Multilocus sequence typing of Cronobacter sakazakii and Cronobacter malonaticus reveals stable clonal structures with clinical significance which do not correlate with biotypes

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Abstract

Background: The Cronobacter genus (Enterobacter sakazakii) has come to prominence due to its association with infant infections, and the ingestion of contaminated reconstituted infant formula. C. sakazakii and C. malonaticus are closely related, and are defined according their biotype. Due to the ubiquitous nature of the organism, and the high severity of infection for the immunocompromised, a multilocus sequence typing (MLST) scheme has been developed for the fast and reliable identification and discrimination of C. sakazakii and C. malonaticus strains. It was applied to 60 strains of C. sakazakii and 16 strains of C. malonaticus, including the index strains used to define the biotypes. The strains were from clinical and non-clinical sources between 1951 and 2008 in USA, Canada, Europe, New Zealand and the Far East.

Results: This scheme uses 7 loci; atpD, fusA, glnS, gltB, gyrB, infB, and pps. There were 12 sequence types (ST) identified in C. sakazakii, and 3 in C. malonaticus. A third (22/60) of C. sakazakii strains were in ST4, which had almost equal numbers of clinical and infant formula isolates from 1951 to 2008. ST8 may represent a particularly virulent grouping of C. sakazakii as 7/8 strains were clinical in origin which had been isolated between 1977 - 2006, from four countries. C. malonaticus divided into three STs. The previous Cronobacter biotyping scheme did not clearly correspond with STs nor with species.

Conclusion: In conclusion, MLST is a more robust means of identifying and discriminating between C. sakazakii and C. malonaticus than biotyping. The MLST database for these organisms is available online at http://pubmlst.org/cronobacter/.

Background
The genus Cronobacter is composed of Gram-negative, facultative aerobic rods, which are members of the Enterobacteriaceae Family. It was formerly known as Enterobacter sakazakii and was divided into 15 biotypes [1]. The biotyping scheme was based on Voges-Proskauer, methyl red,
indole, ornithine decarboxylase, motility, reduction of nitrate to nitrite, production of gas from D-glucose, malonate utilization and production of acid from myo-inositol and dulcitol. Based on 16S rDNA sequence analysis, we extended this further to 16 biotypes [2,3] which has contributed to the recent taxonomic revisions. Initially the Cronobacter genus was composed of 4 species; C. sakazakii, C. turicensis, C. mputjensii, C. dublinensis, plus a possible fifth species [4]. More recently, the species C. malonaticus sp. nov. was proposed [5]. This was initially regarded as a subspecies of C. sakazakii as the two species could not be distinguished according to 16S rDNA sequence analysis however DNA-DNA hybridisation studies revealed a <70% DNA relatedness. Consequently C. sakazakii consists of biotypes 1-4, 7 & 8, 11 & 13, and C. malonaticus contains biotypes 5, 9 and 14 [5].

Cronobacter spp. have come to prominence due to their association with infant infections, and cases linked to the ingestion of contaminated reconstituted infant formula [6-8]. However not all cases have been linked to formula ingestion. The organism is ubiquitous in the environment (water and soil) and food [9,10].

Cronobacter spp. cause infections across all age groups [11]. However neonates, particularly those of low-birth weight, are the major identified group at risk with a high mortality rate [6,11]. The organism is a rare cause of neonatal meningitis, necrotising enterocolitis (NEC) and septis. A number of outbreaks of C. sakazakii have been reported in neonatal intensive care units around the world [12-16]. The International Commission for Microbiological Specifications for Foods (2002) [17] has ranked Cronobacter spp. as ‘severe hazard for restricted populations, life-threatening or substantial chronic sequelae or long duration’. The FAO/WHO [6,7,11] have undertaken three risk assessments of the organism in powdered infant formula, and the WHO [18] have published recommended procedures for the reconstitution of powdered infant formula to reduce the risk of infection to neonates. Together with the ubiquitous nature of the organism, and the high severity of infection for the uncompromised, there is a need for a technique that enables fast and reliable classification and identification of Cronobacter strains worldwide.

