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Identification and Characterization of *Cronobacter* 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 231 *Cronobacter* 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 five receptors for heterologous siderophores. A ferric dicitrate transport system (*fec* system) is encoded specifically by a subset of *Cronobacter 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.**

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 seven species: *C. sakazakii*, *C. malonaticus*, *C. turicensis*, *C. muytjensii*, *C. dublinensis* (*C. dublinensis* subsp. *dublinensis*, *C. dublinensis* subsp. *lausannensis*, and *C. dublinensis* subsp. *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 are 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 foodborne pathogen. Recently, we reported that 98% of 229 *Cronobacter* isolates possessed 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 (18, 54). Once in the cytoplasm of the cell, the

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TABLE 1 Bacterial strains used for *in silico* analysis in this study[a]

Species	Strain ID	Biological source	Geographical source	Relevant characteristic	Reference
Bacterial strains used in *in silico* analysis					
C. sakazakii	ATCC BAA-894	Infant	Tennessee, USA		32
C. sakazakii	2151	CSF	United States		This study
C. turicensis	z3032	Blood	Zurich, Switzerland		74
C. malonaticus	LMG 23826	Breast abscess	United States		34
C. dublinensis subsp. *dublinensis*	LMG 23823	Milk powder production facility	Dublin, Ireland		34
C. dublinensis subsp. *lactaridi*	LMG 23825	Milk powder production facility	Zimbabwe		34
C. dublinensis subsp. *lausennensis*	LMG 23824	Water fountain	Lausanne, Switzerland		34
C. universalis	NCTC 9529	Water	London, England		34
C. muytjensii	ATCC 51329	Unknown	Unknown		34
Additional bacterial strains used for experimental analysis					
C. turicensis	3032.2A			*C. turicensis* z3032 cured of pCTU1	23
C. sakazakii	BAA-894.3			*C. sakazakii* ATCC BAA-894 cured of pESA3	23
C. muytjensii	E488	Unknown	Lausanne, Switzerland	Lacks pESA3-/pCTU1-like plasmid	23, 75
C. muytjensii	E456	Unknown	Lausanne, Switzerland	Lacks pESA3-/pCTU1-like plasmid	23, 75
C. dublinensis subsp. *dublinensis*	CDC 0743-75	Human, clinical	Wisconsin, USA	Lacks pESA3-/pCTU1-like plasmid	23
C. sakazakii	CDC 9363-75	Unknown	New York, USA	Lacks pESA3-/pCTU1-like plasmid	23
C. muytjensii	CDC 3523-75	Human, clinical	Arizona, USA	Harbors pESA3-/pCTU1-like plasmid	23
Vibrio vulnificus	UNCC913	Unknown	Environmental	Hydroxamate/catechol siderophores	73
E. coli	ESA-C01			*E. coli* EPI300 fosmid clone containing *viuB-shiF* and *iucABCD-iutA* from pESA3	43
E. coli	ESA-M04			*E. coli* EPI300 fosmid clone lacking *viuB-shiF* and *iucABCD-iutA*	43

[a] Strain metadata for these and those screened by PCR can be found in PATRN (http://www.patrn.net). ID, identity; CSF, cerebrospinal fluid.

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 or low-pH growth conditions, the iron equilibrium shifts from the ferric (Fe^{3+}) to ferrous (Fe^{2+}) form, which 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* 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* strains screened for iron acquisition system gene targets consisted of 180 *C. sakazakii*, 25 *C. malonaticus*, 12 *C. muytjensii*, 6 *C. turicensis*, 6 *C. dublinensis*, and 2 *C. universalis* strains 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 gene, respectively, were obtained from a *C. sakazakii* BAA-894 fosmid library (43). Presence and absence of *viuB*, *shiF*, and *iucABCD-iutA* in ESA-C01 and ESA-M04 were confirmed by PCR using primers derived from each gene. *Cronobacter* 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: 40 μg/ml for chloramphenicol and 100 μg/ml for ampicillin. Results were submitted to the Pathogen-Annotated Tracking Resource Network

(PATRN) system, which is located at http://www.patrn.net and is accessible to users after a free registration process.

***In silico* analysis.** The genomes of all nine *Cronobacter* strains used in this study were annotated using the RAST server (4). They include *C. sakazakii* ATCC BAA-894, CP000783; the genome of *C. turicensis* z3032, FN543093; *C. sakazakii* 2151, AJKT01000000; *C. universalis* NCTC 9529, AJKW01000000; *C. malonaticus* LMG 23826, AJKV01000000; *C. dublinensis* subsp. *dublinensis* LMG 23823, AJKZ01000000; *C. dublinensis* subsp. *lactaridi* LMG 23825, AJKX01000000; *C. dublinensis* subsp. *lausanensis* LMG 23824, AJKY01000000; and *C. muytjensii* ATCC 51329, AJKU01000000. Additionally, plasmid sequences for pESA3 (NC_009780) and pCTU1 (NC_013383) were used in this study. Iron acquisition genes and gene clusters were identified by intrinsic RAST subsystem profiling for each genome as well as manual gene homologue BLAST searches. Comparative genomics in the SEED viewer (60) was used to confirm identification and conservation of putative iron acquisition genes within *Cronobacter* 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 supplemental 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 1,000 replicates 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 (1,000 replicates) are shown next to each branch (20). Trees are drawn to scale, with branch lengths in units similar to 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 gene clusters. Except for the primers for cronobactin genes, *fecR*, *fecB*, *fecE*, *fcuA*, and *fct*, all primers were derived from common sequences of the nine sequenced *Cronobacter* 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, and LMG 23824 and *C. muytjensii* ATCC 51329. The sequences of the primers, targeted genes, and the amplification parameters used for each set of primers are shown in Table 2. In each PCR, the positive control consisted of DNA preparations of the nine strains sequenced; the negative controls were DNA preparations of the closely related sister species, *Enterobacter helveticus* z513 and *Enterobacter 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 of DNA/25-μl reaction mixture) or 5 μl of boiled genomic DNA sample (approximately 50 ng of DNA/25-μl reaction mixture) as the DNA template. In all PCRs, the polymerase was activated by using a 3-min incubation step at 94°C, followed by 30 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* 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 Biosystems), 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 controls to detect DNA contamination. PCR conditions were selected to permit detection of the PCR products in the linear range of the reaction, and PCRs were performed using the same conditions as 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 intensities of the PCR products are interpreted as differences in transcription and/or stability of the iron acquisition gene 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 5-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 18 h in LB broth containing 300 μM 2,2′-dipyridyl (Sigma-Aldrich). After incubation at 37°C for 4 to 8 h, the presence of an orange halo around a hole indicated that a culture was positive for siderophore production.

