within the *Cronobacter* genus challenge the reliability of some detection and identification methods. As all *Cronobacter* species must be excluded from PIF, the assays employed by a manufacturer or food testing laboratory must be both accurate (able to correctly identify positive samples) and specific (able to correctly identify negative samples). The reclassification of species once used as negative control organisms therefore necessitated a re-evaluation of commonly used detection and identification assays. This chapter discusses this re-evaluation through the phenotypic and genotypic characterization of *Cronobacter* strains, with a particular focus on the reclassified species of *C. helveticus*, *C. pulveris*, and *C. zurichensis*. Though alternative methods such as MALDI-TOF MS, the Vitek 2.0 system, and real-time PCR are available for *Cronobacter* spp., they were not included in this re-evaluation. Cultural, biochemical, and PCR-based detection and identification methods are recommended by both the ISO and FDA standard methods and are, therefore, more widely used *[23-25, 71, 101]*. Additionally, these methods do not require specialized equipment or trained personnel, making them more accessible to PIF manufacturers and food testing laboratories.

## 3.2 *Cronobacter* detection and identification assays utilized in this study

### 3.2.1. Species and strains

As given in Table 3.1, 27 bacterial strains were used for laboratory re-evaluation of *Cronobacter* detection and identification assays. This selection of strains consisted of the type strains of the seven original *Cronobacter* species, and multiple strains of *C. helveticus* (n = 8), *C. pulveris* (n = 4) and *C. zurichensis* (n = 3). Strains of *Escherichia hermanii* (n = 2), *Pantoea* spp. (n = 2), and *Buttiauxella nokiae* (n = 1) were used as negative control organisms. These strains were selected as negative controls as they had previously produced false positive results in one or more of the assays selected for laboratory evaluation.

All culture media were prepared as described in Chapter 2. Strains were stored and resuscitated as described in Section 2.2.

**Table 3.1. Bacterial species and strains used in this study**

| Species | Strain | Source | Country of isolation (Year) |
|---|---|---|---|
| *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 (unknown) |
| *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 | United Kingdom (2011) |
| | 1392 | Ingredients | United Kingdom (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 | United Kingdom (2004) |
| *Escherichia hermanii* | 159 | Dried food | United Kingdom (2004) |
| | 162 | Rice | United Kingdom (2004) |
| *Pantoea* spp. | 44 | Baby food | Korea (2004) |
| | 1318 | Environment | France (2009) |

### 3.2.2. Selective enrichment broths

*Enterobacteriaceae enrichment (EE) broth.* A single colony from each stock plate was inoculated into 10 ml of EE broth, followed by incubation at 37° C for up to 72 hours. A positive result was indicated by turbidity due to growth of the organism.

*Modified lauryl sulphate broth (mLSB).* A single colony from each stock plate was inoculated into 10 ml of mLSB, in duplicate, followed by incubation at either 37° C or 45° C for up to 72 hours *[9, 53, 71]*. A positive result was indicated by turbidity due to growth of the organism.

*Cronobacter screening broth (CSB).* A single colony from each stock plate was inoculated into 10 ml of CSB, in duplicate. Tubes were incubated at 37° C or 45° C for up to 72 hours. A positive result was indicated by a colour change from purple to yellow, indicating a decrease in pH due to the utilization of sucrose *[70, 76]*.

### 3.2.3. Selective and differential agars

*Tryptic soy agar (TSA).* An individual colony from each stock plate was streaked for isolation on TSA in duplicate. Colony morphology on TSA was examined after incubation at 30° C or 37° C for up to 72 hours. Typical *Cronobacter* colonies on TSA are yellow and may be either glossy or rugose *[23]*.

*Violet red bile glucose agar (VRBGA).* An individual colony from each stock plate was streaked for isolation on VRBGA. These plates were incubated at 37° C for up to 72 hours. Typical colonies are are pink to purple, and may be surrounded by a pink to purple halo, indicating fermentation of glucose. Additionally, *Cronobacter* species may produce mucoid colonies on this agar *[149]*.

*Druggan-Forsythe-Iversen agar (DFI).* A single colony from the stock plate was streaked for isolation on DFI. Colony morphologies were examined after incubation at 37° C for up to 72 hours. Typical *Cronobacter* colonies are blue-green due to the activity of $\alpha$-glucosidase *[74]*.

### 3.2.4. Phenotyping with biochemical test kits

All strains were analysed with the API20E and ID32E biochemical test kits (bioMérieux, France), according to the manufacturer's instructions and as described in Chapter 2. Each profile is linked to a species identification in the associated online database. The profiles were analysed in 2013 using version 4.0 of the API20E database and version 3.0 of the ID32E database. Analysis of the profiles was repeated following a 2015 update to the databases and species identifications were then determined using version 5.0 of the API20E database and version 4.0 of

the ID32E database. Also following the 2015 update, the profile numbers from archived culture collection records at NTU were reanalysed using the updated database versions. The identifications from previous version(s) of the database were compared to the identifications from the updated versions for over 240 strains. These strains were collected from 21 different countries over 65 years (1950 – 2015) and were obtained from a variety of sources including PIF and PIF manufacturing environments, foods, herbs and spices, and clinical samples.

For the older versions of both databases, a result of '*Enterobacter sakazakii'* was considered to be a match for all *Cronobacter* strains. A result of '*Cronobacter* spp.' was considered a match for all *Cronobacter* strains with the updated version of the API20E database; however, with the updated ID32E database, a result was only considered to be a match if the identified species matched the species as determined by one or more DNA sequence-based methods. Strains giving a result of 'Unacceptable profile' without a species identification were considered to be mismatches. Identifications with the older versions of the databases were not available for all strains in the archived culture collection records. These results were assigned as 'unknown' and were not considered to be either matches or mismatches. As the previous version(s) of the database were no longer available, some analyses were conducted on different numbers of strains. A $X^2$ analysis was used to determine if the percentage of strains producing matches were significantly different between the old and new versions of the databases.

### 3.2.5. PCR-based identification methods

In order to select PCR-based identification methods for laboratory analysis, the BLAST function of the online *Cronobacter* Bacterial Isolate Genome Sequence Database (BIGSdb) (www.pubMLST.org/*Cronobacter*) was used to search the genome sequences of 34 *Cronobacter* strains *[42]*. These genomes were examined for the presence of target gene sequences used in the original design of PCR primers from a variety of PCR-based identification methods. Target genes and accession numbers are shown in Table 3.2. 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, it was considered absent. These thresholds were arbitrarily defined and absent genes were confirmed by genome sequence alignment using WebAct (http://www.webact.org/WebACT/home) *[1, 2]*. Based on these results, PCR assays targeting *ompA* and *rpoB* were selected for laboratory evaluation *[104, 119, 147]*.

**Table 3.2. Target genes and accession numbers used for genome searching**

| Gene | Reference | Genbank accession number[a] |
|---|---|---|
| *cgcA* | *[17]* | ESA_01230 |
| *gluA* | *[107]* | AM075208[b] |
| *gluB* | *[107]* | AM075208[b] |
| *gyrB* | *[65]* | JX088572 |
| *dnaG* | *[141]* | L01755 |
| *ompA* | *[119]* | DQ000206 |
| *rpoB* | *[104]* | JQ316670 |
| | *[147]* | FJ717638 |
| | *[147]* | FJ717652 |
| | *[147]* | FJ717656 |
| | *[147]* | FJ717657 |
| | *[147]* | FJ717658 |
| | *[147]* | FJ717659 |
| *zpx* | *[99]* | EF061082 |

[a]Sequences were used for *Cronobacter* BIGSdb BLAST searches. [b]Sequences for *gluA* and *gluB* were extracted from the partial genome sequence available with this accession number.

The PCR assay targeting *ompA* was first described by Mohan-Nair and Venkitanarayanan *[119]*. This assay was designed before the establishment of the *Cronobacter* genus and therefore detects '*Enterobacter sakazakii.*' As the *Cronobacter* genus encompasses all organisms previously called '*E. sakazakii*,' this method should identify all *Cronobacter* spp. This method was performed as described in Section 2.4.1. A result was only considered to be positive if an amplicon of the expected size of 469 bp was detected *[119]*.

The PCR assay targeting *rpoB* was described by Stoop *et al. [146]* and expanded by Lehner *et al. [104]*. The method utilizes a unique set of primers for each of the seven previously defined *Cronobacter* species. Only the target species should produce an amplicon of the expected size. Cross-reactivity was reported for *C. malonaticus* with the *C. sakazakii* primers; therefore, the original method specified that isolates testing positive with the *C. sakazakii* primers must also be tested with the *C. malonaticus* primers to differentiate these closely related species *[147]*. The assays were carried out as described in Section 2.4.1. Table 3.3 shows the expected amplicon sizes for each primer set. As the primers were designed before *Cronobacter* genomospecies I was renamed *C. universalis*, the primers for this species are named Cgenomof and Cgenomor. A result was considered positive if an amplicon of the expected size was produced.

**Table 3.3. Expected amplicon sizes produced by PCR primers targeting *rpoB***

| Primer names | Expected amplicon size (bp) | Reference |
|---|---|---|
| Cdublf/Cdubr | 418 | *[147]* |
| Cmalf/Cmalr | 251 | *[147]* |
| Cmuyf/Cmuyr | 289 | *[147]* |
| Csakf/Csakr | 514 | *[147]* |
| Cturf/Cturr | 628 | *[147]* |
| Cgenomof/Cgenomor[a] | 506 | *[147]* |
| Ccon-f/Ccon-r | 689 | *[104]* |

[a]*Cronobacter* genomospecies I was later renamed *C. universalis [93]*.

### 3.2.6. *In silico* analysis of the *ompA* PCR identification method

For a more thorough analysis of the PCR-based method targeting *ompA*, the genomes of 223 strains were examined for the presence of the PCR primer binding sites. Using *in silico* analysis, a large number of strains from diverse sources could be examined, despite the fact that they are not centrally available in a single culture collection. This analysis used 186 genomes from the seven original species of *Cronobacter*, 18 genomes from the three reclassified species, and 19 genomes from the closely-related *Citrobacter, Enterobacter,* and *Yersinia* genera *[42]*.

The BLAST function of the BIGSdb was used to search the genomes for *ompA* (GenBank accession number: DQ000206) *[42, 119]*. These sequences were extracted with 500 flanking nucleotides as primer binding sites do not necessarily fall within the target gene. Gene sequences were aligned with MEGA6 and the primer binding sites were identified *[153]*. The number of mismatches to each primer was counted for each strain. By comparing these results to the results of the laboratory analyses, the number of mismatches detected was used to predict if amplification would occur for each strain and to determine the expected size of the amplicon. A similar analysis could not be performed for the method targeting *rpoB* because the genomes of strains producing negative results with certain primers sets were not available for analysis. Thus, it was not always possible to determine how many mismatches would result in lack of amplification, making it impossible to predict all results based solely on the DNA sequences.

## 3.3. Re-evaluation of *Cronobacter* detection and identification assays

### 3.3.1. Growth of *Cronobacter* spp. in selective enrichment broths

Table 3.4 shows the growth of all 27 strains in the tested enrichment broths. As expected, pure cultures of all species and strains were capable of growth in EE broth at 37° C; however, this broth supports the growth of not only *Cronobacter* spp., but all *Enterobacteriaceae*. This is particularly important as *Cronobacter* spp. are often present at low levels in PIF and may be overgrown by competing microorganisms during enrichment *[40, 73, 122, 123, 142]*. Thus, mLSB and CSB were developed for selective enrichment and subsequent isolation of *Cronobacter* from mixed cultures and food samples.

**Table 3.4 Growth of *Cronobacter* and related species in common enrichment broths**

| | | Growth in broth culture | | | | |
|---|---|---|---|---|---|---|
| | | EE | mLSB | | CSB[a] | |
| Bacterial species | Strain | (37° C) | (37° C) | (45° C) | (37° C) | (41.5° C) |
| *C. condimenti* | LMG 26250$^T$ | + | + | - | + | + |
| *C. dublinensis* | LMG 23823$^T$ | + | + | - | + | + |
| *C. malonaticus* | LMG 23826$^T$ | + | + | + | + | + |
| *C. muytjensii* | ATCC 51329$^T$ | + | + | + | + | + |
| *C. sakazakii* | ATCC 29544$^T$ | + | + | - | + | + |
| *C. turicensis* | LMG 23827$^T$ | + | + | - | + | + |
| *C. universalis* | NCTC 9529$^T$ | + | + | + | + | + |
| *C. helveticus* | LMG 23732$^T$ | + | - | - | - | - |
| | 1204 | + | + | + | - | - |
| | 1208 | + | + | - | - | - |
| | 1344 | + | + | - | - | - |
| | 1373 | + | + | - | - | - |
| | 1374 | + | + | - | - | - |
| | 1387 | + | + | - | - | - |
| | 1392 | + | + | - | - | - |
| *C. pulveris* | LMG 24057$^T$ | + | + | - | + | + |
| | LMG 24059 | + | + | - | + | + |
| | 1390 | + | + | - | + | + |
| | 1393 | + | + | - | + | + |
| *C. zurichensis* | LMG 23730$^T$ | + | + | - | - | - |
| | LMG 23731 | + | + | - | - | - |
| | 1383 | + | + | - | - | - |
| Negative control strains | | | | | | |
| *B. noakiae* | 53 | + | + | - | - | - |
| *Esch. hermanii* | 159 | + | + | - | - | - |
| | 162 | + | + | - | - | - |
| *Pantoea* spp. | 44 | + | + | - | + | - |
| | 1318 | + | + | - | + | - |

[a]Positive reaction in CSB was indicated by turbidity and a colour change from purple to yellow, as prescribed by the original method *[76]*. Strains exhibiting turbidity but no colour change were considered negative

mLSB utilizes high salt content, vancomycin and incubation at 45° C for selective enrichment of *Cronobacter* spp. *[53]*. Except for one *C. helveticus* strain (LMG 23732$^T$), all ten *Cronobacter* spp. were able to grow in mLSB at 37° C, but the prescribed incubation temperature for this broth is 45° C *[9, 71]*. Incubation at the increased temperature did not permit growth of the type strains of *C. condimenti*, *C. dublinensis*, *C. sakazakii*, or *C. turicensis*. Of the reclassified species, only *C. helveticus* strain 1204, was able to grow in mLSB at 45° C.

It has been reported that higher incubation temperatures may inhibit the growth of some *Cronobacter* strains. For example, up to 6% of '*E. sakazakii*' strains were unable to grow at 44° C in mLSB and the maximum growth temperature for of *'E. sakazakii'* was found to be between 41º C and 45º C *[72, 73]*. Thus, incubation at these temperatures may slow or even prevent growth of *Cronobacter* spp., particularly when using a selective medium. For mLSB, an increased incubation period of 48 h at 45° C did not improve recovery and resulted in only the *C. sakazakii* type strain exhibiting slightly greater turbidity. This broth was intended for use with selective and/or differential agars to identify suspect isolates, but the absence of turbidity after the prescribed 24

hour incubation period indicates that *Cronobacter* spp. are not able to grow much, if at all, in mLSB at 45° C. Thus, the bacterial population may not reach a high enough concentration to result in detection on agar plates when following the prescribed protocols *[9, 53, 71]*. As a result, CSB was designed as an alternative selective enrichment broth for *Cronobacter* species.

Like mLSB, CSB utilizes a raised incubation temperature (41.5° C) to increase the selectivity of the enrichment procedure *[71, 76]*. High levels of sucrose select for strains resistant to osmotic stress and vancomycin inhibits the growth of Gram positive bacteria *[76]*. This broth is also differential due to the inclusion of the pH indicator bromocresol purple to detect the decrease in pH resulting from utilization of sucrose *[76]*. Iversen *et al. [76]* indicated that presumptive positive samples (yellow colour) require further testing, including cultural isolation and biochemical or molecular identification, but negative results (purple colour) do not. Thus, use of CSB reduces the time to a negative result, allowing PIF manufacturers to release tested batches sooner.

In the current study, results for the CSB enrichment were considered positive when the colour change from purple to yellow was observed after 24 hours of incubation, as specified by Iversen *et al. [76]*. The two *Pantoea* strains did produce positive results at 37° C, but not at 41.5° C (Table 3.4), demonstrating that the increased incubation temperature is able to inhibit the growth of some non-*Cronobacter* species in CSB. The other negative control strains were unable to grow in this broth at either temperature.

The results for the ten *Cronobacter* species were the same at both 37° C and 41.5° C. Notably, *C. helveticus* and *C. zurichensis* were unable to grow in this medium, regardless of incubation temperature. During development of CSB, *'E. helveticus,' 'E. pulveris*,' and '*E. turicensis'* were used as negative control species for exclusivity testing. Iversen *et al. [76]* reported positive results for '*E. pulveris'* and negative results for *'E. helveticus'* and *'E. turicensis,'* matching the observations in the current study. Prior to 2013, a positive result associated with '*E. pulveris'* in CSB would have been considered a false-positive (colour change occurring with a non-*Cronobacter* isolate), but after the reclassification of this species as *C. pulveris*, this result would be considered a true positive. Meanwhile, the lack of growth and colour change for *C. helveticus* and *C. zurichensis* strains in CSB would constitute false-negative results (no colour change associated with strains identified as *Cronobacter* species) following the 2013 taxonomic revision.

### 3.3.2. Colony morphology of *Cronobacter* spp. on selective and differential agars

Colony morphologies on TSA, VRBGA and DFI are shown in Table 3.5. Most strains of the reclassified species produced non-pigmented colonies on TSA after incubation at 37° C for 24 hours; however, all but one of these strains (*C. helveticus* 1344) showed some yellow colour after incubation at 25° C for 72 hours. Some strains did show a slight darkening of the yellow colour after 72 hours at 37° C, but five stains of *C. helveticus*, two strains of *C. pulveris* and one strain of *C. zurichensis* did not produce yellow pigment at this temperature, regardless of incubation time.

Yellow pigmentation on TSA was stated in the 2006 ISO standard protocol for detection and identification of *Cronobacter* spp., but is no longer recommended for characterization of suspect isolates due to the unreliability of the test *[9, 70, 71, 72]*. It has been shown that up to 21.4% of *Cronobacter* strains do not produce yellow colonies on TSA after 72 hours of incubation at 25° C *[72]*. Additionally, pigment production can be affected by incubation temperature and exposure to light in those strains capable of producing it *[31, 34, 85, 123]*. Thus, yellow pigment production on TSA is inconsistent and unreliable for identification of *Cronobacter* spp. and should not be recommended for identification of *Cronobacter* species.

While VRBGA and DFI are more selective, growth of typical colonies on these agars should not be considered a definitive result either. All strains, including the negative control strains, were able to grow on VRBGA with a variety of colony morphologies. As shown in Table 3.5, some strains produced large, mucoid colonies with very little pink colour, while others produced small, pink to purple, non-mucoid colonies. Like EE broth, VRBGA was designed to allow for the growth of all *Enterobacteriaceae,* not only *Cronobacter* species. Thus, further characterization of suspect isolates would be required to identify the species of these isolates.

Though more selective, DFI does allow for the growth of some non-*Cronobacter* species. All *Cronobacter*, *Esch. hermanii,* and *B. noakiae* strains in this study showed typical blue-green colonies on DFI after 24 hours at 37° C. The observation of typical colonies on DFI for the negative control strains again demonstrates the need for further characterization of suspect isolates by biochemical, molecular or DNA sequence-based methods. When DFI was first developed typical, blue-green colonies were observed for other species, including *Esch. hermanii* and *Pantoea* spp., which were used as negative controls in the current study *[74]*. The false negative results on TSA, false positive results on DFI and the low specificity of VRBGA, indicate that *Cronobacter* spp. cannot be reliably identified based solely on colony morphology.

**Table 3.5. Colony morphology of *Cronobacter* spp. on selective and differential agars**

| Bacterial species | Strain | Colony appearance: 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 |
| *Esch. 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 |

### 3.3.3. Re-evaluation of biochemical identification and characterization methods

Biochemical methods are often used for species identification and confirmation of suspect isolates. Panels of biochemical tests, such as the API20E and ID32E, are popular among testing laboratories for identification of *Enterobacteriaceae*, and are used in conjunction with online databases to identify the bacterial species based on 20 or 32 biochemical tests, respectively. As mentioned previously, both ISO and the US FDA recommend the use of such biochemical tests for identification of suspect *Cronobacter* isolates in current and previous versions of their standard methods *[9, 23-25, 70, 71 101, 158]*. The FDA BAM method previously recommended the use of the API20E test kit, but it has since been updated to recommend the ID32E biochemical test panel or the Vitek 2.0 system *[23-25, 101, 158]*. Additionally, the 2015 draft ISO standard specifies more traditional biochemical test methods for identification of *Cronobacter* isolates; however, this standard also states that 'miniaturized galleries' can` be used in place of the more traditional biochemical test methods *[70]*. As six of the seven required biochemical tests are included in the ID32E test panel, it is likely that these test kits will continue to be used for identification of suspect *Cronobacter* isolates (see Box 3.1).

The results of the API20E assays are shown in Table 3.6. Where indicated, the API20E assays had been performed previously by N. Masood or H. Sonbol. Profile numbers for these strains were retrieved from the archived records of the NTU culture collection. All species identifications were determined in both 2013 and 2015, using version 4.0 or 5.0 of the API20E database, respectively. Both versions of the database produced the same species identifications, but some percent identifications did differ slightly between versions. Six of the seven original species type strains were identified as '*Enterobacter sakazakii'* (the former name for members of the *Cronobacter* genus) in 2013 and as *Cronobacter* spp. in 2015; however, three of these identifications were based on 'doubtful' profiles (*C. condimenti, C. dublinensis,* and *C. muytjensii*). This test identified *C. turicensis* as *Enterobacter gergoviae* with both versions of the database. All *C. zurichensis* strains were identified as *Klebsiella pneumoniae* ssp. *ozaenea* or *Buttiauxella agrestis* using both versions 4.0 and 5.0 of the API20E database. *C. helveticus* 1208 was identified as *Yersinia pseudotuberculosis* and *C. pulveris* 1390 was identified as *Citrobacter freundii*. The remaining *C. helveticus* and *C. pulveris* strains were identified as *Escherichia vulneris*. While three *C. pulveris* strains were identified as '*E. sakazakii'* with a 0.8% identification in 2013, no other strains of the reclassified species gave possible identifications as '*E. sakazakii*.' Of the negative control strains, only the *Pantoea* spp. were correctly identified with the API20E test kit.

**Table 3.6. Biochemical identification using the API20E test kit**

| Bacterial species | Strain | API20E Profile | API20E species identification (v. 4.0; 2013) (% identification; t-value) | API20E Report | API20E species identification (v. 5.0; 2015) (% identification; t-value) | API20E Report |
|---|---|---|---|---|---|---|
| *C. condimenti* | LMG 26250$^T$ | 3367373[a] | *Enterobacter sakazakii* (99.9; 0.26) | Doubtful profile | *Cronobacter* spp.(99.9; 0.26) | Doubtful profile |
| *C. dublinensis* | LMG 23823$^T$ | 7347373[a] | *Enterobacter sakazakii* (61.3; 0.26) | Doubtful profile | *Cronobacter* spp.(63.0; 0.26) | Doubtful profile |
| *C. malonaticus* | LMG 23826$^T$ | 3305173[a] | *Enterobacter sakazakii* (51.1; 0.92) | Excellent identification to the genus | *Cronobacter* spp.(53.1; 0.92) | Low discrimination |
| *C. muytjensii* | ATCC 51329$^T$ | 3365373 | *Enterobacter sakazakii* (99.9; 0.42) | Doubtful profile | *Cronobacter* spp.(99.9; 0.42) | Doubtful profile |
| *C. sakazakii* | ATCC 29544$^T$ | 3305373[a] | *Enterobacter sakazakii* (98.4; 1.0) | Good identification | *Cronobacter* spp.(98.5; 1.0) | Good identification |
| *C. turicensis* | LMG 23827$^T$ | 7315373[a] | *Enterobacter gergoviae* (91.2; 0.36) | Doubtful profile | *Enterobacter gergoviae* (90.6; 0.36) | Doubtful profile |
| *C. universalis* | NCTC 9529$^T$ | 3205373[a] | *Enterobacter sakazakii* (98.0; 0.84) | Good identification | *Cronobacter* spp.(98.1; 0.84) | Good identification |
| *C. helveticus* | LMG 23732$^T$ | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1204 | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1208 | 1014153[a] | *Yersinia pseudotuberculosis* (97.5; 0.92) | Good identification | *Yersinia pseudotuberculosis* (97.5; 0.92) | Good identification |
| | 1344 | 1024153 | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1373 | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1374 | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1387 | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| | 1392 | 1024153[a] | *Escherichia vulneris* (61.5; 0.50) | Doubtful profile | *Escherichia vulneris* (61.4; 0.50) | Doubtful profile |
| *C. pulveris* | LMG 24057$^T$ | 3004173[a] | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | *Escherichia vulneris* (73.4; 0.75) | Identification not valid |
| | LMG 24059 | 3004173[a] | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | *Escherichia vulneris* (73.4; 0.75) | Identification not valid |
| | 1390 | 3004573[a] | *Citrobacter freundii* (48.8; 0.73) | Low discrimination | *Citrobacter freundii* (48.6; 0.73) | Low discrimination |
| | 1393 | 3004173[a] | *Escherichia vulneris* (73.5; 0.75) | Identification not valid | *Escherichia vulneris* (73.4; 0.75) | Identification not valid |
| *C. zurichensis* | LMG 23730$^T$ | 3204153[a] | *Klebsiella pneumoniae* ssp. *ozaenae* (66.7; 0.71) | Identification not valid | *Klebsiella pneumoniae* ssp. *ozaenae* (66.3; 0.71) | Identification not valid |
| | LMG 23731 | 1224153 | *Buttiauxella agrestis* (63.0; 0.30) | Doubtful profile | *Buttiauxella agrestis* (62.9; 0.30) | Doubtful profile |
| | 1383 | 3204153[a] | *Klebsiella pneumoniae* ssp. *ozaenae* (66.7; 0.71) | Identification not valid | *Klebsiella pneumoniae* ssp. *ozaenae* (66.3; 0.71) | Identification not valid |
| Negative control strains | | | | | | |
| *B. noakiae* | 53 | 0004153[a] | *Pantoea* spp. 4 (53.2; 0.78) | Doubtful profile | *Pantoea* spp. 4 (53.2; 0.78) | Doubtful profile |
| *E. hermanii* | 159 | 1204153[a] | *Buttiauxella agrestis* (63.0; 0.80) | Low discrimination | *Buttiauxella agrestis* (62.9; 0.80) | Low discrimination |
| | 162 | 1004153[a] | *Escherichia vulneris* (61.5; 1.0) | Low discrimination | *Escherichia vulneris* (61.4; 1.0) | Low discrimination |
| *Pantoea* spp. | 44 | 1005333 | *Pantoea* spp. 3 (99.8; 0.95) | Very good identification | *Pantoea* spp. 3 (99.7; 0.95) | Very good identification |
| | 1318 | 0221133 | *Pantoea* spp. 3 (NS[b]; NS) | Unacceptable profile | *Pantoea* spp. 3 (NS[b]; NS) | Unacceptable profile |

[a]API20E profiles were determined previously. Profile numbers for these strains were retrieved from the archived records of the NTU culture collection for identification in 2013 and 2015.

Strains were identified with the ID32E test kit in 2013, using version 3.0 of the database and in 2015, using version 4.0. The results of these analyses are shown in Table 3.7. Where indicated, the ID32E assays had been performed previously by N. Masood or H. Sonbol. Profile numbers for these strains were retrieved from the archived records of the NTU culture collection. With the ID32E kit, identifications with doubtful or unacceptable profiles did not return percent identifications. The type strains of *C. condimenti*, *C. dublinensis*, *C. malonaticus*, *C. sakazakii*, *C. turicensis*, and *C. universalis* were identified as '*E. sakazakii'* in 2013; however, three of these identifications (*C. condimenti, C. dublinensis*, and *C. sakazakii*) were the results of 'doubtful' or 'unacceptable' profiles. Following the 2015 update, the ID32E database purports to identify members of the *Cronobacter* genus to the species level. Of the original *Cronobacter* species, only the type strain of *C. malonaticus* was identified as the correct species. Five of the six other original species were correctly identified to the genus level, but not to the species level. The type strain of *C. muytjensii* (ATCC 51329$^{T}$) was unidentified with both versions of the ID32E database.

With regard to the reclassified species, the profiles for the type strain of *C. helveticus* and two strains of *C. pulveris* returned '*E. sakazakii'* as the top species in 2013, but these identifications were the results of 'unacceptable profiles'. After the database was updated in 2015, the type strain of *C. helveticus* was identified as *C. sakazakii,* while the two strains of *C. pulveris* were identified as *E. cloacae. C. zurichensis* 1383 was identified as '*E. sakazakii'* in 2013, but as *B. agrestis* in 2015, though both identifications were the results of unacceptable profiles. The other two *C. zurichensis* strains were identified as *B. agrestis* with both versions of the database. None of the negative control strains were correctly identified to the species level with the ID32E kit. Of particular concern was the misidentification of *Esch. hermanii* strain 162 as '*E. sakazakii'* (99.9%) in 2013 and as *C. sakazakii* in 2015. This strain could easily be mistaken for a *Cronobacter* species because it produces blue-green colonies on DFI and yellow colonies on TSA, as described above.

When comparing the two test kits, 15 of the 22 *Cronobacter* strains (68.1%) gave contradictory identifications in 2013, while 16/22 (72.7%) resulted in contradictions in 2015. For example, the type strain of *C. turicensis* was correctly identified as '*E. sakazakii'* with the ID32E test panel, but was misidentified as *E. geroviae* with the API20E kit in 2013. Similarly, Iversen *et al. [75]* found that while 90% of *Cronobacter* isolates were correctly identified as '*E. sakazakii'* with the ID32E biochemical test panel, only 70% of these same isolates were correctly identified with the API20E test kit. In addition to the misidentifications described above, these discrepancies between the two test kits bring the reliability of such methods into question.

**Table 3.7. Biochemical identification using the ID32E test kit.**

| | | | ID32E species identification (v. 3.0; 2013) | | ID32E species identification (v. 4.0; 2015) | |
|---|---|---|---|---|---|---|
| Bacterial species | Strain | ID32E Profile | (% identification; t-value)[a] | ID32E Report | (% identification; t-value)[a] | ID32E Report |
| *C. condimenti* | LMG 26250$^T$ | 34217360051 | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Cronobacter muytjensii* (NS; NS) | Unacceptable profile |
| *C. dublinensis* | LMG 23823$^T$ | 34256166211[a] | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Cronobacter malonaticus* (NS; NS) | Unacceptable profile |
| *C. malonaticus* | LMG 23826$^T$ | 34276763251[a] | *Enterobacter sakazakii (99.9; 0.83)* | Excellent identification | *Cronobacter malonaticus* (84.4; 0.95) | Acceptable identification |
| *C. muytjensii* | ATCC 51329$^T$ | 34217041041 | Unidentified | Unacceptable profile | Unidentified | Unacceptable profile |
| *C. sakazakii* | ATCC 29544$^T$ | 32276767051[a] | *Enterobacter sakazakii* (99.9; 0.12) | Doubtful profile | *Cronobacter turicensis* (70.1; 0.32) | Doubtful profile |
| *C. turicensis* | LMG 23827$^T$ | 34276767211[a] | *Enterobacter sakazakii* (99.9; 0.62) | Very good identification | *Cronobacter malonaticus* (64.5; 0.94) | Excellent identification to the genus |
| *C. universalis* | NCTC 9529$^T$ | 24276777051[a] | *Enterobacter sakazakii* (99.9; 0.35) | Good identification | *Cronobacter malonaticus* (65.1; 0.5) | Doubtful profile |
| *C. helveticus* | LMG 23732$^T$ | 30675567010[a] | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Cronobacter sakazakii* (NS; NS) | Unacceptable profile |
| | 1204 | 04675561001[a] | *Aeromonas hydrophila/caviae/sobria* (NS; NS) | Unacceptable profile | *Aeromonas hydrophila/caviae/sobria* (NS; NS) | Unacceptable profile |
| | 1208 | 34215461041[a] | Unidentified | Unacceptable profile | Unidentified | Unacceptable profile |
| | 1344 | 00674563011[a] | *Buttiauxella agrestis* (86.2; 0.32) | Doubtful profile | *Buttiauxella agrestis* (86.0; 0.32) | Doubtful profile |
| | 1373 | 06635771041[a] | Unidentified | Unacceptable profile | Unidentified | Unacceptable profile |
| | 1374 | 04677563011[a] | *Leclercia adecarboxylata* (NS; NS) | Unacceptable profile | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile |
| | 1387 | 35275663311[a] | *Enterobacter cloacae* (NS; NS) | Unacceptable profile | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| | 1392 | 06675563011[a] | *Leclercia adecarboxylata* (NS; NS) | Unacceptable profile | *Leclercia adecarboxylata* (NS; NS) | Unacceptable profile |
| *C. pulveris* | LMG 24057$^T$ | 04275773310[a] | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| | LMG 24059 | 04075773310[a] | *Klebsiella pneumoniae* ssp. *ozaenae* (NS; NS) | Unacceptable profile | *Klebsiella pneumoniae* ssp. *ozaenae* (NS; NS) | Unacceptable profile |
| | 1390 | 04075763310[a] | *Enterobacter cloacae* (NS; NS) | Unacceptable profile | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| | 1393 | 04275763310[a] | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Enterobacter cloacae* (NS; NS) | Unacceptable profile |
| *C. zurichensis* | LMG 23730$^T$ | 14475563310[a] | *Buttiauxella agrestis* (98.3; 0.28) | Doubtful profile | *Buttiauxella agrestis* (99.8; 0.41) | Good identification |
| | LMG 23731 | 14074561041 | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile |
| | 1383 | 04077563310[a] | *Enterobacter sakazakii* (NS; NS) | Unacceptable profile | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile |
| Negative control strains | | | | | | |
| *B. noakiae* | 53 | 04134563410[a] | *Buttiauxella agrestis* (NS; NS) | Unacceptable profile | *Buttiauxella agrestis* (99.5; 0.2) | Doubtful profile |
| *E. hermanii* | 159 | 34074703051[a] | *Enterobacter cancerogenus* (96.9; 0.51) | Good identification | *Enterobacter cancerogenus* (96.8; 0.51) | Good identification |
| | 162 | 34676767050[a] | *Enterobacter sakazakii* (99.9; 0.67) | Doubtful profile | *Cronobacter sakazakii* (90.8; 0.78) | Good identification |
| *Pantoea* spp. | 44 | 04476563051[a] | *Buttiauxella agrestis* (89.7; 0.46) | Acceptable identification | *Buttiauxella agrestis* (89.4; 0.46) | Acceptable identification |
| | 1318 | 30014601001[a] | *Aeromonas sobria* (NS; NS) | Unacceptable profile | *Aeromonas sobria* (NS; NS) | Unacceptable profile |

[a]ID32E profiles were determined previously. Profiles numbers for these strains were retrieved from the archived records of the NTU culture collection for identification in 2013 and 2015.

