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Transcriptional regulation of a second flavodoxin gene from *Klebsiella pneumoniae*

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Abstract

A second flavodoxin gene, distinct from the *nifF* gene encoding nitrogenase flavodoxin, has been isolated from *Klebsiella pneumoniae*. This flavodoxin gene is a homologue of the *E. coli fldA* gene and is located 286 bp upstream of the *K. pneumoniae fur* gene. Primer extension analysis revealed an unusual promoter region upstream of the *K. pneumoniae fldA* gene that does not match the −35/−10 consensus sequence. Transcriptional analyses using a Fur titration assay and *fldA* gene fusions demonstrated that, unlike *E. coli fldA*, the *K. pneumoniae fldA* gene is not constitutively expressed. Rather, the *fldA* gene of *K. pneumoniae* is repressed in high iron conditions by the ferric uptake regulator (Fur) protein. Expression of *K. pneumoniae fldA* is also induced by heat shock but not by salt stress. © 1997 Elsevier Science B.V.

Keywords: *fldA*; *nifF*; Heat shock; Fur protein; Iron; Sequence

1. Introduction

Flavodoxins are small FMN-containing proteins that function as electron carriers. Two distinct classes of flavodoxins are known, based on molecular mass. Short-chain flavodoxins have molecular masses from 14.5 to 16 kDa and are found in several bacterial species including *Desulfovibrio* sp., *Clostridium* sp., and *Megasphaera elsdenii* (Kitamura et al., 1994; Geoghegan et al., 1995). Long-chain flavodoxins have molecular masses ranging from 18.5 to 23 kDa and comprise all of the other flavodoxins described to date including those from red and green algae, cyanobacteria, enterics, and photosynthetic and nitrogen-fixing bacteria (Peleato et al., 1994; Razquin et al., 1994; Osborne et al., 1991; Gennaro et al., 1996).

In nitrogen-fixing organisms such as *Klebsiella pneumoniae*, flavodoxins can function as electron donors to nitrogenase and their synthesis is controlled by the fixed-nitrogen status of the cell. The *nif*-specific flavodoxin gene *nifF* of *K. pneumoniae* is part of the *nif* regulon and is activated when concentrations of fixed nitrogen and oxygen are low (Drummond, 1985). Homologues of the *nifF* gene have been reported from *Azotobacter* sp., *Enterobacter agglomerans* and *Rhodobacter capsulatus*.

In *Escherichia coli*, flavodoxin is the product of the *fldA* gene and is reported to be involved in the activation of anaerobic ribonucleotide reductase (Bianchi et al., 1993), the conversion of dethiobiotin to biotin (Ifuku et al., 1994), and the activation of pyruvate formate-lyase (Knappe and Schmitt, 1976). Flavodoxin synthesis in *E. coli* has been reported to be constitutive (Osborne et al., 1991). Although an *fldA* homologue has been identified in *Haemophilus influenzae* (Genbank accession No. L42023), there is no information regarding the regulation of flavodoxin expression in this species.

Flavodoxin synthesis in several cyanobacterial species is regulated by iron (Ferreira and Straus, 1994). In low

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Abbreviations: aa, amino acid(s); βGal, β-galactosidase; bp, base pairs; ca, approximately; CAT, chloramphenicol acetyltransferase; DEAE, diethylaminoethyl; ds, double strand(ed); ELISA, enzyme-linked immunosorbent assay; *fldA*, flavodoxin-encoding gene; FldA, flavodoxin protein; FMN, flavin mononucleotide; *fur*, gene encoding ferric uptake regulator protein; Fur, ferric uptake regulator protein; IPTG, isopropyl-β-D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; kDa, kilodaltons; LB, Luria-Bertani; *lacZ*, gene encoding β-galactosidase; *nifF*, gene encoding nitrogenase flavodoxin; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; *phoA*, gene encoding alkaline phosphatase; RBS, ribosome binding site(s); X, any aa; XGal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ::, fusion.

