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Expression of *fnr* Is Constrained by an Upstream IS*5* Insertion in Certain *Escherichia coli* K-12 Strains

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FNR is a global transcriptional regulator that controls anaerobic gene expression in *Escherichia coli*. Through the use of a number of approaches it was shown that *fnr* gene expression is reduced approximately three- to fourfold in *E. coli* strain MC4100 compared with the results seen with strain MG1655. This reduction in *fnr* expression is due to the insertion of IS*5* (*is5F*) in the regulatory region of the gene at position −41 relative to the transcription initiation site. Transcription of the *fnr* gene nevertheless occurs from its own promoter in strain MC4100, but transcript levels are reduced approximately fourfold compared with those seen with strain MG1655. Remarkably, in strains bearing *is5F* the presence of Hfq prevents IS*5*-dependent transcriptional silencing of *fnr* expression. Thus, an *hfq* mutant of MC4100 is devoid of FNR protein and has the phenotype of an *fnr* mutant. In strain MG1655, or a derivative of MC4100 lacking *is5F*, mutation of *hfq* had no effect on *fnr* transcript levels. This finding indicates that IS*5* mediates the effect of Hfq on *fnr* expression in MC4100. Western blot analysis revealed that cellular levels of FNR were reduced threefold in strain MC4100 compared with strain MG1655 results. A selection of FNR-dependent genes fused to *lacZ* were analyzed for the effects of reduced FNR levels on anaerobic gene expression. Expression of some operons, e.g., *focA-pfl* and *fdnGHJI*, was unaffected by reduction in the level of FNR, while the expression of other genes such as *ndh* and *nikA* was clearly affected.

The genomes of *Escherichia coli* K-12 strains contain variable numbers of insertion sequences (IS). These are mobile bacterial DNA elements that can transpose to many sites on the chromosome, and their activity can result in various genetic rearrangements. One of the most common IS elements is the 1,195-bp IS*5* (9), which can be localized to a number of conserved positions in the genome. IS*5* elements range in copy number from 11 in the sequenced *E. coli* strain MG1655 (1) to 23 in strain W3110 (45). Indeed, the locations of the IS*5* elements in the chromosome of W3110 have been named *is5A* through *is5W*.

This study demonstrates that an IS*5* element (termed *is5F*) is present in the regulatory region of the *fnr* gene of some *E. coli* strains, including MC4100, which, remarkably, causes an approximately threefold reduction in FNR concentration. FNR is a global transcriptional regulator that controls gene expression in response to the transition between aerobic and anaerobic growth and is a member of the CRP-FNR superfamily (11, 18). Current evidence indicates that FNR senses dioxygen directly through a redox-sensitive [4Fe-4S] cluster (14, 17). Global gene expression profiling analysis of MC4100 suggests that as many as 700 genes in *E. coli* are regulated directly or indirectly by FNR (26).

Expression of *fnr* is more or less constitutive and is achieved through negative autogenous control (25, 30). The consequence of this is that cellular FNR levels remain relatively constant. Differential responses to anoxia of particular gene sets are therefore determined by the binding affinities and locations of FNR recognition sequences in the promoters of the respective genes. Furthermore, many FNR-regulated genes are also subject to dual regulation by a further regulator. For example, expression of the structural gene operon *narGHJI*, encoding respiratory nitrate reductase, is activated by FNR anaerobically and expression is further increased in response to nitrate (41). Altering the concentration of FNR in the cell by increasing or decreasing expression of the *fnr* gene might therefore have significant impacts on global gene expression and ultimately on general cell physiology.

The synthesis of one of the key anaerobic enzymes, pyruvate formate-lyase (PFL), is controlled by FNR (reviewed in reference 32). The *pfl* gene is transcribed in an operon with the *focA* gene. Multiple, coordinately regulated promoters control transcription of the operon, and expression is subject to regulation by a number of transcription factors, including FNR (6, 7, 15, 16, 28, 31, 37). In an *fnr* mutant, induction of anaerobic *focA-pfl* gene expression is significantly reduced.

