Genome Sequence of *Cronobacter sakazakii* BAA-894 and Comparative Genomic Hybridization Analysis with Other *Cronobacter* Species

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Abstract

**Background:** The genus *Cronobacter* (formerly called *Enterobacter sakazakii*) is composed of five species; *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muysjensii*, and *C. dublinensis*. The genus includes opportunistic human pathogens, and the first three species have been associated with neonatal infections. The most severe diseases are caused in neonates and include fatal necrotizing enterocolitis and meningitis. The genetic basis of the diversity within the genus is unknown, and few virulence traits have been identified.

**Methodology/Principal Findings:** We report here the first sequence of a member of this genus, *C. sakazakii* strain BAA-894. The genome of *Cronobacter sakazakii* strain BAA-894 comprises a 4.4 Mb chromosome (57% GC content) and two plasmids; 31 kb (51% GC) and 131 kb (56% GC). The genome was used to construct a 387,000 probe oligonucleotide tiling DNA microarray covering the whole genome. Comparative genomic hybridization (CGH) was undertaken on five other *C. sakazakii* strains, and representatives of the four other *Cronobacter* species. Among 4,382 annotated genes inspected in this study, about 55% of genes were common to all *C. sakazakii* strains and 43% were common to all *Cronobacter* strains, with 10–17% absence of genes.

**Conclusions/Significance:** CGH highlighted 15 clusters of genes in *C. sakazakii* BAA-894 that were divergent or absent in more than half of the tested strains; six of these are of probable prophage origin. Putative virulence factors were identified in these prophage and in other variable regions. A number of genes unique to *Cronobacter* species associated with neonatal infections (*C. sakazakii*, *C. malonaticus* and *C. turicensis*) were identified. These included a copper and silver resistance system known to be linked to invasion of the blood-brain barrier by neonatal meningitic strains of *Escherichia coli*. In addition, genes encoding for multidrug efflux pumps and adhesins were identified that were unique to *C. sakazakii* strains from outbreaks in neonatal intensive care units.

Introduction

*Cronobacter* spp. (formerly *Enterobacter sakazakii*) are Gram-negative, motile, non-sporeforming, peritrichous rods of the *Enterobacteracea* family. *Cronobacter* is a ubiquitous organism present in a wide range of environments, including water, soil, and a variety of processed foods and fresh produce [1]. The bacterium has been isolated from factory production lines including powdered infant formula factories and households [2] as well as from a wide range of clinical samples including cerebrospinal fluid, blood, bone marrow, sputum, urine and faeces [3]. The organism is an opportunistic pathogen of humans that can cause infections in all age groups. However, low birth weight neonates are most at risk. In this host group *Cronobacter* has been associated with outbreaks of necrotizing enterocolitis, meningitis and septicemia. Infections with these presentations result in exceptionally high mortality rates ranging from 40 to 80 percent [4]. In recent years, some outbreaks of bacterial infection in neonatal intensive care units (NICU) have been traced to powdered formula contaminated with *Cronobacter* [5–8].

*Cronobacter* was defined as ‘yellow-pigmented *Enterobacter cloaca*’ until 1980, when it was designated a new species, *Enterobacter*...
sakazakii, by Farmer et al [9]. Analysis of both partial 16S rDNA and hlyB60 sequences showed that E. sakazakii isolates formed at least four distinct clusters, and it was proposed that clusters 2, 3, and 4 could be unique species [10]. Based on DNA-DNA hybridization and phenotyping, Enterobacter sakazakii was subsequently proposed to be re-classified into a new genus Cronobacter, composed of five distinct species: Cronobacter sakazakii, C. malonaticus, C. turicensis, C. muytjensii and C. dublinensis [11]. Due to their close relatedness C. sakazakii and C. malonaticus are difficult to distinguish by 16S rDNA sequence analysis. However, multilocus sequence typing (MLST) differentiates between the two species, and also reveals a strong clonal nature of the organism [12]. Previous studies on ‘E. sakazakii’ will therefore be difficult to interpret unless the strains are re-examined and re-classified according to the current taxonomic structure.

Cronobacter strains vary in their virulence, as determined by epidemiological studies and in-house mammalian tissue culture [6,13,14], but their virulence mechanisms are unknown. The bacteria can attach to intestinal cells and survive in macrophages [13], but the specific receptors involved remain to be determined. To date, only strains from C. sakazakii, C. malonaticus and C. turicensis have been associated with neonatal infections. Recently it was shown that the disruption of tight junctions significantly enhances association of C. sakazakii with Caco2 cells [15]. Some reports suggest a similarity between the tropism of Cronobacter and Citrobacter koseri for invasion and infection of the central nervous system [16,17]. It was noted that brain abscesses due to Cronobacter and Citrobacter koseri were morphologically similar and may be due to similar virulence mechanisms [18]. The first putative Cronobacter virulence factors were enterotoxin-like compounds produced by four of eighteen strains [19]. The genes encoding the putative toxins have yet to be identified, however.

Here, we present the genome sequence of C. sakazakii strain BAA-894, isolated from powdered formula associated with a NICU outbreak [7], and use that sequence for comparative analysis of physiological and virulence related traits across the Cronobacter genus. Due to the severity of infant infection, a better understanding of the genomic variation between Cronobacter spp. is needed, and will be of interest to manufacturers of powdered infant formula, regulatory bodies, as well as those studying the evolution and diversity of pathogenicity.

