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Functional Interactions between the Carbon and Iron Utilization Regulators, Crp and Fur, in *Escherichia coli*

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In *Escherichia coli*, the ferric uptake regulator (Fur) controls expression of the iron regulon in response to iron availability while the cyclic AMP receptor protein (Crp) regulates expression of the carbon regulon in response to carbon availability. We here identify genes subject to significant changes in expression level in response to the loss of both Fur and Crp. Many iron transport genes and several carbon metabolic genes are subject to dual control, being repressed by the loss of Crp and activated by the loss of Fur. However, the *sodB* gene, encoding superoxide dismutase, and the *aceBAK* operon, encoding the glyoxalate shunt enzymes, show the opposite responses, being activated by the loss of Crp and repressed by the loss of Fur. Several other genes including the *sdhA-D*, *sucA-D*, and *fumA* genes, encoding key constituents of the Krebs cycle, proved to be repressed by the loss of both transcription factors. Finally, the loss of both Crp and Fur activated a heterogeneous group of genes under σ^S control encoding, for example, the cyclopropane fatty acid synthase, Cfa, the glycogen synthesis protein, GlgS, the 30S ribosomal protein, S22, and the mechanosensitive channel protein, YggB. Many genes appeared to be regulated by the two transcription factors in an apparently additive fashion, but apparent positive or negative cooperativity characterized several putative Crp/Fur interactions. Relevant published data were evaluated, putative Crp and Fur binding sites were identified, and representative results were confirmed by real-time PCR. Molecular explanations for some, but not all, of these effects are provided.

Processes that alter the rates of transcriptional initiation, elongation and termination, mRNA degradation, and mRNA translation control gene expression in all living organisms. Of these, regulation of transcriptional initiation is of greatest physiological importance in bacteria (34). About one-fourth of all *Escherichia coli* proteins interact functionally with nucleic acids, and about one-fourth of these are pleiotropic transcriptional regulators that control expression of multioperon regulons, primarily by influencing the transcriptional initiation step (57, 72). In fact, each regulon is defined by the activity of a transcription factor that coordinately controls expression of the operons included within that regulon (28). The global regulator, responsive to select cellular stimuli, binds to specific sites in the control regions of these operons. Examples of such pleiotropic transcription factors in *E. coli* include Crp, a primary sensor of carbon availability (8, 15, 23, 65), NtrBC, a sensor of nitrogen availability (32, 63, 77), CysB, the sensor of sulfur availability (38, 39, 41, 42), and Fur, a dominant sensor of iron availability (30, 31, 50, 71). Crp when complexed with cyclic AMP (cAMP) binds to a consensus sequence in the DNA and activates transcription of many genes while repressing transcription of a few others. Some genes are subject to both positive and negative regulation by the cAMP-Crp complex due to the presence of multiple promoters (36, 64). Fur generally represses iron transport and iron siderophore biosynthetic genes when complexed with ferrous iron. Under iron limiting conditions, iron dissociates from Fur, and increased transcription ensues (6, 30, 31).

Functional interactions between these regulons are believed to coordinate the activities of the different metabolons so that the supply of one type of nutrient matches the supply of other essential types of nutrients (28). Thus, for example, multiple links between carbon and nitrogen metabolism have been identified (43, 59). Moreover, data providing evidence for functional links between carbon and sulfur metabolism (62) as well as carbon and iron metabolism (18) have been presented. In the case of the iron-carbon connection, Crp, the primary transcriptional regulator of carbon metabolism, modulates transcription of the *fur* gene which encodes the ferric uptake regulator, Fur (18). These and other potential global interrelationships serve as the bacterial "nervous system," coordinating the various activities of the cell (28, 44, 66).

In this paper we use a whole-transcriptome approach to provide evidence for previously unidentified potential interactive mechanisms involving Fur and Crp. We show that many iron metabolic genes are subject to dual Crp/Fur control although our transcriptome results cannot distinguish between direct and indirect effects of the loss of these transcription factors. For many iron transport genes (class A genes), the consequence of the loss of Crp (transcriptional down regulation) is opposite to that of the loss of Fur (transcriptional up regulation). We also show that the loss of Crp and Fur regulates expression of the genes encoding the oxidative stress enzyme, superoxide dismutase, and the *aceBAK* operon encoding the glyoxalate shunt enzymes (class B genes) in directions opposite to those observed for the iron transport genes. These

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genes are directly or indirectly repressed by the loss of Fur and activated by the loss of Crp in a *fur* background. Moreover, other genes were identified that are either repressed (class C gene) or activated (class D genes) by the loss of both transcription factors. For example, several enzyme complexes of the Krebs cycle are directly or indirectly repressed by the loss of both Fur and Crp. The transcriptome results were confirmed in representative cases by using the real-time PCR. Putative factor binding sites were identified when present, and based in part on published data, mechanisms of transcriptional regulation could sometimes be proposed. Physiological relevance was also considered. This work serves to suggest a previously unrecognized transcriptional regulatory connection between iron and carbon metabolism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains used in this study were BW25113 and isogenic *crp* (LJ3017), *fur* (LJ3019), and *crp fur* (LJ3027) mutant derivatives, constructed as described by Zhang et al. (78). Strains were grown at 37°C with agitation at 250 rpm in Luria-Bertani (LB) broth containing 50 mM potassium phosphate, pH 7.4, and 0.2 mM L-cysteine with or without 0.4% glucose. Cells were grown in 25 ml of medium in 250-ml shake flasks starting at an optical density at 600 nm (OD_{600}) of 0.05 (transcriptome experiments) or 0.1 (growth studies). Each determination was performed three times, and the results were averaged as described by Gosset et al. (23).

Growth studies in minimal media were performed using M9 medium (67) with glucose (0.4%) as the sole carbon source. Growth was in 6 ml of medium in 25-ml glass test tubes rotating at 250 rpm. Growth on solid plates was performed with the LB media described above, to which 1.5% agar was added.

Cell harvesting and preparation of RNA. Cells from each triplicate experiment were harvested in the exponential growth phase when cultures reached an OD_{600} of 0.5. The total contents of each shake flask (25 ml) were poured into a Millipore vacuum filtration apparatus (catalog no. 1004700) using a Millipore 0.8-μm-pore-size filter (catalog no. AAWPO4700). The collected cells with filter were immediately transferred to a 200-ml glass beaker containing liquid nitrogen. RNA was extracted from each sample, following the procedure described by Caldwell et al. (9).

