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

Susan Joseph[1], Esin Cetinkaya[2], Hana Drahovska[3], Arturo Levican[4], Maria J. Figueras[4] and Stephen J. Forsythe[1]

[1]School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK, NG11 8NS.

[2] Food Engineering Department, Engineering Faculty, Ankara University, Turkey

[3] Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovakia

[4] Unit of Microbiology, Rovira i Virgili University, IISPV, Sant Llorenc¸ 21, 43201 Reus, Spain

Running title: Two new *Cronobacter* species.

Subject category: New taxa

Author for correspondence: Stephen J Forsythe. School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK, NG11 8NS. Tel: +115 8483529, Fax: +115 8486636. Email: stephen.forsythe@ntu.ac.uk.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene (1361 bp) of strains 1330^T, NCTC 9529^T, 1435, 731 and 96 are FN539031, EF059877, JN205049, JN205050 and JN205051 respectively; the accession numbers for the genes *atpD*, *fusA*, *glnS, gltB, gyrB*, *infB, ppsA* of strains 1330^T, NCTC 9529^T, *C. sakazakii* ATCC 29544^T, *C. malonaticus* LMG 23826^T, *C. turicensis* LMG 23827^T, *C. muytjensii* ATCC 51329^T, *C. dublinensis* LMG 23823^T and *Citrobacter koseri* CDC 3613-63^T used in MLSA are from JF268258 to JF268314; they can also be directly accessed from the *Cronobacter* MLST database website (http://www.pubMLST.org/cronobacter) developed by Keith Jolley and sited at the University of Oxford (Jolley *et al*. 2004, *BMC Bioinformatics*, **5**:86). The development of this site has been funded by the Wellcome Trust.

Abstract

A re-evaluation of the taxonomic position of five strains, one assigned to *Cronobacter sakazakii* (1330^T), two previously identified as *Cronobacter* genomospecies 1 (strain NCTC 9529^T and strain 731), and two as *Cronobacter turicensis* (strain 96 and 1435) was carried out using a polyphasic approach. The analysis included a phenotypic characterization, sequencing of the 16S rRNA and a Multilocus sequence analysis (MLSA) of seven housekeeping genes (*atpD*, *fusA*, *glnS, gltB, gyrB*, *infB, ppsA*; 3036 bp). The 16S rRNA and MLSA analyses showed strain 1330^T, isolated from spiced meat purchased in Slovakia, to form an independent phylogenetic line. *Cronobacter dublinensis* was the closest neighbour species on the basis of the MLSA. DNA-DNA reassociation experiments and phenotypic analysis revealed that strain 1330^T represented a novel species, for which the name *Cronobacter condimenti* sp. nov. is proposed, type strain 1330^T =CECT 7863^T, =LMG 26250^T). The 4 bacterial strains NCTC 9529^T, 731, 96, and 1435, isolated from water, a leg infection and two food ingredients; onion powder and rye flour, respectively, showed on the MLSA phylogenetic tree to cluster together within an independent phylogenetic line, with *Cronobacter turicensis* as the closest species. The DNA-DNA hybridization data and the phenotypic characterization confirmed that these strains represented a novel species, for which the name *Cronobacter universalis* sp. nov. is proposed with type strain NCTC 9529^T =CECT 7864^T,=LMG 26249^T.

The genus *Cronobacter* was defined and created originally by the reclassification of the species *Enterobacter sakazakii* (Iversen *et al.*, 2007) and belongs to the class *Gammaproteobacteria* and to the family *Enterobacteriaceae*. It includes facultatively anaerobic, Gram-negative, oxidase negative, catalase positive, non-spore-forming rods which are generally motile, able to reduce nitrate to nitrite, produce acetoin (Voges-Proskauer test) and are negative for the methyl red test (Iversen *et al.*, 2007). Species of this genus are primarily inhabitants of plant material, and are often associated with human diseases most notably severe neonatal infections (Iversen & Forsythe 2004; Osaili & Forsythe 2009). The genus includes 5 species, which were differentiated according to the division of the 16 *E. sakazakii* biogroups (Farmer *et al.*, 1980; Iversen *et al.*, 2006b) as *Cronobacter sakazakii* (biogroups 1-4, 7, 8, 11 and 13), *Cronobacter malonaticus* (biogroups 5, 9 and 14), *Cronobacter turicensis* (biogroups 16, 16a and 16b), *Cronobacter muytjensii* (biogroup 15), and *Cronobacter dublinensis* (biogroups 6, 10 and 12) (Iversen *et al.*, 2007, 2008). Each biogroup being defined by their phenotype based on 10 tests. However, not all *E. sakazakii* strains were accommodated in the taxonomic revision into named *Cronobacter* species. Strain NCTC 9529^{T} (sole member of biogroup 16c) was deemed a separate *Cronobacter* species, however insufficient strains and biochemical tests were available to define the species and consequently it was called *Cronobacter* genomospecies 1 (Iversen *et al.*, 2007).