Results and Discussion

Cronobacter sakazakii BAA-894 Genome

The complete sequencing of the genome of C. sakazakii BAA-894 revealed that it was composed of 1 chromosome (4.36837 Mb, 57% GC) and 2 plasmids (pESAK2 31 kb, 51% GC; pESAK3 131 kb, 56% GC); (Genbank accessions CP000783-5). A largely automated annotation of the genome resulted in the identification of 4,392 genes, covering 87% of the chromosome, 38 genes covering 83% of pESAK2 and 127 genes, covering 87% of pESAK3. The genome is aligned to many other enterobacterial genomes in the Enterix [20,21] server (http://enterix.cbcb.umd.edu/enteric/enteric.html).

Genome Cluster Analysis

In order to compare closely related genomes to the sequence of C. sakazakii BAA-894, we designed a set of 304,030 50-mer oligonucleotides that tiled the whole genome in both strands at an average density of about one oligonucleotide every 12 bases. An array was then manufactured by Roche NimbleGen (www.nimblegen.com).

Genomic diversity of 10 strains of Cronobacter representing the five different recognized species of this genus (Table 1) was analyzed by CGH on this tiled DNA microarray against the sequenced strain C. sakazakii BAA-894. Cronobacter genes were classified as present, absent or of intermediate status, as defined in the Materials and Methods section. The raw data is deposited in GenBank GEO, accession number GSE19908.

To determine the presence or absence of genes, the median log2 difference of the genome relative to the reference strain for all the oligonucleotides in that gene was calculated. Then GAPCK analysis [22] was used, which sets a floating threshold for presence and absence of genes for every hybridization (see below). Using Cluster and Treeview softwares [23], Cronobacter strains formed two distinct phylogenetic clusters. All C. sakazakii strains formed one cluster (Figure 1). C. malonaticus, C. turicensis, C. dublinensis and C. malonaticus formed a second, separate cluster. Within C. sakazakii, strains 701

Table 1. Bacterial strains used in this study.

| Organism ID | Organism Type | Comment | Source | % 16S rDNA sequence difference from C. sakazakii BAA-894 | MLST sequence type | Reference |
|-------------|---------------|---------|--------|---------------------------------------------------|-------------------|-----------|
| C. sakazakii strain BAA-894 | Csak658 | Genome sequenced strain | Powdered formula | - | 1 | [7,12] |
| C. sakazakii ATCC 29544T | Csak1 | Species type strain | Child’s throat | 0.38 | 8 | [11,12] |
| C. sakazakii ATCC 12688 | Csak2 | Unknown | 0.57 | 3 | [12] |
| C. sakazakii strain 20 | Csak20 | Clinical | 0.57 | 4 | [12] |
| C. sakazakii strain 701 | Csak701 | Fatal NEC III case | Peritoneal fluid | 0.47 | 4 | [6,12] |
| C. sakazakii strain 767 | Csak767 | Fatal meningitis case | Trachea | 0.47 | 4 | [6,12] |
| C. sakazakii strain 696 | Csak696 | NECT II case | Stools | 0.57 | 12 | [6,12] |
| C. malonaticus LMG 23826T | Cmal1212 | Species type strain | Breast abscess isolate | 0.57 | NA | [11] |
| C. turicensis LMG 23827T | Ctur1211 | Species type strain | Blood isolate | 1.80 | NA | [11] |
| C. muytjensii ATCC 51329T | Cmuy3 | Species type strain | Unknown | 2.94 | NA | [11] |
| C. dublinensis LMG 23823T | Cdub1210 | Species type strain | Environmental. Milk powder production plant | 3.69 | NA | [11] |

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and 767 were the most closely related and clustered together with strain 20. Previously, strains 701 and 767 were shown to belong to the same pulse field gel electrophoresis restriction digestion type [12]. Although the clinical details of the source of C. sakazakii strain 20 are unknown, the strain belongs to MLST sequence type 4 (as do 701 and 767), which is a stable clone of C. sakazakii strain ATCC 29544 T (species type strain) formed a separate branch within the C. sakazakii cluster. The remaining Cronobacter species formed sub-clusters: C. malonaticus clustered with C. dublinensis and C. muytjensii grouped with C. muytjensii. The tree remained identical when adjacent genes were collapsed into a single phylogenetic character if they had the same pattern of presence and absence.

Core Genome

Of the 4,382 unique annotated gene sequences represented on the microarray, 54.9% (2404) were common to all C. sakazakii strains and 43.3% (1899) were common to all five Cronobacter species. The vast majority of these shared genes are predicted to encode cellular essential functions such as energy metabolism, biosynthesis, DNA, RNA and protein synthesis, cell division and membrane transport. The proportion of genes absent from test strains compared with C. sakazakii BAA-894 ranged from 10.3% (453) in C. sakazakii strain 20 to 17.1% (751) in C. muytjensii (Table 2). In total, 5.1% (224) of BAA-894 genes were absent in all "C. sakazakii" strains, and 3.1% (137) genes were absent in all Cronobacter strains (Table 2). Even though C. muytjensii and C. malonaticus are classified as separate species, the proportion of absent genes was only 11.3% and 11.9%, respectively, when compared to C. sakazakii BAA-894. This is in concordance with the previous 16S sequence comparison studies which showed that all Cronobacter strains are closely related (Table 1).

C. sakazakii Unique Genes

CGH analysis showed there were 21 genes present in all five C. sakazakii strains which were absent in C. malonaticus, C. muytjensii, C. turicensis and C. dublinensis. The unique genes to C. sakazakii strains were in two separate clusters of proteins involved in pilus assembly (ESA_02540–ESA_02542 and ESA_02796–ESA_02799), pilin FimA proteins (ESA_02541, ESA_02542, ESA_02796 and ESA_02799), porin PapC (ESA_02797) and the chaperone PapD (ESA_02798). The genes unique to C. sakazakii also included proteins for the phosphotransferase system (ESA_03303 and ESA_03305), a putative sialic acid transporter (ESA_03611), N-acetylmuramidase lyase (ESA_03612) and ReICB from a toxin/antitoxin system (ESA_00527).