Target preparation. RNA harvested from a given *E. coli* strain and at a given time point was reverse transcribed into biotin-labeled cDNA by the method of de Saizieu et al. (19). Total RNA (18 μg) was incubated at 37°C overnight in a 80-μl reaction mixture consisting of 1× GIBCO first-strand buffer (50 mM Tris-HCl [pH 8.3], 75 mM KCl, 3 mM $MgCl_2$), 10 mM dithiothreitol, 40 μM random hexamer, 0.3 mM concentrations (each) of dCTP, dGTP, and dTTP, 0.12 mM dATP, 0.3 mM biotin-dATP (NEN catalog no. NEL999), and 1800 U of SuperScript II reverse transcriptase. To remove RNA, the reaction was brought to 0.25 M NaOH and incubated at 65°C for 30 min. The reaction mixture was neutralized with HCl, and the nucleic acid was precipitated at −20°C in ethanol with 2.5 M ammonium acetate and 20 μg of glycogen. The pellet was washed, air dried, resuspended in water, and quantitated by UV spectroscopy. The yield was approximately 10 to 12 μg of biotin-labeled cDNA. This cDNA (10 μg) was fragmented in 33 μl of 1× One-Phor-All buffer (Amersham-Pharmacia catalog no. 27-0901-02) with 3.75 mU of DNaseI at 37°C for 10 min. After the DNase had been heat killed, fragmentation was validated by running 1.5 μg of the fragmented cDNA on a 1.2% agarose gel. Biotin-containing cDNA routinely ranged in size from 25 to 400 nucleotides. The remaining fragmented cDNA (8.5 μg) was hybridized to an Affymetrix (Santa Clara, Calif.) *E. coli* GeneChip array.

Array description. The Affymetrix *E. coli* array has been described in detail previously (69). Briefly, each microarray contains 295,936 25-mer oligonucleotide probes. Half of the probes are a perfect match to the corresponding *E. coli* chromosomal sequences while the other half have a single mismatch at the 13th base position. The array includes 4,327 genes and intergenic regions (4). The complete set of averaged data are provided on our website (www-biology.ucsd.edu/~msaier/supmat; see supplemental Table S1).

Hybridization, scanning, and data collection. The procedure described by Caldwell et al. (9) was followed.

Real-time PCR studies. Bacterial cells were cultured in LB broth exactly as for the microarray analyses. When the OD_{600} reached 0.5, cells were collected by centrifugation at 4°C. Total RNA was subsequently isolated using an RNeasy Mini kit (QIAGEN) following the manufacturer's protocol. Residual DNA present in the RNA preparations was removed by RNase-free DNase (Stratagene). cDNAs were synthesized using a superscript first-strand synthesis kit (Invitrogen) according to the manufacturer's instructions and stored at −20°C prior to use. *polA*, encoding DNA polymerase I, was included as an internal control (50). Real-time PCR was carried out on a LightCycler instrument (Roche Diagnosis Corporation) following the manufacturer's recommended protocol. Primers used for the real-time PCR were as shown in the supplemental Table S1 on our website.

Data analysis. Raw expression intensity values were determined from array images analyzed using the Affymetrix Microarray Suite 5.0 software. These data were then scaled, normalized to a target value of 1,000, and imported into a Microsoft Excel spreadsheet for further analysis. The intensity values in the data sets ranged from about 3×10^{-1} to 8×10^{4}. Three data sets were obtained for each of eight experimental condition: (i) wild-type (wt) LB, (ii) wt LB plus glucose, (iii) *crp* LB, (iv) *crp* LB plus glucose, (v) *fur* LB, (vi) *fur* LB plus glucose, (vii) *crp fur* LB, and (viii) *crp fur* LB plus glucose. Pairwise comparisons were performed with the three data sets for each strain and condition to determine the Pearson correlation coefficient using the following formula: $r = [n(\Sigma XY) - (\Sigma X)(\Sigma Y)]/\{[n\Sigma X^2 - (\Sigma X)^2][n\Sigma Y^2 - (\Sigma Y)^2]\}^{1/2}$. For each triplicate data set, the two sets with the highest Pearson correlation coefficient were retained for further analysis. The statistical significance of *n*-fold changes between data sets from different strains and conditions was determined as described by Caldwell et al. (9). Briefly, for each pair of replicate data sets, a table was generated from the average of the logs of the two replicate signals (ALS) and their log ratio (LR). From this table, using a series of sliding windows with a size of 201 ALS values, the mean ALS values and the standard deviation of the corresponding LR were calculated (SD_{LR}).

Phenotypic studies. Phenotypic analyses were performed using 96-well Biolog microtiter plates including GN2 and Phenotypic Microarray plates PM1 and PM2 (5). These Biolog plates are designed to measure oxidation of various carbon sources by gram-negative bacteria.

RESULTS

Identification of genes whose expression is influenced by the loss of both Fur and Crp. Analysis of transcriptome data allowed the identification of genes that showed significant changes in expression level in response to the loss of both Crp and Fur. Most of the genes presented in Table 1 met the following criteria. (i) They showed statistically significant changes in gene expression upon introduction of both the *crp* and *fur* mutations. The *n*-fold change was at least 4 times higher than the SD_{LR} for the ALS of the gene when comparing wt and *fur* or wt and *crp fur* (see reference 23 for explanation of the statistical analyses of the microarray data). (ii) A large signal was observed in at least one of the mutant strains (signal intensity value, ≥1,000). (iii) The ratio for *crp fur*/*fur* was ≤0.5 or ≥2 following growth in LB broth. Table 1 also indicates whether or not putative Crp and/or Fur binding sites in the control regions of these operons could be found using the GRASP-DNA program (68). The putative binding site sequences and their locations can be found in supplemental Table S2A on our website (http://www.biology.ucsd.edu/~msaier/supmat/crp_fur/index.html). Published regulatory data for these operons are summarized in Table S2B in the supplemental material. It is important to note that some of the genes in Table 1 are in operons, and not all of the genes in a specific operon always met these criteria. However, in the discussion below, all of the cistrons of each operon are usually considered.

As shown in Table 1, genes meeting these criteria can be divided into four categories: A, genes that appear to be repressed by Fur but activated by Crp in a *fur* background (i.e., genes involved in iron sequestration and transport); B, genes that appear to be activated by Fur but repressed by Crp in a *fur* background (i.e., the *sodB* gene and the *aceBAK* operon); C,

TABLE 1. Genes subject to regulation in response to the loss of both Crp and Fur[a]

