JOURNAL OF BACTERIOLOGY, Aug. 1995, p. 4628–4637 Vol. 177, No. 16
0021-9193/95/$04.00+0

Fur Regulon of *Salmonella typhimurium*: Identification of New Iron-Regulated Genes

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Received 20 March 1995/Accepted 1 June 1995

In order to identify genes belonging to the Fur regulon of *Salmonella typhimurium*, a bank of 10,000 independent *S. typhimurium* Mud*J* insertion mutants was screened for *lacZ* fusions regulated by the iron response regulator Fur. In parallel, a plasmid gene bank of *S. typhimurium* consisting of 10,000 independent clones was screened for Fur-regulated promoters or iron binding proteins by the Fur titration assay (FURTA). Fur-regulated Mud*J* insertions and Fur-regulated promoters were mapped. In addition, iron-regulated promoter activities of transcriptional fusions from Mud*J* insertions and FURTA-positive clones were quantified. The nucleotide sequences of 11 FURTA-positive plasmids and of short fragments of DNA flanking three Mud*J* insertions were determined. By these methods we identified 14 Fur-regulated genes of *S. typhimurium*. For 11 of these genes, Fur-regulated homologs have been described in *Escherichia coli* or *Yersinia enterocolitica*, including *fhuA*, *fhuB*, *fepA*, *fes*, *fepD*, *p43*, *entB*, *fur*, *foxA*, *hemP*, and *fhuE*. In addition, we identified three genes with homologs in other bacteria which have not previously been shown to be Fur regulated.

Iron availability is an environmental stimulus to which bacteria respond by regulating expression of genes. The set of genes which is regulated in response to changes in iron concentration forms the iron stimulon. Included in this group are genes which may be under the control of several different regulators; of these, the best characterized is Fur. By using ferrous ions as corepressors, Fur has been shown to sense the intracellular iron concentration (9). The Fe(II)-Fur complex binds a regulatory DNA sequence, designated Fur-box, which is located in the promoter region of Fur-regulated genes (11, 16). Fur regulation seems to be a general feature of iron metabolism in gram-negative bacteria, as numerous homologs of Fur have been described to date. Genes whose expression are under the control of this regulatory protein form the Fur regulon.

The Fur regulon contains genes which are involved in iron acquisition, a function which is essential for bacterial multiplication. For animal pathogens, efficient strategies for iron uptake are important adaptations for growth in the host, since iron availability is limited. Nonspecific host defense mechanisms, collectively known as the iron withholding response, further lower iron levels in the body during bacterial infection (54). Although it is clear that iron-uptake systems are a prerequisite for bacterial multiplication, the genes involved in iron uptake during growth of *Salmonella typhimurium* in its host have not been described. A further host adaptation mechanism which requires Fur for regulation is the acid-tolerance response (17). *S. typhimurium*, in order to cause infection, must withstand the acid pH of the stomach and possibly of the intracellular compartment in which it resides. As a first step in identifying factors involved in iron uptake and acid tolerance within the host, we attempted to identify Fur-regulated genes in *S. typhimurium*.

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For the identification of genes belonging to a regulon, the isolation of operon fusions to a reporter gene created by transposon mutagenesis has been used most frequently. An alternative approach has recently been developed which is useful for the identification of Fur-regulated genes of a wide variety of gram-negative and even some gram-positive bacteria (48). This technique, called FURTA (for Fur titration assay), is based on the observation that a Fur-box introduced on a multicopy plasmid is able to compete with chromosomal Fur-boxes for the Fe(II)-Fur repressor. A plasmid-encoded Fur-box is introduced into a strain carrying a chromosomal reporter gene, which is expressed from a Fur-regulated promoter. Upon introduction, the plasmid-encoded Fur-boxes titrate the Fe(II)-Fur complex, allowing expression of the reporter gene.

In this study we use two approaches, the isolation of transcriptional fusions created by transposon mutagenesis and FURTA, for the identification of Fur-regulated genes of *S. typhimurium*. Our results allow a direct comparison of these approaches and their usefulness in identifying genes of the Fur regulon.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains used are listed in Table 1. All bacteria were cultured aerobically at 37°C. Antibiotics, when required, were included in the culture medium or plates at the following concentrations: kanamycin, 60 mg/liter; chloramphenicol, 20 mg/liter; and carbenicillin, 100 mg/liter. In order to create iron-limiting or iron-sufficient growth conditions, 0.2 mM 2,2′-dipyridyl or 0.04 mM $FeSO_4$ was added, respectively.

The bacteria were tested for auxotrophy on M9 agar plates (36). If required, histidine was included at a concentration of 0.04 mg/ml.

Iron-regulated *lacZ* operon fusions of *S. typhimurium* were screened on MacConkey agar plates containing either 0.04 mM $FeSO_4$ (M+Fe) or 0.2 mM 2,2′-dipyridyl, as described earlier (22). MacConkey agar plates used for the FURTA contained 0.04 mM $FeSO_4$.

Chrome azurol S (CAS) plates used to assay for siderophore production were prepared according to Schwyn and Nielands (44). Evans Blue-uranine plates were prepared according to Bochner (6).

The medium for Mn^{2+} selection of *fur* mutants reported previously (24) was prepared with one modification: the chelator desferal was replaced by 2,2′-dipyridyl, since the former can be utilized as a siderophore by *Salmonella* spp. (25).

Utilization of ferrioxamine was detected by an agar diffusion assay. The strain

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference[a]
S. typhimurium		
ATCC 14028	Wild-type strain	ATCC
IR 715	Nalidixic acid-resistant derivative of ATCC 14028	49
AR396	14028 Mud*J* mutant	This study
AR895	14028 *sidK*::*mudJ*	This study
AR1258	14028 *entB*::*mudJ*	This study
AR3675	14028 *fhuB*::*mudJ*	This study
AR6687	14028 Mud*J* mutant	This study
AR8412	14028 Mud*J* mutant	This study
AR8439	14028 *ent*::*mudJ*	This study
SF895	AR895 *fur*	This study
SF1	14028 *fur*	This study
SL1346	*aroA554*::Tn*10*	B. Stocker
CL 1509	14028 *aroA554*	This study
E. coli		
S17-1 λ*pir*	*prp thi recA hsdR*, chromosomal RP4-2 (Tn*1*::ISR*1* *tet*::Mu Km::Tn*7*), λ*pir*	Laboratory collection
DH5α	*endA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 recA1 gyrA relA1* Δ(*lacZYA-argF*)*U169 deoR* [ϕ80 *dlac* Δ(*lacZ*)*M15*]	Laboratory collection
H1717	*araD139* Δ*lacU169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR aroB fhuF*::λplacMu	25
CC118	*araD139* Δ(*ara leu*) Δ*lacY74 phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*	34
Plasmids		
pGP704	R6K ori, *bla*	37
pMAP	pGP704, *Bln*I site in polylinker	This study
pMAP1	pMAP, *'lacZ'*	This study
pBluescriptKS	colE1, *bla*	Stratagene
pMH152	pACYC184, *fur* mutant	9
pMH15	pACYC184, *E. coli fur*	24
pUJ10	*'lacZ' phoA*, *bla*	14
pSUKS1	pSU19 with *Hae*II fragment of pBCSK+	40

[a] ATCC, American Type Culture Collection.

to be tested was poured in 3 ml of 2% Noble agar onto a nutrient broth-dipyridyl agar plate. Filter paper disks impregnated with ferrioxamine (10 μl of a 1-mg/ml solution of desferal in 0.1 M $FeCl_3$) were laid onto the top agar, and after incubation overnight at 37°C, the zone of growth around the filter disk was measured.

