The Journal of Microbiology, December 2007, p. 583-589
Vol. 45, No. 6
Copyright © 2007, The Microbiological Society of Korea

Effect of *fur* on *pyrC* Gene Expression

Sangho Chai, Chang Kyu Song, Seong Kwun Kim, Jun Ho Park, and Sechan Wee*

Department of Biomedical Sciences, Hallym University, Chun Cheon 200-702, Republic of Korea

(Received October 31, 2006 / Accepted December 17, 2007)

The promoter region of *pyrC* (dihydroorotase) gene of *Escherichia coli* was shown to have Fur protein binding properties by gel retardation assay. *In vivo* regulation of the *pyrC* expression was studied by measuring dihydroorotase activity and β-galactosidase level in the *fur*$^+$ and *fur*$^-$ genetic background. The expression of chromosomal dihydroorotase activity and β-galactosidase activity of *pyrC-lacZ* fusion plasmid was repressed to about 30% and 17%, respectively in the *fur*$^+$ strain compared to those in the *fur*$^-$ strain. Divalent ions such as Fe^{2+} and Zn^{2+} were not required for the repression. *PyrC* expression was also reduced to one half by 1 mM uracil. The effect of uracil was independent on the *fur* gene.

Keywords: Fur, *pyrC* expression, iron, dihydroorotase

Mode of negative regulation by Fur (ferric uptake regulation) protein is essentially the same as typical negative control of tryptophan paradigm. When the intracellular iron concentration is high, iron binds to the Fur protein (aporepressor) to make active repressor. Then the activated repressor binds to the promoter region of the genes, aerobactin operon, for instance to abolish transcription of the genes for synthetic enzymes of siderophore (aerobactin) and outer membrane receptor (Bagg and Neilands, 1987). The repression of the genes consequently results in decrease of siderophore production, and iron uptake into the cell. Since the repressor has relatively weak affinity to iron, the metal dissociates from the protein to produce an inactive aporepressor in iron deficient condition. If intracellular iron becomes below a critical level, the iron-regulated genes become derepressed and begin to transcribe genes for aerobactin synthetic enzymes and receptor of the ferri-aerobactin (Bagg and Neilands, 1987).

Action of the Fur protein does not seem to confine only to the iron metabolism. From the result of 2-D gel electrophoresis, Foster and Hall (1992) showed that 36 proteins of *Salmonella typhimurium* were affected by iron availability, most of which were under the control of *fur*. They found some proteins were regulated by Fur without iron and some iron regulated proteins did not require Fur (Foster and Hall, 1992). Some Fur-regulated genes apparently are negatively controlled, while some are positively controlled. However, the genes coding for the proteins were not identified. Stojiljkovic *et al.* (1994) developed a genetic screening method called as Fur titration assay (FURTA) for detecting iron-regulated and iron-storage/binding proteins. They identified 24 cosmid clones that respond to the system. Nine of them may contain Fur-box sequences in yet unidentified genes. From newly developed genome analyses, wide variety of gene group was identified as Fe^{2+}-Fur regulated in *Salmonella enterica* (Bjarnason *et al.*, 2003). Positively or negatively regulated genes were identified which are involved in iron metabolism, energy metabolism, and miscellaneous phenomena such as metal assimilation, phage related function, and nucleic acid metabolism (McHugh *et al.*, 2003). Since Fur is involved in regulation of genes of large variety of cellular functions, it is regarded as a global regulator such as Fnr and Arc.

In our previous work using nitrocellulose filter binding assay (Oh *et al.*, 1999), we isolated and cloned five DNA fragments possessing putative Fur binding site some of which are located in the promoter region. *PyrC* (dihydroorotase) was the one of the genes identified by the above method suggesting that it is a member of the Fur regulon. In this study, dihydroorotase activity and β-galactosidase level of the *pyrC-lacZ* fusion were tested in the presence or absence of *fur* background to determine whether the gene, *pyrC*, is really under the control of *fur* gene in their expression.

Materials and Methods

Bacterial strains and growth conditions

Strains and plasmids used in this study are listed in Table 1. Bacteria were routinely cultured in Luria-Bertani (LB) broth or LB plates. Minimal media M9 and MM9 (Schwyn and Neilands, 1987) was supplemented with tryptophan (40 μg/ml), proline (40 μg/ml), uracil (40 μg/ml), and thiamine (40 μg/ml) for the strains of KWC and KWT. For AB402 and AB4020, histidine (40 μg/ml), arginine (40 μg/ml), threonine (40 μg/ml), tryptophan (40 μg/ml), and thiamine·HCl (40 μg/ml) were supplemented. For iron supplementation, 20 mM ferric chloride in 10 mM HCl was added. Antibiotics concentrations were as follow; ampicillin (100 μg/ml), chloramphenicol (25 μg/ml), kanamycin (50 μg/ml), streptomycin (50 μg/ml), and nalidixic acid (250 μg/ml). Numbers in parentheses indicate final concentration in the medium.