Category and gene designation	Absolute signal values[b]								Ratios		Binding sites[c]		Function
	wt	wt+G	*crp*	*crp*+G	*fur*	*fur*+G	*crp fur*	*crp fur*+G	*fur*/wt	*crp fur*/*fur*	Crp	Fur	
Category A													
aceE[d]	1,880	8,177	4,520	4,447	5,107	5,046	1,787	2,144	2.7	0.3			Enzyme I component of pyruvate dehydrogenase complex
aceF	1,712	5,255	3,134	2,758	3,920	3,321	1,271	1,596	2.3	0.3	X		Dihydrolipoamide acetyltransferase of pyruvate dehydrogenase complex
ackA	1,249	3,307	2,355	2,237	2,691	2,775	823	1,144	2.1	0.3	X		Propionate kinase 2/acetate kinase A
fepA	848	948	527	800	2,119	5,339	708	1,958	2.5	0.3	X	X	Outer membrane receptor for ferric enterobactin (enterochelin) and colicins B and D
fes	765	518	319	513	1,563	1,818	663	1,470	2.0	0.4	X	X	Enterochelin esterase
entF	360	233	194	174	1,676	1,488	832	1,212	4.7	0.5			Serine activating enzyme
entC	1,026	581	639	574	2,884	3,132	1,277	1,966	2.8	0.4	X	X	Isochorismate synthase, enterobactin specific
entE	430	130	329	207	2,413	2,340	1,138	2,213	5.6	0.5			Enterobactin synthase multienzyme complex
entB	1,224	816	851	666	3,394	4,318	2,062	2,727	2.8	0.6			Isochorismate lyase
entA	226	437	309	414	1,201	2,128	481	993	5.3	0.4			2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase
ybdB	486	530	577	513	3,027	3,862	1,670	2,435	6.2	0.6			Orf, hypothetical protein
ybiI	238	212	244	211	792	1,020	514	529	3.3	0.6	X		Orf, hypothetical protein
ybiX	372	327	437	278	1,365	1,922	549	682	3.7	0.4			Putative enzyme
ybiL	427	313	361	166	3,778	6,264	1,084	1,182	8.8	0.3	X	X	Putative outer membrane receptor for iron transport (*fiu*)
yncE	2,039	1,543	1,440	1,239	7,647	9,318	4,427	6,066	3.8	0.6		X	Putative receptor
fecE	318	212	285	225	581	1,049	396	732	1.8	0.7			Iron dicitrate ABC transporter
fecB	507	394	271	378	1,223	1,244	531	869	2.4	0.4	X		Iron dicitrate ABC transporter
fecA	905	1,327	751	764	3,224	4,578	1,532	2,075	3.6	0.5		X	Outer membrane receptor; citrate-dependent iron transporter
cirA	911	941	720	806	3,000	5,735	851	1,501	3.3	0.3	X	X	Outer membrane receptor for iron-regulated colicin 1 receptor
yhaN	652	443	403	285	1,377	407	458	432	2.1	0.3	X		Orf, hypothetical protein
ptsG	735	2,387	1,584	1,390	2,144	1,601	444	449	2.9	0.2	X		Enzyme II^{Glc} of PTS
Category B													
sodB	1,926	1,618	1,923	1,647	318	277	1,493	306	0.2	4.7			Superoxide dismutase (Fe)
aceB	1,432	296	339	237	374	438	2,029	1,767	0.3	5.4	X	X	Malate synthase A
aceA	2,747	498	711	462	676	472	3,449	2,244	0.2	5.1	X		Isocitrate lyase monomer
Category C													
cspA	9,076	15,827	3,899	7,603	3,693	8,366	1,054	1,661	0.4	0.3	X		Cold shock protein A
ompF	4,794	2,710	9,040	7,334	1,536	629	598	324	0.3	0.4	X		Outer membrane protein F
sdhA	3,503	614	1,591	682	2,321	488	760	431	0.7	0.3			Succinate dehydrogenase flavoprotein
sdhB	2,620	389	1,424	571	1,796	323	703	233	0.7	0.4			Succinate dehydrogenase iron-sulfur protein
sdhC	1,136	386	592	358	905	297	404	227	0.8	0.4	X	X	Succinate dehydrogenase membrane protein
sdhD	2,304	401	1,019	462	1,979	355	587	180	0.6	0.4			Succinate dehydrogenase membrane protein
sucA	3,920	822	1,345	813	1,598	874	1,124	921	0.4	0.7			Subunit enzyme 1 of 2-oxoglutarate dehydrogenase
sucB	5,734	802	1,725	923	2,450	943	1,187	810	0.4	0.5	X		Dihydrolipoamide succinyltransferase of 2-oxoglutarate dehydrogenase
sucC	6,684	1,250	1,866	1,700	2,702	1,461	1,280	1,622	0.4	0.5	X		Succinyl-CoA synthetase
sucD	3,815	526	1,057	753	1,134	748	833	681	0.3	0.7			Succinyl-CoA synthetase
fumA	3,406	819	1,051	469	1,328	530	908	340	0.4	0.7	X		Fumarase A monomer
Category D													
yedR	104	304	393	537	211	356	1,045	1,037	2.0	5.0	X		Orf, hypothetical protein
cfa	130	143	311	289	301	170	2,008	2,163	2.3	6.7	X		Cyclopropane fatty acid synthase
glgS	223	222	388	329	550	154	1,955	2,605	2.5	3.6	X		Glycogen biosynthesis protein
rpsV	663	637	1,472	1,960	1,453	998	9,670	14,453	2.2	6.7	X		30S ribosomal subunit protein S22
yjbJ	679	749	1,182	875	1,381	747	7,864	8,834	2.0	5.7			Orf, hypothetical protein
ytfK	396	426	873	604	1,346	310	5,180	3,458	3.4	3.9	X		Orf, hypothetical protein
osmE	587	328	1,562	919	1,145	352	4,087	4,720	2.7	2.6	X		Activator of *ntrL* gene
yggB	598	2,048	1,462	2,645	536	1,454	6,361	5,826	2.4	4.3	X		Mechanosensitive channel (MscS family)
tktB	209	227	440	415	280	194	2,070	2,258	2.1	4.7			Transketolase II

[a] Genes tabulated gave high reproducibility for replicate values, large signals for at least one of the strains assayed, *fur*/wt ratios greater than 2 or less than 0.6, and *crp fur*/*fur* ratios of greater than 2 or less than 0.6 for most of the genes in an operon. Brackets to the left of the gene designations indicate genes included within a single operon. Note that transcriptome data do not by themselves allow direct and indirect effects of the loss of a transcription factor to be distinguished. Category A, genes apparently repressed by Fur and activated by Crp in a *fur* background; category B, genes apparently activated by Fur and repressed by Crp in a *fur* background; category C, genes apparently activated by Fur and activated by Crp in a *fur* background; category D, genes apparently repressed by both Crp and Fur.

[b] Values are the average signal values provided as described in Materials and Methods. The abbreviations are as follows: wt, wild type; *crp,* the isogenic *crp* null mutant; *fur,* the isogenic *fur* null mutant; *crp fur,* the isogenic double mutant; G, grown in LB medium plus glucose. When G is not indicated, cells were grown in LB medium.

[c] The presence of putative binding sites for Fur and Crp is indicated by an X. The predicted binding sites are present within or close to the regulated gene in most cases (see our website). Putative sigma-70 promoters (34) were identified for all tabulated genes. The putative Crp and Fur binding sites and their positions are presented on our website (Table S2A). The occurrence of other established binding sites (72) within these control regions is also reported on our website (Table S2B).

[d] Two putative Crp binding sites are present upstream of *pdhR*.

genes that appear to be activated by Fur and activated by Crp in a *fur* background (i.e., the *cspA* cold shock regulatory gene and the *ompF* outer membrane porin gene as well as several genes encoding Krebs cycle enzymes); and D, genes that appear to be repressed by both Crp and Fur (e.g., the *glgS* glycogen biosynthetic gene and the *rpsV* ribosomal protein gene). It should be noted that the transcriptome data alone do not distinguish direct from indirect effects of the loss of a transcription factor.

