Identification and Characterization of Cronobacter spp. Iron Acquisition Systems

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Cronobacter spp. are emerging pathogens that cause severe infantile meningitis, septicemia, or necrotizing enterocolitis. Contaminated powdered infant formula has been implicated as the source of Cronobacter spp. in most cases, but questions still remain regarding the natural habitat and virulence potential for each strain. The iron acquisition systems in Cronobacter spp. strains isolated from different sources were identified and characterized. All Cronobacter spp. have both the Feo and Efe systems for acquisition of ferrous iron and all plasmid-harboring strains (98%) have the aerobactin-like siderophore, cronobactin, for transport of ferric iron. All Cronobacter spp. have the genes encoding an enterobactin-like siderophore, although it was not functional under the conditions tested. Furthermore, all Cronobacter spp. have genes encoding for five receptors for heterologous siderophores. A ferric dicitrate transport system (fec system) is encoded specifically by a subset of C. sakazakii and C. malonaticus strains, of which a high percentage were isolated from clinical samples. Phylogenetic analysis confirmed that the fec system is most closely related to orthologous genes present in human pathogenic bacterial strains. Moreover, all strains of C. dublinensis and C. muytjensii encode two receptors, FcuA and Fct, for heterologous siderophores produced by plant pathogens. Identification of putative Fur boxes and expression of the genes under iron-depleted conditions revealed which genes and operons are components of the Fur regulon. Taken together, these results support the proposition that C. sakazakii and C. malonaticus may be more associated with the human host and C. dublinensis and C. muytjensii with plants.
**INTRODUCTION**

*Cronobacter* spp. are gram-negative, rod-shaped bacteria within the family *Enterobacteriaceae*. The genus *Cronobacter* has been shown to be phenotypically and genetically diverse (45) and has been proposed to comprise of seven species, *C. sakazakii*, *C. malonicus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis* (subsp. *dublinensis*, *lausannensis*, and *lactaridi*), *C. universalis* and *C. condimenti* (34, 36). These emerging pathogens cause severe meningitis, septicemia, or necrotizing enterocolitis in neonates and infants (40, 57). Although the disease frequency is very low, the mortality rate ranges from 40%, to as high as 80% (29, 57). Meningitis caused by *Cronobacter* spp. occurs both as sporadic cases and as outbreaks, and contaminated powdered infant formula (PIF) has been epidemiologically implicated as the source of the pathogen in most cases (14, 44, 71, 79). However, extrinsic contamination of opened PIF cans and bottled water supplies has also been reported (59). *Cronobacter* spp. have been also isolated from a wide spectrum of environmental sources and food products (25, 38) but their natural habitat and whether all strains have the capacity to produce disease is unclear. An environmental niche of eukaryotic plant material has been proposed for *Cronobacter* spp. due to the ability to produce a yellow pigment that protects the cell against the effects of UV radiation from sunlight, and expression of capsules and fimbriae to aid in adherence to surfaces, and promote survival under high osmotic and desiccated stressful growth conditions (35, 69).

Identification of virulence markers to distinguish pathogenic from nonpathogenic strains will improve our understanding of the epidemiology of *Cronobacter* spp., which will in turn help elucidate potential contamination risks associated with this food-borne pathogen. Recently, we reported that 97% of the 229 *Cronobacter* spp. isolates possess a plasmid that was closely related or identical to pESA3 and pCTU1 (pESA3-/pCTU1-like plasmid) (23); plasmids harbored by *C.
sakazakii ATCC BAA-894 and C. turicensis z3032, respectively (43, 74). Furthermore, we found that pESA3-/pCTU1-like plasmids encode common virulence factors, including an aerobactin-like siderophore and an ABC ferric-iron transporter eitABCD (23).

Iron is an essential cofactor for many enzymes involved in cellular respiration, electron transfer, and superoxide metabolism (28). Iron is also an important factor for bacterial pathogenesis (9, 77, 82). Although the concentration of iron in the environment is sufficient to sustain the viability of microbes, under aerobic conditions most iron is present as ferric hydroxide (Fe$^{3+}$), which is insoluble and biologically inaccessible to bacteria (58). Under iron starvation conditions, bacteria produce small iron-chelating molecules termed siderophores (54). Siderophores bind to the six coordinate sites of ferric ions by forming water-soluble hexadentate ferric complexes. Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates-phenolates (e.g. enterobactin), hydroxamates (e.g. aerobactin and ferrichrome), and carboxylates (e.g. citric acid and derivatives) (54). In gram-negative bacteria, the Fe$^{3+}$-siderophore complex is recognized and transported into the periplasm via TonB-dependent receptors, and is transferred into the cytoplasm by ABC transporters formed by a permease, and an ATPase protein (54, 18). Once in the cytoplasm of the cell, the Fe$^{3+}$-siderophore complex is usually reduced to Fe$^{2+}$ to release the iron, especially in the case of "weaker" siderophore ligands such as hydroxamates and carboxylates (53, 81). Siderophore decomposition or other biological mechanisms can also release iron, especially in the case of catecholates such as ferric-enterobactin, whose reduction potential is too low for reducing agents (48). Under anaerobiosis, (reducing) or low pH growth conditions, the iron equilibrium shifts from the ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) form that is more
easily accessible to microorganisms. This allows several permeases of different protein families to also contribute to overall iron uptake (11, 64).

The presence of iron acquisition systems is advantageous for the growth of bacteria under low iron availability stress conditions. Pathogens, in particular, require efficient iron acquisition mechanisms to enable them to compete successfully for iron in the highly iron-restricted environment of the human host. There is considerable variation in the type of iron transporters and iron sources utilized by different microbial species. This may reflect the diversity of various niches occupied by particular strains and the nature of the source of iron available in a specific environment. In this study, we performed a comparative in silico analysis of putative iron acquisition systems found in the genomes of nine strains of *Cronobacter*, representing six species. Furthermore, we identified the iron acquisition systems profile of a collection of 231 *Cronobacter* spp. strains isolated from clinical, food, and environmental sources and from diverse geographical locations. Expression of the putative iron acquisition genes under different iron concentrations and functionality of identified siderophores were also determined.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** The bacterial strains used in the present study are described in Table 1. The *Cronobacter* spp. strains screened for iron acquisition system gene targets consisted of 180 *C. sakazakii*, 25 *C. malonicicus*, 12 *C. muytjensii*, 6 *C. turicensis*, 6 *C. dublinensis*, and 2 *C. universalis* from our laboratory culture collection; these strains represent isolates obtained from clinical (50, 22%), food (48, 21%), and environmental (122, 53%) sources (4% unknown) and from diverse geographical locations. Assignment of the *Cronobacter* species nomenclature to the strains was performed according to the proposed classification scheme suggested by Iversen et al. (34) and identification was confirmed using the species-specific *rpoB*
PCR assay described by Stoop et al. (75). Fosmid clones ESA-C01 and ESA-M04 containing and lacking the cronobactin genes, respectively were obtained from a *C. sakazakii* BAA-894 fosmid library (43). Presence and absence of *viuB/shiF* and *iuABCD/iutA* in ESA-C01 and ESA-M04 was confirmed by PCR using primers derived from each gene. *Cronobacter* spp. and *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth with shaking (175 rpm) or on LB agar. Antibiotics were added when required at the following concentrations: chloramphenicol (40 µg/ml) and ampicillin (100 µg/ml). Results were submitted to the Pathogen-Annotated Tracking Resource Network (PATRN) system located at http://www.patrn.net and it is accessible to users after a free registration process.

*In silico analysis.* The genomes of all nine *Cronobacter* spp. strains used in this study were annotated using the RAST server (4). They include, the genome of *C. sakazakii* ATCC BAA-894, CP000783; the genome of *C. turicensis* z3032, FN543093; pESA3, NC_009780; and pCTU1, NC_013283; *C. sakazakii* 2151, AJKT01000000; *C. universalis* NCTC 9529, AJKW01000000; *C. malonaticus* LMG 23826, AJKV01000000; *C. dublinensis* subsp. dublinensis LMG 23823, AJKZ01000000; *C. dublinensis* subsp. lactaridii LMG 23825, AJKX01000000; *C. dublinensis* subsp. lausanensis LMG 23824, AJKY01000000; *C. muytjensii* ATCC 51329, AJKU01000000. Iron acquisition genes and gene clusters were identified by intrinsic RAST subsystem profiling for each genome as well as manual gene homolog BLAST searches. Comparative genomics in the SEED viewer (60) was used to confirm identification and conservation of putative iron acquisition genes within *Cronobacter* spp. genome sequences.

