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Interplay between Iron Homeostasis and the Osmotic Stress Response in the Halophilic Bacterium *Chromohalobacter salexigens*▽†

Montserrat Argandoña,[1] Joaquín J. Nieto,[1] Fernando Iglesias-Guerra,[2] Maria Isabel Calderón,[1] Raúl García-Estepa,[1] and Carmen Vargas[1]*

Department of Microbiology and Parasitology[1] and Department of Organic and Pharmaceutical Chemistry,[2] Faculty of Pharmacy, University of Seville, Profesor García González 2, Seville 41012, Spain

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In this study, the connection between iron homeostasis and the osmostress response in the halophile *Chromohalobacter salexigens* was investigated. A decrease in the requirement for both iron and histidine and a lower level of siderophore synthesis were observed at high salinity, and these findings were correlated with a lower protein content in salt-stressed cells. A six-gene operon (*cfuABC-fur-hisI-orf6* operon) located downstream of the *ectABC* ectoine synthesis genes was characterized. A *fur* strain (in which the ferric iron uptake regulator Fur was affected) had the Mn resistance phenotype typical of *fur* mutants, was deregulated for siderophore production, and displayed delayed growth under iron limitation conditions, indicating that *fur* encodes a functional iron regulator. *hisI* was essential for histidine synthesis, which in turn was necessary for siderophore production. Fur boxes were found in the promoters of the *cfuABC-fur-hisI-orf6* and *ectABC* operons, suggesting that Fur directly interacts with DNA in these regions. Fur mediated the osmoregulated inhibition of *cfuABC-fur-hisI-orf6* operon expression by iron and functioned as a positive regulator of the *ectABC* genes under high-salinity conditions, linking the salt stress response with iron homeostasis. Excess iron led to a higher cytoplasmic hydroxyectoine content, suggesting that hydroxyectoine protects against the oxidative stress caused by iron better than ectoine. This study provides the first evidence of involvement of the iron homeostasis regulator Fur as part of the complex circuit that controls the response to osmotic stress in halophilic bacteria.

The halophilic bacteria are an important group of microorganisms that have evolved to live and thrive in hypersaline habitats (75). Most of these organisms need salt to grow due to a specific requirement for Na^+ (i.e., there are Na^+ gradients that drive transport, or there are respiration-driven Na^+ pumps) (75), whereas a few others, such as *Halobacillus halophilus*, *Salinibacter ruber*, and the anaerobic *Halanaerobiales* (64), have a specific requirement for Cl^-. Despite their NaCl-dependent growth, halophilic bacteria must use osmoadaptation strategies to maintain turgor under hyperosmotic conditions. A second strategy ("salt-out"), which is used by most halophilic bacteria, by halophilic methanogenic *Archaea*, and by halotolerant representatives of the three domains of life, is based on intracellular accumulation (via uptake or *de novo* synthesis) of compatible solutes (10, 21, 52). These solutes are small organic compounds, mainly amino acids or carbohydrates, that do not interfere with cell metabolism. This osmostress response is more versatile and flexible and does not require much evolutionary adjustment of cytoplasmic proteins and cellular processes to high salt concentrations (20, 21, 52).

Chromohalobacter salexigens is a moderately halophilic gammaproteobacterium that shows remarkable versatility in salt tolerance (75); it is able to grow with 0.5 to 3 M NaCl in a minimal medium, and optimal growth occurs in the presence of 1.5 M NaCl (13). It requires Na^+ (at least 0.3 M), Cl^- (>0.1 M), and high ionic strength for optimal growth (13, 54) and has very acidic periplasmic binding proteins in its ABC transport systems, which are exposed to the full salinity of the medium (58). *C. salexigens* adjusts its compatible cytoplasmic solute pool in order to cope with high salinity and supraoptimal temperatures (11, 30). This adjustment is achieved by highly hierarchical accumulation of solutes, which is dominated by uptake of external osmoprotectants such as betaine or its precursor choline (11, 14), and is followed by synthesis of endogenous solutes, mainly ectoines (ectoine and hydroxyectoine) and minor amounts of glutamate, glutamine, trehalose, and glucosylglycerate (13, 16, 30). Ectoines have biotechnological applications as agents that protect enzymes and whole cells (74). Thus, elucidation of the mechanisms that control the synthesis of ectoines is crucial for generating modified strains with improved ectoine production for prospective industrial use. Ectoine and hydroxyectoine are essential for osmoprotection and thermoprotection, respectively (30), and can be used as nutrients as well (73).

In previous work, we characterized the *C. salexigens ectABC* (12, 15) and *ectD* (30) genes, which encode the main routes for ectoine synthesis and hydroxyectoine synthesis, respectively.

Regulation of ectoine synthesis occurs, at least in part, at the transcriptional level. Thus, S1 protection assays and transcriptional fusions with *lacZ* demonstrated that the *ectABC* genes can be expressed from two promoter regions. One promoter region is located upstream of *ectA* and is composed of four promoters (*ectAp1* to *ectAp4*), and the

* Corresponding author. Mailing address: Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Profesor García González 2, Seville 41012, Spain. Phone: (34) 954-553811. Fax: (34) 954-628162. E-mail: cvargas@us.es.

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second promoter region is an internal promoter located upstream of *ectB* (*ectBp*) (11). *In silico* analysis of the −10 and −35 sequences of these regions showed that *ectAp1* and *ectAp2* may be dependent on the main vegetative factor σ^{70}, whereas *ectAp3* and *ectBp* were similar to σ^{S}- and σ^{32}-dependent promoters, respectively. In agreement with these predictions, expression of an *ectAp*(*1-4*)::*lacZ* fusion was osmoregulated and depended in part on the general stress factor σ^{S}, whereas *ectBp* was induced by continuous growth at a high temperature. Very interestingly, expression from *ectAp* was reduced when excess iron (50 μM $FeCl_3$) was added to the medium, suggesting that there is a regulatory link between iron homeostasis and the osmostress response in *C. salexigens* (11).

Iron is an essential nutrient for almost all microorganisms as it is a structural and functional component of various important enzymes. Cells have developed several systems for iron uptake under iron-limited conditions. On the other hand, elevated amounts of iron are toxic because iron is able to generate very reactive free radicals, which may react with any cellular macromolecule (31). Thus, maintenance of iron homeostasis in the cell is crucial, and iron uptake and storage are strictly regulated in response to external iron availability (3, 17, 51). Uptake of ferric iron by bacteria is generally mediated by siderophores, which are excreted from the cell and form complexes with iron; then the complexes are transported through specific membrane receptors (28, 71). Despite the fact that most siderophore receptors are highly specific, certain receptors have lower affinities than others and can recognize different siderophores that have closely related structures and similar iron coordination sites (32).

The key regulator for iron homeostasis is Fur (ferric uptake regulator), a histidine-rich protein that is widespread in bacteria and controls expression of many genes in response to iron availability. Fur is a global regulator that can act both as a repressor and as a positive regulator. The role of Fur as a negative regulator is thought to be direct. For example, when intracellular iron levels are high enough, Fur-Fe^{2+} binds to Fur box sequences of iron-regulated genes (i.e., genes encoding siderophore synthesis or transport), creating a steric impediment for RNA polymerase binding and therefore repressing transcription (26, 34). However, the role of Fur as a positive regulator seems to be mostly indirect. Thus, by using Fe^{2+} as a corepressor (when Fur acts as a repressor), Fur represses transcription of small regulatory RNAs (sRNAs) (e.g., for RyhB in *Escherichia coli*, FrrF in *Pseudomonas aeruginosa*, and FsrA in *Bacillus subtilis*), which act at the posttranscriptional level by pairing with the mRNAs encoding iron-requiring proteins (29, 46, 76). In addition to genes involved in iron storage, intermediate metabolism, and respiration, this sRNA-mediated positive regulation by Fur has been observed for genes involved in the acid response, oxidative stress, and quorum sensing in enterobacteria (55), *P. aeruginosa* (56, 76), *Vibrio cholerae* (77), and *B. subtilis* (29).

Interestingly, proteome and transcriptome analyses of salt-adapted *B. subtilis* cells revealed a connection between iron regulation and salt stress (37, 69). In a *B. subtilis* strain defective in the synthesis of bacillibactin (the major iron siderophore) (48), members of the Fur regulon encoding bacillibactin and putative iron uptake systems were induced by high salinity, suggesting that cells grown with osmotic stress experience severe iron limitation (37, 69). This finding, together with our finding that excess iron represses transcription of the ectoine synthesis genes in *C. salexigens*, led us to investigate in detail the relationship between iron metabolism and the osmoadaptive response in this halophilic microorganism. Our findings show that there is a two-way interaction between these two mechanisms. On the one hand, *C. salexigens* challenged by osmotic stress produces less protein and adapts its iron metabolism, probably to optimize compatible solute synthesis, and, on the other hand, excess iron influences the relative proportions of ectoine and hydroxyectoine. The regulatory protein Fur has a dual role as a regulator of iron homeostasis and an activator of the *ectABC* genes for ectoine synthesis. To our knowledge, this is the first time that a regulatory link between the mechanisms of an osmostress response and iron homeostasis has been established in a halophilic bacterium.

MATERIALS AND METHODS

Strains, media, growth conditions, and chemicals. CHR61, a spontaneous rifampin-resistant mutant of *C. salexigens* DSM 3043^T (5), was used as the wild-type strain. The *C. salexigens* wild-type strain and derivatives of this strain were routinely grown in SW-10 medium containing 10% (wt/vol) total salts and 0.5% (wt/vol) yeast extract (53). *C. salexigens* does not form aggregates under any growth conditions. Mating was done on SW-2 medium (similar to SW-10 medium but containing 2% [wt/vol] total salts) (72). *E. coli* was grown aerobically in complex LB medium (50). M63 medium (19) containing 20 mM glucose as the sole carbon source and 1.8 μM $FeSO_4$ was used as the minimal medium for *C. salexigens* and *E. coli*. Cells grown overnight in SW-2 medium were subcultured 1:100 in M63 medium having the salinity required for the experiment. The osmotic strength of M63 medium was increased by addition of 0.75 to 2.5 M (final concentrations) ultrapure NaCl (≥98% titration; Sigma), which was not contaminated with iron as it contained only traces of heavy metals (≤5 ppm). Although *C. salexigens* can grow in M63 medium with 0.5 M NaCl, growth at this salinity is extremely slow (doubling time [*g*], 24 h), and cells take a very long time to reach exponential phase (13). Therefore, we used M63 medium with 0.75 M NaCl as the standard medium for growth with a low salt concentration in all experiments. All of the glassware used was washed with HCl and rinsed several times with MilliQ water to avoid iron contamination. The pH of each medium was adjusted to 7.2 with KOH. Solid media contained 2% Bacto agar (Difco). Liquid cultures were incubated at 37°C in an orbital shaker at 200 rpm. When antibiotics were used, filter-sterilized antibiotics were added to LB or SW-2 medium at the following final concentrations: ampicillin (Ap), 150 μg ml^{-1}; tetracycline (Tc), 10 μg ml^{-1} (for M63 medium with 0.75 M or 1.5 M NaCl) and 1.2 μg ml^{-1} (for M63 medium with 2.5 M NaCl); chloramphenicol (Cm), 25 μg ml^{-1}; rifampin (Rf), 25 μg ml^{-1}; gentamicin (Gm), 25 μg ml^{-1}; and streptomycin (Sm), 20 μg ml^{-1}. When appropriate, the following compounds were added to the media (final concentrations): X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) (40 μg/ml), IPTG (isopropyl-β-D-1-thiogalactopyranoside) (Sigma) (25 μg/ml), sucrose (Sigma) (10%, wt/vol), histidine (Sigma) (1 mM), 2,2′-dipyridyl (Sigma) (0 to 90 μM), deferoxamine (Sigma) (100 to 900 μM), $FeCl_3$ (Sigma) (50 μM), and iron-free ferrichrome (Sigma) (1 mg/ml). Growth was monitored by determining the optical density at 600 nm (OD_{600}) with a Perkin-Elmer 551S UV/visible spectrophotometer.