The taxonomy of the genus *Cronobacter* is complex due to the high inter-species similarity of the 16S rRNA gene sequences, which ranges from 97.8% to 99.7%, the overlap of biochemical profiles and a poor correlation between genotypic and phenotypic identification (Duaga & Breuwer, 2008; Kucerova *et al.*, 2010). Furthermore confusions with other members of the *Enterobacteriaceae* have been described i.e. a number of *Enterobacter cloacae* and *Enterobacter hormaechei* strains isolated from human infections have been misidentified as *Cronobacter* using phenotypic tests (Caubilla-Barron *et al.*, 2007; Townsend *et al.*, 2008). The existence of microheterogeneities in the 16S rRNA gene sequence is another factor that can generate misidentifications as has been reported for certain strains of *C. sakazakii* and *C. malonaticus* that could not be differentiated (Iversen *et al.* 2007). Analysis based on the sequences of housekeeping genes (multilocus sequence analysis, MLSA) has proven to be a useful tool for *Enterobacteriaceae* (Lacher *et al.*, 2007, Ibarz Pavón & Maiden 2009). Baldwin *et al.* (2009) applied a MLSA based on 7 housekeeping genes (*atpD*, *fusA*, *glnS, gltB, gyrB*, *infB* and *ppsA*) to *C. sakazakii* and *C. malonaticus* and demonstrated a robust phylogenetic frame to separate the two species. The latter study also showed that some previous confusion between the two species may have been due to incorrect speciation of some biotype index strains (Baldwin *et al.*, 2009). Further MLSA has revealed a clear differentiation between all the closely related *Cronobacter* species and the association of *C. sakazakii* sequence type 4 with neonatal meningitis (Joseph & Forsythe, in press; Kucerova *et al.*, in press). The scheme has open access at the web site http://www.pubMLST.org/cronobacter.

The present investigation was initiated to determine the taxonomic position of 5 *Cronobacter* strains recovered from: a leg infection, spiced meat, water and two food ingredients (onion powder and rye flour). A polyphasic approach based on MLSA of 7 genes, DNA-DNA reassociation experiments and phenotypic analysis was performed to establish the taxonomic status of these strains in the genus *Cronobacter*.

Cronobacter strain 1330^T was previously isolated from spiced meat (strain 040407/32; Turcovský *et al.*, 2011). Phenotypic analysis placed it in biogroup 1 and therefore as a strain of *C. sakazakii* (Iversen *et al.*, 2007). However partial 16S rRNA sequence analysis (657 bp) showed the nearest match was *C. dublinensis* (Turcovský *et al.*, 2011). Strain 1330^T was isolated from spiced meat purchased in Slovakia using selective enrichment at 45°C that consisted in a modified Lauryl Sulfate Tryptose (mLST) broth that included NaCl (0.5 mol l^{-1}) and vancomycin (10 mg l^{-1}). Colonies were recovered from *Cronobacter* chromogenic agar, as previously described (Turcovský *et al.*, 2011). Strain 1330^T was phenotypically verified as a member of the *Cronobacter* genus using a phenotyping kit (API 20E, bioMerieux), and additional recommended tests (Farmer *et al.*, 1980; Iversen *et al.*, 2006b) that enabled the strain to be classified as *Cronobacter* biogroup 1.

NCTC 9529^T was previously referred to as *E. sakazakii* biogroup 16c as defined by Iversen *et al.* (2006b) i.e. non-motile, able to produce acid from inositol and dulcitol, and to utilize malonate. The strain was later renamed as *Cronobacter* genomospecies 1 (Iversen *et al.*, 2007). The remaining strains within biogroup 16 were defined as *C. turicensis* based on phenotyping, DNA-DNA hybridization, and amplified fragment length polymorphism fingerprints (Iversen *et al.*, 2007).

Strain 731 was isolated in 2005 from post-operative mixed infection of a 9 year old boy with humeral fracture treated by osteosynthesis using intra medullar nailing. The strain was phenotypically identified as *E. sakazakii* and not further characterised. *Staphylococcus aureus* was also isolated from the infected site (Marie-Françoise Prère, pers. comm.).

Strain 96 was isolated from onion powder purchased in the UK using Enterobacteriaceae enrichment broth and *E. sakazakii* chromogenic agar, as previously described (Iversen & Forsythe 2004). The strain was phenotypically identified as a member of *E. sakazakii* and assigned to biogroup 16 (motile, acid production from inositol and dulcitol, positive for malonate and ornithine utilization; Iversen *et al.* 2006b). It was designated *E. sakazakii* cluster 2 according to partial 16S rRNA (528 bp) (GenBank accession number AY579172) and *hsp60* sequence analysis (GenBank accession number AY579197) by Iversen *et al.* (2004). Cluster 2 was later renamed, without further analysis of strain 96, as *C. turicensis* in the taxonomic revision of *E. sakazakii* (Iversen *et al.* 2007).