*Phylogenetic analysis.* Phylogenetic analyses of iron acquisition system nucleotide sequences, a total of 16, were conducted in MEGA5 (76), using the entire iron acquisition gene or gene cluster for each system, retrieved from the SEED viewer and NCBI (See Supplementary
material for those not shown in Fig. 3). The evolutionary history was inferred by using the neighbor-joining method (65). The bootstrap consensus tree inferred from 1000 replicates (20) is taken to represent the evolutionary history of the taxa analyzed (20). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to each branch (20). Trees are drawn to scale, with branch lengths in similar units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed by using the Poisson correction method (84) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the data set.

**PCR assays.** PCR primers were designed that targeted the different putative iron acquisition genes and genes clusters. Except for the primers for cronobactin genes fecR, fecB, fecE, fcuA and fct, all the primers were derived from common sequence of the nine sequenced Cronobacter spp. strains tested in this study. Primers for the cronobactin genes were derived from common sequences of pESA3 and pCTU1, primers for fecR, fecB and fecE were designed from C. sakazakii 2151, and primers for fcuA and fct were derived from common sequences of C. dublinensis strains LMG 23823, LMG 23825, LMG 23824 and C. muytjensii ATCC 51329. The sequences of the primers, targeted gene, and the amplification parameters used for each set of primers are shown in Table 2. In each PCR reaction, the positive control consisted of DNA preparations of the 9 strains sequenced; the negative controls were DNA preparations of the closely related sister species, Enterobacter helveticus z513 and E. turicensis z508, as well as water (no template control). All PCR mixtures were prepared using the GoTaq Green master mix (Promega Corp., Madison, WI), according to the manufacturer’s instructions using 1 μl of the plasmid preparation (approximately 90 ng DNA/25-μl reaction mixture) or 5 μl of boiled...
genomic DNA sample (approximately 50 ng DNA/25-μl reaction mixture) served as DNA template. In all PCRs, the polymerase was activated by using a 3-min incubation step at 94°C, followed by 25 cycles of denaturation at 94°C for 30 s and annealing and extension steps according to the PCR parameters described in Table 2. For each reaction, a final extension step of 7 min at the cycle extension temperature, as described for each PCR, was used (Table 2).

**RT-PCR.** Expression of the iron acquisition genes was determined by reverse transcription (RT)-PCR. Total RNA of *Cronobacter* spp. strains was obtained using Trizol reagent (Invitrogen) according to the manufacturer’s protocol. Trace levels of genomic DNA were removed by treatment with DNase I using a DNA-free kit (Applied Biosystem) and cDNA was synthesized from 1 μg of total RNA using the SuperScript III First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer’s instructions with the primers listed in Table 2. RNA samples lacking reverse transcriptase were used as control to detect DNA contamination. PCR conditions were selected to permit detection of the PCR products in the linear range of the reaction and were performed using the same conditions described above to screen for detection of the various iron acquisition systems (Table 2). The intensity of the PCR products was quantified using ImageJ, version 1.45s (1). Differences in the intensity of the PCR products are interpreted as differences in transcription and/or stability of the iron acquisition genes mRNA. The level of expression of *Cronobacter* 16S rRNA using primers P0 and P6 (12) served as an internal control.

**Siderophore detection.** Siderophore production was determined using the chrome azurol S (CAS) agar diffusion (CASAD) assay as described previously (23). Briefly, the CAS agar plate was punched with five-mm-diameter holes by using a gel puncher. Each hole was filled in a two-step process with 70 μl (35 μl twice) of cell-free culture supernatant of the test bacteria grown for
181 18 h in LB broth containing 300 μM 2, 2′-dipyridyl (Sigma Aldrich). After incubation at 37°C
182 for four to 8 h, the presence of an orange halo around a hole indicated a culture was positive for
183 siderophore production.
184
185 The presence of phenolic-type and/or hydroxamate-type siderophores were detected in
186 cell-free culture supernatants or whole cell lysate preparations obtained from-iron depleted
187 cultures using the colorimetric assays described by Arnow (3) and Csáky (15), respectively. Cell-
188 free culture supernatants were lyophilized and concentrated 10-folds for the Arnow’s test. The
189 Csáky assay was carried out with and without the sulfuric acid digestion step. Cell-free culture
190 supernatants of Vibrio vulnificus UNCC913 (73) and catechol (6 µg) were used as positive
191 controls in the Csáky’ and Arnow’s tests, respectively.
192
193 **Statistical analysis.** Data were analyzed by the Student’s t-test (paired); a P-value of
194 <0.05 was considered statistically significant.
195
196 **RESULTS AND DISCUSSION**
197
198 **Identification of Cronobacter spp. iron acquisition systems.** Targeted *in silico*
199 sequence analysis of the genomes of nine Cronobacter spp. strains, including, three *C.*
200 dublinensis, two *C. sakazakii*, and single strains of *C. malonaticus, C. turicensis, C. muytjensii*
201 and *C. universalis* revealed the presence of shared iron acquisition systems, with additional
202 systems in some *Cronobacter* spp. strains. The identified iron acquisition systems and their
203 locations in the genome of the nine *Cronobacter* spp. strains are listed in Supplementary Table
204 S1. These systems include genes encoding ferric and ferrous transporters, heme-iron extractors,
205 as well as putative TonB-dependent iron receptors and ferric reductases.
206
207 **i) Ferric iron transporters.** For acquisition of ferric iron (Fe³⁺), all nine *Cronobacter*
208 spp. genomes contain genes homologous to the hydroxamate-type siderophore aerobactin
(named cronobactin in this study) and catechol-type siderophore enterobactin genes, except for
C. muytjensii 51329, which did not have the cronobactin gene cluster because it does not harbor
the common virulence plasmid. The cronobactin locus consists of five genes homologous to
biosynthetic genes iucABCD and the receptor gene iutA (Fig. 1A). In agreement with a previous
report (23), this gene cluster is localized on a pESA3-/pCTU1-like plasmid.

In contrast, to the single enterobactin locus encoded by E. coli and other bacteria, the
genes encoding the enterobactin-like siderophore in seven of the nine Cronobacter spp. genomes
analyzed are localized in three different loci in the chromosome (Fig. 1B). The first locus
contains a cluster of five genes, entD/fepA/feS/ybdz/entF, the second locus contains only fepE,
and the third locus comprises 10 genes, entH/entA/entB/entE/entC/fepB/entS/fepD/fepG/fepC. C.
dublinensis lactaridii LMG 23825 and C. dublinensis lausenensis LMG 23824 contain loci one and
three, but lack the second locus containing fepE. In E. coli, entA/B/C/D/E/F/H are involved in
enterobactin biosynthesis, entS is involved in enterobactin export, fepA encodes the ferric
enterobactin receptor, fep/B/C/D/E/G allows the transport of the ferric enterobactin complex
inside of the cell and fes encodes an esterase that catalyzes hydrolytic cleavage of the ferric
enterobactin backbone, leading to the intracellular release of iron (26, 48, 62).

All nine Cronobacter spp. genomes harbor genes homologous to the fhuACDB operon
(Fig. 1C). The fhuA gene encodes the TonB-dependent outer-membrane receptor specific for
hydroxamate-type siderophore ferrichrome (19), suggesting that Cronobacter spp. are able to
incorporate ferrichrome produced by other species. The fhuCDB genes encode an ABC
transporter for a range of hydroxamate siderophores (19, 63). It has been determined that
mutations in the fhuCDB operon abolish the ability of bacteria to use ferrichrome, aerobactin,
and coprogen (22, 42). Thus, the FhuCDB transport system must be involved in the import of the
iron-cronobactin complex into the cell. Furthermore, the eight *Cronobacter* spp. genomes harboring pESA-3/ pCTU1-like plasmids contain an *eitABCD* operon with homology to ABC transporters that mediate translocation of ferric iron, siderophores and heme (Fig. 1D), which was previously reported (23).

