Lack of continuity between *Cronobacter* biotypes and species as determined using multilocus sequence typing

Susan Joseph, Sumyya Hariri, Stephen. J. Forsythe

Pathogen Research Centre

School of Science and Technology

Nottingham Trent University

Clifton Lane

Nottingham, UK

NG11 8NS

Corresponding author: Prof SJ Forsythe.

Email: Stephen.forsythe@ntu.ac.uk. Tel: 0115 8483529

Abstract

The accuracy of the *Cronobacter* biotyping scheme was compared with the 7-loci multilocus sequence typing scheme. Biotyping did not reliably assign species level identification, as only half (17/31) of the biotype variants were unique to any of the seven *Cronobacter* species and the remaining biotypes were shared across the genus.

Main text

Cronobacter is a Gram-negative bacterial genus belonging to the family *Enterobacteriaceae* and currently comprises of seven species: *C. sakazakii, C. malonaticus, C. universalis, C. turicensis, C. muytjensii, C. dublinensis,* and *C. condimenti* [1]. *Cronobacter* spp. are opportunistic pathogens, and of special note due to the severe form of meningitis that can occur in neonates [2,3].

Initially Farmer et al. [4] used combinations of ten phenotyping tests to define 15 biotypes for the organism, then known as *Enterobacter sakazakii*. The ten defining traits were motility, acid production from inositol, ornithine utilization, malonate utilization, indole production, gas production from glucose, nitrate reduction, acid production from dulcitol, methyl-red and Voges-Proskaeur. We later expanded the definitions to include new strains in a 16th biotype, which with subdivisions gave a total of 31 biotype variants [5]. In 2007, the taxonomic reclassification of the species *E. sakazakii* into the genus *Cronobacter* [6] described 5 species, which were differentiated according to the biotyping scheme; *C. sakazakii* (biotypes 1-5, 7-9, 11, 13 & 14), *C. turicensis* (biotypes 16, 16a and 16b), *C. muytjensii* (biotype 15), *C. dublinensis* (biotypes 6, 10 and 12), and *Cronobacter* genomospecies 1. Soon afterwards, Iversen et al. [7] proposed the additional species *C. malonaticus*. This was composed of three biotypes (5, 9 & 14) which had previously been assigned to *C. sakazakii;* Table 1. However, some malonate utilizing strains (biotypes 8b & 8c) remained in the *C. sakazakii* species, hence causing some confusion in the use of phenotyping for speciation. This short communication reviews the limitations of the continued use of the biotyping scheme in the light of an improved understanding of the phylogeny of the *Cronobacter* genus as revealed by multilocus sequence typing (MLST), and supported by recent whole genome sequence analysis across the genus[8,9].

To overcome the subjectivity of phenotyping and microheterogeneties in 16S rDNA sequence analysis, we established a 7-loci multilocus sequence typing (MLST) scheme for the *Cronobacter* genus [8,10]. The MLST scheme is hosted by the University of Oxford (UK) as an open access, curated database at http://www.pubmlst.org/cronobacter and currently consists of 136 defined sequence types (ST)[2]. The MLST scheme was recently used in a polyphasic study, but without reference to biotypes, to describe two novel species *C. universalis* and *C. condimenti* [1]. The MLST scheme has also revealed a high level of clonality within the genus, the most notable being the strong association of *C. sakazakii* clonal complex 4 (composed of seven sequence types) with cases of neonatal meningitis [3,8,11]. Phylogenetic analysis based on 14 whole genome sequences across the seven *Cronobacter* species supported the phylogeny generated by MLST [8,9].

Despite the robustness of MLST, some researchers have continued to use biotyping to speciate their isolates [12,13,14]. Presented here is a study investigating the reliability of the biotyping scheme for identifying the *Cronobacter* species. For this study, 163 biotyped and sequence typed *Cronobacter* spp. strains covering all the seven species were analysed. The PCR primers and conditions used for the MLST were as previously described [8]. Background information on the strains and their MLST details are available with open access at http://www.pubmlst.org/cronobacter. The biotyping was as previously described, and were obtained by downloading from the *Cronobacter* PubMLST database and [4, 5]. A population distribution of the biotypes was plotted against the STs of the strains using the goeBURST algorithm in Phyloviz [15] and is presented in Fig. 1. This map clearly shows that each of the seven species contained unique STs, but overlapping biotypes.