The presence of phenolic-type and/or hydroxamate-type siderophores was detected in cell-free culture supernatants and whole-cell lysate preparations obtained from iron-depleted cultures using the colorimetric assays described by Arnow (3) and Csáky (15), respectively. Cell-free culture supernatants were lyophilized and concentrated 10-fold for the Arnow test. The Csáky assay was carried out with and without the sulfuric acid digestion step. Cell-free culture supernatants of *Vibrio vulnificus* UNCC913 (73) and catechol (6 μg) were used as positive controls in the Csáky and Arnow tests, respectively.

Statistical analysis. Data were analyzed by the Student *t* test (paired); a P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Identification of *Cronobacter* iron acquisition systems. Targeted *in silico* sequence analysis of the genomes of nine *Cronobacter* strains, including three *C. dublinensis* strains, two *C. sakazakii* strains, and single strains of *C. malonaticus*, *C. turicensis*, *C. muytjensii*, and *C. universalis*, revealed the presence of shared iron acquisition systems, with additional systems in some *Cronobacter* strains. The identified iron acquisition systems and their locations in the genome of the nine *Cronobacter* strains are listed in Table S1 in the supplemental material. These systems include genes encoding ferric and ferrous transporters and heme-iron extractors, as well as putative TonB-dependent iron receptors and ferric reductases.

(i) Ferric iron transporters. For acquisition of ferric iron (Fe^{3+}), all nine *Cronobacter* 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 carried by *Escherichia coli* and other bacteria, the genes encoding the enterobactin-like siderophore in seven of the nine *Cronobacter* 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,* and *entF*, the second locus contains only *fepE*, and the third locus comprises 10 genes, *entH, entA, entB, entE, entC, fepB, entS, fepD, fepG*, and *fepC*. *C. dublinensis* subsp. *lactaridi* LMG 23825

TABLE 2 PCR primers used in this study

Target	Primer pairs[a]	Sequence (5′–3′)	Amplicon size (bp)	Annealing/extension cycle parameters[b]
viuB	viuB_108F	RCATGAAGCGCCCGATCAGCG	445	58°C for 30 s/72°C for 45 s
	viuB_552R	CGCCAGCGGCACTTCCAGAAA		
shif	ShiF_761R	CGGAGATCGCCATGAAACAG	490	54°C for 30 s/72°C for 60 s[c]
	ShiF_272F	TGCTGAGTTTCGCCGTGATG		
iucA	IucA_352F	GAGAGCCACCGCCATACCTG	657	58°C for 30 s/72°C for 60 s
	IucA_1008R	CACCCAGCCATCTTCCTGCA		
iucB	IucB_319F	CGTGCGGGAATACAGTTTGACG	574	55°C for 30 s/72°C for 60 s
	IucB_892R	GCTTGTGCGGGAAATCGAACTC		
iucC	IucC_389F	TGCAGTGCCTGATGTCAGGCCAT	660	58°C for 30 s/72°C for 60 s
	IucC_1049R	ACGCCAAACATCTCCTGATAGCG		
iucD	IucD_327F	GAGCAATCTGTCGTTCAGCC	484	52°C for 30 s/72°C for 60 s
	IucD_810R	GATAGCGAGCAGCGATTCGC		
iutA	IutA_943F	CGCGATGAGAGCCTGACCTA	598	55°C for 30 s/72°C for 60 s
	IutA_1540R	CAAGACGATAGGTGCCGGAG		
eitA	EitA_904R	CCTTTTTCACGGCGTCGAGCTG	281	60°C for 30 s/72°C for 30 s
	EitA_624F	TCTCTTCTGGTTCTCCAGCGCG		
eitD	EitD_358F	CCGTCGATTGAATCGCTGCTG	536	56°C for 30 s/72°C for 60 s
	EitD_893R	GCCACGCTGACAAACGAGGC		
fepA	FepA_840F	GTTTGAAGCGGGCTACAGCC	597	55°C for 30 s/72°C for 60 s
	FepA_1436R	GGGCTCCAGTTGTTGCCAAC		
entF	EntF_1936F	AACCGGCTGCTGTGGATGCAAA	280	58°C for 30 s/72°C for 30 s
	EntF_2215R	TACAGAACACCTGRCGCAGCGA		
entE	EntE_1496R	AGTTTGAAATCGGCGACGCCCAG	244	58°C for 30 s/72°C for 30 s
	EntE_1253F	CASGGCTACATCACCGTTCAGGG		
fepE	FepE_272F	GCTGCCGCAGAAATGGACCAG	339	58°C for 30 s/72°C for 30 s
	FepE_610R	AGCGTCCAGGAGCTGTAAGGC		
entC	EntC_1149R	GTGGAGCATGGTGGAGAGCT	711	55°C for 30 s/72°C for 60 s
	EntC_439F	TCCCGCCTTATCGACATCAC		
fepG	FepG_230F	GCGCGATTTTCCAGTCGCTGATG	263	58°C for 30 s/72°C for 30 s
	FepG_492R	CCASGTATTGAACGCCACCAGC		
entS	Ents_127F	CAGATCCAGACGCTCACCGG	369	56°C for 30 s/72°C for 30 s
	Ents_495R	GAGCAGCGGCGAAATYACCG		
fepB	FepG_36F	CGCCAGCCTGCTGSTTTTAGG	600	58°C for 30 s/72°C for 60 s
	FepG_635R	GCGAATCTCTGGACCGCCGA		
fhuA	Fhua_4F	GCGCGTTCYACTCACACTCAG	275	55°C for 30 s/72°C for 30 s
	Fhua_278R	TCCATCTCTTCSCGCGTCAC		
fhuB	Fhub_1313F	TGATGCTGCTGTTCGTGCCG	493	58°C for 30 s/72°C for 45 s
	Fhub_1805R	AGCATSCGCGTAATGTGCGG		
fecR	FecR_108F	GCGCTGGCAACAGTGGTATG	487	56°C for 30 s/72°C for 60 s
	FecR_594R	CTGCACGTCAAGCTGCGTGA		
fecB	FecB_286F	GCTGCCCTGAAACCAGACCT	324	56°C for 30 s/72°C for 60 s
	FecB_609R	CAGAGAAGCCAGCACGCTGC		
fecE	FecE_283F	GAACTGGTTTCCTACGGCCG	435	56°C for 30 s/72°C for 60 s
	FecE_717R	CGCTTCTACGCTGAACACCG		
feoB	Feob_895F	CTGCTCGCCATTAACATCGGCG	296	58°C for 30 s/72°C for 30 s
	Feob_1190R	ACGAACGATTTGCCCGGCAG		
efeB	Efeb_167F	AGACGCAGCCGTTTTACGGCG	431	58°C for 30 s/72°C for 45 s
	Efeb_597R	GTCGCGCAGCGCATGGATMAC		
efeO	Efeo_26F	CGCTTTCCGTGACGCTGCTG	312	58°C for 30 s/72°C for 30 s
	Efeo_337R	GCTTGCCTTTCGGGTTGCTCAG		
fhuF	FhuF_270F	GCTGAAATCGCTCTGGGCGCAG	351	58°C for 30 s/72°C for 30 s
	FhuF_620R	ACGGTCTCKTCGCCGAGCCA		
fhuE	FhuE_156F	CACGCCTGACGAGTCRCAGGA	291	58°C for 30 s/72°C for 30 s
	FhuE_446R	GCTGGAACTTTGGCGATACCGC		
pfeA	PfeA_286F	CGCCAGATTGACATTCGCGG	494	56°C for 30 s/72°C for 45 s
	PfeA_779R	AATACCGCGCAGAACGGCTC		
foxA	FoxA_10F	GCTTTAACCCTGAAACGCTCCGC	400	58°C for 30 s/72°C for 45 s
	FoxA_409R	GAGCGACGGCGGCAGCTATA		
yncD	YncD_1726F	CTTGACGCCACCTACCGCRC	243	58°C for 30 s/72°C for 30 s
	YncD_1968R	AAATTCCAGCGCGGCAACTGG		