Genes homologous to the ferric dicitrate transport genes *fecIRABCDE* (*fec* genes) were found solely in the genome of *C. sakazakii* strain 2151 (Fig. 1E). This iron transport system has been well characterized in *E. coli* K-12 and other bacteria (50, 51, 80). The *fec* system is capable of maintaining bacterial growth under iron-limited conditions in the absence of other iron uptake systems (50, 51, 80). Like the *E. coli fec* system, the *C. sakazakii* 2151 *fec* locus consists of genes located within two operons carrying the regulatory genes *fecI* and *fecR*, and the downstream structural genes *fecABCDE*. The *fecA* gene encodes a TonB-dependent receptor for the ferric-dicitrate complex and *fecBCDE* encodes the transport system (50, 51, 80). In *C. sakazakii* 2151, the *fec* genes are contained in a plasmid, named pCSA2151, not previously identified in *Cronobacter* spp. Similar to other bacteria (50, 51, 80) the *fec* genes of *C. sakazakii* 2151 are flanked by insertion sequences (IS), indicating their mobility (Fig. 1E).

**ii) Ferrous iron transporters.** All nine *Cronobacter* spp. genomes analyzed have both the genes encoding the Feo and Efe systems to acquire the ferrous (Fe^{2+}) form of iron from their environment. The Feo system is the major ferrous transporter in *E. coli* and is widely distributed among bacteria (37). This system operates anaerobically at pH ≥ 7. Like all *Enterobacteriaceae*, the *Cronobacter* Feo system is comprised of three genes *feoABC* (Fig. 2). In *E. coli*, FeoA is a small soluble SH3-domain protein probably located in the cytosol (11). **FeoA of *Cronobacter* spp. conserves the FeoA-SH3 domain of *E. coli*, suggesting that the *Cronobacter* spp. and *E. coli* Feo systems are regulated by the same mechanisms.** FeoB is most likely a permease and
FeoC is a small protein apparently functioning as an [Fe-S]-dependent transcriptional repressor (11).

The Efe system is a ferrous transport system that operates aerobically under low pH, conditions in which the ferrous iron remains stable (10). The Bacterial Efe system in general has similarities to the well-studied high affinity Fe$^{2+}$ transporter (Ftr1p) of the *Saccharomyces cerevisiae* (10). Like *S. cerevisiae* Fe$^{2+}$ transporter, the Efe system consists of three genes, *efeUOB* (Fig. 2). EfeU is homologous to the high-affinity iron permease, Ftr1p, of *S. cerevisiae*, EfeO is periplasmic with a cupredoxin N-terminal domain; EfeB is also periplasmic and is a heme peroxidase-like protein (10). The Efe system in *E. coli* K-12 is not functional due to a frameshift mutation in *efeU* (10). The *efeU* gene of *Cronobacter* spp. lacks any frameshift suggesting that the Efe system is functional in this genus.

**iii) Heme-iron extractors.** In the mammalian host, most of the iron is sequestered and contained within heme proteins. Many pathogens have the ability to use these host compounds directly, and heme utilization genes have been identified in numerous pathogens (55, 56, 78). No genes homologous to heme receptors were identified in any of the nine *Cronobacter* spp. genomes analyzed; however, all nine *Cronobacter* spp. have a common gene whose predicted encoded protein has significant identity with *E. coli* YfeX. It has been reported that YfeX and EfeB of the Efe system in *E. coli* promote iron extraction from heme (46). Both YfeX and EfeB are widespread and highly conserved in bacteria.

**iv) Putative TonB-dependent iron receptors.** In addition to the TonB-dependent receptors IutA, FepA, FhuA and FecA specific for cronobactin, enterobactin, ferrichrome, and ferric dicitrate respectively, the nine *Cronobacter* spp. genomes contain five common genes whose predicted encoded protein have significant identity with TonB-dependent iron receptors.
These outer membrane receptors include the siderophore receptor YncD (83), the vitamin B12/cobalamin outer membrane transporter BtuB (30), the ferroxamine receptor FoxA (5), the ferric-rhodotorulic acid/coprogen receptor FhuE (67), and the ferric-enterobactin receptor PfeA. All of these TonB-dependent receptors are required for the virulence of different bacteria, with the exception of BtuB (39, 61, 66, 83). PfeA is 60% identical to the ferric-enterobactin receptor FepA encoded by the enterobactin gene cluster (Fig 1B) and the presence of two putative ferric-enterobactin receptors in Cronobacter spp suggests that there may be siderophore receptor redundancy.

The chromosomes of the C. muytjensii and the three C. dublinensis strains analyzed encode TonB-dependent iron receptors FcT and FcuA, not encoded by the other Cronobacter spp. strains. FcT has significant homology with ferrichrysobactin receptor (FcT), encoded by the plant pathogen Erwinia chrysanthemi and other gram-negative bacteria (68). Siderophore chrysobactin is an important virulence factor of E. chrysanthemi (16). FcuA is also a hydroxamate receptor encoded by Yersinia enterocolitica and other gram-negative bacteria (41). The presence of these TonB-dependent outer membrane receptors suggests that Cronobacter spp. can incorporate many heterologous siderophores of bacterial or fungal origin.

v) Ferric reductase. Upstream of the cronobactin gene iucA in all of the pES3A/pCTU1-like plasmid harboring Cronobacter spp. genomes analyzed, are two ORFs, named shiF and viuB, due to their similarity to shiF and viuB of Shigella and Vibrio spp., respectively (Fig. 1A). Similar shiF-like genes lie upstream of the aerobactin locus in a number of bacteria (22), suggesting a possible role in the aerobactin system. Annotations of shiF-like genes indicate it is a member of the COG0477 permeases of the major facilitator superfamily (MFS). The role of ShiF in the aerobactin system has not been determined, but similar MFS
systems have been involved in the export of siderophores (26, 54). The putative protein encoded by viuB has significant identity with ViuB and YqiH encoded by *V. cholerae* and *E. coli* (8, 81). These two proteins reduce the bound iron from the ferric state to the ferrous state, resulting in the loss of affinity of the ferrous iron for the siderophore. Results reported by Wang et al. (81), suggest that the function of YqiH is to aid in the release the iron from the siderophore into the cytosol.

Furthermore, the chromosomes of all *Cronobacter* spp. genomes analyzed have a common gene encoding the ferric reductase FhuF. The homologous ferric reductase proteins found in *E. coli* and described by Matzanke et al. (53) are thought to promote the release of iron from hydroxamate siderophores, specifically coprogen, ferrichrome and ferroxamine B.

**Phylogenetic analysis of *Cronobacter* spp. iron acquisition systems.** Phylogenetic analysis was performed on the nucleotide sequence of each putative iron acquisition operon or gene identified in the in silico analysis, to infer evolutionary relationships. Phylogenetic analyses of 16S rDNA and housekeeping genes typically places the group of *Citrobacter, Klebsiella, Escherichia* and *Shigella, Salmonella*, and other *Enterobacter* spp. as the nearest neighbors to *Cronobacter* spp., followed by *Erwinia, Serratia* and *Yersinia*. This general trend was also observed for the majority of iron acquisition genes in *Cronobacter* spp. In the majority of instances, homologues in the genome of *Klebsiella pneumoniae*, strain MGH 78578, were the most closely related by nucleotide sequence, for example, as that for the larger of the three enterobactin gene clusters containing *entH-fepC* (Fig. 3A). After *Klebsiella*, typically, homologues in the genomes of *Enterobacter* sp., such as strain 638, several *Salmonella enterica* serovar strains, and *Citrobacter koseri* ATCC BAA-895 were the next closely related sequences, followed by homologous sequences present in various strains of *E. coli* and *Shigella* spp.
Typically, homologous genes in the genomes of *Serratia*, *Yersinia*, *Erwinia*, and *Edwardsiella* spp. and other enterics formed a separate cluster from that containing *Cronobacter* spp. This is indeed true for the observed phylogenetic evolutionary reconstruction of both ferric transporters, enterobactin (large and small clusters) and the *fhu* operon, the *fhuF* ferric reductase, the *feo* ferrous transporter, and the putative heme-iron extractor encoding gene, *yfeX* (Fig. 3A and supplemental material Fig. S1).

In two instances, homologues in *E. coli* and *Shigella* spp. were more closely related than those in *Salmonella* spp.; i.e., the TonB receptor encoding genes, *pfeA* and *yncD* (supplemental material Fig. S1). In a few cases, homologues were not present in all members of this enteric clade, which contained *Cronobacter* spp., such as *Salmonella* and *Citrobacter* spp. for the ferrous transporter, *efe* (supplemental material Fig. S1), also for which genes of *Klebsiella pneumoniae* MGH 78578 are the most distantly related in the cluster; and the TonB receptor encoding gene, *fhuE* (supplemental material Fig. S1), for which there is no apparent homologue in *Klebsiella* and a homologue in the genome of *Erwinia carotovora* strain SCRI1043 is most closely related to *Cronobacter* spp.