Only seventeen out of 31 biotype variants were unique to five of the seven *Cronobacter* species; Table 1. There were no biotypes unique to *C. muytjensii* or *C. dublinensis*. The remaining biotypes were shared between at least two *Cronobacter* species; Table 1. Five of these were found to be between the closely related species *C. sakazakii* and *C. malonaticus*. Almost half (16/31) of the biotypes were in *C. sakazakii*, the most frequent ones being biotypes 1, 2 and 13. Biotype 1 strains were only found in *C. sakazakii,* and were spread across 11 STs. Forty strains were in *C. sakazakii* clonal complex 4 (associated with neonatal meningitis). These were primarily biotypes 1(18/40), and 13 (13/40), as well as biotypes 3, 4, and 7. These five biotypes were unique to the *C. sakazakii* species and not the clonal complex; Table 1. Clonal complex

4 also contained biotype 6, which was also found in *C. turicensis* and *C. muytjensii*. Therefore the clinically significant *C. sakazakii* CC4, associated with severe neonatal meningitis, cannot be described using biotyping as the biotypes are not unique to the *C. sakazakii* species, let alone sequence types. All the *C. sakazakii* ST1 and ST3 strains were biotype 2, and this biotype was also observed in *C. dublinensis*. Fourteen biotypes were distributed across the *C. malonaticus* STs, with ST7 (associated with adult infections) being dominated by biotype 9; Table 1. Although *C. malonaticus* was originally defined as *Cronobacter* biotypes 4, 9 and 13, MLST analysis revealed strains in biotypes 4a and 13a were in both *C. sakazakii* and *C. malonaticus*. Biotype 12 included *C. malonaticus* strains from three STs (84, 102, & 112). This also included the only non-*C. sakazakii* isolate from the 15 reported infant cases of *Cronobacter* infection in the US in 2011 [3]. Interestingly, this biotype is also shared with the *C. dublinensis* type strain LMG 23823 ^{T} (NTU strain 1210; ST 106).

The *C. universalis* type strain NCTC 9529^{T} is biotype 16c [16], yet this biotype was also shared with the species *C. turicensis*. The later species comprised 6 different biotypes, four of which were also shared with *C. sakazakii, C. malonaticus* or *C. universalis*. The *C. dublinensis* strains were in five different biotypes, all of which were shared with *C. sakazakii* strains. The lone *C. condimenti* strain was previously reported to be biotype 1 [17]. However this was found to be incorrect and was re-assigned to biotype 10a. All strains of *C. muytjensii*, except for one, belonged to biotype 15, which was also shared with *C. sakazakii* and *C. turicensis*. The lone exception was *C. muytjensii* strain 1129 which belonged to biotype 6. Interestingly, *C. muytjensii* has previously been reported to be very genetically diverse [6] and yet was dominated by only a single biotype 15. This indicates that the biochemical tests do not reflect the diversity within the species.

This study has revealed the lack of reliability in using the biotyping scheme for species level identification of the *Cronobacter* genus. It is undeniable that the initial biotyping scheme consisting of 10 differential tests chosen by Farmer et al [4] played a crucial role in the initial definition of the species *E. sakazakii*. However, with more sophisticated and accurate DNA based methods available for typing, the dependency on the biotyping has declined and therefore removes the subjectivity involved in the reporting of the results of these phenotypic tests.

