A method for direct cloning of Fur-regulated genes: identification of seven new Fur-regulated loci in *Escherichia coli*

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A strain that allows the cloning of Fur-regulated loci was constructed. The strain, named FUR-SEL1, contains a chromosomal *fhuA'*-*cat* transcriptional fusion that is expressed from the Fur-regulated promoter, *fhuA_p*. Therefore, Fur boxes introduced on a multicopy plasmid can cause derepression of the fusion by titrating the Fur repressor and thereby confer chloramphenicol resistance, which can be used as a selectable phenotype for cloning Fur-regulated loci. However, a number of additional mutations had to be introduced before FUR-SEL1 could be used for cloning Fur-regulated genes. The principal approach consisted of introducing a leaky *fur* mutation that ensures a more than 10⁶-fold increase in chloramphenicol resistance for FUR-SEL1 transformants carrying FUR-box-containing plasmids. To verify that the cloning procedure selects Fur-regulated genes, 10 recombinant plasmids chosen at random among the ones selected with FUR-SEL1 were analysed by FURTA (Fur-titration assay), a method for identification of Fur-regulated genes. In addition, the nucleotide sequences of their chromosomal inserts were determined. Besides known Fur-regulated genes, seven *Escherichia coli* loci which have not previously been shown to be Fur regulated were found, including the *pgmA* and *nrdHIEF* genes, predicted ORF *yhhY* and four promoters identified first in this study. Three of the promoters preceded the *nohA* gene, and ORFs *ygaC* and *yhhX*. The fourth was located upstream of *orf78* predicted in this work. The regulation of the promoter activities by iron and the involvement of Fur in this regulation were shown. Employing FUR-SEL1 for cloning Fur-regulated loci from other enterobacteria is discussed.

Keywords: Fur regulon, Fur repressor, Fur box, iron regulation, *Escherichia coli*

INTRODUCTION

Fur (ferric uptake regulator) is a global transcriptional regulator, using ferrous iron as a corepressor, that controls the expression of a great many operons in *E. coli* (Hantke & Braun, 1998). Fur-like proteins have also been identified in many other bacterial species. By binding regulatory DNA sequences, Fur boxes, located in the promoter regions of Fur-controlled genes, Fur regulates not only the genes responsible for iron uptake, but also genes involved in aerobic respiration, chemotaxis, synthesis of amino acids and DNA precursors, sugar metabolism, protecting the cell from oxidative damage, and genes encoding bacterial toxins

(Stojiljkovic *et al.*, 1994; Compan & Touati, 1993; Prodromou *et al.*, 1992; Calderwood & Mekalanos, 1987). A number of new Fur-regulated loci of unknown function has recently been found in the *Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa* genomes (Stojiljkovic *et al.*, 1994; Tsois *et al.*, 1995; Ochsner & Vasil, 1996).

A method, called FURTA (Fur-titration assay), for the identification of Fur-regulated genes has been developed (Stojiljkovic *et al.*, 1994) that is based on multiple plasmid-encoded Fur boxes derepressing chromosomal Fur-regulated genes by titrating the Fur protein. Both FURTA and strategies employing tandem *lacZ* fusions demand labour-intensive analysis of many clones. Identification of seven Fur-regulated genes of *S. typhimurium* required screening 10000 independent MudJ insertion mutants. Screening 10000 clones of a *Salmonella*

Abbreviations: FURTA, Fur-titration assay; Lac-FURTA, FURTA using *lacZ* as a reporter; Cm-FURTA, FURTA using *cat* as a reporter.

plasmid bank by FURTA allowed the isolation of eight Fur-regulated loci (Tsolis *et al.*, 1995).

An alternative *in vitro* method, the cycle-selection procedure, is based on the specific interaction of the Fur repressor with chromosomal fragments containing Fur boxes, purification of Fur-DNA complexes and PCR amplification of the enriched DNA pool for the next cycle (Ochsner & Vasil, 1996). This method was applied to the isolation of Fur targets in *P. aeruginosa*. This is a very laborious procedure requiring the purification of the Fur protein. In addition, the percentage of isolated fragments for which the specificity of the Fur-DNA interaction was not verified by subsequent analysis was high.

In this study we describe an approach for cloning Fur-regulated loci that is based on direct *in vivo* selection.

METHODS

Strains, media and growth conditions. The bacterial strains and the plasmids used in this study are listed in Table 1. Bacteria were grown in LB broth or on LB agar plates unless indicated otherwise. Antibiotics, when required, were added at the following concentrations: ampicillin, 100 mg l⁻¹; kanamycin, 25 mg l⁻¹; streptomycin, 100 mg l⁻¹; tetracycline, 3.0–12.5 mg l⁻¹; chloramphenicol, 2.5–7.5 mg l⁻¹. M9 minimal medium supplemented with threonine, histidine, arginine (40 mg l⁻¹) and 0.2% (w/v) glucose as the carbon source was used for selection after conjugation.

For selection of *fur* mutants, the medium described previously (Hantke, 1987; Silver *et al.*, 1972) was used except that desferal was removed.

For quantitative assay of β -galactosidase activity in the FURTA technique, strains were grown on EMBO agar medium (10 g tryptone, 1 g yeast extract, 5 g NaCl, 2 g KH₂PO₄ and 12 g agar l⁻¹) supplemented with 0.025 mM (NH₄)₂Fe(SO₄)₂. The β -galactosidase and alkaline phosphatase activities of the iron-regulated fusions carried on pUJ10 derivatives were determined using cells grown on iron-rich and iron-deficient agar medium. LB broth was used as iron-rich medium (de Lorenzo *et al.*, 1988). To create conditions of iron starvation, LB broth was supplemented with 0.2 mM 2,2'-dipyridyl. β -Galactosidase activity was determined by the method of Miller (1972) and alkaline phosphatase activity by the method of Brickman & Beckwith (1975), using cells treated with chloroform and 0.1% SDS.

Lac-FURTA was carried out on Lac-EMBO agar plates (Miller, 1972) containing (NH₄)₂Fe(SO₄)₂.

Genetic techniques. Phage P1vir was used for generalized transduction of operon fusions into different genetic backgrounds. Transductants were selected on plates containing 25 mM sodium citrate and the appropriate antibiotic. Selection for *fur* mutants of *E. coli* was carried out according to Hantke (1987).