Invasion of Brain Microvascular Endothelial Cells

Because Cronobacter is associated with often fatal cases of neonatal meningitis, the status of genes identified in other organisms as associated with invasion of brain microvascular endothelial cells (BMEC) (ibaA, ibaB, yipP and omplA) in the sequenced isolate was of particular interest [24–27]. The gene encoding OmpA was present in all tested strains. This protein is associated with the invasive ability of neonatal meningitic E. coli. While genes ibaA and yipP produced no match in the reference strain C. sakazakii BAA-894, ibaB (synonymous to cusC) was found. CusC belongs to a cluster of genes encoding a copper and silver resistance cation efflux system which allows bacteria to invade BMEC [28]. The complete cation efflux system cusC (ESA_04242), cusB (ESA_04241), cusF (ESA_04239) and its

Table 2. Number of absent or divergent and intermediate genes in Cronobacter strains when compared to the sequenced C. sakazakii BAA-894.

| Strain                        | Absent genes (%) | Intermediate genes (%) |
|-------------------------------|------------------|------------------------|
| C. sakazakii ATCC 29544        | 582 (13.3)       | 449 (10.2)             |
| C. sakazakii ATCC 12868       | 461 (10.5)       | 314 (7.2)              |
| C. sakazakii strain 20         | 453 (10.3)       | 580 (13.2)             |
| C. sakazakii strain 701        | 521 (11.9)       | 569 (13.0)             |
| C. sakazakii strain 767        | 546 (12.5)       | 649 (14.8)             |
| C. sakazakii strain 696        | 497 (11.3)       | 593 (13.5)             |
| C. malonaticus LMG 23826      | 522 (11.9)       | 608 (13.9)             |
| C. turicensis LMG 23827       | 495 (11.3)       | 591 (13.5)             |
| C. muytjensii ATCC 51329      | 751 (17.1)       | 522 (11.9)             |
| C. dublinensis LMG 23823      | 682 (15.6)       | 549 (12.5)             |
| Absent in at least one C. sakazakii | 835 (19.1) |                     |
| Absent in at least one Cronobacter | 1081 (24.7) |                     |

1The numbers in brackets indicate the percentage of absent genes out of 4,382 annotated genes analyzed by comparative genomic hybridization. doi:10.1371/journal.pone.0009556.t002
regulatory gene csoR (ESA_04238) was present in strains associated with neonatal infections (C. sakazakii ATCC 29544T, 701, 767, 696, C. turicensis and C. malonaticus), and absent in the other tested strains.

Other Physiological Traits
The presence of genes conferring physiological traits commonly associated with Cronobacter spp. was examined. Seventy genes involved in desiccation resistance [29], the metalloprotease zps which causes rounding of Chinese hamster ovary (CHO) cells [30] and yellow pigment production genes [31] were present on the arrays. All these genes were present in all 10 Cronobacter strains tested.

Comparison of C. sakazakii Neonatal Intensive Care Unit (NICU) Outbreak Strains with C. sakazakii Type Strain ATCC 29544T

The genes that were shared by the three strains associated with C. sakazakii outbreaks in NICUs (BAA-894, 701 & 767) were compared with the C. sakazakii species type strain ATCC 29544T, which showed decreased virulence properties compared to strains 701 and 767 in tissue culture studies [14]. One hundred and forty-four genes present in the three NICU strains were absent in the type strain, 66 (46%) in clusters of consecutive genes based on the annotation of BAA-894. In most of these clusters, genes encoding proteins associated with resistance to different forms of stress were identified, including multidrug efflux systems, genes involved in resistance to oxidative stress, and those with a putative function in resistance to metals. The complete list of genes present in NICU outbreak strains C. sakazakii BAA-894, 701 and 767 and absent in the C. sakazakii type strain ATCC 29544T is in Table S1; genes of interest are listed below.

Category I. Genes encoding proteins associated with resistance to different forms of antibiotics: (i), a transcriptional regulator (ESA_01938) from the TetR family of protein repressors that control the level of susceptibility to hydrophobic antibiotics and detergents; (ii), a homologue of the CpmG protein involved in carbapenem resistance (ESA_pESP3p05435); (iii), a protein conferring resistance to antimicrobial peptides Mig-14 (ESA_pESP3p05439); and (iv), a transcriptional regulator (ESA_pESP3p05448) involved in tetracycline resistance.

Category II. Genes encoding multidrug efflux systems: (i), a cationic drug transporter (ESA_01940) from the family of proteins that confer resistance to a wide range of toxic compounds; (ii), genes for complete bacterial ABC-transport systems involved in active transport across the cytoplasmic membrane (ESA_01944–ESA_01945); and (iii), a variety of genes encoding multidrug efflux components located on the plasmid pES3A in BAA-894 (Table S1).

Category III. Genes involved in resistance to oxidative stress and genes with a putative function in resistance to metals: (i), a redox-sensitive transcriptional activator SoxS (ESA_00115); (ii), a glutathione S-transferase (ESA_00116); (iii), ADP-ribose pyrophosphatase involved in oxidative stress protection (ESA_pESP3p05446); (iv), an arsenate reductase (ESA_pESP3p05485); and (v), a predicted transcriptional regulator involved in mercuric resistance ESA_pESP3p05463.