Two of the iron acquisition genes that are present in all genome of *Cronobacter* spp. analyzed present a phylogenetic evolutionary history quite different from the conserved enteric features described above. For the TonB-receptor encoding gene, *foxA*, again the homologue of *K. pneumoniae* MGH 78578 is most closely related, in fact, this homologue clusters among *Cronobacter* spp (Fig. 3B). The next closest homologues are contained in a cluster comprised of *Serratia proteamaculans* strain 568 and various *Yersinia* spp. strains, other than *Y. pestis*. For *btuB*, it is clear that this gene is under considerably different evolutionary pressure within the *Enterobacteriaceae* (supplementary material Fig. S1). The *btuB* genes of *Cronobacter* spp. form
three clusters, as compared to other conserved iron acquisition genes, which typically display a
two clade phylogeny within *Cronobacter* spp. genomes. Likewise, other enterics demonstrate a
mixture of species clustering and monophyletic divergence.

Of the iron acquisition elements analyzed, six are not conserved among all species of
*Cronobacter*. Previously, we performed an *in silico* analysis on the cronobactin (aerobactin)
gene cluster, *iucABCD/iutA*, and the *eit* ferric transporter, *eitCBAD*, which are harbored on a
non-mobile F-type plasmid (23). The reconstructed evolutionary history of *sfiF/viuB* is
considerably different to that of the genes downstream, namely the cronobactin operon. The
cronobactin operon clusters with homologous sequences from two *Enterobacter* spp. and that of
*Escherichia fergusonii* and this cluster is closely related to homologus systems found in *Serratia*
and *Yersinia* spp (23). The *Cronobacter* spp. *viuB* gene is also similar to the same in *Serratia*
and *Yersinia* spp.; however, it is more closely related to homologues in several plant-associated
species, including *Erwinia* spp., *Stenotrophomonas maltophilia*, *Pectobacterium carotovorum*,
and *Pantoea ananatis* (Fig. 3C). Differences in G+C% content between *viuB/shiF* (66) and
*iucABCD/iutA* (59.7), together with differences in inferred phylogenetic history, strongly suggest
operon evolution in which the genomes of *Cronobacter* spp., as well as *Serratia* and *Yersinia*
spp., have acquired these two accessory genes, most likely, in two separate events.

For the two TonB-receptor encoding genes, *fcT* and *fcuA*, present in the genomes of *C.
dublinensis* and *C. muytjensis*, inferred phylogenetic history reveals that, as for the majority of
conserved iron acquisition genes, these genes are most closely related to homologues present in
the genomes of *Enterobacter* spp., *Klebsiella* spp., and *Citrobacter koseri*. For *fcuA*, this cluster
containing *Cronobacter* spp. is then most similar to homologues in water- and plant-associated
species, *Rahnella*, *Pantoea*, and *Erwinia*, followed by *Yersinia* spp (Fig. 3D). For *fcT*, the
cluster containing *Cronobacter* spp. is most similar to homologues in water- and plant-associated 

species, *Serratia*, *Pantoea*, and *Erwinia chrysanthemi* (supplemental material Fig. S1).

The plasmid-encoded ferric dicitrate system present in the genome of *C. sakazakii* strain 2151 is almost identical in sequence to the same found on plasmid pKPN-IT of *K. pneumoniae* strain ST258, and in the chromosome of *Enterobacter cloacae* subspp. *cloacae* NCTC 9394, and highly similar to homologous sequences in several pathogenic strains of *E. coli* and *Shigella* spp.

Distantly related are homologues from the plant-associated species, *Erwinia carotovora* and  
*Photorhabdus* spp. (supplemental material Fig. S1)

Within the genus, *Cronobacter*, phylogenetic analysis revealed two sub-clades for most iron acquisition genes and systems analyzed (Fig. 3A and supplemental material Fig. S1). One sub-clade is comprised of the species, *C. sakazakii*, *C. malonaticus*, *C. universalis*, and *C. turicensis*; the other sub-clade comprised *C. muytjensii* and *C. dublinensis*. This phylogenetic relationship is suggestive of the fact that these two groups of *Cronobacter* spp. are under different evolutionary pressure, and most likely reflects differences in ecological niches. This clustering was in agreement with that reported for the *rpoB* sequence (47), virulence markers on pESA3/pCTU1-like plasmids (23) and for whole genome phylogeny (C. J. Grim and B. D. Tall, unpublished data).

**Detection of *Cronobacter* species iron acquisition systems by PCR.** In order to determine the distribution of iron acquisition genes and systems identified by the *in silico* analysis, a total of 231 strains of *Cronobacter* spp. isolated from different sources were screened by PCR (Table 3). Previously, it was reported that 97% (226) of the same 231 *Cronobacter* spp. isolates harbor a pESA3/pCTU-1 like plasmid that has the genes encoding the cronobactin siderophore and EitABC/D transporter (23). In this study, PCR results showed that all 226
Cronobacter spp. containing the plasmid encoded iucABCD/iutA operon, also have the viuB gene, suggesting that this gene has a possible role in cronobactin activity. PCR analysis of the enterobactin genes entF (locus 1), fepE (locus 2), and entE and fepG (locus 3) show that all 231 Cronobacter spp. strains are positive for locus 1, and locus 3 while only 98% are positive for locus 2 due to only two of the six C. dublinensis isolates being positive for the fepE gene (Table 3).

PCR results also showed that all 231 Cronobacter spp. isolates possess the genes encoding the ferric transporter FhuACDB, the ferrous transporters FeoABC and EfeUOB, as well as the heme-iron extractor, YfeX, and ferric reductase, FhuF (Table 3). The ferric dicitrate genes fecR and fecE were only found in 14% of C. sakazakii and 16% of C. malonaticus strains tested. These genes were not found in any of the other Cronobacter species (Table 3).

Interestingly, of the 30 Cronobacter spp. strains containing the fec transport system, 19 were isolated from clinical samples, suggesting that this iron system may be a virulence marker and play a role in the virulence of Cronobacter spp. Further analysis of this locus using repA and repB origin of replication genes found that three of the C. sakazakii and one C. malonaticus strains harbor the fec genes on a pCSA2151-like plasmid and the remaining 26 strains have these genes on their chromosome (Table 4). The presence of insertion sequences flanking the fec genes suggests that this transport system is mobilizable and possibly integrated into the chromosome at different regions of the Cronobacter genome.

Independent of the source of isolation, all Cronobacter spp. tested have the putative genes encoding the TonB-dependent iron receptors YncD, BtuB, FoxA, FhuE, and PfeA; however, the genes encoding the TonB-dependent receptor FcuA are unique to C. dublinensis and C. muytjensii strains tested (Table 3). Furthermore, the gene encoding the TonB-dependent
receptor FcT was specific to all six *C. dublinensis*, and 12 *C. muytjensii*, as well as three of six *C.*

turicensis* strains tested (Table 3). It is known that most TonB-dependent iron receptors are

substrate specific (54). Even though, *Cronobacter* spp. do not produce the siderophores specific

for some of their TonB-dependent receptors, in polymicrobial communities *Cronobacter* spp.

strains may interact with ferric siderophores produced by other bacteria or fungi. This suggests

that the presence of FcuA and/or FcT in *C. dublinensis*, *C. muytjensii* and some *C. turicensis*

strains may give them an advantage to compete more successfully for iron in certain ecological

niches where they may encounter the specific siderophores of FcuA and FcT, such as

chrysoabactin, which is produced by plant pathogen *E. chrysanthemi*.