Selected strains of Cronobacter spp. have been shown to invade human intestinal cells, replicate in macrophages, and invade the blood brain barrier [19,20]. Based on the clinical outcome of different pulsetypes during a neonatal intensive care unit outbreak it was proposed that certain types of C. sakazakii are particularly virulent [16,20]. Whether the virulence was linked to a particular genotype or phenotype warranted further investigation.

16S rDNA sequences can be useful to determine phylogeneies between distantly related Enterobacteriaceae [21]. However it is less discriminatory and unclear for more closely related organisms. An alternative to rDNA sequence analysis is the partial sequencing of protein-encoding genes. Additionally, for determining phylogenetic relationships, sequence data from more than one gene should be used to reduce the possibly of ambiguities caused by genetic recombination or specific selection [21,22]. A number of such genes have been used as phylogenetic markers for members of the Enterobacteriaceae. Genes which have been analysed include rpoB, gyrB, mdh, infB and recA [23,24]. These results can be more reliable for species identification and determining intra- and intergeneric relationships than 16S rDNA gene sequencing. Recently, Kuhnert et al. [25] used three loci (recN, rpoA and thdF) for 30 species of Enterobacteriaceae including Cronobacter spp. Whereas our work is focussed on a higher resolution analysis of C. sakazakii and C. malonaticus using 7 loci. The genes under study were atpD, fusa, gns, gltB, gyrB, infB, and pps. These genes encode for ATP synthase B subunit, elongation factor EF-2, glutamyl-tRNA synthetase, glutamate synthase large subunit, type IIA topoisomerase, initiation translation factor 2 and phosphoenolpyruvate synthase, respectively. These were compared with the previous biotyping scheme for Cronobacter spp. and also considered the source and isolation date of the strains.

Results

Development of the Cronobacter MLST scheme

Several criteria were used in the selection of all potential loci. Genes included were those encoding for putative housekeeping products necessary for biological roles in DNA repair, replication and amino acid biosynthesis. Genes that were either a) located near to or b) implicated as being, putative virulence factors and mobile elements were avoided as these may come under greater selective evolutionary pressures than other genes. The selected loci were distributed as much as possible across the chromosome to ensure that each locus was genetically unlinked. Each gene fragment was also required to be approximately 500 bp in length to facilitate the design of universal nested primers for each locus preferably in conserved flanking regions around a variable central core. Using these criteria, genes were selected and the chromosomal locations of all these loci were confirmed by bioinformatic analysis of the C. sakazakii BAA-894 genome sequence (Genbank accession number CP000785).

The genes selected for the MLST scheme (atpD, fusa, gns, gltB, gyrB, infB and pps) are shown in Table 1 along with putative gene products, gene sizes, primers and location within the C. sakazakii ATCC BAA-894 genome.
Allelic variation

Novel sequence information for all seven loci was obtained from a collection of 60 \textit{C. sakazakii} and 16 \textit{C. malonaticus} strains. To assess the performance of the MLST scheme, \textit{Cronobacter} strains were selected to be representative of the different biotypes (most of which were previously derived in an earlier study \cite{3}), and were also distributed both temporally and geographically in terms of their isolation (See Additional file 1). \textit{In silico} sequence data was also obtained for all the loci from \textit{C. sakazakii} strain ATCC BAA-894 (Accession No. \textit{CP000785}), \textit{Citrobacter koseri} strain ATCC BAA-895 (Accession No. \textit{CP000822}), and \textit{Enterobacter} species strain 683 (Accession No. \textit{CP000653}). The latter strain sequence data was used to root the data set. The seven alleles obtained for the \textit{C. sakazakii} genome reference strain BAA-894 were identical to the online genome sequence (\textit{CP000785}).