Genetic techniques. P22 was used for generalized transduction of transposon insertions into different genetic backgrounds. Transductants were routinely streaked on Evans Blue-uranine plates to detect phage contamination before use in further experiments.

Mud*J* insertion mutants of *S. typhimurium* were isolated by the method of Hughes and Roth (27). In brief, the strain 14028 was transduced with a P22 lysate of TT10288, and Mud*J* insertions were selected on Luria-Bertani agar plates containing kanamycin. The donor (TT10288) carries Mud*J* inserted in *hisD*. The transductants were therefore screened for homologous recombination of Mud*J* into the *hisD* gene by comparison of the growth on M9 and M9-histidine agar plates. Only histidine prototrophs were further investigated. To detect differences in β-galactosidase expression under different iron concentrations, mutants were streaked both on plates containing M+Fe and on MacConkey agar plates containing 0.2 mM 2,2′-dipyridyl. Mutants with differences in color on these two media were characterized further by cross-streaking on M+Fe and overlaying with a filter strip soaked in a solution of 0.02 mM dipyridyl, as described previously (22). The quantitative determination of β-galactosidase units has been published elsewhere (36).

The selection for *fur* mutants of *S. typhimurium* was carried out according to a protocol of Hantke (24). The dependence of β-galactosidase expression on Fur was determined by transforming mutants with plasmid pMH152 (9). The promoter activity of DNA fragments identified by FURTA was investigated by cloning fragments into the promoter probe vector pUJ10 (14). The quantitative determination of alkaline phosphatase activity has been described elsewhere (10).

FURTA. Chromosomal DNA of *S. typhimurium* 14028 was digested partially with the restriction enzyme *Sau*3A or to completion with *Eco*RV and *Hin*dII. Fragments between 0.5 and 3 kb were cloned into the vector pSUKS1 (40), digested with *Bam*HI or *Sma*I, respectively, and transformed into *Escherichia coli* DH5α. A total of ca. 10,000 independent colonies were pooled into four groups, and the plasmid DNA was isolated from these pools. FURTA was performed as previously described (48). In brief, plasmid DNA from each pool was used to transform *E. coli* H1717, transformants that were red rather than white on M+Fe were selected, and plasmid DNA was isolated and retransformed into H1717 to confirm the FURTA-positive phenotype.

Construction of strain derivatives for mapping with pulsed-field gel electrophoresis. For physical mapping of Mud*J* insertion sites and FURTA clones, a set of suicide vectors was used to introduce *Xba*I and *Bln*I restriction sites at the map location of the cloned DNA. A *Bln*I site was introduced into the polylinker of pGP704 (37) by creating a linker with the oligonucleotides 5′-TCGATCC TAGG-3′ and 5′-TCGACCTAGGA-3′. The oligonucleotides were denatured and annealed, and the resulting linker was cloned into the *Sal*I site of pGP704 to create pMAP (Fig. 1). For mapping of Mud*J* mutants, a 1.8-kb *Eco*RI-*Eco*RV fragment of the *E. coli lacZ* gene was isolated from pHSS6 and cloned into *Eco*RI-*Eco*RV-cut pMAP to yield pMAP1 (Fig. 1). *E. coli* S17-1λpir was used for the propagation of all suicide vector constructs and as a donor for the introduction of these constructs into *S. typhimurium* IR715 (49) by conjugation.

Pulsed-field gel electrophoresis. The preparation of agarose-embedded chromosomal DNA for pulsed-field gel electrophoresis was based on a protocol from Liu and Sanderson (32). In brief, cells were harvested from 5 ml of an early-log-phase bacterial culture, resuspended in 0.5 ml of prewarmed (37°C) cell suspension buffer (10 mM Tris–HCl [pH 7.2], 20 mM NaCl, 100 mM EDTA), and mixed with 0.5 ml of 2% InCert agarose which was dissolved in PBS (phosphate-buffered saline) and precooled to 37°C. The mixture was poured into molds (Bio-Rad) and allowed to solidify. The molds were digested with lysozyme (1 mg/ml; New England Biolabs) in a volume of 7.5 ml for 1 h at room temperature. The lysozyme solution was replaced by 7.5 ml of proteinase K (1 mg/ml; New England Biolabs) and incubated at 55°C for 48 h with shaking. Agarose-embedded DNA prepared in this manner can be stored in 7.5 ml of TE buffer (10 mM Tris-HCl [pH 7.2], 5 mM EDTA) at 4°C for several months without degradation.

For restriction digestion, an agarose block equivalent to about 0.05 to 0.1 ml was incubated for 15 min at room temperature in 0.2 ml 1× KGB buffer (35) in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1× KGB buffer containing 20 U of a restriction endonuclease (*Xba*I or *Bln*I), and the samples were incubated for 4 h at 37°C. The agarose blocks were then loaded on an agarose gel for pulsed-field gel electrophoresis.

Pulsed-field gel electrophoresis of a 1% agarose gel was performed in 0.5% Tris-borate-EDTA at 200 V with a pulse length of 6 to 150 s for 6 h, 6 to 12 s for 6 h, 26 to 36 s for 6 h, and 120 to 180 s for 6 h with a CHEF-DRII apparatus from Bio-Rad.

Recombinant DNA techniques. Plasmid DNA was isolated with ion-exchange

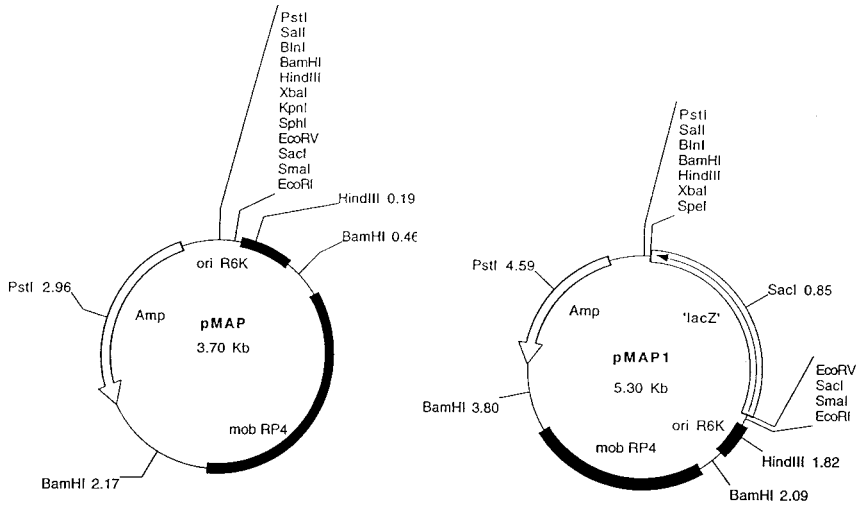

FIG. 1. Restriction maps of suicide vectors pMAP and pMAP1.

columns from Qiagen. Standard methods were used for restriction endonuclease analyses, ligation, and transformation of plasmid DNA (36). Sequencing was performed by the dideoxy-chain-termination method according to a protocol of Kraft et al. (30) with [α-^{35}S]dATP (Amersham) for labeling or with the ALF automated sequencer (Pharmacia).