(Continued on following page)

TABLE 2 (Continued)

Target	Primer pairs[a]	Sequence (5′–3′)	Amplicon size (bp)	Annealing/extension cycle parameters[b]
btuB	BtuB_1953F	GTCAGCCTGTGGGATGTCGC	156	58°C for 30 s/72°C for 30 s
	BtuB_2108R	TGTCAGGCAGCTACACCTTCTGA		
fcuA	fcuA_356F	CCTACCGCATTCGCGGCTTTG	198	58°C for 30 s/72°C for 30 s
	fcuA_553R	CGTCGGCATGTTTCGGCTCAAG		
fct	fct_311F	GCTCCAACCGCAACGACGAAG	554	58°C for 30 s/72°C for 60 s
	fct_864R	GTCGCTGACGTTGAAATCGCGC		
yfeX	YfeX_125F	TAGCGACCTTTCAGGCGCAGT	281	58°C for 30 s/72°C for 30 s
	YfeX_405R	CACGGTTTTCGCTGGGTGGAA		
repA[d]	repA_185F	CAGACGCGACTGAGGAGCTTG	784	56°C for 30 s/72°C for 60 s
	repA_968R	AGAGGATCGATGCCAGCAGCC		
repE[e]	repE_46F	CTGCAGGAACATGACGGCATC	380	56°C for 30 s/72°C for 60 s
	repE_425R	CCTGAGCCATCAGGTTTACGG		
16S rRNA	P0	AGAGTTTGATCCTGGCTCAG	1,503	55°C for 30 s/72°C for 90 s
	P6	GTACGGCTACCTTGTTACGA		

[a] Number in primer name corresponds to 5′ nucleotide position of the ClustalW alignments for each gene (see Table S1 in the supplemental material).
[b] All PCRs were performed with 30 cycles, except for that for *Cronobacter* 16S rRNA, which was performed with 25 cycles.
[c] Dimethyl sulfoxide (DMSO) at a 7% (final concentration) was added to the PCR mix.
[d] The *repA* gene of plasmid pCS2151.
[e] The *repE* gene of plasmid pCS2151.

and C. *dublinensis* subsp. *lausennensis* LMG 23824 contain loci one and three but lack the second locus containing *fepE*. In *E. coli*, *entABCDEFH* are involved in enterobactin biosynthesis, *entS* is involved in enterobactin export, *fepA* encodes the ferric enterobactin receptor, *fepBCDEG* allow the transport of the ferric enterobactin complex inside 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* 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* 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