### Table 1: Oligonucleotide nested primer sequences for the amplification and sequencing of the seven loci from genes in \textit{C. sakazakii} and \textit{C. malonaticus}, with gene number and location of genes within the genome of the \textit{C. sakazakii} strain ATCC BAA-894.

| Gene (Gene label) | Putative Gene Product | Chromosome location (bp) | Gene Size (bp) | Locus Primers (5'→3') |
|------------------|------------------------|--------------------------|----------------|----------------------|
| \textit{atpD}   | ATP synthase β chain   | 3,689,177 - 3,690,559    | 1,382          | CGACATGAAGGCGA CAT  |
|                  |                        |                          |                | CGAAATGACCGACTC CAA |
| (\textit{ESA}_04006) |                      |                          |                |                      |
| \textit{fusA}   | Elongation factor      | 3,275,843 - 3,277,957    | 2,114          | GAAACCGTGATGGCGT CAG |
| (\textit{ESA}_04401) |                      |                          |                | GCTGATGCGGTAAT TGA  |
| \textit{glnS}   | Glutaminyl-tRNA synthetase | 660,368 - 662,035        | 1,667          | GCATCTACCCGATGT ACG |
| (\textit{ESA}_02658) |                    |                          |                | GGGTGCTGGATAAACA TCA |
| \textit{gltB}   | Glutamate synthase     | 3,538,713 - 3,542,921    | 4,208          | CATCTGCCCCAGCTGC TTC |
| (\textit{ESA}_03606) |                    |                          |                | CGGAATACCCACCGCT ACA |
| \textit{gyrB}   | DNA gyrase B           | 3,719,848 - 3,722,262    | 2,414          | TGCCACCACATGGTAT TCG |
| (\textit{ESA}_03973) |                    |                          |                | TCGGGGGTCACTGT AAA  |
| \textit{infB}   | Translation initiation factor IF-2 | 4,139,051 - 4,141,762 | 2,711          | GAAGAAGCGGTAATG AGC |
| (\textit{ESA}_03561) |                    |                          |                | TGACCAACGGTTAAAAC CTC |
| \textit{pps}    | Phosphoenol-pyruvate synthase | 1,218,599 - 1,220,977    | 2,378          | GTCCAACAATGGCTGTC GTC |
| (\textit{ESA}_02102) |                    |                          |                | ACCCTGACGAAATCT AGC |
|                  |                        |                          |                | CAGACTCAAGCCAGGTT TTG  |
|                  |                        |                          |                | CAGATCGGCGCATGTT ATC  |
The mean allele length was 434 bp for the scheme and ranged between 363 bp (glnS) and 507 bp (gltB) in length (Table 2). All alleles within a particular locus were found to be of an identical length for all Cronobacter strains examined. Nucleotide sequence diversity at all seven loci is shown in Table 2. The proportion of variable sites varied from 10.8% (atpD) to 27.6% (gyrB) which extended over the whole section of the sequenced allele.

Allele variation is not necessarily equally likely at every nucleotide of each locus. If a locus does not have a role affected by a selective pressure (such as antibiotic exposure) then nucleotide substitutions would frequently not be expected to change the amino acid sequence (synonymous) as changes are likely to be eliminated by purifying selection. By calculating the $d_N/d_S$ ratio (non-synonymous substitutions to synonymous substitutions) the degree of selection operating on each locus can be estimated. The $d_N/d_S$ ratio for all seven loci within Cronobacter strains was found to be significantly less than 1, ranging from 0.006 (atpD) to 0.079 (infB) (Table 2), indicating that no strong positive selective pressure was present at any of the loci selected, validating their suitability for inclusion in the MLST scheme.

**Assignment of allele and sequence types**

The number of different alleles resolved from this Cronobacter MLST scheme at each locus ranged from 11 (gltB) to 15 (pps) alleles. The mean number of allele types per locus was found to be 13.4, providing the potential to distinguish >7 × 10¹⁰ different genotypes and also making it highly unlikely to obtain identical sequence types (ST) by chance.

For the development of the MLST scheme, it was important to use a diverse collection of strains to obtain primarily different STs based upon known 16S rRNA gene sequencing and biotype to validate the scheme’s effectiveness across the two Cronobacter species. For each unique allelic profile in the order atpD, fusA, glnS, gltB, gyrB, infB and pps, a unique ST was designated; See Additional file 1.