### 3.3.4. Comparison of old and new versions of the API20E and ID32E databases

One of the major problems with the API20E and ID32E test kits is that the associated databases are not frequently updated to reflect taxonomic changes. Though the *Cronobacter* genus was first described in 2007, the databases reported results of '*Enterobacter sakazakii*' until 2015. Problems with the databases have been noted by other authors, suggesting that these assays are not sufficient for identification of *Cronobacter* spp. *[33, 72, 75]*. Updating the databases more frequently may help to increase their accuracy. For example, Fanjat *et al. [33]* examined '*E. sakazakii'* isolates in 2007 and found that only 71.4% 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 the isolates *[33]*. It should be noted, however, that updates to the databases are slow and can be inaccurate with regard to changes in bacterial taxonomy.

In 2015, both the API20E and ID32E databases were updated, including the replacement of the former name '*Enterobacter sakazakii*.' As described above, while version 5.0 of the API20E database returns a result of *Cronobacter* spp., version 4.0 the ID32E database claims to identify isolates to the species level within the *Cronobacter* genus. In order to examine the reliability of the updated databases, API20E and ID32E profiles and identifications from the archived records of the NTU culture collection were re-analysed using the updated databases. The results for each individual profile can be found in Appendix A.

With previous version(s) of the API20E database, an identification of '*Enterobacter sakazakii*,' was considered to be a match for all *Cronobacter* species. There was a significant difference ($p < 0.05$, $X^2$ test) between the percentage of matches identified in the archived records and matches produced with version 5.0 of the API20E database. Following the update, only 82.3% of strains resulted in a match, while 90.0% produced matches with previous database version(s). Approximately two-thirds of strains (68.8%) were correctly identified with both old and new versions of the database; however, 6.3% of strains were correctly identified in the culture collection records but incorrectly identified using version 5.0 of the API20E database. Only one strain (0.4%) was identified incorrectly in the archived records, but correctly identified with the updated database.

Strains of *Cronobacter* were misidentified as *Enterobacter aerogenes, E. amnigenus,* and *E. cloacae* in the culture collection records and as *E. aerogenes, E. amnigenus, E. cancerogenus, E. cloacae,* and *Serratia liquefaciens* with the updated version of the API20E database. Strains of *E. cloacae* and *E. hormaechei* were misidentified as *Cronobacter* spp. (or '*E. sakazakii*') in both the archived records and with version 5.0 of the API20E database. Seven profiles did not return a

percent identification with the updated database, instead returning a result of 'Unacceptable profile'. These results incorporate further uncertainty into the species identification.

Confirmed *Cronobacter* strains (n = 268) produced 61 unique API20E profiles, but, overall, the biochemical profiles were fairly consistent. All 61 of the observed profiles and, therefore 100% of *Cronobacter* strains, were positive for amygdalin fermentation and negative for hydrogen sulphide production. More than 90% of strains were positive for β-galactosidase (98.1%), arginine dihydrolase (97.8%), ornithine decarboxylase (91.4%), citrate utilization (98.5%), indole production (95.2%), acetoin production (90.7%), and D-glucose (98.9%), D-mannitol (98.1%), D-sorbitol (91.0%) L-rhamnose (94.4%), D-saccharose (98.5%), D-melibiose (99.3%), and L-arabinose (98.5%) fermentation. Additionally, the majority of strains were negative for urease (98.9%) and lysine decarboxylase (94.8%) activity. The observed reactions for arginine dihydrolase, lysine decarboxylase, citrate utilization, hydrogen sulphide production, urease activity, and acid production from glucose, D-mannitol, L-rhamnose, D-saccharose, D-melibiose, and L-arabinose match the original description of the *Cronobacter* genus, given in Section 1.2 *[77, 78]*. The other tests included in the API20E panel were not used in the definition of the *Cronobacter* genus.

The most commonly observed API20E profiles were 3305373, 3307173, and 3305173 which represented 60, 41 and 40 strains, respectively, and were observed for more than half of the strains analysed (141/260; 54.2%). These profiles differed from one another by two traits; gelatinase activity and inositol fermentation. Each profile resulted in a species identification of '*E. sakazakii'* with version 4.0 of the database and *Cronobacter* spp. with version 5.0. Profile 3307173 was only observed for confirmed strains of *Cronobacter,* belonging to four different species*; C. sakazakii* (n = 34), *C. malonaticus* (n = 4), *C. turicensis* (n = 2), and *C. dublinensis* (n = 1). In addition to *C. sakazakii* (n = 32) and *C. malonaticus* (n = 8), strains of *E. hormaechei* (n = 3) and *E. cloacae* (n = 1) were identified with profile 3305173. Strains of *C. sakazakii* (n = 49), *C. malonaticus* (n = 7)*, C. turicensis* (n = 4) and *E. hormaechei* (n = 6) produced profile 3305373. Therefore, while these profiles were frequently observed for *Cronobacter* spp., they are not necessarily specific to members of this genus and may be assigned to *Enterobacter* spp.; however, *Enterobacter* spp. and *Cronobacter* spp. can be differentiated from one another based on colony morphology on DFI *[76]*.

Using version 3.0 of the ID32E database, results were considered to be a match if a strain was identified as the nearest match of '*E. sakazakii.'* Following the update to version 4.0, however, strains were required to be correctly identified to the species level in order to be considered a match. Updating the ID32E database to version 4.0 resulted in a significantly higher percentage of

mismatches for strains of *Cronobacter* species ($p < 0.05$, $X^2$ test). In the archived culture collection records, 88.9% of strains were identified as '*E. sakazakii*' with the ID32E test kit and this result was considered to be a match; however, the percentage of strains producing matches drops to only 43.2% when version 4.0 of the ID32E database is used. The major problem with the update to the ID32E database is that it attempts to identify strains to the species level, but speciation of *Cronobacter* isolates can be difficult without highly specific methods. *C. sakazakii* and *C. malonaticus*, for example, are so closely related that they cannot be differentiated using 16S rDNA sequencing methods *[6]*. The ID32E biochemical test panel is simply not specific enough to differentiate members of the *Cronobacter* genus. If these results are examined to the genus level, 82.3% of strains were correctly identified as *Cronobacter* spp. using the updated ID32E database. Though this also represents a significant decrease ($p < 0.05$, $X^2$ test) in the accuracy of identification from the archived culture collection records, it is more accurate than identification to the species level. Of the strains analysed, 37.9% produced matches both in the culture collection records and with version 4.0 of the ID32E database; however, 36.6% of strains produced matches in the culture collection records, but mismatches after the database was updated.

With the ID32E test kit, *Cronobacter* strains were misidentified as *Enterobacter cancerogenus, E. cloacae,* and *Stenotrophomonas maltophilia* in the culture collection records and as *Buttiauxella agrestis, Citrobacter freundii, Cit. koseri, Enterobacter aerogenes, E. cancerogenus, E. cloacae, Escherichia vulneris, Leclercia adecarboxylata, Serratia liquefaciens, S. rubidea,* and *Stenotrophomonas maltophilia* with the updated database. Meanwhile, strains of *Cit. freundii, Cit. koseri, Enterobacter hormaechei, Escherichia hermanii, Leclercia adecarboxylata,* and *Pantoea* spp. were misidentified as *Cronobacter* species with previous versions of the database while *Cit. freundii, Cit. koseri, Enterobacter hormaechei, E. pyrinus, Escherichia hermanii,* and *Leclercia adecarboxylata* were misidentified as *Cronobacter* species with the updated version of the database. Additionally, several strains of *Cronobacter* produced 'Unacceptable profiles' with no species identification using both versions of the ID32E database. Forty-eight profiles returned a species identification with a description of 'Unacceptable profile' instead of a percent identification with the updated version of the database. As with the API20E profiles, this produces more uncertainty in the species identification of strains producing these profiles.

A total of 155 unique ID32E profiles were observed for confirmed strains of *Cronobacter* species (n = 292). Nearly all (99.7%) strains gave a positive result for β-glucuronidase activity and a negative reaction for rhamnose acidification (99.7%). More than 90% of known *Cronobacter* strains

produced positive results for ornithine decarboxylase (94.5%), arginine dihydrolase (96.2%), β-glucosidase (99.0%), malonate utilization (97.6%), indole production (91.8%) and acidification of D-mannitol (94.2%), maltose (94.2%), D-glucose (94.5%), D-saccharose (98.6%), L-arabinose (96.6%) and D-arabitol (94.2%). Additionally, more than 90% of strains were negative for lysine decarboxylase (96.2%), urease (93.5%), L-aspartic acid arylamidase (98.6%), α-galactosidase (95.9%), β-galactosidase (94.5%), the phenol red test (92.1%), and acidification of L-arabitol (97.4%), adonitol (90.4%), and 5-ketogluconate (96.6%).

The observed reactions for ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase, maltose utilization and acidification of D-glucose and L-arabinose match the original genus description of *Cronobacter*; however, the results for the acidification of L-rhamnose and D-arabitol do not match the original description of the genus (see Section 1.2) *[77, 78]*. Additionally, the results for 4-nitrophenyl-α-D-glucopyranoside, and acidification of D-arabitol and D-sorbitol differ significantly from the expected phenotypes specified in the updated ISO standard (see Box 3.1) *[70]*. While only 62.3% of *Cronobacter* strains were positive for 4-nitrophenyl-α-D-glucopyranoside using the ID32E kit, the ISO standard states that 100% of strains produced a positive result for this test *[70]*. Similarly, with the ID32E test kit, 84.6% of strains were found to be positive for the acidification of D-sorbitol; however, the draft ISO standard indicates that all *Cronobacter* spp. are negative for this test *[70]*. Finally, nearly all of the *Cronobacter* strains (94.2%) were positive for the acidification of D-arabitol, but only a few strains of *C. dublinensis, C. turicensis,* and *C. universalis* are reported to produce positive results in the ISO standard *[70]*.

The differences in the biochemical identification described in the draft ISO standard and the *Cronobacter* genus description may be due to the use of more traditional biochemical methods when defining the genus and preparing the standard. The presence of such discrepancies suggests that the ID32E biochemical test panel may not be completely accurate in its characterization of suspect isolates. Importantly, biochemical test panels are suggested as an alternative to more traditional biochemical test methods in the draft ISO standard. The differences between the expected results in the standard and the results reported here suggest that the ID32E test kit is not an acceptable alternative for biochemical identification of suspect *Cronobacter* isolates, even though 'miniaturized galleries' are permitted by the 2015 draft ISO standard method *[70]*.

The most common ID32E profile was 34276767250 (n = 34) and was observed for only confirmed *C. sakazakii* strains. Version 4.0 of the ID32E database correctly identified isolates with this profile to the species level. The next two most common profiles were 34276767050 (n = 22) and

34276763050 (n = 19). Each of these profiles was observed for three *Cronobacter* species. Profile 34276767050 was produced by *C. sakazakii* (n = 16), *C. malonaticus* (n = 4), and *C. turicensis* (n = 2). According to the updated ID32E database, these strains were identified as *C. sakazakii*; however this identification was correct for only 16/22 strains (72.3%). Similarly, profile 34276763050 was observed for *C. sakazakii* (n = 16), *C. malonaticus* (n = 2), and *C. turicensis* (n = 1), but the species identification using version 4.0 of the ID32E database was *C. dublinensis*. Though correct to the genus level, none of the strains with this profile were correctly identified to the species level.

In summary, the subjectivity of biochemical test panels, the possibility of misidentifications, and the lack of updated databases shows that these test kits are not sufficient to correctly identify suspect *Cronobacter* isolates to the species level. While these phenotyping tests are recommended or permitted by both ISO and the US FDA standard methods, they have been shown here to be unreliable for species identification.

### 3.3.5. Presence of PCR target genes in *Cronobacter* spp.

As demonstrated above, cultural and biochemical identification of suspect *Cronobacter* isolates can be unreliable and alternative methods are needed for species identification. Many such methods, including the Vitek 2.0 system, MALDI-TOF MS, and real-time PCR methods require trained personnel to perform the analyses and/or specialized equipment, usually with a large up-front cost. DNA sequence-based identification methods are also labour intensive and time consuming. These factors limit the availability of such alternative methods, making them inaccessible to some PIF manufacturers or food testing laboratories, including the microbiology laboratory at NTU. On the other hand, conventional PCR methods require no additional equipment beyond a PCR thermocycler and gel electrophoresis capabilities. As most microbiology laboratories already possess this equipment, conventional PCR methods are a much more accessible alternative to biochemical speciation of suspect *Cronobacter* isolates.

PCR probe-based detection and identification assays rely on the presence of particular gene sequences that are recognized by PCR primers. Amplicons of the expected size must be produced in order for the test to be considered positive. To select PCR assays for laboratory evaluation, the BLAST function of the online *Cronobacter* BIGSdb facility was used to search the full genome sequences of 34 *Cronobacter* laboratory strains that were available at the time *[42]*. The target gene sequences that were used in the original design of PCR primers and probes were used for this search, as described above. 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. Genes were considered absent if <50% of the target gene was detected. The results of this analysis are shown in Table 3.8.

The gene *gluA*, encoding an α-glucosidase, was present in nearly all *Cronobacter* spp., but only a partial *gluA* sequence was detected in *C. universalis* NCTC9529$^T$. Similarly, partial sequences of the gene *gluB* were detected in *C. malonaticus* LMG23726$^T$ and *C. sakazakii* 701. As such, the assays targeting these genes were not selected for laboratory evaluation. Interestingly, the type strains of both *C. malonaticus* and *C. universalis* produced typical, blue-green colonies on DFI. Both *gluA* and *gluB* encode α-glucosidases which are responsible for producing this colony morphology, suggesting that there may be errors in the genome assemblies for these strains. This was later confirmed by searching the reassembled genomes of these strains, which identified the full-length sequences of both genes in the three strains which previously showed partial matches.

The gene *dnaG*, part of the macromolecular synthesis operon, was detected in all *Cronobacter* species, except *C. condimenti* (n = 1) and *C. zurichensis* (n = 4), which contained only a fragment of the target sequence. This sequence was only 319 bp long and analysis of a larger fragment may allow for the design of PCR primers targeting *dnaG* that will produce amplicons with all *Cronobacter* spp. Similarly, *cgcA* was found to be absent or partially present in all strains of the reclassified species, indicating that it would be unable to detect all ten *Cronobacter* species.

Though present in all *Cronobacter* species, partial *zpx* sequences were detected in *C. malonaticus* 507 and *C. sakazakii* 701. The zinc-containing metalloprotease encoded by *zpx* may serve as an indicator of pathogenicity *[99]*; however, the presence of partial sequences in some strains suggested that it is not suitable for identification of all *Cronobacter* species or strains. Again, the partial sequences may have been the result of errors in the genome assemblies for these strains. The full-length sequence of *zpx* was later identified in the reassembled genome of strain 701, supporting the hypothesis of errors in the original genome assembly.

The genes *gyrB*, *ompA*, and *rpoB* were present in all 34 strains of *Cronobacter* examined by genome searching. The *gyrB* primers used by Huang *et al. [65]* were designed for identification of only *C. sakazakii* and *C. dublinensis* and this gene is part of the seven loci *Cronobacter* multilocus sequence typing (MLST) scheme *[6]*. Therefore, such a restricted assay was not included in this study. As the gene sequences encoding the outer membrane protein A (*ompA*), and the ß-subunit of RNA polymerase (*rpoB*) were found in all species and strains, the PCR assays targeting these genes were selected for laboratory analysis.

**Table 3.8. Results of genome searching for PCR target genes.** Shaded boxes indicate PCR assays selected for laboratory evaluation.

| Species | Strain | *ompA* DQ000206 | *rpoB* Multiple[a] | *cgcA* ESA_01230 | *gluA* AM075208[b] | *gluB* AM075208[b] | *gyrB* JX088572 | *dnaG* L01755 | *zpx* EF061082 |
|---|---|---|---|---|---|---|---|---|---|
| *C. condimenti* | LMG26250$^T$ | Present | Present | Present | Present | Present | Present | ***Partial***[c] | Present |
| *C. dublinensis* | LMG23823$^T$ | Present | Present | Present | Present | Present | Present | Present | Present |
| | LMG23824 | Present | Present | Present | Present | Present | Present | Present | Present |
| | LMG23825 | Present | Present | Present | Present | Present | Present | Present | Present |
| | NCTC9844 | Present | Present | ***Partial*** | Present | Present | Present | Present | Present |
| *C. malonaticus* | LMG23826$^T$ | Present | Present | Present | Present | ***Partial***[c] | Present | Present | Present |
| | 507 | Present | Present | Present | Present | Present | Present | Present | ***Partial*** |
| *C. muytjensii* | ATCC 51329$^T$ | Present | Present | Present | Present | Present | Present | Present | Present |
| *C. sakazakii* | ATCC 29544$^T$ | Present | Present | Present | Present | Present | Present | Present | Present |
| | ATCC BAA-894 | Present | Present | Present | Present | Present | Present | Present | Present |
| | E764 | Present | Present | Present | Present | Present | Present | Present | Present |
| | ES15 | Present | Present | Present | Present | Present | Present | Present | Present |
| | ES35 | Present | Present | Present | Present | Present | Present | Present | Present |
| | ES713 | Present | Present | Present | Present | Present | Present | Present | Present |
| | G-2151 | Present | Present | Present | Present | Present | Present | Present | Present |
| | SP291 | Present | Present | Present | Present | Present | Present | Present | Present |
| | 377 | Present | Present | Present | Present | Present | Present | Present | Present |
| | 680 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | 696 | Present | Present | Present | Present | Present | Present | Present | Present |
| | 701 | Present | Present | Present | Present | ***Partial***[c] | Present | Present | ***Partial***[c] |
| *C. turicensis* | LMG23827$^T$ | Present | Present | Present | Present | Present | Present | Present | Present |
| | 564 | Present | Present | Present | Present | Present | Present | Present | Present |
| *C. universalis* | NCTC9529$^T$ | Present | Present | Present | ***Partial***[c] | Present | Present | Present | Present |
| *C. helveticus* | LMG23732$^T$ | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | LMG23733 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | 1204 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | 1392 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| *C. pulveris* | LMG24059 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | G-1160/04 | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| | G-601/05$^T$ | Present | Present | *Absent* | Present | Present | Present | Present | Present |
| *C. zurichensis* | LMG23730$^T$ | Present | Present | ***Partial*** | Present | Present | Present | ***Partial*** | Present |
| | z508 | Present | Present | ***Partial*** | Present | Present | Present | ***Partial*** | Present |
| | z610 | Present | Present | ***Partial*** | Present | Present | Present | ***Partial*** | Present |
| | 1383 | Present | Present | ***Partial*** | Present | Present | Present | ***Partial*** | Present |

[a]*rpoB* sequences from all 7 original species were examined and found in all species and strains. Accession numbers of *rpoB* sequences: JQ316670, FJ717638, FJ717652, and FJ717656-FJ717659. [b]*gluA* and *gluB* sequences were extracted from the partial genome sequence with this accession number. [c]Full-length sequence later identified in reassembled genome.

### 3.3.6. Results of PCR probe-based assays targeting *ompA* and *rpoB*

Though BLAST searching of 34 whole genomes showed that *ompA* is present in all species (Table 3.8), the *ompA* PCR assay described by Mohan-Nair and Venkitanarayanan *[119]* produced bands of the expected size (469 bp) for the type strains of only the seven original *Cronobacter* spp. None of the reclassified species produced amplicons with these primers. Jaradat *et al. [82]* also reported false negative results with these primers for two strains previously identified as *Cronobacter* spp. using biochemical and 16S rRNA sequencing methods. Sequence variability was suggested to explain the lack of detection with the *ompA* primers, but the presence of *ompA* in all ten species is of significance as it has been identified as an important trait in the invasion of host brain cells *[82, 96]*.

Later, a more thorough, *in silico* analysis of the *ompA* method was carried out by searching 223 genomes for the primer binding sites. This method enabled the analysis of a large cohort of internationally derived strains that are not centrally available. Table 3.9 compares the results from the laboratory and *in silico* analyses for the ten *Cronobacter* species. *C. helveticus* strains 1344, 1373, and 1374 were excluded as their genomes were not available at the time of analysis. As stated above, no *C. helveticus, C. pulveris,* or *C. zurichensis* strains produced amplicons during the laboratory analysis. *C. helveticus* strains showed seven mismatches to the ESSR-R primer and this was the lowest number of mismatches observed for any strain producing negative results in the laboratory analysis. Therefore, this was considered to be the threshold for amplification. Strains with seven or more mismatches to the ESSR-R primer were predicted to produce no amplicon.

**Table 3.9. Comparison of laboratory and *in silico* analyses of the *ompA* PCR method for identification of *Cronobacter* spp.**

| | | | # mismatches | |
|---|---|---|---|---|
| Bacterial species | Strain | Laboratory *ompA* result | ESSR-F (22 bp) | ESSR-R (19 bp) |
| *C. condimenti* | LMG 26250$^T$ | + | 0 | 2 |
| *C. dublinensis* | LMG 23823$^T$ | + | 0 | 3 |
| *C. malonaticus* | LMG 23826$^T$ | + | 0 | 2 |
| *C. muytjensii* | ATCC 51329$^T$ | + | 0 | 0 |
| *C. sakazakii* | ATCC 29544$^T$ | + | 0 | 2 |
| *C. turicensis* | LMG 23827$^T$ | + | 0 | 2 |
| *C. universalis* | NCTC 9529$^T$ | + | 0 | 2 |
| *C. helveticus* | LMG 23732$^T$ | - | 2 | 7 |
| | 1204 | - | 2 | 7 |
| | 1208 | - | 2 | 7 |
| | 1387 | - | 2 | 7 |
| | 1392 | - | 2 | 7 |
| *C. pulveris* | LMG 24057$^T$ | - | 1 | 8 |
| | LMG 24059 | - | 1 | 8 |
| | 1390 | - | 1 | 8 |
| | 1393 | - | 1 | 8 |
| *C. zurichensis* | LMG 23730$^T$ | - | 4 | 10 |
| | LMG 23731 | - | 4 | 10 |
| | 1383 | - | 4 | 10 |

The results of the *in silico* analysis of all species and strains are shown in Table 3.10. As with the laboratory analysis, only the seven original *Cronobacter* species were expected to produce amplicons of approximately the expected size. Mohan-Nair and Venkitanarayanan *[119]* reported an expected size of 469 bp, while the *in silico* analysis predicted amplicons of 468-469 bp. While the non-*Cronobacter* species were predicted to produce a negative result with this assay, the reclassified species of *C. helveticus, C. pulveris,* and *C. zurichensis* were also predicted to produce negative results. The predicted results for these three species were confirmed by the laboratory analysis, as shown in Table 3.9. Thus, this method would not be able to ensure the exclusion of all ten *Cronobacter* species from PIF, as it was unable to detect the species added to genus in 2013.

**Table 3.10. *In silico* analysis of the *ompA* PCR method for *Cronobacter* spp. and members of related genera**

| | | # mismatches | | |
|---|---|---|---|---|
| Species | n | ESSR-F[a] (22 bp) | ESSR-R (19 bp) | Predicted amplicon size (bp)[b] |
| *Cronobacter sakazakii* | 100 | 0 | 1-2 | 468 |
| *Cronobacter malonaticus* | 45 | 0 | 2 | 468 |
| *Cronobacter dublinensis* | 14 | 0 | 2 | 469 |
| *Cronobacter turicensis* | 10 | 0 | 2 | 468 |
| *Cronobacter muytjensii* | 9 | 0 | 0 | 469 |
| *Cronobacter universalis* | 7 | 0 | 2 | 468 |
| *Cronobacter condimenti* | 1 | 0 | 2 | 469 |
| *Cronobacter pulveris* | 7 | 1 | 8 | NA[c] |
| *Cronobacter helveticus* | 6 | 2 | 7 | NA |
| *Cronobacter zurichensis* | 5 | 4 | 10 | NA |
| *Enterobacter asburiae* | 2 | 4-6 | 8-11 | NA |
| *Enterobacter hormaechei* | 2 | 6-7 | 8 | NA |
| *Enterobacter* spp. | 2 | 5-6 | 7 | NA |
| *Citrobacter amalonaticus* | 1 | 7 | 9 | NA |
| *Citrobacter freundii* | 1 | 6 | 12 | NA |
| *Citrobacter koseri* | 1 | 7 | 13 | NA |
| *Citrobacter rodentium* | 1 | 7 | 13 | NA |
| *Citrobacter youngae* | 1 | 6 | 13 | NA |
| *Enterobacter aerogenes* | 1 | 5 | 13 | NA |
| *Enterobacter cancerogenus* | 1 | 5 | 7 | NA |
| *Enterobacter cloacae* | 1 | 7 | 8 | NA |
| *Enterobacter ludwiggi* | 1 | 5 | 7 | NA |
| *Enterobacter massilensis* | 1 | 5 | 10 | NA |
| *Enterobacter mori* | 1 | 5 | 7 | NA |
| *Enterobacter sacchari* | 1 | 7 | 15 | NA |
| *Yersinia regensburgii* | 1 | 5 | 7 | NA |

[a]Primer sequences described previously *[119]*. [b]Expected amplicon size: 469 bp *[119]*. [c]NA, no amplicon

The *rpoB* multiple primer assays were designed, not for detection of the *Cronobacter* genus, but for speciation of *Cronobacter* isolates *[104, 147]*. The species-specific primer sets were designed so that amplification should only occur with the target species. Hence cross-reactivity of the primers with the three reclassified species was considered. The *C. sakazakii* primers produced amplicons of a slightly smaller size (approx. 490 bp) for all of the *C. helveticus* strains, indicating slight sequence variation between the two species. Faint bands of both the expected (514 bp) and smaller size (approx. 490 bp) were observed for two *C. pulveris* strains, which were also positive

with the *C. malonaticus* primers (Table 3.11). As the *C. malonaticus* primers are intended for use only with strains producing positive results with the *C. sakazakii* primers, these strains could be misidentified as *C. malonaticus*. Additionally, the primers intended to identify *C. malonaticus* and *C. muytjensii* gave positive results for all strains of the reclassified species except *C. helveticus* 1373 (Table 3.11). Except for the two *C. pulveris* strains described above, none of the newly reclassified species would be tested with the *C. malonaticus* primers. The *C. dublinensis* primers also produced positive results with the reclassified species, except *C. zurichensis* 1383. Thus, these results indicate that the reclassified species could be misidentified with this method.

**Table 3.11. Detection and identification of *Cronobacter* spp. with PCR assays targeting *rpoB***

| Bacterial species | Strain | 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[a] | + | 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$^T$ | - | + | - | + | + | -[b] | - |
| | 1204 | - | + | - | + | + | -[b] | - |
| | 1208 | - | + | - | + | + | -[b] | - |
| | 1344 | - | + | - | + | + | -[b] | - |
| | 1373 | - | + | - | - | - | -[b] | - |
| | 1374 | - | + | - | + | + | -[b] | - |
| | 1387 | - | + | - | + | + | -[b] | - |
| | 1392 | - | + | - | + | + | -[b] | - |
| *C. pulveris* | LMG 24057$^T$ | - | + | - | + | + | - | - |
| | LMG 24059 | - | + | - | + | + | +[c] | - |
| | 1390 | - | + | - | + | + | - | - |
| | 1393 | - | + | - | + | + | +[c] | - |
| *C. zurichensis* | LMG 23730$^T$ | - | + | - | + | + | - | - |
| | LMG 23731 | - | + | - | + | + | - | - |
| | 1383 | - | - | - | + | + | - | - |
| Negative control strains | | | | | | | | |
| *B. noakiae* | 53 | - | + | - | - | + | - | - |
| *Esch. hermanii* | 159 | - | + | - | + | + | - | - |
| | 162 | - | + | - | + | + | - | - |
| *Pantoea* spp. | 44 | - | - | - | - | - | - | - |
| | 1318 | - | - | - | + | - | - | - |

[a]NT: Not tested. [b]Expected is 514 bp, however an amplicon of a slightly smaller size (approx. 490 bp) was detected. [c]Faint bands of both 514 bp and the slightly smaller size were detected.

As shown in Table 3.11, some negative control strains also produced amplicons of the expected size when tested with the *C. dublinensis*, *C. malonaticus* and *C. muytjensii* primer sets, adding to the confusion of species identification when using this method. No amplification was observed with the *C. turicensis* primers in this study (Table 3.11). Though the remaining primer sets were specific to their target species, the possibility of misidentification with the *C. dublinensis, C. malonaticus, C. muytjensii,* and *C. sakazakii* primer sets suggests that the Stoop *et al. [147]* and Lehner *et al. [104]* multiple primer *rpoB* method is not effective for speciation of *Cronobacter* isolates.

### 3.4. Conclusions and implications of this re-evaluation

Cultural and biochemical assays are limited by variation among species and strains. Since these tests detect one or more biochemical reactions, variation in these traits can lead to false negative or false positive results. For example, though yellow pigment production is used for presumptive identification of *Cronobacter* spp., not all strains produce the pigment and some non-*Cronobacter* species may also appear yellow on TSA *[72, 75]*.

Biochemical identification of suspect isolates can also cause confusion. Though easily distinguished by colony morphology on DFI, *Enterobacter* isolates have been misidentified as members of the *Cronobacter* genus when using biochemical test panels. For example, Townsend *et al. [157]* used 16S rDNA sequencing to identify 10 clinical strains of *E. hormaechei* that had been previously identified as *C. sakazakii* by phenotyping. That study indicated that *Enterobacter* isolates have been mistaken for *C. sakazakii* and though infectious, these organisms are not addressed in the microbiological criteria applied to PIF *[26, 40, 41]*.

While more specific than cultural and biochemical methods, PCR assays are limited by the presence of the target gene and sequence variation among strains. Both of these factors may affect the binding of the primers, thereby preventing amplification and producing false negative results. Currently, cultural, biochemical, and molecular detection and identification assays must be used in combination with one another to identify *Cronobacter* spp; however, as demonstrated above, even a combination of these methods could result in misidentifications.

Conversely, the recent developments in DNA sequence-based methods, including MLST, allow for highly specific species and strain identification *[129]*. These methods are more reliable than subjective biochemical and morphological tests or identification based on amplification of a particular DNA fragment. Additionally, other alternative methods, including the Vitek 2.0 system, real-time PCR, and MALDI-TOF MS are available for identification of *Cronobacter* spp., but, as discussed previously, such alternative methods are not accessible to all laboratories.