In order to obtain *pyrC*$^-$ and *lacZ*$^-$ double mutant strain, KWC, CSH59 was conjugated with CSH63. Donor (CSH63)

* To whom correspondence should be addressed.
(Tel) 82-33-248-2111; (Fax) 82-33-256-3420
(E-mail) swee@hallym.ac.kr

Table 1. Bacterial strains and plasmids used

Strain and plasmid	Descripton	Source or reference
DH5α		Lab stock
AB402	*his, arg, thr, lac U169, galK, trp, thi*	Lab stock
AB4020	*fur, his, arg, thr, lac U169, galK, trp, thi*	Lab stock
JRB45	*lacΔU169, lonD, ara, strA, supF*	Lab stock
CSH29	*trpB, thi*	CSH lab
CSH59	F^-, *pyrC, trp, strA, thi*	CSH lab
CSH63	HfrH, *val^r, thi Δ(lac pro)*	CSH lab
KWC	*pyrC, trp, Δ(lac pro), thi, val^r, strA*	This work
KWT	*fur, pyrC, trp, Δ(lac pro), thi, val^r, strA*	This work
pMON2064	Fur expression vector	Wee *et al.*, 1988
pOX38	F plasmid derivative carrying mTn3-leu	Lab stock
pGEM-T	Cloning vector	Promega Co.
pBluescript II SK(+)	Cloning vector (pBS)	Stratagene Co.
pMFT	868 bp mutagenic fur DNA fragment was inserted in pGEM-T	This work
pBpyrC	Complete *pyrC* gene is inserted in pBS	This work
pBlacZ	BamHI fragment of *lacZ* gene is inserted in pBS	This work
pBpyrZ	*pyrC* promoter and promoterless *lacZ* gene were fused in pBS II SK(+) vector	This work

and recipient (CSH59) bacteria were grown in nutrient broth at 37°C to about 2×10^8 cells/ml. Same volumes (0.5 ml) were mixed and incubated for 20 min at 37°C in an oven incubator. The cell mixture was plated after appropriate dilutions on M9 minimal agar plate containing required amino acids. Valine (40 μg/ml) and streptomycine (50 μg/ml) were used for selection and counter selection respectively.

DNA manipulations

Genomic DNA was isolated from strain CSH29 according to Ausubel *et al.* (1994). Most of the other DNA manipulations were performed as described in Sambrook *et al.* (1989). Labelling of DNA fragment with radioactive isotope was performed by end-filling technique with restriction digested 5'-protruding ends.

Cloning of pyrC gene and pyrC-lacZ fusion

Complete sequence of dihydroortase gene was amplified by polymerase chain reaction (PCR). The PCR primer sequences of pyr 1 and pyr 2 are shown below and are designed to contain all the regulatory sites as well as coding region in the amplified gene.

pyr 1: 5'-gagccGAATTCggttaccctaaccggagacgcc-3'

pyr 2: 5'-aaacgggAAGCTTacttcaattcgcataatagcccc-3'

(Capital letters indicate restriction sites introduced for easy cloning.)

According to the Genbank sequence data, the restriction sites introduced in the primers are not present in the *pyrC* gene (ecopyrca, m16752). PCR reaction mixture contains 2 μg of genomic DNA from CSH29, 10 μl of 2 mM dNTPs, 10 μl of 10× PCR buffer, 3 μl of 20 μM of the two PCR primers and distilled water to fill up 100 μl of total reaction volume. Portions of 2.5 units of *Pfu* DNA polymerase and 50 μl of light mineral oil were added to the mixture. The reaction mixture was pretreated at 98°C for 5 min and applied 35 heating cycles (1 min at 55°C, 1 min at 72°C, and 1 min at 95°C). The amplified DNA was digested by restriction endonucleases *Eco*RI and *Hind*III. The 1,591 bp DNA fragment of *pyrC* gene containing nucleotides from the number of 11 to 1,602 was cloned into the plasmid vecter, pBluescript II SK(+) (pBS, Stratagene Co.) digested with the same restriction endonucleases and produced recombinant plasmid pBpyrC. Competent cells of *E. coli* strain DH5α were transformed with the pBpyrC and were plated on LB agar containing X-gal (40 μg/ml) and ampicillin (200 μg/ml). White colony was selected.

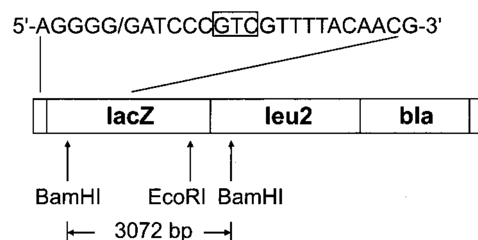


Fig. 1. Structure of mTn3-leu2. Codon in the box indicates the 9th codon of *lacZ* gene. Drawing not in scale.