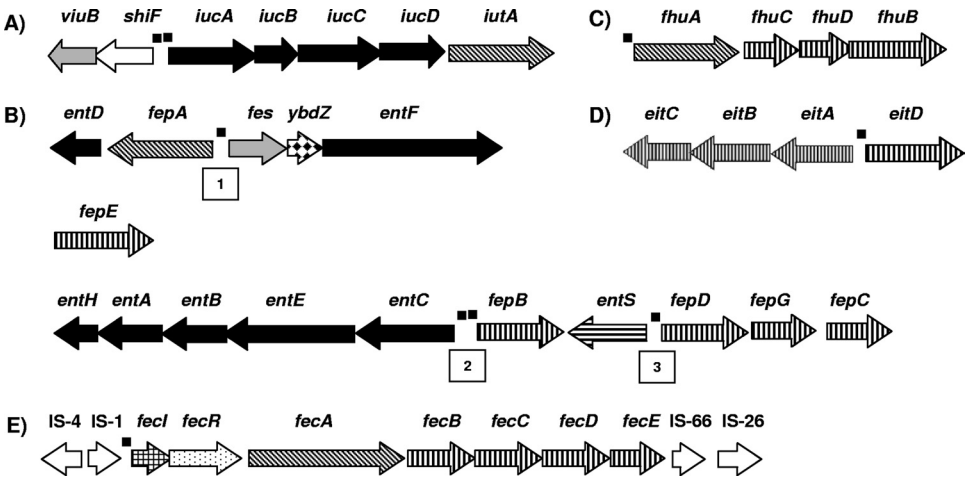

FIG 1 Ferric iron transporters encoded by *Cronobacter* spp. (A) Cronobactin siderophore; (B) enterobactin-like siderophore; (C) hydroxamate ABC transporter encoded by *fhuACDB*; (D) ferric iron/siderophore/heme ABC transporter encoded by *eitCBAD*; (E) ferric dicitrate transport system. Arrows show 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 of the iron from siderophore-iron complex (gray), 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 locations of the three putative bidirectional promoter-operator regions. The small filled boxes upstream of some genes or operons show locations of putative Fur boxes.

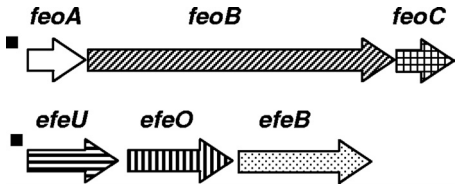

FIG 2 Genes for ferrous iron transporters, *feoABC* and *efeUOB*, carried by the chromosome of *Cronobacter* spp. Arrows show the direction of transcription, and arrow fills identify genes encoding GTP-binding protein (probably permease) (diagonal lines), Fe-S-dependent transcriptional regulator of FeoABC expression (square), high-affinity iron permease (horizontal lines), transporter periplasmic protein (vertical lines), periplasmatic peroxidase protein (dots), and unknown function (white). The small filled boxes upstream of the operons show location of putative Fur boxes.

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 the case with 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* 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 for 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* 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 *Saccharomyces cerevisiae* (10). Like the *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; and 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 receptor-encoding genes were identified in any of the nine *Cronobacter* 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 genes for the TonB-dependent receptors IutA, FepA, FhuA, and FecA, specific for cronobactin, enterobactin, ferrichrome, and ferric dicitrate, respectively, the nine *Cronobacter* genomes contain five common genes whose predicted encoded proteins have significant identity with TonB-dependent iron receptors. These outer membrane receptors include the siderophore receptor YncD (83), the vitamin B_{12}/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* strains. Fct has significant homology with the 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 both bacterial and fungal origins.

(v) Ferric reductase. Upstream of the cronobactin gene *iucA* in all of the pESA3-/pCTU1-like plasmid-harboring *Cronobacter* genomes analyzed are two open reading frames (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 that 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 YqjH encoded by *Vibrio 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 YqjH is to aid in the release of the iron from the siderophore into the cytosol.

Furthermore, the chromosomes of all *Cronobacter* 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.