**Expression of iron acquisition systems.** The expression of most iron transport systems

are repressed by iron via interaction of the Fur protein at the promoter site. Their promoters

contain the consensus Fur binding, or Fur box. In the presence of iron, Fur binds to the Fur box

and block transcription (17). *In silico* analysis identified potential Fur boxes matching the *E. coli*

Fur box consensus sequence 5’-GATAATGATAATCATTATC-3’ in the upstream region of all

putative *Cronobacter* spp. iron acquisition systems, except in the heme iron extractor, *yfeX*, and

*btuB*, a predicted vitamin B12/cobalamine receptor (Table 5). In order to confirm if the

*Cronobacter* spp. iron acquisition systems are regulated by Fur, their expression was determined

after growing the bacterial cells for 16 h in LB medium (iron-replete conditions) and LB

supplemented with 300 µM of the ferrous iron chelator 2,2’-dipyridyl (DIP) (iron depleted-

conditions). Of 25 genes tested, 18 exhibited higher expression in iron-depleted conditions as

compared to iron-replete conditions, indicating derepression of these genes (Table 5). On

average, their expression increased 2.7-fold when grown in LB medium amended with DIP

(Table 5).
RT-PCR results of *C. turicensis* z3032 showed that all four biosynthetic cronobactin genes, *iucABCD*, and the ferric-cronobactin receptor gene, *iutA*, were expressed at higher levels under iron-depleted growth conditions (Fig. 4A). Expression of *iucC* and *iutA* increased 4.1 and 2.9 fold, respectively, under iron-depleted conditions (Table 5). A potential Fur-binding site is located 19 bp upstream of the putative start of *iucA* (Table 5). This Fur box matches the Fur box consensus sequence in 17 out of 19 bases (17). Furthermore, supernatants of *C. turicensis* z3032 had siderophore activity in CASAD assay only when it was grown in the presence of DIP (data not shown), which confirms that production of cronobactin siderophore is inducible and expressed only under iron depleted growth conditions. The genes, *viuB* and *shiF*, were also expressed at higher levels under iron-depleted conditions, confirming that these genes are also components of the Fur regulon (Table 5; Fig 4A). The stop codon of *shiF* overlaps the start codon of *viuB*, suggesting that these two genes are transcribed together. These two genes are located 136 bp upstream of *iucA* and transcribed in opposite directions to that of the *iucABCD/iutA* operon (Fig. 1A). This sequence structure suggests that similar to the *E. coli* enterobactin operons, the transcription of *shiF/viuB* and *iucABCD/iutA* operons may be Fur-controlled by a bidirectional promoter-operator region (7). In addition to the putative Fur box located 19 bp upstream of *iucA*, there is a potential Fur-binding site located 43 bp upstream of the putative start of *shiF* (Table 5). Presence of two Fur boxes between *iucA* and *shiF* suggest that the promoters of each operon may be independently expressed and controlled by Fur from distinct operator sites. This sequence displays a resemblance to the *E. coli* bidirectional intercistronic region controlling the expression of enterobactin *fepB-entC* genes (7). In contrast to *Cronobacter* spp., a single Fur box is located between *iucA* and *shiF* in *Y. pestis*, and likely serves as an iron- and Fur-regulated promoter for expression of aerobactin and *shiF* (22).
The Cronobacter eitD and eitA genes, which are part of the operon encoding the ABC transporter EitABC/D localized on pCTU1 in C. turicensis z3032 (Fig 1D), were not derepressed in iron-depleted conditions (Table 5). The eitA gene was poorly expressed and eitD was not expressed under both iron-replete and iron-depleted growth conditions, despite the presence of putative Fur boxes 73 bp upstream of eitA and 150 bp upstream of eitD (Table 5). Similar RT-PCR results were obtained for C. dublinensis LMG23825 (data not shown). One explanation for the lack of expression in Cronobacter spp. could be the gene arrangement of the cluster eitABC/D, which is different from the typical arrangement of eitABCD in other Enterobacteriaceae (Fig. 1D) (23).

In E. coli, the enterobactin gene cluster includes 14 genes organized into six contiguous operons originating from three Fur-controlled bidirectional promoter-operator regions (7, 33, 70). The bidirectional control regions include: fepA/entD and fes/entF/fepE; entCEBAH and fepB and entS and fepDGC. These three control regions possess distinct regulatory architectures (7, 33, 70), suggesting that control by the Fur repressor is manifested through different regulatory strategies. In Cronobacter spp., with the exception of fepE, which forms a single locus separate from the fes/entF/fepE operon, we found the same gene organization as E. coli even though the enterobactin genes are found in three distinct chromosomal loci (Fig. 1B). Potential Fur boxes were identified in all three putative bidirectional promoter-operator regions, but not upstream of fepE (Table 5). As in E. coli, single putative Fur boxes were identified in the first and third putative bidirectional promoter regions between fepA-fes and entS-feD, respectively (Table 5). In E. coli, the operons controlled by these two bidirectional promoter regions transcribe from overlapping promoters regulated by the binding of Fur to a single binding site (13, 33). In contrast, two putative Fur boxes were identified in the second regulatory region between entC-
fepB (Table 5). In E. coli, the promoters for fepB and the entCEBA/ybdB operon are situated back to back and each one is controlled by Fur from distinct operator sites (7).

To test the expression of Cronobacter enterobactin-like system, we determined the expression of representative genes from each promoter region in C. turicensis z3032 (Table 5, Fig. 4B). We found that fepA and entF comprising the two operons of the first putative bidirectional promoter region (Fig. 1B) were expressed at higher levels, 1.5 and 4.4 fold, respectively, under iron-depleted conditions. Expression of fepE was higher under iron-replete conditions, most likely due to its genomic rearrangement to a distant locus without a putative Fur box in the promoter region. For genes of the second bidirectional promoter region (Fig. 1B), fepB and entC were expressed at higher levels under iron limiting conditions; however entS and fepG of the third regulatory region (Fig. 1B) were poorly or not expressed respectively when grown under both iron-depleted and iron-replete conditions (Table 5, Fig. 4B).

Expression of the fec transport genes start with the binding of diferric citrate to the FecA protein (21) which causes substantial structural changes in FecA, triggering a signal cascade (6). FecA interacts with FecR in the periplasm which in turns transmits the signal across the cytoplasmic membrane into the cytoplasm and activates the FecI sigma factor, which binds to the RNA polymerase core enzyme and directs the RNA polymerase to the promoter upstream of the fecABCDE transport genes to initiate transcription (52). Promoters of the fecIR regulatory genes and fecABCDE transport genes are repressed by the Fur protein loaded with Fe^{2+} (2). Therefore, transcription of the fec transport genes is subjected to a double controlled regulation scheme. In silico analysis in C. sakazakii 2151 identified a putative Fur box 44 bp upstream of fecI start codon (Table 5). Accordingly, fecB and fecE were expressed at higher levels under iron-depleted growth conditions and in the presence of 1 mM citrate, as compared to iron-replete growth.
conditions lacking citrate, confirming that expression of the fec transport genes are induced by citrate and derepressed by low-iron growth conditions (Table 5).

Putative Fur boxes were identified in the upstream region of ferrous transporter systems feo and efe (Table 5). Even though in E. coli, the expression of the feoABC operon is active under anaerobic conditions (37), we observed that genes of these iron transport systems were expressed at higher levels under aerobic iron limiting conditions (Table 5).

Putative Fur binding sites were identified upstream of the putative TonB-dependent iron receptors fhuA, yncD, foxA, fhuE, and pfeA (Table 5, Fig. 4C), suggesting that these receptors are repressed by iron via the interaction of Fur protein. Expression of these genes was repressed when C. turicensis z3032 was grown in the presence of iron, confirming that they are regulated by iron concentration (Table 5). It has been reported that in addition to iron concentration, some of these genes are also positively regulated by the presence of their cognate siderophore (49).

Furthermore, recently it has been determined that YncD plays an important role in the survival inside of the host and the gene is over expressed under stress conditions such as heat and acid (83). In contrast to the TonB-dependent iron receptors mentioned above, no putative Fur boxes were found upstream of btuB and yfeX and expression of these genes was similar under iron-replete and depleted conditions (Table 5; Fig. 4C, lanes 10-11), indicating that these genes are constitutively expressed in Cronobacter spp.

Expression of fct and fcuA was determined in C. dublinensis LMG23825. RT-PCR results showed that expression of both genes was repressed under iron-replete conditions suggesting that these two genes are also part of the Fur regulon (Table 5; Fig. 4C, lanes 12-13 and 14-15). Interestingly, a gene encoding an enterochelin esterase, named fesA in this study, is located 269 bp upstream of the fct start codon. The two genes are oppositely transcribed and a potential Fur
box is located intergenically, 62 bp upstream of fct start codon (Table 5). This organizational structure is similar to that described for the E. coli fepA-fes bidirectional promoter, where fepA encodes the enterobactin receptor and fes encodes an esterase involved in the release of iron from ferric enterobactin inside of the cell. Furthermore, the same organization is found for the fct promoter region of E. chrysanthemi, where overlapping promoters are controlled by the binding of Fur to a single binding site (68).