Genes apparently repressed by Fur and activated by Crp in a *fur* background. Fur is known to repress many iron transport system-encoding genes under conditions of iron sufficiency (50). For representative genes of this group, the detected transcript levels increased 1.8- to 8.8-fold as a result of *fur* inactivation when the wild-type and mutant strains were grown in LB medium (Table 1). Simultaneous inactivation of *crp* and *fur* caused a substantial decrease in transcript level for all genes in this group relative to the *fur* single mutant. The *crp fur*/*fur* ratio for these genes ranged from 0.2 to 0.7 (Table 1). Putative Crp and/or Fur binding sites could usually be identified in these operons, suggesting that many of the mRNA level changes observed were direct consequences of factor binding.

The 21 genes that comprise category A are included within several different transcription units (Table 1). Some of these will be discussed. Genes *aceE* and *aceF* encode pyruvate dehydrogenase (E1) and lipoate acetyltransferase (E2), respectively. These two proteins and lipoamide dehydrogenase (E3), encoded by gene *lpd*, constitute the pyruvate dehydrogenase (PDH) complex. Synthesis of the PDH complex is induced by pyruvate, repressed during anaerobic growth, and partially repressed by growth in media containing excess glucose, citric acid intermediates, or acetate (20). Genes encoding PDH subunits are part of the complex operon *pdhR-aceEF-lpd* where the *pdhR* gene encodes a transcriptional repressor. Two different transcripts originate from this group of genes, one including *pdhR-aceEF-lpd* and another only *lpd*. The larger transcript originates from a single promoter in front of the *pdhR* gene. Weak activation of the *pdhR* promoter by Crp and Fnr and corresponding putative binding sites upstream of the −35 promoter sequence have been reported (61).

The *ackA* and *pta* genes form an operon and encode acetate kinase and phosphotransacetylase, respectively. These enzymes constitute a pathway for the generation of acetate and ATP from acetyl-coenzyme A (CoA). The *ackA-pta* operon is part of a group of genes regulated by the two-component regulator CreCB. Expression of the *ackA-pta* operon is induced during growth in minimal medium containing glucose (2).

The *fepA* gene, encoding the outer membrane receptor for enterobactin, forms an operon with *entD*, encoding an enzyme involved in enterobactin biosynthesis. The genes *fes*, *entF*, and *fepE* form a second operon and encode two more biosynthetic enzymes and a ferric enterobactin transport protein, respectively. The *fepA* and *fes* operons, divergently transcribed from overlapping promoters, are controlled by the binding of Fur to a single binding site (35).

The *entCEBA-ybdB* operon, encoding enterobactin biosynthetic enzymes, is transcribed from a bidirectional promoter region shared with *fepB* that encodes a protein involved in enterobactin transport. Transcriptome data for *fepB* showed low levels of transcript expression. Therefore, it did not meet our selection criteria. Divergent promoters for the *entC* and *fepB* operons are separated by 31 bp, and for each, a functional Fur binding sequence has been identified (7).

Genes *ybiL*, *ybiX*, and *ybiI* are clustered and possibly form an operon. The gene *ybiL*, also known as *fiu* (ferric iron uptake), encodes an outer membrane protein having a putative TonB box (29, 51, 52, 56). The *ybiX* gene encodes a protein similar to an iron-regulated hydroxylase-encoding gene from *Pseudomonas aeruginosa* (*piuC*), and *ybiI* encodes a probable C_4-Zn finger protein (50, 54). Sequence and functional analyses of the promoter region of *fiu* have revealed the presence of four overlapping Fur binding sites (52).

The gene *yncE* potentially encodes a protein with sequence similarity to a pyrolo-quinoline quinone containing periplasmic oxidase. A putative Fur binding site has been found in the promoter region of this gene (50).

Iron-citrate import depends on the genes of the *fecABCDE* operon. *fecA* encodes an outer membrane iron-citrate receptor, while *fecBCDE* encodes components of an ABC iron-citrate transporter. A separate operon, *fecIR*, encodes the alternative sigma factor, FecI, and an anti-sigma factor, FecR. When iron concentrations are low and iron citrate is present, the receptor protein FecA transduces a signal to the transmembrane FecR protein, which causes FecI to become active and promote transcription from the *fecA* promoter (6). Functional Fur binding sites have been detected in the promoter regions of both the *fecABCDE* and the *fecIR* operons (1). According to our transcriptome data, the *fecI* gene showed a twofold increase in expression in the *fur* mutant relative to the wild type, and deletion of the *crp* gene caused a slight decrease in *fecI* expression in both the wild-type and *fur* mutant backgrounds (data not shown). *fecR* gave a low signal. Real-time PCR data confirmed that the *fur* mutation caused a substantial increase (5×) in *fecI* expression (data not shown). Our results are in general agreement with those of Braun et al. (6). The effects of the *fur* mutation on *fecI* gene expression could partially explain the data reported in Table 1 for the *fec* structural genes. Other genes in category A include genes encoding an additional outer membrane receptor for iron transport, enzymes involved in carbon metabolism, and two proteins of unknown function.

The *cirA* gene encodes the colicin I receptor. This protein may bind a catechol-type siderophore (14, 29, 56). *cirA* is transcribed from two overlapping promoters. Footprinting analysis revealed the presence of a Fur binding site overlapping both promoters. Transcription from *cirA* is significantly reduced in *cya* and *crp* mutants, and a potential Crp binding site has been identified downstream of the tandem promoters (25).

Genes apparently activated by Fur and repressed by Crp in a *fur* background. The second group of genes showing significant changes in expression level when *crp* was inactivated in a *fur* background includes the *sodB* and the *aceBA* genes (Table 1). The *sodB* gene encodes iron-dependent superoxide dismutase (3) while the *aceB* and *aceA* genes encode malate synthase and isocitrate lyase, respectively, the enzymes of the glyoxalate shunt (12). Fur has been shown to activate the *sodB* gene, at least in part by promoting mRNA stabilization (21). Our results showed that in the *fur* mutant, the *sodB* transcript level decreased fivefold compared to the wild-type strain. In

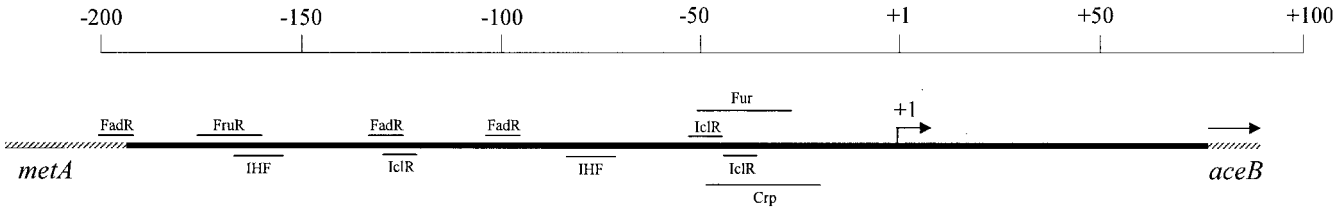

FIG. 1. The *aceBAK* upstream regulatory region showing all known binding sites for protein transcription factors known to influence operon expression as well as the putative Crp and Fur binding sites. The relative positions and lengths of all binding sites are drawn to scale. See the text for discussion of the regulators.

the *crp fur* double mutant, the *sodB* transcript level increased 4.7-fold over that observed in the *fur* mutant although the *crp* mutation in the wild-type background had no effect on gene expression (Table 1). It is surprising that in the double mutant, *sodB* expression is repressed by glucose, although this is not true in the wild-type or single mutant genetic backgrounds.