Conjugal transfer of plasmids. Plasmids were transferred from *E. coli* to *C. salexigens* by triparental mating on SW-2 medium, using pRK600 as a helper plasmid (40) as described by Vargas et al. (72).

Siderophore production assay. The assay to determine siderophore production by *C. salexigens* strains was based on the procedure described by Schwyn and Neilands (65) to prepare Chrome Azurol S (CAS) agar plates, but it was modified as follows. MM9 basal medium was replaced by M63 minimal medium in which $FeSO_4 \cdot 7H_2O$ was omitted and a reduced KH_2PO_4 concentration (<0.03%, wt/vol) was used to eliminate any interference of the phosphate present in M63 medium with iron. Histidine (1 mM) was added when necessary. Each strain was grown at 37°C in M63 medium with different salinities until mid-logarithmic phase. One milliliter of each culture was centrifuged, washed with the same medium to remove siderophores and histidine from the supernatant, and resuspended in 30 μl of M63 medium containing 0.75 M NaCl. Aliquots

(10 μl) were placed on the modified CAS agar plates supplemented with different concentrations of NaCl and incubated for 72 h at 37°C. CAS is both a low-iron-affinity chelating agent and an indicator; it is blue-green when it is chelated with iron and turns orange when Fe is removed from it by higher-affinity chelating agents present in the medium, like siderophores. As controls, 1 μl of the commercial siderophore ferrichrome (1 mg/ml) and 2 μl of the iron chelators deferoxamine and 2,2′-dipyridyl (4 mg/ml) were placed on the same modified CAS agar plates containing 0.75, 1.5, or 2.5 M NaCl, and the diameters of the haloes produced were measured.

Determination of protein content. To estimate total cell protein contents, *C. salexigens* was grown at 37°C in 5 ml of liquid M63 medium with 0.75 M, 1.5 M, or 2.5 M NaCl until late exponential phase. The cell protein content in 1 ml of a culture was determined in triplicate by using a bicinchoninic acid (BCA) protein assay kit (Pierce) as described by García-Estepa et al. (30).

Methods used with nucleic acids and construction of *C. salexigens* mutants. Plasmid DNA was isolated from *E. coli* with a Wizard *Plus* SV miniprep kit (Promega), and genomic DNA was isolated with a Quantum Prep Aquapure genomic DNA kit (Bio-Rad). Restriction enzyme digestion and ligation were performed as recommended by the manufacturers (Amersham and Promega). DNA sequencing of the *cfuABC-fur-hisI-orf6* region and deletion clones was performed by Newbiotechniques (Seville, Spain).

To clone the *C. salexigens cfuABC-fur-hisI-orf6* region, a 5.7-kb HindIII fragment from pDE9 (a pVK102-derived cosmid clone from a *C. salexigens* gene library containing a ca. 35-kb SalI insert, including *ectABC* [see Fig. S1 in the supplemental material]) (15) was inserted into pKS(−) (Stratagene) to obtain plasmid pMH2. In addition to the *cfuABC-fur-hisI-orf6* region, pMH2 carries the 3′ end of *ectB* (265 bp) and the complete *ectC* gene (see Fig. S1 in the supplemental material).

To construct mutant CHR134 (Δ*fur*), a 3.0-kb PstI-HindIII fragment from pMH2 containing the 3′ end of *cfuC* (288 bp) and the complete *fur* and *hisI* genes was cloned in pKS, and the resulting plasmid (pMM1) (see Fig. S1 in the supplemental material) was used as the template to generate a 336-bp in-frame deletion of *fur* by using a PCR-based QuikChange site-directed mutagenesis kit (Stratagene). The primers used were *Fur-fw* (5′-GCGTGCTGGAAATGATCG CCAATCGGCGTAATCCTTTTCCAC-3′) and *Fur-rv* (5′-GTGGAAAAGGA TTACGCCGATTGGCGATCATTTCCAGCACGC-3′), and the PCR was performed using *Pfu* Turbo (Stratagene) and the following conditions: 3 min 95°C and then 18 cycles of 30 s at 95°C, 1 min at 70°C, and 11 min at 68°C, followed by 10 min at 68°C. Before transformation of DH5α competent cells (Stratagene), the amplified mutated plasmids containing staggered nicks were treated with DpnI at 37°C for 1 h to digest the parental DNA template and to select for mutation-containing synthesized DNA. The mutation in one of the resulting plasmids, pMM2 (see Fig. S1 in the supplemental material), was confirmed by using PCR and the restriction enzyme pattern. A 2.65-kb ApaI fragment from pMM2 with an in-frame deletion of *fur* was cloned into pJQSK200, and the plasmid generated (pHS333) (see Fig. S1 in the supplemental material) was transferred to *C. salexigens* strain CHR61 by triparental mating.

Recombinant strains resulting from a single recombination event were first selected on SW-2 medium plates with Gm and checked by PCR to confirm that there was correct insertion of the complete plasmid into *orf4*. From one Gm^r colony (CHR133) a *fur* mutant was isolated by Mn^r selection as described by Hantke (33); however, instead of minimal medium, we used fresh plates containing a modified SW-2 medium containing less than 1 mM $MgSO_4$ to avoid interference of Mg^{2+} with the selection by Mn (66). After testing $MnCl_2$ concentrations ranging from 2.5 to 10 mM, we selected 2.5 mM as the concentration most suitable for the assay with *C. salexigens*. In addition, the medium was supplemented with 10% sucrose to select double recombinant strains carrying the *fur* mutation. Aliquots of an overnight culture of CHR133 in SW-2 medium were spread on the Mn–SW-2 medium plates, which were incubated at 37°C for 4 days. Several sucrose- and Mn-resistant colonies were selected, and the in-frame deletion in *fur* was confirmed by PCR and sequencing. One strain, CHR134, was selected as a *fur* mutant strain.

To construct mutant CHR100 (Δ*cfuABC fur*::Ω), a 3.9-kb StuI region of pMH2 containing the 3′ end of *cfuA*, the complete *cfuB* and *cfuC* genes, and 120 bp of *fur* was replaced by a 1.95-kb SmaI fragment from pHP45-Ω, which contained the streptomycin-spectinomycin resistance gene (59). The resulting plasmid was designated pMH5 (see Fig. S1 in the supplemental material). To recombine the deletion in the *C. salexigens* chromosome, a 7-kb XbaI-ApaI fragment from pMH5 was cloned into the suicide vector pJQSK200 (60) to obtain plasmid pMH6 (see Fig. S1 in the supplemental material), which was mobilized into *C. salexigens* CHR61 by triparental mating. Mutant strains resulting from a double homologous recombination event were identified as St^r Gm^s colonies on SW-2 medium plates containing 10% sucrose. One of these colonies was purified for further analysis and was designated CHR100. Insertion of the omega cassette into CHR100 was confirmed by hybridization with an omega cassette probe (not shown).

Construction of a *cfuAp*::*gfp* transcriptional fusion and assay for green fluorescent protein (GFP) activity. To check whether there was a promoter upstream of the *cfuABC-fur-hisI-orf6* operon, a transcriptional fusion of the region upstream of *cfuA* with the *gfp* reporter gene was constructed in low-copy-number plasmid pMP92 (67) as follows. A 574-bp region upstream of *cfuA* was PCR amplified as a BglII/ClaI fragment from *C. salexigens* genomic DNA by using primers *cfuAp-fw* (5′-ATGTACTTGCTCGATCAGCACGACGA-3′) and *cfuAp-rv* (5′-GAGGAGGAACGAACGAGTAGGCATA-3′). The 574-bp promoter region was subsequently subcloned in the pHS332 vector (pMP92::*gfp* [M. Argandoña and C. Vargas, unpublished data]) digested with BglII/ClaI to obtain plasmid pHS378 (see Fig. S1 in the supplemental material). Plasmids pHS378 and pHS332 (negative control) were transferred to wild-type *C. salexigens* by conjugation.

To determine GFP activity, *C. salexigens* wild-type cells carrying plasmids pHS378 (*cfuAp*::*gfp*) and pHS332 (promoterless negative control) were grown overnight in SW-2 complex medium with Rf and Tc and diluted 1:100 in 25 ml of M63 minimal medium containing 0.75 M NaCl supplemented with Tc. As the cultures reached late exponential phase, two 1-ml aliquots of each culture were harvested by centrifugation, washed with buffer (10 mM Tris-HCl, 600 mM NaCl; pH 8.0), and resuspended in 1 ml of the same buffer. Fluorescence was measured immediately using a Perkin-Elmer LS-5 fluorimeter that was set up to excite the cells at 428 nm and detect emission at 511 nm. The fluorescence values for *C. salexigens*(pHS332) were subtracted from those determined for cells carrying the *cfuAp*::*gfp* fusion. The protein content was determined as described above. Promoter activity was expressed in units of fluorescence per mg of protein.

RNA extraction, RT-PCR, and real-time PCR. *C. salexigens* cultures were grown in M63 medium at 37°C (with 0.75 M or 2.5 M NaCl) until late exponential phase, and cells were harvested by centrifugation. Total RNA was extracted with an RNeasy minikit (Qiagen) used according to the manufacturer's instructions. After isolation, the integrity of the RNA samples was assessed by agarose gel electrophoresis. Chromosomal DNA was removed by DNase digestion (Promega) and subsequent enzyme inactivation by incubation with 2.5 μM diethyl pyrocarbonate (DEPC)-treated EDTA for 10 min at 65°C. The absence of DNA contamination was checked by performing PCR using 16S rRNA primers *16S-RT-fw* (5′-GGCCGCAAGGTTAAAACTCAAATG-3′) and *16S-RT-rv* (5′-GA AGGCACCCCGGAATCTCT-3′). The RNA concentration was determined spectrophotometrically at 260 nm, and RNA was stored at −80°C until it was used. cDNA was synthesized by using a Transcriptor first-strand cDNA synthesis kit (Roche) according to the manufacturer's recommendations. Two micrograms of total RNA was denatured at 65°C for 10 min, and then random hexamers (60 μM), protector RNase inhibitor (20 U), reverse transcriptase (RT) (10 U), and reaction buffer were added to a 20-μl (final volume) mixture. The reaction mixture was incubated at 25°C for 10 min and then at 50°C for 60 min. Finally, the mixture was incubated for 5 min at 85°C to inactivate the reverse transcriptase. The cDNA synthesized was kept at −20°C until it was used.