A total of 17 STs were found for the 78 strains examined (See Additional file 1); 12 STs for for C. sakazakii (n = 60), 3 C. malonaticus (n = 16), 1 Cit. koseri (n = 1) and 1 Entero bacter sp. 638 (n = 1). The sequences of each allele type at all seven loci, along with the allelic profiles and sequence types used for the multilocus sequence sequence analysis (MLSA) of the Cronobacter strains examined are available at http://pubmlst.org/cronobacter/.

The close genetic relationship between C. sakazakii and C. malonaticus was evident in that atpD allele 3 was identified both in C. sakazakii (ST3, ST17) and C. malonaticus (ST10). Apparently ‘species specific’ alleles were found across different STs e.g. the GlnS allele 3 was identified in C. sakazakii ST 3, 4,15 and 16, fusA allele 1 was in C. sakazakii ST1, 4, and 14, and three C. malonaticus STs had fusA allelic profile 7, and ST7 and ST10 had gltB allelic profile 7.

**Comparison of sequence type with source and biotype**

In total 60 C. sakazakii and 16 C. malonaticus strains were analysed. Most strains analysed were associated with previous publications (See Additional file 1). The earliest isolate (NCIMB 8272) was from a can of dried milk powder, which was deposited in the culture collection in 1951, and the earliest clinical isolate (NCTC 9238) was deposited in 1953 [1].

C. sakazakii ST1 contained infant formula isolates from 1988-2003 from Russia, Netherlands, USA and UK. It included the ATCC BAA-894 strain from the Tennessee NICU outbreak [13] which has been sequenced (Accession number CP000785). Two strains were from milk powder and faeces. There were no known clinical outbreak isolates in ST1. C. sakazakii ST14 was a single strain from infant formula in France (1994) [16]. This ST varied by just a single nucleotide polymorphism from ST1 with respect to the pps locus. C. sakazakii ST3 strains were from infant formula, follow up formula, weaning food, and neonatal enteral feeding tubes. The strains were from 1988-2008, and were isolated in the Netherlands, UK, and Korea. There were no known clinical isolates, however

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**Table 2: Analysis of the seven MLST loci in the Cronobacter strains sampled.**

| Gene  | Size (bp) of fragment analysed | No. of alleles | No. of polymorphic sites | Proportion of fragment as polymorphic sites (%) | $d_N/d_S$ |
|-------|-------------------------------|----------------|--------------------------|-----------------------------------------------|-----------|
| atpD  | 390                           | 12             | 42                       | 10.8                                          | 0.006     |
| fusA  | 438                           | 12             | 69                       | 15.8                                          | 0.061     |
| glnS  | 363                           | 12             | 72                       | 19.8                                          | 0.062     |
| gltB  | 507                           | 11             | 118                      | 23.3                                          | 0.059     |
| gyrB  | 402                           | 13             | 111                      | 27.6                                          | 0.055     |
| infB  | 441                           | 12             | 87                       | 19.7                                          | 0.079     |
| pps   | 495                           | 15             | 123                      | 24.8                                          | 0.033     |
C. sakazakii ST4 was the major (22/60) sequence type among the isolates. It contained almost equal numbers of clinical (n = 9) and infant formula (n = 7) isolates. This ST also included the Betty Hobbs 1951 isolate from a can of dried milk (NCIMB 8272) [1]. In contrast, strains in C. sakazakii ST8 were predominantly (7/8) clinical isolates from USA, Canada, and Czech Republic. The isolation dates ranged from 1977-2003, and included the C. sakazakii type strain (NCTC 11467). The remaining strain was isolated in 2006 from infant formula in France. C. sakazakii ST12 included 5 strains from UK, USA, France and Czech Republic, at least 3 of which were clinical in origin.

C. malonaticus ST7 contained 11 strains which were primarily clinical in origin from the Czech Republic, isolated between 1977 and 2004. C. malonaticus ST11 contained 3 clinical strains from the Czech Republic, biotypes 2a, 14a, and 13b which were isolated in 1983 [30]. C. malonaticus ST10 was composed of two strains from Chinese herbs which were both isolated in 2005.