The *lacZ* deletion $\Delta(lacZ)$ M15 was introduced into strain VK-6C using a two-step procedure. First, F'::Tn10 *proA*⁺*B*⁺ *lacI*ⁿ $\Delta(lacZ)$ M15 was introduced into VK-6C by conjugation with XL-1 Blue. The VK-6C(F') exconjugants were selected on medium containing streptomycin and tetracycline. To transfer $\Delta(lacZ)$ from F' to the chromosome, ~10³ cells of strain VK-6C(F') were inoculated into 1 ml LB broth and 3.8 mg ethidium

bromide l⁻¹ was added to induce homologous recombination between F' and the chromosome, and then to eliminate F'. After overnight incubation at 37 °C, the bacterial culture was diluted and plated on X-Gal and IPTG-containing medium to allow Lac⁻ colonies to be selected. After streaking on X-Gal medium, several Lac⁻ clones were transformed with pUC19 and their β -galactosidase activity was found to be complemented by the *lac* α peptide of pUC19. The data indicated that the Lac⁻ clones carry only the $\Delta(lacZ)$ M15 allele of the *lac* gene. One of them was designated FUR-SEL1.

Recombinant DNA techniques. Preparations of plasmid and chromosomal DNA, restriction digestions and ligations were performed according to standard techniques (Sambrook *et al.*, 1989). To prepare plasmid DNA for digestion with *Bcl*I the *E. coli* *dam* strain WA321 was used. Plasmid DNA was introduced into bacteria by transformation of CaCl₂-treated cells (Cohen *et al.*, 1973) or by electroporation (Dower *et al.*, 1988).

Construction of *lacZ* and *phoA* gene fusion plasmids. Isolation of transcriptional fusions between the Fur-regulated genes found in this work and the *lacZ* or *phoA* genes was accomplished using plasmid pUJ10 (de Lorenzo *et al.*, 1990) in strain CC118. This vector contains several unique sites flanked by promoterless β -galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) genes oriented in opposite directions. The *Sau*3A chromosomal insertions from pEF7, pEF19, pEF20, pEF29, pEF34 and pEF63, and the 585 bp *Sau*3A fragment containing the promoter of the *bla* gene encoding β -lactamase from pUC19 were isolated after separation in 5% acrylamide gels and ligated into *Bgl*II-digested and dephosphorylated pUJ10. Transformants were selected on LB medium with ampicillin. The structures of pUJ10 derivatives and the orientations of their *Sau*3A inserts were verified by restriction enzyme mapping.

Construction of the promoter probe vector pBR- Δ . pBR- Δ is a pBR322 deletion derivative with the promoterless tetracycline-resistance gene *tet*. For construction of this vector, the 29 bp *Eco*RI-*Hind*III fragment of pBR322 containing the -35 element of the *tet* promoter was replaced by the 0.6 kb *Eco*RI-*Hind*III fragment including the 3' portion of the kanamycin resistance gene from pUC-4K (Vieira & Messing, 1982). In this study, the vector was used for the generation of transcriptional *tet* fusions by replacing its truncated kanamycin resistance gene by the *Eco*RI-*Hind*III fragments including the chromosomal insertions from plasmids of the pEF series.

Construction of pEF-A plasmid series. To obtain pEF derivatives pEF19-A, pEF34-A and pEF63-A that contain *Sau*3A chromosomal insertions in the opposite orientation to pEF19, pEF34 and pEF63, respectively, the corresponding *Sau*3A fragments were recloned in pUC19 with strain FUR-SEL1. The desired constructions were identified by restriction analysis.

Construction of strain VK-0C containing the *fhuA*'-*cat* operon fusion located on the chromosome. Strain VK-0C carrying the chromosomal transcriptional fusion *fhuA*'-*cat* was constructed in a two-step procedure. First, the *fhuA*'-*cat* fusion was constructed in plasmid pFC2 (Fig. 1) and transferred from the plasmid to the chromosome of strain CA77 by *in vivo* homologous recombination. To enhance recombination frequency, CA77 cells transformed with pFC2 were UV irradiated. The recombinants resulting from double crossovers were selected with phage T5 which uses *FhuA* for infection. To distinguish between the recombinants selected in this way

Table 1. *E. coli* strains and plasmids used in this study

Strain/plasmid	Description	Source/reference
Strains		
AB1157	F ⁻ <i>supE44 thr leu proA his-4 thi argE lacY1 galK ara-14 xyl-6 mtl tsx-33 rpsL31</i>	Laboratory stock
803-8	F ⁻ <i>supE supF hsdR gal metB thyA</i>	Laboratory stock
803-8A	As 803-8 but <i>fhuA2</i>	This study
WA321	F ⁻ <i>dam-4 thr-1 leu-6 thi-1 proA2 his-4 ara lacY1 gal xyl mtl rpsL31</i>	Laboratory stock
XL-1 Blue	F ['] ::Tn10 <i>proA⁺B⁺ lacI^q Δ(lacZ)M15/recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac</i>	Laboratory stock
CA77	HfrC wild-type	Laboratory stock
H1443	<i>aroB araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR</i>	Hantke (1983)
H1717	As H1443 but <i>fhuF::λplacMu53</i>	Hantke (1987)
H1698	<i>araD139 Δ(argF-lac)U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR fhu::λplacMu53</i>	Hantke (1987)
H1698-1	As H1443 but <i>fhu::λplacMu53 lamB</i>	This study
VK-6	As AB1157 but <i>fur-6</i>	This study
VK-12	As AB1157 but <i>fur-12</i>	This study
VK-0C	As AB1157 but <i>fhuA'-cat</i>	This study
VK-6C	As VK-6 but <i>fhuA'-cat</i>	This study
FUR-SEL1	As VK-6C but <i>Δ(lacZ)M15</i>	This study
Plasmids		
pUC19	Amp ^r , <i>α-lac</i>	Yanisch-Perron <i>et al.</i> (1985)
pCM4	Amp ^r , <i>cat</i> cartridge	Close & Rodriguez (1982)
pBR322	Amp ^r , Tet ^r	Laboratory stock
pUJ10	Amp ^r ' <i>phoA trp'</i> lacZ	de Lorenzo <i>et al.</i> (1990)
pLC19-19	ColE1 derivative, <i>fhuA</i> gene source	Clarke & Carbon (1976)
pBC.FUR1	Oligonucleotide FUR1, consensus Fur box, inserted as a 29 bp <i>EcoRI</i> – <i>Bam</i> HI fragment into pBC	Stojilkovic <i>et al.</i> (1994)
pUCFUR1	Oligonucleotide FUR1 inserted into pUC19 as a 29 bp <i>EcoRI</i> – <i>Bam</i> HI fragment	This study
pBR-Δ	Amp ^r , pBR322 derivative with deleted <i>tet_p</i>	This study
pFHU2	Tet ^r , 4.5 kb <i>Pst</i> I fragment containing <i>fhuA</i> from pLC19-19 inserted in pBR322	This study
pFC2	Tet ^r , pFHU2 derivative containing <i>fhuA'-cat</i>	This study
pEF7	259 bp <i>Sau</i> 3A fragment containing <i>fepA-fes</i> promoter region inserted in pUC19	This study
pEF10	818 bp <i>Sau</i> 3A fragment containing <i>entC-fepB</i> promoter region inserted in pUC19	This study
pEF19	682 bp <i>Sau</i> 3A fragment containing <i>nrdH-ygaC</i> promoter region inserted in pUC19	This study
pEF19-A	As pEF19 but 682 bp <i>Sau</i> 3A fragment inserted in the opposite orientation	This study
pEF20	595 bp <i>Sau</i> 3A fragment containing <i>pgm</i> promoter region inserted in pUC19	This study
pEF23	389 bp <i>Sau</i> 3A fragment containing <i>cir</i> promoter region inserted in pUC19	This study
pEF29	115 bp <i>Sau</i> 3A fragment, belonging to cryptic prophage Qin and lying near <i>nohA</i> , inserted in pUC19	This study
pEF33	As pEF23, but 389 bp <i>Sau</i> 3A fragment inserted in the opposite orientation	This study
pEF34	237 bp <i>Sau</i> 3A fragment containing <i>yhbX-yhbY</i> promoter region inserted in pUC19	This study
pEF34-A	As pEF34 but 237 bp <i>Sau</i> 3A fragment inserted in the opposite orientation	This study
pEF63	293 bp <i>Sau</i> 3A fragment containing <i>fhuF-orf78</i> promoter region inserted in pUC19	This study
pEF63-A	As pEF63 but 293 bp <i>Sau</i> 3A fragment inserted in the opposite orientation	This study
pEF67	As pEF7, but 259 bp <i>Sau</i> 3A fragment inserted in the opposite orientation	This study
pUJ-F7	259 bp <i>Sau</i> 3A fragment from pEF7 inserted in pUJ10	This study
pUJ-F19	682 bp <i>Sau</i> 3A fragment from pEF19 inserted in pUJ10	This study
pUJ-F20	595 bp <i>Sau</i> 3A fragment from pEF20 inserted in pUJ10	This study
pUJ-F29	115 bp <i>Sau</i> 3A fragment from pEF29 inserted in pUJ10	This study
pUJ-F34	237 bp <i>Sau</i> 3A fragment from pEF34 inserted in pUJ10	This study
pUJ-F63	293 bp <i>Sau</i> 3A fragment from pEF63 inserted in pUJ10	This study
pUJ-BLA	585 bp <i>Sau</i> 3A fragment with <i>bla</i> promoter from pUC19 inserted in pUJ10	This study
pBR-ΔF7	<i>EcoRI</i> – <i>Hind</i> III fragment including chromosomal insertion from pEF7 inserted in <i>EcoRI</i> / <i>Hind</i> III sites of pBR-Δ	This study
pBR-ΔF19	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF19	This study
pBR-ΔF19-A	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF19-A	This study
pBR-ΔF20	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF20	This study
pBR-ΔF23	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF23	This study
pBR-ΔF34	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF34	This study
pBR-ΔF34-A	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF34-A	This study
pBR-ΔF63-A	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF63-A	This study
pBR-ΔF67	As pBR-ΔF7 but <i>EcoRI</i> – <i>Hind</i> III insertion from pEF67	This study