As demonstrated in this chapter, the reclassification of three *Enterobacter* species into the *Cronobacter* genus limited the utility of some *Cronobacter* isolation and detection methods. Many published methods utilized these reclassified species as negative controls and, thus, these methods will be unable to detect these species. This is particularly important as international microbiological criteria require an absence of all *Cronobacter* species in PIF, but it will be impossible to ensure exclusion of *Cronobacter* spp. without accurate and specific detection and identification assays *[26]*. Both false negative and false positive results can be costly to industry. A batch of infant formula may

be rejected due to a false-positive identification of *Cronobacter* spp., leading to lost profits for the company. Conversely, infant formula containing *Cronobacter* may be mistakenly released due to false-negative identifications. This latter scenario could result in the release of contaminated formula, leading to illnesses, product recalls, lost consumer confidence, and lost profits for the manufacturer. Given that only three *Cronobacter* species have been epidemiologically-linked to neonatal infections the possibility of revising the international microbiological criteria to only those species should be considered; however, such a revision requires methods that are capable of differentiating these closely-related species. The work presented here demonstrates that commonly used cultural, biochemical, and PCR-based detection and identification assays were not sufficient to correctly identify all *Cronobacter* strains to the species level following the 2013 taxonomic revision of the genus. Thus, it would be impossible to meet a standard requiring the absence of only three *Cronobacter* species using only cultural, biochemical, or conventional PCR-based methods.

### 3.5. Further taxonomic revisions of the *Cronobacter* genus

As *Cronobacter* is an emergent genus, its taxonomy is subject to change and the development of DNA sequence-based identification methods allows for more reliable identification than cultural or molecular methods. Phenotyping tests were not the primary means of characterization in the naming of *C. condimenti*, *C. helveticus*, *C. pulveris* and *C. zurichensis [15, 93]*. Instead, MLST and 16S rDNA sequencing were used to differentiate the species; however, 16S rDNA sequencing has limited application to the *Cronobacter* genus due to high interspecies similarity and microheterogeneities in the gene *[6, 77, 78, 94]*. Therefore, *fusA* sequence analysis is commonly used for *Cronobacter* species identification and is included in the *Cronobacter* MLST scheme. The phylogenetic analysis of this gene reflects the whole genome phylogeny of the *Cronobacter* genus, indicating that it is sufficient for speciation of *Cronobacter* strains *[44, 90, 92]*.

MLST, which will be discussed in more detail in Chapter 4, has proven to be a useful tool for the taxonomic analysis of *Enterobacteriaceae* and was found to be more effective than phenotyping for *Cronobacter* speciation *[86, 90]*. In 2012, Joseph *et al. [93]* used seven loci MLST (*atpD, fusA, glnS, gltB, gyrB, infB, ppsA*; 3036 bp concatenated sequence length) for the definitions of two new *Cronobacter* species: *C. universalis*, and *C. condimenti*. Brady *et al. [15]*, however, only used four loci (*atpD, gyrB, infB* and *rpoB*) to support their reclassification of *Enterobacter helveticus*, *E. pulveris* and *E. turicensis* to *Cronobacter helveticus*, *C. pulveris* and *C. zurichensis*, respectively. This reclassification was disputed by Stephan *et al. [146]* in 2014 who proposed two new genera

containing the species *Franconibacter helveticus*, *F. pulveris* and *Siccibacter turicensis* for the same former *Enterobacter* species (Figure 3.2). This taxonomic revision occurred after the majority of the laboratory analyses described in this chapter had been completed; therefore, the most recent taxonomic designations will be used from this point forward.

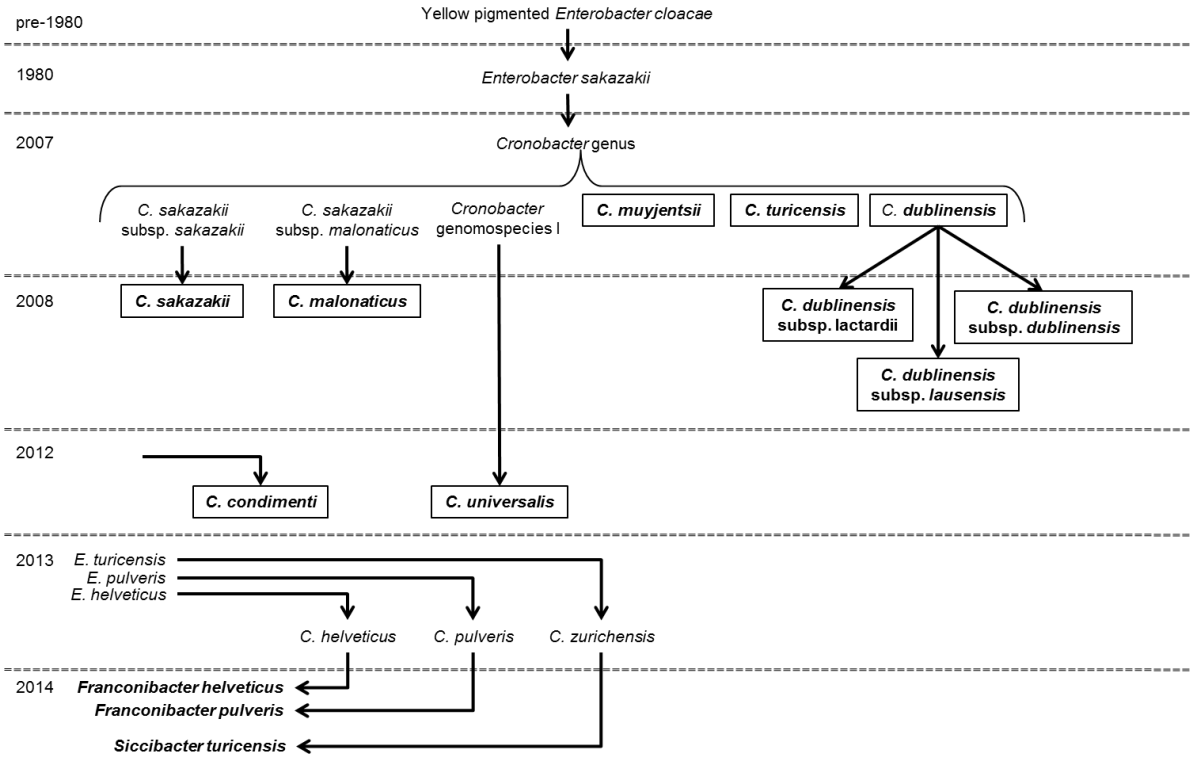


**Figure 3.2. A summary of taxonomic revisions of the *Cronobacter* genus (1980-2014)**

As *F. helveticus, F. pulveris*, and *S. turicensis* were removed from the *Cronobacter* genus, their absence is no longer required by the microbiological criteria applied to PIF. Though some methods used in this re-evaluation did exclude these species, other methods were able to detect them along with the seven original *Cronobacter* species. Detection of *Franconibacter* and *Siccibacter* species using those methods would now be considered a false positive result, while lack of detection of these species would now be considered a true negative.

When using cultural methods, all strains now known as *Franconibacter* and *Siccibacter* were able to grow in EE broth and strains now known as *F. pulveris* produced positive results in CSB. Additionally, all of these strains produced blue-green colonies on DFI and many produced yellow colonies on TSA; therefore, it is possible that these species could be mistaken for *Cronobacter* spp. when using cultural detection methods. While strains of *F. helveticus, F. pulveris,* and *S. turicensis* were identified as '*E. sakazakii'* with the ID32E test kit in the culture collection

records, only strains of *F. helveticus* were identified as members of the *Cronobacter* genus following the 2015 update to this database. These misidentifications are important as the ID32E test kit could be used by PIF manufacturers and food testing laboratories who may observe false positive results. Finally, the false positive results observed for *Franconibacter* and *Siccibacter* species when using the PCR method targeting *rpoB* suggest that isolates from these genera may be misidentified as multiple *Cronobacter* species. Such misidentifications could be costly to PIF manufacturers who may reject a batch of safe product based on these results, as no cases of illness have been linked to members of the *Franconibacter* or *Siccibacter* genera. Thus, biochemical test panels and the PCR method targeting *rpoB* are not sufficient for accurate identification of *Cronobacter* spp. and exclusion of members of the *Franconibacter* and *Siccibacter* genera.

Prior to the definition of the *Franconibacter* and *Siccibacter* genera, the PCR method targeting *ompA* was predicted to exclude the strains now considered to be members of these genera. Thus, the exclusion of the species now known as *F. helveticus, F. pulveris,* and *S. turicensis* is considered to be a true negative result. Based on the *in silico* and laboratory analyses described in this chapter, the *ompA* method was predicted to be capable of identifying the seven original species of *Cronobacter* to the genus level, while excluding members of the *Citrobacter, Enterobacter, Franconibacter, Siccibacter,* and *Yersinia* genera. While additional analyses would be necessary to identify positive strains to the species level, the *ompA* PCR method would be sufficient for PIF manufacturers to ensure the exclusion of *Cronobacter* spp. from their products, as specified in the international microbiological criteria applied to PIF *[26]*.

As demonstrated above, the application of cultural, biochemical, and PCR-based detection and identification methods is limited, particularly when the taxonomy is changed and species are added to or removed from a genus. Alternative methods are needed to ensure accurate identification of species within the *Cronobacter* genus. While some methods, such as MALDI-TOF MS, real-time PCR, and the Vitek 2.0 system, have been developed, they are not accessible to all laboratories due to the high cost of equipment and need for trained personnel to perform the analyses. Developments in sequence-based methods, including MLST, allow for highly specific species and strain identification that can adapt to taxonomic revisions *[129]*. While these methods are more reliable than subjective biochemical and morphological tests or molecular identification methods, they are not necessarily accessible to all PIF manufacturers or food testing laboratories. As DNA sequencing becomes cheaper and easier, it may be possible to incorporate such techniques into standard test methods, but this is not currently feasible for all laboratories.

Regardless, the use of DNA sequence-based methods has greatly increased our understanding of *Cronobacter* and related genera. In addition to identification of suspect isolates, DNA sequence-based methods, such as MLST, have a variety of uses, including outbreak tracking and identification of novel species and genera. The application of MLST to strains of *Cronobacter* will be discussed in more detail in the next chapter.

# CHAPTER 4

# Applications of multilocus sequence typing for characterization and differentiation of *Cronobacter* species and strains

*Portions of the work presented in this chapter have been published in peer-reviewed journals.*
***Jackson, E. E.,*** et al. *(2015)*. Journal of Food Protection. *78:6.*
***Jackson, E. E.,*** et al. *(2015).* IJSEM. *65:4.*

*Two genome sequences presented in this chapter have also been published.*
*Masood, N.,* ***Jackson, E.*** et al. *(2014).* Genome Announcements. *2:2.*
***Jackson E. E.,*** et al. *(2016).* Genome Announcements. *4:1.*

*See Appendix B for full text versions of these publications.*

*A portion of the data presented in this chapter was presented as a poster at the Congress of European Microbiologists (FEMS 2015) in Maastricht, the Netherlands.*

## 4.1. Introduction

As shown in the previous chapter, cultural, biochemical, and molecular identification methods can result in the misidentification of *Cronobacter* spp. Both false negative and false positive identifications can be costly to powdered infant formula (PIF) manufacturers and these misidentifications should be minimized. Additionally, accurate identification methods are essential during illness and outbreak investigations. Misidentifications in these situations can lead to incorrect treatments and can mask other potential hazards. For example, *Enterobacter hormaechei* was misidentified as '*E. sakazakii'* during an outbreak of neonatal sepsis in a California hospital in 1996 and 1997 *[157]*. The misidentification of these isolates concealed a nosocomial *E. hormaechei* outbreak that had occurred among neonates at the hospital *[157]*.

*E. hormaechei* harbours many virulence factors and has been linked to multiple outbreaks; however, as described in Section 1.4, only *Cronobacter* and *Salmonella* species are identified as Category A organisms ('clear evidence of causality') in the microbiological criteria applied to PIF *[40, 41, 157, 161]*. Other *Enterobacteriaceae*, including *Enterobacter* spp., are considered Category B organisms ('causality plausible, but not yet demonstrated') and are, therefore, permitted in PIF *[40, 41]*. While transmission of *E. hormaechei* from PIF to infants has not yet been reported, this species is permitted in the product and is known to cause illness, presenting a possible risk of infection.

Chapter 3 discussed some of the issues associated with cultural, biochemical, and molecular detection and identification assays. It was shown that these methods are not sufficient for accurate and specific identification of suspect *Cronobacter* isolates, as is necessary to meet the criteria requiring the absence of all *Cronobacter* species in PIF. DNA sequence-based

identification methods, on the other hand, are regarded as more accurate and reliable for species identification of bacteria. In particular, a 7-loci, laboratory-based multilocus sequence typing (MLST) scheme has been developed for *Cronobacter* spp. *[6]*. This method utilizes the sequences of seven housekeeping genes to assign strains and isolates to sequence types (STs) *[6]*. Each allele is sequenced individually and each unique sequence is assigned an allele number. Together, the allele numbers define the ST. Strains from related STs are further clustered into clonal complexes (CCs) when they share four or more identical alleles *[90]*. Members of a CC can be identified as single locus variants (SLVs), double locus variants (DLVs), or triple locus variants (TLVs), depending on the number of alleles differing from the founder ST for the complex *[90]*. Additionally, allele sequences can be analysed individually or as a concatenated sequence (3,036 bp) to determine the phylogenetic relationships of the strains in question *[6]*.

The genes included in the *Cronobacter* MLST scheme, their functions, and allele sizes are shown in Table 4.1. These genes were selected for inclusion in the scheme because they are housekeeping genes required for survival of the organism *[6]*. Thus, the genes will be present in all species and strains. In addition, the selected genes are scattered around the genome to ensure that they are not genetically linked or subjected to the same evolutionary pressures *[6]*. As of July 2016, 440 unique STs had been defined, covering all seven *Cronobacter* species and members of some closely related genera, including *Enterobacter, Franconibacter,* and *Siccibacter.* All sequences and MLST profiles are available at http://www.pubmlst.org/Cronobacter *[42]*.

**Table 4.1. Genes in the *Cronobacter* MLST scheme with their functions and allele sizes**

| Gene | Function | Allele size (bp) |
|---|---|---|
| *atpD* | ATP synthase beta chain | 390 |
| *fusA* | Elongation factor G | 438 |
| *glnS* | Glutaminyl-t-RNA synthase subunit | 363 |
| *gltB* | Glutamate synthase large subunit | 507 |
| *gyrB* | DNA gyrase subunit B | 402 |
| *infB* | Translocation initiation factor IF-2 | 441 |
| *ppsA* | Phosphoenolpyruvate synthase | 495 |

Importantly, phylogenetic relationships can be determined using multilocus sequence analysis (MLSA) *[44, 100, 129]*. The phylogenetic tree shown in Figure 4.1 illustrates the diversity of the *Cronobacter* genus and the relationship of this genus to members of the *Enterobacter, Franconibacter,* and *Siccibacter* genera based on the 440 STs identified with the *Cronobacter* MLST scheme *[42]*. Each ST is represented once, but some STs are more prevalent in the PubMLST database *[42]*. This may be due to the natural variation within the *Cronobacter* population or overrepresentation of some STs in the database, particularly those associated with

illnesses and outbreaks. Clinically relevant STs (*C. sakazakii* ST1, ST4, ST8 and ST12; *C. malonaticus* ST7) are also indicated in Figure 4.1. The phylogenetic relationships determined by MLSA are consistent with the phylogenetic relationships determined using whole genome sequencing (WGS), indicating that MLST and MLSA can be used to examine phylogeny in situations where WGS is not available or accessible *[44].*

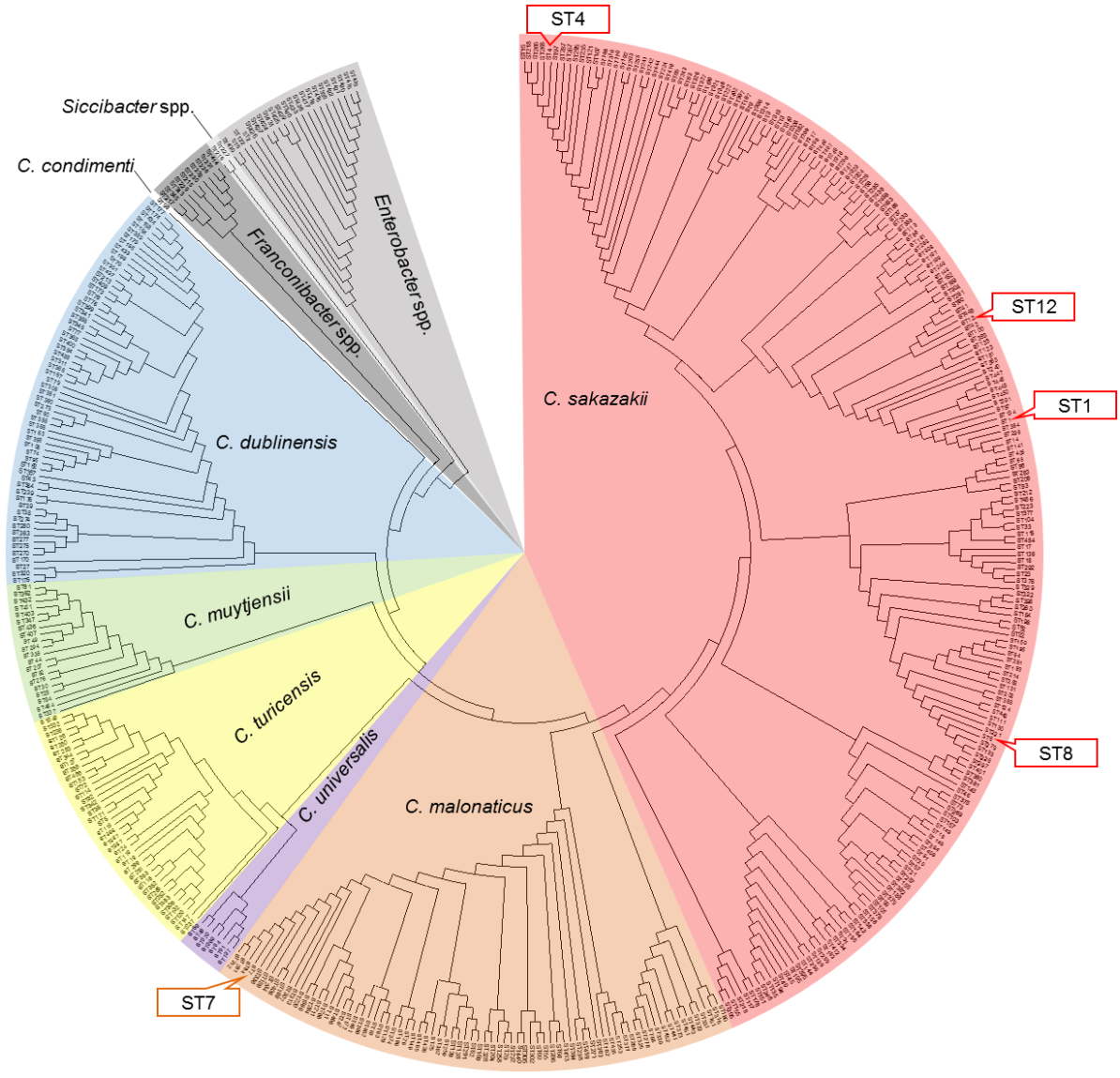


**Figure 4.1. Maximum likelihood tree of 440 sequence types identified using the *Cronobacter* MLST scheme.** Each branch represents a single ST. Each ST is represented once to show the diversity of the *Cronobacter* genus. Clinically relevant STs are indicated on the tree.

Ribosomal MLST (rMLST) can also be used for characterization and differentiation of strains *[87, 88, 111].* This method works similarly to the 7-loci MLST technique, but utilizes the sequences of 53 ribosomal genes (concatenated length: approx. 22,511 bp) to differentiate species and strains. rMLST data have been found to be consistent with current bacterial

nomenclature and the phylogenetic relationships determined by 7-loci MLSA and WGS *[44, 88]*. This is illustrated by the rMLST tree in Figure 4.2, which is very similar to the 7-loci tree shown in Figure 4.1. Importantly, ribosomal MLSA shows that some STs defined by the 7-loci scheme can be further subdivided. For example, CC4 strains are represented by eight rMLST profiles, even though only three STs belonging to CC4 are included in the tree. This demonstrates that diversity exists not only between STs, but also within them. While more precise than 7-loci MLST, rMLST can only be applied to strains for which a whole genome sequence is available. Thus, it is a useful tool for characterization of strains, but is currently somewhat limited in its application.

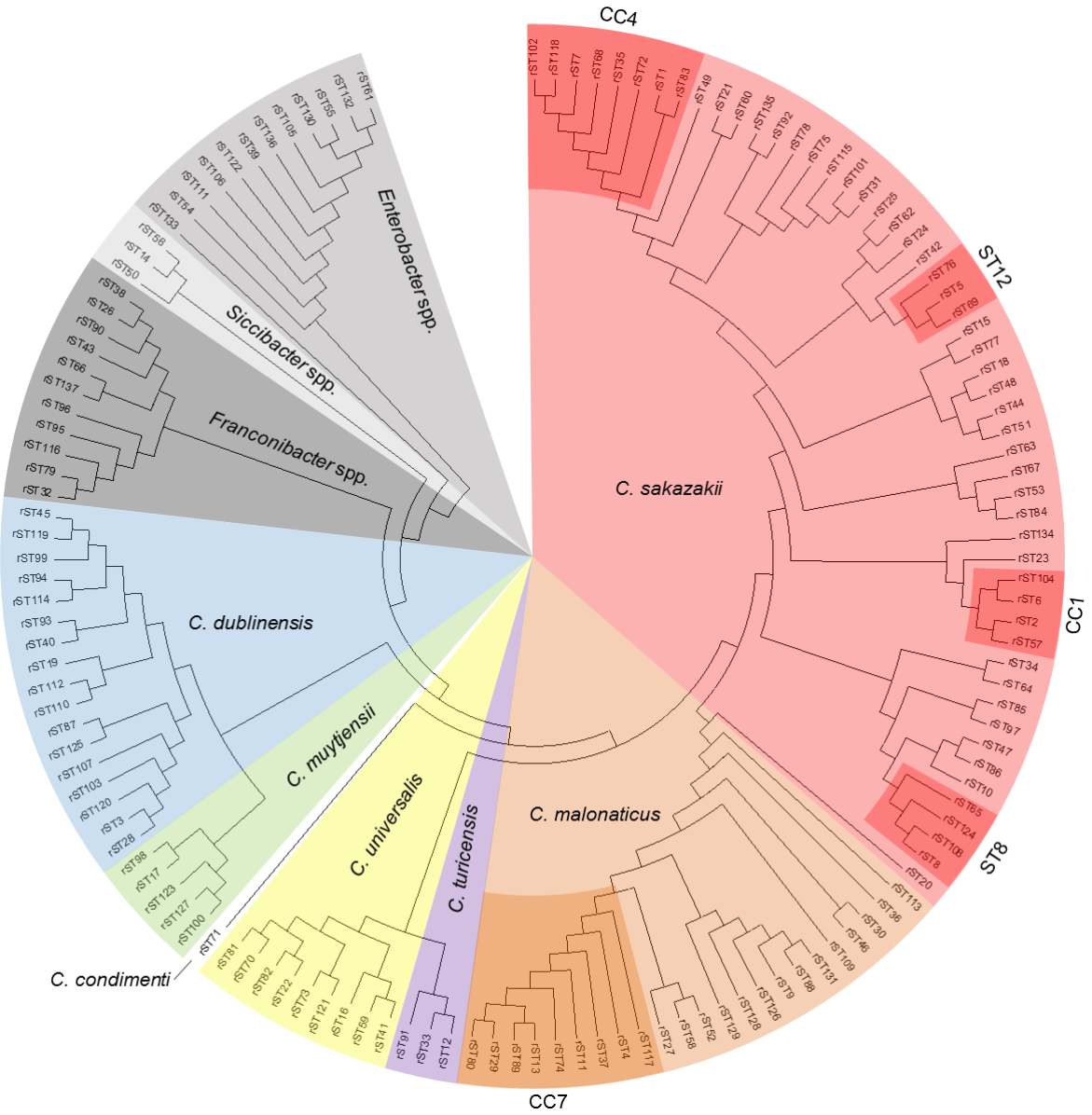


**Figure 4.2. Maximum likelihood tree of 136 ribosomal sequence types (rSTs).** Each branch represents a unique combination of 53 ribosomal gene sequences. Each unique combination is represented once in the tree. rST numbers were assigned arbitrarily and do not correspond to STs assigned using the 7-loci MLST scheme. Clinically relevant STs and CCs (as assigned by the 7-loci MLST scheme) are indicated by the darker shaded sections.

As stated in Chapter 3, MLST and MLSA have been used to identify and describe novel species. *Cronobacter condimenti* and *C. universalis* were both defined using MLSA and multiple reclassifications of the species now known as *Franconibacter helveticus, F. pulveris*, and *Siccibacter turicensis*, were based on 4- or 7-loci MLST *[15, 93, 146]*. WGS would be preferable for the identification of novel species, but these studies show that MLST and MLSA can also be used for this purpose. As shown in Figures 4.1 and 4.2, the genera *Franconibacter* and *Siccibacter* form distinct clusters, separate from the *Cronobacter* and *Enterobacter* genera. This supports the claim that *Franconibacter* and *Siccibacter* should be considered unique genera. The taxonomy of the *Siccibacter* genus will be discussed in detail in Section 4.3.

Additionally, MLSA has revealed certain associations within the *Cronobacter* genus. For example, ST4 strains have been linked to cases of neonatal meningitis *[55, 91]*. Conversely, while ST12 strains have been linked to cases of necrotizing enterocolitis (NEC), they have not been isolated from any cases of neonatal meningitis *[42, 43]*. Other *C. sakazakii* STs linked to neonatal illnesses include ST1 and ST8 *[6, 90]*. While most invasive cases of *Cronobacter* infection have occurred in infants, neonates, and children under the age of four years, adults can also be infected with *Cronobacter* spp. The elderly are considered to be an at-risk population, but the symptoms of infection in adults differ from those observed in infants and neonates *[128]*. While Holy *et al.* collected the majority of adult isolates from throat swabs, both Patrick *et al.* and Holy *et al.* identified urine as a major source of *Cronobacter* isolates in adults *[61, 62, 128]*. Gosney *et al. [49]* also found an association of *Cronobacter* spp. (then known as *'Enterobacter sakazakii'*) with pneumonia in elderly patients who had previously suffered a stroke. With regard to adult infections, Joseph and Forsythe reported an association of *C. malonaticus* ST7 with adult infections, but this association was not observed for the hospital isolates examined by AIsonosi *et al. [3, 90].* These studies demonstrate that identification and characterization of *Cronobacter* isolates using MLST may provide insights into the potential hazards posed by a strain or isolate.

The aim of the work described in this chapter was to apply the *Cronobacter* MLST scheme to isolates from a variety of sources. Identification of these isolates increased the number of strains and STs in the *Cronobacter* PubMLST database *[42]*. Emphasis was placed on the typing of isolates from the PIF factory environment, as this source is under-represented in the database. Isolates from three separate outbreaks were also analysed, including misidentified strains from an outbreak in Mexico. This chapter describes not only the MLST of these strains, but also the associated analyses and conclusions that could be drawn based on these data.

## 4.2. MLST of selected strains belonging to *Cronobacter* and related genera

The strains used in this work, their sources, MLST profiles, STs and CCs are shown in Table 4.2. As stated previously, CCs are defined by STs that are identical at four or more of the seven loci in the MLST scheme. Strains used in previous publications are also indicated in the table. Strains from France, Belgium and Switzerland were provided by N. Gnanou-Besse at the Agence française de sécurité sanitaire de aliments (AFSSA). Strains from Mexico and Chile were provided by J. Flores at the Universidad del Bio-Bio in Chile and the strain from Portugal was provided by C. Quintas at Universidade do Algarve in Portugal. The remaining strains were available in the NTU culture collection. Where indicated, strains were characterized culturally and/or biochemically, as described in Section 2.2. Strains were preserved by freezing at -80° C in TSB with 20% glycerol.

DNA extractions, MLST, and WGS were carried out as described in Chapter 2. Species identifications were based on the *fusA* sequence and ST of each strain. Speciation is often based on the *fusA* allele as this gene was found to be stable and these alleles are rarely shared between species *[92].* Where indicated, rMLST sequences were extracted with the Genome Comparator tool of the Bacterial Isolate Genome Sequence Database (BIGSdb) at http://www.pubmlst.org/Cronobacter *[42, 87, 88, 111]*. DNA sequences were aligned using MEGA6 *[153]*. Phylogenetic trees were also constructed with MEGA6, using the neighbour joining or maximum likelihood method, as indicated *[153]*. Average nucleotide identity (ANI) analysis was carried out using the tool available at http://enve-omics.ce.gatech.edu/ani/ *[48]*.

For convenience, each set of results will be discussed individually below, in the context of a particular application of MLST.

**Table 4.2. Bacterial strains analysed by 7-loci MLST**

| Strain | Alias | Country | Source | Reference | *atpD* | *fusA* | *glnS* | *gltB* | *gyrB* | *infB* | *ppsA* | Species | ST | CC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2106 | - | Belgium | Clinical; blood | *[160]* | 3 | 11 | 13 | 116 | 11 | 17 | 13 | *Cronobacter sakazakii* | 257 | 21 |
| 2107 | - | Belgium | Clinical; blood | *[160]* | 18 | 17 | 10 | 12 | 18 | 24 | 18 | *Cronobacter sakazakii* | 12 | - |
| 2089 | 08HMPA08 | France | Clinical; CSF | *[27, 116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2090 | 08HMPA09 | France | Clinical; CSF | *[27, 116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2091 | 08HMPA10 | France | Clinical; fecal | *[27, 116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2092 | 08HMPA11 | France | PIF | *[27, 116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2224[a] | 8107 | Mexico | Clinical; fecal | *[38]* | 99 | 75 | 118 | 150 | 132 | 138 | 176 | *Enterobacter hormaechei* | 339 | - |
| 2118[a] | 8706 | Mexico | Clinical; fecal | *[38]* | 100 | 81 | 132 | 151 | 133 | 139 | 177 | *Enterobacter* spp. | 340 | - |
| 2161[a] | 8756 | Mexico | Hospital environment | *[38]* | 3 | 1 | 48 | 84 | 130 | 36 | 178 | *Cronobacter sakazakii* | 297 | - |
| 2122 | 8710 | Mexico | PIF | *[38]* | 3 | 1 | 48 | 84 | 130 | 36 | 178 | *Cronobacter sakazakii* | 297 | - |
| 2129 | 8718 | Mexico | PIF | *[38]* | 3 | 1 | 48 | 84 | 130 | 36 | 178 | *Cronobacter sakazakii* | 297 | - |
| 2140 | 8731 | Mexico | PIF | *[38]* | 3 | 1 | 48 | 84 | 130 | 36 | 178 | *Cronobacter sakazakii* | 297 | - |
| 2142 | 8733 | Mexico | PIF | *[38]* | 3 | 1 | 48 | 84 | 130 | 36 | 178 | *Cronobacter sakazakii* | 297 | - |
| 2033 | 05CHPL18 | France | Environment | *[116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2034 | 05CHPL27 | France | Environment | *[116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2035 | 05CHPL29 | France | Environment | *[116]* | 5 | 1 | 3 | 3 | 5 | 123 | 4 | *Cronobacter sakazakii* | 255 | 4 |
| 2041 | 05CHPL40 | France | Environment | *[116]* | 3 | 13 | 102 | 8 | 77 | 141 | 154 | *Cronobacter malonaticus* | 392 | 250 |
| 2045[a] | 05CHPL46 | France | Environment | *[116]* | 98 | 7 | 67 | 23 | 10 | 123 | 154 | *Cronobacter malonaticus* | 302 | - |
| 2046[a] | 05CHPL47 | France | Environment | *[116]* | 98 | 7 | 67 | 23 | 10 | 123 | 154 | *Cronobacter malonaticus* | 302 | - |
| 2048[a] | 05CHPL50 | France | Environment | *[116]* | 11 | 8 | 7 | 5 | 8 | 15 | 10 | *Cronobacter sakazakii* | 8 | 8 |
| 2051[a] | 05CHPL53 | France | Environment | *[116]* | 16 | 8 | 13 | 40 | 15 | 15 | 10 | *Cronobacter sakazakii* | 64 | 64 |
| 2052 | 05CHPL54 | France | Environment | *[116]* | 10 | 17 | 30 | 59 | 57 | 66 | 83 | *Cronobacter sakazakii* | 125 | 100 |
| 2053 | 05CHPL56 | France | Environment | *[116]* | 16 | 8 | 13 | 40 | 15 | 15 | 10 | *Cronobacter sakazakii* | 64 | 64 |
| 2064[a] | 05CHPL78 | France | Environment | *[116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2065 | 05CHPL82 | France | Environment | *[116]* | 5 | 1 | 3 | 3 | 5 | 5 | 4 | *Cronobacter sakazakii* | 4 | 4 |
| 2070 | 05CHPL97 | France | Environment | *[116]* | 5 | 1 | 3 | 3 | 5 | 61 | 4 | *Cronobacter sakazakii* | 295 | 4 |
| 2071 | 05CHPL99 | France | Environment | *[116]* | 5 | 1 | 3 | 3 | 5 | 5 | 4 | *Cronobacter sakazakii* | 4 | 4 |
| 2072 | 05CHPL101bis | France | Environment | *[116]* | 5 | 1 | 3 | 3 | 5 | 5 | 4 | *Cronobacter sakazakii* | 4 | 4 |
| 2087[a] | 08HMPA06 | France | Environment | *[116]* | 10 | 17 | 30 | 59 | 57 | 66 | 63 | *Cronobacter sakazakii* | 100 | 100 |
| 2109[a] | 07HMPA87A | France | Environment | *[116]* | 10 | 13 | 67 | 7 | 131 | 124 | 174 | *Cronobacter malonaticus* | 300 | 300 |
| 2110 | 07HMPA87B | France | Environment | *[116]* | 3 | 11 | 13 | 18 | 11 | 17 | 13 | *Cronobacter sakazakii* | 21 | 21 |
| 2111 | 07HMPA87F | France | Environment | *[116]* | 1 | 1 | 30 | 1 | 1 | 125 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2112 | 07HMPA93A | France | Environment | *[116]* | 10 | 17 | 1 | 59 | 57 | 66 | 63 | *Cronobacter sakazakii* | 393 | 100 |
| 2032 | 05CHPL10 | France | PIF | *[116]* | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2108 | 07HMPA41A | France | PIF | *[116]* | 3 | 8 | 37 | 22 | 29 | 36 | 32 | *Cronobacter sakazakii* | 31 | 31 |
| 2059 | 05CHPL63 | France | Food | *[116]* | 35 | 115 | 124 | 8 | 138 | 39 | 181 | *Cronobacter muyjentsii* | 337 | - |
| 2257 | - | Saudi Arabia | Milk powder | Unpublished | 1 | 1 | 1 | 1 | 1 | 1 | 1 | *Cronobacter sakazakii* | 1 | 1 |
| 2023 | - | UK | Soup mix | Unpublished | 16 | 1 | 19 | 19 | 26 | 5 | 26 | *Cronobacter sakazakii* | 22 | - |
| 2024 | - | UK | Soup mix | Unpublished | 3 | 8 | 37 | 117 | 29 | 36 | 32 | *Cronobacter sakazakii* | 254 | - |
| 2284 | - | Portugal | Soil | Unpublished | 3 | 8 | 37 | 22 | 29 | 36 | 32 | *Cronobacter sakazakii* | 31 | 31 |
| 2285 | - | Portugal | Soil | Unpublished | 3 | 8 | 37 | 22 | 29 | 36 | 32 | *Cronobacter sakazakii* | 31 | 31 |
| 2249 | - | UK | Tea | Unpublished | 96 | 97 | 115 | - | 129 | 136 | - | *Siccibacter colletis* | - | - |
| 2085 | 08HMPA02 | Switzerland | Clinical (collection strain) | *[116]* | 22 | 22 | 14 | 16 | 24 | 18 | 24 | *Cronobacter turicensis* | 19 | 24 |
| 2030[a] | 05CHPL02 | France | Collection strain | *[116]* | 59 | 20 | 71 | 138 | 83 | 137 | 175 | *Cronobacter dublinensis* | 301 | - |
| 2055 | 05CHPL59 | France | Collection strain | *[116]* | 5 | 1 | 3 | 3 | 5 | 5 | 4 | *Cronobacter sakazakii* | 4 | 4 |
| 2061 | 05CHPL65 | France | Collection strain | *[116]* | 15 | 15 | 80 | 126 | 124 | 56 | 159 | *Cronobacter sakazakii* | 256 | - |

[a]MLST profile completed by extraction of allele sequences from whole genome sequence. [b]ST, sequence type. [c]CC, clonal complex

## 4.3. Use of MLST for identification of a novel species

As discussed previously, MLST was used to help identify and define the novel species *Cronobacter condimenti, C. universalis, Franconibacter helveticus, F. pulveris,* and *Siccibacter turicensis [93, 146]*. Strains of *S. turicensis* had been identified with three unique *fusA* alleles (70, 76, and 91). While *fusA* 76 was attributed to seven strains in the *Cronobacter* PubMLST database, the other allele sequences were each assigned to only a single strain *[42]*. Comparison of the three *fusA* alleles found that *fusA* 76 and 91 differed from one another by only 6 nucleotides. In contrast, *fusA* 70 differed from alleles 76 and 91 by 21 and 27 nucleotides, respectively.