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iron conditions, flavodoxin production is induced presumably because flavodoxin is able to substitute for ferredoxin in certain biochemical reactions during periods of iron stress. Flavodoxin expression was first shown to be regulated by iron at the transcriptional level in *Anacystis nidulans* (Laudenbach et al., 1988). In at least one cyanobacterium, this transcriptional regulation has been demonstrated to be controlled by the global iron regulator protein, Fur (Ghassemian and Straus, 1996). In high iron conditions, Fur uses iron as a cofactor to bind to the operator sequences of genes under Fur control. Flavodoxin production in diatoms is also induced under iron limitation; this feature of flavodoxin expression is currently being exploited for use as a molecular probe of iron limitation in marine ecosystems (LaRoche et al., 1996).

Although most organisms apparently synthesize a single flavodoxin species, the existence of different molecular forms of flavodoxin in one organism has been reported for two *Azotobacter* species and for *Chlorella fusca*, a green alga (Klugkist et al., 1986; Bagby et al., 1991; Peleato et al., 1994). However, the nature of the regulatory mechanisms controlling these different flavodoxin species is unknown. In this paper, we report the existence of a second flavodoxin gene from *K. pneumoniae* that is distinct from the *nifF* nitrogenase flavodoxin gene and that, unlike the *nifF* gene, is transcriptionally regulated by iron and heat shock.

2. Materials and methods

2.1. Bacterial strains and media

K. pneumoniae 13883 and *E. coli* strains CC118 (*lacY*74 *phoA*20), H1717 (*fhuF*::λ*plac*Mu), H1780 (*fur fiu*::λ*plac*Mu53) and JB1698 (*fur*$^+$ parent of H1780) were grown at 37°C with agitation in LB. Cells were grown in LB supplemented with 200 μM of 2,2′-dipyridyl (Sigma Chemical Co., St. Louis, MO) for iron-restricted conditions, 75 μM $FeSO_4$ for iron-replete (adapted) conditions, or 684 mM NaCl for high salt (adapted) conditions. Cells were iron stressed by growing in LB to early-log phase, adding 75 μM $FeSO_4$ and incubating at 37°C to mid-log ($A_{600}=0.4$). Cells were heat shocked by growing in LB to early-log phase then incubating at 42°C until mid-log. Cells were salt shocked by growing in LB to early-log phase, adding 684 mM NaCl, and incubating at 37°C to mid-log. 60 μl of $FeSO_4$ were added to the salt-adapted cells during the final growth phase to compensate for the possible role of iron deficiency. Ampicillin (100 μg/ml), chloramphenicol (20 μg/ml) or zeocin (25 μg/ml) was added to the growth media as required for plasmid maintenance.

2.2. fldA sequencing and promoter analysis

Previous work by our laboratory resulted in the isolation of a *K. pneumoniae fur* clone; sequence analysis of the *fur* upstream region revealed a partial ORF that was identified as an *fldA* homologue (Achenbach and Yang, 1997). Sequencing of dsDNA and PCR amplification products was performed by gel purification with GeneClean III (BIO 101, Vista, CA) or on DEAE membrane and sequencing as described by Nickrent (1994). Complete sequencing of the *fldA* gene and flanking regions was performed.

Total RNA was isolated from *K. pneumoniae* cells grown in iron-restricted conditions. A primer specific to a region immediately upstream of the *fldA* start codon (5′-GTGTCGC-TGCCAAAAAAGATGCCGA-3′) was synthesized. Primer extension was performed as previously described (DiRuggiero and Robb, 1991) except that 4.6 μCi of [^{35}S]dATP was substituted for the 2 μCi of [^{32}P]dATP.

Promoter probe analysis utilized a *fldA–lacZ* gene fusion constructed as follows. Primers fldA.−131FH (5′-CGGATCCAAGCTTCCGCGAAA-AACCCGCAT-3′) and fldA.1RH (5′-CGGATCCAA-GCTTGAGTAAAATAACC-3′) were used to amplify a 176 bp fragment containing the *K. pneumoniae fldA* promoter region. The amplification product was digested with *Bam*HI and ligated into promoter probe vector pUJ10 (de Lorenzo et al., 1990) to produce plasmid pUJ.FldA. The resulting plasmid was used to transform *E. coli* strain CC118 (Manoil and Beckwith, 1985). Selection for insertion of the *fldA* promoter in the same orientation as the *lacZ* reporter gene on the vector was performed by plating CC118(pUJ.FldA) transformants on LB+XGal/IPTG plates; CC118 transformed with nonrecombinant vector was used as a negative control. Plasmid DNA from a positive (blue) colony was sequenced to verify the proper orientation of the *fldA* promoter. βGal assays using CC118(pUJ.FldA) cells grown under low iron, high iron, heat shock and high salt conditions were performed as previously described (Miller, 1992). CC118 and CC118(pUJ10) were used as negative controls.