and spontaneous *fhuA* mutants, the *fhuA'-cat* fusion was transferred from several T5-resistant clones into AB1157 via conjugation. As the *fhuA* locus is at about 4 min on the *E. coli*

physical map, it is closely linked with the *leu*⁺ (2 min) and *pro*⁺ (6 min) markers. *Leu*⁺ *Pro*⁺ exconjugants were selected and tested for resistance to chloramphenicol, phage T5 and for

Fig. 1. Construction of *fhuA'*-*cat* gene fusion. Plasmid pFHUA2 was made by cloning the 4.5 kb *Pst*I fragment including the *fhuA* gene from pLC19-19 into the single *Pst*I site of pBR322. pFC2 is a result of insertion of the promoterless portion of *cat* gene into the coding sequence of *fhuA* in plasmid pFHUA2. The 0.78 kb *Bam*HI fragment of plasmid pCM4 was used as the source of the *cat* cartridge. The fragment was inserted into the *Bcl*I(b) site of pFHUA2 located within the *fhuA* gene in the appropriate orientation to produce a *fhuA'*-*cat* fusion where the transcription of *cat* is dependent on the *fhuA* promoter. To distinguish between the desired construction and the *cat* insertions into the *Bcl*I(a) and *Bcl*I(c) sites positioned beyond the *fhuA* gene, the selection of recombinant plasmids was performed in strain 803-8A (*fhuA* mutant) with bacteriophage T1 and the appropriate antibiotics. Only the clones harbouring plasmids carrying the disrupted *fhuA* gene could exhibit the resistance to T1, which uses FhuA for infection. Gene fusion plasmids from several Tc^r Cm^r T1^r clones were analysed by restriction mapping to verify they had the required structure.

sensitivity to tetracycline. To obtain a strain more isogenic with AB1157, the *fhuA'*-*cat* fusion of a Cm^r/T5^r/Tc^s exconjugant was transferred into AB1157 by P1vir transduction, yielding strain VK-0C that was used further as the parental strain for constructing FUR-SEL1.

RESULTS

Construction and characterization of strain FUR-SEL1

The procedure used for cloning Fur-regulated loci is based on the FURTA. As shown previously, a Fur box introduced on a high-copy-number plasmid can compete with Fur boxes located on the chromosome for the Fur repressor (Stojiljkovic *et al.*, 1994). Therefore, if a plasmid-encoded Fur box is introduced into a strain carrying a reporter gene, whose transcription is under control of Fur, it will titrate the repressor, causing expression of the reporter gene. The FURTA technique employs a Fur-regulated *lacZ* fusion as a reporter gene that allows the detection of transformants carrying multicopy Fur-binding sites as Lac⁺ colonies on MacConkey agar plates. In contrast to the traditional FURTA, we used a strain (FUR-SEL1) carrying a

Table 2. Plating efficiency (PE) of strains VK-0C and VK-6C

Values given are means of five independent experiments $\pm$ standard error.

Strain	Plasmid	PE*	PE(pUCFUR1): PE(pUC19)
VK-0C	pUC19	$< 2.0 \times 10^{-7}$	$(4.2 \pm 0.7) \times 10^3$
VK-0C	pUCFUR1	$(8.5 \pm 1.4) \times 10^{-4}$	
VK-6C	pUC19	$< 1.5 \times 10^{-7}$	$(4.5 \pm 1.5) \times 10^6$
VK-6C	pUCFUR1	0.7 ± 0.2	

* PE is the proportion of cells capable of forming colonies on agar medium containing chloramphenicol at a concentration of $7.5 \mu\text{g ml}^{-1}$ after 36 h incubation at 37°C .

fhuA'-*cat* fusion expressed from the Fur-regulated *fhuA* promoter. This approach allows plasmid-encoded Fur boxes to confer chloramphenicol resistance on FUR-SEL1, which can be used as a selectable phenotype for cloning Fur-regulated loci.