Strain 1435 was isolated from rye flour purchased in Turkey using Enterobacteriaceae enrichment broth and *Cronobacter* chromogenic agar. The strain was phenotypically identified as a member of the *Cronobacter* genus.

The phenotypic tests evaluated on strains 1330^T, NCTC 9529^T, 731, 96 and 1435 in the present study were selected from Iversen *et al*. (2006a, 2006b, 2007, 2008) and were the following: catalase and oxidase activity, nitrate reduction, acid production from sugars, malonate utilisation, production of indole from tryptophan, motility, gas from D-glucose, Voges-Proskauer (VP), methyl red, α-glucosidase activity, pigment production on TSA (21 and 37°C), aerobic and anaerobic growth on TSA (37°C), growth on MacConkey agar, and hydrolysis of DNA. Acid production from carbohydrates was determined in nutrient broth at a final concentration of 1% (w/v), supplemented with phenol red in the following substrates: sucrose, L-arabinose, cellobiose, lactose, raffinose, L-rhamnose, inositol, D-mannitol, D-sorbitol, N-acetylglucosamine and salicin. These tests were performed at least twice using conventional methods and additionally some tests (production of indole and hydrogen sulphide, VP test, α-glucosidase, β-galactosidase, presence of ODC, hydrolysis of gelatine and urea and acid production from D-mannitol, D-sorbitol, L-rhamnose, *myo*-inositol, sucrose and L-arabinose) were performed in parallel using commercial identification kits (API 20E, ID32E; bioMérieux). Fermentation/oxidation of 49 carbohydrates were tested using API50CH (bioMérieux) following the manufacturer's instructions. Appropriate positive and negative controls were included. All tests were evaluated for 48 hours and performed at 37°C. Type strains belonging to all species of the genus *Cronobacter* were evaluated under identical conditions to those for strains 1330^T, NCTC 9529^T, 731, 96 and 1435, for the selected differential tests included in Table 1. Between 3 to 12 of these tests are able to distinguish the new strains from other *Cronobacter* species.

Strains 1330^T, NCTC 9529^T, 731, 96, and 1435 were Gram-negative, oxidase negative, catalase positive, facultative anaerobic rods, positive for acetoin production (Voges-Proskauer), negative for methyl red and, produced yellow pigmentation on TSA at 21°C after 48 h incubation. They fermented glucose, saccharose, cellobiose, arabinose, mannitol, amygdaline, and galacturonic acid, reduced nitrate, utilised citrate, malonate and ornithine and produced delayed DNase activity. They did not hydrolyse urea, nor produced acid from sorbitol, 5-ketogluconate, or adonitol. These traits are common in the *Cronobacter* genus (Iversen et al. 2007, 2008).

Strain 1330^T was found to be biochemically different from all other species of the genus *Cronobacter* by at least 6 different tests (Table 1). As indicated above using criteria of Farmer *et al.* (1980), this strain has been classified as *C. sakazakii* (biogroup 1), but on the basis of the present results it can be differentiated from this species biogroup because it is not motile, by the ability to produce indole from tryptophan, to utilize malonate and the no production of acid from turanose, inositol, lactulose, putrescine, *cis*-aconitate, 4-aminobutyrate, maltitol and palatinose. Strain 1330^T was relatively similar to strains NCTC 9529^T, 731, 96, and 1435 but could also be differentiated from them by several tests i.e. indole production, and non acid production from dulcitol, melezitose, inositol, lactulose or maltitol (Table 1).

Strains NCTC 9529^T, 731, 96, and 1435 were found to be biochemically similar and different from all other species of the genus *Cronobacter* by at least 3 tests (Table 1). They can be differentiated from the species *C. turicencis* (biogroups 16, 16a and 16b) because they do not produce acid from turanose, putrescine, or 4-aminobutyrate.

The susceptibility of strains 1330^T, NCTC 9529^T, 731, 96 and 1435 to 17 antibiotics was assessed according to the standards and procedures of the British Society for Antimicrobial Chemotherapy (BSAC, 2010). The strains were classified as susceptible, intermediate, or resistant according to BSAC criteria. The following antibiotic-containing discs obtained from Mast Diagnostics: amikacin, (AK30), ampicillin (AP10), amoxicillin plus clavulanic acid (AUC30), cefotaxime (CTX30), cefuroxime (CXM30), cefpodoxime (CPD10), ceftazidime (CAZ30), chloramphenicol (C30), ciprofloxacin (CIP1), doxycycline (DXT30), gentamicin (GM10), imienem (IMI10), piperacillin plus tazobactam (PTZ85), tripethoprim (TM2.5), ceftazidime plus clavulanic acid (CAZ-CV), cefotaxime plus clavulanic acid (CTX-CV) and cefpodoxime plus clavulanic acid (CPD-CV) were tested.