Category IV. Other genes of interest include: (i), putative adhesins which are recognized as virulence factors in enteric bacteria [32] (ESA_00983–ESA_00986); (ii), the universal stress protein UspA (ESA_01955) which can enhance the rate of cell survival during prolonged exposure to stress conditions [33]; (iii), a gene encoding a Type VI secretion lysosome-related protein (ESA_02735); (iv), a gene for a predicted virulence SciE-type protein (ESA_02736) which affects the ability of bacteria to enter eukaryotic cells [34]; and (v), genes involved in pilus assembly (ESA_00315 and ESA_00316).

C. sakazakii Plasmids
The sequenced strain C. sakazakii BAA-894 contains two plasmids, pESA2 (31 kb) and pES3A (131 kb). Thirty-eight genes were annotated on pESA2 and 127 genes on pES3A.

The copy number of the plasmids was estimated from the median hybridization signals of oligonucleotides representing the plasmid compared to the sequenced genome. The ratio was (1:1.13:6) for the chromosome versus pESA2 versus pES3A. Thus, pESA2 exists as low copy, and pES3A appears to be a moderate copy number plasmid.

The genes on pESA2 were absent in all other strains tested except C. turicensis, which had 19 (61.3%) genes present, and C. sakazakii 696, which had 4 (12.9%) genes present. The results for genes on pES3A are summarized in Table 3.

Note that it is possible that some or all of the detected genes are on the chromosome in other strains. In addition, genes on a multicopy or medium copy plasmid may require a different degree of divergence to be identified as absent or divergent by comparative hybridizations. Plasmid profiling was performed on the Cronobacter strains analyzed by comparative hybridization. A plasmid of a size similar to pESA2 (31 kb) was detected in C. sakazakii 696 and in C. turicensis, which is in accordance with our CGH results. A large plasmid similar in size to pES3A (131 kb) was visible in C. sakazakii strains ATCC 29544T, ATCC 2868, 20, 696, 701, 767 and C. malonaticus (Table 3).

Genomic Regions Absent in Some Strains of Cronobacter
Genes that were absent in more than half of the Cronobacter strains relative to the sequenced strain C. sakazakii BAA-894 were selected for further analysis. These genes form 15 clusters of contiguous genes (based on the annotation of the reference genome). These are shown on Figure 2, where the number of strains in which a particular gene was classified as absent is plotted against the gene locus. The clusters were designated as regions GR1–GR15.

Table 3. pES3A genes present in Cronobacter strains when compared to the sequenced strain of C. sakazakii BAA-894.

| Strain          | Present genes (%) | Plasmid profile |
|-----------------|-------------------|-----------------|
| C. sakazakii ATCC 12868 | 103 (88.8) | + |
| C. sakazakii 696 | 81 (69.8) | + |
| C. sakazakii 767 | 77 (66.4) | + |
| C. sakazakii 20 | 73 (62.9) | + |
| C. sakazakii 701 | 61 (52.6) | + |
| C. malonaticus LMG 23826 | 57 (49.1) | + (3) |
| C. sakazakii ATCC 29544 | 51 (44.0) | + (3) |
| C. turicensis LMG 23827 | 35 (30.2) | – |
| C. dublinensis LMG 23823 | 19 (16.4) | – |
| C. muytjensii ATCC 51329 | 0 (0.0) | – |

1Of the 127 annotated genes on pES3A, 116 were analyzed by comparative genomic hybridization. The other genes did not have a sufficient number of probes that passed the filters for manufacture on the array.

2Plasmid of a size similar to pES3A (131 kb) was detected by gel electrophoresis of plasmid isolations.

3The detected plasmid was smaller in size than pES3A (110 kb). doi:10.1371/journal.pone.0009556.t003
Prophages

Of the 15 clusters, three putative prophage genomes and one prophage fragment were identified by Prophinder [35], and two additional regions are probable prophage fragments based on the presence of phage protein homologues identified by BLASTX (Table 3).

In the three prophage gene clusters (prophages 1, 2 and 3), genes encoding close homologues of known phage genes involved in integration, lysis and termination as well as head and tail structure were identified based on amino acid identity searches in IMG-JGI (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi). The average GC content of the sequenced C. sakazakii BAA-894 genome is 56%, the GC content of prophages 1, 2 and 3 was 53, 49 and 51%, respectively. The complete list of annotated prophage genes is available Table S2. In addition, Figure S1. shows the status of all putative prophage genes in the 10 Cronobacter strains.

Prophage 1 (GR4; ESA_00990–ESA_01052). In the 46-kb putative prophage 1 (Figure 3A), 30/63 (48%) hypothetical proteins were similar to known phage proteins. Homologues of phage genes involved in integration, lysis, head morphogenesis, tail assembly and phage regulation were identified. Prophage 1 also contains a gene (ESA_00997) encoding a protein homologous to the eae-like adhesion protein associated with the attaching and effacing phenotype. The eae gene is carried by some other bacteriophages of enteric pathogens: Salmonella phage epsilon34, E. coli O157:H7 bacteriophage PhiV10, enterobacterial phage P22 and enterobacterial phage epsilon15.

Prophage 2 (GR6; ESA_01608–ESA_01644). Putative prophage 2 (Figure 3B) contains 37 genes, out of which 25 (68%) were homologous to known phage proteins. Prophage 2 contains several lambdoid phage genes encoding the following proteins: repressor CII (ESA_01613), replication proteins O and P (ESA_01614 and ESA_01615), the antitermination protein Q (ESA_01622), small and large subunits of the phage terminase (ESA_01632 and ESA_01633) as well as head and tail morphogenesis proteins. Head morphogenesis genes (ESA_01635–ESA_01637) were similar to head proteins of bacteriophage HK97 from the family of lambda phages. Two gene clusters have very low average GC content; 33% (ESA_01616–ESA_01620) and 44% (ESA_01627–ESA_01631). Both these clusters contain hypothetical proteins that showed no similarity with known phage proteins or functions.