*S. turicensis* strain 1383 (formerly *C. zurichensis*) was previously assigned *fusA* allele 70 *[42]*. This is the only strain in the *Cronobacter* pubMLST database with this particular *fusA* allele *[42]*. The genome of this strain was sequenced at Exeter University and assembled by N. Masood, with assistance from K. Ibrahim and S. Hariri. Average nucleotide identity (ANI) analysis was used to compare the genome of *S. turicensis* strain 1383 (GenBank Accession Number: JMSQ00000000) to the type strains of all species in the *Siccibacter, Franconibacter,* and *Cronobacter* genera *[113]*. An ANI value of $\geq$95% is commonly used as the threshold for species differentiation, but there is no accepted threshold for genus demarcation *[48, 131]*. As shown in Table 4.3, strain 1383 was found to have an ANI value of 87.2% when compared to the type strain of *S. turicensis*, suggesting that strain 1383 belongs to a separate species in the *Siccibacter* genera.

**Table 4.3. Average nucleotide identity of strain 1383 with members of the *Siccibacter, Franconibacter,* and *Cronobacter* genera**

| Species | Strain | Strain 1383 |
|---|---|---|
| *S. turicensis* | LMG 23730$^T$ | 87.2% |
| *F. helveticus* | LMG 23732 $^T$ | 83.4% |
| *F. pulveris* | LMG 24057$^T$ | 83.3% |
| *C. sakazakii* | ATCC 29544$^T$ | 83.6% |
| *C. malonaticus* | LMG 23826$^T$ | 84.2% |
| *C. universalis* | NCTC 9529$^T$ | 84.4% |
| *C. turicensis* | LMG 23827$^T$ | 83.6% |
| *C. muytjensii* | ATCC 51329$^T$ | 83.5% |
| *C. dublinensis* | LMG 23823$^T$ | 83.9% |
| *C. condimenti* | NCTC 9529$^T$ | 83.4% |

A second strain (2249) was isolated from tea by N. Urvoy in 2014. This strain was identified with *fusA* allele 97 (Table 4.2). This *fusA* allele differs from *fusA* 70 (assigned to strain 1383) by only one nucleotide. The nearly identical sequences of these alleles suggest that strain 2249 also belongs to the same novel species as strain 1383. This species was named *Siccibacter colletis* and will be referred to as such for the remainder of this discussion.

The MLST alleles for strain 1383 were extracted from the whole genome sequence and this strain was assigned to ST227 *[42, 113]*; however, the MLST of strain 2249 was carried out in the laboratory, as described in Section 2.4.3. Due to sequence variation between the genera, only five of the seven loci (*atpD, fusA, glnS, gyrB, infB*) were able to be sequenced during the laboratory analysis. Even though a sequence type could not be assigned to this strain at the time, five alleles can be used for phylogenetic analysis. The concatenated length of these allele sequences was 2,034 bp. Figure 4.3 shows the relationship of *S. colletis* strains 1383 and 2249 to members of the *Siccibacter, Franconibacter, Cronobacter, Enterobacter, Citrobacter*, and *Yokenella* genera, based on these sequences.

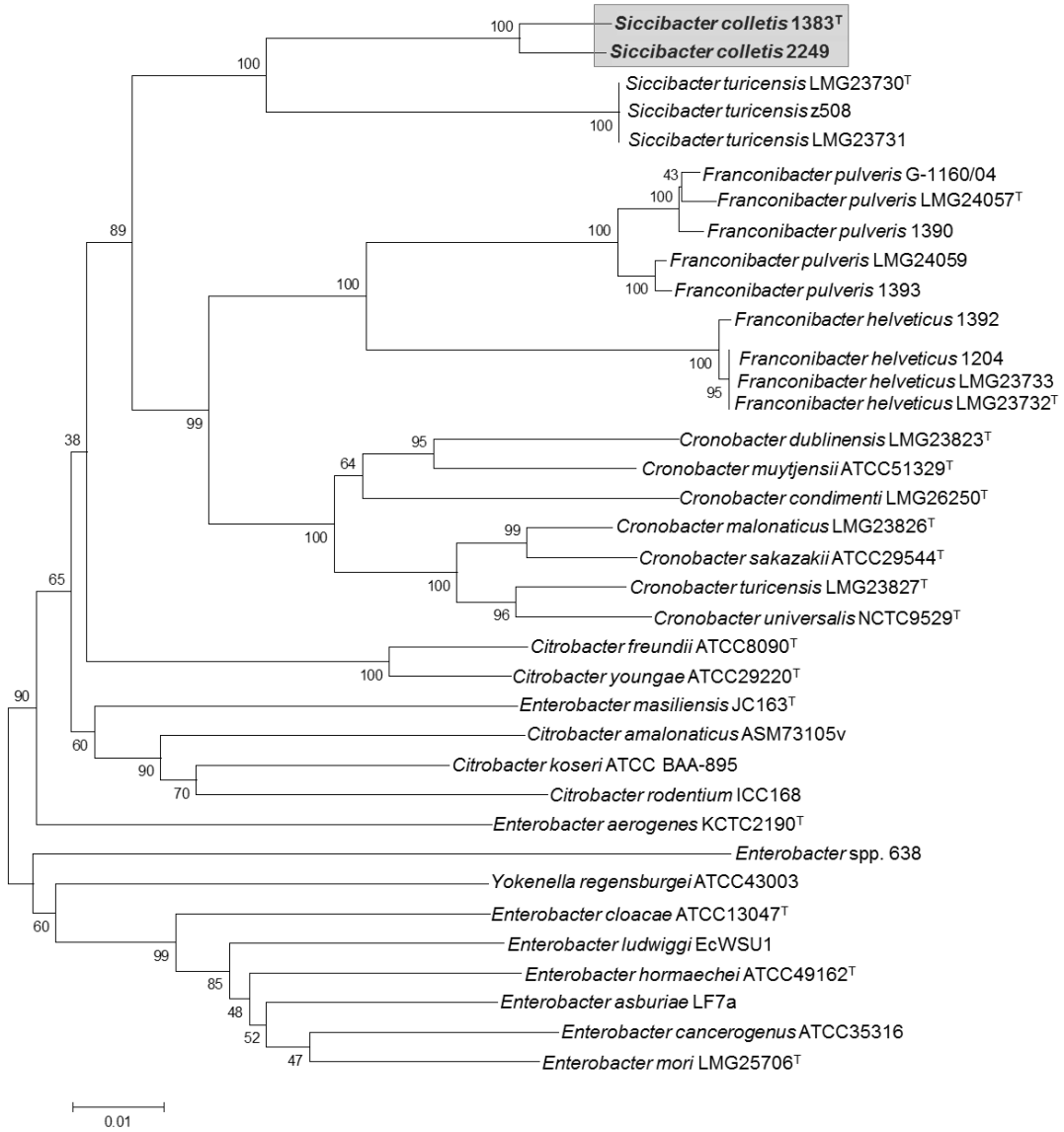


**Figure 4.3. Neighbour-joining, 5-loci MLST phylogenetic tree showing the position of *S. colletis* strains 1383 and 2249.** This tree is based on concatenated *atpD, fusA, glnS, gyrB,* and *infB* sequences (concatenated length: 2,034 bp) and shows the relationship of *S. colletis* (shaded box) to members of the *Siccibacter, Franconibacter, Cronobacter, Enterobacter, Citrobacter,* and *Yokenella* genera. Bootstrap values based on 1,000 replications are indicated at the branch nodes. Scale bar = 1 substitution per 100 nucleotides.

The 5-loci MLSA supports the identification of the *Franconibacter* and *Siccibacter* genera as each genus formed a discrete cluster, separate from the *Cronobacter* genus. This is in agreement with the taxonomy proposed by Stephan *et al. [146]* and refutes the inclusion of these species in the *Cronobacter* genus, as proposed by Brady *et al. [15]*. Interestingly, the 4-loci MLST reported by Brady *et al. [15]* did show members of the *Franconibacter* and *Siccibacter* genera clustering near the *Cronobacter* genus, but on separate branches. It seems that these authors drew the line for species demarcation closer to the root of the phylogenetic tree than Stephan *et al. [146]* resulting in the disagreement over the inclusion of these species in the *Cronobacter* genus. Though similar phylogenies were observed by both authors, the classification by Stephan *et al.* is more reliable, as it is supported by ANI, DNA-DNA hybridization, and single nucleotide polymorphism analyses *[146]*.

Additionally, the 5-loci MLST in the current study showed that strains 1383 and 2249 clustered near to the other members of the *Siccibacter* genus (Figure 4.3); however, strains 1383 and 2249 appear on their own branch, with strong bootstrap support (100%). In addition to the ANI analysis described above (Table 4.3), this observation provides support for the hypothesis that strains 1383 and 2249 should be considered a separate *Siccibacter* species.

The identification of the novel species *Siccibacter colletis* was further supported by rMLST analysis. Only strain 1383 could be included in this analysis as a genome sequence for strain 2249 was not available at the time. Figure 4.4 shows the position of strain 1383 in relationship to members of the *Siccibacter, Franconibacter, Cronobacter, Enterobacter, Citrobacter,* and *Yokenella* genera. The phylogenetic relationships determined using rMLST (53-loci) are similar to those observed with the 5-loci MLST. The *Siccibacter, Franconibacter,* and *Cronobacter* genera again clustered separately from one another, supporting the claim by Stephan *et al. [146]* that these strains should be considered distinct genera. Additionally, strain 1383 again clustered near the *S. turicensis* strains, but on its own branch. As with the 5-loci MLSA, this supports the claim that strain 1383 is not *S. turicensis,* but should instead be considered to be a member of a novel species.

This work highlights one of the major advantages of MLST: the ability to adapt to taxonomic revisions. Though the species names have changed over time, the allele numbers and STs do not change when the taxonomy is updated *[111].* Consequently, only the name of the species needed to be changed in the database when *S. colletis* was defined as a novel species. In contrast, the databases associated with biochemical test kits are not updated so easily, as evidenced in the previous chapter *[80].* Not only do the species names need to be changed, but the biochemical profiles themselves must be taken into consideration. One or more profiles will need to be assigned

to the new species, but identical or highly similar profiles may be produced by other species already included in the database. As demonstrated in Chapter 3, there is a possibility that misidentifications will result following an update to the database because the biochemical profiles included in the database do not accurately reflect the diversity that exists both between and within species. MLST also has the advantage in that each unique allele is assigned its own number, giving rise to many possible combinations. Meanwhile, each test in a biochemical panel has only two possibilities (positive or negative), leading to a limited number of possible profiles. This adaptability is one of many traits that makes MLST more reliable than other methods for identification, characterization, and differentiation of *Cronobacter* spp.

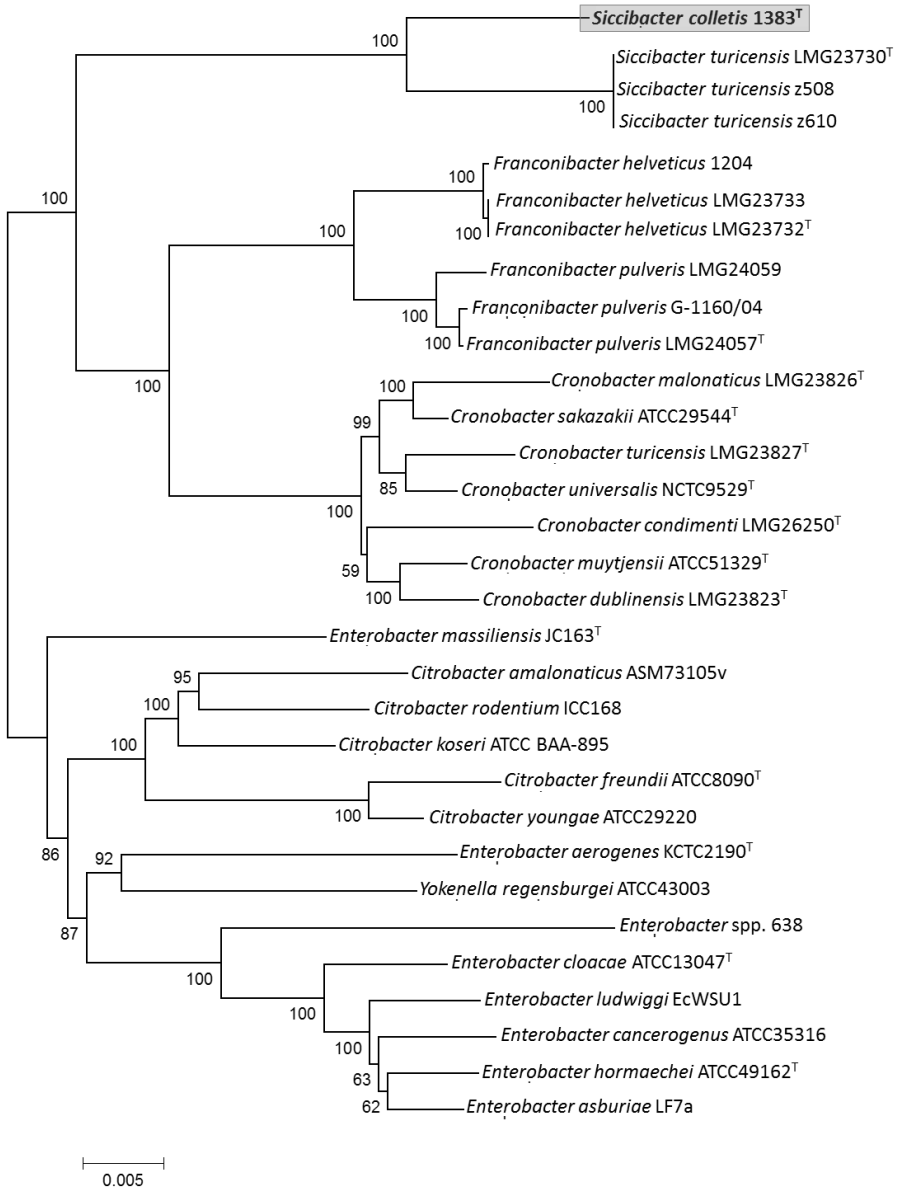


**Figure 4.4. Neighbour-joining rMLST phylogenetic tree showing the position of *S. colletis* strain 1383.** This tree is based on 53 ribosomal protein genes (concatenated length: 22,511 bp) and shows the relationship of *S. colletis* (shaded box) to members of the *Siccibacter, Franconibacter, Cronobacter, Enterobacter, Citrobacter,* and *Yokenella* genera. Bootstrap values based on 1,000 replications are indicated at the branch nodes. Scale bar = 1 substitution per 5000 nucleotides.

In order to define the novel species *S. colletis,* strains were subjected to phenotypic characterization as described in detail in Chapter 2. Acid production from *myo*-inositol, putrescine, lactulose, 4-aminobutyrate, maltitol, and *trans*-aconitate was evaluated in phenol red broth. This broth was also used to test for acid and gas production from glucose with the inclusion of an inverted Durham tube. Catalase and oxidase activity, motility, and utilization of sialic acid were assessed using the traditional methods described in Chapter 2. Strains were also analysed using the biochemical test panels API20E, ID32E, 50CHE, and APIZYM (bioMérieux, France), according the manufacturer's instructions, which are summarized in Chapter 2.

Strains were also subjected to antimicrobial resistance testing, as described by the British Society for Antimicrobial Chemotherapy (BSAC) and in Section 2.3.10 *[16]*. BSAC criteria were used to designate strains as resistant, susceptible or intermediate *[16]*. The following antibiotic discs (MAST Diagnostics, Bootle, UK) were used for analysis: amikacin (AK30), ampicillin (AP10), amoxicillin plus clavulanic acid (AUC30), cefotaxime (CTX30), cefuroxime (CXM30), ceftazidime (CAZ30), chloramphenicol (C30), ciprofloxacin (CIP1), doxycycline (DXT30), gentamicin (GM10), imipenem (IMI10), and trimethoprim plus sulfamethoxazole (TS25). The type strains of all *Siccibacter, Franconibacter,* and *Cronobacter* species were assessed with both *S. colletis* strains, under identical conditions.

The results of the phenotypic analyses are shown in Table 4.4. Four traits were identified which can differentiate *S. colletis* from *S. turicensis.* These traits are acid phosphatase and N-acetyl-ß-glucosaminidase activities, utilization of sialic acid, and production of gas from D-glucose. Additionally, both strains 1383 and 2249 exhibited resistance to doxycycline. Intermediate resistance to cefotaxime was also observed for strain 1383. Both strains showed susceptibility to all the other antibiotics used in this analysis.

**Table 4.4. Phenotypic characteristics differentiating *Siccibacter colletis* from members of the *Siccibacter, Franconibacter* and *Cronobacter* genera.** Key traits for differentiation of *Siccibacter* species are shown in bold. Reactions of the type strains are shown in parentheses.

| Characteristic | *Siccibacter colletis* (n=2)[a] | *Siccibacter turicensis* (n=2)[b] | *Franconibacter helveticus* (n=2) | *Franconibacter pulveris* (n=6) | *Cronobacter sakazakii* (n=163) | *Cronobacter malonaticus* (n=22) | *Cronobacter turicensis* (n=8) | *Cronobacter universalis* (n=4) | *Cronobacter muytjensii* (n=7) | *Cronobacter dublinensis* (n=8) | *Cronobacter condimenti* (n=1) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Acid phosphatase** | **-(-)[c]** | **+(+)** | v(-) | +(+) | - | + | + | + | - | - | - |
| **N-acetyl-ß-glucosaminidase** | **+(+)** | **-(-)** | (-) | +(+) | + | + | + | + | + | + | + |
| Motility | +(+) | +(+) | +(+) | +(+) | +(+) | v(+) | +(+) | v(-) | +(+) | +(+) | - |
| Voges-Proskauer | -(-) | -(-) | -(-) | -(-) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | + |
| $H_2S$ production | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | - |
| Indole production | -(-) | -(-) | -(-) | -(-) | -(-) | +(+) | -(-) | -(-) | +(+) | +(+) | + |
| Carbon utilization | | | | | | | | | | | |
| **Sialic acid** | **-(-)** | **+(+)** | -(-) | +(+) | +(+) | -(-) | -(-) | -(-) | -(-) | -(-) | - |
| **D-glucose, gas production** | **-(-)** | **+(+)** | ND | ND | ND | ND | ND | v(-) | ND | ND | - |
| D-glucose, acid production | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | + |
| Sucrose, acid production | -(-) | -(-) | -(-) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | +(+) | + |
| Dulcitol | +(+) | +(+) | + | - | -(-) | -(-) | +(+) | +(+) | +(+) | -(-) | - |
| Malonate | -(-) | -(-) | +(-) | -(-) | -(-) | +(+) | v(+) | +(+) | +(+) | +(+) | + |
| D-melezitose | -(-) | -(-) | - | - | -(-) | +(+) | +(+) | +(+) | -(-) | +(+) | - |
| Inositol | -(-) | -(-) | -(-) | -(-) | v(+) | +(+) | +(+) | +(+) | +(+) | +(+) | - |
| trans-Aconitate | -(-) | (-) | +(+) | +(+) | -(-) | +(+) | -(-) | -(-) | +(+) | +(+) | - |
| Maltitol | -(-) | -(-) | -(-) | v | +(+) | +(+) | +(+) | +(+) | -(-) | +(+) | - |
| D-Arabitol | -(-) | -(-) | -(-) | +(+) | -(-) | -(-) | -(-) | -(-) | -(-) | -(-) | - |
| Lactulose | -(-) | -(-) | -(-) | v | +(+) | v(+) | +(+) | +(+) | +(+) | +(+) | - |
| Putrescine | -(-) | -(-) | +(+) | v | +(+) | +(+) | +(+) | -(-) | +(+) | +(+) | - |

[a]Data obtained in this study. [b]Data in columns 2-11 obtained from Iversen *et al. [77, 78]*, Stephan *et al. [144-146],* Joseph *et al. [93]*, and Brady *et al. [15]* and the current study. [c]+, Positive; v, variable (25-75% positive); -, negative; ND, not determined.

### 4.3.1. Description of the novel species *Siccibacter colletis* (as given by Jackson *et al. [81]*)

Cells are straight, Gram-negative, non-spore-forming, motile rods approximately 2 µm x 1 µm in size. *S. colletis* is facultatively anaerobic and produces opaque, circular cream-coloured (strain 1383) or yellow (strain 2249) colonies with a diameter of 2-3 mm on tryptic soy agar incubated at 37° C for 24h. At 25° C, colonies are pale yellow to yellow and glossy. *S. colletis* produces pink/purple colonies with large halo on MacConkey agar. The species grows at 42° C (optimum 37° C), but not at 5° C in tryptic soy broth.

*S. colletis* produces catalase, α-glucosidase, and β-galactosidase. The organisms give a weakly positive reaction for oxidase. *S. colletis* does not produce gas from D-glucose, which is one trait that allows for differentiation from its closest relative *S. turicensis*. *S. colletis* does not produce hydrogen sulphide, indole from tryptophan, or acetoin (Voges-Proskauer negative). The species does not hydrolyse gelatine or urea and is negative for lysine decarboxylase and ornithine decarboxylase activities. *S. colletis* is positive for the methyl red test and can reduce nitrate. *S. colletis* shows resistance to doxycycline.

*S. colletis* can utilize the carbohydrates dulcitol, aesculin, D-melibiose and L-rhamnose, but not malonate, D-melezitose, D-turanose, inositol, lactulose, trans-aconitate, putrescine, 4-aminobutyrate, maltitol or sialic acid. The species produces acid from the carbohydrates glucose, dulcitol, L-arabinose, cellobiose, lactose, L-rhamnose, D-mannitol, N-acetylglucosamine, sialicin, 2-ketogluconate, but does not produce acid from inositol, melezitose, sucrose, D-fucose, L-fucose adonitol, turanose, D-sorbitol, and 5-ketogluconate. *S. colletis* is negative for acid phosphatase, but positive for N-acetyl-ß-glucosaminidase. The API20E and ID32E profiles of the type strain ($1383^T$) are 3204153 and 04077563310, respectively.

Strain 1383 was submitted to three culture collections as the type strain of *S. colletis* ($1383^T$ = $NCTC14934^T$ = $CECT8567^T$ = $LMG28204^T$). This strain was isolated from poppy seeds. The whole genome sequence of *Siccibacter colletis* strain $1383^T$ is deposited in the *Cronobacter* PubMLST database and GenBank (accession number JMSQ00000000) *[42, 113]*.

## 4.4. Characterization of outbreak strains using MLST

One of the main applications of MLST is outbreak tracing. As described in Section 1.3.2, three pulsotypes (PTs) were identified among patient isolates during a 1994 outbreak in France *[19]*. Importantly, strains belonging to multiple PTs were isolated from the same patient, indicating co-infection with multiple strains of the same species. Further phenotypic analysis showed variation both between and within these identified PTs. For example, the PT3 strain showed lower attachment to and invasion of endothelial cells than strains from PT1 and PT2 *[156]*. Variation in attachment, invasion and antibiotic resistance profiles were also observed within PT2, with two of the 16 strains in PT2 showing extended spectrum β-lactamase activity *[19, 156]*. These variations suggest that further genotypic differences also exist within the identified PTs. Later analysis with MLST was carried out to genetically characterize these isolates and revealed three STs among patient isolates: ST4, ST12, and ST13 *[91]*. Not only did this characterization more accurately identify the species responsible for this outbreak, but this characterization also helped to show the importance of ST4, which has since been linked to cases of neonatal meningitis *[55, 91]*. As MLST has been successfully applied to outbreaks in the past, strains from three additional outbreaks were analysed with this method. These results and the implications of the characterization of the outbreak isolates are described in detail below.

### 4.4.1 Belgium, 1998

At a Belgian hospital, 12 infants developed NEC, including two fatal cases *[160]*. The original investigation utilized arbitrarily primed PCR (AP-PCR) for characterization of the isolates. Infant formula was suspected as a source; however, only a single AP-PCR profile was identified in the prepared formula. While this profile did match some of the patient isolates, strains with other AP-PCR profiles were isolated from patient samples as well *[160]*. Additionally, as with the previously described outbreak in France, multiple strains with different profiles were isolated from individual patients, suggesting that co-infection with multiple strains of *C. sakazakii* had occurred *[19, 160]*. Therefore, it was not possible to determine which strain(s) were responsible for the illnesses or to determine the source of the infections.

Though 23 isolates were originally collected during this outbreak (nine patient isolates and 14 PIF isolates), only two (strains 2106 and 2107) were available for DNA sequence-based analysis. The remaining isolates from the outbreak have been lost. Strains 2106 and 2107 were both isolated from the blood of the same infant, who died during the outbreak *[160]*. Both strains were identified as *C. sakazakii,* but belong to different STs (Table 4.2). Strain 2106 was identified as ST257, a SLV of ST21, making strain 2106 a member of CC21. This CC includes four STs (ST21, ST257, ST382, and ST156). Strain 2106 is the only clinical strain within CC21 and the only ST257 strain in the *Cronobacter* PubMLST database *[42]*. Strain 2107 was identified as ST12. As mentioned previously, ST12 strains have been linked to cases of neonatal NEC, but not to cases of meningitis *[42, 43].* All 12 patients in this outbreak exhibited symptoms of NEC, but no cases of meningitis were reported. Thus, this outbreak supports the association of ST12 strains with NEC. It is possible that ST12 strains are unable to cause meningitis, though further work is necessary to confirm this hypothesis.

MLST of the isolates from the Belgian outbreak confirms the results of the AP-PCR profiling, which indicated that a single infant had been infected by multiple strains of *C. sakazakii*. This suggests that two or more outbreaks had occurred simultaneously. The effects of co-infection with multiple strains of *C. sakazakii* have not yet been determined, but it is possible that this is an important trait related to the virulence of the organism. As mentioned previously, co-infection with multiple strains of the same *Cronobacter* species has been reported in the past. Caubilla-Barron *et al. [19]* reported that three of the 18 patients from the 1994 French outbreak were infected with more than one strain of *C. sakazakii*. Similarly, MLST and AP-PCR showed that at least one patient in the Belgian outbreak had been infected with multiple strains of *C. sakazakii*; however, the full story of this outbreak cannot be determined because the remaining strains are not available for analysis.

### 4.4.2. France, 2004

Nine cases of *C. sakazakii* infection were observed in multiple hospitals in France in 2004 *[27]*. Two cases of meningitis, one case of NEC, one case of conjunctivitis, and five asymptomatic colonisations were reported *[27]*. Both meningitis cases were fatal. Isolates from patients and PIF displayed identical pulsed field gel electrophoresis (PFGE) profiles, suggesting that PIF was the source of the infections *[27]*. All patients consumed PIF from the same producer, despite their different geographical locations, supporting the claim that PIF was the source *[27]*.

Four strains were obtained from this outbreak (2089, 2090, 2091, and 2092). Strains were isolated from cerebrospinal fluid (n = 2), faeces (n = 1), and PIF (n = 1). All four strains were identified as ST1, as shown in Table 4.2. As of August 2016, 114 strains belonging to ST1 were present in the *Cronobacter* PubMLST database, excluding strains from this outbreak *[42]*. These other ST1 strains were isolated from food, PIF, environmental, and clinical samples *[42, 44]*. Clinical isolates belonging to ST1 have been collected from the Netherlands, the Czech Republic, the United States, the United Kingdom, Canada, and France *[42, 44, 92]*. The clinical strains in ST1 have been linked to illnesses, including at least one additional case of meningitis *[59]*. The presence of the same ST in both patients and PIF in the 2004 outbreak in France again supports the claim of PIF as the source of these infections, as proposed in the original outbreak investigation *[27]*.

### 4.4.3. Mexico, 2010

In 2010, two infants in a hospital in Mexico who were fed rehydrated powdered infant formula (R-PIF) developed bloody diarrhoea *[38]*. Both infants had been admitted to the hospital for other reasons. The first case was a 4-month-old infant exhibiting psychomotor difficulties *[38]*. The second case was a 4-month-old infant admitted the hospital with pneumonia *[38]*. Both infants recovered after treatment with antibiotics. Based on the analysis of isolates from PIF, R-PIF and patient faecal samples, Flores *et al. [38]* concluded that the illnesses were caused by *Cronobacter sakazakii*, despite the fact that bloody diarrhoea is not a typical symptom of *C. sakazakii* infection *[38]*. PIF was suspected as the source and it was found that the water used to prepare the formula was not hot enough to destroy microorganisms that might have been present *[38]*.

The suspect isolates were identified using cultural isolation, phenotyping, and PCR probe-based identification methods during the original outbreak investigation *[38, 104, 105, 147]*. Yet, difficulties with these identification methods have been reported by other authors and in the previous chapter *[22, 79, 82, 157]*. The current study further characterized the strains isolated during this

outbreak by colony morphology and MLST. In this discussion, all strains will be referred to using the strain number assigned in the original outbreak investigation to facilitate comparison of the results.