In vitro analysis of a *fldA*–CAT gene fusion utilized the 176 bp *fldA* promoter region cloned into the *Bam*HI site of promoter probe vector pKK232-8 (Pharmacia Biotech, Piscataway, NJ). The resulting plasmid (pKK.FldA) was used to transform isogenic Fur$^+$ and Fur$^-$ *E. coli* strains JB1698 and H1780 (Hantke, 1987). Crude protein extracts were obtained from transformants grown under low and high iron conditions. CAT assays were performed using an ELISA method (5 Prime - 3 Prime, Boulder, CO). JB1698 and H1780 cells transformed with nonrecombinant pKK232-8 were included as negative controls.

2.3. Fur titration assay and Northern blot analysis

Primers specific to regions flanking the *K. pneumoniae fldA* promoter region were synthesized with *Bam*HI restriction sites at the 5′ ends. Primers fldA.−131FH and fldA.1RH were used to amplify the 176 bp fragment from *K. pneumoniae*. The amplification product was digested with *Bam*HI and ligated into pZErO DNA (Invitrogen, Carlsbad, CA). This clone was used to transform the Fur titration assay (FURTA) assay strain, *E. coli* strain H1717 (Stojiljkovic et al., 1994); transformants were plated on MacConkey-lactose + zeocin + 75 μM $FeSO_4$ + IPTG. The Lac^+ phenotype was confirmed by isolating plasmid DNA from positive (red) colonies, re-transforming strain H1717 and plating on MacConkey-iron plates.

Total RNA from *K. pneumoniae* cells grown in low iron, high iron (adapted and stressed), and high salt (adapted and stressed) conditions and from heat shocked cells was isolated and electrophoresed in an agarose–formaldehyde gel. RNA was transferred to nylon membrane (Duralon-UV; Stratagene Cloning Systems, La Jolla, CA) by overnight capillary transfer and the membrane was baked at 80°C under vacuum for 90 min. A hybridization probe was constructed by using *fldA*-specific primers in a PCR reaction incorporating digoxigenin-11-dUTP (Boehringer Mannheim Biochemical, Indianapolis, IN) as a label (Lanzillo, 1990). The primers used in the labeling reaction were fldA.85F (5′-GATGTTGCTGATGTTCAC-GACATTG-3′) and fldA.490R (5′-GCAGCTCTTCGGCAAC-3′). The Northern blot was hybridized overnight in a 50% (v/v) formamide hybridization solution that contained 5 μl of nonradioactive probe per ml (Boehringer Mannheim Biochemical Genius™ protocols). Chemiluminescent detection with CDP-Star was performed and the blot was exposed to X-ray film for 15 min.

3. Results and discussion

3.1. Sequence analysis of the K. pneumoniae fldA gene

A 1 kb region upstream of the *K. pneumoniae fur* gene was sequenced and revealed an ORF of 533 bp. The deduced aa sequence of the ORF exhibited high sequence similarity to the *E. coli fldA* gene encoding flavodoxin. The *K. pneumoniae fur* gene is located 286 bp downstream of the *fldA* gene. Thus, in *E. coli*, *K. pneumoniae* and *H. influenzae*, the *fldA* homologues are all located upstream of the *fur* (ferric uptake regulator) coding sequence and in the same orientation. There is a reasonable RBS located 11 bp upstream of the *K. pneumoniae fldA* start codon with the sequence GAGG. There is also a palindromic region immediately downstream of the two *fldA* stop codons capable of forming a stem–loop structure that could function as a rho-dependent terminator. The complete sequence of the *K. pneumoniae fldA* gene and flanking regions has been deposited in GenBank under accession No. U67169.