Prophage 3 (GR12; ESA_03025–ESA_03102) was the largest (47 kb) putative prophage identified (Figure 3C). Thirty-four genes (39%) genes had close homology to known phage proteins or functions. The rest of the annotated genes are conserved proteins of unknown functions or hypothetical proteins. Similarly to prophage 2, several regulatory genes characteristic for lambdoid
phages were identified: repressor proteins CI, CII and CIII, early gene regulator protein, replication proteins O and P as well as N independence proteins NinBFGZ. A cluster of three O-antigen conversion genes (ESA_03026–ESA_03028) was found in putative prophage 3 between phage integrase and tail morphogenesis genes (Figure 3C). The putative colicin uptake protein TolA (ESA_03048) may be involved in the internalization of the bacteriophage, as the Tol pathway can be also used for the translocation of phages into the bacterial cell [36]. CGH showed that the entire genome of prophage 3 or its close relatives are absent from the genomes of all other Cronobacter strains except C. turicensis. In this species, 18 prophage genes (mostly annotated as hypothetical proteins) were classified as present (Figure S1). Interestingly, the CII regulator protein (ESA_03094), the Kil protein (ESA_03095), and both large and small terminase subunits (ESA_03052 and ESA_03053) were present in two C. sakazakii strains (701 and 767), possibly as a part of different bacteriophages. These two strains were isolated from two fatal neonate cases of C. sakazakii infection [6]. A cluster of putative phage tail proteins (ESA_03029–ESA_03034) and a cluster containing phage head morphogenesis genes and a putative colicin uptake gene (ESA_03039–ESA_03051) were homologous to genes of the S. enterica serovar Typhi Vi type II phage E1 which may use virulence-associated capsular antigen as entry. This antigen was present on the surface of clinical Typhi isolates [37]. Although most genes of the putative prophage 3 were absent in all 10 Cronobacter strains tested by CGH, the region corresponding to the phage Vi genes (ESA_03041–ESA_03048) was present in C. turicensis and partially present in C. sakazakii strains 20, 701 and 767 (Figure S1). Strains 701 and 767 were both associated with fatal outbreaks, and are in MLST sequence type 4 with strain 20 [12].

**Prophage fragment 1 (GR3; ESA_00604–ESA_00630).** Eight of 19 genes in this region encode proteins associated with phages: plasmid and phage DNA primase (ESA_00620), a protein from Ash phage family (ESA_00624), the phage transcriptional regulator AlpA (ESA_00625), a putative phage capsid protein (ESA_00626), the phage transcriptional activator Ogr/Delta (ESA_00627) and phage integrase (ESA_00630). ESA_00618 was homologous to ea59 of lambda bacteriophage and ESA_00622 was homologous to a P4 phage protein. This cluster is most probably a phage remnant and may not encode a functional phage due to the absence of homologues of known structural tail proteins. The phage cluster was absent in all other strains. However, a short region, ESA_00609–ESA_00617, was present in C. sakazakii strains 701 and 767. A group of restriction endonucleases belonging to this cluster encoding a restriction-modification methyltransferase subunit (ESA_00614), a restriction endonuclease S subunit (ESA_00615), a hypothetical protein (ESA_00616) and a type I site-specific deoxyribonuclease (ESA_00617) were homologous to genes api49, api50, api51 and api52, respectively, from the Yersinia pseudotuberculosis adhesion pathogenicity island [38].

Figure 3. Gene maps of putative prophages. A. Gene map of putative prophage 1. B. Gene map of putative prophage 2. C. Gene map of putative prophage 3. Annotation of the putative prophage genes is available in Table S2. doi:10.1371/journal.pone.0009556.g003
**Prophage fragment 2 (GR10):** The region ESA_02304–ESA_02339 is likely to represent another prophage remnant. The region mostly contains hypothetical proteins with unknown functions. However, eight genes showed some degree of homology to proteins of phage origin. The cluster is flanked by a gene homologous to phage methyltransferase (ESA_02304) and a gene containing a site-specific recombinase domain that is found in putative integrases/recombinases of mobile genetic elements of diverse bacteria and phages (ESA_02339). It also includes genes homologous to phage lysozyme (ESA_02309), a phage tail component (ESA_02311), a putative phage tape measure protein (ESA_02313), another unspecified phage protein (ESA_02316), a major capsid protein (ESA_02319) and a phage portal protein (ESA_02320). GR10 might be another remnant of a prophage that has previously integrated into *C. sakazakii* BAA-894 genome. The presence of putative integrase flanking the cluster suggests introduction of this cluster into the genome by horizontal gene transfer. This cluster was absent in all *Cronobacter* strains except the genes encoding phage lysozyme (ESA_02309) and a hypothetical protein (ESA_02310), present in *C. sakazakii* strains 2 and 20, probably as a part of different prophages.

**Prophage fragment 3 (GR11; ESA_02740–ESA_02755):**

The gene cluster of the 8 kb putative prophage fragment 3 comprises 16 hypothetical proteins, 10 of which (63%) may be associated with phage functions. As it lacks genes for head and tail morphogenesis as well as phage regulatory genes, it is likely to be a non-functional phage remnant.