Inverse PCR was performed with agarose-embedded chromosomal DNA from Mud*J* mutants, as described previously (5). In brief, an agarose block equivalent to approximately 0.05 to 0.1 ml was incubated for 15 min at room temperature in 0.2 ml 1× KGB buffer (35) in which sodium acetate was replaced by potassium acetate. The buffer was replaced with 1× KGB buffer containing 50 U of *Alu*I, and the samples were incubated for 4 h at 37°C. The samples were then incubated for 20 min at 72°C (to inactivate the restriction enzyme and to melt the agarose block). ATP (10 mM) and T4 DNA ligase (2 U) were added at 37°C, and ligation was performed overnight at 15°C. Inverse PCR was performed under standard conditions (29) in a total volume of 0.1 ml with 0.005 ml of the above-described ligation as a template and 0.1 ng of each of the following primers: 5′-CCAATTCTGCCCCGAATTAC-3′ and 5′-GTCGTGTAAAATAT CGAGTTCG-3′. These primers are complementary to the left end of Mud*J* (52). PCR products were cloned into the vector pCRII and transformed into the *E. coli* strain TA One Shot (TA cloning kit; Invitrogen).

Southern hybridization. Southern transfer of DNA onto a nylon membrane was performed as previously described (2). Labeling of DNA probes, hybridization, and immunological detection were performed with the DNA labeling and detection kit (nonradioactive) from Boehringer Mannheim. The DNA was labeled by random-primed incorporation of digoxigenin-labeled dUTP. Hybridization to the blot was performed at 68°C in solutions without formamide. Hybrids were detected by an enzyme-linked immunoassay with an antibody conjugate (anti-digoxigenin alkaline phosphatase conjugate) and the substrate AMPPD [3-(2′-spiroademantane)-4-methoxy-4-(3″-phosphoryloxy)phenyl-1,2-dioxethane; Boehringer-Mannheim]. The light emitted by the dephosphorylated AMPPD was detected with X-ray film.

Computer analysis. The nucleotide sequences were compared with those of SWISS-PROT, PIR(R), and GenPept at the National Center for Biotechnology Information with the program blastX and with sequences in GenBank and the EMBL Data Library with the program blastN (1, 20).

RESULTS

Screening for Mud*J* insertions in Fur-regulated genes. A bank of 10,000 independent Mud*J* insertion mutants of *S. typhimurium* 14028 was screened for differences in *lacZ* expression in response to variations in the available iron concentration on MacConkey agar plates. Of these mutants, 75 showed elevated levels of β-galactosidase expression in response to iron restriction. These fusions define genes belonging to the iron stimulon.

In order to identify fusions regulated by Fur, we compared expression of β-galactosidase in Fur-positive and Fur-negative strains. Negative complementation, which has been described by Braun et al. (9), was used to create a Fur-negative phenotype in the Mud*J* mutants analyzed. By introducing a mutated *fur* gene carrying a point mutation in the DNA binding domain on a multicopy plasmid (pMH152), inactive heterodimers are formed which are unable to bind DNA (9). As a result, the merodiploid strain behaves as a *fur* mutant with respect to expression of *fur*-regulated genes. Plasmid pMH152 was introduced by electroporation into each of the iron-responsive Mud*J* mutants. Both the Fur-negative transformant and the original mutant were streaked across dipyridyl on M+Fe plates in order to compare β-galactosidase expression, as described previously (48). We identified seven mutants in which the expression of β-galactosidase appeared to be Fur dependent. To confirm these data, the expression of β-galactosidase was quantified according to Miller (36) (Table 2). A comparison of β-galactosidase activity in the presence and absence of pMH152 showed that these seven transcriptional fusions were indeed regulated by Fur. Regulation of these seven fusions was affected to varying degrees by iron availability or the introduction of pMH152 (Table 2). The expression of fusions in two strains, AR1258 and AR8439, was regulated about 30-fold in response to iron availability. The experiments described below show that these mutations are in genes involved in siderophore biosynthesis. In contrast, fusions in the mutants AR895 and AR3675, later shown to be in the ferrichrome-uptake locus (*fhu*), were found to be regulated only three- to fourfold in response to iron concentration. Experiments described below show that in these two mutants, Mud*J* is inserted in homologs of the first and last gene of the *E. coli fhuACDB* operon, respectively.

Since pMH152 contains the *E. coli fur* gene, the effect of the negative complementation method on gene expression was compared with the effect of the inactivation of the chromosomal copy of *fur* in *S. typhimurium*. For this purpose, a *S. typhimurium fur* mutant was selected by growth of strain 14028 on media containing high concentrations of Mn^{2+} and low concentrations of iron (24). Under these conditions, a Fur-Mn^{2+} repressor is formed so that *fur*$^+$ cells cannot grow because they are starved for iron. In contrast, *fur* mutants can express their iron-uptake systems and can therefore form colonies. One such mutant was designated SF1. The Mud*J* insertion in strain AR895 was introduced into SF1 by P22 transduction, yielding strain SF895. The *fur* mutation in SF895 was complemented with the cloned *fur* gene of *E. coli* (pMH15) (24). The expression of β-galactosidase in strains SF895 and

TABLE 2. Iron-dependent expression of *lacZ* in Mud*J* mutants in *fur*-positive and *fur*-negative backgrounds

Strain	Relevant genotype	β-Galactosidase activity of Mud*J* mutants (Miller U)[a]:			
		Without pMH152		With pMH152	
		With Fe[b]	Without Fe[c]	With Fe	Without Fe
S. typhimurium					
AR396	ND[d,e]	16 ± 6.2	182 ± 77	93 ± 44	248 ± 107
AR895	*sidK*::Mud*J*	81 ± 18	329 ± 22	316 ± 79	379 ± 53
AR1258	*entB*::Mud*J*	13 ± 2	375 ± 64	94 ± 37	352 ± 71
AR3675	*fhuB*::Mud*J*	33 ± 13	138 ± 23	83 ± 21	91 ± 30
AR6687	ND[d]	38 ± 0.7	163 ± 24	104 ± 8.5	118 ± 3.5
AR8412	ND[d]	4.8 ± 0.8	134 ± 22	38 ± 19	140 ± 39
AR8439	*ent*::Mud*J*	9.8 ± 3.2	319 ± 79	152 ± 76	541 ± 363
SF895	*fur sidK*::Mud*J*[f]	212 ± 59	227 ± 65	ND	ND
SF895 (pMH15)	*fur sidK*::Mud*J*[f], *E. coli fur* gene on plasmid	99 ± 26	158 ± 48	ND	ND
E. coli H1717	*fhuF*::Mud*J*	60 ± 44	699 ± 539	668 ± 49	630 ± 23

[a] Calculated according to Miller (36). Values given are averages of at least three independent experiments ± standard error.
[b] Grown in Luria-Bertani broth with 40 μM $FeSO_4$.
[c] Grown in nutrient broth with 0.2 mM 2,2′-dipyridyl.
[d] Uncharacterized Mud*J* insertion.
[e] ND, not determined.
[f] Chromosomal *fur* mutant.