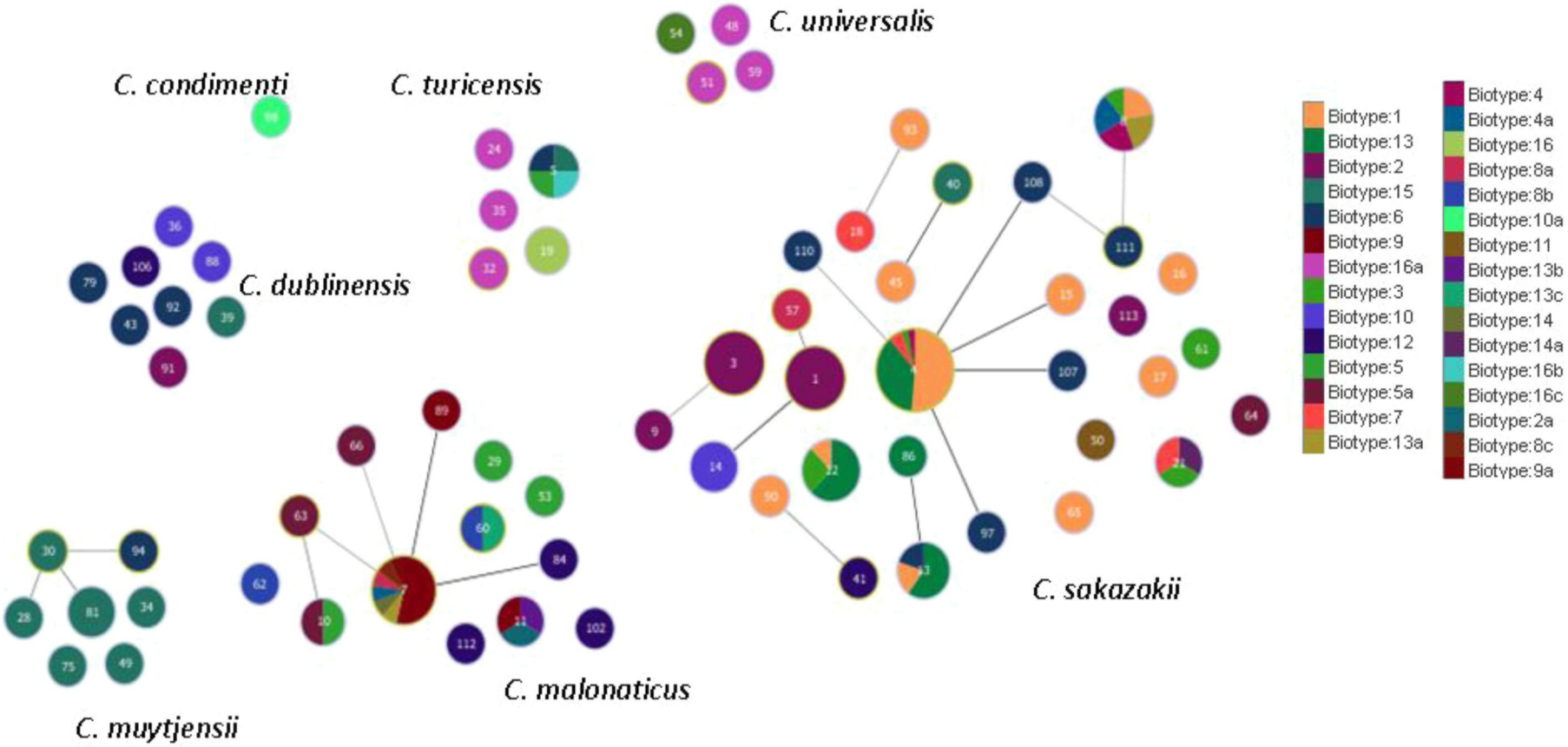

Fig. 1. Population snapshot graph created using the goeBURST algorithm to plot the *Cronobacter* biotypes against the STs. The dataset comprised of 163 *Cronobacter* isolates distributed across 68 STs.

References

1. Joseph S, Cetinkaya E, Drahovska H, Levican A, Figueras MJ, Forsythe SJ. *Cronobacter condimenti* sp. nov., isolated from spiced meat and *Cronobacter universalis* sp. nov., a novel species designation for *Cronobacter* sp. genomospecies 1, recovered from a leg infection, water, and food ingredients. Int J Syst Evol Microbiol 2012;62:1277-83.

2. Joseph S, Forsythe SJ. Insights into the emergent bacterial pathogen *Cronobacter* spp., generated by multilocus sequence typing and analysis. Frontiers in Food Microbiol 2012;3:397.

3. Hariri S, Joseph S, Forsythe SJ. Predominance of *Cronobacter sakazakii* ST4 clonal complex strains in *Cronobacter* neonatal meningitis infections in US 2011. Emerg Infect Dis 2012; 19:175-177.

4. Farmer JJ III, Asbury MA, Hickman FW, Brenner DJ & The Enterobacteriaceae study group. *Enterobacter sakazakii*: a new species of "Enterobacteriaceae" isolated from clinical specimens. Int J Syst Bact 1980;30:569-84.