Biotypes did not always correspond with sequence types or Cronobacter species (See Additional file 1). For example, biotype 2 was primarily distributed over C. sakazakii ST1 and 3, with two other strains in ST4. The index strain for biotype 2a was in C. malonaticus ST11, and a second strain was in C. sakazakii ST12. Biotype 1 was in C. sakazakii ST4, 8, 13, 15, 17 and 18. C. malonaticus is defined as biotypes 5, 9 and 14 [5]. However biotype 5 was in C. malonaticus ST7 and 10, and C. sakazakii ST16. Biotype 9 was only in C. malonaticus ST14. Whereas biotype 14 and 14a were in C. malonaticus ST7, and ST11. Biotypes 2a, 4a, 13a, and 13b are conventionally assigned to C. sakazakii [3]. However, C. malonaticus ST7 included the index strains for biotypes 4a and 13a, and C. sakazakii ST11 included the index strains for biotypes 2a and 13b.

Relationships of C. sakazakii, C. malonaticus, Cit. koseri and Enterobacter sp. 638 using concatenated nucleotide sequences

In order to assess all the loci together in one tree, concatenated nucleotide sequences were used. Concatenated nucleotide sequences (3,036 bp) for the 15 Cronobacter STs, Cit. koseri and Enterobacter sp. 638 were analysed using the UPGMA method (Figure 1). The Cronobacter species were fully resolved, falling into distinctive clusters of strains. The Cronobacter species were clearly separated from the Enterobacter sp. strain 638 and also by a lesser extent from the Cit. koseri strain (100% bootstraps). C. sakazakii and C. malonaticus separated from each other at 2.6% divergence (100% bootstrap value), and from Citrobacter koseri at 13% divergence. Figure 1 also shows the distribution of biotypes across the sequence types.

Analysis of recombination among C. sakazakii

Bacterial existing as clonal populations evolve diversity by the accumulation of point mutations, while non-clonal populations evolve more through recombination within or between species. In this study identical alleles were found within species and between the two Cronobacter species (See Additional file 1).

Evidence for clonal or recombining populations can be estimated by assessing the level of linkage between alleles at different loci around the chromosome. The index of association (I_{AS}) measures the extent of linkage. An I_{AS} not significantly greater than zero after 1,000 computer randomizations would suggest that a single species population (monophyletic) is in linkage equilibrium (freely recombining), while a population with an I_{AS} significantly greater than zero (p < 0.001) is considered to be in linkage disequilibrium (clonal). C. sakazakii examined had an I_{AS} value of 0.28 (p value < 0.01) and therefore indicates a more clonal that freely recombining population. Further analysis will be undertaken as part of a subsequent study, along with other Cronobacter spp..

Discussion and Conclusion

The diversity of Enterobacter sakazakii was well acknowledged prior to the taxonomic revision to the Cronobacter genus, which was based on DNA-DNA hybridisation, 16S rDNA sequence analysis, and biotyping [5]. The earlier biotyping scheme was extremely useful in aiding the definition of the various Cronobacter species, especially due to the close genetic relationship of C. sakazakii and C. malonaticus which initially was regarded as a subspecies of C. sakazakii [4].

Nevertheless, phenotyping is in part subjective, and a DNA based scheme is preferred for its robustness. This study has used 7 loci for a MLST scheme for C. sakazakii and C. malonaticus. Strains were chosen to represent the diversity of C. sakazakii and C. malonaticus based on biotype, geographic and temporal distribution, and source (environmental, formula, clinical). The strains were from Europe, USA, Canada, Russia, New Zealand, Korea and China. The isolation dates ranged over 57 years from 1951 to 2008.