For the phylogenetic studies of the 16S rRNA gene and for the seven mentioned housekeeping genes (MLSA), strains were cultured on tryptone soya agar (TSA) at 37°C. DNA was extracted from a single colony by using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) following the manufacturer's instructions. Primers and conditions for amplification and sequencing of the 16S rRNA (1361 bp), *atpD* (390 bp), *fusA* (438 bp), *glnS* (363 bp), *gltB* (507 bp), *gyrB* (402 bp), *infB* (441 bp) and *ppsA* (495 bp) genes have been described previously (Iversen *et al.*, 2007; Baldwin *et al.,* 2009). Amplified genes were sequenced with an ABI sequencer (Applied Biosystems). The sequences obtained were independently aligned with sequences of the type strains of all the species of the genus *Cronobacter* using the CLUSTALW2 program (Larkin *et al.*, 2007) and MEGA (Molecular Evolutionary Genetics Analysis) software version 4 (Tamura *et al.*, 2007). Genetic distances and clustering were determined using Kimura's two-parameter model (Kimura, 1980) and evolutionary trees were reconstructed by the neighbour-joining method (Saitou & Nei, 1987). Stability of the relationships was assessed by the bootstrap method (1000 replicates). The phylogenetic trees (Figs 1, 2) were constructed using new and available 16S rRNA gene sequences (1361 bp) for all *Cronobacter* spp. at GenBank and for the MLSA the existing sequences at the MLST database created by Baldwin *et al.* (2009) and the new ones obtained in this study. The 16S rRNA gene sequence similarities (1361 bp) were determined using Eztaxon tool (Chun *et al.*, 2007).

The 16S rRNA gene phylogenetic tree (Fig.1.) showed strain 1330^T and NCTC 9529^T as independent phylogenetic lines within the genus *Cronobacter* and strains 731, 96 and 1435^T. This last strain clustered within the branch that grouped the species *C. dublinensis* and *C. turicensis*, while strain 1330^T formed a totally independent branch with respect to the other species.

Similarly the 16S rRNA gene sequence similarity (1361 bp) between each other for strains 1330^T, and NCTC 9529^T and the other species of the genus *Cronobacter* ranged from 98.2% to 99.7%, corresponding from 24 to 4 bp differences respectively. The highest value was obtained between strain NCTC 9529^T and strain LMG 23827^T *C. turicencis* (99.7%, 4 bp) this being also the most similar species to strain 1330^T (98.6%) with 19 bp differences. Similarities between the strain NCTC 9529^T and strains 731, 96 and 1435 were 99.4 (8 bp), 99.6% (5 bp) and 99.7% (4 bp), respectively.

The MLSA analysis showed strains 1330^T, NCTC 9529^T, 731, 96 and 1435 were within the cluster of the *Cronobacter* spp., but represented two independent branches (Fig. 2). The MLSA tree revealed strain 1330^T to be more phylogenetically closer neighbour to *C. dublinensis* despite the highest 16S rRNA gene similarity was obtained with *C. turicencis*. However, the latter species was the closest neighbour to strains NCTC 9529^T, 731, 96 and 1435, as also shown with 16S rRNA gene sequence analysis.

DNA-DNA hybridisation (direct and reciprocal) experiments were performed between strain 1330^T and NCTC 9529^T and between the former and the type strains of the currently accepted *Cronobacter* species. DNA was extracted using the method described by Marmur (1961) and DNA-DNA hybridisation was conducted using the method described by Urdiain *et al.*, (2008). Renaturalisation was performed under optimal conditions at 68°C, single- and double-stranded DNA molecules were separated by the use of hydroxyapatite and colour development was measured at 405 nm using a Bio Whittaker Kinetic-QCL microplate reader. Values of DNA-DNA reassociation were determined at least three times. The DNA-DNA hybridisation results between strain 1330^T, and NCTC 9529^T, and of the former with the type strains of all other species are shown in Table 2. All results were below the 70% limit for the species definition (Wayne *et al.,* 1987; Stackebrandt & Goebel, 1994) as did previously obtained DNA-DNA results (also shown in table 2) for strain NCTC 9529^T (Iversen *et al.,* 2008) as shown in Table 2. Despite DNA-DNA reassociation being considered to give information on the DNA similarity between entire bacterial genomes, it has been criticized because of the high number of experimental errors, lack of reproducibility and failure to generate collective databases (Rosselló-Mora, 2006). Moreover, DNA-DNA reassociation values do not provide any information concerning phylogenetic relationships (Harayama & Kasai, 2006), in contrast to the phylogenetic reconstruction with the MLSA (Baldwin *et al.,* 2009) applied in the present study.

The polyphasic approach using the 16S rRNA gene sequencing, MLSA, DNA-DNA reassociation results and phenotypic characterisation all clearly differentiate strains 1330^T, NCTC 9529^T, 731, 96 and 1435 from the existing *Cronobacter* species and therefore *Cronobacter condimenti* (type strain 1330^T, =LMG 26250^T, =CECT 7863^T) and *Cronobacter universalis* (type strain NCTC 9529^T, =CECT 7864^T, =LMG 26250^T) are proposed as new species.