Regulation of *sodB* expression has been shown to be dependent on the small RNA encoded by the *ryhB* gene (45). RyhB RNA exerts posttranscriptional down regulation of *sodB* and other genes involved in iron metabolism, apparently by an antisense RNA mechanism (45). *ryhB* expression is repressed by Fur (73). If RyhB decreases the stability or causes mRNA truncation of the *sodB* mRNA, its effect could in part explain the transcriptional results reported in Table 1.

Other genes known to be regulated by RyhB (*acnB*, *fumA*, *bfr*, *fln*, and *sdhC*) also show a general trend of a decrease in transcript level in response to this small RNA. The *bfr* (bacterioferrin) gene shows increased expression in the *crp* mutant, slightly decreased expression in the *fur* mutant, but dramatically increased expression in the *crp fur* double mutant (data not shown). Particularly in this last respect, the *bfr* gene behaves the same as the *sodB* gene. Also, the *acnA* gene shows similar behavior although the changes in gene expression levels are substantially less dramatic (data not shown).

The *aceBAK* operon encodes the glyoxalate shunt enzymes required for growth of the bacterium on acetate as a carbon source (11, 12). Regulation of this operon is extremely complex, as suggested by the transcription factor binding sites presented in Fig. 1. Putative overlapping Crp and Fur binding sites were identified upstream of the *aceB* gene, and these also overlap with two IclR binding sites (Fig. 1; see Discussion). Expression of this operon is subject to glucose repression which is largely abolished by either the *crp* or the *fur* mutation, either of which also reduces the expression level to a low value. The double mutant shows greatly enhanced expression, comparable to that of the wild-type strain grown in the absence of glucose, and this expression is no longer subject to strong glucose repression (Table 1).

Genes apparently activated by Fur and activated by Crp in a *fur* genetic background. The *cspA* gene encodes a cold shock transcriptional regulator (22, 27). Expression of this gene appeared to be activated by glucose, but also by Fur, and was further activated by Crp (Table 1). *cspA* showed depressed expression when either the *crp* or the *fur* mutation was introduced, and the double *crp fur* mutant showed a more than additive effect. Glucose activation was not abolished by either the *crp* mutation or the *fur* mutation although expression in the double mutant was largely glucose independent.

Another gene, *ompF*, encoding an outer membrane porin, showed apparent repression by Crp in the wild-type genetic background and activation by Fur in the same background (Table 1). However, while the *crp* mutation enhanced *ompF* gene expression in the wild-type genetic background, it reduced expression in the *fur* background. *ompF* expression is known to be subject to complex regulation by multiple factors, but regulation by Fur had not been reported previously (16, 17, 40, 48, 49, 53, 60). Our results suggest that Fur is required for Crp-dependent repression and that in the absence of Fur, Crp activates gene expression.

The four genes of the *sdh* operon (*sdhCDAB*), encoding the four subunits of iron-dependent succinate dehydrogenase, showed strong glucose repression, mild apparent Fur activation in a wild-type background, and strong apparent activation by Crp both in the wild-type background and in the *fur* genetic background. Expression of the *sdh* operon is known to be subject to negative control by the RyhB antisense RNA that promotes mRNA truncation at a region of complementarity in the first gene of the operon (*sdhC*). RyhB requires the RNA binding protein, Hfq, for this activity. It seems that RyhB provides a general mechanism for the down regulation of nonessential iron-containing genes when iron is limiting (45).

The *sucABCD* operon that clusters together with the *sdh* operon showed similar regulation in response to the loss of Crp and Fur. Genes *sdhCDAB-sucABCD* form a complex operon. Three promoters have been identified in this cluster, one in front of the *sdhC* gene, a second within *sdhC*, and a third in front of the *sucA* gene. Transcription starting from the promoter in *sdhC* usually terminates in the *sdhB-sucA* intergenic region. However, a large 10-kb transcript including *sdhCDAB-sucABCD* has been detected. The transcript starting in front of *sucA* terminates at the 3′ end of *sucD* (13, 75). Expression from the *sdhC* promoter is depressed by anaerobiosis, inclusion of glucose in the growth medium, and inactivation of either the *crp* or the *cya* gene. Binding sites for ArcA, Crp, and Fnr have been identified in this promoter region (55). The promoter upstream of the *sucA* gene is repressed by integration host factor (IHF) and partially activated by σ^S. It is not repressed by glucose or anaerobiosis. Binding sites for ArcA, Fnr, and IHF have been identified in this promoter region. It is believed that the strong glucose repression (Table 1) is due to read-through from the *sdh* operon (13).

As for the *cspA* and *ompF* genes, Crp appeared to activate transcription of the *sdh* and *suc* genes although the activation by Fur was less pronounced. As noted above, the *sdh* operon is subject to regulation by RyhB, and the same could be true of the *suc* operon. The consequences of the loss of both Crp and

TABLE 2. Real-time PCR analysis of gene expression for genes that are regulated by both Crp and Fur in *E. coli*

Category and gene	Gene expression ratios measured by:							
	Microarray				Real-time PCR			
	fur/wt	*crp*/wt	*crp fur*/wt	*crp fur*/*fur*	*fur*/wt	*crp*/wt	*crp fur*/wt	*crp fur*/*fur*
Category A								
fepA	2.5	0.62	0.8	0.33	64.5 ± 8.6	1.3 ± 0.2	5.7 ± 0.6	0.1 ± 0.01
entC	2.8	0.62	1.2	0.44	85.0 ± 10.2	1.3 ± 0.1	9.7 ± 0.8	0.1 ± 0.01
ybiL	8.9	0.85	2.5	0.29	53.8 ± 5.4	1.1 ± 0.1	5.0 ± 0.4	0.1 ± 0.01
fecA	3.4	0.83	1.7	0.48	11.6 ± 2.1	0.8 ± 0.1	4.1 ± 0.4	0.4 ± 0.03
cirA	3.3	0.79	0.9	0.28	41.9 ± 5.8	1.0 ± 0.01	3.9 ± 0.5	0.1 ± 0.01
Category B								
sodB	0.2	1.0	0.8	4.7	0.03 ± 0.01	0.8 ± 0.1	0.2 ± 0.04	6.3 ± 1.5
Category C								
cspA	0.41	0.43	0.12	0.29	0.4 ± 0.04	0.5 ± 0.1	0.2 ± 0.03	0.4 ± 0.1
ompF	0.32	1.9	0.12	0.39	0.1 ± 0.01	0.2 ± 0.01	0.01 ± 0.003	0.1 ± 0.04
Category D								
glgS	2.5	1.7	8.8	3.6	1.7 ± 0.2	6.0 ± 0.7	20.8 ± 2.0	12.5 ± 1.2
rpsV	2.2	2.2	14.6	6.7	2.5 ± 0.2	12.8 ± 1.3	37.5 ± 4.8	14.8 ± 1.9

Fur appear to be less than the additive effects of the loss of either one alone for both of these operons.