As MLST uses multiple loci, a greater degree of variation and better resolution for MLSA and for inferring evolutionary and epidemiological relatedness can be obtained than by a single locus alone. Twelve sequence types of C. sakazakii were assigned. ST4 contained the largest number of strains, both clinical, infant formula, and milk powder isolates, from USA, Canada, Europe and Russia. The earli-
Figure 1

Phylogenetic tree of concatenated nucleotide sequences from the seven loci, using the UPGMA method, Jukes-Cantor. Bootstrap values are shown for 1,000 replicates.

| Species    | Strain     | ST | Biotype |
|------------|------------|----|---------|
| Cronobacter sakazakii | 471 | 17 | 1       |
| Cronobacter sakazakii | 580 | 18 | 1       |
| Cronobacter sakazakii | 7  | 1  | 2       |
| Cronobacter sakazakii | 12 | 1  | 2       |
| Cronobacter sakazakii | 27 | 1  | 2       |
| Cronobacter sakazakii | 537 | 1  | 2      |
| Cronobacter sakazakii | 541 | 1  | 2      |
| Cronobacter sakazakii | 555 | 1  | 2      |
| Cronobacter sakazakii | 561 | 1  | 2      |
| Cronobacter sakazakii | 658 | 1  | 2      |
| Cronobacter sakazakii | 716 | 14 | 2      |
| Cronobacter sakazakii | 520 | 12 | 2a     |
| Cronobacter sakazakii | 567 | 12 | 13     |
| Cronobacter sakazakii | 690 | 12 | 3      |
| Cronobacter sakazakii | 696 | 12 | 1      |
| Cronobacter sakazakii | 1108 | 12 | 3      |
| Cronobacter sakazakii | 6  | 4  | 1      |
| Cronobacter sakazakii | 14 | 4  | 1      |
| Cronobacter sakazakii | 30 | 4  | 1      |
| Cronobacter sakazakii | 377 | 4 | 1      |
| Cronobacter sakazakii | 425 | 4 | 1      |
| Cronobacter sakazakii | 425 | 4 | 1      |
| Cronobacter sakazakii | 467 | 4 | 1      |
| Cronobacter sakazakii | 470 | 4 | 1      |
| Cronobacter sakazakii | 538 | 4 | 13     |
| Cronobacter sakazakii | 548 | 4 | 1      |
| Cronobacter sakazakii | 551 | 4 | 1      |
| Cronobacter sakazakii | 552 | 4 | 2      |
| Cronobacter sakazakii | 553 | 4 | 2      |
| Cronobacter sakazakii | 557 | 4 | 1      |
| Cronobacter sakazakii | 558a | 4 | 1     |
| Cronobacter sakazakii | 559 | 4 | 1      |
| Cronobacter sakazakii | 685 | 4 | 7      |
| Cronobacter sakazakii | 695 | 4 | 1      |
| Cronobacter sakazakii | 701 | 4 | 1      |
| Cronobacter sakazakii | 709 | 4 | 1      |
| Cronobacter sakazakii | 767 | 4 | 1      |
| Cronobacter sakazakii | 1105 | 4 | 1     |
| Cronobacter sakazakii | 4  | 15 | 1      |
| Cronobacter sakazakii | 532 | 13 | 1     |
| Cronobacter sakazakii | 693 | 13 | 4     |
| Cronobacter sakazakii | 2  | 3  | 2      |
| Cronobacter sakazakii | 25 | 3  | 2      |
| Cronobacter sakazakii | 26 | 3  | 2      |
| Cronobacter sakazakii | 120 | 3 | 2      |
| Cronobacter sakazakii | 121 | 3 | 2      |
| Cronobacter sakazakii | 545 | 3 | 2      |
| Cronobacter sakazakii | 978 | 3 | 2      |
| Cronobacter sakazakii | 984 | 3 | 2      |
| Cronobacter sakazakii | 1105 | 3 | 2     |
| Cronobacter sakazakii | 1107 | 9 | 2     |
| Cronobacter sakazakii | 1 | 8  | 1      |
| Cronobacter sakazakii | 5 | 8  | 4a     |
| Cronobacter sakazakii | 424 | 8 | 1      |
| Cronobacter sakazakii | 511 | 8 | 13a    |
| Cronobacter sakazakii | 513 | 8 | 1      |
| Cronobacter sakazakii | 536 | 8 | 13a    |
| Cronobacter sakazakii | 680 | 8 | 3      |
| Cronobacter sakazakii | 683 | 8 | 4      |
| Cronobacter sakazakii | 150 | 16 | 5     |
| Cronobacter maltonaticus | 507 | 11 | 13b    |
| Cronobacter maltonaticus | 512 | 11 | 2a     |
| Cronobacter maltonaticus | 514 | 11 | 14a    |
| Cronobacter maltonaticus | 90 | 10 | 5a     |
| Cronobacter maltonaticus | 156 | 10 | 5      |
| Cronobacter maltonaticus | 8 | 7 | 14a    |
| Cronobacter maltonaticus | 18 | 7 | 9      |
| Cronobacter maltonaticus | 35 | 7 | 8a     |
| Cronobacter maltonaticus | 510 | 7 | 9      |
| Cronobacter maltonaticus | 515 | 7 | 4a     |
| Cronobacter maltonaticus | 521 | 7 | 8a     |
| Cronobacter maltonaticus | 522 | 7 | 9      |
| Cronobacter maltonaticus | 524 | 7 | 9      |
| Cronobacter maltonaticus | 535 | 7 | 13a    |
| Cronobacter maltonaticus | 565 | 7 | 14     |
| Cronobacter maltonaticus | 631 | 7 | 9      |
| Citrobacter koseri | 6   |
| Enterobacter sp | 2   |
est isolate dates from 1951 and demonstrates the ubiquity of this sequence type. Many (18/22) of these strains were biotype 1, which was previously shown to be the most numerous biotype (60/189) [3].