In the present study, while screening mutations for their effects on *focA-pfl* expression it was shown that an *E. coli* MC4100 *hfq* mutant also exhibited reduced levels of anaerobic *focA-pfl* induction. Hfq has RNA chaperone activity, and it has recently been implicated in the regulation of gene expression through mediating the action of small RNAs (44, 49, 50). Surprisingly, however, detailed analysis of the MC4100 *hfq* mutant revealed that the effect of the *hfq* mutation on *focA-pfl* expression was indirect and that in fact the mutation prevented *fnr* expression. Consequently, the lack of FNR in the MC4100 *hfq* mutant was the actual cause of impaired anaerobic induction of *focA-pfl* expression. Moreover, Hfq-dependent expression of *fnr* in strain MC4100 was due to the IS*5* insertion in the *fnr* regulatory region. Levels of FNR in *E. coli* strains lacking IS*5* upstream of the *fnr* gene were unaffected by the *hfq* mutation. Taken together, these findings indicate that there is

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TABLE 1. Strains, plasmids, and phages used in this study

Strain, phage, or plasmid	Genotype	Reference or source
Strain		
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 ptsF25 deoC1 relA1 flbB530 rpsL150* λ$^-$	3
MG1655	Also known as NCM3629 prototroph	38
MG1655	Also known as NCM3105 *ilvG rfb-50 rph-1 fnr-267 eut*	38
GS081	Like MC4100, but *hfq-1*::Ω(Cm^r)	49
RM4100	Like MC4100, but with wild-type *fnr* gene regulatory region	This work
RM101	Like MC4100, but Δ *fnr*	31
RM123	Like MC4100, but *recA56* λRM123	29
RM135	RM102 λRM123	29
RM1010	Like MC4100, but *hfq-1*::Ω (Cm^r)	This work
RM1011	Like MG1655 (NCM3629), but *hfq-1*::Ω (Cm^r)	This work
HYD72K1	MC4100 *nikA*::Mu*d*I (Kan^r *lacZ*)	M. Mandrand-Berthelot
JRG1941	RK4353 λRS5 *aspA′-′lacZ*	47
JRG1988	MC1000 λG211 (λRS5 *ndh-lacZ*)	39
Plasmid or phage		
pCH21	Ap^r *fnr*$^+$	13
pCrp	Ap^r *crp*$^+$	S. Busby
p29	Cm^r *focA*$^+$ *pfl*$^+$ *act*$^+$	4
pRS551	Ap^r Kan^r *lacZ′ lacY*$^+$ *lacA*$^+$	36
pRM900	pRS551 Φ*fnr′-lacZ* 246-bp insert including *fnr* upstream regulatory region	This work
pRM901	pRS551 Φ*fnr′-lacZ* 94-bp insert from the *fnr* regulatory region	This work
pNK120	pRS551 Φ*fdnG′-lacZ*	T. Palmer
λRS45	*lacZ′ lacY*$^+$ *lacA*$^+$ *imm*^{21} *ind*$^+$	36
λRM123	λRS45 Φ(*pfl′-′lacZ*)*1397*(Hyb)	29
λRM900	λRS45 Φ*fnr246′-′lacZ*	This work
λRM901	λRS45 Φ*fnr94′-lacZ*	This work
λNK120	λRS45 Φ*fdnG′-lacZ*	This work

strain-dependent variability in FNR levels in *E. coli* K-12 caused by *is5F* insertion and that only in those strains containing this insertion sequence is *fnr* transcription dependent on Hfq.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phage and culture conditions. Bacterial strains, plasmids and phages used in this study are listed in Table 1. Bacteria were grown routinely in Luria broth (LB) (27). When required, glucose was added to achieve a final concentration of 20 mM. Growth in minimal medium was performed in M9 (23) supplemented with 0.001% (wt/vol) thiamine and 5 mM $MgSO_4$. The carbon source was either glycerol added to achieve a final concentration of 0.8% (wt/vol) or L-arabinose added to achieve a final concentration of 0.5% (wt/vol). When required, nitrate was added to achieve a final concentration of 10 mM. Aerobic cultures were grown in flasks filled maximally to 1/10 of their volume, while anaerobic cultures were grown in stoppered bottles filled to the top with medium. Media and buffers for work with lambda and P1 were prepared as described previously (29). Cells for enzyme assays and RNA preparation were harvested in the mid-exponential phase of growth.