A putative Fur box was found five bp upstream of the fcuA start codon, but the sequence is only identical to 8 of the 19 nucleotides of the Fur box consensus sequence (Table 5). Sixty three bp upstream of fcuA, there is an uncharacterized gene, yncE, which is transcribed in the same orientation as fcuA. A potential Fur box, matching the consensus sequence at 14 of the 19 bp, is located upstream of yncE (Table 5). It is unclear if fcuA is co-transcribed with yncE under control of one promoter-Fur box region, or, alternatively, both genes transcribe independently under control of its own promoter-Fur box region.

Cronobactin promotes the growth of Cronobacter spp. under iron-limiting conditions. Previously, we found that wild-type C. sakazakii BAA-894 and C. turicensis z3032, but not their plasmid-cured derivatives (strains BAA-894.3 and 3032.2A, respectively) produce active siderophores (23). To further confirm that the siderophore activity was due to the production of cronobactin encoded by the plasmids, we assayed for siderophore production in the cell-free culture supernatant of an E. coli strain, ESA-C01, harboring a fosmid containing the complete pESA3 iucABCD/iutA operon. Using the CASAD assay, siderophore production was detected in the cell-free culture supernatant of ESA-C01 but not in the supernatant of a fosmid clone control strain, ESA-M04, containing a different region of pESA3 (Fig. 5), indicating that the iucABCD synthesis genes do encode for active siderophores. However, larger orange halos
were produced by wild-type \textit{C. sakazakii} BAA-894 harboring pESA3 as compared to \textit{E. coli} ESA-C01, suggesting that \textit{C. sakazakii} BAA-894 has additional factors that may increase the cronobactin activity or result in a greater amount of cronobactin being secreted. In order to determine whether \textit{iucABDCD/iutA} operon enhances growth of \textit{Cronobacter} spp. under iron-depleted growth conditions, \textit{C. turicensis} z3032 harboring plasmid pCTU1 and its plasmid-cured derivative strain 3032.2A were grown in LB medium and LB supplemented with 300 µM of DIP. While no difference in growth was observed when both strains were grown in LB medium alone, 3032.2A grew significantly ($P < 0.001$) slower than the wild-strain between 7 and 24 h in LB supplement with DIP (Fig. 6). Similar results were obtained when wild-type \textit{C. sakazakii} BAA-894 and its plasmid-cured derivative, 894.3, were grown in low-iron conditions (data not shown). RT-PCR analysis showed no difference in the expression of \textit{iucC} after 5 hours and 18 hours of growth in LB supplement with DIP but larger halo was produced after 18 hours than after 5 hours of growth suggesting that greater amount of siderophore accumulates in the cell-free culture supernatant during the stationary phase (data not shown). Overall, these results strongly suggest that cronobactin encoded by pESA3- and pCTI-1-like plasmids play a critical role to promote growth of \textit{Cronobacter} spp. under iron-limiting conditions.

\textbf{Enterobactin-like siderophore is not functional.} Even though the chromosomes of most of the \textit{Cronobacter} spp. isolates tested have the enterobactin genes, we were not able to detect siderophore activity in \textit{C. sakazakii} BAA-894 and \textit{C. turicensis} z3032 lacking pESA3 and pCTU-1, respectively (Fig. 5, 23). These results suggest that the bacteria do not encode active enterobactin siderophores and the activity identified by the CASAD assay is solely due to the cronobactin siderophore encoded by the virulence plasmids. To determine whether the
enterobactin inactivity encoded by the chromosome of C. sakazakii BAA-894 and C. turicensis z3032 was defective only in these strains, we performed the CASAD assay of the cell-free culture supernatants of C. muytjensii ATCC 51329 and other four Cronobacter strains, of our culture collection, lacking pESA3-/pCTU1-like plasmids (strains listed in Table 1). Siderophore activity was detected in the cell-free culture supernatants of 2 control Cronobacter spp. strains harboring a pESA3/ pCTU1-like plasmids (strains listed in Table 1), but not in the supernatants of any of the five Cronobacter spp. strains lacking the plasmids (data not shown). These results suggest that Cronobacter spp. do not produce active enterobactin-like siderophores. To confirm these results, the colorimetric tests described by Arnow (3) and Csáky (15) were used to identify catechol-type (such as enterobactin) and hydroxamate-type (aerobactin) siderophores, respectively. The results showed that cell-free culture supernatants of C. turicensis z3032 harboring pCTU1 produce hydroxamate-type but not catechol-type of siderophores, while its plasmid-cured derivative does not produce any type of siderophore (Fig. 7), confirming that C. turicensis z3032 solely produces the hydroxamate-type cronobactin siderophore encoded by pCTU1.

Closely related plasmids pESA3 and pCTU1 encode some common transport genes, including permeases of the MFS that may be involved in the secretion of siderophores, and some transcriptional regulators that may influence the expression of enterobactin synthesis genes. To test whether siderophore inactivity identified in plasmid-less strains was due to defects in secretion, we tested siderophore activity in whole-cell-lysate preparations of wild-type C. turicensis z3032 and its plasmid-cured derivative 3032.2A. Using the CASAD assay, we did not find siderophore activity in either of the whole-cell lysate preparations of C. turicensis z3032 or 3032.A (data not shown). Furthermore, we did not observe catechol-type siderophore activity in
any of the lysate preparations using the Arnow’s tests (data not shown), indicating that the enterobactin inactivity is not due to a secretion defect in the wild-type or plasmid-cured derivative strain. In order to determine whether siderophore inactivity identified in plasmid-less strains was due to defects in the expression of enterobactin, we compared the expression of enterobactin genes in strains z3032 and 3032.2A. RT-PCR of representative enterobactin synthesis gene including entC and entE showed no difference in the expression between strains z3032 and 3032.2A (data not shown) indicating that pCTU1 is not involved in the regulation of expression of enterobactin synthesis genes.

Sequence analysis of the enterobactin cluster genes revealed that the enterobactin inactivity was not due to an obvious gene truncation; however, in contrast to other bacteria the enterobactin genes in Cronobacter spp. are localized in three separated loci in the chromosome (Fig. 1B). Our expression analysis showed expression of fepE was not repressed by iron (Fig. 4B, Table 5). In addition, entS and fepG localized in the third regulatory region were poorly or not expressed and the level of expression was not modified under conditions of restricted iron availability (Fig. 4B, Table 5). In E. coli, fepG is involved in ferric enterobactin transport and entS plays a role in the export of enterobactin outside of the cell (26, 62). However, our results, presented above, indicate that the enterobactin inactivity is not due to a secretion defect. Taken together, these results indicate that the rearrangement of enterobactin in three different regions of the chromosome is the most likely cause for the defect in expression of the enterobactin genes.

We do not rule out the possibility that Cronobacter spp. produce active enterobactin in the host. It has been determined that enterobactin and other iron acquisition systems are upregulated two- to five-fold in vivo in comparison to growth in LB-broth (72). It is possible...
that the expression of enterobactin genes, under laboratory conditions, is not sufficient to be
detected by the CASAD or Arnow’s assays; i.e., below the level of detection. In support of this,
we observed that the cronobactin genes were more highly expressed than enterobactin genes, by
RT-PCR (Fig. 4). It is also possible that the inactivity of enterobactin expression may be due to
amino acid variation in the biosynthetic enzymes compared to known functional enzymes from
*E. coli* and other gram-negative bacteria. Alternately, enterobactin genes may have post-
transcriptional modifications that affect the activity of the siderophore.

We observed that the ferric-enterobactin receptor FepA was expressed and its expression
was upregulated under iron-depleted conditions (Table 2), suggesting that this receptor is
functional. However, the presence and expression of specific siderophores receptors genes does
not reliably predict activity of the complex protein assemblies involved in synthesis of
siderophores. For example, it has been reported that uropathogenic *E. coli* strains contain the
genes encoding enterobactin, aerobactin, yersinabactin and salmochelin siderophores but only
yersinabactin and salmochelin are produced (31). In polymicrobial communities pathogenic
bacteria may benefit from inactivated siderophore production if they retain the ability to sense
and “steal” ferric-siderophore complexes in which the siderophore is produced by a neighboring
competing cell, thereby avoiding the metabolic cost of siderophore biosynthesis (27).