Two thirds of all gamma-proteobacteria and low GC Gram-negative bacteria harbor prophages [39]. There is an increasing body of evidence that phages play a pivotal role in the diversification of bacterial species. Some phages can carry additional cargo genes, which are not required for the phage cycle and are suspected or proven virulence factors [39]. Such genes are typically located near prophage ends, downstream of phage tail genes or next to Q or N-like antiterminator genes [40]. Putative prophages identified in this study contain genes which are not similar to any other known prophage genes. Moreover, prophage 1 contains a gene encoding a protein homologous to the *eae*-like adhesion protein, which is a recognized virulence factor in enteropathogenic *E. coli* associated with the attaching and effacing phenotype [41]. It is hypothesized that most prophages are lost by horizontal gene transfer. GR7 was found to be carried on plasmid pK29 of *Klebsiella pneumoniae* strain NK29, plasmid pEC-IMPQ of *Enterobacter cloacae*, plasmid R478 of *Serratia marcescens* and plasmid pAPEC-O1-R of *Excherichia coli* APEC O1, which is further evidence of horizontal gene transfer. As the gene cluster was entirely absent from all other *Cronobacter* strains in our study, the reference strain BAA-894 probably acquired the tellurite resistance cluster recently.

**GR8** (ESA_01970–ESA_01976) contains seven genes encoding pilus assembly proteins. Fimbriae (or pili) enable bacteria to colonize the epithelium of specific host organs and are therefore considered major virulence factors [45]. This cluster of genes was absent in all *Cronobacter* strains except *C. sakazakii* strain 20.

**GR9** (ESA_02032–ESA_02041) contains genes encoding hypothetical proteins and four proteins involved in Type VI secretion system (ESA_02037–ESA_02040). These four genes were present in *C. sakazakii* strains 1 and 20 and absent in all other strains.

**GR13** (ESA_03887–ESA_03912) is a cluster of 16 hypothetical proteins without homology to known proteins or functions. This cluster was absent in all other *Cronobacter* strains.

**GR14** (ESA_04248–ESA_04255) is a cluster of genes involved in copper resistance (Table 4). This cluster was found in *C. sakazakii* strains 1 and 696, as well as *C. turicensis* and *C. malonicus*.

**GR15** (ESA_pESA3p05493–ESA_pESA3p05505) involves genes located on *C. sakazakii* plasmid pESA3 and includes components of type IV and type VI secretion pathways (Table S2) as well as a gene encoding an outer membrane protein from the OmpA family (ESA_pESA3p05495). GR15 was absent from all strains except *C. sakazakii* strains 1 and 696.

Most of the described regions contain suspected or proven virulence factors. The genes in GR1, GR9 and GR15 are involved in a type VI secretion system, a newly described mechanism for protein transport across the cell envelope of Gram-negative bacteria.

**Non-Phage Regions That Differ among the Strains**

The complete list of genes belonging to the variable non-phage regions and their presence in tested strains is available in Table S2.

**GR1** (ESA_00140–ESA_00145) is a small cluster of type VI secretion system genes. ESA_00142 shares a conserved region with a family of IcmF-related proteins proposed to be involved in increased *Vibrio cholerae* adherence to epithelial cells [42]. ESA_00143 is a secretion protein belonging to the VC_A0110 family; mutations in proteins of this family are associated with impaired virulence [43]. ESA_00145, a secretion lipoprotein from a VC_A0113 family was present in *C. sakazakii* strains 701 and 767 associated with fatal outbreaks. The rest of GR1 was absent in all tested strains except *C. sakazakii* 696.

**GR2** (ESA_00292–ESA_00310) mostly contains uncharacterized conserved proteins. It also contains the gene encoding a protein from a family of beta-lactamases (ESA_00299).

**GR5** (ESA_01179–ESA_01189) contains a cluster of proteins involved in cell wall biogenesis and nucleotide sugar metabolism. GR5 corresponds to the *C. sakazakii* O-antigen gene locus used to distinguish the two *Cronobacter* serotypes O:1 and O:2 [44]. DNA microarray analysis revealed that GR5 is highly divergent; its genes were not sufficiently similar to be detected by microarray hybridization in any other *Cronobacter* strains. The O-antigen locus contains two more genes (homologues of ESA_01177 and ESA_01178) which were present in all strains except *C. sakazakii* 696.

**GR7** (ESA_01775–ESA_01804) Most genes in GR7 were predicted to be involved in tellurite and stress resistance. It contains homologues of tellurite resistance proteins TerA, TerC, TerD, TerY and TerZ. The cluster contains two putative transposase genes, which suggests that the cluster was acquired by horizontal transfer. GR7 was found to be carried on plasmid pK29 of *Klebsiella pneumoniae* strain NK29, plasmid pEC-IMPQ of *Enterobacter cloacae*, plasmid R478 of *Serratia marcescens* and plasmid pAPEC-O1-R of *Excherichia coli* APEC O1, which is further evidence of horizontal gene transfer. As the gene cluster was entirely absent from all other *Cronobacter* strains in our study, the reference strain BAA-894 probably acquired the tellurite resistance cluster recently.

**Table 4. Putative prophages identified in the genome of *C. sakazakii* BAA-894 by Prophinder [35] or BlastX.**

| Designation | Prophinder ID | Size (kb) | Coordinates |
|-------------|---------------|-----------|-------------|
| Prophage 1 (GR4) | 48917 | 45.7 | 969835–1015482 |
| Prophage 2 (GR6) | 48912 | 26.7 | 1561010–1587672 |
| Prophage 3 (GR12) | 48916 | 46.9 | 2974743–3021625 |
| Prophage fragment 1 (GR3) | 48908 | 8.1 | 2693304–2701447 |
| Prophage fragment 2 (GR10) | BlastX | 33.4 | 2237406–2270858 |
| Prophage fragment 3 (GR11) | BlastX | 8.2 | 2693304–2701446 |

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bacteria that can increase adherence to epithelial cells [46,47]. GR3 contains four genes (ESA_00614–ESA_00617) that are homologous to a restriction-modification gene cluster (api49–api52) in the Yersinia pseudotuberculosis pathogenicity island (YAPI) [30]. As these genes were present in strains 701 and 767 isolated from two neonates that died as a result of infection by Cronobacter during an outbreak in France [8,17], and are absent in all other strains tested by CGH, these genes may be important virulence factors contributing to the pathogenicity of Cronobacter.