To demonstrate that the *cfuABC-fur-hisI-orf6* gene cluster is an operon, intergenic regions were amplified by using 2 μl of *C. salexigens* cDNA synthesized from RNA isolated from cells grown in M63 medium with 2.5 M NaCl at 37°C as the template. The cycling conditions for the PCR were as follows: 5 min at 95°C, followed by 30 cycles of 35 s at 95°C, 35 s at 60°C, and 35 s to 2 min (depending on the amplicon size) at 72°C, followed by 10 min of extension at 72°C. The following primer pairs were used: *cfuA-RT-fw* (5′-GAACTGCTGCG TGTGCAA-3′) and *cfuB-RT-rv* (5′-GATAGATAACTGCGGATGACCAG-3′) for the *cfuA-cfuB* intergenic region, *cfuB-RT-fw* (5′-GCTCAATTTCTCGATTC ACAGCC-3′) and *cfuC-RT-rv* (5′-CTTCTCGATCAGGTCCAGACG-3′) for the *cfuB-cfuC* intergenic region, *cfuC-RT-fw* (5′-CAGCTGCTACGAGATGAC GG-3′) and *fur-RT-rv* (5′-GAAAGCCATGCGCGTGTTCG-3′) for the *cfuC-fur* intergenic region, *fur-RT-fw* (5′-GTGCTGGAAATGATCGCCAC-3′) and *hisI-RT-rv* (5′-TATGACACTGATCGACGCCG-3′) for the *fur-hisI* intergenic region, and *hisI-RT-fw* (5′-TCTGATGATGGCGTGGATGAAC-3′) and *orf6-RT-rv* (5′-GTCTTTCGCGGTGTCTCGTG-3′) for the *hisI-orf6* intergenic region. As a control for individual expression of each gene, intragenic regions were amplified by using the primers described above and other primers as follows: *cfuA-RT-fw* and *cfuA-RT-rv* (5′-ATTCGTTGCGACCCAGCATTT-3′) for *cfuA*, *cfuB-RT-fw* and *cfuB-RT-rv* for *cfuB*, *cfuC-RT-fw* and *cfuC-RT-rv* for *cfuC*, *fur-RT-fw* and *fur-RT-rv* for *fur*, *hisI-RT-fw* and *hisI-RT-rv* for *hisI*, and *orf6-RT-fw* (5′-ATCGGGTTATTGCGTGTCTACC-3′) and *orf6-RT-rv* for *orf6*.

Two sets of specific primers that amplified internal regions of *cfuA* and *ectA* were used for real-time PCR. These primers were *ectA-qRT-fw* (5′-ACGAACT CGTCAAGGCATGC-3′) and *ectA-qRT-rv* (5′-TGCCATAGAAAGTAGGTG

TCCG-3′) for *ectA* and *cfuA-RT-fw* and *cfuA-RT-rv* for *cfuA* (see above). Primers were designed with the Primer3 software (63) so that they were about 20 to 25 bases long, had G+C contents of >50%, and had melting temperatures of about 60°C. The lengths of the PCR products ranged from 134 to 210 bp. Secondary structures and dimer formation were controlled using Sigma-Aldrich web analyzer. Real-time PCR was performed in 96-well plates using an ABI Prism 7000 sequence detector (Applied Biosystems) and a FastStart Master (Rox) (Roche). Each reaction mixture contained 5 μl of diluted cDNA, 20 μl of PCR mixture, 10 μl of FastStart SYBR green master mixture, 6 pmol of each primer, and 3.8 μl of RNase-free water. A melting curve was generated at the end of every run to ensure product uniformity (62). For each assay, the amplification efficiency was determined by constructing a standard curve with different amounts of cDNA. In all cases, the slope of the curve indicated that the PCR conditions were adequate (slopes, 3.2 to 3.4). Amplification data were analyzed with the ABI Prism 7000 software (Applied Biosystems). Gene expression was expressed in relative units and was calculated by the $2^{-\Delta CT}$ method using the 16S rRNA gene as an endogenous control to normalize expression in each sample.

Extraction and determination of intracellular solutes by ^{13}C-NMR spectroscopy. *C. salexigens* wild-type and *fur* strains were grown in 200 ml of M63 medium with 2.5 M NaCl at 37°C in the absence or presence of 50 μM $FeCl_3$ until late exponential phase. Cell pellets were collected by centrifugation and washed with the same medium without any carbon source. Each cell pellet was resuspended in 10 ml of an extraction mixture (methanol-chloroform-water, 10:5:4) and extracted with gentle shaking for 30 min at 37°C. The cell debris was removed by centrifugation, and supernatants were extracted once with chloroform-water (1:1) and freeze-dried. The solids were dissolved in D_2O (0.6 ml). ^{13}C nuclear magnetic resonance (^{13}C-NMR) spectra were recorded at 25°C with a Brucker AV500 spectrometer at 125 MHz. The chemical shifts are reported below in ppm on a δ scale relative to trimethylsilane. Signals were assigned by comparison with previously described chemical shift values (16, 30) and were confirmed by comparison with ^{13}C-NMR spectra of pure compounds.

LC-MS quantification of ectoine and hydroxyectoine. Cells used for liquid chromatography-mass spectrometry (LC-MS) analysis of ectoine and hydroxyectoine were extracted by using a modified Bligh-Dyer technique described by Kraegeloh and Kunte (43). Chromatographic separation was performed using a series 200 high-performance liquid chromatography (HPLC) system (Perkin-Elmer, Wellesley, MA) coupled to a QTRAP LC-tandem mass spectrometry (MS/MS) system (Applied Biosystems Foster City, CA) consisting of a hybrid triple-quadrupole linear ion trap (QqQ_{lit}) mass spectrometer equipped with an electrospray ion source. Chromatographic separation was achieved using a binary gradient consisting of water and methanol with 0.1% formic acid (vol/vol). Samples (20 μl) of the water-soluble fraction containing the compatible solutes were separated isocratically with 80% methanol on a Spherisorb S3 NH2 column (150 by 4.6 mm; Waters). The flow rate was 0.4 ml min^{-1}. A multiple reaction monitoring (MRM) experiment was performed, in which the parent ions and fragment ions were monitored at Q1 and Q3, respectively. For HPLC-electrospray ionization (ESI)-MS/MS analyses, the mass spectrometer was set to the following optimized tune parameters: curtain gas pressure, 35 lb/in^2; ion spray voltage, 5,500 V; source temperature, 350°C; and source gas pressure, 60 lb/in^2. MRM transitions were performed with the following parameters: $[M+H]^+$, 143.2 (ectoine) and 159.1 (hydroxyectoine); Q1→Q3/CE (V), 143.2→97.0/20 and 143.2→68.0/35 (ectoine) and 159.1→113.0/18 and 159.1→83.0/35 (hydroxyectoine); and DP(V), 85 (ectoine) and 90 (hydroxyectoine). The dwell time and collision cell exit potential (CXP) were set at 300 ms and 5 V, respectively, for each transition. The time retention (TR) was 4.30 min for ectoine and 4.70 min for hydroxyectoine. Transitions 143.2/68.0 (ectoine) and 159.1/83.0 (hydroxyectoine) were used to confirm identifications. MRM transitions 143.2/97.0 and 159.1/113.0 were used for quantification of ectoine and hydroxyectoine, respectively. The solute concentration was expressed in μmol/mg protein.

DNA and protein sequence analysis. The sequence of the *C. salexigens* genome is available in the NCBI microbial genome database (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) under accession number NC_007963. Sequence data were analyzed using BLAST (NCBI; http://www.ncbi.nlm.nih.gov/BLAST). Promoter sequences were predicted using BGDP Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). The signal peptides and topology of protein sequences were predicted using the LipoP 1.0 and Signal 3.0 (http://www.cbs.dtu.dk/services/) (8, 39), TMpred (http://www.cbs.dtu.dk/services) (38), TMHMM (http://www.cbs.dtu.dk/services;), and SOSUI (http://sosui.proteome.bio.tuat.ac.jp) (35) servers.

Nucleotide sequence accession number. The nucleotide sequence data for the *cfuABC-fur-hisI* region have been deposited in the DDBJ/EMBL/GenBank database under accession number FM998812.

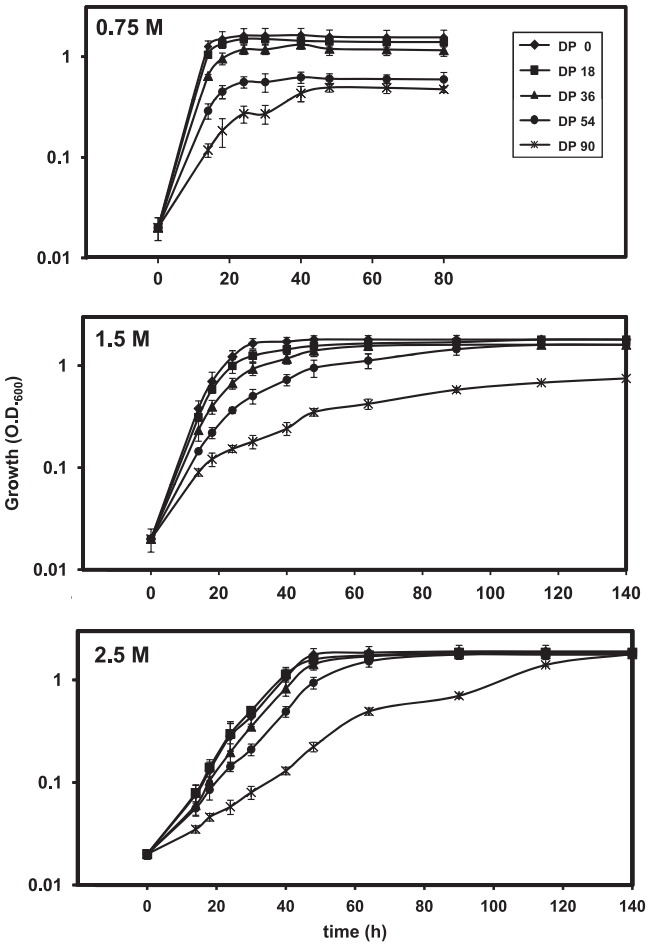

FIG. 1. Titration of salt-dependent inhibition of *C. salexigens* growth by 2,2′-dipyridyl (DP). Cells were grown at 37°C in M63 minimal medium with 0.75, 1.5, or 2.5 M NaCl and different concentrations 2,2′-dipyridyl (μM), as indicated in the top panel. The values are the means ± standard deviations of two replicates for each condition in three independent experiments.