Finally, the *fumA* gene, encoding fumarase, shows strong apparent activation by both Crp and Fur, but the loss of both of these transcription factors together, as for the *sdh* and *suc* operons, gave a much less than additive effect. *fumA* gene expression, like *sdh* operon expression, is negatively regulated by RyhB (45).

Genes apparently repressed by both Crp and Fur. Nine genes appeared to be strongly repressed by both Crp and Fur, and in all such cases loss of both Crp and Fur resulted in greater than additive enhancement of gene expression (Table 1). Three of these genes encode proteins of unknown function, but the *cfa* gene encodes a cyclopropane fatty acid synthase (CFA synthase); the *glgS* gene encodes a glycogen biosynthetic protein, and *rpsV* encodes a 30S ribosomal protein, S22 or SRA (see below). The *osmE* gene, an osmotically induced gene, encodes an envelope protein of unknown function; the *tktB* gene encodes transketolase II, and the *yggB* gene encodes a mechanosensitive channel of the MscS family (58). Several of these genes are subject to σ^S control as noted below.

During growth of *E. coli*, CFA synthase activity is detected throughout the growth cycle, but the highest activity is observed upon entering the stationary phase. The *cfa* gene is transcribed from two promoters, one requiring σ^{70} and the other requiring σ^S. No binding sites for transcriptional regulators have been reported for this gene. CFA synthase activity is unstable in vivo, explaining a decrease in activity when cells progress into stationary phase (74).

The *glgS* gene encodes a 7.9-kDA protein that stimulates glycogen synthesis. However, the biochemical function of GlgS has not been elucidated. Two promoters have been identified and transcription from both promoters requires functional *cya* and *rpoS* genes (33).

Gene *rpsV* (*sra*) encodes the ribosome-associated protein D, the abundance of which increases upon entry into stationary phase. For this reason, protein D has been renamed the SRA (stationary phase-induced ribosome-associated) protein. This gene possesses a single promoter that is partly dependent on σ^S. Transcription from this promoter is decreased in mutants lacking σ^S, FIS (factor for inversion stimulation), HNS (histone-like nucleoid structuring protein), cAMP, and ppGpp (37).

The *osmE* gene displays biphasic expression when cells are grown at elevated osmotic pressure. Induction is detected during exponential growth, but stronger induction occurs at the onset of stationary phase. Transcription of *osmE* starts at a single promoter. DNA supercoiling is involved in *osmE* expression during exponential growth in media of high osmolarity, but stationary phase induction depends on σ^S (10).

All nine genes respond similarly to the loss of either Crp or Fur, showing about twofold enhancement of gene expression in either case. The simultaneous loss of both proteins showed synergistic enhancement, with increased expression, relative to the wild-type strain, of about 10-fold. These results suggest that both Crp and Fur somehow antagonize the repressive effects of the other transcription factor. While all but one of these genes exhibit putative Crp binding sites in their control regions, none could be shown to have a recognizable Fur binding site, suggesting an indirect mechanism, possibly involving interaction of Fur with the Crp binding site (see Discussion and Table 4).

Confirmation of transcriptome results using real-time PCR. Ten genes from various categories of Table 1 were selected for confirmation using real-time PCR. Table 2 shows the results of these analyses. The five genes analyzed from category A are *fepA*, *entC*, *ybiL*, *fecA*, and *cirA*. When expression levels are measured by real-time PCR, a much more dramatic increase in transcript level is observed for these genes when *fur* is inactivated compared with the levels measured with the microarrays. In the *crp fur* mutant, the transcript levels of these genes are dramatically decreased compared to those of the *fur* mutant, regardless of the method used to measure relative expression level. Nevertheless, relative values are in good agreement. In a similar fashion, expression levels for *sodB* from category B, *cspA* and *ompF* from category C, and *glgS* and *rpsV* from category D were analyzed by real-time PCR. Qualitative agree-

TABLE 3. Oxidation of various carbon sources by *E. coli* wild type and isogenic *crp, fur,* and *crp fur* mutants[a]

Carbon source	Reaction with[b]			
	wt	*crp*	*fur*	*fur crp*
Adenosine	+	+	+	±
β-Methyl-D-glucoside	+	+	+	±
D-Galactose	+	±	+	±−
D-Gluconic acid	+	+	+	±−
D-Mannitol	+	−	+	±
D-Serine	+	±	+	−
Glucose-1-phosphate	+	+	+	±−
Glycerol	+	−	+	−
Inosine	+	±	+	+
L-Alanine	+	±	+	−
L-Alanyl-glycine	+	+	+	±−
N-Acetyl-D-glucosamine	+	+	+	±−
Pyruvic acid methyl ester	+	+	+	±
Thymidine	+	±	+	±−

[a] Oxidation of all compounds was determined in 96-well Biolog or Phenotypic Microarray plates.

[b] +, positive reaction (blue or dark blue); ±, fairly positive reaction (light blue); ±−, poorly positive reaction (very faint blue); and −, negative reaction (transparent, no blue color).

ment when using the two methods for measuring relative expression levels was always obtained (Table 2). The results obtained by real-time PCR are thus in good agreement with the data obtained from the transcriptome analysis presented in Table 1 although a systematic quantitative difference was consistently observed.

Phenotypic studies. Table 3 summarizes the results when substrate oxidation was measured for wild-type, *crp*, *fur*, and *crp fur* mutant strains. In general, the *fur* mutation by itself showed no effect on the apparent rates of oxidation although the *crp* mutation alone frequently had dramatic effects. The double mutant usually showed depressed oxidation relative to the *crp* mutant although the opposite was sometimes observed (Table 3). For several substrates, the *crp* mutant strain showed positive responses while the *crp fur* double mutant strain showed significantly reduced responses. However, for D-mannitol and inosine the *crp* mutant gave significantly reduced oxidation responses while the double mutant gave more-positive responses. Since the wild-type and *fur* mutant strains showed fully positive responses, the results suggest that Fur and Crp may interact to regulate cellular carbon metabolic activities in previously unrecognized ways. For a more complete description of the phenotypic analyses, see Table S3 in the supplemental material.

The phenotypic data cannot be compared directly with the transcriptome data because the substrates listed in Table 3 are not subject to metabolism by enzymes and transporters encoded by genes listed in Table 1 and the conditions are not comparable. However, there is agreement in the following respects: (i) the *crp* mutation often depresses the rate of oxidation as expected; (ii) the *fur* mutation sometimes has no effect by itself, but usually modifies the phenotype of the *crp* mutant; and (iii) for a majority of substrates, a cooperative effect of the two mutations is observed, but (iv) for a minority of the substrates (D-mannitol and inosine), apparently antagonistic effects are observed. It seems that Fur and Crp can somehow act either cooperatively or antagonistically to produce a phenotype. Whether these effects represent direct or indirect consequences of the loss of Crp and Fur cannot be ascertained.