Strain VK-0C was used to determine the levels of chloramphenicol resistance achieved for transformants with a high-copy-number plasmid containing a Fur box. The plating efficiency of the strain transformed with pUC19 and pUCFUR1 carrying a synthetic Fur box consensus sequence was tested on iron-rich medium containing chloramphenicol (7.5 mg l^{-1}). The survival of strain VK-0C containing pUCFUR1 was found to be increased by about 4×10^3 -fold compared to that of the strain with pUC19 (Table 2). However, in spite of the fact that VK-0C with pUC19 virtually completely loses its ability to grow on medium with chloramphenicol (its plating efficiency becomes less than 2.0×10^{-7}), which excludes undesirable background in cloning, the 4×10^3 -fold increase in the survival of VK-0C caused by plasmid-encoded Fur boxes is not enough for the strain to be applied successfully for cloning Fur-regulated loci. The reason for this is that the absolute value of survival still remains low ($\sim 10^{-3}$).

To enhance the difference in the level of *cat* expression between the cells transformed with pUC19 and Fur-box-containing plasmids we tried to find a leaky *fur* mutation that would reduce the affinity of Fur for the *fhuA* promoter and, thereby, allow Fur to be titrated more easily by a Fur box introduced on a multicopy plasmid.

On analysing the effect of pUCFUR1 on chloramphenicol resistance for a collection of 24 *fur* mutants of strain AB1157 (see Methods), we found that strain VK-6C exhibited the greatest increase in resistance. The survival of strain VK-6C containing plasmid pUCFUR1 on chloramphenicol medium was increased by 4.5×10^6 -fold compared to that of the same cells transformed with pUC19 (Table 2). This increased difference in chloramphenicol resistance accompanied by the increase in the plating efficiency (up to 0.7) of the pUCFUR1-containing

Table 3. Induction of fusion operons *fhuF'*-*lac* and *fiu'*-*lac* caused by pEF plasmids

Plasmid	Induction of chromosomal <i>lac</i> fusions		Promoter region§	Co-ordinates of <i>Sau</i> 3A insertions	
	<i>fhuF'</i> - <i>lac</i>				<i>fhu'</i> - <i>lac</i> Lac-FURTA‡
	Lac-FURTA*	β-Gal activity†			
pUC19	—	61 ± 3 (1)	—		
pUCFUR1	++	170 ± 7 (2.8)	++		
pEF7	+++	192 ± 9 (3.1)	++	<i>fes-fepA</i> 611806–612065	
pEF10	++++	437 ± 33 (7.2)	+++	<i>entC-fepB</i> 623530–624348	
pEF19	+	134 ± 9 (2.2)	+	<i>nrdH-ygaC</i> 2798161–2798843	
pEF20	+	123 ± 12 (2.0)	+	<i>pgmA</i> 786730–787325	
pEF23	++++	323 ± 18 (5.3)	++	<i>cir</i> 2244898–2245287	
pEF29	++	167 ± 12 (2.7)	+	<i>nobA</i> 1634593–1634708	
pEF33	++++	303 ± 17 (5.0)	++	<i>cir</i> 2244898–2245287	
pEF34	+++	232 ± 15 (3.8)	++	<i>yhbX-yhbY</i> 3578643–3578880	
pEF63	+++	195 ± 6 (3.2)	++	<i>fhuF-orf78</i> 4603166–4603459	
pEF67	+++	187 ± 3 (3.1)	++	<i>fes-fepA</i> 611806–612065	

* Lac phenotype detected after 24 h incubation at 30 °C on Lac-EMBO medium supplemented with 50 μ M (NH₄)₂Fe(SO₄)₂. Lac[−] phenotype (−), pink colonies; Lac⁺: wine-coloured (+) to dark-wine-coloured with a green halo (++++) colonies.

† Calculated according to Miller (1972). Values given are means of at least two independent experiments ± standard error. The fold increase in β -galactosidase activity caused by plasmids pEF is indicated in parentheses.

‡ Lac phenotype detected after 36 h incubation at 30 °C on Lac-EMBO medium supplemented with 5 μ M (NH₄)₂Fe(SO₄)₂; Lac[−] phenotype (−), pink colonies; Lac⁺: wine-coloured (+) to dark-wine-coloured with a green halo (++++) colonies.

§ Promoter activities preceding *ygaC*, *nobA*, *yhbX* and *orf78* were identified in the F19, F29, F34 and F63 fragments, respectively (see text for details).

|| Co-ordinates of the *Sau*3A chromosomal fragments cloned in pEF plasmids are given in bp on the *E. coli* genome sequence (Blattner *et al.*, 1997).

strain made it likely that strain VK-6C would be suitable for cloning chromosomal Fur boxes.

In addition, the $\Delta(lacZ)$ M15 mutation was introduced into strain VK-6C to allow screening for recombinants as Lac[−] clones on X-Gal medium in contrast to clones containing pUC19 exhibiting a Lac⁺ phenotype. The resulting strain was named FUR-SEL1 and was used for cloning Fur-box-containing fragments of the *E. coli* chromosome.

Cloning and functional analysis of Fur-regulated loci

*Sau*3A fragments of the AB1157 chromosome were inserted into the *Bam*HI site of pUC19. FUR-SEL1 cells were transformed by electroporation, the transformants were selected on ampicillin- and chloramphenicol-containing plates and then screened for the presence of recombinant plasmids on X-Gal medium.

The chloramphenicol resistance of the clones selected in this way may be caused not only by recombinant plasmids carrying Fur boxes but also by chromosomal mutations, for instance in *cmlAB*. Therefore, the recombinant plasmids from the clones selected with chloramphenicol were retransformed into FUR-SEL1 and tested

by repeating the FURTA in order to distinguish the clones containing recombinant plasmids with Fur boxes from those whose chloramphenicol resistance is due to a chromosomal mutation. All plasmids examined gave positive results in the second Cm-FURTA. The plating efficiencies of FUR-SEL1 cells transformed by the plasmids were 10⁵–10⁶ times higher than the FUR-SEL1 cells containing pUC19.

It is possible that recombinant plasmids carrying chromosomal fragments other than Fur boxes could cause a nonspecific increase in chloramphenicol resistance, not connected with *fhuA'*-*cat* derepression. To confirm the reliability of the selection technique, *fhuF'*-*lacZ* and *fiu'*-*lacZ* operon fusions, in which expression of β -galactosidase emanates from Fur-regulated promoters *fhuF*_p and *fiu*_p, respectively, were tested in the FURTA. If the recombinant plasmids obtained carry Fur boxes they should cause derepression not only of the *fhuA* promoter but also of other Fur-regulated operons.