Description of *Cronobacter condimenti* sp. nov.

Cronobacter condimenti (con.di.men'ti. L. gen. n. condimenti, of spice, seasoning)

Cells of strain 1330^T are straight, Gram-stain-negative, non-spore-forming rods, non- motile. Colonies on TSA incubated at 37°C for 24h are 2-3 mm in diameter, opaque, circular and pigmented yellow in colour at 37°C. Strain 1330^T grows on MacConkey agar. Optimal growth occurs at 37°C after 24h in TSB and also at 45°C but no growth is observed at 5°C. No haemolysis is observed on sheep blood agar at 37°C. The strain produces catalase, α-glucosidase, β-galactosidase, and DNase activities, indole from tryptophan, acetoin (Voges-Proskauer positive), hydrolyses gelatine and reduces nitrate. Strain 1330^T does not produce oxidase activity, does not produce hydrogen sulphide, does not hydrolyse urea, and does not produce gas from glucose. Strain 1330^T is able to utilize ornithine, citrate, lysine, and malonate produce acid from 1-0-methyl α– D-glucopyranoside, glycerol, L-arabinose, ribose, D-xylose, galactose, D-glucose, D-fructose, D-manose, L-rhamnose, D-mannitol, N-acetyl glucosamine, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, myo-inositol, D-raffinose, β-gentiobiose, D-fucose, L-fucose or galacturonic acid. The strain does not ferment dulcitol, inositol, melezitose, turanose, lactulose, putrescine, *cis*-aconitate, *trans*-aconitate, 4-amino-butyrate, maltitol, palatinose, D-erythritol, D-arabinose, adonitol, β-methyl-D-xyloside, L-sorbose, dulcitol, sorbitol, α-methyl-D-glucoside, inulin, glycogen, xylitol, D-lyxose, D-tagatose, D-arabitol, L-arabitol, gluconate, or (2&) 5-ketogluconate. Strain 1330^T is resistant to doxycycline and susceptible to the rest of the antimicrobials tested. The API 20E and ID32E profiles obtained for strain 1330^T were 3367373 and 342137610030 respectively.

The type strain is 1330^T (=CECT 7863^T, = LMG 26250^T), isolated from spiced meat purchased in Slovakia.

Description of *Cronobacter universalis* sp. nov.

Cronobacter universalis (u.ni.ver.sa'lis. L. masc. adj. universalis, of or belonging to all, universal)

Cells of strains NCTC 9529^T, 731, 96 and 1435 are straight, Gram-stain-negative, non-spore-forming rods, variable motility (strain NCTC 9529^T is non-motile). Colonies on TSA incubated at 37°C for 24h are 2-3 mm in diameter, opaque, circular and pigmented yellow in colour at 37°C. All strains grow on MacConkey agar. Optimal growth occurs at 37°C after 24h in TSB and also at 45°C, but no growth is observed at 5°C. No haemolysis is observed on sheep blood agar at 37°C. Strains produce acetoin (Voges-Prokauer positive), catalase, α-

glucosidase, β-galactosidase and DNase activities, and reduce nitrate. Strains do not produce oxidase activity, do not produce indole from tryptophan, do not produce hydrogen sulphide, hydrolyse gelatine or urea. Strains are able to use malonate, ornithine or citrate and to produce acid from glucose, 1-0-methyl α-D-glucopyranoside, dulcitol, inositol, melezitose, lactulose, sucrose, L-arabinose, cellobiose, lactose, myo-inositol, L-rhamnose, D-mannitol, N-acetyl glucosamine, salicin, maltitol, D-fucose, amygdaline or galacturonic acid, but not able to produce acid from turanose, D-sorbitol, putrescine, *trans*-aconitate, L-fucose, adonitol, 5-ketogluconate or 4-aminobutyrate. Variable results are obtained for the acid production from *cis*-aconitate (NCTC 9529^T is negative), palatinose (NCTC 9529^T is negative), production of gas from glucose (NCTC 9529^T is negative). Strains are resistant to doxycycline and susceptible to the rest of the antimicrobials tested. The API 20E and ID32E profiles obtained for strains NCTC 9529^T were 3205373 and 24276777051 respectively.

The type strains is NCTC 9529^T (= CECT 7864^T, =LMG 26250^T), isolated from freshwater and deposited at the NCTC (London) in 1954.

Acknowledgements

We are grateful to Nottingham Trent University for their financial support. We are also grateful to Dr. Roxana Beaz-Hidalgo for her assistance with the DNA-DNA hybridisation and also thank Jean Euzeby for helping to correct the species name.

References

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

British Society for Antimicrobial Chemotherapy. (2010). BSAC methods for antimicrobial susceptibility testing, version 9.
http://www.bsac.org.uk/Resources/BSAC/Version_9.1_March_2010_final.pdf.