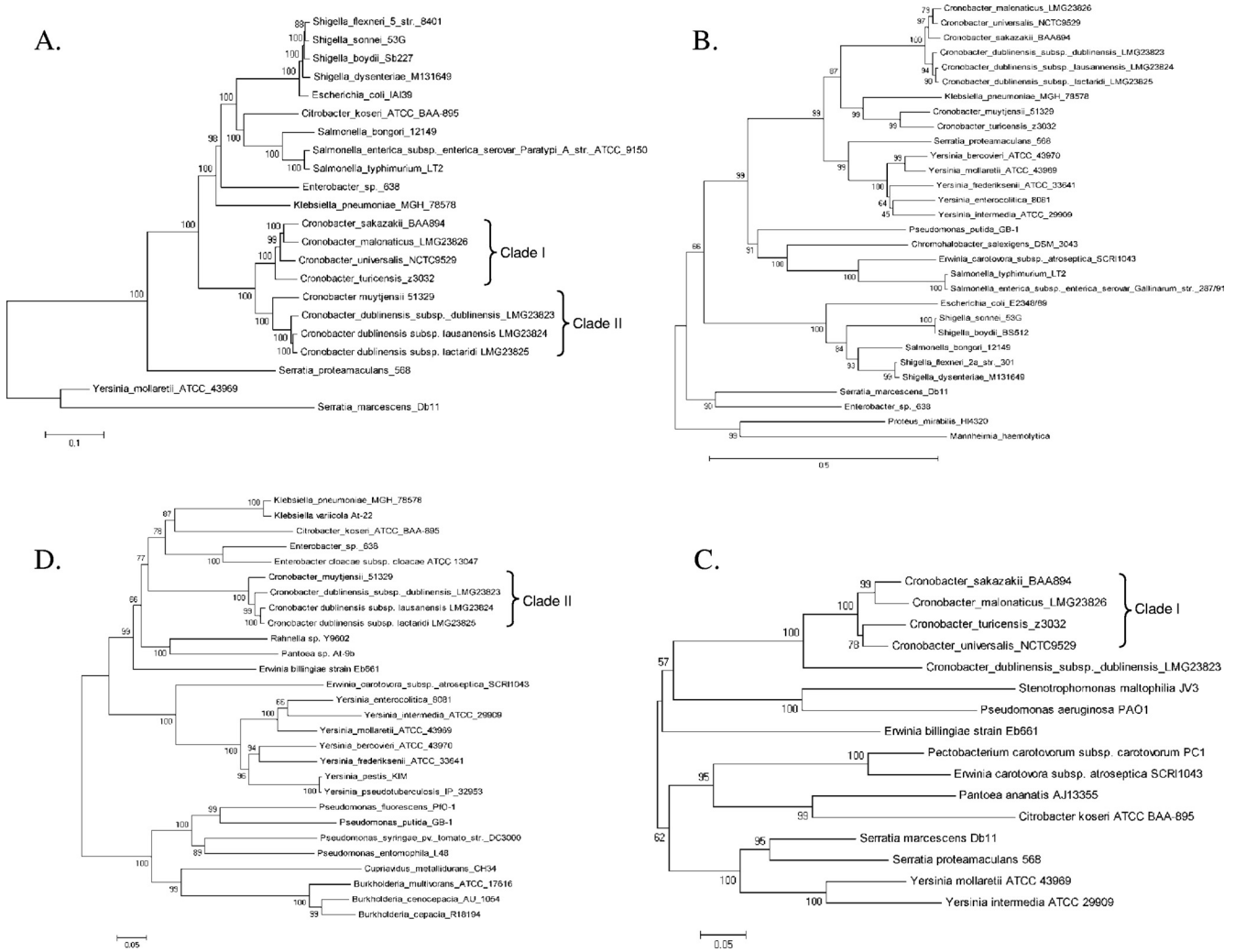

FIG 3 Evolutionary history of iron acquisition system genes. (A) Enterobactin gene cluster in *Cronobacter* spp., *entHABEC-fepB* and *entS-fepDGC*; (B) the TonB receptor-encoding gene *foxA*; (C) the TonB receptor-encoding gene *fcuA*; (D) the ferric reductase gene, *viuB*.

Phylogenetic analysis of *Cronobacter* iron acquisition systems. To infer evolutionary relationships, phylogenetic analysis was performed on the nucleotide sequence of each putative iron acquisition operon or gene identified in the *in silico* analysis. Phylogenetic analyses of 16S rRNA genes and housekeeping genes typically place the group consisting of *Citrobacter*, *Klebsiella*, *Escherichia*, *Shigella*, *Salmonella*, and *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, to the three-enterobactin gene cluster containing *entH* to *fepC* (Fig. 3A). After *Klebsiella*, typically, homologues in the genomes of *Enterobacter* spp., such as strain 638, several *Salmonella enterica* serovar strains, and *Citrobacter koseri* ATCC BAA-895 were the next most 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 cluster separate 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; see also Fig. S1 in the supplemental material).

In two instances, homologues in *E. coli* and *Shigella* spp. were more closely related than those in *Salmonella* spp.: the TonB receptor-encoding genes, *pfeA* and *yncD* (see 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* (see 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* (see 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 genomes of *Cronobacter* spp. analyzed present a phylogenetic evolu-

tionary 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* species other than *Y. pestis*. For *btuB*, it is clear that this gene is under considerably different evolutionary pressure within the *Enterobacteriaceae* (see Fig. S1 in the supplemental material). The *btuB* genes of *Cronobacter* spp. form three clusters, in contrast to other conserved iron acquisition genes, which typically display a two-clade phylogeny within *Cronobacter* 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 nonmobile F-type plasmid (23). The reconstructed evolutionary history of *shiF-viuB* is considerably different from 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 homologous systems found in *Serratia* and *Yersinia* spp. (23). The *Cronobacter viuB* gene is also similar to the same gene 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* and *shiF* (66%) and between *iucABCD* and *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. dublinenisis* and *C. muytjensii*, 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* (see Fig. S1 in the supplemental material).

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* subsp. *cloacae* NCTC 9394, and it is 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. (see Fig. S1).

Within the genus *Cronobacter*, phylogenetic analysis revealed two subclades for most iron acquisition genes and systems analyzed (Fig. 3A; see also Fig. S1 in the supplemental material). One subclade is comprised of the species *C. sakazakii*, *C. malonaticus*, *C. universalis*, and *C. turicensis*; the other subclade is comprised of *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 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 98% (226) of the same 231 *Cronobacter* isolates harbor a pESA3-/pCTU-1-like plasmid that has the genes encoding the cronobactin siderophore and EitABCD transporter (23). In this study, PCR results showed that all 226 *Cronobacter* strains 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* 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* isolates possess the genes encoding the ferric transporter FhuACDB and 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 found in only14% of the *C. sakazakii* and 16% of the *C. malonaticus* strains tested. These genes were not found in any of the other *Cronobacter* species (Table 3). Interestingly, of the 30 *Cronobacter* 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* strains and one *C. malonaticus* strain harbor the *fec* genes on a pCSA2151-like plasmid, and the remaining 26 strains have these genes on their chromosomes (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 the *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* strains 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* 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

TABLE 3 Distribution of iron acquisition systems in 231 *Cronobacter* strains

Iron acquisition system	Gene	No. (%) of isolates PCR positive for target gene					
		C. sakazakii ($n = 180$)	*C. malonaticus* ($n = 25$)	*C. turicensis* ($n = 6$)	*C. muytjensii* ($n = 12$)	*C. dublinensis* ($n = 6$)	*C. universalis* ($n = 2$)
Ferric transporter							
Cronobactin	*iucC*	179 (99)	25 (100)	6 (100)	9 (75)	5 (83)	2 (100)
Enterobactin	*entF*	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	fepE	180 (100)	25 (100)	6 (100)	12 (100)	2 (33)	2 (100)
	entE	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	fepG	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
FhuABCD	*fhuA*	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	fhuB	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
EitABCD	*eitA*	179 (99)	25 (100)	6 (100)	9 (75)	5 (83)	2 (100)
Ferric dicitrate	*fecR*	26 (14)	4 (16)	0 (0)	0 (0)	0 (0)	0 (0)
	fecE	26 (14)	4 (16)	0 (0)	0 (0)	0 (0)	0 (0)
Ferrous transporter							
FeoABC	*feoB*	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
EfeUOB	*efeB*	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	efeO	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
Ferric reductase	*viuB*	179 (99)	25 (100)	6 (100)	9 (75)	5 (83)	2 (100)
	fhuF	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
TonB receptor	*fcuA*	0 (0)	0 (0)	0 (0)	12 (100)	6 (100)	0 (0)
	fct	0 (0)	0 (0)	3 (50)	12 (100)	6 (100)	0 (0)
	yncD	179 (99)	25 (100)	6 (100)	12 (100)	5 (83)	2 (100)
	btuB	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	foxA	175 (97)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	fhuE	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
	pfeA	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)
Heme iron extractor	*yfeX*	180 (100)	25 (100)	6 (100)	12 (100)	6 (100)	2 (100)

Fct, such as chrysobactin, which is produced by plant pathogen *E. chrysanthemi*.