Ten recombinant plasmids (pEF7, 10, 19, 20, 23, 29, 33, 34, 63, 67) chosen at random among those containing putative Fur boxes were introduced into strains H1717 and H1698-1. Plasmids pUCFUR1 and pUC19 were used

as positive and negative controls, respectively. Derepression of the operon fusions caused by the pEF plasmids was detected on Lac-EMBO agar medium supplemented with $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2$. All 10 recombinant plasmids and the control plasmid pUCFUR1 were positive in this Lac-FURTA. They conferred a Lac⁺ phenotype (wine-coloured colonies) to cells carrying reporter fusions (Table 3). In contrast, the cells containing pUC19 exhibited a Lac⁻ phenotype (pink colonies on Lac-EMBO plates).

In addition, pEF-stimulated derepression of *fhuF'*-*lacZ* was confirmed by measuring the activity of β -galactosidase in strain H1717 harbouring pEF plasmids. As seen in Table 3, the level of β -galactosidase activity reached in the strain carrying any of the 10 pEF plasmids was increased by two- to sevenfold compared to that reached in the same strain containing the vector control.

Sequence analysis and mapping of Fur-regulated loci cloned in FUR-SEL1

Automated sequencing of the 10 recombinant plasmids shown to be positive in the Cm-FURTA and the Lac-FURTA was performed. Comparison of their nucleotide sequences with genes known to be Fur-regulated revealed that six of the plasmids contained promoter regions of genes responsible for iron uptake. The inserts of two of these plasmids (pEF23 and pEF33) contained the promoter region of the *cir* gene, encoding the colicin I receptor responsible for 2,3-dihydroxybenzoate-promoted iron uptake (Hancock *et al.*, 1977). Three overlapping Fur-binding sites are located in the *cir* promoter region (Griggs & Konisky, 1989). Two FURTA-positive plasmids (pEF7 and pEF67) contained overlapping, divergently oriented promoters governing the expression of *fes-entF* and *fepA-entD* belonging to the enterochelin gene cluster. A single operator region consisting of two overlapping Fur boxes co-ordinately regulates the expression of the genes (Hunt *et al.*, 1994). One plasmid (pEF10) contained another bidirectional promoter from the enterochelin region that contacts the *fepB* and *entCEBA* operons. Three Fur boxes are characterized in the bidirectional control region: one located in the *fepB* promoter and a pair of contiguous Fur boxes in the 5' untranslated region of the *entC* message (Brickman *et al.*, 1990). One plasmid (pEF63) contained a *Sau3A* fragment with the promoter region of *fhuF*, which is responsible for ferrioxamine B-promoted iron uptake (Patzer & Hantke, 1999).

The remaining four plasmids contained promoter regions of genes which are not at present known to be Fur regulated in *E. coli*. The insert of plasmid pEF20 contained the promoter of the *pgm* gene encoding phosphoglyceromutase 1. The position of the corresponding *Sau3A* fragment on the *E. coli* genome sequence (Blattner *et al.*, 1997) is given in Table 3. Two putative overlapping Fur-binding sites matching the consensus sequence in 15 of 19 bases spanned positions -13 to +12 with respect to the *pgm* transcriptional-start site (Fig. 2b). pEF19 contained the promoter region

of the *nrdHIEF* operon responsible for synthesis of deoxyribonucleotides from the corresponding ribonucleotides (Jordan *et al.*, 1996). A sequence matching the consensus site for Fur binding in 11 of 19 bases was found to precede the promoter at position -61 relative to the transcription-start site (Fig. 2c). pEF34 contained two potential promoters located upstream of an ORF, designated *yhbY* (or *o162*), defining a gene product of unknown function. Two putative overlapping Fur boxes identical to the consensus sequence in 16 and 13 of 19 bases were present at the periphery of the proposed promoters, spanning positions -68 to -94 with respect to the transcriptional start site of *yhbY* (Fig. 2a). pEF29 contained a 115 bp chromosomal fragment where no promoter or ORF has previously been identified. Two putative overlapping Fur boxes, each of which matched the consensus sequence in 15 of 19 bases, were present in the *Sau3A* fragment. The nearest ORF that is preceded by the Fur boxes is the *nohA* gene belonging to cryptic prophage Qin, which many strains of *E. coli* K-12 contain (Kotani *et al.*, 1992). The Fur-binding region is located approximately 230 bp upstream of the coding sequence of *nohA*.

In summary, sequence analysis of 10 recombinant plasmids chosen at random among the ones selected with FUR-SEL1 permitted us to identify eight distinct Fur-regulated loci, four of which have not previously been shown to be Fur-regulated in *E. coli*. The promoter regions of *fepA-fes* and *cir* were found in several plasmids.

Fur-dependent promoter activity of new genes belonging to the Fur regulon of *E. coli*

To prove that the chromosomal fragments cloned in FUR-SEL1 possess promoter activities regulated by Fur, the inserts of pEF19, pEF20, pEF29 and pEF34 were transferred into the promoter probe vector pBR- Δ . This vector is a pBR322 deletion derivative with the promoterless tetracycline resistance gene *tet*. The orientations of the proposed Fur-regulated promoters in pEF19, pEF20 and pEF29 were determined from sequence data to be opposite to that of the *lac* promoter of the vector. That permitted us to conclude that transfer of their *EcoRI-HindIII* fragments, including chromosomal insertions, into pBR- Δ would result in transcriptional fusions to *tet*. The fact that all the resulting constructs (pBR- Δ F19, pBR- Δ F20 and pBR- Δ F29) were selected on tetracycline medium by transforming strain 803-8 confirmed this prediction. The direction of the *yhbY* promoter in pEF34 coincided with the *lac* promoter and hence the same strategy could not be employed for creating a *yhbY'*-*tet* fusion. However, the F34 fragment contained a region of the genome upstream of the *yhbY* promoter where no regulatory sites or ORFs are identified according to the NCBI (National Center for Biotechnology Information) database. Therefore, the pBR- Δ derivative of pEF34 was also constructed to examine whether the F34 fragment contains promoter activity oriented oppositely to *yhbY* in this region. The

Fig. 2. Organization of promoter-regulatory regions in *Sau3A* chromosomal inserts of FURTA-positive clones. Arrangement of genetic elements in cloned *Sau3A* fragments and contiguous regions of the *E. coli* genome is shown. (a), fragment F34 (pEF34); (b), fragment F20 (pEF20); (c), fragment F19 (pEF19); (d), fragment F29 (pEF29); (e), fragment F63 (pEF63). The 5'–3' nucleotide sequences are shown on the DNA strand which is an antisense strand for loci transcribed from left to right and a sense one for loci transcribed in the opposite direction on the *E. coli* chromosome. The distances between two represented DNA sequences or/and between a regulatory region and the corresponding ORF are given in base pairs and designated with numbers preceded by N. Proposed promoters and putative Fur-protein-binding sites are marked. White arrows denote positions and orientations of ORFs. Figures in parentheses indicate identity (in base pairs) of putative Fur boxes with the Fur box consensus sequence. Fur boxes were identified on the basis of similarity to the consensus sequence for Fur binding. The potential promoters and transcriptional start points for *yhhX*, *ygaC*, *nohA*, *orf78* and *fhuF*, consistent with the positions of the corresponding Fur boxes and statistically most similar to the consensus sequence, were determined on the basis of the algorithm described by Staden (1984).

selection of pBR-ΔF34 in strain 803-8 with tetracycline confirmed the existence of the opposing promoter activity. The nearest ORF which is preceded by the promoter activity is the coding sequence for *yhhX*, which lies 205 bp beyond the F34 fragment on the *E. coli* genome (Fig. 2a).