Plasmid pOX38, a F-plasmid derivative carrying mTn3-*LEU2* (Fig. 1), was digested with *Bam*HI and 3,072 bp fragment containing *lac*Z coding region was purified with 0.8% low melting agarose gel. The DNA fragment was ligated into the pBS(+) digested with the same restriction endonucleases and produced recombinant plasmid pBlacZ. In order to construct *pyrC-lacZ* fusion, large fragment of pBS(+) digested with *Eco*RI and *Bam*HI, 711 bp DNA fragment of pBpyrC digested with the same restriction endonucleases, and small

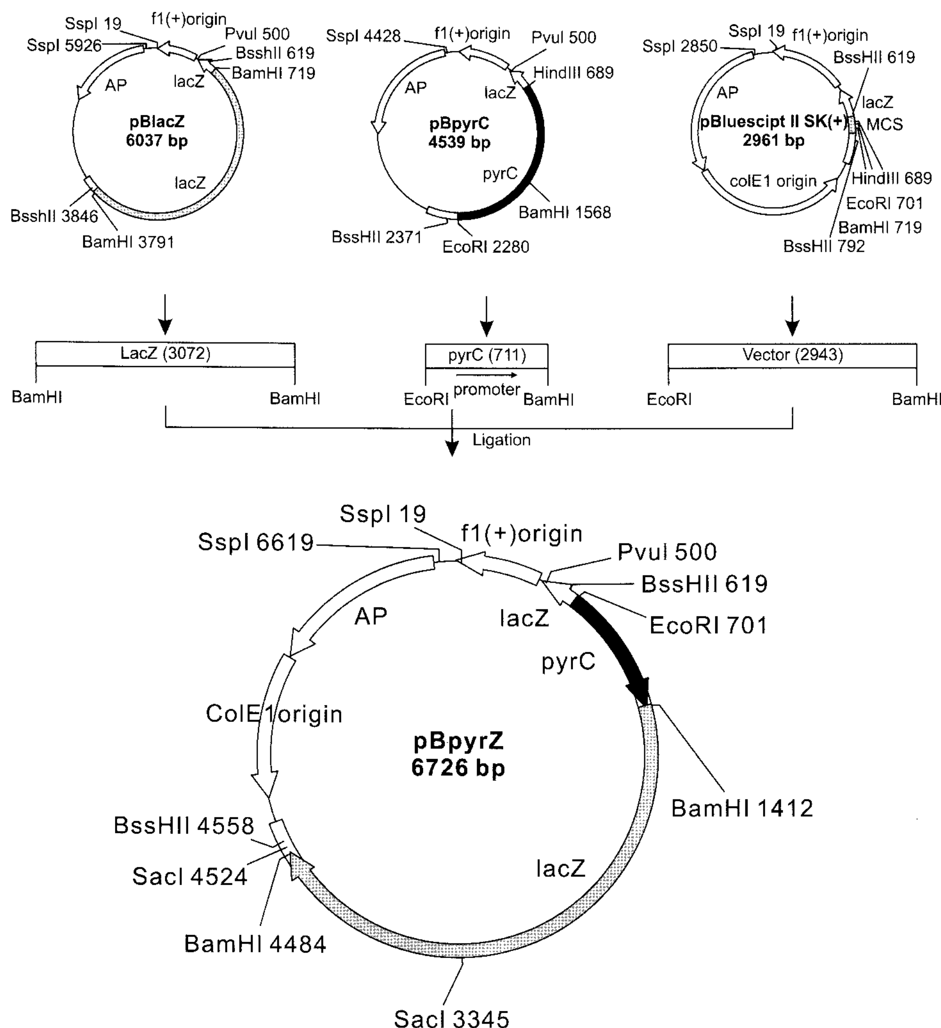


Fig. 2. Construction of pBpyrZ. Large fragment of pBS(+) digested with *Eco*RI and *Bam*HI, 711 bp DNA fragment of pBpyrC digested with the same enzymes, and small fragment of pBlacZ digested with *Bam*HI were mixed together in equal amounts and ligated. The resulting recombinant plasmids of expected size and orientation was selected (See text).

fragment of pBlacZ digested with *Bam*HI were mixed together in equal amounts and ligated (Fig. 2). Competent cells of DH5α were transformed with the ligation products and plated on LB agar containing X-gal (40 μg/ml) and ampicillin (200 μg/ml). Plasmids of expected size produced from the culture of white colony on this selection plate were tested for the orientation of the *lacZ* by the position of *Sac*I restriction site. The *pyrC-lacZ* fusion plasmid was named as pBpyrZ.

Mutagenesis of fur

Site-directed mutagenesis was performed in order to introduce *fur* mutation in the KWC strain. Mutagenic PCR was performed as described by Chamberline (1998). The sequences of the oligonucleotide primers are as follow.