Expression of iron acquisition systems. The expression of most iron transport systems is repressed by iron via interaction of the Fur protein at the promoter site. Their promoters contain the consensus Fur binding site, or Fur box. In the presence of iron, Fur binds to the Fur box and blocks 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* iron acquisition systems, except in the genes for the heme iron extractor, *yfeX*, and *btuB*, which encodes a predicted vitamin B_{12}/cobalamin receptor (Table 5). In order to confirm if the *Cronobacter* iron acquisition systems are regulated by Fur, their expression was determined after the bacterial cells were grown for 16 h in LB medium (iron-replete conditions) and LB supplemented with the ferrous iron chelator 2,2′-dipyridyl (DIP) at 300 μM (iron-depleted conditions). Of 25 genes tested, 18 exhibited higher expression under iron-depleted conditions than under iron-replete conditions, indicating derepression of these genes (Table 5). On average, their expression increased 2.7-fold when cells were 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 the 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 condi-

TABLE 4 Distribution of ferric dicitrate transport system and plasmid pCSA2151 replication genes among 231 strains of *Cronobacter* spp.[a]

Species	No. of isolates	No. (%) PCR positive			
		fecR	*fecE*	*repA*	*repE*
C. sakazakii	180	3 (1.7)	3 (1.7)	3 (1.7)	3 (1.7)
		23 (13)	23 (13)	0 (0)	0 (0)
C. malonaticus	25	1 (4)	1 (4)	1 (4)	1 (4)
		3 (12)	3 (12)	0 (0)	0 (0)
		0 (0)	0(0)	1 (4)	1 (4)
C. turicensis	6	0 (0)	0 (0)	0 (0)	0 (0)
C. muytjensii	12	0 (0)	0 (0)	0 (0)	0 (0)
C. dublinensis	6	0 (0)	0 (0)	0 (0)	0 (0)
C. universalis	2	0 (0)	0 (0)	0 (0)	0 (0)

[a] Multiple profiles were found among isolates of *C. sakazakii* and *C. malonaticus*.

TABLE 5 Putative Fur boxes identified in *Cronobacter* iron acquisition systems and fold increase in expression under iron-depleted conditions

Gene or operon(s)	Putative phenotype	Sequence (5′– 3′)[a]	No. of identical bases	Location[b]	Fold increase in expression[c]
iucABCD-iutA	Cronobactin biosynthesis and transport	C**ATAACGATAATCATTATC**	17	19 bp of *iucA*	4.1 ± 0.2 (*iucC*), 2.9 ± 0.1 (*iutA*)
shiF-viuB	Ferric reductase and MFS permease	A**ATA**TT**T**G**T**TT**TCATTA**AT	11	44 bp of *shiF*	2.2 ± 0.2 (*viuB*)
fepA-entD-fes-ybdZ-entF	Enterobactin biosynthesis, receptor and iron release	**GATAAT**AA**C**T**ATCATTATC**	17	18 bp of *fepA*, 71 bp of *fes*	1.5 ± 0.05 (*fepA*), 4.4 ± 0.3 (*entF*)
fepE	Enterobactin transport	Not present			0.8 ± 0.03 (*fepE*)
entCEBAH-fepB	Enterobactin transport and biosynthesis	**GA**A**A**T**G**A**G**AA**G**C**ATTATT** ACA**AATGATAA**CA**ATTATC**	16, 14	26 bp of *fepB*, 29 bp of *entC*	1.5 ± 0.08 (*fepB*), 1.7 ± 0.3 (*entC*)
entS-fepDGC	Enterobactin transport and biosynthesis	**GATAAT**AA**C**T**ATCATTATC**	16	19 bp of *fepD*, 107 bp of *entS*	No expression (*fepG*), 1.0 ± 0.04 (*entS*)
fhuACDB	Ferrichrome receptor and hydroxamate transport	**G**CGC**ATAATAATAATTC**T**C**	13	42 bp of *fhuA*	1.8 ± 0.2 (*fhuA*)
fecIRABCDE	Ferric dicitrate receptor and transport	TG**TAATGATAACCATTCTC**	16	45 bp of *fecI*	3.4 ± 0.2 (*fecB*)[d]
eitABC-eitD	ABC ferric transporter	A**AGAATGAT**TT**TCATT**TG**C**	13	75 bp of *eitA*, 141 bp of *eitD*	0.7 ± 0.05 (*eitA*), no expression (*eitD*)
feoABC	Ferrous transporter	A**AAAA**CC**ATT**C**TCATTACC**	12	103 bp of *feoA*	1.8 ± 0.3 (*feoB*)
efeUOB	Ferrous transporter	**GGTAATGATAATCA**C**TTTC**	16	59 bp of *efeU*	1.9 ± 0.3 (*efeO*)
yncD	Siderophore receptor	**GAGAATAATAATCATTAT**T	16	164 bp of *yncD*	3.4 ± 0.4 (*yncD*)
foxA	Ferroxamine receptor	**GATAAT**AA**TT**CG**CATT**C**TT**	13	60 bp of *foxA*	3.0 ± 0.4 (*foxA*)
fhuE	Ferric rhodotorulic acid/coprogen receptor	ACA**AATGATTATATTTCTC**	12	138 bp of *fhuE*	2.7 ± 0.3 (*fhuE*)
pfeA	Ferric enterobactin receptor	**GATAATTATTATCATTATC**	17	47 bp of *pfeA*	4.3 ± 0.4 (*pfeA*)
fcuA	Hydroxamate receptor	A**ATAATGA**C**A**GGACAACCT	8	6 bp of *fcuA*	2.8 ± 0.3 (*fcuA*)
		G**CAAATGATTATTAGTAAC**	14	116 bp of *yncE*	
fct	Ferrichrysobactin receptor	**GGTAATGATTTTCAATATC**	16	63 bp of *fct*, 189 bp of *fesA*	2.8 ± 0.2 (*fct*)
fhuF	Ferric reductase	AGG**ATTGGCAATCATTATC**	13	28 bp of *fhuF*	2.1 ± 0.4 (*fhuF*)
btuB	Vitamin B_{12}/cobalamin receptor	Not present			1.0 ± 0.04 (*btuB*)
yfeX	Heme iron extractor	Not present			0.76 ± 0.2 (*yfeX*)