RESULTS

Iron requirement, siderophore production, and protein content of *C. salexigens* are lower at high salinity. In previous work, we found that in *C. salexigens* cells grown with 0.75 M NaCl in the presence of excess iron (50 μM $FeCl_3$), expression of the *ectABC* genes was reduced 1.8-fold, suggesting that there is a connection between the osmostress response and iron homeostasis (11). To test if the iron requirement of *C. salexigens* is influenced by salinity, the wild-type strain was grown in M63 minimal medium (a low-iron medium) with 0.75, 1.5, and 2.5 M NaCl and different concentrations (18 to 90 μM) of the nonmetabolizable high-affinity iron-chelating agent 2,2′-dipyridyl, which reduces the availability of iron in a culture medium. Figure 1 shows that in the absence of the chelator the wild-type strain grew slightly better with 1.5 M NaCl (change in the optical density of the wild-type culture per hour [$\Delta OD/h_{wt}$], 0.079) than with 0.75 M ($\Delta OD/h_{wt}$, 0.065); addition of 2.5 M NaCl resulted in delayed growth, but eventually the optical density was the same as that obtained with 1.5 M NaCl ($\Delta OD/h_{wt}$, 0.07). Remarkably, the capacity of 2,2′-dipyridyl to inhibit

growth was progressively attenuated as the NaCl concentration was increased from 0.75 M (change in the optical density of the wild-type culture per hour with 18 μM 2,2′-dipyridyl [$\Delta OD/h_{wt\_18DP}$], 0.06; $\Delta OD/h_{wt\_36DP}$, 0.052; $\Delta OD/h_{wt\_54DP}$, 0.018; $\Delta OD/h_{wt\_90DP}$, 0.010) to 2.5 M ($\Delta OD/h_{wt\_18DP}$, 0.062; $\Delta OD/h_{wt\_36DP}$, 0.06; $\Delta OD/h_{wt\_54DP}$, 0.056; $\Delta OD/h_{wt\_90DP}$, 0.040), suggesting that the requirement for iron for growth of *C. salexigens* is lower at high salinity. In fact, with 2.5 M NaCl only the highest chelator concentration tested resulted in impairment of growth. We also tested a second standard iron chelator, deferoxamine, at concentrations ranging from 100 to 900 μM to determine its ability to inhibit growth at different salinities. However, deferoxamine enhanced the growth of the wild-type strain, especially at an NaCl concentration of 0.75 M (data not shown). This finding, which suggested that *C. salexigens* can use deferoxamine as a siderophore, was supported by subsequent findings (see below).

If *C. salexigens* requires less iron at high salt concentrations, we predicted that siderophore production by this microorganism should exhibit the same pattern. To test this hypothesis, the *C. salexigens* wild-type strain was grown at 37°C in M63 minimal medium with 0.75, 1.5, or 2.5 M NaCl, and siderophore production was investigated on modified CAS agar plates with the salinities indicated above, as described in Materials and Methods. On these plates, secreted siderophores formed orange haloes surrounding the colonies, and the diameters of the haloes were a measure of the quantity secreted. In agreement with our prediction, the largest siderophore halo was produced with 0.75 M NaCl, whereas with 1.5 M NaCl the diameter of the halo was smaller and with 2.5 M NaCl the level of siderophore production was even lower (Fig. 2A).

To rule out the possibility that high salt concentrations could interfere with the chelating capacity of chemical chelators or a siderophore(s), we performed control experiments with the iron chelators 2,2′-dipyridyl and deferoxamine and the commercial siderophore ferrichrome. As shown in Fig. 2A, the haloes resulting from iron retrieval by the same amount of the control iron chelators and the siderophore were similar at each salinity tested. These findings indicate that (i) the chelator 2,2′-dipyridyl (as well as deferoxamine or ferrichrome) does not lose activity at high salinity and (ii) at high salinity the modified CAS assay is quantitative and reliable.

From the findings described above, we concluded that the iron requirement of *C. salexigens* decreases as the salinity increases. As many proteins contain iron at their active site, one possible reason for this is that the protein content is lower under hypersaline conditions. To test this hypothesis, the total protein content was determined for the *C. salexigens* wild-type strain grown in M63 minimal medium with different salinities. When the cell cultures reached late exponential phase, serial dilutions were prepared, and cells were plated on SW-10 medium to confirm that the cultures contained the same number of viable cells/ml regardless of the NaCl concentration (1.15×10^5 CFU/ml with 0.75 M NaCl, 1.4×10^5 CFU/ml with 1.5 M NaCl, and 1.33×10^5 CFU/ml with 2.5 M NaCl). As shown in Fig. 2B, the cell protein content gradually decreased as the NaCl concentration increased from 0.75 M to 2.5 M NaCl, confirming that *C. salexigens* cells synthesize smaller amounts of proteins under saline stress conditions.

DNA region downstream of the *ectABC* genes encodes functions related to iron metabolism. The data presented above suggested that the iron requirement and siderophore production of *C. salexigens* are inversely related to the salinity during growth. We have previously described isolation of a cosmid clone from a *C. salexigens* gene bank, pDE9 carrying a ca. 35-kb region containing the ectoine synthesis genes *ectABC* (12) (see Fig. S1 in the supplemental material). Sequence analysis of the DNA region downstream of *ectABC* (cloned in pMH2 [see Fig. S1 in the supplemental material]) revealed the presence of five complete open reading frames which were oriented in the same direction. *orf1*, *orf2*, *orf3*, *orf4*, and *orf 5* (designated *cfuABC*, *fur*, and *hisI*, respectively) were 834, 1,509, 918, 450, and 441 nucleotides long, respectively, and were preceded by putative ribosome binding sites. Computer searches revealed a moderate level of similarity (ca. 35% amino acid sequence identity) between Orf1 (CfuA) and a family of unknown periplasmic lipoproteins. Orf2 (CfuB) and Orf3 (CfuC) showed high levels of similarity (ca. 60% amino acid sequence identity) to hypothetical proteins with a predicted permease function and proteins included in the superfamily containing the ATP-grasp enzymes, respectively. All these findings suggested that the proteins encoded by *orf1*, *orf2*, and *orf3* (*cfuABC* [*Chromohalobacter* ferric uptake] [see below]) may form part of a membrane transporter. Orf4 (Fur) showed a high level of identity to iron uptake regulator proteins belonging to the Fur superfamily, such as the Fe^{2+} uptake regulators of *Pseudomonas putida* KT2440 (accession no. NP_742289; 48% amino acid sequence identity) and *Rhodopseudomonas palustris* CGA009 (accession no. NP_945870; 42% amino acid sequence identity). Orf5 (HisI) exhibited significant similarity to phosphoribosyl AMP-cyclohydrolase enzymes and had a phosphoribosyl-AMP-cyclohydrolase (PRA-CH) domain, which is typical of these proteins. The highest levels of similarity were the levels of similarity with the HisI proteins of *P. putida* (67% amino acid sequence identity) and *Pseudomonas syringae* (55% amino acid sequence identity). During this work, the *C. salexigens* genome sequence became available at NCBI, and we observed that there was a sixth open reading frame (*orf6*) downstream of *orf5* that was the last gene of an *orf1-orf2-orf3-orf4-orf5-orf6* gene cluster. Orf6 had a DUF37 domain with an unknown function that is found in short hypothetical proteins annotated as alpha-hemolysin-like proteins in *Aeromonas hydrophila* (63% amino acid sequence identity) and *Idiomarina loihiensis* (67% amino acid sequence identity). The short distances between the six open reading frames and the presence of a promoter region upstream *cfuA* (see below) suggested that *cfuABC*, *fur*, *hisI*, and *orf6* may be organized in one operon (see Fig. S1 in the supplemental material). This was confirmed by amplification of the *cfuA-cfuB*, *cfuB-cfuC*, *cfuC-fur*, *fur-hisI*, and *hisI-orf6* intergenic regions by RT-PCR, as described in Materials and Methods (see Fig. S2 in the supplemental material).

***fur* is involved in the regulation of iron metabolism.** To confirm that *orf4* encodes an iron uptake regulator, we designed an experiment to test if an *orf4* mutant was able to grow in the presence of manganese. *fur* mutants of other gammaproteobacteria, such as *E. coli*, *Vibrio parahaemolyticus*, or *V. cholerae*, have been isolated by using the same manganese selection assay, which is based on the fact that manganese

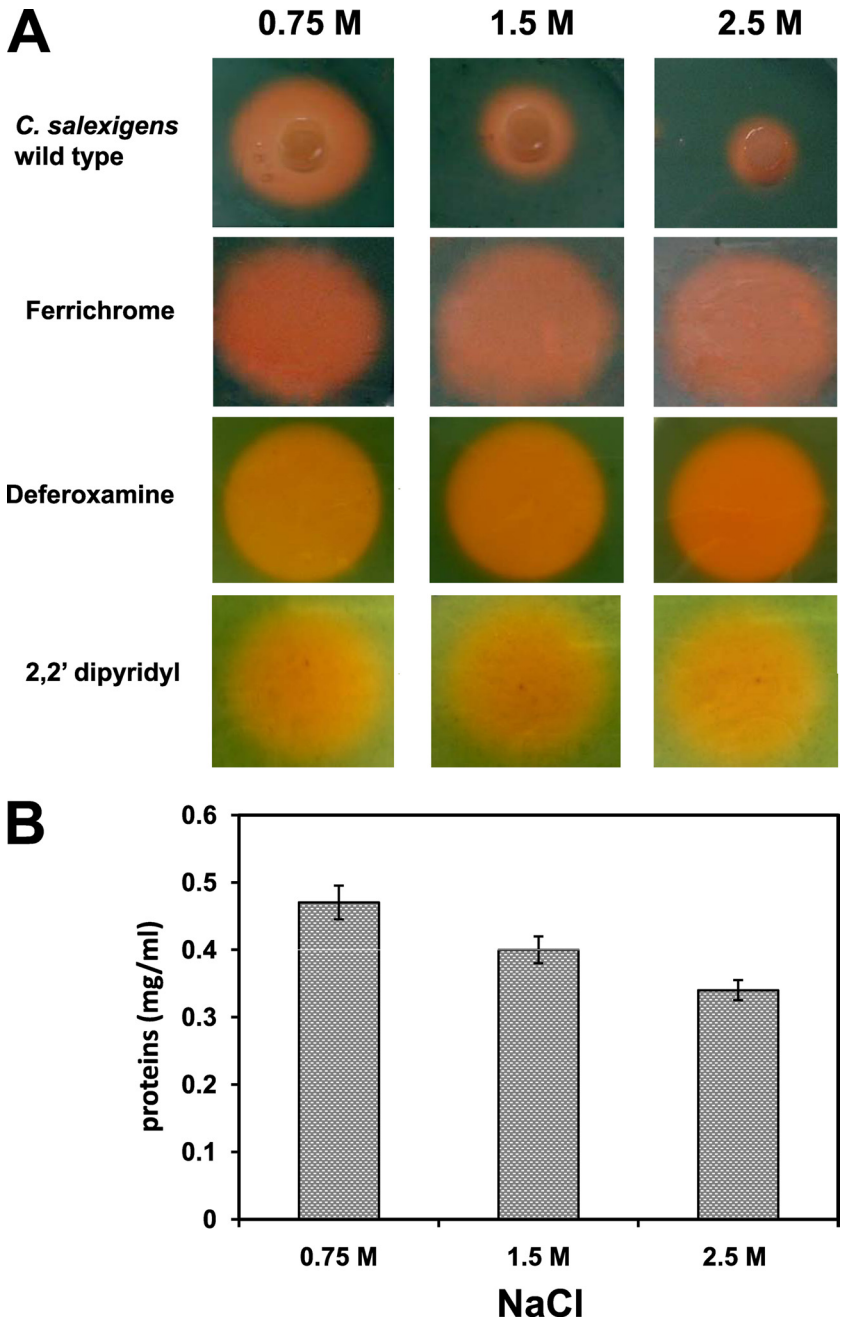

FIG. 2. Siderophore production and protein production by *C. salexigens* are lower at high salinity. (A) Siderophore production assay. Ten-microliter bacterial suspensions were placed on modified CAS agar plates with 0.75, 1.5, or 2.5 M NaCl, and siderophore production by the *C. salexigens* wild-type strain was observed after 72 h of incubation at 37°C. One microliter of the commercial siderophore ferrichrome (1 mg/ml) and 2 μl of the iron chelators deferoxamine and 2,2′-dipyridyl were used as controls. (B) Protein content of *C. salexigens* wild-type cells grown at 37°C in M63 minimal medium with different NaCl concentrations. The values are the means ± standard deviations of two replicates for each condition in three independent experiments.