Strains from the original outbreak investigation were provided by J. Flores. The 21 strains used in the current study (except strains 8731, 8733, and 8756) were included in the original outbreak investigation *[38]*. The excluded strains were isolated from PIF and the hospital environment and were not included in the original outbreak investigation for unknown reasons. These strains were included in the current study as they were isolated at approximately the same time as strains included in the original outbreak investigation *[38]*. Strains were assigned to three PTs based on the PFGE profiles in the original publication (Table 4.5) *[37, 38]*. Strains were cultured on TSA and DFI and incubated at 37° C for 24 hours. Plates were examined for typical colony morphologies before DNA sequence-based analyses were performed.

As shown in Table 4.5, re-evaluation of the outbreak strains revealed that only PT1 strains produced typical colonies on TSA or DFI. This suggests that while the PT1 isolates may belong to the *Cronobacter* genus, the isolates from PT2 and PT3 likely do not. This is particularly important as no patient isolates belonged to PT1. Based on colony morphologies, it did not appear that the illnesses were caused by *C. sakazakii,* as originally reported. Thus, further analysis was performed to characterize strains from this outbreak. The MLST profiles were determined for all PT1 strains as described in Section 2.4.3 and strain 8756 was selected for WGS as the representative strain of PT1. Strains 8701 and 8706 were selected as representative strains for PT3 and PT2, respectively. These two strains were also subjected to WGS and the MLST alleles from these strains were extracted from the genome sequences. The genomes from these three strains were sequenced at Swansea University and genome sequences were assembled by P. Ogrodzki. The genome for strain 8701 was uploaded to GenBank with the accession number LLXN00000000. The other genome sequences and MLST profiles are available via the *Cronobacter* PubMLST database *[42]*.

MLST profiles for PT1 strains and representative strains of PT2 and PT3 are shown in Table 4.5. PT1 strains were identified as *C. sakazakii* ST297. This ST is unrelated to other *C. sakazakii* STs and no other ST297 strains can be found in the *Cronobacter* PubMLST database *[42]*. No clinical strains were included in PT1 and strains from this PT were isolated approximately six months before the outbreak. No illnesses were reported at the time, but the presence of *C. sakazakii* in the hospital is a cause for concern. The original outbreak investigation found that the PIF was reconstituted with water heated to only 40° C *[38]*. This not sufficient to destroy microorganisms that may be present. Thus, infants in the hospital at the time could have been exposed to *C. sakazakii*.

**Table 4.5. Summary of the re-evaluation of a suspected *C. sakazakii* outbreak in Mexico**

| Strain number | Source | Date of isolation | Colony morphology | | MLST | | | | | | | | Identity |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | TSA | DFI | *atpD* | *fusA* | *glnS* | *gltB* | *gyrB* | *infB* | *ppsA* | ST | |
| Pulsotype 1[a] | | | | | | | | | | | | | |
| 8710 | PIF unopened | 21-Jul-09 | Yellow, glossy | Blue-green | 3 | 1 | 48 | 84 | 130 | 36 | 178 | 297 | *C. sakazakii* |
| 8731[b] | PIF unopened | 21-Jul-09 | Yellow, glossy | Blue-green | 3 | 1 | 48 | 84 | 130 | 36 | 178 | 297 | *C. sakazakii* |
| 8718 | PIF unopened | 28-Jul-09 | Yellow, glossy | Blue-green | 3 | 1 | 48 | 84 | 130 | 36 | 178 | 297 | *C. sakazakii* |
| 8733[b] | PIF unopened | 28-Jul-09 | Yellow, glossy | Blue-green | 3 | 1 | 48 | 84 | 130 | 36 | 178 | 297 | *C. sakazakii* |
| **8756[b,c]** | **Bottle washing area** | **1-Aug-09** | **Yellow, glossy** | **Blue-green** | **3** | **1** | **48** | **84** | **130** | **36** | **178** | **297** | ***C. sakazakii*** |
| Pulsotype 2 | | | | | | | | | | | | | |
| 8740[b] | Washing zone | 10-Aug-09 | Cream, glossy | Cream | nd[d] | nd | nd | nd | nd | nd | nd | nd | nd |
| 8715 | Rehydrated PIF | 19-Aug-09 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8705 | PIF unopened | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8720 | PIF unopened | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8708 | Rehydrated PIF | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8716 | Rehydrated PIF | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8724 | Rehydrated PIF | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8721 | Rehydrated PIF | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8707[b] | Rehydrated PIF | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **8706** | **Faeces (infant 1)** | **24-Jan-10** | **Cream, glossy** | **Cream** | **100** | **81** | **132** | **151** | **133** | **139** | **177** | **340** | ***Enterobacter* spp.** |
| 8709 | Faeces (infant 2) | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| Pulsotype 3 | | | | | | | | | | | | | |
| 8700 | Faeces (infant 1) | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8719 | Faeces (infant 1) | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| **8701** | **Faeces (infant 2)** | **24-Jan-10** | **Cream, glossy** | **Cream** | **99** | **75** | **118** | **150** | **132** | **138** | **176** | **339** | ***E. hormaechei*** |
| 8702 | Faeces (infant 2) | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| 8703 | Faeces (infant 2) | 24-Jan-10 | Cream, glossy | Cream | nd | nd | nd | nd | nd | nd | nd | nd | nd |

[a]PT, pulsotype. In the original outbreak investigation, strains were assigned to groups A-D, based on date of isolation. In the current study, strains were assigned to PTs based on the original PFGE results *[37, 38]*.
[b]PT determined from unpublished data *[37]*. [c]Representative isolates shown in bold were subjected to whole genome sequencing.

Based on the *fusA* allele sequences, strain 8701 (PT3) was identified as *E. hormaechei*, while strain 8706 (PT2) was identified as a member of the *Enterobacter* genus. It was not possible to identify strain 8706 to the species level based only on the *fusA* allele. Similarly, the 7-loci MLST profiles identified strain 8701 (PT3) as *E. hormaechei* and strain 8706 (PT2) as an unknown member of the *Enterobacter* genus. To further characterize these strains, phylogenetic analysis of the MLST sequences was carried out. Figure 4.5 shows the PT1 strains clustered nearest to the type strain of *C. sakazakii*, with strong bootstrap support. This supports the identification of PT1 strains as *C. sakazakii.* As expected based on the *fusA* alleles and MLST profiles, strain 8701 (PT3) and strain 8706 (PT2) clustered with members of the *Enterobacter* genus. Unsurprisingly, strain 8701 (PT3) clustered near *E. hormaechei,* but strain 8706 (PT2) clustered closest to *E. ludwigii* with low bootstrap support. Thus, while this analysis supports the identification of strain 8701 as *E. hormaechei,* it was not possible to identify strain 8706 to the species level.

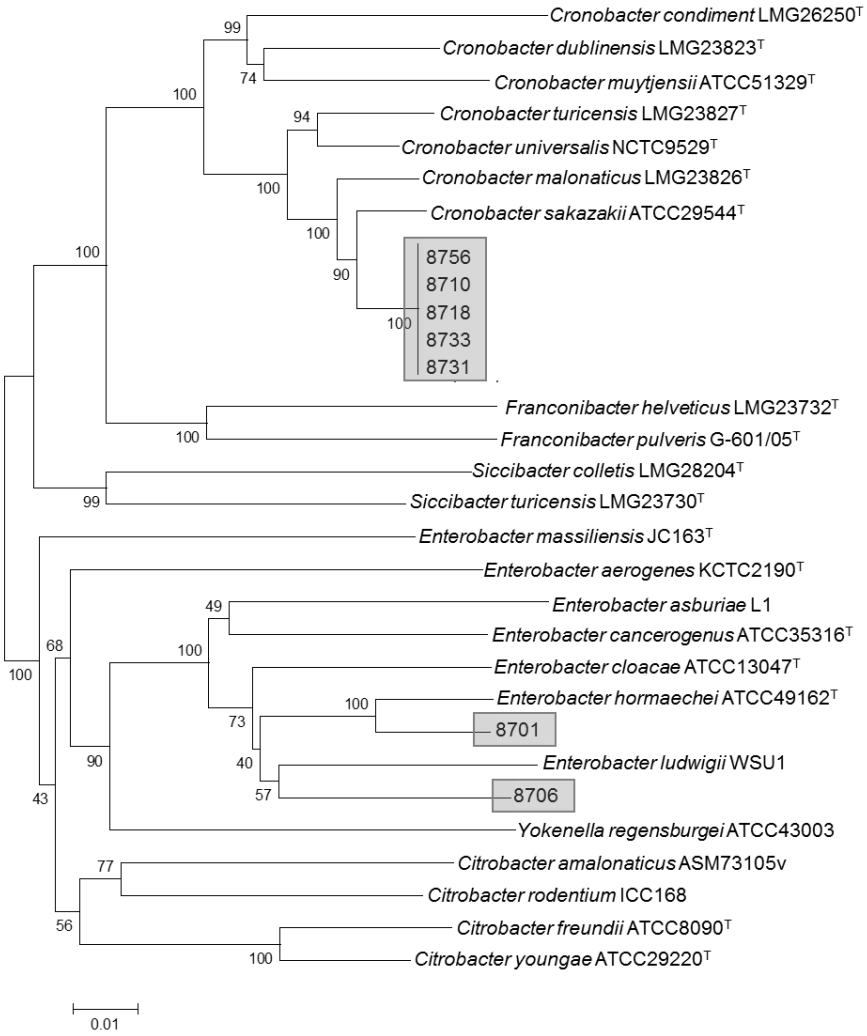


**Figure 4.5. Neighbour-joining phylogenetic tree based on the 7-loci MLST scheme** Concatenated length: 3,036 bp. Tree shows the positions of strains 8701, 8706, 8710, 8718, 8731, 8733, and 8756 relative to the *Cronobacter, Citrobacter, Enterobacter, Franconibacter,* and *Siccibacter* genera. Bootstrap values based on 1000 replications are shown at branch nodes. Scale bar = 1 substitution per 100 nucleotides.

Similarly, phylogenetic analysis of the sequences from the 53-loci rMLST was also performed (Figure 4.6). As with the 7-loci MLST, strain 8756 (PT1) clustered close to the type strain of *C. sakazakii*, with strong bootstrap support. This again supports the identification of this strain as *C. sakazakii.* Likewise, strain 8701 (PT3) again clustered nearest to *E. hormaechei*, supporting the identification of this strain as determined by the *fusA* allele sequence and 7-loci MLST profile. While there was stronger bootstrap support for the position of strain 8706 (PT2) in this phylogenetic tree, it clustered nearest to *E. hormaechei*, on a separate branch from the type strain of the species and strain 8701 (PT3). Consequently, it was still not possible to identify this strain to the species level.

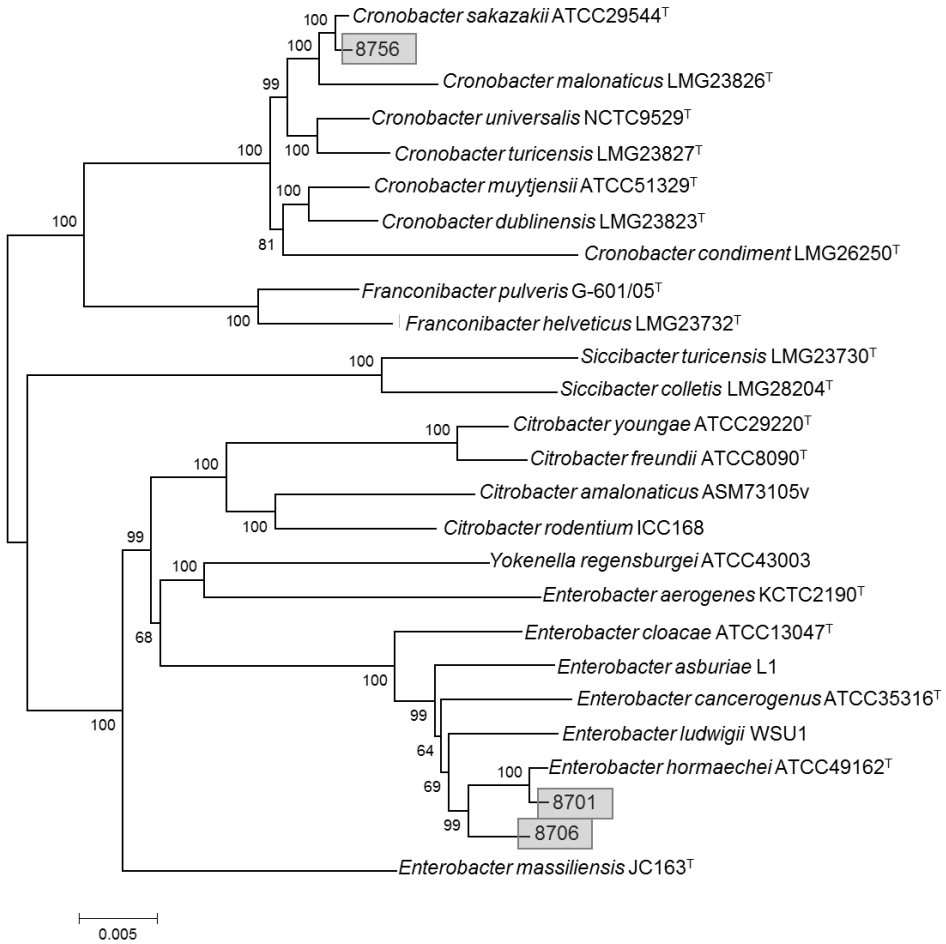


**Figure 4.6. Neighbour-joining phylogenetic tree based on 53 concatenated rMLST gene sequences.** Concatenated length: 22,511 bp. Tree shows the positions of strains 8701, 8706, and 8756 relative to the *Cronobacter, Citrobacter, Enterobacter, Franconibacter,* and *Siccibacter* genera. Bootstrap values based on 1000 replications are shown at branch nodes. Scale bar = 5 substitution per 1,000 nucleotides.

In an attempt to positively identify the species of strains 8701, 8706 and 8756, 2-way average nucleotide identity (ANI) analysis was performed. These results are shown in Table 4.6. As expected strain 8756 (PT1) showed a high ANI value (98.2%) when compared to the type strain of *C. sakazakii*. The commonly accepted threshold for species demarcation is 95% and this result, therefore confirms the identity of strain 8756 (PT1) as *C. sakazakii.* Likewise, strain 8701 (PT3) was identified as *E. hormaechei,* based on an ANI value of 95.6% when compared to the type strain of the species. When combined with the strong support from the phylogenetic and ANI analyses, this result led to the identification of strain 8701 (PT3) as *E. hormaechei.* Importantly, PT3 strains, including strain 8701, were reported to be resistant to several antibiotics *[38]*. This is not uncommon and high antimicrobial resistance was reported for strains of *E. hormaechei* that had been previously misidentified as *C. sakazakii [157].* Strain 8701 and other PT3 strains were isolated only from patient faecal samples. Thus, the source of these strains could not be determined.

**Table 4.6. Average nucleotide identity analysis of strains from the outbreak in Mexico**

| Species | Strain | 8701 | 8706 | 8756 |
|---|---|---|---|---|
| | 8701 | | | |
| | 8706 | 88.9% | | |
| | 8756 | 83.0% | 82.9% | |
| *C. sakazakii* | ATCC29544$^T$ | 83.7% | 84.0% | ***98.2%*** |
| *E. hormaechei* | ATCC49162$^T$ | ***95.6%*** | 88.9% | 83.1% |
| *E. asburiae* | L1 | 89.2% | ***91.3%*** | 83.2% |
| *E. cancerogenus* | ATCC35316 | 88.1% | 87.8% | 83.0% |
| *E. cloacae* | ATCC13047$^T$ | 88.5% | 89.2% | 82.8% |
| *E. ludwiggi* | ECWSU1 | 87.9% | 88.8% | 82.8% |
| *E. aerogenes* | KCTC2190$^T$ | 83.6% | 83.7% | 83.1% |

Even after ANI analysis it was not possible to identify the species of strain 8706 (PT2). The highest ANI value produced by this strain was 91.3% with *E. asburiae* strain L1. This value is not high enough for a positive species identification of this strain, though it does appear to be a member of the *Enterobacter* genus. Additionally, the species could not be determined by 16S rDNA sequencing due to the heterogeneity within the *'Enterobacter cloacae* complex' *[60]*. It is possible that this strain represents a new species within the *Enterobacter* genus, but it is also possible that there are simply not enough genomes available for comparison to allow for identification of this species. Strains from PT2, including 8706, were isolated from patient samples, PIF, and R-PIF, suggesting that PIF could have been the source for these organisms. It should be noted that this is possibly the first demonstrated transmission of an *Enterobacter* species from PIF to infants.

The transmission of an *Enterobacter* species from PIF to infants is significant because members of the *Enterobacter* genus are currently permitted in PIF. In this outbreak, it was found that

the hospital was reconstituting the PIF with water heated to only 40° C *[38]*. Preparing infant formula with water below 70° C can allow for the survival and growth of opportunistic pathogens, like *Enterobacter* spp., that may be present *[40, 41]*. Thus, this outbreak brings the microbiological standards applied to PIF into question. *Cronobacter* spp. and *Salmonella* spp. are the only organisms in Category A ("clear evidence of causality") with regard to illnesses linked to PIF *[40]*. *Enterobacter* spp. fall in Category B ("causality plausible, but not yet demonstrated") *[40]*. It is possible that the microbiological criteria applied to PIF may need to be revised to reflect the possible hazards posed by some Category B organisms, as demonstrated by the transmission of an *Enterobacter* spp. from PIF to infants in this outbreak.

To further characterize the isolates from the outbreak in Mexico, the PubMLST *Cronobacter* database was used to examine the genomes of strains 8701, 8706, and 8756 for potential virulence associated genes. Previously reported virulence genes encoding iron uptake systems (*entABCDEFS, fepA1, fepA2, fepCDEG, fhuABCD*), fimbriae (*fimA1, fimA2, fimD1, fimD3, fimD4, fimG*), and hemolysins (*hemABDEFGHKLNX*) were included in this analysis *[94]*. The $\alpha$-glucosidase encoding genes *palQ* and *palZ* were also examined as they are involved in the production of typical blue-green colonies on DFI *[107]*. The accession number for *palQ* and *palZ* is ASM075208, while the accession numbers for *fhuABCDE* are ESA_03187-90 and ESA_02242. The remaining genes were accessed via the Genome Comparator tool of the BIGSdb *[42, 111]*. Genes were considered present if $\geq$90% of the target gene sequence was detected.

Examination of the genome sequences of *E. hormaechei* strain 8701 and *Enterobacter* spp. strain 8706 revealed that they lack the genes *palQ* and *palZ* (GenBank accession number: AM075208), which encode an $\alpha$-glucosidase. This enzyme is responsible for producing the blue-green colonies on DFI, which is the typical colony morphology of *Cronobacter* spp. on this agar *[74]*. Thus, it is unsurprising that strains 8701 and 8706 did not produce blue-green colonies on this agar. Meanwhile, *C. sakazakii* strain 8756 matched the full sequences for both genes, as expected based on the observation of blue-green colonies for this strain on DFI.

The genes encoding fimbriae were detected in all three strains; however, *fimD1* was only partially present in *E. hormaechei* strain 8701. The majority of the haemolysin genes were also found to be present in all three strains. The genes *hemF, hemG, hemH,* and *hemK*, however, were found to be absent in strain 8706. Similarly, the *fhu* gene cluster was detected in all three strains, but a partial sequence was detected for ESA_03190 in *Enterobacter* spp. strain 8706. The absence of some of the virulence-associated genes in *Enterobacter* spp. strain 8706 suggests that it may be

less capable of causing illness than *E. hormaechei* strain 8701. It is suspected that *E. hormaechei* was the cause of the infant illnesses reported in Mexico, but it was not possible to confirm this hypothesis based on the results reported here. *E. hormaechei* has been implicated in previous outbreaks and has been misidentified as a member of the *Cronobacter* genus (then known as '*E. sakazakii*), leading to the suspicion that this species was responsible for the illnesses reported in Mexico *[79, 122, 157, 161]*.

The re-evaluation and characterization of strains from these three outbreaks highlights the importance of accurate detection and identification assays. As discussed in Chapter 3, the phenotypic and molecular identification assays utilized in the original outbreak investigation can result in misidentifications of suspect isolates. While the true cause of the illnesses cannot be determined from these results, DNA sequence-based identification did show that the reported illnesses were not caused by *C. sakazakii*, as originally reported.

The original outbreak investigation suggested that improved sanitation practices should be employed at the hospital and it is suspected that cross-contamination may have contributed to the reported illnesses *[38]*. Strains of *C. sakazakii* ST297 were recovered not only from PIF, but also from the bottle washing sink in the hospital, approximately six months before the outbreak. Though no illnesses were reported at the time, the presence of *C. sakazakii* in the PIF and hospital, combined with the use of insufficiently heated water, suggests that there was potential for an outbreak to occur. Cross-contamination has been reported in other neonatal outbreaks. During an *E. hormaechei* outbreak in Pennsylvania, for example, heath care workers were not changing their gloves as frequently as required and handwashing facilities were not always properly stocked *[161]*. These conditions may have contributed to the spread of *E. hormaechei* between infants in the unit.

In conclusion, re-analysis of the strains from a suspected *C. sakazakii* outbreak in Mexico revealed that the reported illnesses were in fact caused by one or more species of *Enterobacter*, which were found to contain multiple virulence genes. Though the species responsible for the illnesses could not be determined, this outbreak did demonstrate possible transmission of *Enterobacter* spp. from PIF to infants. Characterization of isolates from the three outbreaks reported in this section also shows how MLST can be used in outbreak tracing. Finally, genomic characterization of outbreak strains can lead to a better understanding of the outbreaks, virulence mechanisms, and transmission of *C. sakazakii* and related species.

## 4.5. MLST to resolve discrepancies in species identification

In 2010, Miled-Bennour *et al. [116]* published the genotypic and phenotypic characterization of a collection of *Cronobacter* isolates. This characterization included identification by phenotyping, PFGE, ribotyping, and 16S rDNA sequencing *[116]*. As shown in Table 4.7, seven strains produced divergent results with at least one of the methods used by Miled-Bennour *et al. [116]*. The species identifications from the ribotyping and 16S rDNA sequencing methods are based on the clustering of strains in phylogenetic trees *[116]*. The ribotyping method only indicated whether or not a strain had been identified as *C. sakazakii [116]*.

**Table 4.7. Identification of *Cronobacter* strains based on phenotyping, ribotyping, 16S rDNA sequencing and MLST.** Shaded boxes indicate species identifications that do not match the species identification as determined by MLST.

| | Species identification[a] | | | MLST (this study) | |
|---|---|---|---|---|---|
| Strain | Phenotyping | Ribotyping | 16S rDNA sequencing | ST | Species identification |
| 05CHPL02 | *C. sakazakii* | Not *C. sakazakii* | *C. dublinensis* | 301 | *C. dublinensis* |
| 05CHPL40 | *C. malonaticus* | Not *C. sakazakii* | *C. sakazakii* | 392 | *C. malonaticus* |
| 05CHPL46 | *C. malonaticus* | Not *C. sakazakii* | *C. sakazakii* | 302 | *C. malonaticus* |
| 05CHPL53 | *C. malonaticus* | *C. sakazakii* | *C. sakazakii* | 64 | *C. sakazakii* |
| 05CHPL65 | *C. sakazakii* | Not *C. sakazakii* | *C. dublinensis* | 256 | *C. sakazakii* |
| 07HMPA87A | *C. malonaticus* | Not *C. sakazakii* | *C. sakazakii* | 300 | *C. malonaticus* |
| 08HMPA03 | *C. dublinensis* | Not *C. sakazakii* | *C. muytjensii* | 106 | *C. dublinensis* |

[a]Species identifications by phenotyping, ribotyping, and 16S rDNA sequencing were taken from Miled-Bennour *et al. [116]*.

Strains were obtained from N. Gnanou-Besse at AFSSA in France and were stored and resuscitated as described in Section 2.2. MLST was applied to these strains in order to determine the correct species identification and the procedure was carried out as described by Baldwin *et al. [6]* and in Section 2.4.3. As strain 08HMPA03 is the type strain of *C. dublinensis*, its MLST profile had already been determined and was obtained from the PubMLST *Cronobacter* database *[42]*. The STs and species identifications are shown in Table 4.7, along with the previously reported results from the other identification methods. For the purposes of this discussion the AFSSA strain numbers are used to facilitate comparisons to the original publication.

The results from the phenotyping methods did not match the identifications based on the MLST profile for strains 05CHPL02 and 05CHPL53. Based on the results reported in the previous chapter, phenotyping was expected to perform worse than the other methods; however, in the original study, Miled-Bennour *et al. [116]* selected only four biochemical tests for species identification. These tests had been identified as important for differentiation of *Cronobacter* species, based on the original species descriptions of Iversen *et al. [77, 78, 116]*. By selecting biochemical tests in this manner, the authors were able to focus on tests that are known to differentiate the species, increasing the accuracy of the phenotyping method.

Strain 05CHPL65 was misidentified as a non-*C. sakazakii* strain using the ribotyping method. Miled-Bennour *et al. [116]* had used automated ribotyping during their analysis. Though this method does reduce the labour required, automated ribotyping methods use a smaller gel than traditional ribotyping methods, making them less discriminatory *[13]*. This factor may have contributed to the misidentification of this strain in the original study.

Of the strains showing discrepant results in the original study, only 05CHPL02 and 05CHPL53 were identified as the same species using both 16S rDNA sequencing and MLST. Three strains identified as *C. sakazakii* by 16S rDNA sequencing were identified as *C. malonaticus* by MLST. 16S rDNA sequencing has been shown to be insufficient for discrimination of *C. sakazakii* and *C. malonaticus* due to the presence of multiple copies of the gene, which contain microheterogeneties *[6]*. This limits the effectiveness of 16S rDNA sequencing for identification of *Cronobacter* species.

MLST, on the other hand, has been shown to be more reliable than other methods for species identification *[44, 129]*. By applying MLST to strains that showed discrepant results using other identification methods, the results can be clarified to reach a more reliable species identification. This leads to more accurate identification of individual strains, more reliable characterization of each species, and a better understanding of the genus as a whole.

### 4.6. Lack of correlation between sequence type and growth rate

In addition to resolving the discrepancies in species identification described above, strains from the AFSSA collection were selected for analysis based on their growth rates or source. Clinical isolates are overrepresented in the PubMLST *Cronobacter* database and this work increased the number of environmental strains in the database *[42]*. Additionally, strains 05CHPL02, 05CHPL65, 05CHPL101bis, 05CHPL47, 05CHPL54, 05CHPL99, 05CHPL10, 05CHPL97, 05CHPL29 were selected for analysis due to their growth rates, as reported by Miled-Bennour *et al. [116]*. Strains 05CHPL02, 05CHPL65, 05CHPL101bis have some of the slowest growth rates, while 05CHPL47, 05CHPL54, and 05CHPL99 are the fastest *[116]*. The remaining three strains were selected as they have growth rates near the mean value observed by Miled-Bennour *et al. [116]*. In total, 32 strains from the AFSSA collection were analysed by MLST (Table 4.2).

An examination of the AFSSA collection strains attempted to link the observed growth rates with the ST. Only *C. sakazakii* strains (n = 24) were included in this analysis to minimize variation due to the differences in species. Growth rates were determined by Miled-Bennour *et al. [116]* and

STs were determined as described previously. The STs and growth rates for all strains used in this analysis are shown in Table 4.8.

Though strains were selected to represent a range of growth rates, some STs and CCs were more frequently observed among the strains utilized for this investigation. Therefore, statistical analysis focused on the most common CCs observed; CC1 (n = 9) and CC4 (n = 6), both of which have been linked to cases of human illness. The Wilcoxon rank sum test was used to determine if the growth rates of the CC1 or CC4 strains differed significantly from the growth rates of the remaining strains *[164]*. Calculations were carried out using the Statistics Computational Online Resource (http://socr.ucla.edu/SOCR.html) *[30]*.

**Table 4.8. Growth rates and sequence types of strains from the AFSSA collection**

| | | Growth rate ($hr^{-1}$)[a] | | | |
|---|---|---|---|---|---|
| Strain | Source | 25° C | 37° C | ST | CC |
| 08HMPA08 | Clinical | 0.99 | 2.16 | 1 | 1 |
| 08HMPA09 | Clinical | 1.02 | 2.30 | 1 | 1 |
| 08HMPA10 | Clinical | 0.91 | 2.07 | 1 | 1 |
| 05CHPL10 | PIF | 1.00 | 1.95 | 1 | 1 |
| 05CHPL18 | PIF | 0.95 | 2.07 | 1 | 1 |
| 08HMPA11 | PIF | 0.93 | 2.17 | 1 | 1 |
| 05CHPL27 | Environmental | 1.02 | 1.90 | 1 | 1 |
| 05CHPL78 | Environmental | 0.92 | 2.20 | 1 | 1 |
| 07HMPA87F | Environmental | 0.99 | 2.00 | 391 | 1 |
| 05CHPL59 | Collection strain | 0.84 | 1.99 | 4 | 4 |
| 05CHPL101bis | Environmental | 0.63 | 1.46 | 4 | 4 |
| 05CHPL82 | Environmental | 0.95 | 2.23 | 4 | 4 |
| 05CHPL99 | Environmental | 0.97 | 2.60 | 4 | 4 |
| 05CHPL29 | Environmental | 0.99 | 2.06 | 255 | 4 |
| 05CHPL97 | Environmental | 0.99 | 2.03 | 295 | 4 |
| 05CHPL50 | Environmental | 0.91 | 1.88 | 8 | 8 |
| 07HMPA87B | Environmental | 0.97 | 1.89 | 21 | 21 |
| 07HMPA41A | PIF | 1.03 | 1.83 | 31 | 31 |
| 05CHPL53 | Environmental | 1.01 | 2.09 | 64 | 64 |
| 05CHPL56 | Environmental | 1.03 | 2.29 | 64 | 64 |
| 07HMPA93A | Environmental | 0.99 | 2.13 | 100 | 100 |
| 08HMPA06 | Environmental | 1.01 | 2.02 | 100 | 100 |
| 05CHPL54 | Environmental | 1.17 | 2.43 | 125 | 100 |
| 05CHPL65 | Collection strain | 0.76 | 1.63 | 256 | -- |

[a]Growth rates determined by Miled-Bennour *et al.* (2010)

No significant differences ($p > 0.05$) were identified between the growth rates of the CC1 strains and those of the remaining STs at either 25° C or 37° C. Similarly, no significant differences ($p > 0.05$) were found when comparing the growth rates of the CC4 strains to the growth rates of the other STs (including CC1) at either temperature. It is interesting to note that the slowest and fastest growing strains at both temperatures belonged to CC4, highlighting the natural variation that exists among even closely related *C. sakazakii* strains.

Though based on a small subset of strains, this analysis suggests that ST or CC is not strongly correlated to growth rate of *C. sakazakii* strains. This is likely due to the fact that MLST is

based on housekeeping genes *[6]*. While useful for typing and differentiating strains, these genes are not responsible for specific, observable phenotypes *[95]*. Though strains belonging to the same ST or CC are closely related, variations in phenotype are expected *[95]*. These observed differences suggest that it may be possible to use DNA sequence-based analysis to further subdivide STs for more accurate differentiation of strains. As more genome sequences become available, it will be possible to better characterize strains and isolates. This could lead to the development of a variety of typing schemes for different purposes, including characterization of strains and isolates based on traits important for virulence or environmental survival *[87, 111]*. A proposed capsular typing scheme is discussed in Chapter 6.

## 4.7. Conclusions

This chapter has highlighted some of the uses and benefits of MLST with regard to *Cronobacter* species and their identification. It was demonstrated that this technique can be used for identification of novel species and for characterization of outbreak strains. Unfortunately, while MLST is useful for the differentiation of strains, it cannot necessarily be used for predicting phenotypes. The attempt to correlate ST to growth rate highlights one of the major issues with MLST: the use of housekeeping genes. The seven loci sequenced are not directly related to the phenotype of the organism. They were selected because, as housekeeping genes, they are required for the organism to remain alive. Thus, they will be present in all strains examined. This is beneficial for differentiation of strains and phylogenetic analyses, but less reliable for characterization of strains, particularly with regard to potential virulence.

While previous studies have linked ST4 strains to cases of neonatal meningitis, the genes used to determine the ST are not directly responsible for the phenotype of these strains *[55, 91, 95]*. In order to better understand the *Cronobacter* genus and how certain species cause illness while others do not, it is necessary to link genotype and phenotype together. In the future, it may be possible to predict a strain's phenotype from its genome sequence *[87, 111]*. Currently, individual genomic data can be used to guide laboratory analysis and particular genes can be linked to observable phenotypes. This approach to characterization of *Cronobacter* species and strains will be discussed in more detail in the next chapter.