The *fldA* gene of *K. pneumoniae* would code for a protein product of 19.6 kDa (assuming cleavage of the N-terminal methionine) with an aa sequence similarity of 96% to the *E. coli* FldA flavodoxin. The *fldA*-encoded flavodoxin in *K. pneumoniae* is distinct from the 19 kDa NifF flavodoxin, showing only 41.7% aa sequence similarity, and thus represents a second flavodoxin in this organism. Although the existence of different molecular forms of flavodoxin in one organism has been reported for two *Azotobacter* species and for *Chlorella fusca*, a green alga, this work is the first such report for *K. pneumoniae*. Thorneley et al. (1992) discovered two forms of the NifF flavodoxin from *K. pneumoniae*, one of which was a post-translationally modified NifF with coenzyme A attached to the cysteine residue at position 68. And although this group reported the existence of a third FMN-containing protein (protein X) that eluted from the FPLC at high KCl concentrations, this may not be the *fldA* gene product as the M_r for protein X is 28 kDa, much larger than that predicted for *K. pneumoniae* FldA (19.6 kDa).

With 13 long-chain flavodoxin protein sequences now available from GenBank, it is possible to identify with some confidence those regions of the protein that are potentially required for flavodoxin function. Five conserved motifs identified from the long-chain flavodoxin sequence alignment are: (1) GlFfG (*E. coli* aa 5–9); (2) LilGXPT (*E. coli* aa 50–56); (3) GXGDQ (*E. coli* aa 87–91); (4) WptXGY (*E. coli* aa 120–125); and (5) FvGLalD (*E. coli* aa 139–145), in which the upper case are conserved in all 13 long-chain flavodoxin sequences and the lowercase in at least 7 of the 13 sequences. (The flavodoxin sequence alignment is available on request.)

3.2. Primer extension to identify the fldA transcription start site

Osborne et al. (1991), while reporting the sequence of the *E. coli fldA* gene, noted the similarity of the sequence TATGAT, located 64 bp upstream of the *fldA* start codon, to the −10 promoter consensus sequence. This sequence is also present upstream of the *K. pneumoniae fldA* gene at position −65. However, our primer extension results do not indicate that this sequence functions as the *fldA* promoter in *K. pneumoniae*. Primer extension analysis using a primer specific to the 5′ end of the *K. pneumoniae fldA* coding region resulted in a single intense signal at position −76 (Fig. 1), upstream of the region similar to the −10 consensus. Therefore, the sequence TATGAT does not appear to function as the −10 promoter sequence. However, there is no sequence similar to the −10 or −35 consensus located

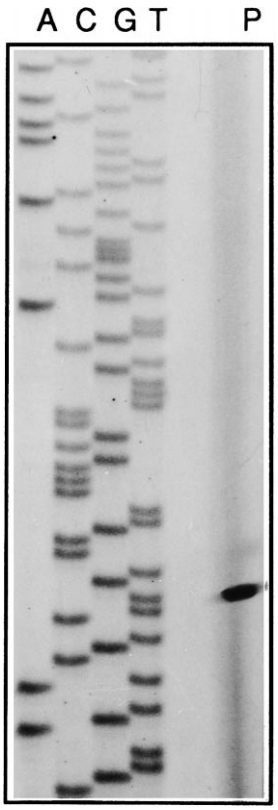

Fig. 1. Primer extension analysis of the *K. pneumoniae fldA* promoter region. Lanes A, C, G, and T: DNA sequencing ladder. Lane P: primer extension reaction mixture.

within 100 bp upstream of the primer extension signal. There is 75.9% sequence identity between the *K. pneumoniae* and *E. coli fldA* upstream regions; similarity stops at position −134 of the *K. pneumoniae* sequence.

To establish that the region upstream of the *K. pneumoniae fldA* gene contains a functional promoter, a promoter probe assay was performed. Promoter probe vector pUJ10 contains promoterless *lacZ* and *phoA* genes positioned in opposite orientations away from a multiple cloning site (de Lorenzo et al., 1990). If a DNA fragment containing a functional promoter is inserted into the multiple cloning site, either the *lacZ* or the *phoA* gene is expressed, depending on the orientation of the inserted promoter. A PCR amplification product of 176 bp extending 150 bp upstream of the *K. pneumoniae fldA* gene was cloned into pUJ10 and used to transform *E. coli* strain CC118 (Manoil and Beckwith, 1985). Transformants were plated onto LB+XGal/IPTG plates to screen for the production of βGal. Four blue colonies were chosen and plasmid DNA was prepared. The insert was then sequenced to verify that the fragment containing the putative *fldA* promoter was cloned in the same orientation as the *lacZ* gene on the vector. Plasmids from the selected blue colonies were then used to transform *E. coli* strain CC118 and re-tested to confirm the Lac^+ phenotype. These results demonstrate that the region within 150 bp upstream of the *K. pneumoniae fldA* gene contains a functional promoter.