mimics iron by binding to the Fur protein, which leads to binding of the Mn-Fur complex to the promoter of iron uptake genes (33, 66). As a result, bacteria with a wild-type copy of the *fur* gene repress iron uptake systems and are starved for iron in the presence of manganese, whereas *fur* mutants do not repress the iron uptake system and survive (9). First, a 3.0-kb PstI-HindIII fragment from plasmid pMH2 containing the complete *fur* and *hisI* genes (see Fig. S1 in the supplemental material), carried in plasmid pMM1 (see Fig. S1 in the supplemental material), was subjected to site-directed mutagenesis to create an in-frame deletion of *orf4* (in plasmid pMM2 [see Fig. S1 in the supplemental material]) as described in Materials and Methods. Second, this in-frame deletion was transferred into the suicide plasmid pJQSK200 (in clone

pHS333 [see Fig. S1 in the supplemental material]) and then transferred into the *C. salexigens* wild-type strain by conjugation. A *fur* mutant was isolated from one Gm^r colony (resulting from a single homologous recombination event) by Mn^r selection on low-Mg SW-2 medium plates with 10% sucrose (to select double recombinants) and 2.5 mM $MnCl_2$ (to select *fur* mutants). Whereas the wild-type strain, which was used as a negative control, could not grow on low-Mg SW-2 medium plates with manganese, some Mn^r colonies were isolated on plates containing Mn and sucrose, and they were tested using PCR and sequencing to confirm that they carried the in-frame deletion in *orf4*. One of these colonies, strain CHR134 (*fur*), was selected for further experiments. Figure 3A shows the differences in growth between the wild type and the *fur* strain on low-Mg SW-2 medium plates with manganese. As expected, the mutant was not auxotrophic for histidine, confirming that the in-frame deletion carried by CHR134 did not affect the downstream gene *hisI* (data not shown). When the wild-type and *fur* strains were tested for siderophore production on modified CAS agar plates with 0.75 M NaCl, the halo surrounding strain CHR134 was much larger than the halo around the wild-type strain, indicating that siderophore production was deregulated in the *fur* mutant (Fig. 3B).

Kiphati and coworkers (42) found that a *fur* mutant of *Agrobacterium tumefaciens* is sensitive to the iron chelator 2,2′-dipyridyl, suggesting that Fur is essential for survival under iron-limiting conditions. To test this hypothesis with *C. salexigens*, we assessed the growth of the wild type and strain CHR134 (*fur*) in M63 medium with 36 μM 2,2′-dipyridyl as an iron-chelating agent. This 2,2′-dipyridyl concentration was selected to keep the iron concentration very low but not completely inhibit wild-type growth (Fig. 1). The results of these experiments are shown in Fig. 3C. As previously observed, the growth of the wild-type strain in the presence of the chelator was impaired in the presence of 0.75 M NaCl ($\Delta OD/h_{wt\_36DP}$, 0.053) compared to the growth in the presence of 2.5 M NaCl ($\Delta OD/h_{wt\_36DP}$, 0.06). The growth of strain CHR134 (*fur*) was delayed at both NaCl concentrations compared to the growth of the wild-type strain ($\Delta OD/h_{CHR134\_36DP}$ with 0.75 and 2.5 M NaCl, 0.034 and 0.045, respectively). However, when we tested the growth of the *fur* mutant (CHR134) in the low-iron M63 medium with the salinities described above in the absence of the chelator, no differences were observed compared to the growth of the *C. salexigens* wild-type strain (not shown). These findings suggest that (i) *fur* also has a role in regulating the cellular demand for iron in *C. salexigens* and (ii) a *fur* mutant is not salt sensitive. From all these results, we concluded that *orf4* (*fur*) indeed encodes a functional central iron regulator.

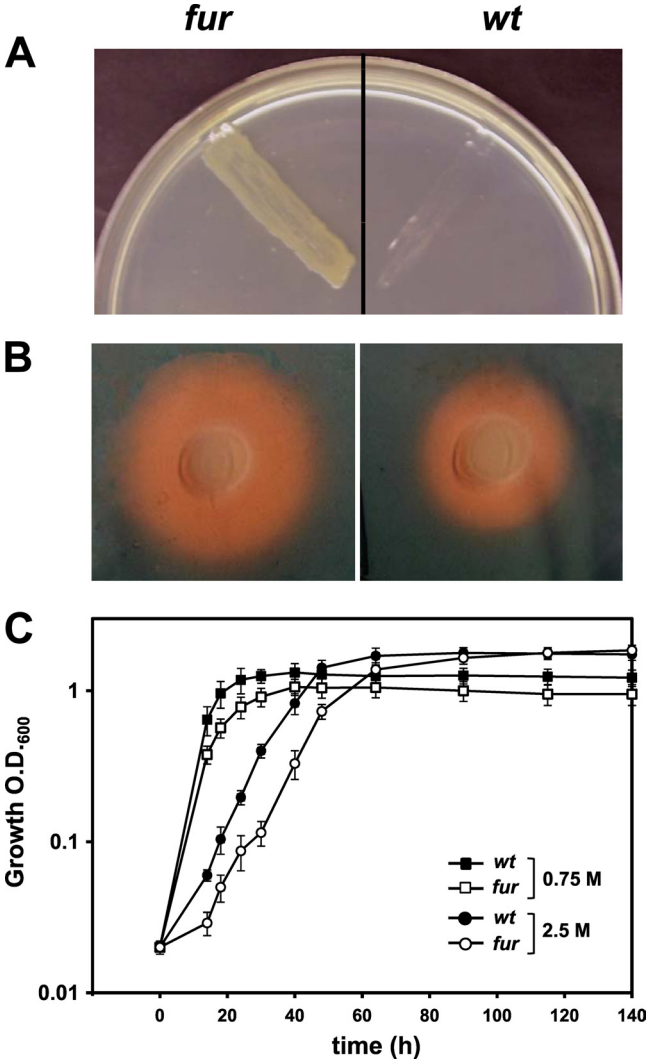

FIG. 3. Phenotype of *C. salexigens fur* mutant CHR134. (A) The *fur* strain grew on SW-2 medium plates with 2.5 mM $MnCl_2$, whereas the wild-type strain (*wt*) did not. (B) The *fur* strain exhibited deregulation of siderophore production. Strains were incubated in M63 minimal medium with 0.75 M NaCl, and after removal of the supernatant from 500 μl of each sample, cells were resuspended in 30 μl of M63 medium. Five microliters of each cell suspension was deposited on a modified CAS agar plate with 0.75 M NaCl and incubated at 37°C for 72 h. (C) Fur is important for *C. salexigens* growth under iron-limiting conditions. The *C. salexigens* wild-type strain and *fur* mutant CHR134 were grown at 37°C in M63 minimal medium with 0.75 and 2.5 M NaCl in the presence of 36 μM 2,2′-dipyridyl, an iron chelator. The values are the means ± standard deviations of two replicates for each condition in three independent experiments.

Histidine is essential for siderophore production by *C. salexigens*. HisI is a cytoplasmic enzyme that is involved in the third step of the histidine synthetic pathway. The amino acid histidine is present in the structure of some hydroxamate-type siderophores (e.g., in some mycobacteria and fungi) (7, 24) or is used as a siderophore precursor (e.g., in *Vibrio anguillarum* or *Acinetobacter baumannii*) (49, 70). The *C. salexigens* genome has only one *hisI* homolog. The fact that *hisI*, which was predicted to be involved in histidine biosynthesis, was clustered with the gene encoding the iron regulator Fur (see Fig. S1 in the supplemental material), suggests that histidine might be involved in the synthesis of a *C. salexigens* siderophore. To test this hypothesis, we generated a mutant (strain CHR100 [Δ*cfuABC fur*::Ω]) in which a fragment containing *cfuABC* and the 5′ end of *fur* was replaced by the omega interposon (see Fig. S1 in the supplemental material). We anticipated that insertion of the omega cassette into strain CHR100 would affect histidine biosynthesis due to a polar effect on the *hisI* gene. Strain CHR100 was tested to determine its siderophore production and histidine auxotrophy.

To examine if *hisI* was involved in siderophore synthesis, siderophore production by strain CHR100 was tested by using

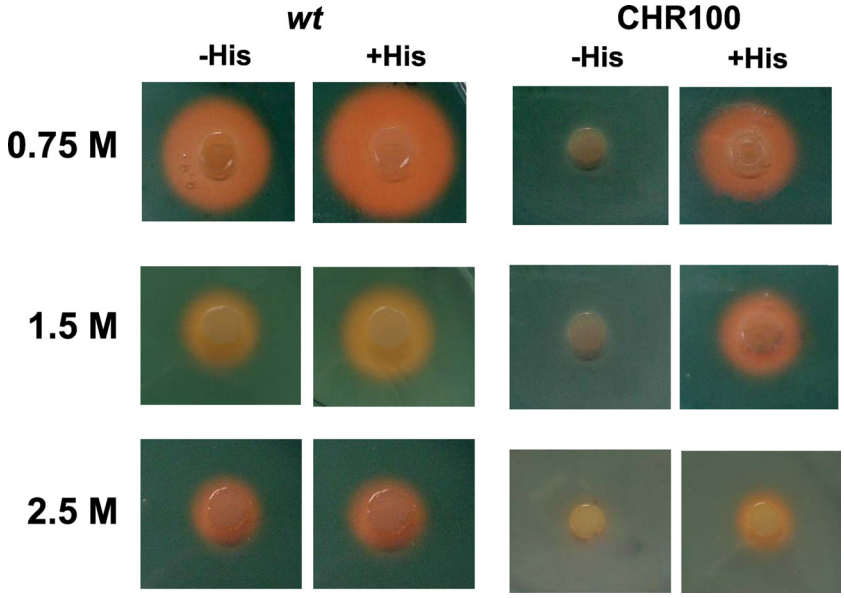

FIG. 4. Histidine is essential for siderophore production by *C. salexigens*. Siderophore production by the *C. salexigens* wild-type strain and *cfuABC-fur* mutant CHR100 at different salinities was determined. Strains were incubated in M63 minimal medium with 0.75, 1.5, or 2.5 M NaCl until exponential phase. Aliquots (500 μl) were removed and centrifuged. Each cell pellet was washed with M63 medium and resuspended in 30 μl of the same medium. After removal of the supernatant, 10 μl of each cell suspension was deposited on a modified CAS agar plate with 0.75, 1.5, or 2.5 M NaCl in the absence or presence of 1 mM histidine and incubated at 37°C for 72 h. *wt*, wild type.

the CAS agar plate assay in the presence or absence of 1 mM histidine at different salinities. As shown in Fig. 4, in the absence of histidine a siderophore production halo was not observed around CHR100 at any salinity tested, whereas addition of histidine to the growth medium not only reversed the phenotype of strain CHR100 but also strongly enhanced siderophore production by the wild type at any salinity tested, especially 0.75 M NaCl.