# CHAPTER 5

# Linking genotype and phenotype for the characterization of *Cronobacter* species

*A portion of the data presented in this chapter was presented as a poster at ASM Microbe 2016 in Boston, Massachusetts, USA.*

## 5.1. Introduction

As described in the previous chapters, both genotyping and phenotyping methods have disadvantages for the characterization of *Cronobacter* spp. Phenotyping methods are not specific enough for identification and differentiation of species and strains, but genotyping methods, such as multilocus sequence typing (MLST) are not necessarily linked to the behaviour of the organism. This is particularly important with regard to virulence among *Cronobacter* spp. While some species and sequence types (STs) have been linked to types of human illness, identification of the species or ST is not sufficient to accurately assess the risk posed by a particular isolate.

As whole genome sequencing (WGS) becomes cheaper and easier, more and more genomes are becoming available for analysis. Additionally, the Bacterial Isolate Genome Sequence Database (BIGSdb) allows for easy analysis of a large number of genomes *[86]*. Prior analysis of the genome sequences can serve as a guide for planning experiments and analysing the resulting data. For example, a more suitable collection of strains can be selected for analysis based on examination of particular gene sequences or even entire genomes. Additionally, linking genotype and phenotype can provide further insights into the variations observed between species and strains. This chapter discusses two examples where genome sequences were used to direct laboratory experiments or data analysis in an attempt to link genotype to phenotype in *Cronobacter* spp. The results and their implications are discussed in context below.

## 5.2 Analysis of the *mutS-rpoS* genomic region

Among enteric pathogens, significant variation has been reported in the *mutS-rpoS* genomic region *[36, 97, 98]*. This segment ranges in size from 88 bp in *Yersinia enterocolitica* to over 12,000 bp in *Salmonella* subgroups *[97]*. In particular, a unique sequence of 2,700 bp was found in all *Salmonella* strains, but was missing from the *mutS-rpoS* region of some other enteric pathogens, including *Escherichia coli* O157:H7 *[98]*. The functions of the identified open reading frames (ORFs)

in this region were not reported, but a similar sequence was also identified in strains of both *Enterobacter cloacae* and *Klebsiella pneumoniae [98].*

The *mutS-rpoS* region differs not only between, but also within species. Culham and Wood *[28]* found that the *mutS-rpoS* region differed between uropathogenic and enterohaemorrhagic strains of *Esch. coli*. Additionally, Herbelin *et al. [58]* compared reference strains of *Esch. coli* to known enteropathogenic or enterohaemorrhagic strains. By amplifying the *mutS-rpoS* genomic region, segments of 7,800 to 14,800 bp were detected in the various *Esch. coli* strains *[58]*. In particular, a novel sequence of approximately 3,000 bp was found in enterohaemorrhagic strains of *Esch. coli* O157:H7, but not in the non-pathogenic strains of *Esch. coli* K12 *[58, 103]*.

It has been proposed that the variation in the *mutS-rpoS* genomic region in other enteric bacteria is primarily due to horizontal gene transfer (HGT) *[28, 36 97, 98, 103]*. This genetic reshuffling results in a selective advantage that could be related to virulence or environmental survival *[97].* For example, examination of the *mutS-rpoS* region in *Esch. coli* O157:H7 identified a segment that was highly similar to a segment in the *mutS-rpoS* region of *Shigella dysenteriae* and it was suggested that this region was acquired by HGT *[103]*. As *Esch. coli* O157:H7 emerged fairly recently, it was suspected that the genes were transferred from *S. dysenteriae* to *Esch. coli* O157:H7 *[103].* It has also been proposed that acquisition or loss of genes via HGT may contribute to the emergence of new pathogens, like *Esch. coli* O157:H7 *[97, 103]*.

As only three of the seven *Cronobacter* species (*C. sakazakii, C. malonaticus,* and *C. turicensis*) have been linked to cases of neonatal illness, it is possible the the genes in the *mutS-rpoS* region could contribute to these differences in virulence *[77, 78]*. Thus, this work was undertaken to identify the genes present in the *mutS-rpoS* region of *Cronobacter* spp. and to determine what effect, if any, they may have on the observed phenotypes and virulence characteristics of the different species and STs.

### 5.2.1. Gene arrangement

The annotated genomes for 130 strains of *Cronobacter* (71 *C. sakazakii,* 36 *C. malonaticus,* 13 C*. dublinensis,* four *C. turicensis,* three *C. muytjensii,* two *C. universalis*, and one *C. condimenti*), nine strains of *Franconibacter* (five *F. helveticus* and four *F. pulveris*), three strains of *Siccibacter* (two *S. turicensis* and one *S. colletis*), five strains of *Enterobacter* (one *E. asburiae,* one *E. cloacae,* one *E. hormaechei,* one *E. ludwiggi,* and one *Enterobacter* spp.) and three strains of *Citrobacter* (one *Cit. freundii,* one *Cit*. *koseri*, and one *Cit. rodentium*) were obtained from the *Cronobacter*

PubMLST database *[42]*. Sequences of the *mutS-rpoS* region were extracted using Artemis and aligned in MEGA6, as described in Section 2.5.2 *[18, 153]*. The gene organization figures were generated using the Illustrator for Biological Sequences (http://ibs.biocuckoo.org/online.php) *[108]*.

Figure 5.1 shows the *mutS-rpoS* region for members of the *Cronobacter, Enterobacter, Franconibacter, Citrobacter,* and *Siccibacter* genera. The functions of the genes found in this region are shown in Table 5.1. All *Cronobacter* species and strains were found to have the same genes in the *mutS-rpoS* region; therefore it is unlikely that these genes are responsible for the observed differences in virulence between the seven *Cronobacter* species. Even *C. condimenti,* which differs significantly from the other *Cronobacter* species both genotypically and phenotypically, contains the same gene arrangement in the *mutS-rpoS* region.

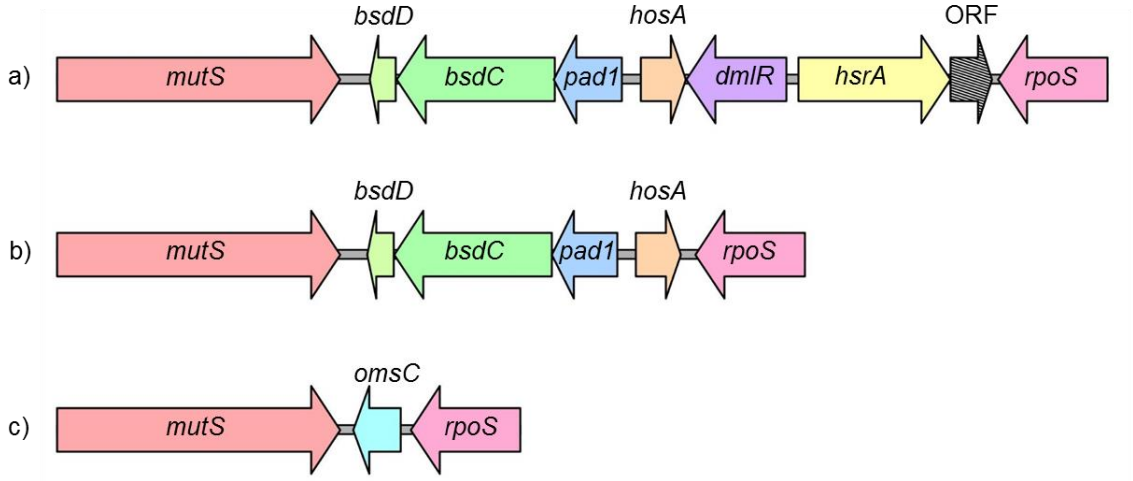


**Figure 5.1. Gene organization of the *mutS-rpoS* genomic region** in a) *Cronobacter* (n = 130) and *Enterobacter* (n = 5); b) *Franconibacter* (n = 9) and *Citrobacter* (n = 3); c) *Siccibacter* (n = 3)

**Table 5.1. Predicted functions of genes found in the *mutS-rpoS* genomic region**

| Gene | Length (bp) | Function |
|---|---|---|
| *mutS* | 2562 | DNA mismatch repair |
| *bsdD* | 237 | Phenolic acid decarboxylase D subunit |
| *bsdC* | 1431 | Phenolic acid decarboxylase C subunit |
| *pad1* | 618 | Aromatic acid decarboxylase |
| *hosA* | 411 | Transcriptional regulator |
| *dmlR* | 906 | Transcriptional regulator |
| *hsrA* | 1380 | Drug efflux pump |
| ORF | 375 | Unidentified open reading frame |
| *rpoS* | 993 | Stress response sigma factor |
| *omsC* | 429 | Osmotically induced peroxidase |

While the genes identified in the *mutS-rpoS* region do not appear to be directly related to virulence, their predicted functions are related to environmental survival. While these traits may help the organism to survive in harsh environments and allow them to reach a host, the presence of the same genes, in the same order in all seven *Cronobacter* species suggests that these genes are not responsible for the differences in observed virulence characteristics between and/or within the species.

The presence of aromatic acid and phenolic acid decarboxylases is particularly interesting for *Cronobacter* spp. These compounds are commonly produced by plants, which are suspected to be a natural reservoir for *Cronobacter* spp. *[47, 139]*. Additionally, the drug efflux pump could be related to survival in the host, but may also combat the antimicrobial activity of essential oils produced by plants *[154]*. Finally, there was one unidentified ORF found in the *mutS-rpoS* region of *Cronobacter* spp. This sequence returned no significant matches in NCBI BLAST *[4]*.

The *mutS-rpoS* region identified in members of the *Franconibacter* and *Citrobacter* genera was very similar to the region found in the *Cronobacter* and *Enterobacter* genera; however, the *Franconibacter* and *Citrobacter* strains were missing three genes: *dmlR, hsrA,* and the unidentified ORF. Because the genes are present in the same order and orientation in the four genera, it seems likely that these three genes were lost in *Franconibacter* and *Citrobacter* due to a deletion event. Examination of the non-coding region between the genes *hosA* and *rpoS* in *Franconibacter* and *Citrobacter* species did not reveal sequences matching segments of any of the three missing genes. If present, these sequences would have indicated that the genes were present at one time, but had been lost in *Franconibacter* and *Citrobacter*; however, this hypothesis could not be confirmed. Interestingly, the *mutS-rpoS* region in members of the *Siccibacter* genus contains only one gene and it does not match any of the genes observed in the other genera. Thus, based on this analysis, it is not possible to determine the history of this region in the *Siccibacter* genus.

For members of the *Cronobacter* genus (n = 130), MEGA6 was used to identify the number of variable sites in each gene in the *mutS-rpoS* genomic region. The percentage of polymorphic nucleotides was calculated as described in Section 2.5.4. It should be noted that this value represents the percentage of nucleotides which differ among 130 strains, over the entire length of the gene. Table 5.2 shows the length, the number of variable sites and the percentage of polymorphic nucleotides for each gene. Among *Cronobacter* spp., the genes in the *mutS-rpoS* region showed very few polymorphisms. Though many variable sites were identified in each gene, a low percentage of polymorphic nucleotides were identified, indicating that the polymorphisms

occurred in relatively few strains. The *bsdD* gene showed the lowest percentage of polymorphic nucleotides (2.36%) while the unidentified ORF showed the highest (5.67%). Regardless, the percentages were all below 6%, indicating that the DNA sequences of these genes are conserved among *Cronobacter* spp.

**Table 5.2. Variable sites and polymorphisms in the genes of the *Cronobacter mutS-rpoS* region**

| Gene | Length (bp) | Variable sites | Polymorphic nucleotides (%) |
|---|---|---|---|
| *mutS* | 2562 | 643 | 2.97 |
| *bsdD* | 237 | 68 | 2.36 |
| *bsdC* | 1431 | 353 | 2.81 |
| *pad*1 | 618-624[a] | 185 | 3.94 |
| *hosA* | 408-411[a] | 12 | 3.88 |
| *dmlR* | 867-906[a] | 318 | 4.89 |
| *hsrA* | 1380 | 513 | 4.55 |
| ORF | 375 | 162 | 5.67 |
| *rpoS* | 985-993[a] | 120 | 2.48 |

[a]Insertions and deletions resulted in variation in the lengths of the genes indicated. Positions with insertions or deletions were considered to be variable sites and were counted as polymorphic nucleotides

Table 5.3 shows the results of the Z-test for selection (Z-statistics and p-values) for each gene in the *Cronobacter mutS-rpoS* genomic region. This test was performed in MEGA6 and as described in Section 2.5.4 *[5, 153]*. This test is used to determine if a population is undergoing positive or purifying selection, based on the ratio of synonymous and nonsynonymous substitutions per site *[5]*. All of the p-values determined for each gene in the *mutS-rpoS* region in *Cronobacter* species are well below 0.05, indicating that the null hypothesis of neutrality should be rejected and that the *mutS-rpoS* region in *Cronobacter* spp. is undergoing purifying selection.

**Table 5.3. Results of the Z-test for selection in the *Cronobacter mutS-rpoS* region**

| Gene | Z-statistic | p-value |
|---|---|---|
| *mutS* | 21.896 | <0.00001 |
| *bsdD* | 4.329 | 0.00002 |
| *bsdC* | 16.598 | <0.00001 |
| *pad*1 | 8.903 | <0.00001 |
| *hosA* | 9.188 | <0.00001 |
| *dmlR* | 12.190 | <0.00001 |
| *hsrA* | 13.360 | <0.00001 |
| ORF | 7.590 | <0.00001 |
| *rpoS* | 8.855 | <0.00001 |

The results of the Z-test indicate that the rate of synonymous substitutions per site is higher than the rate of nonsynonymous substitutions per site in each gene in the *Cronobacter mutS-rpoS* genomic region. Thus, the protein sequences are being conserved even though slight variations exist in the DNA sequences. Herbelin *et al. [58]* found that the *mutS-rpoS* region in *Esch. coli* is also under purifying selection and suggested that this is an indication that the region was not acquired

recently. By extension, the observation that the *mutS-rpoS* region in *Cronobacter* is under purifying selection suggests that the genes in this region were not acquired in the recent evolutionary past. Regardless, the true evolutionary history of this region could not be determined based on these analyses and further work will be necessary to do so.

### 5.2.2. Recombination in the *mutS-rpoS* region of *Cronobacter* spp.

As discussed previously, it has been suggested the the *mutS-rpoS* region evolves via HGT *[28, 97, 98, 103]*. To visualize possible instances of recombination in this region, the sequences from the 130 *Cronobacter* strains were analysed using SplitsTree4, as described in Section 2.5.2 *[69]*. The resulting split network is shown in Figure 5.2.

Though a split network does not explicitly show the evolutionary history of the strains, it can be used to visualize conflicting phylogenies *[69]*. If HGT had occurred in the *Cronobacter mutS-rpoS* genomic region, differing phylogenies would result from analysis of different segments of the region. Therefore, evidence of HGT would appear as splits between the different species in the network. Based on the split network shown in Figure 5.2, there is limited evidence for conflicting phylogenies or recombination in the *mutS-rpoS* region among the different *Cronobacter* species. It is particularly interesting that some sequence types (STs) form very distinct branches. For example, the *C. sakazakii* ST4 strains form a distinct branch in the network (highlighted in Figure 5.2). This indicates that the sequences are very similar to one another and that these strains produce very few conflicting phylogenies. These results suggest that these strains are not undergoing recombination or are only recombining with other members of the same ST or CC.

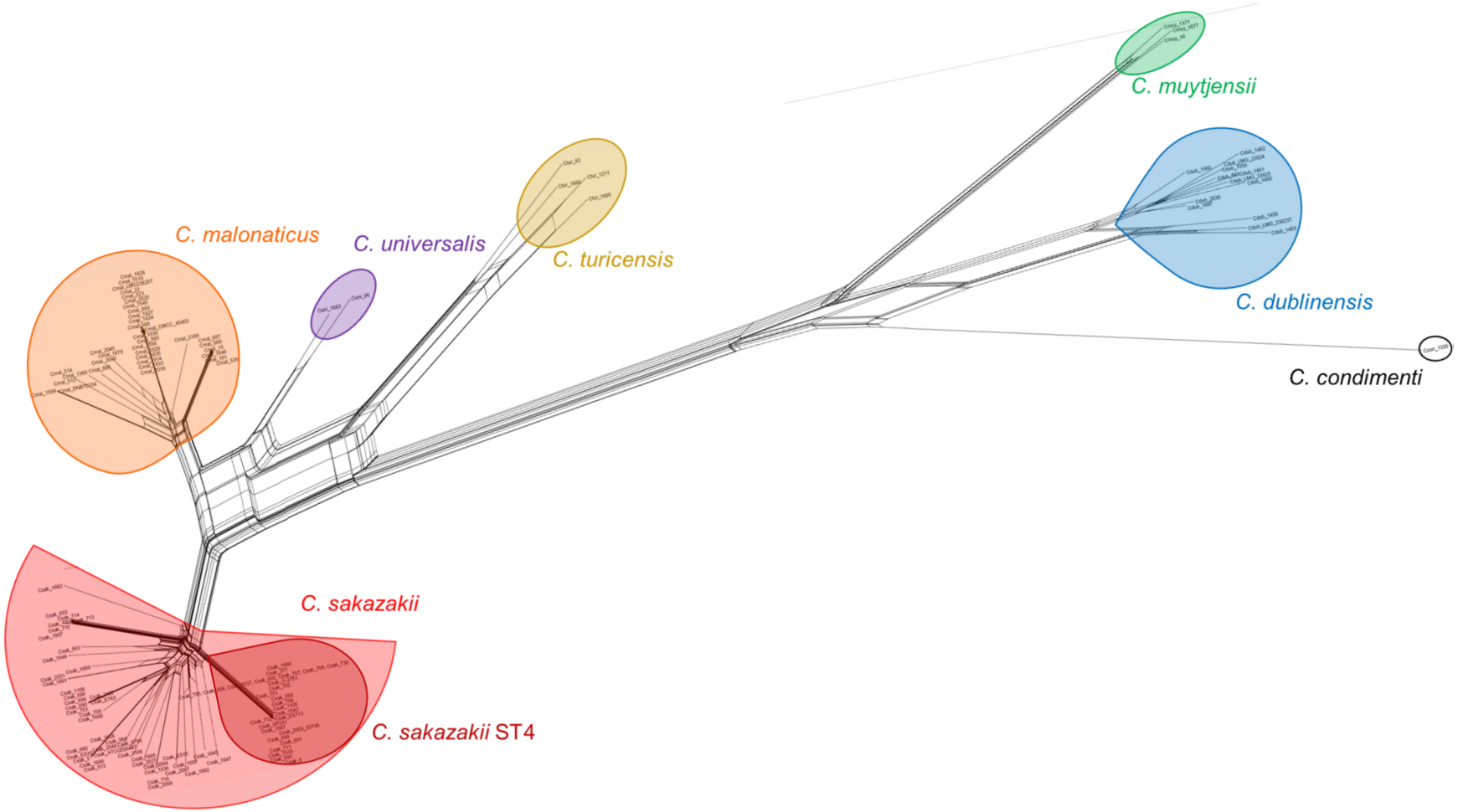


**Figure 5.2. Split network of the *Cronobacter mutS-rpoS* genomic region (9,722 bp) generated using SplitsTree4 *[69]*.**

To better determine if recombination had occurred among *Cronobacter* spp. in the *mutS-rpoS* region, the sequences were analysed using the difference of sum-of-squares method in TOPALi2, as described in Section 2.5.4 *[114, 117]*. Figure 5.3 shows the results of these analyses using a significance value of 0.95. Any peaks above the threshold (designated by a red line), indicate positions where recombination may have occurred. Figure 5.3.a shows no evidence of recombination when the genus was analysed as a whole. Similarly, no evidence of recombination was observed in either *C. sakazakii* or *C. malonaticus* when each species was analysed individually (Figure 5.3 b and c). One possible point of recombination was observed for *C. dublinensis*, but this was the only evidence of recombination within the *Cronobacter* genus (Figure 5.3.d). Four or fewer genomes were available for the remaining species; therefore, reliable analyses of these species were not possible. Regardless, this analysis suggests that little to no HGT is taking place in the *mutS-rpoS* region of *Cronobacter* spp.

Though the *mutS-rpoS* genomic region has been postulated to be evolving via HGT, there is little evidence for this in *Cronobacter* spp. *[28, 97, 98, 103]*. This is not unexpected as *Cronobacter* spp. are known to be clonal *[6]*. Clonal organisms evolve primarily via point mutation and not by HGT *[6]*. Thus, the clonality of *Cronobacter* spp. may account for the lack of evidence for recombination in the *mutS-rpoS* region, despite the fact the HGT has been identified as an important factor for the evolution of this region in other species. The clonality of *Cronobacter* spp. is also highlighted by the split network shown in Figure 5.2. This network is highly similar to a previously published split network of the *Cronobacter* genus. As the tree published by Forsythe *et al. [44]* was constructed using sequences from the 7-loci *Cronobacter* MLST scheme, more sequences were available for analysis, resulting in more branches in their network. Regardless, the species cluster in a similar manner and some evidence of recombination can be seen within each species, but not between them.

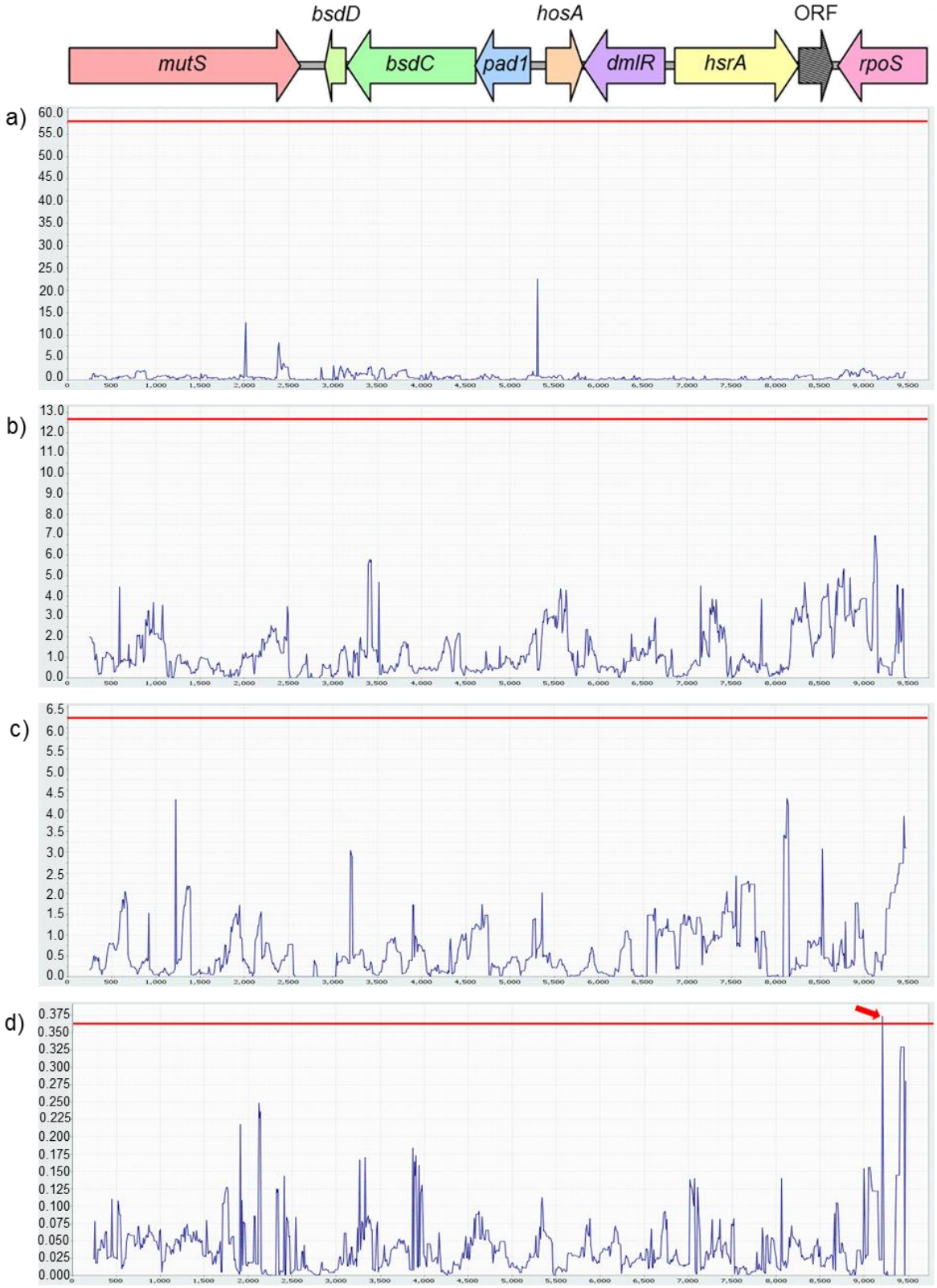


**Figure 5.3. Recombination analysis of the *Cronobacter mutS-rpoS* genomic region** in a) the entire *Cronobacter* genus; b) *C. sakazakii;* c) *C. malonaticus;* d) *C. dublinensis* The positions of the genes in this region are shown. Thresholds for recombination are indicated by the red line. Peaks above this threshold indicate possible positions of recombination (red arrow).

### 5.2.3. Conclusions and implications of genomic analysis of the *mutS-rpoS* region

Analysis of the *Cronobacter mutS-rpoS* region revealed the same genes in the same order in all seven species. In addition, the DNA and protein sequences of the genes in this region were highly conserved. As the same gene composition was identified in all members of the *Cronobacter* genus, it is unlikely that these genes are responsible for the observed differences in virulence characteristics between members of the *Cronobacter* genus. Finally, it was observed that HGT is not occurring between or within most *Cronobacter* spp., probably due to the clonal nature of the organism.

The original hypothesis for this study was incorrect. The same environmental survival genes were found in the *mutS-rpoS* genomic region for all *Cronobacter* species and strains. While these genes may help the organisms to survive in harsh environments until they reach a host, they are unlikely to be responsible for the observed differences in virulence characteristics for the seven species of *Cronobacter*. Thus, this work was discontinued to pursue a more productive line of inquiry.

## 5.4. Cellulose production in *Cronobacter* spp.

The bacterial capsule is composed of multiple polysaccharides and is an important feature for virulence, environmental survival, and biofilm formation *[51, 56, 143, 162, 165]*. The composition of the bacterial capsule is highly variable both between and among species in the *Cronobacter* genus *[126, 151, 152]*. In addition, bacterial species and strains can be characterized by capsular composition. For example, capsular typing schemes exist for both *Esch. coli* and *Salmonella [126, 163]*. Furthermore, the O-antigen, a component of the bacterial capsule, has been studied extensively in *Cronobacter* spp. and can be used for characterization of individual species and strains. PCR methods targeting the O-antigen locus are commonly used, but these assays are limited as they can only detect known O-antigens *[83, 84, 126, 151, 152]*.

In *Cronobacter* spp., five capsular components have been identified: the O-antigen, the K-antigen, colanic acid (CA), cellulose, and the enterobacterial common antigen (ECA) *[126]*. Ogrodzki and Forsythe *[126]* determined the capsular profiles of a collection of *Cronobacter* spp. based on analysis of whole genome sequences. The genes included in this analysis encode for the proteins responsible for the biosynthesis, processing, and transport of the various capsular components *[126]*. This profiling focused on the presence or absence of the genes, but did not examine the variation within the individual gene sequences *[126]*.

The current study took a closer look at the cellulose component of the bacterial capsule. In the *Cronobacter* genus, the *bcs* gene cluster consists of 9 genes. Gene sequences were extracted using Artemis and aligned using MEGA6 *[18, 153]*. The functions of the genes in the *bcs* gene cluster have been published by other authors *[102, 133, 134]*. The structure of the gene cluster in *Cronobacter* spp. is shown in Figure 5.4. Gene organization figures were prepared using the Illustrator for Biological Sequences *[108].* The predicted functions of these genes are shown in Table 5.4. The genes in the figure are drawn to scale, but the lengths of the intergenic regions do differ slightly between species and strains. The same gene organization has been observed in the *bcs* gene cluster of other enteric bacteria, including *Esch. coli* and *Salmonella [134]*.

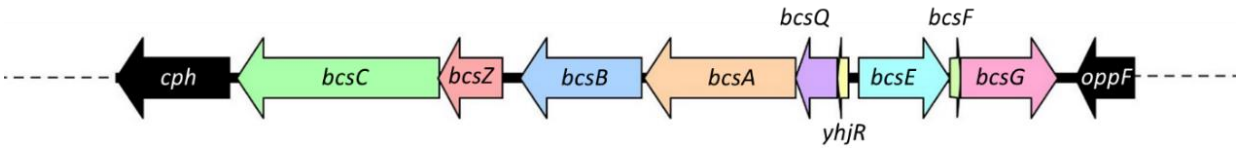


**Figure 5.4. The *bcs* gene cluster of *C. sakazakii.*** Flanking genes *cph* and *oppF* are shown in black.

**Table 5.4. Functions of genes found in the *bcs* cluster**

| Gene | Length (bp) | Function |
|---|---|---|
| *bcsC* | 3504 | Cellulose synthase subunit |
| *bcsZ* | 1116 | Endo-1,4-D-glucanase |
| *bcsB* | 2403 | Cellulose synthase subunit |
| *bcsA* | 2619 | Cellulose synthase subunit |
| *bcsQ* | 729 | Cellulose synthesis protein |
| *yhjR* | 204 | Possible regulatory subunit |
| *bcsE* | 1575 | Cellulose synthase subunit |
| *bcsF* | 198 | Membrane anchored subunit |
| *bcsG* | 1686 | Endogluconase |

The subunits of the synthase responsible for the production of cellulose are encoded by *bcsA, bcsB, bcsC,* and *bcsE [133, 134].* The genes *bcsG* and *bcsZ* encode cellulases that are required for cellulose synthesis and regulate the length of the cellulose chains expressed on the cell surface *[133, 134]*. The gene *bcsQ* was found to be responsible for the polar localization of cellulose production in *Esch. coli [102]*. The product of this gene may be necessary to allow the cell to divide while still producing cellulose and could anchor the cell to a biofilm, while pushing new cells toward the surface after division *[102]*. While its function has not yet been confirmed, it is suspected *yhjR* may be involved in the regulation of cellulose production *[134]*. Additionally, the function of *bcsF* has not been determined, but it is a membrane bound protein and is required for *in vitro* synthesis of cellulose *[134]*.

### 5.4.1. Examination of *bcs* gene sequences

This study was undertaken to examine how the cellulose component of the bacterial capsule affects colony morphology of *C. sakazakii.* The other species were not included in this analysis to reduce the number of variables. The genomes of 78 strains were examined using Artemis and sequences were extracted using the BLAST function of the BIGSdb *[18, 42]*. The *bcs* gene sequences extracted with Artemis from the genome sequence of strain 658 were used to conduct the BLAST searches *[18]*.

Ogrodzki and Forsythe indicated that strains belonging to ST13 and ST100 lacked the *bcs* gene cluster *[126]*. This was confirmed in the current study; however, one ST13 strain (1887) was found to contain all nine genes in the *bcs* cluster. This strain was not included in the analysis by Ogrodzki and Forsythe *[126]*. The *bcs* negative ST13 strains were found to contain a fragment of the gene *bcsC* (2323 bp), while the ST100 strains contain fragments of both *bcsC* (2072 bp) and *bcsG* (1188 bp). The *bcs* gene clusters from ST13 and ST100 are compared to the full gene cluster in Figure 5.5. These results suggest that the *bcs* genes were lost in two separate deletion events in these STs. Additionally, strain 553 was found to be missing a portion of the *bcs* gene cluster as well. This strain was found to contain only 1919 bp of *bcsA* and 556 bp of *bcsE.* The genes *bcsQ* and *yhjR*, located between *bcsA* and *bcsE*, were absent in this strain (Figure 5.5.d). This demonstrates an additional deletion event that resulted in the loss of genes in the *bcs* cluster.