Previously Caubilla-Barron et al. [16] and Townsend et al. [20] reported on C. sakazakii infections in neonatal intensive care unit outbreak, which involved 4 pulsetypes. Only one pulsetype (PT2) was associated with all the deaths and therefore indicated that C. sakazakii strains may vary in their virulence potential. PT2 strains caused necrotizing enterocolitis (NEC), septicemia, and meningitis. These strains were all in ST4. Other strains, associated with non-fatal NEC, neonatal colonisation, and infant formulas were in ST12, 13 and 14.

ST8 is of particular interest as 7/8 strains were clinical in origin, the eighth isolate being isolated from infant formula. These organisms isolated between 1977 and 2006 were from USA, Canada, France and the Czech Republic. ST8 also contains the C. sakazakii type strain (NCTC 11467\(^\text{T}\), equivalent ATCC 29544\(^\text{T}\)) and interestingly the index strains for biotypes 1, 3 and 4. Some of these strains have previously been studied by Pagotto et al. [33] and Postupa and Aldova [35]. ST(8) therefore merits further investigation, as it may represent a particularly virulent type of C. sakazakii strains.

Similarly ST7 in C. malonaticus was dominated (8/11) by clinical isolates, however this grouping may be biased as 5 clinical isolates (510, 515, 521, 522, 524) were epidemiologically linked. There is also a predominance of biotype 9 in this sequence type, which may in part explain why that biotype was previously associated with clinical source; 10/13 strains [3].

The MLST scheme is openly available on the internet for other workers and will assist in the identification and discrimination of C. sakazakii and C. malonaticus based on DNA sequence in place of the far less reliable biotyping approach, which in isolation is essentially of no phylogenetic value and little epidemiological value. The role of biotyping in the identification and discrimination of C. sakazakii and C. malonaticus needs to be seriously reviewed. Even within the sample of isolates examined MLSA has already identified 1 or 2 STs which appear to be associated with enhanced virulence, and this may aid our understanding of the pathogenicity of this ubiquitous organism.

**Methods**

**Source of strains and biotyping**

Strains were chosen on the basis of their species, biotype, geographic and temporal distribution, source and clinical outcome (See Additional file 1). This included the type strains C. sakazakii NCTC 11467\(^\text{T}\), and C. malonaticus CDC 1058-77\(^\text{T}\), biotype index strains, infant formula and clinical isolates, from Europe, USA, Canada, Russia, New Zealand, Korea and China, ranging from 1951 to 2008. The majority of these have associated published articles (See Additional file 1). Biotyping was as according to Iversen et al. [3].

**DNA isolation and PCR**

Genomic DNA was prepared using GenElute™ Bacterial Genomic DNA Kit (Sigma) and 1.5 ml of overnight culture grown in TSB broth as per the manufacturer's instructions.