3.3. *Induction of* fldA *gene expression by low iron and heat shock*

To analyze the transcriptional regulation of the *fldA* gene in vivo, total RNA from *K. pneumoniae* cells grown in low or high iron or from salt adapted, salt shocked and heat shocked cells was hybridized to a *fldA* probe in a Northern blot experiment. Results were verified by re-isolating RNA from freshly grown cells in the appropriate conditions and repeating the hybridization experiment.

A faint signal at approximately 600 nt was detected from the RNA isolated from cells grown in LB+$FeSO_4$; however, a strong signal of the same mobility was produced from RNA-grown LB+2,2′-dipyridyl, an iron chelating agent (Fig. 2). No signal was detected from RNA isolated from iron stressed cells. Transcription of the *K. pneumoniae fldA* gene is therefore iron-regulated. Iron-responsive regulation of flavodoxin expression has been demonstrated for the cyanobacteria *Anabaena* sp. strain PCC 7120 (Razquin et al., 1994; Leonhardt and Straus, 1994), *Synechococcus* sp. strain PCC 7942 (Ghassemian and Straus, 1996), and *Synechocystis* sp. PCC 6803 (Fulda and Hagemann, 1995), the photosynthetic eubacterium *Rhodobacter capsulatus* (Yakunin et al., 1993), and for eukaryotic algae (LaRoche et al., 1993). However, the only flavodoxin examined to date from an enteric bacterium (*E. coli*) is constitutively expressed and is therefore not iron regulated.

To ascertain whether the Fur protein plays a role in the iron-regulated expression of *fldA* from the enteric bacterium *K. pneumoniae*, a Fur titration assay (FURTA) was employed (Stojiljkovic et al., 1994). The FURTA uses βGal expression as an indicator of the presence of a Fur-binding site (or an iron-binding protein gene) in a cloned DNA fragment. An *E. coli fhuF*::*lacZ* strain (H1717) is utilized that cannot synthesize βGal under high iron conditions due to the Fur repressor binding to the weak *fhuF* Fur-binding site. However, introduction of a multicopy plasmid containing a Fur-binding site in the insert titrates the Fur repressor away from the *fhuF* Fur-binding site, thus allowing βGal expression in the transformant. Introduction of a plasmid containing the *K. pneumoniae fldA* promoter into the *E. coli* assay strain H1717 resulted in a Lac^+ phenotype, indicating that the *K. pneumoniae fldA* gene is regulated by Fur protein.

The Fur protein uses Fe(II) as a cofactor to repress gene expression by binding DNA at specific operator sites called Fur boxes (de Lorenzo et al., 1987). In *K. pneumoniae*, a sequence located upstream of the *fur* gene shares high similarity to the Fur box consensus sequence

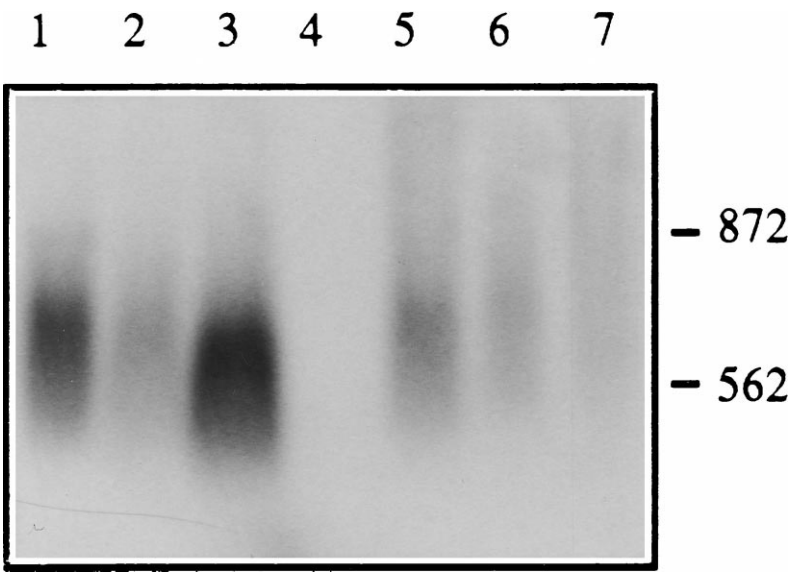

Fig. 2. Northern blot of *K. pneumoniae* RNA probed with *fldA*. Lane (1) low iron, (2) high iron adapted, (3) heat shocked, (4) RNA marker, (5) salt adapted, (6) salt stressed, (7) high iron stressed. The size in nt is indicated on the right.