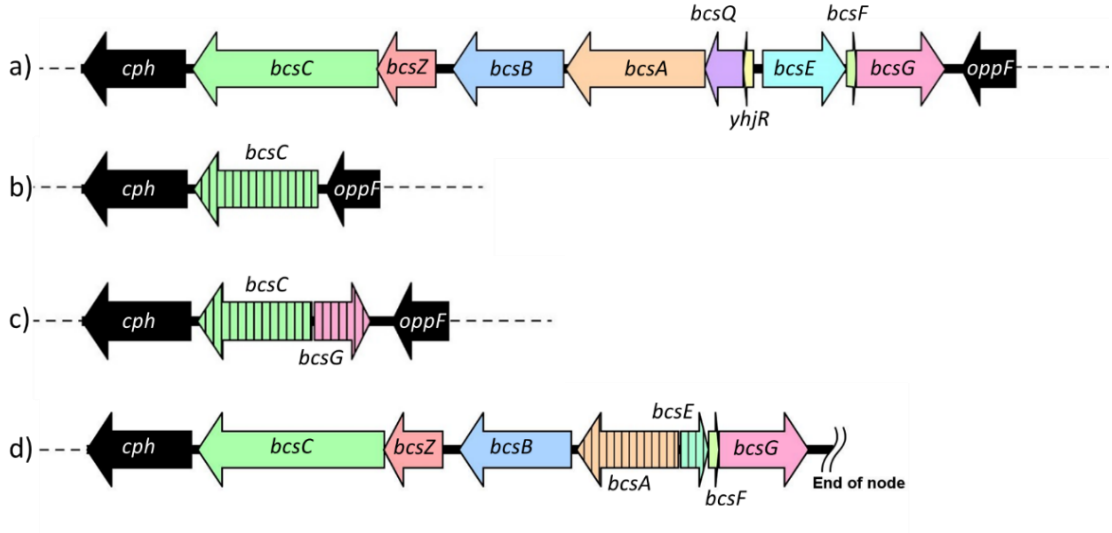


**Figure 5.5. Variation in the gene composition of the *bcs* cluster in *C. sakazakii.*** Truncated genes are indicated by striped arrows. a) Full *bcs* gene cluster; b) *bcs* gene cluster in ST13 strains (excluding strain 1887); c) *bcs* gene cluster in ST100 strains; d) *bcs* gene cluster in strain 553

### 5.4.2. Laboratory analysis of colony morphologies

Based on the genomic analysis described above and the previously published capsular profiles, 22 strains were selected for laboratory analysis (Table 5.5). Genes in the *bcs* cluster that were missing, truncated, or contained nonsense mutations are indicated in Table 5.5 and strains with these features were predicted to be cellulose negative. Strains containing the entire gene cluster with no nonsense mutations were predicted to be cellulose positive.

As discussed above, the ST13 and ST100 strains were predicted to be cellulose negative because of the deletion of the majority of the *bcs* gene cluster. Similarly, strain 553 was also predicted to be cellulose negative due to the deletion of multiple genes in the *bcs* cluster. While strain 553 lacks a smaller fragment of the gene cluster, it has been shown that *bcsQ* is required for cellulose production in *Esch. coli [102]*. Thus, as the deletion event in strain 553 resulted in the loss of this gene, this strain was also expected to be cellulose negative. Strain 20 (ST4) carries a nonsense mutation in *bcsA*, while strain 1992 (ST136) carries a nonsense mutation in *yhjR*. Therefore, both these strains were also predicted to be cellulose negative.

**Table 5.5. Capsular profiles and *bcs* gene clusters for strains selected for laboratory analysis**

| | Capsular profile[a] | | | | | *bcs* gene cluster | | | | | | | | | Predicted cellulose production |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | ST | CC | O-type | K-type | CA type | *bcsC* | *bcsZ* | *bcsB* | *bcsA* | *bcsQ* | *yhjR* | *bcsE* | *bcsF* | *bcsG* | |
| 658 | 1 | 1 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 1536 | 1 | 1 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 2064 | 1 | 1 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 978 | 3 | - | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 984 | 3 | - | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 4 | 15 | 4 | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 20 | 4 | 4 | O2 | K2 | CA2 | N[b] | + | + | + | + | + | + | + | + | - |
| 553 | 4 | 4 | O2 | K2 | CA2 | + | + | + | T | - | - | T | + | + | - |
| 557 | 4 | 4 | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 1533 | 4 | 4 | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 1 | 8 | 8 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 5 | 8 | 8 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 1888 | 8 | 8 | O1 | K1 | CA1 | + | + | + | + | + | + | + | + | + | + |
| 708 | 12 | - | O4 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 1108 | 12 | - | O4 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 2107 | 12 | - | O4 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 713 | 13 | - | O2 | K2 | CA2 | T | - | - | - | - | - | - | - | - | - |
| 1887 | 13 | - | O2 | K2 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 1557 | 23 | 23 | O2 | K1 | CA2 | + | + | + | + | + | + | + | + | + | + |
| 2087 | 100 | 100 | O1 | K2 | CA1 | T | - | - | - | - | - | - | - | T | - |
| 1992 | 136 | - | O2 | K2 | CA1 | + | + | + | + | + | N | + | + | + | - |
| 1844 | 405 | - | O3 | K2 | CA1 | + | + | + | + | + | + | + | + | + | + |

[a]Capsular profiles were published by Ogrodzki and Forsythe *[126]*. [b]Shaded boxes indicate genes that are absent, truncated or contain a nonsense mutation; -, absent; T, truncated; N, nonsense mutation

Strains were streaked for isolation on infant formula (IF) agar, prepared as described in Section 2.1.13. IF agar plates were incubated at 25° C or 37° C for 48 hours. Colony morphologies are given in Table 5.6. Colonies were scored based on how mucoid they appeared. Strains with '-' or '+/-' results were not mucoid, but may have been glossy. Strains with '+' were slightly mucoid, while strains with '++++' were extremely mucoid with material dripping from the surface of the plate. Strains which appeared mucoid were designated '++' and strains which were very mucoid, but not yet dripping from the surface of the plate were designated '+++.'

In the current study, colonies varied from cream-coloured to yellow with a dry, glossy, or mucoid appearance. The different colours observed were due to variation in the production of yellow pigment, as discussed in Chapter 3. The mucoid colony morphology is considered to be indicative of capsule formation on IF agar *[8, 67]*. Notably, colony morphologies were generally more mucoid on plates incubated at 37° C. This is of particular concern for infants being fed via nasogastric feeding tube. A portion of the tube will be inside the infant's body and will, therefore, be at 37° C *[68]*. As the tubes remain in place for hours or even days, feeding regimes will provide the bacteria with fresh nutrients on a regular basis *[67, 68, 115]*. Thus, it is possible that bacteria could be growing in this environment and feeding tubes have been identified as a possible source of neonatal infections *[68, 115]*.

Based on the results observed on IF agar, some *C. sakazakii* strains can produce capsular material when using IF as a source of nutrients. Of importance is the reported link between capsule production and biofilm formation. In particular, reduced biofilm formation has been reported in *Cronobacter* strains with mutations in the cellulose gene cluster, suggesting that cellulose is required to form a biofilm *[56, 63]*. In addition, Solano *et al. [143]* suggested that cellulose is required for biofilm formation when limited nutrients are available. Conversely, Hurrell *et al. [68]* showed that biofilm formation in *C. sakazakii* was not necessarily correlated with colony morphology and capsule production on IF agar, while Ma and Wood *[110]* noted that biofilm formation in *Esch. coli* increased when cellulose production was inhibited. Thus, the link between cellulose production and biofilm formation is complicated and not well understood.

**Table 5.6. Colony morphologies on IF agar after 48 hours**

| Strain | ST | CC | O-type | K-type | CA type | Predicted cellulose phenotype | Colony morphologies | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | 25° C | | 37° C | |
| | | | | | | | Colour | Mucoid[a] | Colour | Mucoid |
| 658 | 1 | 1 | O1 | K1 | CA1 | + | Cream, glossy | - | Cream, glossy | +/- |
| 1536 | 1 | 1 | O1 | K1 | CA1 | + | Yellow, slightly mucoid | + | Yellow, mucoid | + |
| 2064 | 1 | 1 | O1 | K1 | CA1 | + | Pale yellow, mucoid | ++ | Pale yellow, mucoid | +++ |
| 978 | 3 | - | O2 | K2 | CA2 | + | Yellow, mucoid | ++ | Yellow, mucoid | +++ |
| 984 | 3 | - | O2 | K2 | CA2 | + | Yellow, mucoid | ++ | Yellow, mucoid | +++ |
| 4 | 15 | 4 | O2 | K2 | CA2 | + | Cream, glossy | +/- | Pale yellow, mucoid | ++ |
| 20 | 4 | 4 | O2 | K2 | CA2 | - | Yellow, slightly mucoid | + | Yellow, glossy | - |
| 553 | 4 | 4 | O2 | K2 | CA2 | - | Cream, mucoid | ++ | Yellow, mucoid | +++ |
| 557 | 4 | 4 | O2 | K2 | CA2 | + | Cream, mucoid | ++ | Pale yellow, mucoid | ++ |
| 1533 | 4 | 4 | O2 | K2 | CA2 | + | Pale yellow, glossy | - | Yellow, mucoid | ++ |
| 1 | 8 | 8 | O1 | K1 | CA1 | + | Yellow, smooth | - | Yellow, glossy | - |
| 5 | 8 | 8 | O1 | K1 | CA1 | + | Yellow, glossy | - | Yellow, glossy | - |
| 1888 | 8 | 8 | O1 | K1 | CA1 | + | Yellow, dry | - | Yellow, glossy | - |
| 708 | 12 | - | O4 | K2 | CA2 | + | Cream, glossy | +/- | Cream, mucoid | ++ |
| 1108 | 12 | - | O4 | K2 | CA2 | + | Yellow, mucoid | ++ | Yellow, glossy | +/- |
| 2107 | 12 | - | O4 | K2 | CA2 | + | Yellow, dry | - | Yellow, dry | - |
| 713 | 13 | - | O2 | K2 | CA2 | - | Cream, mucoid | +++ | Pale yellow, mucoid | ++++ |
| 1887 | 13 | - | O2 | K2 | CA2 | + | Yellow, glossy | - | Yellow, glossy | - |
| 1557 | 23 | 23 | O2 | K1 | CA2 | + | Yellow, glossy | - | Yellow, glossy | +/- |
| 2087 | 100 | 100 | O1 | K2 | CA1 | - | Yellow, smooth/dry | - | Yellow, dry | - |
| 1992 | 136 | - | O2 | K2 | CA1 | - | Yellow, mucoid | ++ | Yellow, glossy | +/- |
| 1844 | 405 | - | O3 | K2 | CA1 | + | Yellow, mucoid | ++ | Yellow, glossy | +/- |

[a] - : not mucoid; +/- : glossy or slightly mucoid; + : mucoid; ++ : moderately mucoid; +++ : highly mucoid, beginning to drip; ++++ : extremely mucoid, dripping onto lid of plate.

Adhesion to a surface is necessary in the early stages of biofilm production, but capsule formation may provide an advantage in later stages *[29, 138]*. Many authors have suggested that capsule production may inhibit adhesion of the bacteria to a surface, thus preventing the formation of new biofilms on surfaces and adhesion of microbial cells to host tissues *[35, 52, 89, 136-138, 148]*. For example, Schembri *et al. [138]* reported that the thickness of the capsule expressed by *Klebsiella pneumoniae* physically blocked the adhesins expressed on the surface of the cell, limiting the ability of the organism to interact with surfaces and preventing the formation of a biofilm. Reduced biofilm formation has also been associated with cellulose production by other authors, suggesting that the capsule has an inhibitory effect on biofilm formation *[52, 167]*. Thus, increased capsule formation in IF may inhibit the production of new biofilms or enhance the growth of existing biofilms. Further work is needed to determine what role the bacterial capsule plays in the adhesion of cells to surfaces and biofilm formation, particularly as it relates to growth in IF and neonatal nasogastric feeding tubes.

Regardless of its effects on biofilm formation, the bacterial capsule may provide microorganisms with some protection from the host defence systems *[52, 67, 162]*. The gastrointestinal and immune systems of infants are not fully developed, which puts them at an increased risk of infection *[115]*. In particular, the pH of an infant's stomach may not be low enough to destroy pathogens that are introduced during feeding. Hurrell *et al*. *[68]* found that the gastric pH of infants fed with reconstituted PIF ranged from 3.5-5.5, but it has been shown that strains of *C. sakazakii* can survive under these conditions for at least 5 hours *[32]*. Thus, the pathogen can reach the intestines and go on to cause illness *[67, 115]*.

Based on the analysis of selected *C. sakazakii* strains, it does not appear that cellulose production alone is responsible for the observed differences in colony morphology (mucoid or non-mucoid) observed on IF agar. For example, strains 2107 (ST12) and 2087 (ST100) both produced dry, non-mucoid colonies at both temperatures, even though they possess very different capsular profiles. In particular, strain 2107 was predicted to be cellulose positive, while strain 2087 was predicted to be cellulose negative. The lack of correlation between predicted cellulose phenotype and colony morphology on IF agar is also demonstrated by two ST4 strains. Strains 20 and 553 were both predicted to be cellulose negative, but produced different colony morphologies on IF agar. Strain 20 was non-mucoid or slightly mucoid while strain 553 was moderately to highly mucoid. The other ST4 strains, predicted to be cellulose positive, showed moderately mucoid colonies on IF agar at one or both of the incubation temperatures. Again, this suggests that cellulose production (as

predicted based genome analysis) is not solely responsible for the mucoid colony morphology on IF agar.

Finally, Ogrodzki and Forsythe *[126]* indicated that ST1 and ST8 strains have the same capsular profile; however, the colony morphologies differed when strains belonging to these STs were plated on IF agar. Various colony morphologies were observed for strains belonging to ST1, with strains 1536 and 2064 producing mucoid colonies, while strain 658 did not. Meanwhile the ST8 strains were consistently non-mucoid. It should be noted that, though they were predicted to be cellulose positive, ST8 strains carry nonsense mutations in the capsular gene clusters for CA production and the K-antigen *[127]*. It is, therefore, possible that the lack of these two capsular components contributes to the observed colony morphologies for the strains belonging to this ST. The next chapter will discuss a proposed capsular typing scheme that will take all capsular components into account before attempting to link the sequences to observable phenotypes

Farmer *et al. [34]* noted that *Cronobacter* strains (then known as *Enterobacter sakazakii*') could produce rough colonies that smoothed out during refrigerated storage of plates. It has been proposed that cellulose is responsible for the formation of this rough colony morphology *[63, 166]*. This correlates with the observed results for ST8 and ST13. The mutations observed in the K-antigen and CA gene clusters suggest that these components will not be present in the capsule of ST8 strains. Thus, it is likely that cellulose will make up a larger proportion of the capsule, resulting in the non-mucoid colonies observed on IF agar for ST8 strains. Similarly, the cellulose positive ST13 strain (1887) produced rough colonies, while the ST13 strain that was predicted to be cellulose negative (713) produced mucoid colonies. Yet, this hypothesis is contradicted by the observed colony morphologies for strain 2087 (ST100), which was also predicted to be cellulose negative, but still produced rough colonies on IF agar at both temperatures.

As stated above, the results presented here suggest that cellulose production alone cannot account for the different colony morphologies observed on IF agar. Mucoid colony morphologies have been used by other authors as an indicator for capsule formation, but cellulose is just one component of the bacterial capsule *[8, 67]*. Five capsular components, including cellulose, have been identified in *Cronobacter* spp. *[126]*. Therefore, it is not entirely surprising that genomic analysis of the gene cluster responsible for production of single capsular component, cannot explain the variation in colony morphologies on IF agar.

To better investigate cellulose production by *C. sakazakii,* strains were also streaked for isolation on Congo red agar (CRA), prepared as described in Section 2.1.14. Plates were incubated

at both 25° C and 37° C for 48 hours. Colony morphologies on CRA are shown in Table 5.7. Cellulose binds the dye Congo red, resulting in pink or red coloured colonies when cellulose is being produced. Based on the previously reported results for *Salmonella,* species and strains producing both cellulose and curli fimbriae will produce red colonies, while those producing only cellulose will appear pink *[166, 167]*. It has been noted that *C. sakazakii* lacks the genes encoding curli fimbriae *[94]*. Thus, pink colonies were expected for cellulose positive strains on CRA.

**Table 5.7. Colony morphologies on CRA after 48 hours**

| Strain | ST | CC | O-type | K-type | CA type | Predicted cellulose phenotype | 25° C | 37° C |
|---|---|---|---|---|---|---|---|---|
| 658 | 1 | 1 | O1 | K1 | CA1 | + | Pink, glossy | Pink/purple, glossy |
| 1536 | 1 | 1 | O1 | K1 | CA1 | + | Tan, glossy | Dark brown/pink, glossy |
| 2064 | 1 | 1 | O1 | K1 | CA1 | + | Pink/brown, glossy | Dark brown/pink, glossy |
| 978 | 3 | - | O2 | K2 | CA2 | + | Tan, mucoid | Brown, glossy |
| 984 | 3 | - | O2 | K2 | CA2 | + | Tan, mucoid | Brown, glossy |
| 4 | 15 | 4 | O2 | K2 | CA2 | + | Dark pink, glossy | Pink/purple, glossy |
| 20 | 4 | 4 | O2 | K2 | CA2 | - | Tan, glossy | Tan, glossy |
| 553 | 4 | 4 | O2 | K2 | CA2 | - | Tan, glossy | Brown, glossy |
| 557 | 4 | 4 | O2 | K2 | CA2 | + | Pink, glossy | Pink, glossy |
| 1533 | 4 | 4 | O2 | K2 | CA2 | + | Pink, dry | Brown, glossy |
| 1 | 8 | 8 | O1 | K1 | CA1 | + | Tan, glossy | Dark brown, glossy |
| 5 | 8 | 8 | O1 | K1 | CA1 | + | Tan, glossy | Pink/purple, glossy |
| 1888 | 8 | 8 | O1 | K1 | CA1 | + | Pale orange, glossy | Dark pink/purple, glossy |
| 708 | 12 | - | O4 | K2 | CA2 | + | Tan, glossy | Pink/purple, glossy |
| 1108 | 12 | - | O4 | K2 | CA2 | + | Orange, glossy | Pink/brown, glossy |
| 2107 | 12 | - | O4 | K2 | CA2 | + | Dark pink, dry | Pink, glossy |
| 713 | 13 | - | O2 | K2 | CA2 | - | Yellow/brown, glossy | Brown, glossy |
| 1887 | 13 | - | O2 | K2 | CA2 | + | Yellow/brown, glossy | Brown, glossy |
| 1557 | 23 | 23 | O2 | K1 | CA2 | + | Pink/orange, glossy | Brown, glossy |
| 2087 | 100 | 100 | O1 | K2 | CA1 | - | Tan, glossy | Tan/brown, glossy |
| 1992 | 136 | - | O2 | K2 | CA1 | - | Yellow/brown, glossy | Brown, glossy |
| 1844 | 405 | - | O3 | K2 | CA1 | + | Brown, mucoid | Purple, glossy |

Incubation temperature affected the colony morphologies observed on CRA. Pink coloured-colonies were observed for more strains and the colour was generally darker at 37° C than at 25° C. For example, ST8 strains did not produce pink colonies at 25° C, though they were predicted to be cellulose positive. At 37° C, however, two ST8 strains (5 and 1888) did produce pink colonies. Similarly, strain 1536 (ST1) also showed expression of cellulose, as evidenced by pink-coloured colonies, at 37° C, but not at 25° C. These results are similar to those observed for other *Enterobacteriaceae [12, 166]*. For example, a higher percentage of *Esch. coli* strains were found to be producing both cellulose and curli fimbriae at 37° C *[12]*. Thus, it is unsurprising that cellulose production in *C. sakazakii* also appears to be increased at 37° C. As discussed above, this is a concern for organisms growing in neonatal nasogastric feeding tubes, as a portion of the tube will be held at body temperature. Since the bacterial capsule may shield the cells from the acidic conditions in the stomach, increased production of one or more capsular components at body temperature may present a risk to infants *[68, 115]*.

The *C. sakazakii* ST4 strains produced the expected colony morphologies on CRA. The ST4 strains that were predicted to be cellulose positive (strains 4, 557, and 1533) produced pink colonies, while those predicted to be cellulose negative (strains 20 and 553) did not. Similarly, strains 713 (ST13), 2087 (ST100), and 1992 (ST136) were predicted to be cellulose negative and none of these strains produced pink colonies on CRA either temperature. This indicates that these strains were not producing cellulose, which matches the phenotype predicted from the genomic analyses.

Nevertheless, the predicted cellulose phenotype did not always correlate to the observed colony morphologies on CRA. For example, ST3 strains were predicted to be cellulose positive, but produced tan- or brown-coloured colonies on CRA at both temperatures, indicating that cellulose was not being produced. The observed phenotype of the cellulose positive ST13 strain (1887) was also inconsistent with the predicted phenotype. No pink colonies were observed for this strain, though it was predicted to be cellulose positive. While some of the observed results did match the predicted phenotypes, the lack of correlation for some strains suggests that colony morphology on CRA may not be the most reliable indicator of cellulose production.

### 5.4.3. Investigation of multicellular morphotypes on CRA

Though colony morphology on CRA does not appear to be the most effective method for detection of cellulose, some species and strains can produce multicellular morphotypes on this agar. For example, such morphotypes have been observed for *Salmonella [132, 135, 166, 167]*. Strains producing the “rdar” morphology (red, dry, and rough) were found to express both cellulose and curli fimbriae *[132, 135, 166, 167]*. Meanwhile, the “pdar” morphology (pink, dry, and rough) was associated with strains producing only cellulose *[166, 167]*. Zogaj *et al. [166]* also postulated that cellulose production is responsible for the rough appearance of these morphotypes, while curli fimbriae were associated with smooth morphotypes. In order to investigate the formation of similar morphotypes in *C. sakazakii*, the strains were spot plated onto CRA (3 µl per spot). Plates were prepared in duplicate and incubated at 25° C or 37° C. Photos of the observed morphotypes after 48 hours of incubation at 25° C and 37° C are shown in Figures 5.6 and 5.7, respectively. As indicated above, *C. sakazakii* strains have been shown to lack the genes for curli fimbriae. Hence, the “pdar” morphology was expected for cellulose positive strains *[56, 94, 166]*. Cellulose negative strains were predicted to produce smooth morphotypes with no pink colour.

No red morphotypes were observed in the *C. sakazakii* strains, as expected due to the lack of curli fimbriae *[56, 94, 166]*. The colours ranged from tan to dark pink and differed between strains

in a similar pattern to the colony morphologies on the streak plates, with a few discrepancies. For example, at 25° C, strains 708 and 1844 showed some pink colour on the spot plates, but not on the streak plates. Similarly, at 37° C strains 978, 984, 1533, and 1992 also showed pink morphotypes on the spot plates, while producing brown colonies on the streak plates. This may be due to the fact that the pink colour frequently appeared in the centre of the spots. The individual colonies may have been too small to allow for visual detection of the pink colour on the plates that were streaked for isolation. Regardless of these differences, the colours produced on the spot plates for strains predicted to be cellulose negative were consistent with the predicted cellulose phenotype. All strains predicted to be cellulose negative were tan or brown in colour, indicating that cellulose was not being produced.

The observed morphotypes also suggest that regulation may play an important role in cellulose production. For example, rings of varying colours were seen for the ST3 strains at 37° C (Figure 5.7). While the outer rings were tan or brown, the centre was dark pink, suggesting differential expression of cellulose and/or other components of the bacterial capsule in different regions of growth in the spot. More extreme examples of ring structures can been seen for strains 658, 1533, 2107, and 1844 at 25° C. While such unusual morphotypes were not observed for all strains, rings of varying colours were observed for all strains at both temperatures.

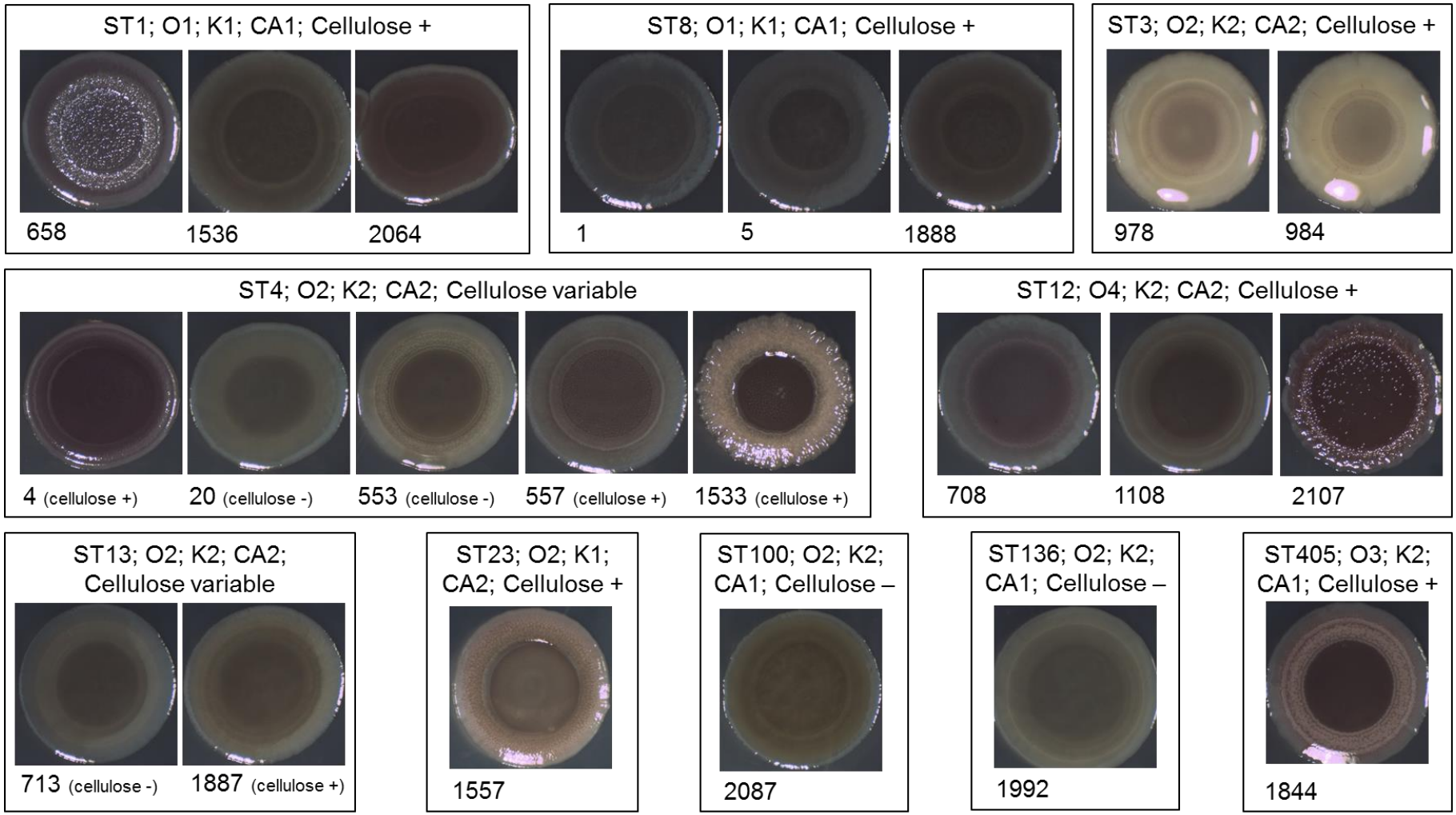


**Figure 5.6. Morphotypes of *C. sakazakii* spot plated on CRA and incubated at 25° C for 48 hours.**

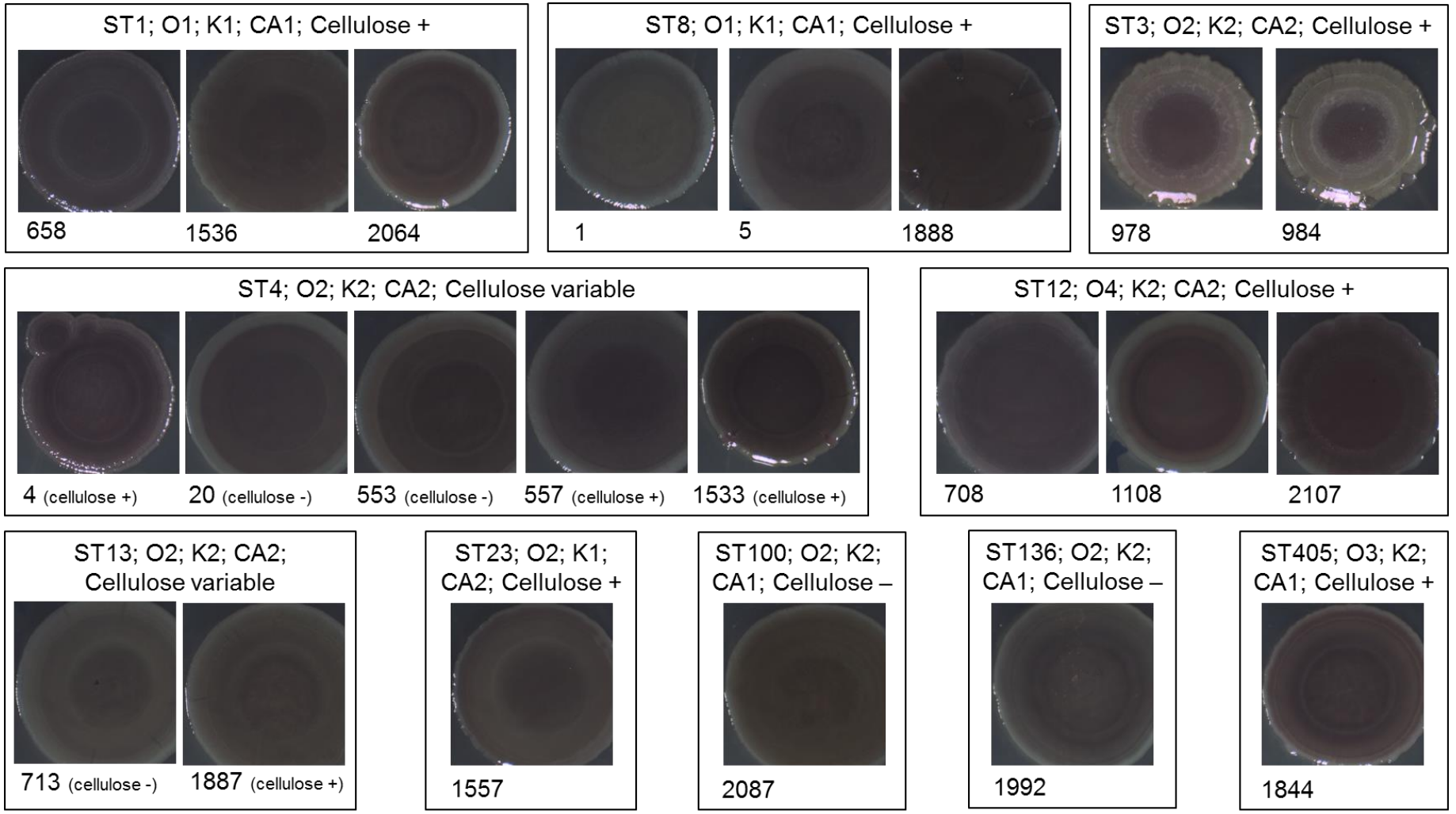


**Figure 5.7. Morphotypes of *C. sakazakii* spot plated on CRA and incubated at 37° C for 48 hour**

Variation in texture was also observed on the spot plates. These variations were more pronounced at 25° C than at 37° C. For the ST1 strains, both 658 and 2064 were pink, indicating cellulose production; however, while strains 1536 and 2064 produced smooth morphotypes, strain 658 showed a rough texture in the centre of the spot. Figure 5.8a shows the growth pattern observed for strain 658 after 24, 48, and 72 hours at 25° C. Similarly, in ST4, strain 1533 produced a unique morphotype, with a pink centre surrounded by a thick, rough, tan-coloured ring at 25° C (Figure 5.8b). Strain 2107 (ST12) also produced a rough, pink morphotype. This strain showed a small rough ring after 24 hours at 37° C, but had changed to a dimpled surface after 48 hours (Figure 5.8c). Interestingly, strains 1533 (ST4) and 2107 (ST12) also produced dry, rough colonies when streaked for isolation on CRA. Conversely, strain 658 (ST1) produced a rough morphotype on the spot plates, but glossy colonies on the streak plates, suggesting differential expression of the capsule under different growth conditions.

Strains 1557 and 1844 showed ring structures on the spot plates at 37° C (Figures 5.8d, e). Despite the formation of these structures, these strains produced smooth morphotypes, matching the smooth colony morphologies on the streak plates. Both ST3 strains (987 and 984) showed mucoid morphotypes at 25° C, matching the colony morphologies observed on the streak plates. While these strains produced smooth spots at 37° C, the edges were crenelated (Figure 5.8f, g).