Effect of glucose on growth of *E. coli* strains. The four strains used in these studies were grown in LB media ± 0.4% glucose in shake flasks under exactly the same conditions used for the microarray and real-time PCR experiments for quantitative measurement of growth rates. The growth curves are shown in Fig. 2. In the absence of glucose (Fig. 2A), the *fur* mutant strain

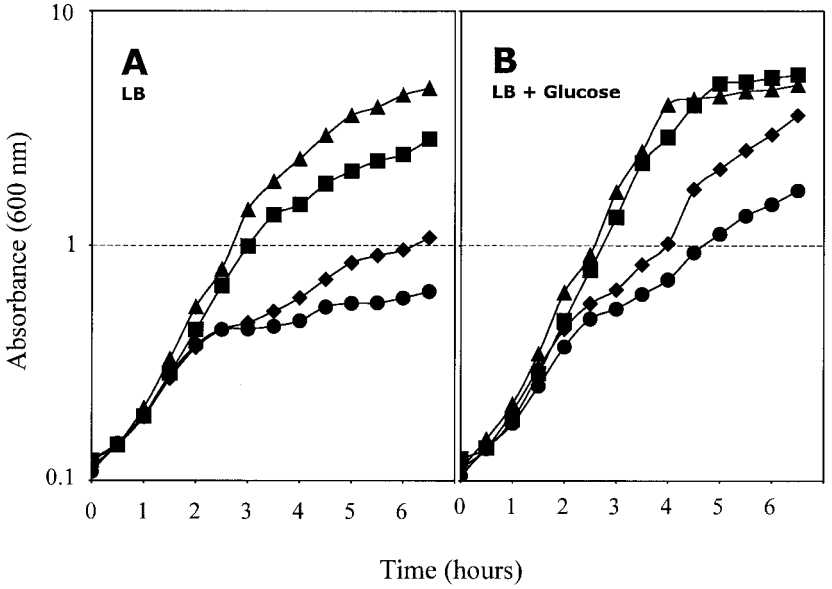

FIG. 2. Effect of glucose on growth of *E. coli* strains used in this study. Cells were grown in LB medium (A) or in LB medium plus 0.4% glucose (B) at 37°C with shaking (250 rpm) in 25 ml of medium in 250-ml Erlenmeyer flasks. These conditions are identical to those used for the transcriptome and real-time PCR experiments. Strains were as follows: triangles, wild type (BW25113); diamonds, *crp* mutant; squares, *fur* mutant; circles, *crp fur* double mutant.

grew almost as well as the isogenic wild-type strain. The *crp* mutant grew decidedly more slowly, and the *crp fur* double mutant grew the slowest. Addition of glucose (Fig. 2B) increased the growth rates of all strains. Thus, the wild-type and *fur* mutants grew equally well in the presence of glucose, significantly faster than observed for the LB grown cells. The *crp* mutant and the *crp fur* double mutant also showed substantial increases in growth rate when glucose was present. These relative growth rates were confirmed by examining colony sizes after growth on LB plus glucose agar media and by measuring growth rates in minimal liquid media (data not shown).

DISCUSSION

The comparative analyses of transcriptome data from wild-type, *crp*, *fur*, and *crp fur* mutant strains allowed the identification of genes that are subject to regulation resulting from the loss of both Fur and Crp. Many of these genes were known to belong to the Fur regulon while others were known to belong to the Crp regulon. Some of these genes, however, had not previously been known to belong to either regulon, and very few of them were known to belong to both.

Since all the genes examined in this report showed changes in transcript levels in the *crp fur* double mutant relative to the *fur* single mutant, it was possible that they would have Crp binding sites in or near their promoters. A search for putative Crp binding sites in the genes shown in Table 1 was performed using the GRASP-DNA program (68). This search yielded significant matches to the reported Crp binding consensus sequence in or near many of these genes (Table 1). Detailed descriptions of Crp and Fur binding sites using the GRASP-DNA program (including their positions, sequences, and scores) are reported in Table S2A and published regulatory information about these genes is summarized in Table S2B in the supplemental material. Some of these data may be relevant to the control of gene expression in response to the availability of Fur and Crp. In a few cases (e.g., the *cirA* gene), direct binding of both Crp and Fur to the upstream control region has been reported (25, 26).

Expression levels of all of the genes listed in Table 1 are significantly altered in response to the lack of Fur and Crp. The major subset of known Fur regulon genes displaying negative regulation by Fur but positive apparent regulation by Crp encode proteins related to iron transport and chelation (Table 1). Most of these genes are repressed by Fur in the presence of excess iron, and therefore showed increased expression in the *fur* mutant under the growth conditions used. These Fur-repressed, Crp-activated genes are grouped into seven transcriptional units that have been shown to be regulated directly by Fur binding to their promoter regions (*fepA-entD*, *fes-entF-fepE*, *entCEBA-ybdB*, *ybiLXI*, *yncE*, *fecABCDE*, and *cirA*). Expression of the *aceEF* operon showed strong glucose activation that was abolished by both the *crp* mutation and the *fur* mutation; while both mutations enhanced operon expression about 2.5-fold, the double mutant showed about threefold-depressed expression relative to the *fur* or *crp* single mutant. The *entC* operon showed mild glucose repression, mild repression by the loss of Crp, about threefold activation by the loss of Fur, and restoration of the wild-type gene expression level in the *crp fur* double mutant with complete loss of glucose repression. The results obtained by real-time PCR were qualitatively similar, but the regulatory effects were generally larger in magnitude. Thus, we observed good qualitative agreement although there were quantitative differences. It should be pointed out that transcriptional initiation of the *fecABCDE* operon is dependent on both Fur clearance from the promoter region and the subsequent binding of RNA polymerase containing the FecI sigma subunit (70). Since *fecI* expression proved to be regulated by Fur and Crp, some of the effects reported for the *fec* structural genes could be indirect.

The *aceBAK* operon and the *sodB* gene (Table 1) appeared to be activated by Fur and repressed by Crp in a *fur* background. The *aceBAK* operon (76) is subject to strong glucose repression, and this repression is abolished by either the *crp* or the *fur* mutation. The *crp fur* double mutant showed about sixfold-enhanced expression over either of the two single mutants. Overlapping putative Crp and Fur binding sites found upstream of the *aceB* gene (Fig. 1 and supplemental Table S2A) suggest direct control of this operon by both global regulators. The fact that activation of the *aceBAK* operon depends on the presence of both Crp and Fur (for glucose-repressible expression) or depends on the absence of both transcription factors (for glucose-independent expression) additionally suggests that protein-protein interactions play a role. However, the *aceBAK* operon has previously been reported to be regulated in a complex fashion by four transcription factors: IclR, IHF, Cra, and FadR (Fig. 1) (12). The operon-specific IclR repressor binds to three sites in the *aceB* promoter region. Two of these sites overlap each other and also overlap the region to which Crp and Fur may bind (Fig. 1). IclR also autoregulates its own expression (76). It is possible, therefore, that Crp and Fur regulate *iclR* expression and/or that IclR interacts with Crp and/or Fur as well as with the C-terminal domain of the RNA polymerase alpha-subunit (76). Glucose-independent high-level expression in the absence of Crp and Fur may occur if IclR repression is dependent on an interaction with either Crp or Fur or both. Additionally, physical interactions between Crp and/or Fur with other regulators such as IHF, Cra, and FadR could be important.