5'-end *fur* primer: 5'-**GTCGAC**GCCGTATTAATAG-3' (*Sal* I site in bold)

3'-end *fur* primer: 5'-**GCGGCCGC**AAATGATCAGGCGG TGAA-3' (*Not*I site in bold)

5'-end mutagenic primer: 5'-CAGGAGCCGGAC**T**A**A**CC ATCACGTCAG-3'

3'-end mutagenic primer: 3'-GTCCTCGGCCTG**A**T**T**GGT AGTGCAGTC-5'

Insertion of thymine and adenine nucleotide (in bold face) in the mutagenic primers causes frame shift mutation and produces nonsense codons (underlined) in the 31st codon and downstream of the gene. Site-directed mutagenesis of *fur* gene was carried out in two steps of polymerization reactions; First step consists of two PCRs. Reaction 1 contains mixture of 10 μl of Ex Taq polymerase buffer, 8 μl of deoxyribonucleotide mix containing 2.5 mM of each deoxyribonucleoside triphosphate in 0.2 mM EDTA (pH 8.0), 2 μg of genomic DNA from KWC strain, 50 pmol of 5'-end *fur* primer, 50 pmol of 3'-end mutagenic primer, 5 units of Ex Taq DNA polymerase (TaKaRa Co.), and deionized H_2O to fill final volume of 50 μl. Heating cycle was 30 sec at 60°C, 2 min at 72°C, and 30 sec at 95°C and repeated 35 times. Reaction 2 was carried out under the same conditions as above except 50 pmol of 5'-end mutagenic primer and 50 pmol of 3'-end *fur* primer. Both PCR products were eluted

```
181 aatgcaaacg gtgatgcaaa cgttgcttcc ctatttgaac caggcattac gcaattactt
241 taaccagcaa cctgcttacg tcctgcgcga agatggcagc cagggcgaag caatggcgaa
301 aaaactggcg aaaggcattg aagtgaagcc aggcgaaatt gtcattccat ttactgatta
361 atcacgaggg cgcattcgcg ccctttattt ttcgtgcaaa ggaaaacgtt tccgcttatc
421 ctttgtgtcc ggcaaaaaca tcccttcagc cggagcatag agattaATGA CTGCACCATC
481 CCAGGTATTA AAGATCCGCC GCCCAGACGA CTGGCACCTT CACCTCCGCG ATGGCGACAT
```

Fig. 3. Sequence of the *pyrC* gene (m16752). Promoter region (-35 and -10 sequences are in box) and putative Fur binding site (in dark gray background) are shown. Coding sequences are written in upper case and PCR primer (5'-end fpyrC primer and 3'-end fpyrC primer) regions are in light gray background.

by Qiaquic Gel Extraction Kit (Qiagen) and named as 5' fragment and 3' fragment respectively. The second step of mutagenic PCR was carried out in 50 μl of reacting mixture containing 10 μl of Ex Taq polymerase buffer, 8 μl of deoxyribonucleotide mix, 2 μl of 10 ng 5' fragment, 2 μl of 10 ng 3' fragment, 50 pmol of 5'-end *fur* primer, 50 pmol of 3'-end *fur* primer, 5 units of *Ex Taq* DNA polymerase, and deionized H_2O. The final mutagenic PCR product was isolated on 2% low temperature melting agarose gel and eluted by Qiaquic Gel Extraction Kit (Qiagen). Competent cells of KWC were transformed with the mutant *fur* fragments and plated on a freshly prepared manganese plate to isolate *fur* mutant strain KWT (Hantke, 1987).

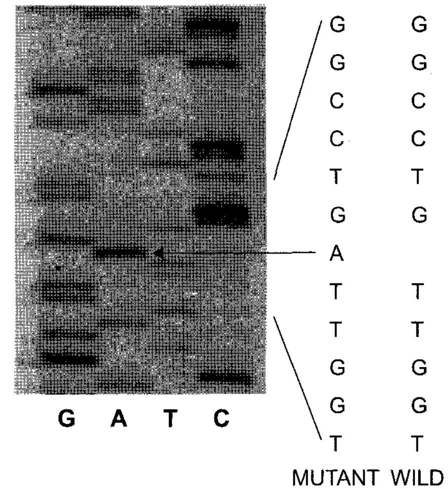
In order to confirm *fur* mutation, some of the manganese resistant colonies were tested for growth capability on succinate and acetate plate and for the control of siderophore production. Also the eluted mutant *fur* DNA fragment was cloned into pGEM-T vector (Promega Co.) yielding pMFT. Competent cells of DH5α strain were transformed with the plasmid and plated on a selective plate containing ampicillin (200 μg/ml), X-gal (20 μg/ml), and IPTG (10 μg/ml). The recombinant plasmid pMFT was prepared by using the plasmid midi kit (Qiagen Co.) and was double digested with restriction enzymes of *Sal*I and *Not*I at 37°C for 3 h. The linear DNA fragment was eluted by Qiaquic Gel Extraction Kit (Qiagen Co.). The 865 bp DNA fragment obtained was cloned into pBS. The recombinant plasmid, pMFB was transformed into *E. coli* strain DH5α and the cells were spreaded on LB plate containing X-gal, IPTG, and ampicillin. One of the white colonies was used to obtain the plasmid which was subsequently sequenced by using T3 primer.