It should be noted that while some *C. sakazakii* strains did produce rough morphotypes, they did not match the description of the rough morphotype of *Salmonella [135].* The morphotypes observed for *C. sakazakii* were similar to the morphotype observed for *Esch. coli* expressing only cellulose *[12, 132]*. This result would be expected as *C. sakazakii* strains lack curli fimbriae and may express cellulose as a component of the bacterial capsule *[94, 126]*. Additionally, the rough morphotypes of *Salmonella* were observed after seven days of incubation, whereas the morphotypes of *C. sakazakii* were observed for only three days *[132]*. The morphotypes for strains 1533 and 2107 did get rougher after 72 hours, but the morphotype for strain 658 began to smooth out with increased incubation time (see Figure 5.8). It is possible that the morphotypes could change with a longer incubation time. Regardless, it is unlikely that *C. sakazakii* strains will produce morphologies similar to the “rdar” morphology as described for *Salmonella*. This morphotype is produced by strains expressing both cellulose and curli fimbriae, but *C. sakazakii* lacks the genes encoding curli fimbriae *[94, 167]*. Based on the amount of variation observed in the morphotypes for *C. sakazakii* strains, it is unlikely that the observed morphotypes will be easily categorized with the acronyms that have been applied to *Salmonella.*

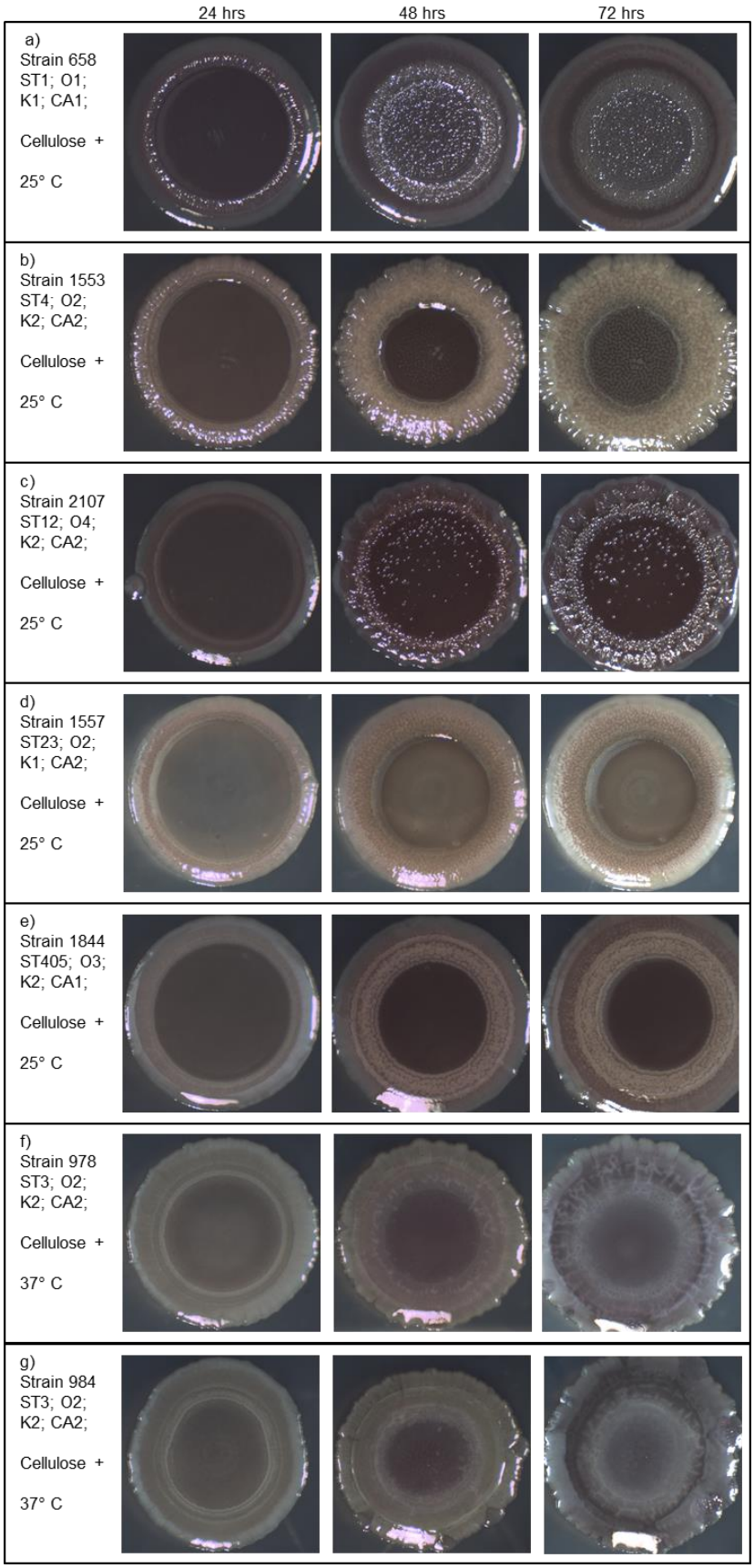


**Figure 5.8. Morphotypes of selected strains spot plated on CRA after incubation for 24, 48 and 72 hours.**

As with the IF agar, temperature appeared to play a role in colony morphology on CRA. On the spot plates the colours were generally darker at 37° C than at 25° C, suggesting that cellulose production was increased at the higher temperature. The effect of temperature on cellulose production has also been reported for *Esch. coli* and was discussed previously *[12].* It has also been proposed that cellulose production is responsible for the production of rough colony morphologies *[166, 167]*; therefore, an increase in rough morphotypes would also be expected at 37° C. Yet, the exact opposite was observed and the morphotypes at the higher temperature were smoother than those observed at 25º C. A similar temperature dependence has also been described for *Salmonella* spp., where the "rdar" morphotype was observed at a lower temperature, but smooth morphotypes were observed when the temperature was increased *[46, 132, 135]*. This result, however, was attributed to the temperature-dependent regulation of curli fimbriae expression, which *C. sakazakii* strains lack *[94, 135]*. Thus, the results presented here suggest that cellulose expression may also be under temperature regulation, with increased production of cellulose at lower temperatures. More work is required to determine what role temperature plays in expression of cellulose and other capsular components in *C. sakazakii.*

The observed colony morphologies on IF agar suggested that capsule production was increased at 37° C and the results from the CRA suggest that cellulose production is also increased at this temperature. Five components in the bacterial capsule have been identified in *Cronobacter* spp., but it is not known if any of them are under temperature-dependent regulation *[126]*. Changes in production of one or more of these capsular components at different temperatures will likely lead to different combinations and levels of each component in the bacterial capsule. Thus, if production of one or more other capsular components is increased, cellulose may constitute a smaller proportion of the capsule, reducing its effects on the observed morphotype. This could then lead to the production of the smoother morphotypes seen at 37° C on CRA.

### 5.4.4. Conclusions and implications of genomic and laboratory analysis of cellulose production

Based on the genomic analysis of the *bcs* gene cluster, predictions were made about the phenotypes that would be produced in the laboratory. While some of these predictions did match the colony morphologies and morphotypes appearing on IF agar and CRA, the observed phenotypes were not always consistent with the predicted phenotypes. This is likely due to the presence of at least five components in the bacterial capsule of *Cronobacter* spp., each having their

own effect on the phenotype. Expression of different combinations and levels of the various capsular components is what determines which colony morphology will appear on the plate. Thus, it is not surprising that analysis of a single gene cluster could not accurately predict the phenotypes of these strains. Further work will be necessary to determine what effect each capsular component and the bacterial capsule as a whole have on a variety of phenotypic characteristics, including colony morphology, biofilm formation, and virulence.

### 5.5. Bringing it all together: Linking genotype and phenotype

This chapter has demonstrated how evaluation of genome sequences can be used to guide laboratory experiments and analysis of collected data. For example, examination of the *mutS-rpoS* genomic region revealed that this region was not likely to be related to the virulence of certain species within the *Cronobacter* genus. Genomic analysis also influenced the selection of strains used for laboratory analysis of cellulose production. Based on the genome sequences, strains predicted to be both cellulose positive and cellulose negative were selected for inclusion in the laboratory experiments. The ST4 strains are an example of how beneficial the genomic analysis was. Since ST4 strains are closely related and were reported to have the same capsular profile, they would be expected to produce similar colony morphologies and morphotypes *[126]*; however, this was not the case. Two of the five ST4 strains had mutations in the *bcs* gene cluster which were predicted to prevent them from expressing cellulose. These strains did not bind Congo red, an observation which matched the prediction based on the genome sequences. Without the analysis of each individual gene sequence, it would have been difficult to explain why these two strains differed from the other ST4 strains when plated on IF agar and CRA.

Similar methods could be applied to other genes, gene clusters, and phenotypic characteristics. As stated previously, this approach could be used to analyse all five capsular components together, as they probably interact with one another to produce the observed phenotypes. A proposed capsular typing scheme will be discussed in more detail in the next chapter.

# CHAPTER 6

# Future Directions

## 6.1. Development of a capsule typing scheme for *Cronobacter* spp.

As discussed in Chapter 5, the bacterial capsule plays an important role in the phenotype of *Cronobacter* spp., particularly as it relates to some virulence associated characteristics such as biofilm formation. It is also possible that the variations in the bacterial capsule may contribute to the differences in virulence that are observed for the different *Cronobacter* spp. Ogrodzki and Forsythe *[126]* published a study on the capsular profiling of *Cronobacter* species, but did not attempt to link these profiles to any particular phenotypic characteristics. Additionally, though Ogrodzki and Forsythe *[126]* did examine all five of the capsular components in *Cronobacter* spp., their analysis was based solely on the presence or absence of certain genes. While this did reveal some differences between and among the seven *Cronobacter* species, these capsular profiles do little to explain the behaviour of the organisms. The analysis of the *bcs* gene cluster presented in Chapter 5 went further by analysing the sequences of the genes, instead of merely their presence; however, the analysis of the *bcs* gene cluster alone is not sufficient to explain all the variation observed in colony morphology and multicellular morphotypes for *C. sakazakii*. As discussed previously, this is likely due to the fact that cellulose is only one of five capsular components observed in *Cronobacter* spp. *[126]*. In order to accurately predict phenotypes affected by capsule formation, the sequences of all five capsular components must be taken into account.

Nonsense mutations have already been identified in the K-antigen and colanic acid gene clusters of *C. sakazakii* ST8 strains *[127]*. As discussed in Chapter 5, these mutations may have contributed to the observed phenotypes on infant formula (IF) agar and Congo red agar (CRA). By analysing all five capsular gene clusters at the sequence level, it may be possible to develop a more accurate capsular typing scheme for *Cronobacter* spp. using the Bacterial Isolate Genome Sequence Database (BIGSdb) *[86]*. Yet, the mutations identified in the ST8 strains stress the importance of examining not only the gene sequences, but also the corresponding protein sequences. In contrast to the gene sequences, the protein sequences will be more closely linked to the functionality of the gene. Analysis of the protein sequences will be able to account for both synonymous and nonsynonymous mutations in the genes and will allow for comparison of strains based only on the differences that will likely alter the observable phenotype.

By assigning allele numbers to the unique gene sequences and unique protein variants, the species and strains could be sorted according to these detailed capsular profiles. From there, phenotypic analyses could be performed to determine if there are traits that are consistent among strains with identical or similar capsular profiles. First, IF agar and CRA could be used to assess the differences in colony morphology as they relate to the entirety of the bacterial capsule. Capsular profiles could also be linked to other phenotypes of interest, such as biofilm formation and resistance to stresses that are likely to be encountered by *Cronobacter* spp. (e.g. desiccation, heat, and acid). By linking these phenotypes to the capsular profiles based on both DNA and protein sequences, it may be possible to predict if a particular strain will be a strong biofilm producer or will be more resistant to environmental stresses before these tests are performed in the laboratory.

Linking the capsular profiles to differences in biofilm formation could lead to a better understanding of biofilm formation in *Cronobacter* spp. and other enteric bacteria. As discussed in Chapter 5, it is unclear what role the bacterial capsule plays in biofilm formation. It is possible that the bacterial capsule may inhibit adhesion to surfaces, leading to lower biofilm formation; however, the capsule may be important for formation and growth of the biofilm after the cells have become attached to the surface. Examining the different stages of biofilm formation within the context of the capsular profile could help to elucidate the role that the capsule plays in this process.

## 6.2. Potential problems with this proposed typing scheme

The major difficulty with the proposal of a sequence-based capsular typing scheme is the number of genes involved in capsule production in *Cronobacter* spp. For example, the O-antigen locus contains anywhere between 7 and 13 genes *[151]*. Additionally, the colanic acid gene cluster contains 20 or 21 individual genes *[126]*. In total, analysis of all five capsular gene clusters could involve up to 66 gene sequences. While it may be possible to generate a laboratory assay to sequence some of these genes, a laboratory assay to sequence all of the capsule genes would be highly inefficient. As genome sequencing becomes cheaper and easier, it will be possible to extract and analyse the desired sequences from the genomes of the desired strains. The BIGSdb does have the capability to perform this type of analysis, but it must be carefully considered.

It may be suggested that it would be easier to use only a few selected genes from each cluster to establish the capsular profile. For example, Ogrodzki and Forsythe *[126]* used the genes flanking the O-antigen locus (*gnd* and *galF*) to determine the O-serotype of *Cronobacter* strains,

rather than analysing all of the genes within the O-antigen locus; however, this approach is problematic as it may miss some of the mutations that could affect phenotype. As described in Chapter 5, *C. sakazakii* strain 553 lacks a small section of the *bcs* gene cluster. If only a few of the genes from this cluster (e.g. *bcsC, bcsB,* and *bcsG*) were used to determine the capsular profile, the deletion in the *bcs* gene cluster in strain 553 would not be reflected in its capsular profile. Based on those three genes, strain 553 would be predicted to be cellulose positive, leading to inconsistent results when comparing the capsular profiles to the observed phenotypes in the laboratory. Thus, in order to accurately determine the capsular profiles of *Cronobacter* strains, it is important to include all of the genes involved in production of the bacterial capsule. To do this, genomic analysis, rather than laboratory assays, would be more efficient. Chapter 5 demonstrated how analysis of the cellulose gene cluster could be linked to colony morphologies. Extending such analyses to all five capsular components will lead to a better understanding of how the bacterial capsule relates to certain phenotypic characteristics of *Cronobacter* spp. and other bacteria. It may be possible to link capsular profiles to not only colony morphologies and biofilm formation, but also other traits, particularly those behaviours that have been linked to virulence or environmental survival. Eventually, it may be possible predict any number of phenotypic traits, based only on an organism's genome sequence. In order to do this, however, phenotypes of interest must first be linked to genotypes. The work presented here is a first step toward this future, but much work is still needed to achieve accurate phenotypic prediction from genome sequences. One day, perhaps phenotypic characterization of bacterial species and strains will become obsolete as genome sequencing becomes cheaper, easier, and more accessible.

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# APPENDIX A

## Species identification using biochemical profiles from the archived NTU culture collection records

**Table A1.** *Cronobacter* spp. identifications using previous and current versions of the API20E database

**Table A2.** *Cronobacter* spp. identifications using previous and current versions of the ID32E database

**Table A1. *Cronobacter* spp. identifications using previous and current versions of the API20E database**

| | Previous identification | | | | Current identification (API20E v. 5.0) | | | |
|---|---|---|---|---|---|---|---|---|
| Profile | Species | %ID | Match[a] | Mismatch | Species | %ID | Match | Mismatch |
| 3305373 | *Enterobacter sakazakii* | 98.4 | 60 | 6 | *Cronobacter* spp. | 98.5 | 60 | 6 |
| 3307173 | *Enterobacter sakazakii* | 99.1 | 41 | 0 | *Cronobacter* spp. | 91.9 | 41 | 0 |
| 3305173 | *Enterobacter sakazakii* | 51.1 | 40 | 4 | *Cronobacter* spp. | 53.1 | 40 | 4 |
| 3205363 | *Enterobacter sakazakii* | 98.0 | 10 | 0 | *Cronobacter* spp. | 79.1 | 10 | 0 |
| 3306373 | *Enterobacter sakazakii* | 99.9 | 8 | 0 | *Cronobacter* spp. | 99.7 | 8 | 0 |
| 3305773 | *Enterobacter sakazakii* | 88.3 | 7 | 1 | *Enterobacter cloacae* | 91.4 | 1 | 7 |
| 3304373 | *Enterobacter sakazakii* | 97.3 | 5 | 0 | *Cronobacter* spp. | 97.4 | 5 | 0 |
| 3347373 | *Enterobacter sakazakii* | 99.9 | 4 | 0 | *Cronobacter* spp. | 99.9 | 4 | 0 |
| 1305373 | *Enterobacter sakazakii* | 82.1 | 3 | 0 | *Cronobacter* spp. | 83.0 | 3 | 0 |
| 3345373 | *Enterobacter sakazakii* | 99.9 | 3 | 0 | *Cronobacter* spp. | 99.9 | 3 | 0 |
| 3345773 | *Enterobacter sakazakii* | 99.5 | 3 | 0 | *Cronobacter* spp. | 96.5 | 3 | 0 |
| 3307773 | *Enterobacter sakazakii* | 98.4 | 3 | 0 | *Enterobacter cloacae* | 51.0 | 0 | 3 |
| 3205173 | *Enterobacter sakazakii* | 69.1 | 2 | 0 | *Cronobacter* spp. | 70.7 | 2 | 0 |
| 3306173 | *Enterobacter sakazakii* | 97.7 | 2 | 0 | *Cronobacter* spp. | 81.2 | 2 | 0 |
| 3205773 | *Enterobacter sakazakii* | 39.4 | 2 | 0 | *Enterobacter cloacae* | 72.0 | 0 | 2 |
| 3304173 | *Enterobacter sakazakii* | 51.1 | 2 | 0 | *Enterobacter amnigenus 1* | 53.9 | 0 | 2 |
| 3305573 | *Enterobacter cloacae* | 95.1 | 0 | 6 | *Enterobacter cloacae* | 97.7 | 0 | 6 |
| 3325373 | NS[b] | NS | - | - | *Cronobacter* spp. | 98.5 | 15 | 0 |
| 7325373 | NS | NS | - | - | *Cronobacter* spp. | UP | 5 | 0 |
| 2225373 | NS | NS | - | - | *Cronobacter* spp. | UP | 4 | 0 |
| 3325173 | NS | NS | - | - | *Cronobacter* spp. | 53.1 | 4 | 0 |
| 7325173 | NS | NS | - | - | *Enterobacter cloacae* | UP | 0 | 4 |
| 3325363 | NS | NS | - | - | *Cronobacter* spp. | 88.0 | 2 | 0 |
| Profiles appearing only once | | | | | | | | |
| 1304373, 3005373, 3105373, 3204373, 3205373, 3207373, 3215373, 3301273, 3305233, 3305253, 3305363, 3307053, 3347173, 3365373, 3367373, 7305373, 7347373, 3345573 | *Enterobacter sakazakii* | - | 17 | 1 | *Cronobacter* spp. | - | 17 | 1 |
| 3001173, 3106173, 1305173 | *Enterobacter sakazakii* | - | 2 | 1 | *Enterobacter amnigenus* I | - | 0 | 3 |
| 3307763 | *Enterobacter sakazakii* | 88.6 | 1 | 0 | *Serratia liquefaciens* | 82.8 | 0 | 1 |
| 3307573 | *Enterobacter sakazakii* | 77.7 | 1 | 0 | *Enterobacter cloacae* | 94.0 | 0 | 1 |
| 3304573, 3325571, 7305173 | *Enterobacter cloacae* | - | 0 | 3 | *Enterobacter cloacae* | - | 0 | 3 |
| 1305773 | *Enterobacter aerogenes* | 49.7 | 0 | 1 | *Enterobacter aerogenes* | 52.5 | 0 | 1 |
| 7315373 | *Enterobacter geroviae* | 91.2 | 0 | 1 | *Enterobacter geroviae* | 90.6 | 0 | 1 |
| 3324273, 3324373, 3325171, 3325353, 3325371, 3327373, 6302373, 3335173 | NS | - | - | - | *Cronobacter spp.* | - | 8 | 0 |
| 3325163, 3325573, 3325773, 3335773, 7324173 | NS | - | - | - | *Enterobacter cloacae* | - | 0 | 5 |
| 3325111 | NS | NS | - | - | *Enterobacter cancerogenus* | 99.7 | 0 | 1 |
| | | | 216 | 24 | | | 237 | 51 |
| | | | 90.0% | 10.0% | | | 82.3% | 17.7% |

[a]With the previous version of the API20E database, a species identification of '*E. sakazakii*' was considered to be correct as this previous designation included all species now known as *Cronobacter.* [b]NS, not specified

**Table A2. *Cronobacter* spp. identifications using previous and current versions of the ID32E database**

| | Previous identification | | | | Current identification (ID32E v. 4.0) | | | |
|---|---|---|---|---|---|---|---|---|
| Profile | Species | %ID | Match[a] | Mismatch | Species | %ID | Match | Mismatch |
| 34276767250 | *Enterobacter sakazakii* | 99.9 | 34 | 0 | *Cronobacter sakazakii* | 90.3 | 34 | 0 |
| 34276767050 | *Enterobacter sakazakii* | 99.9 | 22 | 0 | *Cronobacter sakazakii* | 47.8 | 16 | 6 |
| 34276763050 | *Enterobacter sakazakii* | 99.9 | 19 | 0 | *Cronobacter dublinensis* | 58.1 | 0 | 19 |
| 34276763250 | *Enterobacter sakazakii* | 99.9 | 8 | 2 | *Cronobacter malonaticus* | 59.9 | 0 | 10 |
| 34276763051 | *Enterobacter sakazakii* | 99.9 | 6 | 1 | *Cronobacter malonaticus* | 95.4 | 4 | 3 |
| 34276367250 | *Enterobacter sakazakii* | 99.9 | 6 | 0 | *Cronobacter sakazakii* | 94.2 | 5 | 1 |
| 34274767050 | *Enterobacter sakazakii* | 99.9 | 6 | 0 | *Cronobacter sakazakii* | 69.9 | 4 | 2 |
| 34276767051 | *Enterobacter sakazakii* | 99.9 | 5 | 1 | *Cronobacter turicensis* | 70.1 | 2 | 4 |
| 34277767051 | *Enterobacter sakazakii* | 99.9 | 5 | 0 | *Cronobacter muytjensii* | 90.5 | 3 | 2 |
| 34276767251 | *Enterobacter sakazakii* | 99.9 | 4 | 2 | *Cronobacter turicensis* | 91.6 | 0 | 6 |
| 34274763050 | *Enterobacter sakazakii* | 99.9 | 4 | 0 | *Cronobacter malonaticus* | 88.8 | 1 | 3 |
| 34276763251 | *Enterobacter sakazakii* | 99.9 | 3 | 1 | *Cronobacter malonaticus* | 84.4 | 3 | 1 |
| 10276767250 | *Enterobacter sakazakii* | 99.9 | 3 | 0 | *Cronobacter sakazakii* | 77.9 | 3 | 0 |
| 34277763050 | *Enterobacter sakazakii* | 99.9 | 3 | 0 | *Cronobacter dublinensis* | 99.9 | 1 | 2 |
| 30276763050 | *Enterobacter sakazakii* | 99.9 | 3 | 0 | *Cronobacter dublinensis* | 58.1 | 0 | 3 |
| 34277767050 | *Enterobacter sakazakii* | 99.9 | 3 | 0 | *Cronobacter dublinensis* | 99.3 | 0 | 3 |
| 34274763251 | *Enterobacter sakazakii* | 99.9 | 2 | 1 | *Cronobacter malonaticus* | 99.1 | 2 | 1 |
| 35276767051 | *Enterobacter sakazakii* | 99.9 | 2 | 1 | *Cronobacter turicensis* | 70.1 | 0 | 3 |
| 24276767250 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 98.1 | 2 | 0 |
| 34074763051 | *Enterobacter sakazakii* | 94.4 | 2 | 0 | *Cronobacter malonaticus* | 99.7 | 2 | 0 |
| 34274367250 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 95.9 | 2 | 0 |
| 34276767210 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 96.7 | 2 | 0 |
| 34276767270 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 90.3 | 2 | 0 |
| 34676767250 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 99.0 | 2 | 0 |
| 34274767251 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter malonaticus* | 67.5 | 1 | 1 |
| 34276765050 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter sakazakii* | 82.4 | 1 | 1 |
| 34774563051 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter malonaticus* | UP[c] | 1 | 1 |
| 30474563051 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Escherichia vulneris* | 79.8 | 0 | 2 |
| 34276767211 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter malonaticus* | 64.5 | 0 | 2 |
| 35276763050 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter dublinensis* | 58.1 | 0 | 2 |
| 35276763051 | *Enterobacter sakazakii* | 99.9 | 2 | 0 | *Cronobacter malonaticus* | 95.4 | 0 | 2 |
| 04476563051 | *Enterobacter sakazakii* | 99.9 | 1 | 1 | *Buttiauxella agrestis* | 89.4 | 0 | 2 |
| 34274767051 | *Enterobacter sakazakii* | 99.9 | 1 | 1 | *Cronobacter malonaticus* | 91.4 | 0 | 2 |
| 34474563051 | *Enterobacter sakazakii* | 99.9 | 1 | 1 | *Escherichia vulneris* | 80.3 | 0 | 2 |
| 34674563051 | *Enterobacter sakazakii* | 99.9 | 1 | 1 | *Cronobacter malonaticus* | 99.0 | 0 | 2 |
| 35276763250 | *Enterobacter sakazakii* | 99.9 | 1 | 1 | *Cronobacter malonaticus* | 59.9 | 0 | 2 |
| 34476563051 | *Enterobacter cloacae* | 99.9 | 0 | 2 | *Cronobacter malonaticus* | 94.1 | 0 | 2 |
| 30236747031 | NS[b] | NS | - | - | *Cronobacter malonaticus* | UP | 0 | 3 |
| 34274363060 | NS | NS | - | - | *Cronobacter malonaticus* | UP | 0 | 3 |
| 30074367061 | NS | NS | - | - | *Cronobacter malonaticus* | UP | 0 | 2 |
| 30236761001 | NS | NS | - | - | *Cronobacter malonaticus* | UP | 0 | 2 |
| 30276561001 | NS | NS | - | - | *Cronobacter malonaticus* | UP | 0 | 2 |

**Table A2. *Cronobacter* spp. identifications using previous and current versions of the ID32E database (continued)**

| | Previous identification | | | | Current identification (ID32E v. 4.0) | | | |
|---|---|---|---|---|---|---|---|---|
| Profile | Species | %ID | Match[a] | Mismatch | Species | %ID | Match | Mismatch |
| 24274767250, 24276767050, 24676767250, 30276767050, 30276767250, 30675567010, 34074763210, 34074767210, 34076767250, 34077767250, 34254367040, 34254767250, 34274767250, 34276267240, 34276366210, 34276367050, 34276367210, 34276765250, 34276777050, 34356767010, 34666767250, 34676767050, 35276767050, 75274763250, 75276767250 | *Enterobacter sakazakii* | - | 24 | 1 | *Cronobacter sakazakii* | - | 22 | 3 |
| 04014363051, 14234767010, 24216767050, 24276763051, 24276777051, 30274763051, 30276761051, 31276763051, 34074767051, 34216763050, 34256166211, 34266763251, 34274363050, 34274763010, 34274763051, 34274763150, 34274767451, 34276363040, 34276363250, 34276763010, 34276763011, 34276763211, 34674767251, 71274563011 | *Enterobacter sakazakii* | - | 23 | 1 | *Cronobacter malonaticus* | - | 7 | 17 |
| 34276743050, 34277763051, 34277767250, 34475773050, 75276763050 | *Enterobacter sakazakii* | 99.9 | 5 | 0 | *Cronobacter dublinensis* | - | 0 | 5 |
| 32276767051, 34277767251, 35276366251, 76376767051 | *Enterobacter sakazakii* | 99.9 | 4 | 0 | *Cronobacter turicensis* | - | 0 | 4 |
| 34575517331, 34575537333, 34575773333, 71575512311 | *Enterobacter sakazakii* | 99.9 | 4 | 0 | *Citrobacter koseri* | - | 0 | 4 |
| 34077767051, 34217360051 | *Enterobacter sakazakii* | - | 2 | 0 | *Cronobacter muytjensii* | - | 0 | 2 |
| 04275763310, 04275773310, 14075753330, 35077747231 | *Enterobacter sakazakii* | UP | 2 | 2 | *Enterobacter cloacae* | UP | 0 | 4 |
| 04077563310, 04574563050 | *Enterobacter sakazakii* | - | 1 | 1 | *Buttiauxella agrestis* | - | 0 | 2 |
| 30674773050, 34074767211 | *Enterobacter cloacae* | - | 0 | 2 | *Cronobacter malonaticus* | - | 1 | 1 |
| 04674563051 | *Enterobacter sakazakii* | 99.9 | 1 | 0 | *Escherichia vulneris* | 53.3 | 0 | 1 |
| 04475543011 | *Enterobacter sakazakii* | 99.9 | 1 | 0 | *Leclercia adecarboxylata* | 99.9 | 0 | 1 |
| 24574763022 | *Enterobacter sakazakii* | 99.9 | 1 | 0 | *Citrobacter freundii* | 99.8 | 0 | 1 |
| 43276767250 | *Enterobacter sakazakii* | 99.9 | 1 | 0 | *Serratia rubidea* | UP | 0 | 1 |
| 70075757333 | *Enterobacter sakazakii* | 99.9 | 1 | 0 | *Enterobacter aerogenes* | 99.9 | 0 | 1 |
| 75272763250 | *Citrobacter koseri* | NS | 1 | 0 | *Cronobacter sakazakii* | UP | 0 | 1 |
| 00476763051 | *Serratia plymuthica* | NS | 0 | 1 | *Cronobacter malonaticus* | 69.9 | 0 | 1 |
| 24074513011 | *Enterobacter cancerogenus* | 96.0 | 0 | 1 | *Enterobacter cancerogenus* | 96.0 | 0 | 1 |
| 30216060041 | *Stenotrophomonas maltophilia* | NS | 0 | 1 | *Stenotropomonas maltophilia* | UP | 0 | 1 |
| 34476563050 | *Enterobacter cloacae* | 99.9 | 0 | 1 | *Cronobacter sakazakii* | 88.8 | 0 | 1 |
| 35275777011 | *Citrobacter freundii* | 99.3 | 0 | 1 | *Cronobacter muytjensii* | UP | 0 | 1 |
| 74274777371 | *Enterobacter cloacae* | NS | 0 | 1 | *Enterobacter aerogenes* | UP | 0 | 1 |
| 34217041041 | UP | UP | 0 | 1 | UP | UP | 0 | 1 |
| 30064367071, 30074767070, 30254767010, 30276367070, 34074367070, 34254167050, 34256365060, 34274177060, 34276167060, 34276365060, 34276367051, 34276765110, 34276767010, 34276767070, 34676763000 | NS | NS | - | - | *Cronobacter sakazakii* | UP | 14 | 1 |
| 30074561041, 30076367061, 30076767071, 30274363060, 30276363060, 30276367051, 32274363060, 34076767061, 34254360050, 34254363070, 34274367041, 34274773010, 34276363060, 34276363070 | NS | NS | - | - | *Cronobacter malonaticus* | UP | 1 | 13 |
| 30276767051 | NS | NS | - | - | *Cronobacter turicensis* | 70.1 | 1 | 0 |
| 30030757331, 30034741001, 30036777301, 30236647321, 30236745031 | NS | NS | - | - | *Enterobacter cloacae* | 99.3 | 0 | 5 |
| 71256066200, 71276062200, 75276066200 | NS | NS | - | - | UP | UP | 0 | 3 |
| 30276375060 | NS | NS | - | - | *Serratia liquefaciens* | UP | 0 | 1 |
| 34255367060 | NS | NS | - | - | *Cronobacter dublinensis* | UP | 0 | 1 |
| | | | 240<br>88.9% | 30<br>11.1% | | | 139<br>43.2% | 183<br>56.8% |

[a] With the previous version of the ID32E database, a species identification of '*E. sakazakii*' was considered to be correct as this previous designation included all species now known as *Cronobacter.* [b] NS, not specified. [c] UP, unacceptable profile

# APPENDIX B

## Published works

Masood, N., **E. Jackson**, K. Moore, A. Farbos, K. Paszkiewicz, B. Dickins, A. McNally, and S. Forsythe. 2014. Draft genome sequence of "Candidatus *Cronobacter colletis*" NCTC 14934$^T$, a new species in the genus *Cronobacter. Genome Announc*. 2(3).

**Jackson, E. E.**, H. Sonbol, N. Masood, and S. J. Forsythe. 2014. Genotypic and phenotypic characteristics of *Cronobacter* species, with particular attention to the newly reclassified species *Cronobacter helveticus, Cronobacter pulveris,* and *Cronobacter zurichensis. Food Microbiol*. 44:226-35.

**Jackson, E. E.**, N. Masood, K. Ibrahim, N. Urvoy, S. Hariri, and S. J. Forsythe. 2015. Description of *Siccibacter colletis* sp. nov., a novel species isolated from plant material, and emended description of *Siccibacter turicensis*. *IJSEM*. 65:1335-41.

**Jackson, E. E.**, J. P. Flores, E. Fernandez-Escartin, and S. J. Forsythe. 2015. Reevaluation of a suspected *Cronobacter sakazakii* outbreak in Mexico. *J Food Prot*. 78:1191-6.

**Jackson, E. E.**, P. Ogrodzki, B. Pascoe, S. K. Sheppard, and S. J. Forsythe. 2016. Draft genome sequence of an *Enterobacter* species associated with illnesses and powdered infant formula. *Genome Announc*. 4(1).

**Jackson, E. E.**, and S. J. Forsythe. 2016. Comparative study of *Cronobacter* identification according to phenotyping methods. *BMC Microbiol*. 16(1).

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