SF895(pMH15) was quantified and compared with β-galactosidase expression in strains AR895 and AR895 (pMH152) (Table 2). The creation of a Fur^- phenotype by negative complementation with pMH152 and by mutational inactivation of *fur* abolished regulation of the β-galactosidase fusion in AR895 and SF895, respectively. In the case of SF895, regulation could be restored by the introduction of the cloned *E. coli fur* gene on plasmid pMH15, confirming that SF895 carries a mutation in *fur*.

Cloning and sequence analysis of DNA flanking Fur-regulated Mud*J* insertions. Inverse PCR was used to clone short fragments of DNA flanking the left end of Mud*J* of three mutants. The sequence of DNA immediately flanking the transposon insertion site was determined and compared with sequence entries in the databases at the National Center for Biotechnology Information. The 247-bp nucleotide sequence of transposon-flanking DNA of AR895 was determined, and the deduced amino acid sequence was found to have homology to amino acids 459 to 528 of *E. coli* FhuA, the outer membrane receptor for ferrichrome (Fig. 2A). The deduced amino acid sequence of 77 bp flanking Mud*J* in AR3675 was found to have homology with amino acids 278 to 302 of a second component of the ferrichrome-uptake system in *E. coli*, FhuB, which forms the cytoplasmic membrane permease (Fig. 2B). For mutant AR1258, 189 bp of the sequence flanking Mud*J* was determined. The deduced amino acid sequence was found to have homology with the enterobactin-synthesis enzyme EntB of *E. coli* (Fig. 2C). This comparison revealed that all three Mud*J* insertions were in genes with known homologs in *E. coli*: *fhuA*, *fhuB*, and *entB*. A homolog of *fhuA* is known to exist in *Salmonella* spp., in which it was designated *sidK* (8, 42). For four of the mutants we were unable to clone DNA flanking the insertion site.

Mapping and phenotypic characterization of Mud*J* insertions. Pulsed-field gel electrophoresis was used to determine the locations of the transposon insertions on the *Salmonella* chromosome. Macrorestriction maps of the *S. typhimurium* genome with the enzymes *Bln*I and *Xba*I have been published recently (32, 55). These restriction endonucleases cut the *S. typhimurium* chromosome 11 and 23 times, respectively. In order to map Mud*J* insertions on the *Salmonella* chromosome we constructed a suicide vector derivative of pGP704, termed pMAP, which contains *Xba*I and *Bln*I restriction sites for physical mapping and which can be propagated in the *E. coli* host S17-1λpir (Fig. 1) (46). We then cloned a fragment of the *lacZ* gene into pMAP, giving rise to pMAP1 (Fig. 1). The suicide vector pMAP1 was conjugated into *S. typhimurium* Mud*J* mutants. This vector will integrate into the chromosome by homologous recombination between its internal *lacZ* fragment and the *lacZ* gene of Mud*J*, thereby introducing new *Xba*I and *Bln*I restriction sites into the genome. After restriction digestion of chromosomal DNA from a Mud*J*::pMAP1 mutant, the fragment in which the transposon is inserted will disappear, while two new, smaller fragments can be detected after separation by pulsed-field gel electrophoresis. The map positions calculated for AR895, AR3675, and AR1258 corresponded to the map positions for *fhuA*, *fhuB*, and *entB* on the *E. coli* chromosome, respectively (3). Although these genes have not been sequenced in *Salmonella* spp., homologs are known to be located at 4 to 5 min (*sidK*) and at approximately 14 min (*ent* locus), which is confirmed with the Mud*J* insertions at these loci (42) (Table 3).

All mutants were tested for siderophore production by growth on CAS agar. Colonies which produce the siderophore enterobactin produce a yellow halo on CAS agar plates (44). Only AR1258 and AR8439 failed to produce a halo around colonies on CAS agar plates. These two mutants mapped at 14 min, the location of the enterobactin-synthesis genes (42). Phenotype and map location were further supported by sequence analysis from transposon-flanking DNA from the Mud*J* insertion in AR1258, which showed homology with *entB* from *E. coli* (31, 38). AR6687 formed colonies with halos strongly reduced in size on CAS agar plates, and the corresponding insertion was located at 15 min.

In order to identify the FhuA (SidK) receptor, outer membranes of *S. typhimurium* 14028 and AR895 were purified, and their protein profiles were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A protein of 78 kDa present in the parent strain was missing from the outer membrane of AR895 (data not shown). This result indicated that in AR895, the Mud*J* insertion had inactivated the *Salmonella fhuA* homolog.