**Conclusions.** *Cronobacter* spp. have transport systems for both ferric and ferrous iron.

For acquisition of ferrous iron, all *Cronobacter* spp. have both the Feo and Efe systems and for
transport of ferric iron; all plasmid-harboring strains (97%) have the aerobactin-like siderophore,
cronobactin. All *Cronobacter* spp. have the genes encoding the enterobactin-like siderophore but
this siderophore was not functional under the conditions tested in this study. In addition to
receptors for cronobactin and enterobactin, all *Cronobacter* spp. have 5 common receptors
(FhuA, YncD, FoxA, FhuE, and PfeA) for siderophores produced by other organisms. The ferric
dicitrate transport system was found specifically in a small subset of *C. sakazakii* and *C.
malonaticus* strains, most of which were isolated from clinical samples, suggesting that this iron
acquisition system plays a role in the virulence of *Cronobacter* spp. Furthermore, *C. dublinensis*
and *C. muytjensii* have two receptors, Fct and FcuA, for heterologous siderophores produced by
plant pathogens, indicating that these receptors may give an advantage to those *Cronobacter* spp.
to compete more successfully for iron in a plant niche. *In silico* identification of putative Fur
boxes and expression of the genes under iron-depleted conditions suggest that most of these iron
transport system form part of the Fur regulon. Phylogenetic analysis of TonB-dependent iron
receptors showed that *fcuA* and *fct* are closely related to homologues from water- and-plant-
associated species. In contrast, the ferric dicitrate transport genes specific to *C. sakazakii* and *C.
malonaticus* are more closely related to orthologous genes in several pathogenic strains of *E. coli*
and *Shigella* spp., but distantly related to orthologous genes from plant-associated species.
Moreover, phylogenetic analysis of most of iron acquisition genes and systems separate the
genus *Cronobacter* in two sub-clades. One sub-clade includes the species, *C. sakazakii*, *C.
malonaticus*, *C. universalis*, and *C. turicensis* and the other sub-clade comprises *C. muytjensii*
and *C. dublinensis*. This clustering was in agreement with virulence markers on pESA3/pCTU1-
like plasmids (23), where the *Cronobacter* plasminogen activator (Cpa) apparently involved in
the invasion and serum resistance (24) was found specifically in *C. sakazakii* strains. Overall,
these results suggest that *C. dublinensis* and *C. muytjensii* are more likely inhabitants of an
environmental niche related to eukaryotic plant material; in contrast, *C. sakazakii* and *C.
malonaticus* may be more associated with human host and may explain why most disease is
caused by *C. sakazakii* and *C. malonaticus.*
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FIGURES

**Figure 1.** Ferric iron transporters encoded by *Cronobacter* spp. A) Cronobactin siderophore, B) Enterobactin-like siderophore, C) Hydroxamate ABC transporter *fhuACDB*, D) Ferric iron/siderophore/heme ABC transporter *eitCBA/D*, and E) Ferric-dicitrate transport system. Arrows show the direction of transcription and arrow fills identify genes encoding synthesis of siderophores (black), TonB-dependent outer membrane receptors (diagonal lines), ABC transporters (vertical lines), export of enterobactin (horizontal lines), intracellular release the iron from siderophore-iron complex (grey), sigma factor (small grids), transmembrane signal transducer (dots), IS transposases (white), and unknown function (diamonds). Numbers in boxes shown in the enterobactin-like siderophore diagrams show location of the three putative bidirectional promoter-operator regions. The small filled boxes upstream of some genes/operons show location of putative Fur boxes.
**Figure 2.** Ferrous iron transporters, *feoABC* and *efeUOB* encoded by the chromosome of *Cronobacter* spp. Arrows show the direction of transcription and arrow fills identify genes encoding GTP-binding protein/probably permease (diagonal lines), Fe-S dependent transcriptional regulator of FeoABC expression (square), IM permease (horizontal lines), transporter periplasmatic protein (horizontal lines), periplasmatic peroxidase protein (dots), and unknown function (white). The small filled boxes upstream of the operons show location of putative Fur boxes.

**Figure 3.** Evolutionary history of iron acquisition system genes, A. large enterobactin gene cluster in *Cronobacter* spp., *entHABEC/fepB/entS/fepDGC*; B., the TonB receptor encoding gene, *foxA*; C. the ferric reductase, *viuB*; D. the TonB receptor encoding gene, *fcuA*.

**Figure 4.** Representative RT-PCR of *Cronobacter* iron acquisition systems under iron-replete (even lanes) and iron-depleted (odd lanes) conditions. A) Cronobactin and *shiF/viuB* operons: Lane 1, 1kb plus DNA ladder; lanes 2-3, 16S rRNA; lanes 4-5, *viuB*; lanes 6-7, *shiF*; lanes 8-9, *iucA*; lanes 10-11, *iucB*; lanes 12-13, *iucC*; lanes 14-15, *iucD*; lanes 16-17, *iutA*. B) Enterobactin genes: Lane 1, 1kb plus DNA ladder; lanes 2-3, *fepA*; lanes 4-5, *entF*; lanes 6-7, *fepE*; lanes 8-9, *entC*; lanes 10-11, *fepB*; lanes 12-13, *entS*; lanes 14-15, *fepG*; lanes 16-17, 16S rRNA. C) TonB-dependent iron receptors: Lane 1, 1kb plus DNA ladder; lanes 2-3, *pfeA*; lanes 4-5, *fluE*; lanes 6-7, *foxA*; lanes 8-9, *yncD*; lanes 10-11, *btuB*; lanes 12-13, *fcuA*; lanes 14-15, *fcT*; lanes 16-17, 16S rRNA.
Figure 5. Siderophore activity using the CASAD assay. Wells were filled with cell-free culture supernatants of 1) wild-type *C. sakazakii* BAA-894, 2) plasmid cured derivative 894.3, 3) fosmid clone ESA-C01 containing the cronobactin genes, and 4) fosmid clone ESA-M04 lacking the cronobactin genes.

Figure 6. Growth of wild-type *C. turicensis* z3032 harboring pCTU1 and plasmid-cured derivative, 3032.2A, in LB broth and iron-depleted LB broth containing 300 µM DIP. The data were obtained from 3 independent experiments. * P < 0.001.

Figure 7. Results of Csaky’ (A) and Arnow’s (B) tests used to identify hydroxamate-type and catechol-type of siderophore activity, respectively, using cell-free culture supernatants of wild-type *C. turicensis* z3032 harboring pCTU1[P(+)] and plasmid-cured derivative, 3032.2A [P(-)]. Cell-free culture supernatants of *Vibrio vulnificus* UNCC913 (C) and catechol (6 µg) were used as positive controls in the Csaky’ and Arnow’s tests, respectively.

Supplementary Figure S1. Evolutionary history of iron acquisition system genes, A. small enterobactin gene cluster in *Cronobacter* spp., *entD*/fepA/*fes*/ybdZ/entF; B., the ferric iron siderophore transporter, *fhuACDB*; C., the ferric iron dicitrate transporter, *fecIRABCDE*; D., the ferric iron reductase, *fhuF*; the ferrous iron transporters, E., *efeUOB*; and F., *feoABC*; the TonB receptor encoding genes, G., *btuB*; H., *fct*; I., *fhuE*; J., *pfeA*; K., *yncD*; and L., the heme-iron extractor encoding gene, *yfeX*. 

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Table 1. Bacterial strains used in this study.

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<th>Species</th>
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<th>Biological Source</th>
<th>Geographical Source</th>
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Additional bacterial strains used for experimental analysis

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*Strain metadata of these and those screened by PCR can be found in PATRN (www.patrn.net)
Table 2. PCR primers used in this study.

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- Number in primer name corresponds to 5’ nucleotide position of the ClustalW alignments for each gene (see Supplementary Table 1).
- All PCR were performed with 30 cycles, except for *Cronobacter* 16SrRNA that was performed with 25 cycles.
- 7% (final concentration) dimethyl sulfoxide (DMSO) added to the PCR reaction mix.
- The *repA* gene of plasmid pCS2151.
- The *repE* gene of plasmid pCS2151.
Table 3. Distribution of iron acquisition systems in 231 *Cronobacter* strains.