Sequencing

Correct cloning as well as nucleotide sequences of the *pyrC-lacZ* fusion and the mutant *fur* gene were confirmed by sequencing. Dideoxy chain termination method was used with SequiTherm EXCEL™ II DNA sequencing kit (Epicentre Co.) following manufacture's instruction. Plasmid pBpyrZ and pMFB was isolated and sequenced by using T3 and /or T7 primers.

Enzyme and chemical assays

Dihydroorotase activity was determined with exponential phase cells by the methods described by Prescott and Jones (1969) using dihydroorotate as a substrate. Enzyme activity was expressed as μmol carbamoylaspartate produced per mg protein during 30 min incubation period. β-Galactosidase activity was assayed and expressed as described by Miller



5'-CAGGAGCCGGACTAACCATCACGTCAG-3'
3'-GTCCTC*GGCCTGATTGGT*AGTGCAGTC-5'

Fig. 4. 6% polyacrylamide sequencing gel analysis showing nucleotide sequence of *fur* gene of KWT strain. Plasmid pMFB carrying mutant *fur* gene was isolated and sequenced by dideoxy- mediated chain termination method using T3 primer (See text). Only non-coding strand was read.

(1974) in the strains harboring *pyrC-lacZ* fusion plasmid. Various concentrations of ferric chloride and zinc chloride were supplemented in the growth medium to investigate the effect of the metal ions on the expression of *pyrC* promoter. Effect of uracil (1.0 mM) was also determined.

In order to confirm *fur*$^-$ phenotype, mini-Arnow assay was performed (Arnow, 1937). When cultures reached 0.4 to 0.6 of A_{600}, portions of culture was centrifuged in a microcentrifuge tube for 5 min at room temperature, and 1.0 ml of supernatant was transferred to a small test tube and 0.1 ml of 5 N HCl was added and mixed. Portions of 0.5 ml of Arnow reagent and 0.1 ml of 10 N NaOH were added sequentially. Every addition was followed by thorough mixing. The $O.D._{515nm}$ was measured, and the production of siderophore was expressed as $O.D._{515}/A_{600}$.

Gel retardation assay

Gel retardation assay with purified Fur protein was performed as previously described (Wee *et al.*, 1988). For the protein-DNA interaction, DNA fragment of 185 bp including the promoter site of the *pyrC* gene was produced by PCR since there is no adequate restriction site in this region (Fig. 3). Sequences of the PCR primers were as follow:

5'-end fpyrC primer: 5'-TGCG**TCTAGATGGATCC**CAGG GCG-3' (*Xba*I and *Bam*HI sites in bold)

3'-end fpyrC primer: 5'-TGAAG**GGATCC**TTTGCCGGA CAC-3' (*Bam*HI site in bold).

The amplified fpyrC DNA fragment was digested with restricion enzyme of *Bam*HI at 30°C for 2 h and the DNA was extracted by Qiaquic Gel Extraction Kit (Qiagen). The digested fragments were labelled with [α-^{32}P]ATP (6,000 Ci/mmol; Amersham Pharmacia Biotech, USA) by end-filling reaction of Klenow fragment. The DNA was purified with a Qiaquick nucleotide removal kit (Qiagen). Labeled fragments (10 pmol) were mixed with various concentrations of purified Fur protein in 20 μl reaction volumes containing 10 mM bistris/boric acid (pH 7.5), 5 μg/ml salmon sperm DNA, 5% glycerol, 100 μg/ml BSA, 1 mM $MgCl_2$, and 40 mM KCl. After incubation for 30 min at 37°C, 5 μl of a 5× sample loading solution (50% glycerol, 0.025% bromophenol blue) was added and the mixtures were loaded directly onto a 5.25% BT/B-Mn^{2+} gel containing 5.25% acrylamide:bisacrylamide (30:0.8), 40 mM bistris/boric acid (pH 7.5), 100 μM $MnCl_2$, 0.03% ammonium persulfate, and 30 μl TEMED. Electrophoresis buffer contains 40 mM bistris/boric acid (pH 7.5), 100 μM $MnCl_2$, and 1× TBE.

Results and Discussion

Cloning of pyrC gene and pyrC-lacZ fusion

Recombinant plasmids pPyrC and pPyrZ were sequenced to confirm correct ligation (data not shown). *LacZ* gene can be ligated in two possible orientations. In order to confirm right insertion in the *pyrC-lacZ* fusion, pPyrZ was digested with endonuclease *Sac*I (Fig. 2). The recombinant plasmid produced DNA fragments of 1,179 bp and 5,547 bp on the gel as expected rather than 1,973 bp and 4,753 bp (data not shown). The fusion plasmid (pBpyrZ), will contain putative regulatory site together with promoter region and 261 bp structural gene of *pyrC* fused in-frame into the codon 9 of β-galactosidase.