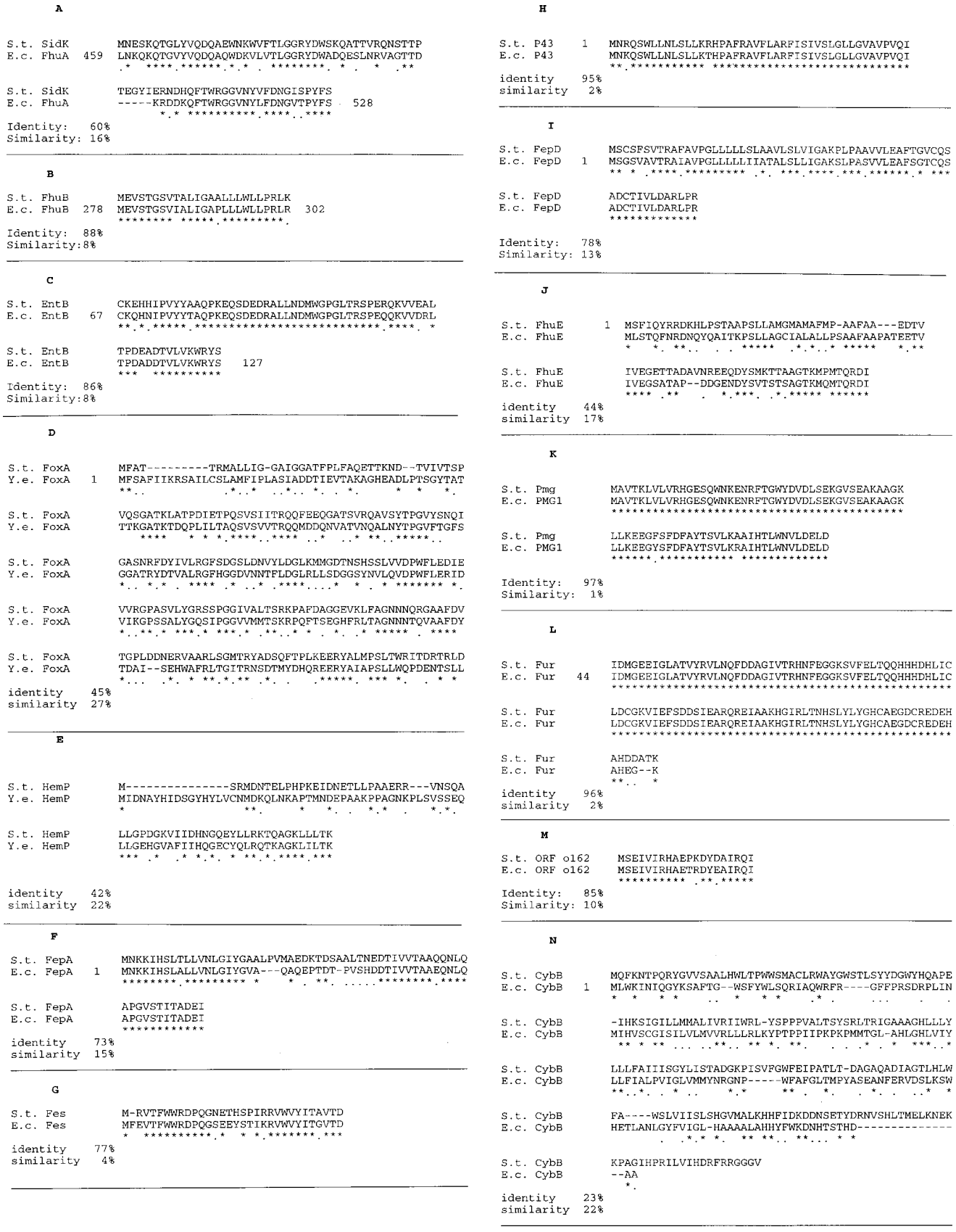

FIG. 2. Deduced amino acid sequences of *S. typhimurium* genes found in this study aligned with the closest homologs from the databases at the National Center for Biotechnology Information. Alignments were performed with the ClustalV program. S.t., *S. typhimurium*; E.c., *E. coli*; Y.e., *Y. enterocolitica*.

TABLE 3. Mapping data of *S. typhimurium* Mud*J* insertions yielding *fur*-regulated *lacZ* fusions

Mud*J* mutant	Mud*J* insertions compared with:				Sequence homology[c]	Phenotype	Map position (min)
	*Xba*I restriction map[a]		*Bln*I restriction map[b]				
	Fragment missing	Size (kb) of new fragments	Fragment missing	Size (kb) of new fragments			
AR396		24	C	400	ND[d]	ND	30
AR895	B	400 + 310	A	700	*fhuA*	FhuA missing	5
AR1258	A	120	A	ca. 1,200	*entB*	Sid$^-$	14
AR3675	B	400 + 310	A	700	*fhuB*	Fox utilization	5
AR6687	A	150 + 650	A		ND	ND	15
AR8412	J	130	B	400	ND	ND	46
AR8439	A	120	A	ca. 1,200	ND	Sid$^-$	14

[a] Based on Liu and Sanderson (32).
[b] Based on Wong and McClelland (55).
[c] Homology to *E. coli* genes.
[d] ND, not determined.

Sequence data indicated that mutant AR3675 carries Mud*J* inserted in another homolog of the *E. coli* ferrichrome-uptake operon *fhuB*. FhuCDB also mediates the transport of ferrioxamine through the cytoplasmic membrane in *E. coli* (7). Since *S. typhimurium* can utilize this siderophore efficiently (33), we examined the effect of a *fhuB* mutation on the utilization of ferrioxamine as a sole iron source. In order to study ferrioxamine uptake in the absence of enterobactin production, an *aroA*::Tn*10* insertion from SL1346 was transduced into strains 14028 and AR3675, giving rise to strains CL1509 and AJB29, respectively. Strain AJB29 and its isogenic parent CL1509 were tested for ferrioxamine utilization by an agar-diffusion assay. While strain CL1509 formed halos 25 mm in diameter around ferrioxamine-soaked filter disks on nutrient broth-dipyridyl agar, no growth stimulation was observed for strain AJB29. These data confirm the involvement of FhuB in ferrioxamine transport in *S. typhimurium*.

In conclusion, by Mud*J* mutagenesis we identified seven distinct Fur-regulated *lacZ* fusions, four of which either resulted in a known phenotype (AR8439) or were located in known Fur-regulated genes (AR895, AR1258, and AR3675). The three other Mud*J* insertion mutants showed no known phenotype. AR6687 mapped to 15 min, the location of *fur* in *Salmonella* spp., but the observed reduction in siderophore production does not agree with an insertion in *fur*. AR8412 mapped to 46 min, the location of *cir*, an outer membrane protein of *E. coli* which has been shown to mediate uptake of siderophore breakdown products (26). AR396 was located at 30 min on the chromosome where no Fur-regulated loci have yet been described for *E. coli* or *S. typhimurium*.

Screen for Fur-regulated promoters by FURTA. Fur-regulated promoters and iron binding proteins carried on a plasmid can be identified by transformation of this plasmid into *E. coli* H1717, which carries *fhuF*::*lacZ*, a Fur-regulated gene fusion sensitive to changes in repressor concentration (48). Transformants carrying plasmids that contain a Fur binding site or encode an iron binding protein form red colonies on M+Fe, while other colonies will be white. A *Salmonella* gene bank consisting of 10,000 independent plasmid clones was screened by this FURTA. Initially, a total of 40 colonies were FURTA positive (showing red colonies on M+Fe) and were further analyzed. After eliminating duplicate clones on the basis of restriction patterns (data not shown), 11 clones were chosen for further characterization. In five clones, pFT5, pFT30, pFT32, pFT35, and pFT36, the insert size was larger than 1 kb, and the clones were subcloned into pBluescriptSK with the enzymes *Rsa*I, *Hin*dII, *Sal*I, or *Eco*RV. The resulting FURTA-positive subclones, pFT501, pFT302, pFT324, pFT3522, and pFT361, respectively, were identified by transformation into H1717 and by plating on M+Fe plates before further characterization.