**Selection of MLST gene loci**

MLST loci were selected by comparing genome sequence data for C. sakazakii (strain ATCC BAA-894; [http://genome.wustl.edu](http://genome.wustl.edu), Cit. koseri (strain ATCC BAA-895; [http://genome.wustl.edu](http://genome.wustl.edu)) and Enterobacter sp. strain 638 [http://www.igi.doe.gov/](http://www.igi.doe.gov/) using the Artemis Comparison Tool (ACT) and the Double ACT program available at [http://www.sanger.ac.uk/Software/ACT/](http://www.sanger.ac.uk/Software/ACT/) and [http://www.hpa-bioinfotools.org.uk/pise/double_act.html](http://www.hpa-bioinfotools.org.uk/pise/double_act.html), respectively.

**Primer design**

 Amplification and nested sequencing primers for the MLST loci were then designed to conserved areas of these genes using Primer3 available at [http://frodo.wi.mit.edu/](http://frodo.wi.mit.edu/) [36].

For each locus, primers were designed to have a similar melting temperature and were found to successfully amplify by PCR a panel of C. sakazakii and C. malonaticus strains (Table 1 and Additional file 1). Reaction conditions for all the primers were as follows: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min, extension at 72°C for 2 min; followed by a final extension step of 72°C for 5 min. Each 50 μl amplification reaction mixture comprised 10 ng chromosomal DNA, 10 μl Q solution (Qiagen, Crawley, UK), 20 pmol forward and reverse primer, 1× PCR buffer (Qiagen) containing 1.5 mM MgCl₂, 0.8 mM deoxynucleotide triphosphates and 1.25 U Taq (Qiagen). The amplification product was then purified using MinElute UF plates (Qiagen) following the manufacturer's protocol before being used in a sequencing reaction.

**Multilocus sequence analysis**

Using the nested sequencing primers, nucleotide sequences were determined at least once on each DNA strand with BigDye Terminator Ready Reaction Mix v3.1 (PE Biosystems, Foster City, US) under standard sequencing conditions according to the manufacturer's protocol.
Unincorporated dye terminators were removed by precipitation with 95% alcohol. The reaction products were separated and detected on an ABI PRISM genetic analyser 3100 (PE Biosystems) using a standard sequencing module with a Performance Optimised Polymer and 5 cm array. The sequences from both strands of a given locus of the same isolate were aligned, trimmed to the desired length and edited using SeqMan II (DNA Star software, Madison, US).

**Allele and Sequence Type designation**

Arbitrary allelic numbers were assigned to each unique allele for a given locus. After sequencing and assigning allele types to all seven loci each isolate was then designated by a combination of seven numbers called an allelic profile that represented a sequence type (ST) for that particular isolate (eg. ST1). A novel sequence type (ST) designation was given to each isolate with a unique allelic profile while subsequent isolates with an identical allelic profile were assigned the same ST identifier and considered to be isogenic strains as they were indistinguishable at all seven loci. All alleles within the MLST scheme were in frame, to aid with analysis.

**Linkage analysis**

Linkage analysis was carried out by using the index of association ($I_a$), as defined previously [37]. We examined whether alleles were randomly associated, that is, at linkage equilibrium, indicating a freely recombining population, or non-randomly associated, that is, at linkage disequilibrium, implying a clonal population structure. If there is linkage equilibrium, i.e., a random association between alleles of different loci, $I_a = 0$. If $I_a$ is significantly different from 0, it indicates that recombination has been rare or absent and that the population has a clonal structure [34].

**Authors’ contributions**

AB designed and carried out the MLST, assisted by EK, JC, ML, GM and CD provided technical expertise. Thanks to Edward Hurrell for additional strain biotyping. AB and SF wrote the manuscript. SF managed the project. All authors read and approved the final manuscript.

**Additional material**

**Additional file 1**

MLST analysis of the Cronobacter isolates showing their source, geographic location and species. The data provided shows the spacial, temporal and source of strains used in this study, and reference where the strains have been used in previous publications. Click here for file

[http://www.biomedcentral.com/content-supplementary/1471-2180-9-223-S1.DOC]  

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