Cronobacter virulence factors have not been extensively studied, although it is known that Cronobacter species vary in their virulence with respect to invasion of intestinal cells, survival in macrophages and serum resistance [13,14]. GR 5 (ESA_01179–ESA_01189) encodes the lipopolysaccharide (LPS) genes. Characterisation of LPS structure and consequently O-antigen can be important in developing identification schemes based on serotyping, and has a role in virulence and serum resistance of the organism. The LPS is one of the few structural features of Cronobacter which has been investigated and it is known that it varies across the Cronobacter spp. In C. sakazakii and C. malonaticus, the LPS are composed of various branched polymers, whereas they are unbranched in C. muytjensii. In C. sakazakii BAA-894 [48] it is a branched polymer of pentasaccharide units composed of 2-acetamido-2-deoxy-D-galactose, 3-(N-acetyl-L-alanylamido)-3-deoxy-D-quinovose, D-glucuronic acid, and D-glucose. C. sakazakii strain 767 is also a branched polymer but of a repeating heptasaccharides composed of 2-acetamido-2-deoxy-D-glucose, and D-galacturonic acid, L-rhamnose, and D-glucose [49]. C. malonaticus LPS [50] is also a branched pentasaccharide unit of 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, 3-deoxy-D-manno-oct-2-ulosonic acid, D-galactose and D-glucose residues. Whereas, C. muytjensii LPS [51] is a unbranched pentasaccharide polymer of 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-3-deoxy-D-quinovose, L-rhamnose and D-glucuronic acid. These considerable differences correspond with the lack of sequence conservation in GR5 as revealed in the microarray analysis. The individual genes encoding these differences in enzymology have yet to be assigned.

Comparison to Other Enterobacterial Genera

The sequenced genome C. sakazakii BAA-894 was compared to the genomes of Citrobacter koseri BAA-885, Klebsiella oxytoca VJ9K009, E. coli K12 MG1655 and Salmonella enterica Typhimurium strain LT2, representing some of the most closely related genera to Cronobacter. Using a threshold of identity of >85% in a 100 base window, 334 genes were present in all Cronobacter but absent or diverged in the four members of other genera (manuscript in preparation). These genes included a cluster of type VI secretion genes (ESA_03943 - ESA_03948) which might be involved in virulence, and a putative palatinose operon (ESA_02709 - ESA_02715). Alpha-glucosidase activity, which has been linked to palatinose metabolism, is considered as one of the major biochemical traits that distinguish Cronobacter from other related Enterobacteriaceae.

Summary and Conclusions

Using a whole-genome ~304,000 oligonucleotide tiling microarray, we analyzed the genomic content of isolates representing all five Cronobacter species by CGH. A dynamic determination of cutoffs GACK [22] was used to minimize the number of incorrectly categorized genes. Among 6 strains of C. sakazakii 2,404 genes (54.9%) represented a core shared genome. Of these genes 1,899 (43.3%) were also in the core genome when compared to four other Cronobacter species. CGH highlighted a copper/silver resistance cluster associated with invasion of BMEC, which were unique to the three Cronobacter species associated with neonatal infections, as well as efflux pumps and adhesins unique to C. sakazakii strains from NICU outbreaks.

The main genetic features that distinguished the Cronobacter strains were putative prophages and several other gene clusters, a pattern of divergence typical among bacteria [52]. A few of the regions present in the sequenced strain and absent in some other Cronobacter strains are found in only a few other Enterobacteriaceae. For example, GR 7 is found in four of the hundreds of Enterobacteriaceae genomes that have been sequenced, which indicates that this region may have been horizontally acquired, possibly from a source outside of the Enterobacteriaceae. We have shown that gene acquisition via integration of plages and other mobile elements and specific gene-loss play a major role in Cronobacter evolution and diversity. Fifteen clusters of genes including three putative prophages and three putative prophage fragments that were absent in more than half tested strains were identified. In most of them, putative virulence genes were identified.

Future studies will focus on the expression of virulence related genes and their role in the pathogenicity of Cronobacter species, particularly the mechanisms of neonatal infection.

Materials and Methods

Strains and Culture Conditions

Cronobacter strains were selected which represented the five recognized species, and included those from reported clinical cases (Table 1). All Cronobacter strains were stored at −80°C in Nutrient Broth (Oxoid, UK) with 10% glycerol, subcultured on Trypticase Soy Agar (Oxoid, UK) and checked for purity. Overnight Trypticase Soy Broth (Oxoid, UK) cultures were used for DNA extraction.

DNA Extraction

Total genomic DNA was isolated using the QIAGEN Genomic tip 100/G and Genomic DNA Buffer Set (www.qiagen.com) with extended cell lysis (1 h) and two additional washes of the precipitated DNA. The DNA samples were checked for fragmentation on 0.8% agarose gels and checked for protein and RNA content by spectrophotometry.