Sequence analysis and mapping of DNA regions identified by FURTA. The DNA sequences of 11 FURTA-positive clones were determined. The insert of two FURTA-positive clones, pFT1S (not shown) and pFT1H, contained the promoter region and the 5′ end of an open reading frame (ORF) with homology to *foxA*, a gene encoding the outer membrane ferrioxamine receptor of *Yersinia enterocolitica* (Fig. 2D and 3) (4). A potential Fur binding site matching the consensus sequence in 12 of 19 bases was located upstream of this ORF (Fig. 4). In contrast to *E. coli*, *Salmonella* spp. can utilize ferrioxamine very efficiently (33). However, the ferrioxamine receptor of *S. typhimurium* has not yet been characterized at the nucleotide sequence level. One FURTA-positive clone, pFT36, contained a small ORF (189 bp) which was preceded by a sequence matching the consensus site for Fur binding in 16 of 19 bases. This ORF showed homology with *hemP* of *Y. enterocolitica* (Fig. 2E and 3). This gene is the first ORF in the Fur-regulated heme-uptake operon of *Y. enterocolitica* (50). Clone pFT17 contained the 5′ ends of two divergently oriented ORFs with homology to *fes* and *fepA*, encoding enterochelin esterase and the ferrienterochelin receptor, respectively, of *E. coli*. The bidirectional promoter region between these two genes has recently been characterized in detail (28), and a sequence identical to the Fur-box determined in this study was also present in the putative promoter region of pFT17 (Fig. 2F and G, 3, and 4). The sequence of clone pFT324 exhibited homology to a second bidirectional promoter region in the *ent-fep* cluster of *E. coli*, the *p43-fepD* region (Fig. 2H and I). The function of P43 is unknown, although it has been suggested to be a cytoplasmic membrane protein (12, 45). FepD forms part of the cytoplasmic membrane permease for ferrienterobactin. Truncated ORFs encoding homologs to these two proteins and a putative Fur binding site with two mismatches to the Fur binding site suggested by Shea and McIntosh (45) and Chenault and Earhart (12) were identified on pFT324. Plasmid pFTE1 contained an ORF with homology to *E. coli fhuE*, the outer membrane receptor for coprogen (23, 43). A putative Fur-box identical to the one identified in the *E. coli fhuE* promoter was present upstream of the *fhuE* homolog on pFTE1 (Fig. 2J, 3, and 4) (43). Upstream of the *fhuE* homolog, and oriented in the same direction, is the 3′ end of a

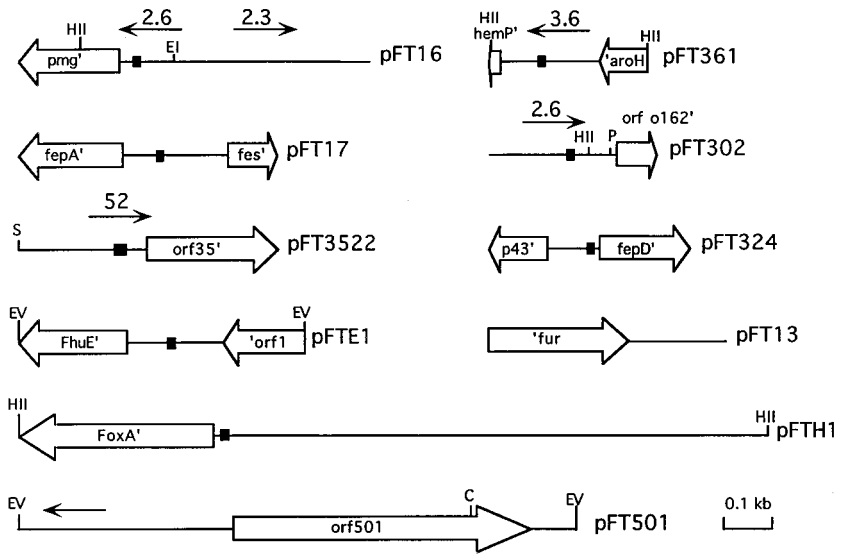

FIG. 3. Inserts of FURTA-positive clones from *S. typhimurium*. Open arrows indicate open reading frames. Thin arrows denote the direction of promoter activity as determined in Table 5. If promoter activity is affected by iron, the fold induction is shown above the arrow. Black boxes indicate the presence of a consensus Fur binding site. HII, *Hin*dII; EI, *Eco*RI; EV, *Eco*RV; P, *Pst*I; S, *Sal*I; C, *Cla*I.

second ORF (designated ′orf1) which had no homology to any entries in the database.

Plasmid pFT16 contained the 5′ end of an ORF with homology to various phosphoglycerate mutases. The predicted protein encoded by this ORF showed homology with PGM1 of *E. coli*. Upstream of this ORF, a potential Fur-box which matched the consensus sequence in 16 of 19 bases was located (Fig. 2K, 3, and 4).

Interestingly, one of the FURTA-positive clones contains a gene fusion between the α-fragment of the *lacZ* gene (contained on the cloning vector) and the *S. typhimurium fur* gene lacking the first 129 bp (Fig. 2L). The resulting LacZ-Fur fusion protein could have caused the FURTA-positive phenotype of this clone. Since the C-terminal part of Fur contains the domain necessary for repressor dimerization but not for DNA binding (13, 51), the LacZ-Fur protein may form defective dimers with wild-type *E. coli* Fur in H1717, thereby causing a Fur$^-$ phenotype. This negative complementation would explain the derepression of the *fhuF*::*lacZ* fusion leading to the FURTA-positive result seen in our screen.

The remaining three FURTA-positive plasmids showed no homology to known genes. Plasmid pFT3522 contained 282 bp of the 5′ end of an ORF, designated orf35. Approximately 50 bp upstream of this ORF was a potential Fur binding site matching the consensus sequence in 15 of 19 bases. Plasmid pFT302 contained 78 bp of the 5′ end of an ORF, designated orf30, with no homology to entries in the database. A primer walk was performed on the parent plasmid pFT30. With this additional sequence data, 342 bp of this ORF were found to be homologous to ORF o162 (NCBI 606376) found in the *E. coli* chromosomal region at 67.4 to 76 min (Fig. 2M). A FURTA-positive clone was also found in a previous study at the corresponding region of the *E. coli* chromosome, 71 min (48). The potential Fur-box upstream of this ORF on pFT302 matched the consensus sequence in 13 of 19 bases, and an identical sequence was found in *E. coli* (Fig. 4). Thus, the truncated ORF contained on pFT302 may define a Fur-regulated gene product of yet unknown function which is common to *E. coli* and *Salmonella* spp. Plasmid pFT501 encoded an ORF (orf501) of 645 bp, whose translated product exhibited homology to the *E. coli* cytochrome b561. No consensus binding sites for Fur were found upstream of this ORF (Fig. 2N and 3). Together, these data suggest that pFT501 may encode an iron binding protein.

Consensus:		GATAATGAT A ATCATTATC		
pFT361:	124	GATAATAAT A ATCATTGAG	106	
pFT324:	229	GATAATGAT A GTAATTATC	211	
pFT16:	273	TATAATGAG A ATTATTATC	255	
pFT3522:	210	GATAATCAT T ATCACTAAC	228	
pFT3522:	204	TGAAGTGAT A ATCATTATC	222	
pFT302:	165	AATAATCAT T CTCATTCGC	183	
pFT17:	294	GCAAATGCA A ATAGTTATC	312	
pFTE1:	334	GCGTATATT T CTCATTTGC	316	
pFTH1:	434	GGTAATAAT T CTTATTTAC	416	

FIG. 4. Comparison of potential Fur binding sites found in FURTA-positive clones with the consensus sequence found by de Lorenzo et al. (16).