In addition, pBR-Δ derivatives for known Fur-regulated promoters *fes_p* and *fepA_p* were constructed to be used as positive controls when analysing promoter activity of new Fur-regulated loci. For this purpose, the *EcoRI*–*HindIII* fragments of pEF7 and pEF67 were inserted into pBR-Δ, giving rise to pBR-ΔF7 and pBR-ΔF67, respectively. Both plasmids contain the same chromosomal fragment carrying the bidirectional promoter region *fes*–*fepA*. In pBR-ΔF7, the region is oriented in such a way that the transcription of *tet* emanates from *fes_p*, but in pBR-ΔF67, the expression of *tet* is driven by *fepA_p*.

The expression of the *tet* gene in pBR-ΔF plasmids was compared in Fur⁺ and Fur[–] backgrounds, in strains AB1157 and VK-12, respectively. Of the six constructs examined, all six exhibited Fur-responsive promoter activity (Table 4). Under Fur[–] conditions, plating efficiency on tetracycline medium of the cells containing the plasmids was increased by greater than 10⁴- to 10⁵-fold compared to Fur⁺ conditions, which confirmed the involvement of Fur in the regulation of the activity of promoters preceding the *nrdHIEF*, *pgm*, *nohA* and *yhhX* genes.

Analysis of DNA regions selected in FUR-SEL1 for divergent promoter activities

Taking into consideration that divergently oriented promoter regions are an organizational scheme utilized often enough in a broad range of organisms we assumed

Table 4. Promoter activity of FURTA-positive fragments

Values shown are means of three independent experiments $\pm$ standard error.

Plasmid	Plating efficiency (PE)		
	Fur ⁺ (strain AB1157)	Fur ⁻ (strain VK-12)	PE(Fur ⁻): PE(Fur ⁺)
pBR- Δ F7*	$< 3.0 \times 10^{-6}$	0.6 ± 0.2	$> 2.0 \times 10^5$
pBR- Δ F67*	$< 3.5 \times 10^{-6}$	0.7 ± 0.2	$> 2.0 \times 10^5$
pBR- Δ *†	$< 2.5 \times 10^{-6}$	$< 3.5 \times 10^{-6}$	1
pBR- Δ F19‡	$< 6.0 \times 10^{-7}$	$(1.0 \pm 0.4) \times 10^{-2}$	$> 1.7 \times 10^4$
pBR- Δ F19-A*	$< 3.5 \times 10^{-6}$	$(2.0 \pm 0.6) \times 10^{-1}$	$> 5.7 \times 10^4$
pBR- Δ F20§	$< 3.0 \times 10^{-6}$	0.5 ± 0.1	$> 1.7 \times 10^5$
pBR- Δ F29†	$< 3.0 \times 10^{-6}$	$(2.0 \pm 0.6) \times 10^{-2}$	$> 0.7 \times 10^4$
pBR- Δ F34‡	$< 3.5 \times 10^{-6}$	0.4 ± 0.2	$> 1.0 \times 10^5$
pBR- Δ F63-A§	$< 3.5 \times 10^{-6}$	0.5 ± 0.2	$> 1.4 \times 10^5$

* Determined after 20 h incubation at 37 °C on agar medium with 10 μ g tetracycline ml⁻¹.

† 5 μ g tetracycline ml⁻¹.

‡ 15 μ g tetracycline ml⁻¹.

§ 20 μ g tetracycline ml⁻¹.

that some chromosomal fragments cloned in plasmids pEF could also possess such promoter activities.

To examine the chromosomal fragments for possible divergent promoter activities, the *Sau*3A inserts of pEF19, pEF20, pEF29, pEF34 and pEF7 (as a control)

were recloned into the promoter probe vector pUJ10. This vector contains a multiple cloning site flanked by promoterless β -galactosidase and alkaline phosphatase genes oriented in opposite directions. Therefore, the introduction of a fragment at this site permits analysis of promoter activity of the insertion simultaneously in both directions. The orientations of *Sau*3A insertions on the pUJ10 derivatives determined by restriction endonuclease mapping are shown in Table 5. By measuring the levels of the β -galactosidase and alkaline phosphatase expressed from the pUJ10 derivatives in response to various iron concentrations in the growth medium, the directions of promoter activities on the *Sau*3A inserts were determined and the influence of iron on the regulation of the promoters driving the synthesis was assessed (Table 5).

Chromosomal insertions F19 and F34 exhibited bidirectional iron-responsive promoter activity. Besides the β -galactosidase expression driven by the promoters of *nrdH* and *ybhX*, whose Fur regulation was estimated with the corresponding pBR- Δ constructs in previous experiments, pUJ-F19 and pUJ-F34 showed iron-regulated alkaline phosphatase expression. The promoter activity driving *phoA* expression in pUJ-F19 was regulated negatively by iron: alkaline phosphatase synthesis was consistently lower in iron-rich conditions compared with that at iron limitation. In contrast, the promoter directing *phoA* synthesis in pUJ-F34 was regulated in a positive fashion by iron: under iron-replete conditions roughly fivefold induction of alkaline phosphatase synthesis was observed while the synthesis fell to near-background levels under low-iron conditions.

Table 5. Bidirectional promoter activity of FURTA-positive fragments

Mean standard deviations did not exceed 15 % of the given values representing means of two experiments.

Alkaline phosphatase activity*			Structure of a bidirectional fusion region in pUJ-F derivatives†	β -Galactosidase activity*		
Induction (-Fe/+Fe)‡	+Fe	-Fe		-Fe	+Fe	Induction (-Fe/+Fe)‡
1:3	17	22	pUJ10: $\langle phoA' 'lacZ \rangle$	1:8	1:3	1:4
	ND		pUJ-BLA: $\langle phoA' bla' -'lacZ \rangle$	62	55	1:1
14:3	442	6314	pUJ-F7: $\langle fepA' -'phoA fes' -'lacZ \rangle$	260	11	23:6
6:7	106	712	pUJ-F19: $\langle ygaC' -'phoA nrdH' -'lacZ \rangle$	324	120	2:7
1:4	36	51	pUJ-F20: $\langle phoA' pgm' -'lacZ \rangle$	214	61	3:5
1:6	110	175	pUJ-F29: $\langle nobA' -'phoA 'lacZ \rangle$	2:5	1:9	1:3
0:18	269	49	pUJ-F34: $\langle ybhY' -'phoA ybhX' -'lacZ \rangle$	246	6:3	39:1
18:0	522	9420	pUJ-F63: $\langle fhuF' -'phoA orf78' -'lacZ \rangle$	340	83	4:1

* Expressed in Miller Units (Miller, 1972; Brickman & Beckwith, 1975).