5. Iversen C, Waddington M, Farmer JJ III, Forsythe S. The biochemical differentiation of *Enterobacter sakazakii* genotypes. BMC Microbiol 2006;6:94.

6. Iversen C, Lehner A, Mullane N, Bidlas E, Cleenwerck I, Marugg J et al. The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter* genomospecies 1. BMC Evol Biol 2007;7:64.

7. Iversen C, Mullane N, McCardell B, Tall BD, Lehner A, Fanning S et al. *Cronobacter* gen. nov., a new genus to accommodate the biogroups of *Enterobacter sakazakii*, and proposal of *Cronobacter sakazakii* gen. nov., comb. nov., *Cronobacter malonaticus* sp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov., *Cronobacter* genomospecies 1, and of three subspecies, *Cronobacter dublinensis* subsp. *dublinensis* subsp. nov., *Cronobacter dublinensis* subsp. *lausannensis* subsp. nov. and *Cronobacter dublinensis* subsp. *lactaridi* subsp. nov. Int J Syst Evol Biol 2008;58:1442-47.

8. Joseph S, Sonbol H, Hariri S, Desai P, McClelland M, Forsythe S. Diversity of the *Cronobacter* genus as revealed by multi locus sequence typing. J Clin Microbiol 2012;50:3031-39.

9. Joseph S, Desai P, Ji Y, Cummings CA, Shih R, Degoricija L et al. Comparative analysis of genome sequences covering the seven *Cronobacter* species. PLoS ONE 2012;7:e49455.

10. Baldwin A, Loughlin M, Caubilla-Barron J, Kucerova E, Manning G, Dowson C et al. Multilocus sequence typing of *Cronobacter sakazakii* and *Cronobacter malonaticus* reveals stable clonal structures with clinical significance which do not correlate with biotypes. BMC Microbiol 2009;9:223.

11. Joseph S, Forsythe SJ. Predominance of *Cronobacter sakazakii* ST4 in neonatal infections. Emerg Infect Dis 2011;17:1713-15.

12. Cruz A, Xicohtencatl-Cortes J, González-Pedrajo B, Bobadilla M, Eslava C, Rosas I. Virulence traits in *Cronobacter* species isolated from different sources. Can J Microbiol 2011;57:735–744

13. Hochel I, Růžičková H, Krásný L, Demnerová K. Occurrence of *Cronobacter* spp. in retail foods. J Appl Microbiol 2012;112:1257–1265.

14. Karamonová L, Junková P, Mihalová D, Javůrková B, Fukal L, Rauch P, Blažková M. The potential of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry for the identification of biogroups of *Cronobacter sakazakii*. Rapid Commun Mass Spectrom. 2013;27:409-18.

15. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carrico JA (2012). PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics 2012;13:87.

16. Iversen C, Waddington M, On SLW, Forsythe S. Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter* species. J Clin Microbiol 2004;42:5368-70.

17. Turcovsky I, Kunikova K, Drahovska H, Kaclikova E. Biochemical and molecular characterization of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) isolated from foods. Ant van Leeuwen 2011;99:257-69.

Table 1. Distribution of biotypes amongst *Cronobacter* species

Cronobacter species	Number of strains	Iversen et al. (2008)	Unique biotypes	Shared biotype	Comments
C. sakazakii	103	1-4, 7, 8, 11, 13	1, 3, 4, 7, 11, 13, 14a	2, 4a, 5a, 6, 8a, 10, 12, 13a, 15	
C. sakazakii ST1	8			2	
C. sakazakii ST3	9			2	
C. sakazakii CC4[a]	40		1, 3, 4, 7, 13	6	
C. sakazakii ST8	9		1		
C. malonaticus	29	5, 9, 14	2a, 8b, 8c, 9, 9a, 13b, 13c, 14	4a, 5, 5a, 8a, 12, 13a	
C. malonaticus ST7	13		9		
C. universalis	4	16c	16c	16a	
C. turicensis	9	16, 16a, 16b	16, 16b	5, 6, 15, 16a	
C. muytjensii	9	15		6, 15	No unique biotypes
C. dublinensis	8	6, 10, 12		2, 6, 10, 12, 15	No unique biotypes
C. condimenti	1	Not recognised	10a		

a CC4= clonal complex 4, composed of STs4, 15, 97, 107,108, 110 and 111 as previously described [8]