In summary, the nucleotide sequences of five FURTA-positive clones were homologous to Fur-regulated promoters described previously in *Salmonella* spp. or other enterobacteria (Fig. 4). The nucleotide sequence of three FURTA-positive clones contained Fur binding motifs but showed no homology to previously described Fur-regulated promoters. One FURTA-positive clone may encode an iron binding protein.

Physical mapping of FURTA-positive clones on the *S. typhimurium* chromosome. In order to find the location of these clones on the *Salmonella* chromosome, *Eco*RV fragments of the inserts of pFT1H, pFT5, and pFT16 were cloned into the suicide vector pMAP (Fig. 1), giving rise to plasmids pMAP1H, pMAP5, and pMAP16, respectively. In order to confirm the reliability of the mapping technique, we also constructed mapping derivatives for *Salmonella* genes which have been mapped previously. For this purpose *Eco*RV fragments of the inserts of pFT1E, pFT17, pFT13, and pFT32 were cloned into the suicide vector pMAP, giving rise to plasmids pMAP1E, pMAP17, pMAP13, and pMAP32, respectively. All pMAP derivatives were introduced into *S. typhimurium* 14028 by conjugation, and exconjugants carrying the suicide vector inserted in the chromosome were selected by plating on carbenicillin. The inser-

TABLE 4. Mapping data of *S. typhimurium* FURTA-positive clones

FURTA clone	Comparison with:				Sequence homology[c]	Map position (min)
	*Xba*I restriction map[a]		*Bln*I restriction map[b]			
	Fragment missing	Size (kb) of new fragments	Fragment missing	Size (kb) of new fragments		
pFT1E	A	185	C	650	*fhuE*	25
pFT1H		210			*foxA*[d]	ND[e]
pFT1S	ND	ND	ND	ND	*foxA*[d]	ND
pFT5	A	510	C	760		23
pFT13	ND	ND	ND	ND	*fur*	15
pFT16	A		A		PMG1	12–22
pFT17	A	120	A	ca. 1,200	*fes-fepA*	14
pFT30	ND	ND	ND	ND	ORF o162	74[f]
pFT32	A	120	A	ca. 1,200	*p43-fepC*	14
pFT35	ND	ND	ND	ND		ND
pFT36	ND	ND	ND	ND	*hemP*[d]	36[f]

[a] Based on Liu and Sanderson (32).
[b] Based on Wong and McClelland (55).
[c] Homology to *E. coli* genes, except where indicated.
[d] Homology to *Y. enterocolitica* genes.
[e] ND, not determined.
[f] Map position was calculated from map position in *E. coli* based on Riley and Krawiec (41a).

tion site of the plasmid was then determined by pulsed-field gel electrophoresis analysis of chromosomal DNA of these derivatives digested with *Xba*I and *Bln*I (Table 4). The map locations calculated for *fhuE*, *fes-fepA*, *fur*, and *p43-fepD* agreed with data published for *Salmonella* spp. or were at positions which corresponded to known loci in *E. coli* (3, 40, 41), thus confirming the reliability of the mapping technique. By using these mapping data or the sequence information we were able to determine the map locations of a total of eight FURTA-positive clones (Table 4).

Iron-responsive promoter activity of FURTA-positive clones. In order to distinguish between plasmids containing Fur-regulated promoters or encoding iron binding proteins, the inserts of FURTA-positive plasmids were cloned, with the restriction enzymes *Xba*I and *Sal*I, into the promoter probe vector pUJ10 (14). This vector contains a multiple cloning site flanked by promoterless β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) genes oriented in opposite directions. Since the 5′ ends of each of these genes flank the multiple cloning site, the introduction of a promoter at this site creates a transcriptional fusion to either *lacZ* or *phoA*. Inserts from FURTA-positive clones were cloned into pUJ10, and the resulting strains were assayed for iron-responsive promoter activity in the *lac phoA E. coli* host CC118 (34). By measuring the activities of β-galactosidase and alkaline phosphatase under both iron-replete and iron-limited conditions, the degree and direction of iron-responsive promoter activity were determined (Table 5).

Although pUJ501 had promoter activity driving *phoA* expression, the level of induction under iron limitation was not higher than that in the vector control. This promoter is therefore not regulated by iron. Of the five constructs examined, four could be shown to exhibit iron-responsive promoter activity (Table 5). An iron-regulated promoter was found to drive expression of *phoA* in pUJ361 and pUJ161. Plasmids pUJ161, pUJ3522, and pUJ302 showed iron-responsive promoter activity driving expression of the *lacZ* gene. The direction of promoter activity of these clones is shown in Fig. 3. Together with the sequence data showing the presence of a Fur-box (Fig. 4), these data indicate that clones pUJ161, pUJ3522, pUJ302, and pUJ361 each carry a Fur-regulated promoter. The absence of an iron-regulated promoter in pUJ501 agrees with sequence analysis data, which indicated a lack of a consensus Fur binding site. The presence of an ORF with a low degree of homology to cytochrome B561, an iron-binding protein of *E. coli*, indicated that this clone may encode an iron binding protein which leads to a FURTA-positive phenotype (39). In conclusion, by FURTA we identified 11 distinct clones, nine of which contained iron-regulated promoters. In addition, one potential iron binding protein and the *Salmonella fur* gene itself were isolated (Fig. 3).

DISCUSSION

In this report we describe the analysis of the *S. typhimurium* Fur regulon by two different techniques, Mud*J* mutagenesis and FURTA (27, 48). By these two methods we screened 10,000 *Salmonella* transposon insertion mutants and 10,000 clones of a *Salmonella* plasmid bank for Fur-regulated genes or promoters, respectively. This approach allowed for a direct comparison of both techniques in identifying members of the *Salmonella* Fur regulon. By screening Mud*J* mutants for Fur-regulated genes we were able to isolate seven Fur-regulated insertions, while eight different Fur-regulated promoters were

TABLE 5. Promoter activity of FURTA-positive fragments

Clone	Mean ± SE of FURTA-positive fragments showing[a]:			
	β-galactosidase activity (Miller U)[b]		Alkaline phosphatase activity (U)[c]	
	With Fe[d]	Without Fe[e]	With Fe	Without Fe
pUJ501	1.59 ± 1.29	3.23 ± 2.22	160 ± 30	228 ± 45
pUJ161	35.5 ± 11	94.8 ± 35	58 ± 54	189 ± 37
pUJ302	5.3 ± 7.5	14 ± 0.7	7.6 ± 2.9	41 ± 24
pUJ352	1.35 ± 0.83	73 ± 41	42 ± 14	43 ± 29
pUJ361	0.36 ± 0.41	2.1 ± 0.67	547 ± 34	1,975 ± 326
pUJ10	2.7 ± 2.53	2.3 ± 0.99	17 ± 1.7	39 ± 11

[a] Averages of at least three independent experiments ± standard error.
[b] Calculated according to Miller (36).
[c] Calculated on the basis of Brickman and Beckwith (10).
[d] Grown in Luria-Bertani broth with 40 μM $FeSO_4$.
[e] Grown in nutrient broth with 0.2 mM 2,2′-dipyridyl.