Expression of the *sodB* gene (Table 1) has been shown to be indirectly regulated by Fur via potential antisense interactions involving a small RNA encoded by the *ryhB* gene (21, 45, 46, 47). This regulatory effect of RyhB, however, does not appear to fully explain the results reported here. Since no good Crp or Fur binding sites could be identified upstream or within the *sodB* gene, *sodB* regulation is likely to be indirect and prove complex.

A few operons listed in Table 1 (such as *sdhC* and *fumA*) that appear to be subject to dual control by Crp and Fur are also known to be regulated by RyhB (24, 45). However, to what extent this small RNA or other small RNAs that influence mRNA stability account for the results reported here has yet to be determined.

In the case of genes included in Table 1, at least one Crp binding site per operon but no Fur sites could be identified. The mechanism of regulation of these genes by Fur may be indirect and may prove to be complex, possibly involving σ^S. A careful analysis of several known Crp and Fur binding sites revealed conserved nucleotides common to both of the binding sequences (Table 4). Therefore, it is possible that Crp binding

TABLE 4. Similarities between the Crp and the Fur binding sequences

Site	Gene[c]	Sequence																				
Crp binding site[a]	Consensus	A	A	T	T	G	T	G	A	T	C	T	A	G	A	T	C	A	C	A	T	T	T
	Alternate										t	c	g	t	t						a	a	
	cir				G	A	**T**	A	**A**	**T**	*T*	G	T	*T*	**A**	**T**	**C**	G	T	T	**T**	G	
	fepA-fes				G	A	**T**	A	**A**	C	*T*	A	T	*T*	*T*	G	**C**	**A**	T	T	**T**	G	
	fepB-entC (1)				A	A	**T**	A	**A**	**T**	G	*C*	T	*T*	C	**T**	**C**	**A**	T	T	**T**	**T**	
	fepB-entC (2)				A	T	A	A	**A**	**T**	G	A	T	A	**A**	**T**	**C**	**A**	T	T	*A*	**T**	
Fur binding site[b]	*fur*				**T**	A	**T**	A	**A**	**T**	G	A	T	A	C	G	**C**	**A**	T	T	*A*	**T**	
	sodA (1)				G	**G**	C	A	T	**T**	G	A	T	A	**A**	**T**	**C**	**A**	T	T	**T**	**T**	
	sodA (2)				G	T	**T**	C	**A**	**T**	*T*	A	T	A	G	**T**	T	**A**	A	T	**T**	*A*	
	tonB (1)				G	A	A	T	**A**	**T**	G	A	T	*T*	G	C	T	**A**	T	T	**T**	G	
	tonB (2)				**T**	**G**	A	A	**A**	**T**	G	A	T	*T*	**A**	**T**	G	**A**	**C**	T	**T**	C	

[a] Forty-one known Crp binding sequences were aligned using the Clustal X program, and the most dominant nucleotide (upper case) at each position was used to compute the consensus sequence. At some positions another nucleotide also occurred in a significant number of sequences, and therefore this nucleotide (lower case) is also shown as the alternate.

[b] Nine known Fur binding sequences were compared with the Crp consensus sequence. Nucleotides in the Fur site that are identical to the most dominant nucleotide in the Crp site are shown in bold, while those that are identical to a second significant residue in the Crp consensus sequence are shown in italics.

[c] Numbers in parentheses are the site numbers (given when two Fur binding sites are present).

sites are recognized by Fur and vice versa. Since the various Crp and Fur binding sites differ in sequence, it is probable that cross-binding specificity will be site specific.

A regulatory interaction between the Fur and Crp regulons had been reported previously. Transcriptional levels of *fur* gene expression were reported to be repressed by Fur and stimulated by Crp (18). Both Fur repression and Crp stimulation of transcription caused modest changes in *fur* gene expression during exponential growth (≤2-fold). In our studies, a total lack of the Fur protein in the *fur* mutant eliminated the possibility that the consequences of the loss of Crp in this genetic background could be explained by regulatory effects of Crp loss on *fur* gene expression. Thus, the effects of the *crp* mutation on the expression of other target genes in a *fur* genetic background must be independent of Fur. The observations of De Lorenzo et al. (18) are evidently not relevant to most of the results reported here. It is possible that the observed effects depend on direct binding of both factors to the DNA, to the binding of one factor to the other, or to regulation of elements higher in the hierarchy of transcriptional regulation.

Examination of transcriptome data for genes encoding proteins involved in transcriptional initiation revealed that expression of the *rpoD* gene, encoding the sigma-70 subunit of RNA polymerase, was significantly lower in the *crp* (fivefold) and *crp fur* mutants (twofold) when compared to wild-type and *fur* transcript levels, respectively (data not shown), and these results were confirmed using real-time PCR (Z. Zhang and M. H. Saier, Jr., unpublished results). It was therefore possible that the reduced transcript levels observed for Fur-repressed genes in the *crp* mutant were the result of decreased availability of sigma-70. Our preliminary experiments, however, suggest that this was not the case for class A genes (Table 1). Overexpression of the *rpoD* gene did not enhance expression of any of the genes listed in Table 2 (Zhang and Saier, unpublished). In the case of the *sodB* gene, the observed increase in transcript level in the *crp fur* double mutant when compared to the *fur* single mutant might be explained assuming that sigma-70 limitation causes a lower expression level of the small RNA RyhB which destabilizes the *sodB* mRNA. This possibility has yet to be examined.

As noted above, many genes subject to simultaneous Fur and Crp regulation are apparently repressed by Fur and activated by Crp in a *fur* mutant. However, the three other possible consequences of the loss of these two transcription factors in a wild-type background indicate that gene expression can be activated and repressed, activated and activated, or repressed and repressed by Fur and Crp, respectively. Although we confirmed the transcriptome results by real-time PCR for representative genes, the detailed regulatory mechanisms have been examined in very few of the genes listed in Table 1.

Crp regulation of Fur-controlled genes and Fur regulation of Crp-controlled genes could allow integration of signals for iron and carbon sufficiency. Because iron is required for some carbon metabolic processes, particularly aerobic Krebs cycle intermediary metabolism and electron flow, but not others, such as anaerobic sugar metabolism, the signals coordinating iron and carbon metabolism are likely to be complex. This report provides a glimpse into the nature and consequences of these interactions.

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