Caubilla-Barron, J., Hurrell, E., Townsend, S., Cheetham, P., Loc-Carrillo, C., Fayet, O., Prere, M. -F., & Forsythe, S.J. (2007). Genotypic and phenotypic analysis of *Enterobacter*

sakazakii strains from an outbreak resulting in fatalities in a neonatal intensive care unit in France. *J Clin Microbiol* **45**, 3979-3985.

Chun, J., Lee, J.H., Jung, Y., Kim, M., Kim, S., Kim, B.K. & Lim, Y.W. (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. *Int J Syst Evol Microbiol* **57,** 2259-2261.

Duaga, C. & Breuwer, P. (2008). Taxonomy and physiology of *Enterobacter sakazakii*. In *Enterobacter sakazakii,* pp 1-26. Edited by J.M. Farber & S.J. Forsythe. Washington, D.C.: ASM Press.

Farmer, J.J. III, Asbury, M.A., Hickman, F.W., Brenner, D.J. and the Enterobacteriaceae study group. (1980) *Enterobacter sakazakii*: a new species of "Enterobacteriaceae" isolated from clinical specimens. *Int J Syt Bacteriol* **30,** 569-584.

Forsythe, S. (2005). *Enterobacter sakazakii* and other bacteria in powdered infant milk formula. *Mother Child Nutrn* **1**, 44-50.

Harayama, S. & Kasai, H. (2006). Bacterial phylogeny reconstruction from molecular sequences. In *Molecular Identification, Systematics, and Populaton Structure of Prokaryotes,* pp. 105-140. Edited by E. Stackebrandt. Berlin, Heidelerg: Springer-Vaerlag.

Ibarz Pavón, A.B. & Maiden, M.C. (2009) Multilocus sequence typing. *Methods Mol Biol* **551**, 129-40.

Iversen, C. & Forsythe, S.J. (2004). Isolation of *Enterobacter sakazakii* and other Enterobacteriaceae from powdered infant formula milk and related products. *Food Microbiol* **21**, 771-776.

Iversen, C., Waddington, M., On, S.L.W. & Forsythe, S. (2004). Identification and phylogeny of *Enterobacter sakazakii* relative to *Enterobacter* and *Citrobacter*. *J Clin Microbiol* **142**, 5368-5370.

Iversen, C., Lancashire, L., Waddington M., Forsythe, S. & Ball, G. (2006a). Identification of *Enterobacter sakazakii* from closely related species: The use of Artificial Neural Networks in the analysis of biochemical and 16S rDNA data. *BMC Microbiol* **6**, 28.

Iversen, C., Waddington, M., Farmer, J.J. III & Forsythe, S. (2006b). The biochemical differentiation of *Enterobacter sakazakii* genotypes. *BMC Microbiol* **6**, 94.

Iversen, C., Lehner, A., Mullane, N., Bidlas, E., Cleenwerck, I., Marugg, J., Fanning, S., Stephan, R. & Joosten, H. (2007). 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* **7**, 64.

Iversen, C., Mullane, N., McCardell, B., Tall, B.D., Lehner, A., Fanning, S., Stephan, R. & Joosten, H. (2008). *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 Microbiol* **58**, 1442-1447.

Jolley, K.A., Chan, M-S., & Maiden, M.C.J. (2004). mlstdbNet-distributed multi-locus sequence typing (MLST) databases**.** *BMC Bioinformatics* **5:**86.

Joseph, S. & Forsythe, S.J. (In press). Predominance of *Cronobacter sakazakii* ST4 with neonatal infections. *Emerging Infectious Disease*.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16, 111-120.

Kucerova, E., Clifton, S.W, Xia, X-Q, Long, F., *et al.* (16 other authors) (2010). Genome sequence of *Cronobacter sakazakii* BAA-894 and comparative genomic hybridization analysis with other *Cronobacter* species. *PLoS ONE* **5**(3): e9556.

Kucerova, E., Joseph, S., & Forsythe, S. (In press). The *Cronobacter* genus: ubiquity and diversity. *Quality Assurance and Safety of Crops & Foods*.

Lacher, D.W., Steinsland, H., Black, T.E., Donnenberg, M.S., & Whittam, T.S. (2007). Molecular evolution of typical enteropathogenic *Escherichia coli*: clonal analysis by Multilocus sequence typing and virulence gene allelic profiling. *J. Bacteriol* **189,** 342-350.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.

Osaili, T., & Forsythe, S. (2009). Desiccation resistance and persistence of *Cronobacter* species in infant formula. *Intl J Food Microbiol* **136,** 214-220.

Rosselló-Mora, R. (2006). DNA-DNA reassociation applied to microbial taxonomy ad their critical evaluation. In *Molecular Identification, Systematics, and Population Structure of Prokaryotes,* pp 23-50. Edited by E. Stackebrandt. Berlin: Springer.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4,** 406-425.

Stackebrandt, E. & Goebel, B.M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846-849.