Sequencing of C. sakazakii ATCC BAA-894 and Assembly

The complete genome of C. sakazakii BAA-894, a strain isolated from a powdered formula used during an NICU outbreak [7], was sequenced using the whole genome shotgun method, supplemented with end sequencing of a fosmid library. Sonicated and size-fractionated DNA was cloned into plasmid vectors (pOTw13). Subclones and fosmids were sequenced using dye-primer and dye-terminator chemistry on ABI 3730 sequencing robots. Using the PCAP assembly software program [53], 384,000 subclones (plasmids and fosmids). Following the auto-finish subclones and fosmids were sequenced using dye-primer and dye-terminator chemistry on ABI 3730 sequencing robots. Using the PCAP assembly software program [53], 51,289 sequence reads, representing 6.2 fold coverage, were assembled. Part of the 6.2X coverage included 1.19X fosmids. Under-represented areas, gaps, and ambiguities were then addressed by performing automated sequence improvement [54] using directed sequencing from the subclones [plasmids and fosmids]. Following the auto-finish process, correcting misassembled regions, resolving ambiguous bases, and filling the remaining gaps by additional directed sequencing and PCR, completed the finishing process. This yielded a product with a final estimated accuracy of 99.99%.

Annotation

AceDB was the primary annotation database. The identification of protein-coding genes used a combination of GeneMark, Glimmer 2.0 and Glimmer 3.0; an evidence-based approach was used to
prioritize genes for inclusion into a final gene set. Genes missed by the two ab initio gene predictors were identified using BlastX.

*C. sakazakii* ATCC BAA-894 Microarray Design and Comparative Genome Hybridization Analysis (CGH)

A 384,030 probe oligonucleotide tiling DNA microarray was designed which comprised the complete genomic sequence of *C. sakazakii* ATCC BAA-894. Probes were designed at an average of less than 12 base spacing on alternating strands, leading to an average of over 100 50-mer oligonucleotide probes per annotated gene.

Every possible 50-base probe from both strands of the *C. sakazakii* genome and two plasmids was tested for the ability to be manufactured by NimbleGen. Those that required too many NimbleGen cycles were shortened. Resulting candidate probes that were less than 35 bases long were thrown out. The remaining 9,061,350 potential probes had an average melting temperature (Tm) of 74 degrees Celsius. Probes that had Tm above the average were shorted, down to a minimum of 35 bases. The probes were selected from the pool of 9,061,350 potential probes by selecting the best probe at 11,375-base increments, alternating between strands each time. The resulting 386,802 candidate probe sequences where analyzed to remove any probes that covered the same region as another probe due to duplications in the genome and the resulting 384,030 unique sequences were chosen for the array. Mappings between the unique probe sequences and their genome/plasmid/gene positions were stored in a separate file.

Sample labeling, CGH and data normalization were performed according to the method described at http://www.nimblegen.com/products/lit/cgh_userguide_v5p1.pdf.

DNA from the sequenced strain, *C. sakazakii* BAA-894, was used as the internal array control. For within-array normalization, a LOWESS method [55] was used as spatial correction and QSPline [56] was used to correct for dye bias. The raw data is deposited in GenBank GEO (accession number GSE19308).

Data Visualization by WebArrayDB

The CGH plotter available at WebArray (www.webarraydb.org/webarray/index.html) was used to create CGH plots. This used the log2 intensity ratio microarray data to calculate the median log2 intensity ratios for each *C. sakazakii* BAA-894 gene. WebArrayDB is a database system and online cross-platform analysis suite for analysis of microarray data, which allows storage of the data in the repository and their online analysis [57].

Dynamic Cut-Off Determination by GACK

Each gene was represented by tens or hundreds of separate oligonucleotides. These measurements were condensed into a single median ratio for each gene. The data were further analyzed using the dynamic cut-off determination tool GACK [22]. The normal probability density function was calculated from the characteristics of the main peak of the data distribution. This was used to calculate the estimated probability of presence that gives a statistical validation of the gene assignment. Each CGH experiment was attributed a specific set of cut-offs to minimize the number of falsely assigned genes.

*Cronobacter* genes were classified according to the most stringent settings of the trinary output of GACK as present, intermediate or absent. Genes classified as ‘present’ had a log2 intensity ratio (test/reference) greater than the cut-off value corresponding to the 100% estimated probability of presence (EPP) calculated by GACK. The ‘absent’ genes had a log2 ratio inferior to the cut-off value corresponding to 0% EPP, and can include genes with sufficient sequence divergence. The ‘intermediate’ category included genes whose status could not be assigned with certainty. The EPP function and cut-offs were determined separately for hybridization data of each strain (Table S3).

Prophage Identification

ACLAME Prophinder was used for prophage identification [35]. This was accessed at http://aclame.ulb.ac.be/Tools/Prophinder/

16S rDNA Sequence Analysis

Partial 16S rDNA sequence analysis was done as previously described [10].

Plasmid Profiling

Plasmid DNA was isolated according to the method described in [58].

Supporting Information

Figure S1 Absence/presence status of prophage genes in *Cronobacter* strains. Red indicates absence/divergence of a particular gene, orange indicates uncertain status and green indicates presence of a gene. Found at: doi:10.1371/journal.pone.0009556.s001 (0.15 MB TIF)

Table S1 Selected genomic regions present in NICU outbreak strains *C. sakazakii* 707 and 767 and absent in *C. sakazakii* type strain ATCC 29544T.

Found at: doi:10.1371/journal.pone.0009556.s002 (0.16 MB DOC)

Table S2 Non-phage genomic regions absent in more than half of the tested strains.

Found at: doi:10.1371/journal.pone.0009556.s003 (0.40 MB DOC)

Table S3 Cut-off values used for the assignment of absent, intermediate or present gene status.

Found at: doi:10.1371/journal.pone.0009556.s004 (0.04 MB DOC)

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Author Contributions

Conceived and designed the experiments: SWC RKW MM. Performed the experiments: EK LAJ CF PJM KK WW RSF DF NS VB WEN. Analyzed the data: EK SWC XQX AW MM SF. Contributed reagents/materials/analysis tools: SWC XQX FL SP KHP MM. Wrote the paper: EK MM SF.
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