To investigate if the *cfuABC-fur* interruption leads to a *hisI* polar mutation and confers histidine auxotrophy, we grew the wild type and mutant CHR100 in minimal medium with NaCl concentrations ranging from 0.75 to 2.5 M in the presence or absence of histidine. In addition, we investigated if deferoxamine could totally or partially restore the growth deficiency of CHR100 in the absence of histidine, as a test of the ability of *C. salexigens* to use deferoxamine as a siderophore. Figure 5 shows that without histidine CHR100 exhibited only residual growth in the presence of 0.75 M NaCl ($\Delta OD/h_{CHR100}$, 0.006), whereas the growth was very delayed in the presence of 1.5 M NaCl ($\Delta OD/h_{CHR100}$, 0.030) and 2.5 M NaCl ($\Delta OD/h_{CHR100}$, 0.021) compared to the growth of the wild type ($\Delta OD/h_{wt}$ with 0.75 M NaCl, 0.061; $\Delta OD/h_{wt}$ with 1.5 M NaCl, 0.079; $\Delta OD/h_{wt}$ with 2.5 M NaCl, 0.064). In contrast, when histidine was added, the level of growth of CHR100 was the same as the level of growth of the wild type at every salinity tested ($\Delta OD/h_{CHR100}$ with 0.75 M NaCl and histidine, 0.06; $\Delta OD/h_{CHR100}$ with 1.5 M NaCl and histidine, 0.08; $\Delta OD/h_{CHR100}$ with 2.5 M NaCl and histidine, 0.065). When 150 μM deferoxamine was added to growing cultures, it changed the growth-impaired phenotype of CHR100, especially with 0.75 M NaCl ($\Delta OD/h_{CHR100}$ with 0.75 M NaCl and deferoxamine, 0.029; $\Delta OD/h_{CHR100}$ with 1.5 M NaCl and deferoxamine, 0.036; $\Delta OD/h_{CHR100}$ with 2.5 M NaCl and deferoxamine, 0.020) (that is, when cell iron's demand was higher) (Fig. 5). Higher concentrations of deferoxamine (up to 600 μM) also had a growth-enhancing effect on CHR100 in the presence of 0.75 M NaCl, but the effect was not as significant at higher salinities (data not shown).

Taken together, these findings strongly suggested that (i) *hisI* is involved in histidine biosynthesis, (ii) histidine is either a component or a precursor of the siderophore(s) produced by *C. salexigens*, and (iii) *C. salexigens* can use deferoxamine as a siderophore, especially when there is high iron demand. On the other hand, the fact that histidine auxotrophy was observed only with 0.75 M NaCl suggests that the requirement of *C. salexigens* for this amino acid is higher at low salinity, as is the cell's demand for iron.

Fur controls osmoregulated repression of the *cfuABC-fur-hisI-orf6* operon by iron. In most bacteria, iron uptake systems and siderophore biosynthesis genes are controlled by Fur (34). Therefore, we investigated if the *cfuABC-fur-hisI-orf6* operon was regulated by Fur. Given that there is a putative Rho-independent transcriptional terminator not far downstream of *ectC* (12) (see Fig. S1 in the supplemental material) and that the intergenic region between *ectC* and *cfuA* is 412 bp long, we predicted that the *cfuABC-fur-hisI-orf6* operon was expressed by its own promoter region. To verify this hypothesis, we constructed a *cfuAp*::*gfp* transcriptional fusion in the *incP* low-copy-number vector pMP92 (plasmid pHS378) (see Fig. S1 in the supplemental material) and transferred it to the *C. salexigens* wild-type strain by conjugation. As a negative control, we transferred the promoterless vector pHS332 (pMP92::*gfp* [M. Argandoña and C. Vargas, unpublished data]). Once the slight background activity of the negative control was subtracted, the promoter activity of the *cfuAp*::*gfp* fusion, measured using GFP activity in cells grown in M63 medium with 0.75 M NaCl, was

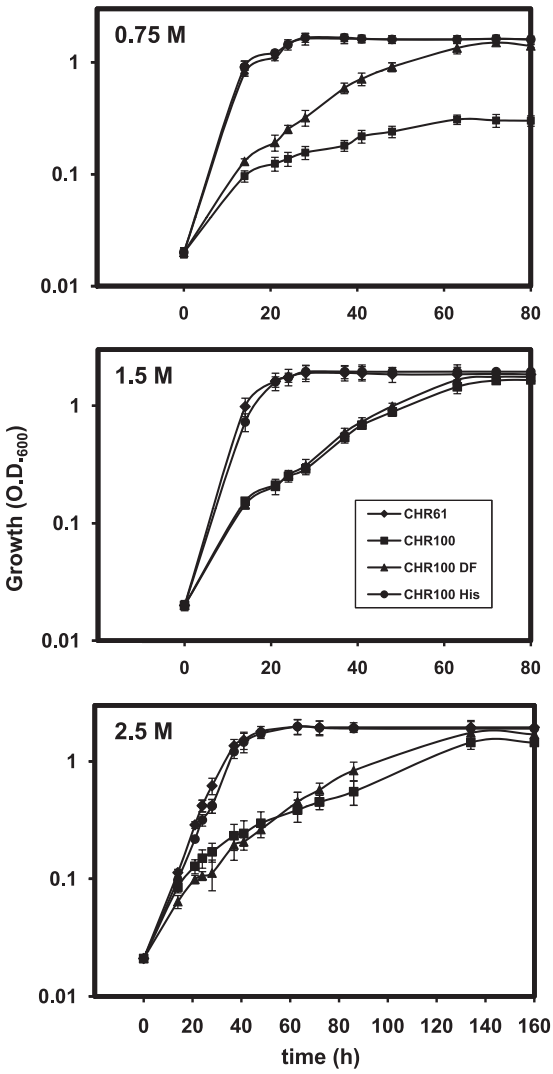

FIG. 5. Histidine requirement of the *C. salexigens* wild-type strain and *cfuABC-fur* mutant CHR100 at different salinities. Strains were grown at 37°C in M63 minimal medium with 0.75, 1.5, or 2.5 M NaCl in the absence (both strains) or in the presence (only CHR100) of 1 mM histidine or 150 μM deferoxamine (DF). The values are the means ± standard deviations of two replicates for each condition in three independent experiments.

about 75 fluorescence units/mg of protein, indicating that there is a promoter region upstream of *cfuA*. An *in silico* analysis of this region showed that there were four putative σ^{70}-dependent promoters and one putative Fur box (21 nucleotides) overlapping the −10 and −35 sequences of the two promoters farthest from the *cfuA* start codon. In the putative Fur box, we found elements corresponding to two different interpretations of the Fur box consensus sequences, as described by Baichoo and Helmann (7) (an inverted repeat heptamer separated by two nucleotides that included the ATAAT motif) and Escolar et al. (26) (several repetitions in different orientations of the GATAAT motif) (Fig. 6A). These findings suggested that the *cfuABC*-encoded putative transport system and the cotranscribed *fur*, *hisI*, and *orf6* genes were regulated by Fur and iron. To test this hypothesis, we used real-time PCR to determine how *cfuA* expression in wild-type and *fur* mutant (CHR134) backgrounds was affected by excess iron. Given that iron demand by *C. salexigens* decreases at high salinity, we also tested the influence of saline stress on *cfuABC-fur-hisI-orf6* operon expression. Figure 6B shows that in the wild type grown in low-iron M63medium, *cfuA* expression was not affected by salinity. However, under high-iron conditions, *cfuA* expression was reduced 1.2- and 6.3-fold in cells grown with 0.75 M and 2.5 M NaCl, respectively, compared to the expression in cells grown in M63 medium. In the *fur* mutant, *cfuA* expression was deregulated regardless of the presence of iron or the salinity of the medium. These findings indicate that Fur is the transcriptional regulator mediating osmoregulated repression by iron of the *cfuABC*-encoded putative transport system and the siderophore synthesis gene *hisI.* They also suggest that Fur autoregulates its own expression in the same way.

Fur functions as a positive regulator of ectoine synthesis. Our previous finding that excess iron in the growth medium reduced the basal expression of the ectoine synthesis genes, *ectABC* (11), led us to investigate if the inhibition was mediated by Fur. An exhaustive analysis of the *ectAp*(*1-4*) promoter region showed the presence of three possible Fur boxes, two of which overlapped, which was interpreted as several repetitions in different orientations of the GATAAT motif (26). These Fur boxes overlapped the P1 and P2 promoters of the *ectABC* genes, which were experimentally mapped and proposed to be σ^{70}-dependent promoters (11) (Fig. 7A). This suggested that Fur has a role in *ectABC* expression. To test this hypothesis, we used real-time PCR to determine how *ectA* expression in the wild-type and *fur* backgrounds was affected by salinity and by excess iron. As expected, in the wild-type strain grown under low-iron conditions, *ectA* expression was clearly upregulated by salinity (Fig. 7B). Compared to the expression in the wild type, the steady-state level of *ectA* expression in the *fur* mutant was similar in cells grown at low salinity, but it was 10-fold lower in cells grown with 2.5 M NaCl, suggesting that Fur functions as a positive regulator of the *ectABC* genes under high-salt conditions. As previously described by Calderón et al. (11) using a *ectAp*(*1-4*)::*lacZ* fusion, *ectA* expression was downregulated by excess iron in wild-type cells grown with 0.75 M NaCl. The repression was even greater (the levels were 5-fold lower than the levels under low-iron conditions) in cells grown at high salinity (Fig. 7B). From these results, we concluded that iron and Fur have opposite effects on *ectABC* expression.