388 **Tamura, K., Dudley, J., Nei, M., & Kumar, S. (2007).** MEGA4: Molecular Evolutionary
389 Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **24**, 1596-1599.

390 **Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H.,**
391 **Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., & Higgins,**
392 **D.G.** (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23,** 2947-8.
393
394 **Townsend, S.M., Hurrell, E., Caubilla-Barron, J., Loc-Carrillo, C. & Forsythe, S.J. (2008).**
395 Characterization of an extended-spectrum beta-lactamase *Enterobacter hormaechei*
396 nosocomial outbreak, and other *Enterobacter hormaechei* misidentified as *Cronobacter*
397 *(Enterobacter) sakazakii. Microbiology* **154**, 3659-3667.

398 **Turcovský, I., Kuniková, K., Drahovská, H., & Kaclíková, E. (2011).** Biochemical and
399 molecular characterization of *Cronobacter* spp. (formerly *Enterobacter sakazakii*) isolated
400 from foods. *Antonie van Leeuwenhoek*. **99,** 257-69.

401 **Urdiain, M., López-López, A., Gonzalo, C., Busse, H. J., Langer, S., Kämpfer, P. & Rosselló-**
402 **Mora, R.** (2008). Reclassification of *Rhodobium marinum* and *Rhodobium pfennigii* as *Afifella*
403 *marina* gen. nov. comb. nov. and *Afifella pfennigii* comb. nov., a new genus of
404 photoheterotrophic Alphaproteobacteria and emended descriptions of *Rhodobium*,
405 *Rhodobium orientis*and *Rhodobium gokarnense*. *Syst Appl Microbiol* **31**, 339–351.

406 **Wayne, L.G., Brenner, D.J., Colwell, R.R., Grimont, P.A.D., Kandler, O., Krichevsky, M.I.,**
407 **Moore, L.H., Moore, W.E.C., Murray, R.G.E. & other authors (1987).** International
408 Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of
409 approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463-464.

410

Table 1. Key tests for phenotypic differentiation of *Cronobacter condimenti* sp. nov. and *Cronobacter universalis* sp. nov. from other species of the genus *Cronobacter*.

Cronobacter species: 1, *C. condimenti* sp. nov. (1330^T) ; 2, *C. universalis* sp. nov. (n=4); 3, *C. sakazakii* (ATCC 29544^T); 4, *C. malonaticus* (LMG 23826^T) ; 5, *C. turicensis* (LMG 23827^T); 6, *C. muytjensii* (ATCC 51329^T); 7, *C. dublinensis* subsp. *dublinensis* (LMG 23823^T) ;8, *C. dublinensis* subsp. *lactaridi*; 9, *C. dublinensis* subsp. *lausannensis*.

Characteristic	**1**	**2**	3*	4*	5*	6*	7*	8*	9*
Motility	-	**v(-)**	+(+)	v (+)	+(+)	+(+)	+(+)	+	+
Carbon utilization:									
Dulcitol	-	**+(+)**	-(-)	-(-)	+(+)	+(+)	-(-)	-	-
Indole	**+**	**-(-)**	-(-)	-(-)	-(-)	+(+)	+(+)	+	v
Malonate	**+**	**+(+)**	-(-)	+(+)	v(+)	+(+)	+(+)	-	-
1-0-Methyl α-D-glucopyranoside (AMG)	**+**	**+(+)**	+(+)	+(+)	+(+)	-(-)	+(+)	+	+
Melezitose	-	**+(+)**	-(-)	-(-)	+(+)	-(-)	+(+)	-	-
Turanose	-	**-(-)**	+(+)	+(+)	+(+)	v(+)	+(+)	v	-
Inositol	-	**+(+)**	v(+)	v(-)	+(+)	+(+)	+(+)	+	-
Lactulose	-	**+(+)**	+(+)	+(+)	+(+)	+(+)	+(+)	+	-
Putrescine	-	**-(-)**	+(+)	v(+)	+(+)	+(+)	+(+)	+	v
Cis-aconitate	-	**v(-)**	+(+)	+(+)	+(+)	+(+)	+(+)	+	+
Trans-aconitate	-	**-(-)**	-(-)	+(+)	-(-)	+(+)	+(+)	+	+
4-Aminobutyrate	-	**-(-)**	+(+)	+(+)	+(+)	v(+)	+(+)	+	+
Maltitol	-	**+(+)**	+(+)	+(+)	+(+)	-(-)	+(+)	+	-
Palatinose	-	**v(-)**	+(+)	+(+)	+(+)	v(+)	+(+)	+	+

*Results from Iversen *et al.* 2007 and 2008, except for *Cronobacter* species type strains (shown in parenthesis) which were obtained in this study. V, 20-80 % variable results.

Table 2. DNA-DNA (mean % ±SD) relatedness of *Cronobacter condimenti* sp. nov. and *Cronobacter universalis* sp. nov. with other species of the genus. Data from Iversen *et al.*, 2008[¶] and from this study.