Mutation of fur gene

To confirm site directed mutation of the fur gene in KWT, the gene was amplified from the genomic DNA using 5'-end *fur* primer and 3'-end *fur* primer. The PCR product was eluted and cloned into plasmid vector pBS as described in Materials and Methods. The recombinant plasmid, pMFB was transformed into *E. coli* strain DH5α and the cells were spreaded on LB plate containing X-gal, IPTG, and ampicillin. One of the white colonies was used to isolate plasmid which was sequenced by using T3 primer. The mutant *fur* sequence is shown in Fig. 4. A new stop codon TAA is created by addition of a thymine nucleotide in wild type *fur* gene.

Phenotype of the KWC and KWT strains was tested. KWC is unable to grow in minimal medium unless supplemented with uracil (*pyrC*$^-$) and produce white colonies (*lacZ*$^-$) on X-gal plate (data not shown). Siderophore producing property and growth behavior was investigated on the KWT strain. As known *fur* mutant strain AB4020, KWT produces large amount of siderophore even in high (100 μM) iron medium. This indicates constitutive expression of siderophore in *fur* mutant (data not shown). Also the strain is unable to grow on succinate and acetate plate in contrast to the *fur*$^+$ counterpart, KWC (data not shown). These results confirm frame shift mutation resulting in *fur*$^-$ phenotype of the KWT.

Effect of fur gene on the pyrC expression

Dihydroorotase activity was reduced to 30% in *fur*$^+$ background compared to the isogenic *fur*$^-$ strain as shown in Table 2. The magnitude of repression by Fur protein for *pyrC* gene regulation appears to be rather moderate. Wild type strain AB402 produced catechol type siderophore according to the iron depletion level. Since siderophore production is a direct indicator of intracellular iron level, iron depletion or repletion conditions were confirmed by Arnow assay. However, dihydrooratase activity of the cell remains constant suggesting that expression of *pyrC* gene is not affected by the iron concentration in the growth medium. This suggests that the *fur* gene, without iron, represses the *pyrC* expression. Since the enzyme contains zinc in it's active center (Porter *et al.*, 2004), and Fur protein also requires zinc ion for structural integrity (Jacquamet *et al.*, 1998) and Fe^{2+} in regulation of iron assimilating genes, various concentration of zinc chloride was supplemented in the growth medium (Table 3). Zinc level also does not affect *pyrC* expression and again *fur* mutant shows higher dihydrooratase activity. This indicates that the action of *fur* on the *pyrC* gene is not dependent on the metal ions such as iron and zinc. While Fur protein requires iron for the action to the most of the regulating gene, in some instances Fur may not require iron for the control of gene expression (Foster and Hall, 1992). Role of iron in the promoter regulation of Fur protein can be diverse in different situation. For instance, the most well known

Table 2. Effect of *Fur* and iron on *pyrC* expression. Dihydroorotase activity is expressed as mmol carbamoylaspartate produced/mg protein during incubation time. Cells were grown in MM9 medium supplemented with various amount of iron. Average of three independent assays±Standard Deviation

Iron (μM)	Strains	Siderophore production	Dihydroorotase activity
0	AB 402	0.3±0.07	1.4±0.62
	AB 4020	0.5±0.06	2.0±0.61
10	AB 402	0.2±0.08	1.2±0.67
	AB 4020	0.7±0.06	1.8±0.95
100	AB 402	0.1±0.03	1.5±0.57
	AB 4020	0.6±0.04	2.3±0.78

Table 3. Effect of *fur* and zinc on *pyrC* expression. Same as in Table 2 except zinc was replaced instead of iron.

Zinc (μM)	Strains	Dihydroorotase activity
0	AB 402	1.4±1.03
	AB 4020	1.9±0.06
10	AB 402	1.3±0.40
	AB 4020	1.8±0.30
100	AB 402	1.4±0.30
	AB 4020	1.8±0.29

Fur action is negative control while iron acts as a corepressor (Bagg and Neilands, 1987). In *Helicobacter pylori* functionally homologous protein, Helicobacter Fur, also regulates non-haem-containing ferritin gene (*pfr*) in a negative control fashion. However, the gene is inducible by iron (Delany *et al.*, 2001).

β-Galactosidase activity of *pyrC-lacZ* fusion was also reduced in *fur*$^+$ background (Table 4). The enzyme activity of AB4020 is about six to seven fold of AB402. The higher degree of repression of the β-galactosidase activity compared to the dihyroorotase activity seems to be due to the multi-copy of the gene in the plasmid. It could be possible that gene product of *pyrC* in the chromosome affects the expression of the β-galactosidase activity of *pyrC-lacZ* fusion plasmid. In order to test this possibility, isogenic *lac*$^-$ *pyrC*$^-$ double mutant strains, KWC and KWT were used for the host of the fusion plasmid. Fur mutant strain shows β-galactosidase activity about 5 fold compared to the wild type (Table 5). Even without chromosomal gene, β-galactosidase activities in the plasmid appear to be similar to the *pyrC*$^+$ chromosomal background. Higher degree of derepression of *pyrC* in the fusion plasmid seems to be copy number effect. However, AB402 strain which is *pyrC*$^+$ shows somewhat lower β-galactosidase activities compared to KWC (Table 4 and 5). This might be due to the end product repression in the *pyrC* pathway (Neuhard and Kellin, 1996).