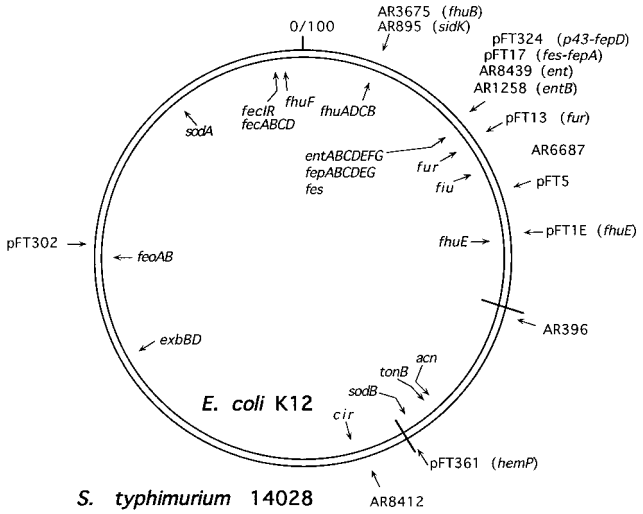

FIG. 5. Comparison of the locations of Fur-regulated genes on the genetic maps of *E. coli* (inner circle) and *S. typhimurium* (outer circle). Lines intersecting the map indicate the endpoints of a region (26′ to 36′ in *S. typhimurium*), which is inverted between the two species. Fur-regulated genes of *S. typhimurium* found in this study are indicated.

isolated by FURTA. In addition, a truncated *fur* gene and a putative iron binding protein were identified by FURTA. Although both approaches were therefore equally efficient in identifying Fur-regulated genes, we found each to have different advantages. Mud*J* mutagenesis had the advantage of immediately providing mutants for phenotypic studies; however, the mutant screen was more labor-intensive than the positive selection for clones used in the FURTA. In contrast, the screening of plasmids by FURTA was rapid and provided clones for immediate sequence analysis. However, this technique would not be suitable for mutational analysis of a larger number of loci.

The Fur regulon has been best studied in *E. coli* K-12, in which more than 36 Fur-regulated genes have been described (7). A thorough analysis by two-dimensional gel electrophoresis identified 19 proteins of *S. typhimurium* whose expression is negatively regulated by Fur (17). By FURTA and Mud*J* mutagenesis we identified 14 *S. typhimurium* genes which are repressed by Fur. Six of these genes, *fhuA*, *fhuB*, *fes*, *fepA*, *fepD*, and *entB*, are located in operons consisting of several genes in *E. coli*. Assuming the same situation in *Salmonella* spp., the Fur-regulated loci identified in this study contain approximately 24 genes. However, since we were unable to find *tonB* and *sodA*, two known Fur-regulated genes of *S. typhimurium*, the number of Fur-repressed genes is likely to be larger (21, 53). Our results show that *S. typhimurium* possesses homologs for many Fur-repressed *E. coli* genes located at similar positions on the genetic map (Fig. 5). However, some differences between *E. coli* and *S. typhimurium* iron-uptake systems exist. For example, *E. coli* has been shown to possess an iron-dicitrate-uptake system, encoded by *fecIR fecABCDE*, whereas *Salmonella* spp. cannot utilize this compound as an iron source (19, 33, 47). Furthermore, *S. typhimurium* is able to utilize the siderophore ferrioxamine very efficiently, while in *E. coli* only a low level of ferrioxamine uptake is mediated by the outer membrane receptor FhuE (33, 43). An *S. typhimurium* gene identified in this study was found to be homologous to *foxA*, the gene encoding the outer membrane ferrioxamine receptor of *Y. enterocolitica* (4). Although it is tempting to speculate that this gene might encode the *Salmonella* ferrioxamine receptor, the possibility that this function is mediated by the *Salmonella fhuE* homolog isolated during this study (Fig. 3) or a third unidentified gene cannot be excluded. However, our data show that, as in *E. coli*, FhuB is necessary for transport of ferrioxamine across the cytoplasmic membrane of *S. typhimurium* (7). In addition to the *foxA* homolog, several other Fur-regulated loci which have previously not been described in *E. coli* or *S. typhimurium* were identified during this study. One such gene showed homology to *hemP*, the first gene in the *Y. enterocolitica* operon encoding a heme-uptake system (50). However, *S. typhimurium* is unable to utilize heme as an iron source (our unpublished results). Therefore, the *hemP* homolog could either be part of a defective heme-uptake system or it may be the first gene in a related but different operon. Interestingly, this ORF was also found in *E. coli* K-12 in the 3′ region of *aroH* and maps close to the *btuCED* locus, encoding a cytoplasmic membrane permease for vitamin B_{12} (18).

By comparing the proteins encoded by the fragments of these *Salmonella* genes with their homologs in *E. coli*, we found that cytoplasmic membrane proteins and cytoplasmic enzymes were highly conserved, with overall similarities (percent identity plus percent conservative amino acid changes) between 81 and 98%. In contrast, the outer membrane receptors were less conserved, with overall similarity scores ranging from 61 to 88%. The bacterial surface is exposed to the immune system of the host, which might have been a selective pressure leading to increased variability of surface-exposed proteins among enterobacteria. Differences in outer membrane receptors of *E. coli* and *Salmonella* have been described previously as differences in susceptibility to phages and colicins (8).

At least one Mud*J* mutation and three FURTA clones appear to define new Fur-regulated loci. However, we found no homology to known proteins; thus, the sequence data do not suggest a function for the proteins encoded by these loci. Since Fur-regulated genes described so far fall into several groups, including genes involved in iron uptake, the acid-tolerance response (17), sugar metabolism, defense against oxygen radicals, and genes encoding bacterial toxins, it is premature to speculate about the function of these genes.

Of particular interest was the finding that *pmg*, encoding the phosphoglycerate mutase, was regulated by Fur. First evidence for the involvement of Fur in the regulation of general metabolic pathways was provided by the finding that *fur* mutants of *E. coli* are unable to utilize succinate as a C source (25). Furthermore, the gene encoding aconitase of *E. coli*, an enzyme of the Krebs cycle, contains a Fur binding site in its promoter region (41). Fur may thus participate in the control of major catabolic pathways, thereby linking bacterial growth to the supply of limiting nutrients like iron. In return, the cyclic AMP receptor protein binding site found in the *fur* promoter region may link expression of this regulator to the availability of C sources (15). That this complex regulatory network has yet other facets is indicated by the observation that *fur* mutants of *Salmonella* spp. are defective in the acid-tolerance response (17).

ACKNOWLEDGMENTS

We thank S. Anic for expert sequencing with the ALF automated sequencer and J. Lipps for technical assistance. We also thank K. Hantke, R. Schönherr, V. de Lorenzo, J. Mekalanos, and R. Taylor for strains and B. Ahmer, P. Valentine, and the reviewers for helpful comments on the manuscript.

This work was supported by U.S. Public Health Service grant AI22933 to F.H. from the National Institutes of Health. A.J.B. was supported by stipend Ba 1337/1-2 from the Deutsche Forschungsgemeinschaft.

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