	C. universalis sp. nov. NCTC 9529^{T}[¶]	*C. condimenti* sp. nov. 1330^{T}
C. sakazakii ATCC 29544^{T}	55.5% ± 1.0	40.3% ± 7.7
C. malonaticus CDC 1058-77^{T}	60.1% ± 1.3	53.0% ± 14.4
C. muytensii ATCC 51329^{T}	53.1% ± 6.6	42.0% ± 9.3
C. dublinensis LMG 23823^{T}	45.9% ± 2.0	54.2% ± 8.7
C. turicencis LMG 23827^{T}	55.0% ± 3.3	47.9% ± 5.9
C. universalis sp. nov. NCTC 9529^{T}		50.7% ± 7.6

Figure 1. Unrooted phylogenetic tree derived from 16S rRNA gene sequences (1361 bp) showing relationships of strains 1330^T, NCTC 9529^T, 96, 731 and 1435 to all other described species of the genus *Cronobacter*. The tree was reconstructed using the neighbour-joining method. Numbers at nodes indicate bootstrap values (>70%) based on 1000 replications. Bar 5 substitutions per 1000 nucleotide positions.

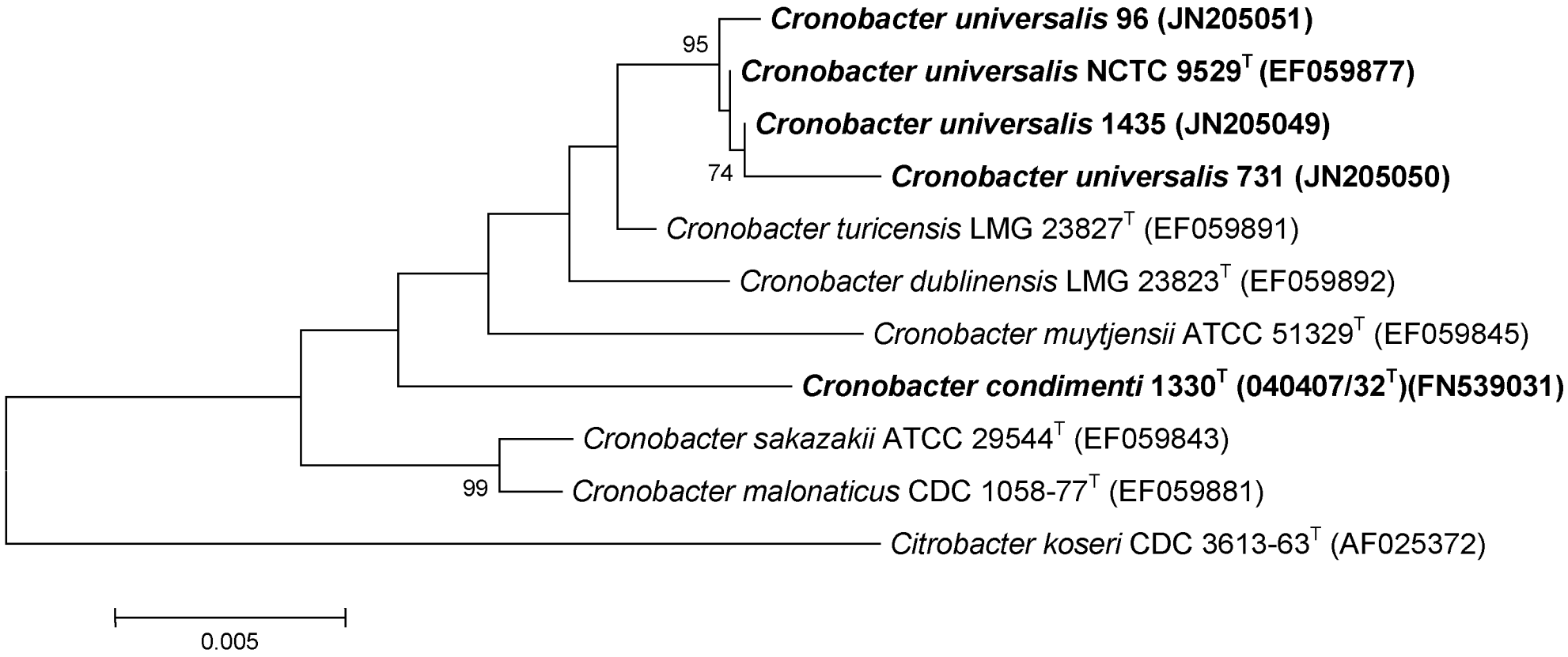

Figure 2. Unrooted neighbour-joining tree based on the concatenated atpD, fusA, glnS, gltB, gyrB, infB, and ppsA (7) gene sequences showing the phylogenetic position of strains 1330T, NCTC 9529T, 96, 731 and 1435 within Cronobacter species. Bootstrap values (>70 %) based on 1000 replications are shown at the nodes of the tree. Bar, 1 substitution per 100 nt.