[a] Comparison with *E. coli* consensus 5′-GATAATGATAATCATTATC-3′ (17). Identical nucleotides are in boldface type.
[b] Number of nucleotides upstream of the start codon.
[c] Average ± standard deviation of three RT-PCRs to determine fold change in the expression of the gene under iron-depleted conditions (LB broth supplemented with 300 μM DIP). All RT-PCRs were performed in *C. turicensis* z3032, except for those for *fhuA*, *fct*, and *fcuA* as well as *fecB*, which were performed in *C. dublinensis* LMG23825 and *C. sakazakii* 2151, respectively.
[d] Growth in LB broth supplemented with 300 μM DIP and 1 mM citrate.

tions, 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 directions opposite to that of the *iucABCD-iutA* operon (Fig. 1A). This sequence structure suggests that similar to the *E. coli* enterobactin operons, transcription of the *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). The presence of two Fur boxes between *iucA* and *shiF* suggests 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 EitABCD localized on pCTU1 in *C. turicensis* z3032 (Fig. 1D), were not derepressed under 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-eitD*, which is different from the typical arrangement 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* to *entF* plus *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

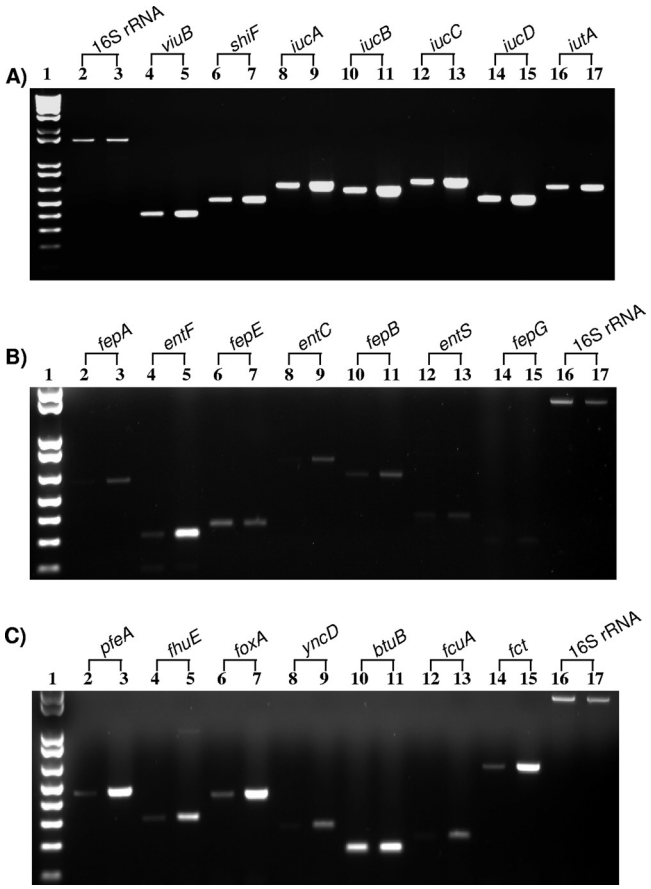

FIG 4 Representative RT-PCR of *Cronobacter* iron acquisition systems under iron-replete (even-numbered lanes) and iron-depleted (odd-numbered lanes) conditions. (A) Cronobactin and *shiF-viuB* operons. Lane 1, 1 kb plus DNA ladder; lanes 2 and 3, 16S rRNA; lanes 4 and 5, *viuB*; lanes 6 and 7, *shiF*; lanes 8 and 9, *iucA*; lanes 10 and 11, *iucB*; lanes 12 and 13, *iucC*; lanes 14 and 15, *iucD*; lanes 16 and 17, *iutA*. (B) Enterobactin genes. Lane 1, 1 kb plus DNA ladder; lanes 2 and 3, *fepA*; lanes 4 and 5, *entF*; lanes 6 and 7, *fepE*; lanes 8 and 9, *entC*; lanes 10 and 11, *fepB*; lanes 12 and 13, *entS*; lanes 14 and 15, *fepG*; lanes 16 and 17, 16S rRNA. (C) TonB-dependent iron receptors. Lane 1, 1 kb plus DNA ladder; lanes 2 and 3, *pfeA*; lanes 4 and 5, *fhuE*; lanes 6 and 7, *foxA*; lanes 8 and 9, *yncD*; lanes 10 and 11, *btuB*; lanes 12 and 13, *fcuA*; lanes 14 and 15, *fct*; lanes 16 and 17, 16S rRNA.

the exception of *fepE*, which forms a single locus separate from the *fes* operon, we found the same gene organization as in *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* and *fes* and between *entS* and *fepD*, 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* and *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 the *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 and not expressed, respectively, when grown under both iron-depleted and iron-replete conditions (Table 5; Fig. 4B).

Expression of the *fec* transport genes starts 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 turn, 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 doubly controlled regulation scheme. *In silico* analysis in *C. sakazakii* 2151 identified a putative Fur box 44 bp upstream of the *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 than under iron-replete growth conditions lacking citrate, confirming that expression of the *fec* transport genes is induced by citrate and derepressed by low-iron growth conditions (Table 5).