Effect of uracil on the pyrC expression

Pyrimidine is known to repress *pyrC* expression (Choi and Zalkin, 1990). Since Fur protein does not require iron or zinc as a corepressor, the effect of uracil in the *fur* action was tested. Addition of 1 mM uracil in the medium decreases about 50% of the *pyrC* activity and the reduction is not affected by *fur* genotype (Table 6). There is no synergistic effect on the repression, indicating the two repression systems are independent each other.

Fur binding to the promoter region of pyrC gene

Since *fur* represses the *pyrC* expression, gel retardation assay was performed with purified Fur protein and 185 bp DNA fragment containing *pyrC* promoter region as well as putative Fur binding region (See Materials and Methods). The DNA changes its mobility from the Fur concentration of 100 nM suggesting that the repression of *pyrC* activity in the *fur*$^+$ genetic background could be due to the direct interaction of the Fur protein with the regulatory region of the *pyrC* promoter (Fig. 5). The Fur-DNA interaction however seems to be unusually weak in regard to the binding with aerobactin promoter where the DNA migration starts to change at the concentration of 5 nM of Fur protein (Wee *et al.*, 1988). It could be possible that there is some other cellular factor acting in concert with Fur protein for the *pyrC* gene control. In *Bacillus subtilis* Fur requires iron for the control of dihydroybenzoate siderophore synthesis (*dhb*) operon *in vivo*. On the contrary, *in vitro*, Fur is not dependent on iron for either DNA binding or transcriptional repression (Bsat and Helmann, 1999). The authors suggested presence of some DNA binding antagonists such as another protein, low-molecular-weight ligand, or another metal ion (Bsat and Helmann, 1999). Or there is some unknown cellular condition that is necessary to give Fur protein full sensitivity to iron. It has been shown that promoter region of *pyrC* gene con-

Table 4. Effect of *fur* and iron on the expression of β-galactosidase activity in *pyrC-lacZ* fusion plasmid, pBpyrZ. Same as in Table 2 except β-galactosidase activity was determined instead of dihydroorotase.

Iron (μM)	Strains	Siderophore production	β-Galactosidase activity (unit)
0	AB 402 (pBpyrZ)	0.3±0.07	22.9±4.16
	AB 4020 (pBpyrZ)	0.4±0.14	169.4±6.50
10	AB 402 (pBpyrZ)	0.1±0.08	24.6±0.43
	AB 4020 (pBpyrZ)	0.4±0.15	160.6±17.35
100	AB 402 (pBpyrZ)	0.1±0.05	23.2±6.20
	AB 4020 (pBpyrZ)	0.3±0.04	138.7±0.93

Table 5. Effect of *fur* and iron on the expression of β-galactosidase activity in *pyrC-lacZ* fusion plasmid, pBpyrZ. Same as in Table 4 except *E. coli* host strains were KWC and KWT

Iron (μM)	Strains	Siderophores production	β-Galactosidase activity
0	SWC (pBpyrZ)	0.11	36
	SWT (pBpyrZ)	0.32	173
100	SWC (pBpyrZ)	0.06	46
	SWT (pBpyrZ)	0.33	208

Table 6. Effect of *fur* and uracil on *pyrC* expression. Essentially the same as in Table 2. Cells were grown in MM9 medium supplemented with 10 μM $FeCl_3$ and with or without 1 mM uracil. Average of three independent assays±Standard Deviation

Uracil (μM)	Strains	Dihydroorotase activity
0	AB 402	2.1±0.42
	AB 4020	3.0±1.06
1000	AB 402	1.0±0.24
	AB 4020	1.6±0.48

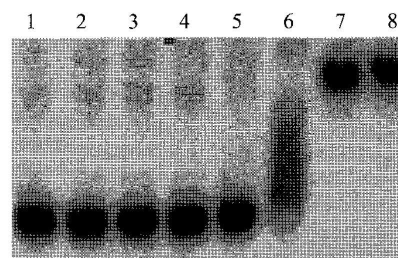


Fig. 5. Gel retardation assay with the 185 bp fpyrC DNA fragment. The DNA fragment containing *pyrC* promoter region was obtained by PCR. Approximately 10 pM solution of ^{32}P-labeled DNA fragment was incubated with various amount of Fur protein. After the binding reaction, each sample was loaded on the 5.25% polyacrylamide gel. Each lane contains the following concentrations of Fur: lane 1, no Fur; 2, 5 nM Fur; 3, 10 nM Fur; 4, 20 nM Fur; 5, 50 nM Fur; 6, 100 nM Fur; 7, 200 nM Fur; 8, 400 nM Fur.

tains *Pur* (purine regulon repressor) control site (Choi and Zalkin, 1990; Wilson and Turnbough, 1990) and the gene is indeed regulated by PurR. Interestingly *purR* is autoregulated and the promoter region contains Fur-box-like sequence (Meng *et al.*, 1990) as pointed by Stojiljkovic *et al.* (1994). These findings suggest that *pyrC* is regulated by *PurR*, which in turn would be controlled by Fur. Together with weak Fur DNA direct interaction, it is also possible that Fur indirectly controls *PyrC* expression via *PurR*.