Given that at high salinity iron represses and Fur activates *ectABC* expression, we used ^{13}C-NMR to compare the total compatible solute pools of wild-type and *fur* strains grown under high-salinity conditions in the absence or presence of excess iron. As shown in Fig. 8A, the wild-type strain grown in low-iron M63 medium accumulated ectoine, hydroxyectoine, glutamate, and trehalose. The presence of excess iron resulted in a change in the ratio of ectoine to hydroxyectoine in favor of hydroxyectoine and in a lack of trehalose accumulation (Fig. 8B). The spectra of the *fur* mutant were comparable to those of the wild type under the same conditions (data not shown). When we used LC-MS to specifically determine the amounts of ectoine and hydroxyectoine in both strains, it was clear that cells grown with excess iron accumulated less ectoine and more hydroxyectoine than cells grown in M63 medium. No differences were found between the wild type and the *fur* mutant

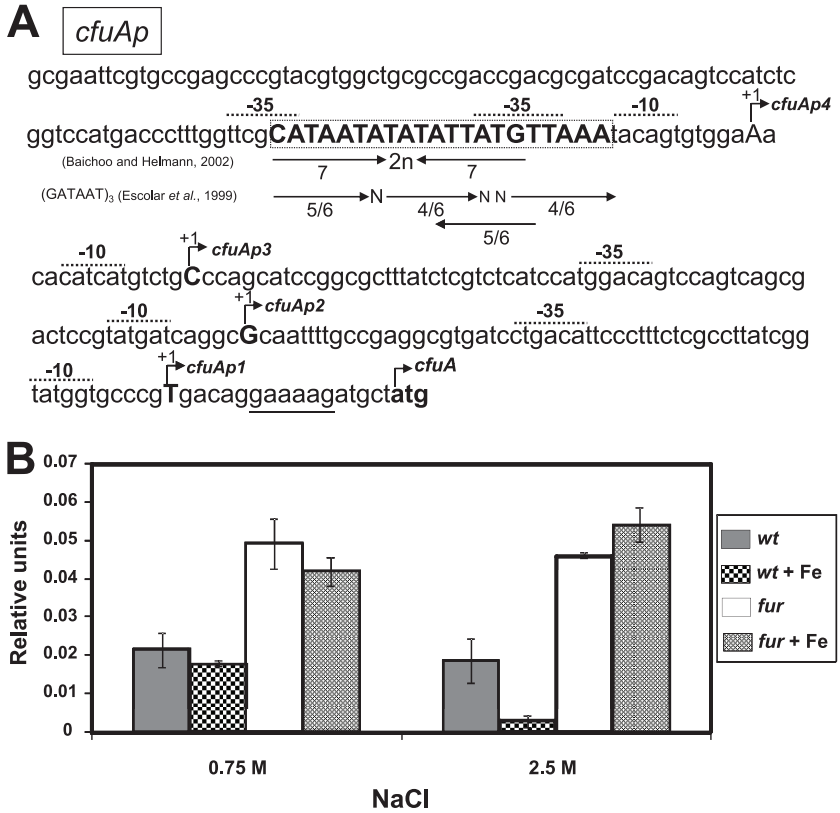

FIG. 6. Fur mediates osmoregulated repression of the *cfuABC-fur-hisI-orf6* operon by iron. (A) *In silico* analysis of *cfuAp*. The transcription start sites (+1) and −10 and −35 regions of each putative promoter are indicated. The ribosome-binding site (RBS) is underlined, and the *cfuA* start codon is indicated by bold type. Below the putative Fur box (box), arrows indicate the characteristic repetitive elements of the Fur boxes, as suggested by Baichoo and Helmann (7) and Escolar et al. (36), and the numbers of nucleotides matching the consensus GATAAT sequence are indicated below the arrows. (B) Determination of *cfuA* expression in wild-type and *fur* strains grown at two different salinities under low- and high-iron conditions. Total RNA was extracted from cells grown at 37°C in M63 medium or M63 medium containing 50 μM $FeCl_3$ with 0.75 or 2.5 M NaCl, and *cfuA* expression was measured by quantitative RT- PCR as described in Materials and Methods. The data are expressed in relative units and were estimated by the $2^{-\Delta C_t}$ method using the 16S rRNA gene as an internal control to normalize expression in each sample. Real-time PCR quantification was performed twice, using RNA samples from independent cultures, and the values are the means ± standard deviations of three replicates from two independent experiments. *wt*, wild type.

(Fig. 8C). Therefore, despite *fur*'s contribution to expression of *ectABC*, a *fur* mutant accumulates ectoine and hydroxyectoine. This may explain the fact that the *fur* strain was not salt sensitive (see above).

DISCUSSION

This study provides the first evidence for the extensive control that salt stress exerts on iron homeostasis in the halophilic bacterium and ectoine producer *C. salexigens*. The extent of this control is such that in salt-stressed *C. salexigens* there is a lower requirement for iron and, concomitantly, siderophore synthesis is repressed. These processes are controlled by the global regulator Fur, the first iron uptake regulator characterized in a moderately halophilic bacterium, which also functions as a positive regulator of ectoine synthesis at high salinity, linking the osmostress response to iron homeostasis in this halophilic microorganism.

In *B. subtilis* members of the Fur regulon were induced by high salinity, suggesting that cells grown under osmotic stress conditions experienced severe iron limitation. In support of this suggestion, addition of excess iron to cells grown with high salt partially reversed the growth defect exhibited by salt-stressed *B. subtilis* cultures and reduced the high-salinity-mediated induction of many of the Fur-regulated genes (37, 69). We observed the opposite effect in *C. salexigens*; that is, greater osmotic stress was correlated with a lower demand for iron. The reason(s) for this difference is unknown. However, the two organisms have different ranges of salinity for growth, and the salinity referred in *B. subtilis* studies as "high" was 0.7 M NaCl, which corresponded approximately to the "low" salinity in our study. In addition, the *B. subtilis* strain for which the effect of salinity on iron demand was observed contains a mutation (sfp^0) that prevents or strongly reduces the synthesis of the main siderophore, bacillibactin. However, when the *B. subtilis* wild-type strain was used, an increase in the salinity of the growth medium had only a marginal effect on the synthesis of 2,3-dihydroxybenzoate, the bacillibactin precursor. Moreover, high-salinity-mediated growth retardation of the wild type could not be rescued increasing the iron concentration of the growth medium (37). Therefore, the wild-type *B. subtilis* strain did not exhibit the iron limitation observed in the mutant strain whose siderophore synthesis was affected at high salinity.

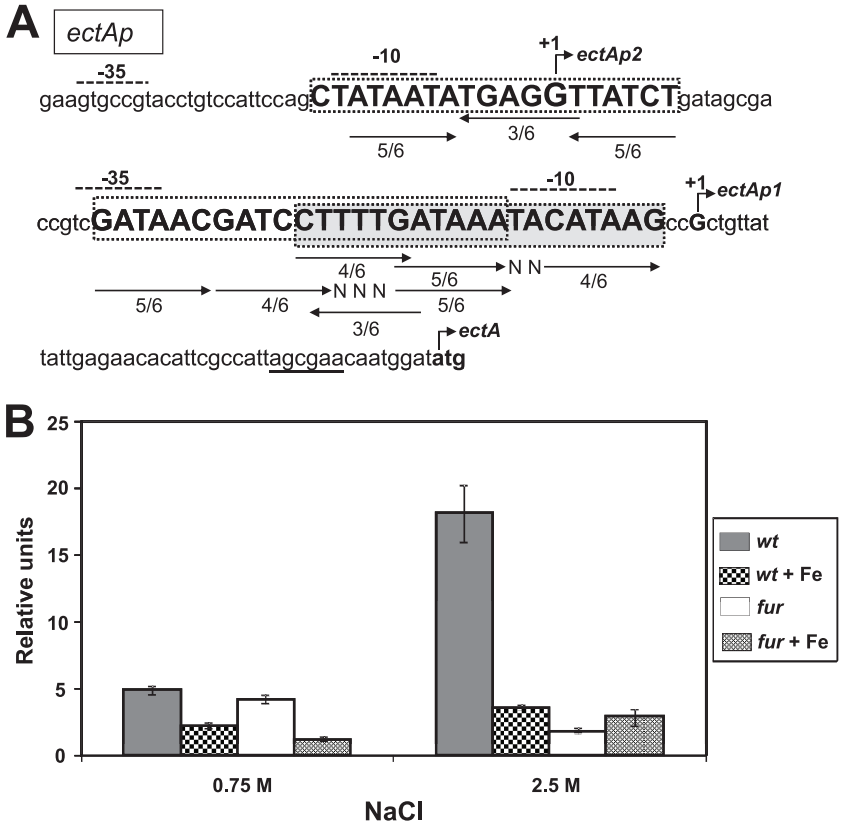

FIG. 7. Fur functions as a positive regulator of ectoine synthesis. (A) *In silico* analysis of *ectAp*. The transcription start sites (+1) and −10 and −35 regions of the *ectAp1* and *ectAp2* promoters of the *ectABC* genes are indicated. The *ectAp3* and *ectAp4* promoters, localized upstream of *ectAp2* by Calderón et al. (11), are not shown. The ribosome-binding site (RBS) is underlined, and the *ectA* start codon is indicated by bold type. The putative Fur boxes are enclosed in boxes, and the repetitions of the GATAAT motif (26) are indicated by arrows; the numbers of nucleotides matches with the consensus sequence are indicated below the arrows. (B) Determination of *ectA* expression in wild-type and *fur* strains grown at two different salinities under low- and high-iron conditions. Total RNA was extracted from cells grown at 37°C in M63 medium or M63 medium containing 50 μM $FeCl_3$ with 0.75 or 2.5 M NaCl, and *ectA* expression was measured by quantitative RT-PCR as described in Materials and Methods. The data are expressed in relative units and were estimated by the $2^{-\Delta C_T}$ method using the 16S rRNA gene as an internal control to normalize expression in each sample. Real-time PCR quantification was performed twice, using RNA samples from independent cultures, and the values are the means ± standard deviations of three replicates from two independent experiments. *wt*, wild type.

At least in part, the regulatory link between salinity and iron metabolism in *C. salexigens* involves the Fur protein, whose roles extend beyond iron homeostasis. The data presented in this study suggest that Fur controls siderophore synthesis (Fig. 3), mediates osmoregulated inhibition by iron of the *cfuABC-fur-hisI-orf6* operon (Fig. 6), and activates *ectABC* expression at high salinity (Fig. 7). In addition, we suggest that Fur plays a role in iron demand in *C. salexigens* cells, as judged by the fact that growth of a *fur* mutant is affected under iron-limiting conditions (Fig. 3C), whereas this strain normally grows with no iron limitation. The role of Fur in regulating iron-requiring systems was elegantly demonstrated for the *E. coli* Fur protein by McHugh and coworkers, who showed that a large number of energy metabolism genes, mainly genes encoding Fe-containing respiratory complexes, were induced by Fe^{2+}-Fur, and hence *fur* mutants were deficient in iron-containing proteins (47). This iron-sparing response (i.e., repression of iron-rich enzymes when iron is limited), currently known to be mediated by sRNAs (29, 46), is likely to occur in *C. salexigens*, although the sRNA mediating it has not been found yet.