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<th><em>C. malonicus</em> (n = 25)</th>
<th><em>C. turicensis</em> (n = 6)</th>
<th><em>C. muytjensii</em> (n = 12)</th>
<th><em>C. dublinensis</em> (n = 6)</th>
<th><em>C. universalis</em> (n = 2)</th>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>yfeX</td>
<td>180 (100)</td>
<td>25 (100)</td>
<td>6 (100)</td>
<td>12 (100)</td>
<td>6 (100)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

a Number of isolates for each *Cronobacter* species.  

b Number of PCR positives for each target gene. Numbers within parentheses are the percent PCR positives for each target gene in relation to the total number of isolates studied for *Cronobacter* species.
Table 4. Distribution of ferric dicitrate transport system and plasmid pCSA2151 replication genes among 231 strains of *Cronobacter* spp. Multiple profiles were found among isolates of *C. sakazakii* and *C. malonaticus*.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of isolates</th>
<th>Target genes</th>
<th>Ferric dicitrate</th>
<th>pCSA2151</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>fecR</td>
<td>fecE</td>
</tr>
<tr>
<td><em>C. sakazakii</em></td>
<td>180</td>
<td>3 (1.7)*</td>
<td>3 (1.7)</td>
<td>3 (1.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23 (13)</td>
<td>23 (13)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. malonaticus</em></td>
<td>25</td>
<td>1 (4)</td>
<td>1 (4)</td>
<td>1 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 (12)</td>
<td>3 (12)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>1 (4)</td>
</tr>
<tr>
<td><em>C. turicensis</em></td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. muytjensii</em></td>
<td>12</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. dublinensis</em></td>
<td>6</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td><em>C. universalis</em></td>
<td>2</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

*Number of PCR positives for each target gene. Numbers within parentheses are the percent PCR positives for each target gene in relation to the total number of isolates studied for each *Cronobacter* species.*
Table 5. Putative Fur boxes identified in *Cronobacter* spp. iron acquisition systems and fold increase in expression under iron depleted conditions.

<table>
<thead>
<tr>
<th>Gene or operon(s)</th>
<th>Putative phenotype</th>
<th>Sequence (5’-3’)a</th>
<th>No. of identical bases</th>
<th>Locationb</th>
<th>Fold increase in expressionc</th>
</tr>
</thead>
<tbody>
<tr>
<td>iucABCD/iutA</td>
<td>Cronobactin biosynthesis and transport</td>
<td>CATAACGATAATCATTATC</td>
<td>17</td>
<td>19 bp of iucA</td>
<td>4.1 ± 0.2 (iucC)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.9 ± 0.1 (iucA)</td>
</tr>
<tr>
<td>shiF/viuB</td>
<td>Ferric reductase and MFS permease</td>
<td>AAATAATTGTPTCATTAT</td>
<td>11</td>
<td>44 bp of shiF</td>
<td>2.2 ± 0.2 (viuB)</td>
</tr>
<tr>
<td>fepA/entD - fes/ybdZ/entF</td>
<td>Enterobactin biosynthesis, receptor and iron release</td>
<td>GATAATCCTATCATTATC</td>
<td>17</td>
<td>18 bp of fepA</td>
<td>1.5 ± 0.05 (fepA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.4 ± 0.3 (entF)</td>
</tr>
<tr>
<td>fepE</td>
<td>Enterobactin transport</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.8 ± 0.03 (fepE)</td>
</tr>
<tr>
<td>enTCBAH - fepB</td>
<td>Enterobactin transport and biosynthesis</td>
<td>GAAAATGATAAAGCATTAT</td>
<td>16</td>
<td>26 bp of fepB</td>
<td>1.5 ± 0.08 (fepB)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.7 ± 0.3 (entC)</td>
</tr>
<tr>
<td>entS - fepD/G/C</td>
<td>Enterobactin transport and biosynthesis</td>
<td>GATAAATATCTATCTATTTC</td>
<td>16</td>
<td>19 bp of fepD</td>
<td>No expression (fepG)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0 ± 0.04 (entS)</td>
</tr>
<tr>
<td>fhuA/C/D/B</td>
<td>Ferrichrome receptor and hydroxamate transport</td>
<td>GGCTGATAATTAATTCTC</td>
<td>13</td>
<td>42 bp of fhuA</td>
<td>1.8 ± 0.2 (fhuA)</td>
</tr>
<tr>
<td>fecI/R/A/B/C/D/E</td>
<td>Ferric dicitrate receptor and transport</td>
<td>TGAAATGATAACATTTC</td>
<td>16</td>
<td>45 bp of fecI</td>
<td>3.4 ± 0.2 (fecB)d</td>
</tr>
</tbody>
</table>

Note: a) Sequence of Fur box sites. b) Location given as number of identical bases after the putative Fur box. c) Fold increase in expression relative to control conditions. d) From reference [1].
<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Sequence</th>
<th>Nucleotides</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>eitABC - eitD</td>
<td>ABC ferric transporter</td>
<td>GGAGTAGGTTTCTATTGTC</td>
<td>75 bp</td>
<td>0.7 ± 0.05 (eitA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>141 bp</td>
<td>No expression (eitD)</td>
</tr>
<tr>
<td>feoA/B/C</td>
<td>Ferrous transporter</td>
<td>AAAACCATTCTCATTACC</td>
<td>103 bp</td>
<td>1.8 ± 0.3 (feoB)</td>
</tr>
<tr>
<td>efeU/O/B</td>
<td>Ferrous transporter</td>
<td>GGAATGATAATGCTATTACC</td>
<td>59 bp</td>
<td>1.9 ± 0.3 (efeoO)</td>
</tr>
<tr>
<td>yncD</td>
<td>Siderophore receptor</td>
<td>GGAATGATAATGCTATTATT</td>
<td>164 bp</td>
<td>3.4 ± 0.4 (yncD)</td>
</tr>
<tr>
<td>foxA</td>
<td>Ferroxamine receptor</td>
<td>GATAATTCCGATTCTTT</td>
<td>60 bp</td>
<td>3.0 ± 0.4 (foxA)</td>
</tr>
<tr>
<td>fhuE</td>
<td>Ferric rhodotorulic acid/</td>
<td>GAAATGATTATATTCTTC</td>
<td>138 bp</td>
<td>2.7 ± 0.3 (fhuE)</td>
</tr>
<tr>
<td></td>
<td>coprogen receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pfeA</td>
<td>Ferric enterobactin</td>
<td>GATAATTATATCATATT</td>
<td>47 bp</td>
<td>4.3 ± 0.4 (pfeA)</td>
</tr>
<tr>
<td></td>
<td>receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fcuA</td>
<td>Hydroxamate receptor</td>
<td>GAAATGACGGACACCT</td>
<td>6 bp</td>
<td>2.8 ± 0.3 (fcuA)</td>
</tr>
<tr>
<td>fcT</td>
<td>Ferrichrysobactin</td>
<td>GATAATTATTCATATTAC</td>
<td>63 bp</td>
<td>2.8 ± 0.2 (fcT)</td>
</tr>
<tr>
<td></td>
<td>receptor</td>
<td></td>
<td>189 bp</td>
<td></td>
</tr>
<tr>
<td>fhuF</td>
<td>Ferric reductase</td>
<td>AGGATGSCATTACATTAC</td>
<td>28 bp</td>
<td>2.1 ± 0.4 (fhuF)</td>
</tr>
<tr>
<td>btuB</td>
<td>Vit. B12/cobalamin</td>
<td>Not present</td>
<td>-</td>
<td>1.0 ± 0.04 (btuB)</td>
</tr>
<tr>
<td></td>
<td>receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>yfeX</td>
<td>Heme iron extractor</td>
<td>Not present</td>
<td>-</td>
<td>0.76 ± 0.2 (yfeX)</td>
</tr>
</tbody>
</table>

*Comparison with *E. coli* consensus 5'-GATAATGATAATCATTATC-3' (17). Identical nucleotides are in boldface type.

*Number of nucleotides upstream of the start codon.
Average ± standard deviation of three RT-PCR fold change in the expression of the gene under iron depleted conditions (LB broth supplemented with 300 µM DIP). All RT-PCR were performed in *C. turicensis* z3032, except for fhuA, fcT and fcuA as well as fecB that were performed in *C. dublinensis* LMG23825 and *C. sakazakii* 2151, respectively.

Growth in LB broth supplemented with 300 µM DIP and 1 mM citrate.