Acknowledgments

This research was supported by Hallym University Research Fund, 2001 (HRF-2001-34).

References

Arnow, L.E. 1937. Colorimetric determination of the components of 3,4-dihydroxyphenyl alanine-tyrosine mixture. *J. Biol. Chem.* 118, 531-537.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1994. *In* Current protocols in molecular biology. John Wiley & Sons, New York, N.Y., USA.

Bagg, A. and J.B. Neilands. 1987. Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiol. Rev.* 51, 509-518.

Bjarnason, J., C.M. Southward, and M.G. Surettle. 2003. Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar typhimurium by high-throughput screening of a random promoter library. *J. Bacteriol.* 185, 4973-4982.

Bsat, N. and J.D. Helmann. 1999. Interaction of *Bacillus subtilis* Fur (ferric uptake repressor) with the *dhb* operator *in vitro* and *in vivo*. *J. Bacteriol.* 181, 4299-4307.

Chamberline, J. 1998. PCR mediated mutagenesis. *In* Multimedia methods in molecular biology, 2nd ed., Chapman and Hall.

Choi, K.Y. and H. Zalkin. 1990. Regulation of *Escherichia coli pyrC* by the purine regulon repressor protein. *J. Bacteriol.* 172, 3201-3207.

Delany, I., G. Spohn, R. Rappuoli, and V. Scarlato. 2001. The Fur repressor controls transcription of iron-activated and -repressed genes in *Helicobacter pylori*. *Mol. Microbiol.* 42, 1297-1309.

Foster, J.W. and H. Hall. 1992. Effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. *J. Bacteriol.* 174, 4317-4323.

Hantke, K. 1987. Selection procedure for deregulated iron transport mutants(*fur*) in *Escherichia coli* K 12: *fur* not only affects iron metabolism. *Mol. Gen. Genet.* 210, 135-139.

Jacquamet, L., D. Aberdam, A. Adrait, J.-L. Hazemann, J.-M. Latour, and I. Michaud-Soret. 1998. X-ray absorption spectroscopy of a new zinc site in the Fur protein from *Escherichia coli*. *Biochemistry* 37, 2564-2571.

McHugh, J.P., F. Rodriguez-Quinones, H. Abdul-Terani, D.A. Svistunenko, R.K. Poole, C.E. Cooper, and S.C. Andrews. 2003. Global iron-dependent gene regulation in *Escherichia coli*. *J. Biol. Chem.* 278, 29478-29486.

Meng, L.M., M. Kilsprup, and P. Nygaard. 1990. Autoregulation of purR repressor synthesis and involvement of *purR* intheregulation of *purB, purC, purL, purMN* and *guaBA* expression in *Escherichia coli*. *Eur. J. Biochem.* 187, 373-379.

Miller, J.H. 1974. Experiments in Molecular Genetics, 2nd ed. Cold Spring Harbor Laboratory Press, USA.

Neuhard, J. and R.A. Kellin. 1996. Biosynthesis and conversions of pyrimidines, p. 580-599. *In* F.C. Neidhardt (eds.) *Escherichia coli* and *Salmonella*: Cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington D.C., USA.

Oh, M., S-H. Chai, and S. Wee. 1999. Isolation and identification of Fur binding genes in *Escherichia coli*. *Mol. Cells* 9, 517-525

Porter, T.N., Y. Li, and F.M. Raushel. 2004. Mechanism of the dihydroorotase reaction. *Biochemistry* 43, 16285-16292.

Prescott, L.M. and M.E. Jones. 1969. Modified methods for the determination of carbamyl aspartate. *Anal. Biochem.* 32, 408-419.

Sambrook, J.C., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, New York, N.Y., USA.

Schwyn, B. and J.B. Neilands. 1987. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem.* 160, 47-56.

Stojiljkovic, I., A.J. Baumler, and K. Hantke. 1994. *Fur* Regulon in Gram-negative bacteria: Identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. *J. Mol. Biol.* 236, 531-545.

Wee, S., J.B. Neilands, M.L. Bittner, B.C. Hemming, B.L. Haymore, and R. Seetharam. 1988. Expression, isolation and properties of Fur (ferric uptake regulation) protein of *Escherichia coli* K 12. *Biol. Metals* 1, 62-68.

Wilson, H.R. and C.L. Turnbough, Jr. 1990. Role of the purine repressor in the regulation of pyrimidine gene expression in *Escherichia coli* K-12. *J. Bacteriol.* 172, 3208-3213.