(Achenbach and Yang, 1997). However, no Fur binding site sequence could be detected upstream of the *K. pneumoniae fldA* gene. Therefore, to confirm the involvement of Fur in the iron-regulated expression of *K. pneumoniae fldA*, a *fldA*-CAT gene fusion was constructed and used to transform isogenic Fur^+ and Fur^- *E. coli* strains. CAT assays were performed on transformants grown in low and high iron conditions. In the Fur^+ transformant (JB1698(pKK.FldA)), CAT activity in high iron conditions was approximately 3-fold lower than in low iron (Table 1), indicating Fur-regulated repression of *fldA* promoter activity. However, in the absence of Fur (transformant H1780(pKK.FldA)), levels of CAT activity were approximately the same in both high and low iron conditions. Thus, as predicted by the Fur titration assay, the Fur protein is responsible for the iron-dependent regulation of the *K. pneumoniae fldA* gene.

The most intense *fldA* hybridization signal in the Northern blot experiments was produced from RNA isolated from heat shocked *K. pneumoniae* cells. Thus, *K. pneumoniae fldA* gene expression is induced by heat shock. This feature is reminiscent of the situation in the cyanobacterium *Synechocystis* sp. PCC 6803 in which flavodoxin synthesis is induced by heat shock and low iron conditions (Fulda and Hagemann, 1995). However, unlike *Synechocyctis*, the *fldA* gene from *K. pneumoniae* does not appear to be salt responsive (Fig. 2).

To further characterize the nature of *K. pneumoniae fldA* transcription and to corroborate the Northern blot analysis, the *fldA* promoter region was fused to a promoterless *lacZ* gene. The resulting construct was used to transform *E. coli* strain CC118 and levels of βGal activity in response to iron availability, temperature and osmolarity were determined. βGal activity was induced by low iron and heat shock (Table 2), confirming that the *K. pneumoniae fldA* promoter is regulated by iron and temperature conditions.

In conclusion, a second flavodoxin gene distinct from the *nifF* gene was isolated from *K. pneumoniae*. The *fldA* gene is preceded by an unusual promoter region that exhibits no similarity to the $-35/-10$ consensus

Table 1
CAT activity from Fur+ (JB1698) and Fur- (H1780) *E. coli* cells transformed with a *K. pneumoniae fldA*–CAT gene fusion (pKK.FldA) and grown in low or high iron conditions

Growth conditions	μg CAT/mg protein			
	JB1698 (pKK232-8)	H1780 (pKK232-8)	JB1698 (pKK.FldA)	H1780 (pKK.FldA)
Low iron	0.006	0.005	21.3	18.3
High iron	0.005	0.005	7.0	14.9

Cells transformed with non-recombinant plasmid (pKK232-8) are included as negative controls.

Table 2
β-Galactosidase activity from a *K. pneumoniae fldA-lacZ* gene fusion in low iron, high iron, heat shock and high salt conditions

Growth conditions	Units of βGal activity		
	CC118	CC118(pUJ10)	CC118(pUJ.FldA)
Low iron	1.6 ± 1.6	2.7 ± 1.6	126 ± 8
High iron	2.1 ± 1.1	2.5 ± 2.4	47 ± 7
Heat shock	2.1 ± 1.4	2.1 ± 2.3	116 ± 12
High salt	2.2 ± 1.4	1.5 ± 0.3	39 ± 14

Data represent the average of two assays. CC118 and CC118(pUJ10) are included as negative controls.

sequences. This flavodoxin gene is regulated at the level of transcription by iron and heat shock. The molecular mechanism involved in the iron-regulatory response is the Fur protein which represses transcription of the *K. pneumoniae fldA* gene in high iron conditions. The specific nature of the heat-regulated response is not yet known.

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