† Symbols $\rangle$ and $\langle$ indicate the orientation of promoterless portions of the *lacZ* and *phoA* genes or the direction of transcription of fusion operons on pUJ-F derivatives.

‡ Ratio of the alkaline phosphatase or β -galactosidase activity in low-iron to that in iron-supplemented conditions.

The F20 insertion was found not to contain other promoters besides that of the *pgm* gene: pUJ-F20 carrying the insertion in orientation yielding the *pgm'*-*lacZ* fusion exhibited iron-regulated β -galactosidase activity (which was consistent with the data from the previous study of the expression of the pBR- Δ F20 construct in *Fur*⁺ and *Fur*⁻ background) but the level of the alkaline phosphatase activity of this clone did not significantly exceed that of the vector control.

The F29 chromosomal fragment exhibited low alkaline phosphatase activity, about 10-fold higher than the background level. The *phoA* synthesis was reproducibly higher under low-iron conditions although the difference never exceeded 60%. The β -galactosidase synthesis observed in cells with pUJ-F29 did not exceed the background level at both low and high iron which led us to conclude that there is not any promoter activity in this direction.

To confirm that the observed iron-responsive induction of the examined operon fusions was not attributable to a difference in plasmid copy number under iron-limited conditions, the expression of the *bla'*-*lacZ* fusion carried on plasmid pUJ-BLA and governed by the Fur-independent promoter of the gene encoding β -lactamase was measured under high- and low-iron conditions. The β -lactamase activities under the two growth conditions were quite similar, which ruled out the involvement of copy number in the modulation of the iron-responsive expression of the fusions carried on pUJ10 derivatives.

To deduce an appropriate coding sequence for the opposing promoter activity observed for the F19 insertion, the DNA sequence of this fragment and the contiguous region of the chromosome were scanned for long ORFs in each of three reading frames in the direction consistent with the promoter activity. Long ORFs other than *ygaC* (noted in the database) were not found. Accordingly, the ORF lying 145 bp upstream of the F19 fragment on the *E. coli* chromosome (Fig. 2c) could be transcribed from the promoter situated within the F19 fragment despite the fact that another potential promoter region preceding the *ygaC* coding sequence and lying beyond the F19 fragment is proposed in the database.

Subsequent transfer of the F19 fragment into pBR- Δ in the orientation yielding the *ygaC'*-*tet* fusion and analysis of the *tet* expression from the pBR- Δ derivative in *Fur*⁺ and *Fur*⁻ strains showed that regulation of the *ygaC* promoter by iron depends on Fur (Table 4; pBR- Δ F19-A). pBR- Δ F34-A carrying the F34 insertion in the orientation giving rise to the *yhhY'*-*tet* fusion was unstable in a *Fur*⁻ strain (perhaps because of a deleterious effect of high-level expression of *bla* from the strong divergently directed *yhhX* promoter). Therefore, in this case the involvement of Fur in regulation was not confirmed by a more direct fashion.

Finally, the F23 insertion was examined for the presence of promoter activity in the direction opposite to the *cir* promoter located in this fragment. For this purpose, the

corresponding pBR- Δ derivative, pBR- Δ F23, was constructed and the plating efficiency of strains AB1157 and VK12 transformed by pBR- Δ F23 was measured at different concentrations of tetracycline (3.0–12.5 mg l⁻¹). pBR- Δ F23 was not found to confer any tetracycline resistance to the *Fur*⁺ host or its *Fur*⁻ derivative: the plating efficiency of the strains carrying pBR- Δ F23 did not exceed that of the same cells containing vector pBR- Δ (data not shown). The results indicated that the F23 fragment does not contain promoter activity opposed to *cir*.

Analysis of the F63 chromosomal fragment for additional genetic loci

A detailed study of the F63 chromosomal fragment was performed to ascertain whether the fragment contains regulatory regions other than the *fhuF* promoter. The basis for such an assumption was the fact that pEF63 provided α complementation of mutation Δ (*lacZ*)M15 located on the chromosome of *FUR*-SEL1 (the clones harbouring the plasmid were light blue on X-Gal medium). This observation could be due to the creation of a functional in-frame fusion between *lacZ* and some coding sequence located on the insertion and containing a functional binding site for ribosomes. However, according to the database, the only coding sequence located in the fragment was the 5' end of the *fhuF* gene oriented oppositely to *lacZ*. The analysis of the sequence of the F63 fragment revealed that it contains the 5' end of a 234 bp ORF which had no homology to any entries in the database. The ORF (designated *orf78*) was oriented in the opposite direction relative to *fhuF* and its amino-terminal portion (30 aa) created an in-phase fusion with the α fragment of LacZ.

To determine whether the promoter driving *orf78* transcription is contained within the F63 insertion or the transcription of the *orf78'*-*lacZ* fusion is governed by the *lacZ* promoter of pUC19, the F63 fragment was transferred into the promoter probe vector pUJ10. Among LacZ⁺ clones the pUJ10 derivatives containing the insertion both in the orientation giving rise to the *fhuF'*-*lacZ* fusion and in the opposite orientation producing *orf78'*-*lacZ* (pUJ-F63) were found. The results pointed to the fact that the F63 insertion contained two divergent promoter activities. Clone pUJ-F63 was chosen to characterize the dependence of the *orf78* promoter activity from iron. As shown in Table 5, growth of CC118(pUJ-F63) under low-iron conditions resulted in marked induction of the alkaline phosphatase expression governed by the *orf78* promoter compared with growth in the presence of iron. To explore the mechanism by which the iron concentration in the growth medium regulates the expression of *orf78* and the involvement of Fur in this regulation, the F63 fragment was inserted in pBR- Δ (plasmid pBR- Δ F63-A) and the expression of the *orf78'*-*tet* fusion was analysed in *Fur*⁺ and *Fur*⁻ backgrounds. Assay of the tetracycline resistance of the pair of isogenic strains AB157 and VK12 containing pBR- Δ F63-A (Table 4) confirmed that the regulation of *orf78* by iron depends on the *fur* locus.