Putative Fur boxes were identified in the upstream region of the ferrous transporter system genes *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, it has recently been determined that YncD plays an important role in survival inside the host and that the gene is overexpressed 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 levels of expression of these genes were similar under iron-replete and iron-depleted conditions (Table 5; Fig. 4C, lanes 10 and 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

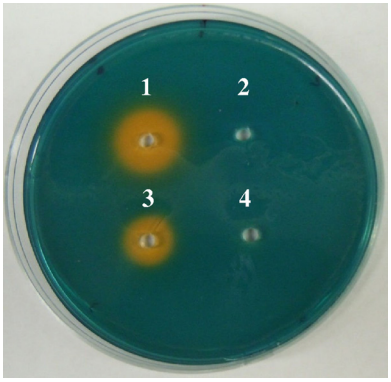

FIG 5 Siderophore activity using the CASAD assay. Wells were filled with cell-free culture supernatants of wild-type *C. sakazakii* BAA-894 (1), plasmid-cured derivative BAA-894.3 (2), fosmid clone ESA-C01 containing the cronobactin genes (3), and fosmid clone ESA-M04 lacking the cronobactin genes (4).

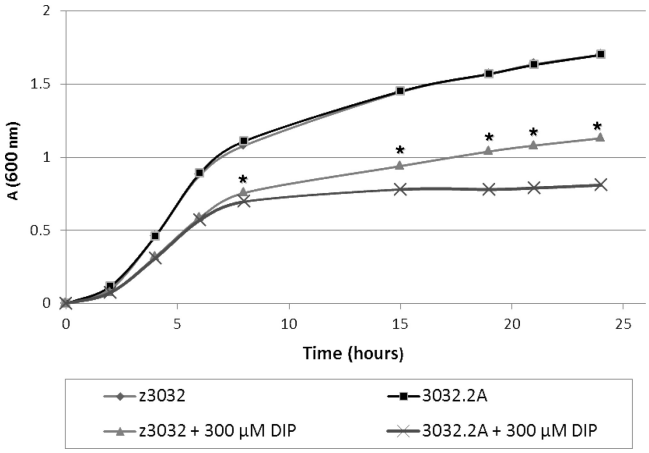

FIG 6 Growth of wild-type *C. turicensis* z3032 harboring pCTU1 and its plasmid-cured derivative, 3032.2A, in LB broth and iron-depleted LB broth containing 300 μM DIP. The data were obtained from 3 independent experiments. *, $P < 0.001$.

that these two genes are also part of the Fur regulon (Table 5; Fig. 4C, lanes 12 to 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 the *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 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 5 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 base pairs 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 *yncD* (Table 5). It is unclear if *fcuA* is cotranscribed with *yncD* under the control of one promoter-Fur box region or, alternatively, both genes transcribe independently under the 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 *iucABDCD* synthesis genes do encode active siderophores. However, larger orange halos were produced by wild-type *C. sakazakii* BAA-894 harboring pESA3 compared to *E. coli* ESA-C01, suggesting that *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 the *iucABDCD-iutA* operon enhances growth of *Cronobacter* spp. under iron-depleted growth conditions, *C. turicensis* z3032 harboring plasmid pCTU1 and its plasmid-cured derivative strain 3032.2A were grown in LB medium and LB medium supplemented with 300 μM 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-type strain between 7 and 24 h in LB medium supplemented with DIP (Fig. 6). Similar results were obtained when wild-type *C. sakazakii* BAA-894 and its plasmid-cured derivative, BAA-894.3, were grown under low-iron conditions (data not shown). RT-PCR analysis showed no difference in the expression of *iucC* after 5 h and 18 h of growth in LB medium supplemented with DIP, but a larger halo was produced after 18 h than after 5 h of growth, suggesting that a 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-/pCTI-1-like plasmids plays a critical role in promoting growth of *Cronobacter* spp. under iron-limiting conditions.

Enterobactin-like siderophore is not functional. Even though the chromosomes of most of the *Cronobacter* isolates tested have the enterobactin genes, we were not able to detect siderophore activity in *C. sakazakii* BAA-894 and *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 a CASAD assay of the cell-free culture supernatants of *C. muytjensii* ATCC 51329 and the other four *Cronobacter* strains, from our culture collection, lacking pESA3-/pCTU1-like plasmids (strains listed in Table

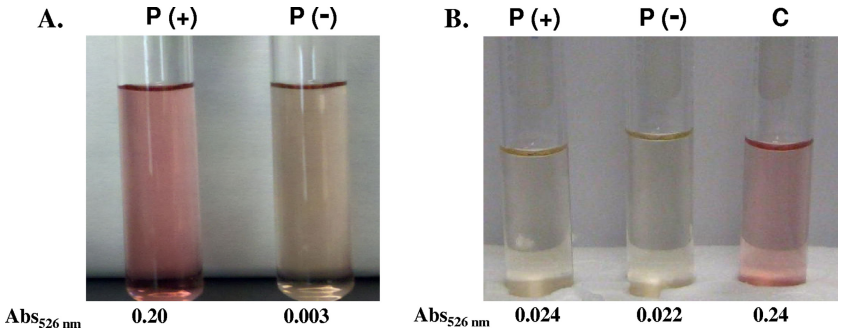

FIG 7 Results of the Csáky (A) and Arnow (B) tests used to identify hydroxamate-type and catechol-type siderophore activity, respectively, using cell-free culture supernatants of wild-type *C. turicensis* z3032 harboring pCTU1 [P(+)] and its plasmid-cured derivative, 3032.2A [P(−)]. Cell-free culture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

1). Siderophore activity was detected in the cell-free culture supernatants of two control *Cronobacter* strains harboring pESA3-/pCTU1-like plasmids (strains listed in Table 1), but not in the supernatants of any of the five *Cronobacter* 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 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 plasmidless 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 test (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 plasmidless 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 genes, 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 the case with other bacteria, the enterobactin genes in *Cronobacter* spp. are localized in three separate loci in the chromosome (Fig. 1B). Our expression analysis showed that 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 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 2- to 5-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 assay, i.e., is 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 posttranscriptional 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 siderophore receptor genes do 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 five 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, which 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 systems 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 the iron acquisition genes and systems separate the genus *Cronobacter* into two subclades. One subclade includes the species *C. sakazakii*, *C. malonaticus*, *C. universalis*, and *C. turicensis*, and the other subclade 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 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 closely associated with the human host, which may explain why most *Cronobacter*-related disease in humans is caused by *C. sakazakii* and *C. malonaticus*.

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