Based on bioenergetic calculations, Oren predicted that the cell yield of halophilic and halotolerant heterotrophic microorganisms should decrease sharply as the salt concentration of the growth medium increases (57). Our finding that the *C. salexigens* protein content decreases linearly with osmotic stress corroborates this prediction and may explain the low demand for iron at high salinity, as less Fe-containing enzymes may be synthesized under salt stress conditions. Paradoxically, ectoine hydroxylase belongs to the Fe(II)- and 2-oxoglutarate-dependent oxygenase superfamily (30), and ectoine synthase was reported to belong to the cupin superfamily of proteins, whose members contain primarily iron as the active site metal (25). In addition, ectoine synthesis is energetically costly, and about 40 ATP molecules are required for the production of one ectoine molecule (57). This suggests that, apart from ectoine biosynthetic enzymes, cell systems responsible for bioenergetics, such as respiratory complexes (many of which are iron-containing proteins) and ATP synthase, should be prioritized and other energy-requiring systems should to be turned off for cells to survive under salt stress conditions. In agreement with this, Fur positively regulated *ectABC* expression under high-salt conditions, sug-

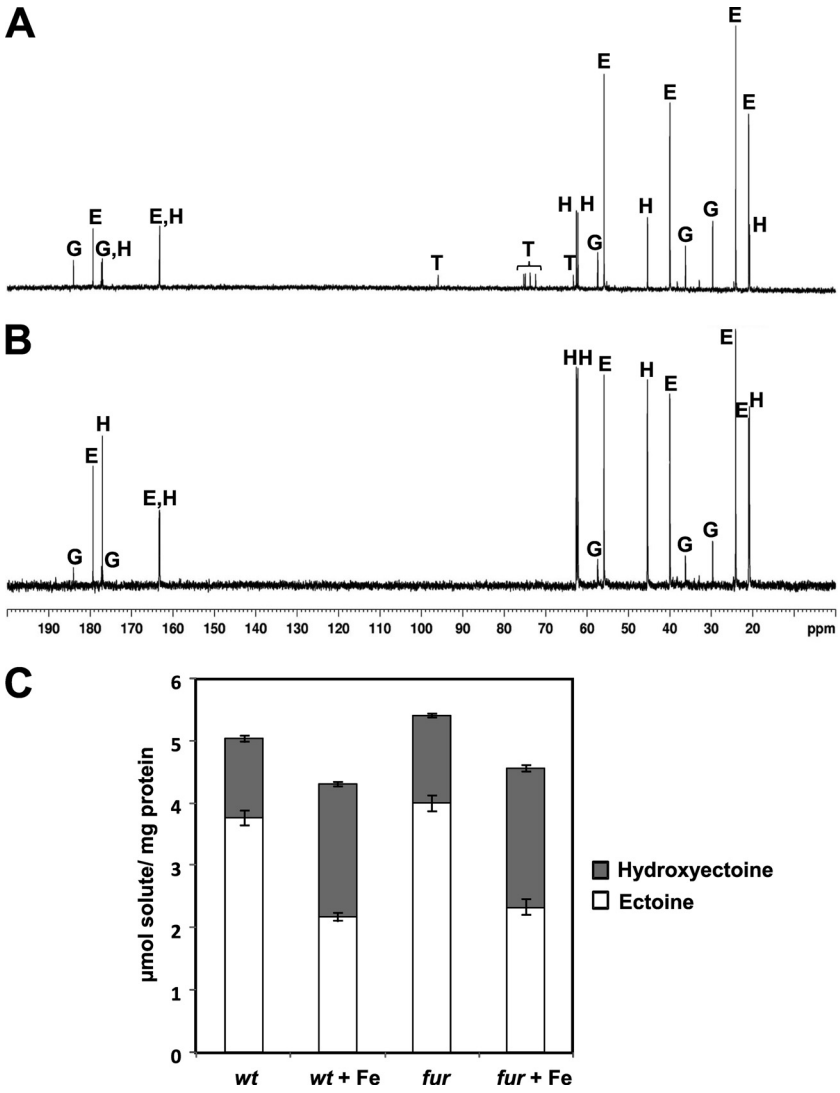

FIG. 8. Effect of excess iron on the pattern of accumulation of compatible solutes by the *C. salexigens* wild-type and *fur* strains. (A and B) Natural abundance ^{13}C-NMR spectra of major cytosolic solutes of the *C. salexigens* wild-type strain grown at 37°C in M63 minimal medium with 2.5 M NaCl in the absence (A) or presence (B) of 50 μM $FeCl_3$. The major solutes were ectoine (E), hydroxyectoine (H), glutamate (G), and trehalose (T). (C) LC-MS quantification of ectoines accumulated by the wild-type and *fur* strains under the growth conditions described above. The values are the means ± standard deviations of three replicates for each condition in two independent experiments. *wt*, wild type.

gesting that it may contribute to optimization of *C. salexigens* metabolism for ectoine synthesis under osmotic stress conditions.

Our discovery that Fur mediates osmoregulated repression of the *cfuAB-fur-hisI-orf6* operon by iron, whereas Fur alone functions as a positive regulator of ectoine synthesis (*ectABC*) at high salinity, was surprising. It is worth mentioning that upstream of *ectAp2* there are two other promoters, one of which (*ectAp3*) is osmoregulated and dependent on the general stress factor σ^S (11). Thus, in both cases, multiple promoters are present, indicating that there is complex transcriptional regulation of these systems. In both cases, a Fur box(es) was found in the regulated promoters, suggesting that Fur directly interacts with DNA in these regions. Whereas this is typical for Fur-Fe^{2+}-repressed systems (e.g., the *cfuABC-fur-hisI-orf6* operon), direct activation by Fur in the absence of iron is rare, but it has been shown for the *norB* gene of *Neisseria meningitidis* (23) and for activation of some genes by the *Bradyrhizobium japonicum* Irr protein, which belongs to the Fur superfamily (78). Interestingly, only one Fur box was found upstream of the classically regulated *cfuABC-fur-hisI-orf6* operon, whereas three Fur boxes were found upstream of *ectABC*. Although it is tempting to speculate that the redundancy of Fur boxes may account for positive regulation of *ectABC* by Fur, this possibility is difficult to reconcile with the fact that the three Fur boxes overlap the *ectAp1* and *ectAp2* σ^{70}-dependent promoters of the *ectABC* genes, a common feature of operator sequences recognized by negative regulators. In fact, activation by Fur is usually associated with a Fur box just upstream of the promoter (44).

Even more intriguing was the fact that repression by iron of *PectA* transcription, which was first documented by Calderón

et al. (11), seemed to be decoupled from Fur. Several factors may account for this. On the one hand, Fur seems to be autoregulated. Thus, repression of *ectABC* transcription by excess iron might be an indirect effect, as Fur-Fe^{2+} represses *fur* transcription and Fur positively regulates *ectABC* expression. On the other hand, Fe^{2+} might function as a corepressor of an unknown regulatory protein and directly downregulate *ectABC* expression. In the *C. salexigens* genome, we have found one homolog of the gene encoding the Fur protein, which shows a high level of similarity to the *P. aeruginosa* Fur regulator. Whether this second copy mediates iron inhibition of *ectABC* transcription has not been determined.

Notably, despite the fact that in the presence of a high salt concentration the *fur* strain showed considerably lower *ectA* expression than the wild type (Fig. 7B), the accumulation of ectoine was similar in the two strains when they were grown continuously under the same conditions. This finding may be explained by two (nonexclusive) factors. First, expression of *ectABC* in *C. salexigens* is semiconstitutive (11) and the level of expression is extremely high (e.g., compare the expression of *ectA* and the expression of *cfuA* in the same cDNA sample with reference to the same endogenous control), reflecting the importance of ectoine synthesis in adaptation to stress. Thus, although lower, the level of *ectA* expression in the *fur* mutant may be high enough to ensure normal ectoine accumulation at high salinity. Second, posttranslational regulatory mechanisms may occur as well. *Halomonas elongata* cells grown in the presence of chloramphenicol, which turns off protein synthesis, were able to respond to a moderate osmotic upshock by synthesizing and accumulating ectoine (43). This was interpreted as major regulation at the level of enzyme activity. The same regulatory scenario may occur in *C. salexigens*, as it is a close relative of *H. elongata* in the family *Halomonadaceae*.

We have shown that *hisI* is essential for histidine synthesis and that histidine is either a precursor or a constituent of the *C. salexigens* siderophore(s). A search of the sequenced *C. salexigens* genome revealed that the rest of the genes involved in histidine biosynthesis are partially scattered, with *hisGD* and *hisBHAF* forming minioperons and *hisC*, *hisE*, *hisI*, and *hisN* distributed throughout the chromosome. The lack of clustering of *his* genes is rare in gammaproteobacteria and more typical of the alphaproteobacterial branch (27). Histidine has been shown to be used as a precursor of hydroxamate-type siderophores, the high-affinity iron chelator anguibactine produced by *Vibrio anguillarum* (1, 70), and the siderophore secreted by *Acinetobacter baumannii* (49). In other microorganisms, such as *Mycobacterium neoaurum* (24) or *Aspergillus* sp. ABp4 (6), histidine forms part of the structure of the siderophores. In the *C. salexigens* genome, we found a cluster of genes whose products showed high levels of similarity to nonribosomal peptide synthetases (NRPS) (18) and ABC transporters of hydroxamate-type siderophores (3). It is plausible that these genes may be involved in the biosynthesis and transport of the siderophore observed in the CAS assay. In the *C. salexigens* genome we also found a homolog of *desA*, which in *Vibrio vulnificus* encodes a specific receptor for deferoxamine located in the outer membrane (41) and might have a similar function in *C. salexigens*.

The exact physiological role of the CfuABC transport system remains to be elucidated. However, the presence of a Fur box sequence upstream of *cfuA* and the Fur-mediated downregulation by iron suggest that *cfuABC* might encode a novel iron transport system different from previously described iron uptake systems, such as FecBD, SfuAB, PvuBD, FepG, AfeABCD, and SirAB (4, 22, 61, 68). *C. salexigens* was also able to transport deferoxamine, a hydroxamate siderophore derived from *Streptomyces pilosus* (36), which can also be used by the closely related organism *V. vulnificus* (41).

Very interestingly, excess iron repressed *ectABC* transcription and favored hydroxyectoine synthesis. Hydroxyectoine was able to protect lactate dehydrogenase from metal-catalyzed oxidation (2) and to protect plasmid DNA from damage by a number of OH radical-producing systems, including 0.1 mM $FeCl_3$ (45). Therefore, our interpretation is that this is a way for *C. salexigens* to respond to the oxidative stress imposed by excess iron (50 μM $FeCl_3$). In summary, the osmoadaptive response through ectoine synthesis in the halophilic bacterium *C. salexigens* seems to be finely controlled at the transcriptional level by at least two global regulators, the general stress response factor σ^S (11) and the central iron regulator Fur (this study). On the other hand, hydroxyectoine synthesis is thermoregulated under control of the heat stress factor σ^{32} (M. Reina-Bueno and C. Vargas, unpublished data), and it is favored when cells are exposed to oxidative stress. All these adaptive responses obviously should involve many more systems than those responsible for compatible solute synthesis. Elucidation of the intricate relationships among the stress response networks and iron homeostasis requires generation of extensive data by transcriptome and proteome approaches, and experiments to do this are currently under way in our laboratory.

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