DISCUSSION

In this study strain FUR-SEL1 that enables the cloning of Fur-regulated loci is described. Unlike the current methods, including the FURTA technique, for identification of genes belonging to a given regulon which are based on screening, the *in vivo* procedure allows positive selection of clones. The introduction of a chromosomal leaky *fur* mutation into FUR-SEL1 allowed cloning of Fur boxes exhibiting weak affinity for the Fur repressor. Indeed, only plasmids pEF10 (*entC-fepB*) and pEF23 or pEF33 (*cir*) were able to impart considerable chloramphenicol resistance to the Fur⁺ strain VK-0C. The plasmids carrying the other Fur-regulated loci found in this work, namely: *fhuF-orf78*, *fepA-fes*, *pgmA*, *nrdHIEF-ygaC* and *ybhY-ybhX* did not bring about sufficient *fhuA* promoter derepression for effective growth of Fur⁺ cells on chloramphenicol medium.

Genes *pgm*, *ybhY* (*o162*), *ybhX*, *ygaC*, *nrdHIEF*, *nohA* and *orf78*, whose Fur regulation has previously not been demonstrated in *E. coli*, were identified during this study. Fur-regulated ORFs exhibiting high similarity with *pgm* and *o162* of *E. coli* (identity at the level of amino acid sequences was 97% and 85%, respectively) were found previously in *S. typhimurium* by FURTA screening of a plasmid bank (Tsolis *et al.*, 1995). *o162* defines a Fur-regulated product of yet unknown function, and no homology to known proteins has been found. The Fur regulation of *pgm* encoding phosphoglyceromutase, an enzyme catalysing one of the phosphorylation reactions in glycolysis, is one more confirmation of the involvement of Fur in the control of general catabolic pathways. The first evidence for this was provided by the findings that the genes encoding aconitase of *E. coli* and fumarase of *P. aeruginosa* contained a Fur box in their promoters (Prodromou *et al.*, 1992; Ochsner & Vasil, 1996).

The *nrdHIEF* operon defines a locus which has not previously been described in *E. coli* or *S. typhimurium* as Fur regulated. Our data, together with the fact that expression of the *purR* repressor, a regulatory protein for the *pur* regulon and for genes involved in pyrimidine synthesis (He *et al.*, 1993), is under Fur control (Stojiljkovic *et al.*, 1994), give evidence in support of the involvement of Fur in the regulation of the synthesis of ribonucleotides and deoxyribonucleotides.

The analytical study of the sequences of the FURTA-positive chromosomal fragment selected in this work permitted identification of promoter-like sequences and putative Fur boxes that could be responsible for the Fur-regulated promoter activities. The results obtained supported a mechanism for the iron-dependent repression of *ybhX*, *ygaC*, *pgm* and *nohA*, and *orf78* transcription that was similar to that utilized for control of other known Fur-regulated genes: the putative Fur-binding sites overlap the -10 (and -35 in the case of *orf78*) region of the promoters. However, the positions of the *ybhY*, *nrdH* and *fhuF* promoters are not consistent with the placement of proposed Fur boxes, which might be caused by either inadequacies in the computer

algorithms employed for detecting the promoters or a more complex scheme of the regulation of the genes supposing involvement of other regulatory mechanisms. Some assumptions that could remove the noted discrepancy are given below.

Among the iron-regulated loci identified in this work there was a locus, *ybhY*, which is regulated in a positive fashion. A putative Fur-binding region consisting of two overlapping Fur boxes was located 32 bp upstream of the *ybhY* promoters and overlapped the opposing *ybhX* promoter (Fig. 2a). In light of the absence of data suggesting a role for Fur as a positive effector, the activation of the *ybhY* promoter under conditions when Fur is also active could be explained by participation of another repressor, which in its turn is negatively regulated by Fur, in the regulation of *ybhY*.

In the F63 fragment, two nearby Fur-binding sites with high similarity (15 and 16 matches) to the Fur box consensus sequence were found. However, neither of them overlapped the promoter region (indicated in Fig. 2e as P'*fhuF*) predicted for *fhuF* in the database. On the other hand, the results obtained from alkaline phosphatase measurements led to the conclusion that expression of the *fhuF'*-*phoA* fusion is highly sensitive to iron: the iron-mediated induction ratio produced by the *fhuF* regulatory region reached values attributable to strongly Fur-regulated promoters. In light of the data the possibility of other potential promoters was investigated. A promoter-like sequence (denoted P'*fhuF* in Fig. 2e) very similar to the consensus promoter sequence and consistent with the position of Fur-binding sites was found. The two putative Fur boxes identified in the F63 fragment encompassed the -10 and -35 elements as well as the transcription-initiation site of P'*fhuF*. Such an arrangement of the *fhuF* regulatory region could abolish the discrepancy in the positions of promoter and operator signals and bring them into line with the significant Fur-mediated modulation of *fhuF* expression.

The Fur-binding regions of all the FURTA-positive fragments identified in this study, except the F19 fragment (*nrdH-ygaC*), were represented by two Fur boxes displaced 6 bp in respect of each other. Such arrangement of the operator region is typical for many Fur-regulated genes, including *fepA-fes* (Hunt *et al.*, 1994), *fur* (de Lorenzo *et al.*, 1988), *sltA* (Calderwood & Mekalanos, 1987), *cir* (Griggs & Konisky, 1989) and *entC-fepB* (Brickman *et al.*, 1990), and indicates that binding of two repressor dimers occurs which contact major groove turns on the DNA helix if Fur conforms to the structural motif observed for other prokaryotic repressors (Pabo & Sauer, 1984; Ptashne, 1986). Perhaps these overlapping sites increase specificity or help to stabilize binding by increasing the site of the target region.

The *fhuF-orf78* operator region consisted of two Fur boxes separated by 4 bp with good similarity to the consensus iron box (15 and 16 matches) and two Fur boxes (not shown in Fig. 2) with less similarity to the consensus (12 matches) which were displaced 6 bp in

respect of the major operators. The placement of the major operators in respect of each other was similar to that for phage λ , where co-operative binding of CI repressor dimers occur (Ptashne, 1986). Therefore, the exceptional sensitivity of the *fhuF* promoter to changes of iron concentration (Stojiljkovic *et al.*, 1994) may be due in part to co-operative effects.

The Fur proteins of Gram-negative bacteria exhibit a high degree of sequence similarity. For example, the Fur repressors of *E. coli*, *Vibrio cholerae* and *Yersinia pestis* have 75% identity (Staggs & Perry, 1991; Litwin *et al.*, 1992). This is the reason why Fur boxes from different Gram-negative bacteria can be detected in *E. coli* strains suitable for FURTA (Stojiljkovic *et al.*, 1994; Tsolis *et al.*, 1995). FUR-SEL1 could, therefore, be employed for cloning Fur-regulated loci, among which there are genes responsible for pathogenicity, not only from *E. coli* but also from other enterobacteria. What is more, the use of *cat* operon fusions whose coding sequence for *cat* is transcribed from promoters regulated by other regulatory proteins could be a general strategy to clone the targets for the regulators.

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