Antibiotics were added to achieve the following final concentrations: ampicillin, 75 μg ml^{-1}; chloramphenicol, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 15 μg ml^{-1}. Media were solidified by the inclusion of 1.5% (wt/vol) agar.

Plasmid and strain construction. PCR amplification of the complete *fnr* regulatory region from within the 3′ end of the *ogt* gene to the 5′ end of the *fnr* gene was performed with oligonucleotides Fnr-Tr1 (5′-GGGGATCCATGAAGGTTATCTTTTGCTG-3′) and Fnr-Tr2 (5′-GGGGATCCGTATAATTCGCTTTTCCGGG-3′). This amplification delivered a 246-bp DNA fragment that was cloned into the BamHI site of pRS551 (36), delivering plasmid pRM900. To construct a derivative with a truncated *fnr* regulatory region extending from position −41 bp relative to the transcription initiation site to the 5′ end of the *fnr* gene, a 94-bp DNA fragment was amplified with oligonucleotides Fnr-Tr2 and Fnr-Tr3 (5′-GGGGATCCTTAGACTTACTTGCTCCC-3′) and cloned into the BamHI site of pRS551. This delivered plasmid pRM901 and lacked any IS*5* DNA sequences. To transfer the transcriptional *lacZ* fusions in strains HYD72K1, JRG1941, and JRG1988 into other *E. coli* genetic backgrounds, phage P1 was grown on the donor strains, lysates were prepared, and the fusions were introduced into recipient strains by P1-mediated transduction according to the method described in reference 23. Successful introduction of the fusion was selected for by acquisition of kanamycin resistance in the case of *nikA*::Mu*d*I and screening for a Lac$^+$ phenotype with λRS5 *aspA′-′lacZ* and *ndh-lacZ*. RM4100 was constructed by transducing RM101 (Δ*fnr*) to an *fnr*$^+$ genotype by the use of a P1 lysate grown on MG1655 and selecting for growth anaerobically on M9 minimal medium with glycerol and nitrate (20). The resulting strain was unable to grow on L-arabinose as the sole carbon source.

Transfer of *lacZ* fusions to λRS45 and thence to the λatt site on the chromosome was performed exactly as described previously (29, 36). All *lacZ* fusions were introduced in single copy into the λatt site, with the exception of the *nikA-lacZ* derivative, which was a single-copy fusion in the *nikA* structural gene.

Analysis of RNA transcripts. Total RNA was isolated from aerobic and anaerobic cultures grown to mid-exponential phase by the use of a QIAGEN RNeasy kit according to the manufacturer's instructions (QIAGEN manual; QIAGEN Ltd., Crawley, United Kingdom). Primer extension analysis was performed exactly as described previously (30) with 25 μg of total RNA and 0.2 pmol of ^{32}P-labeled oligonucleotide Fnr-F2 (5′-GGCTGATGCTGCAATCCTGGCAATGG-3′).

S1 nuclease mapping of *pfl* transcripts was performed according to the method described in reference 12 by the use of 50 μg of total RNA. The DNA fragment used for hybridization was derived from plasmid p29 (4). Labeling of this fragment with [γ-^{32}P]ATP, as well as treatment of the fragment prior to S1 analysis, was carried out exactly as described previously (30).

RT-PCR was performed with 2.5 μg of total RNA by the use of an Access RT-PCR system (Promega, Southampton, United Kingdom). RNA samples were treated with RNase-free DNase and repurified prior to use in RT-PCR experiments. The conditions used for the RT-PCR were exactly as described by the manufacturer. The oligonucleotides used for RT-PCR were as follows: Fnr-1 (5′-GGAAAAGCGAATTATACGGCG-3′) and Fnr-2 (5′-GGGCCAGCGCATCGTTATTTTCG-3′); Fnr-F1 (5′-CACAACCGCCAGACTGAATGCGCC-3′) and Fnr-F3 (5′-GGGTTTTCAAATAGATAGAC-3′); and Crp-1 (5′-GGACGTCACATTACCGTGCAGTACAGTTG-3′) and Crp-2 (5′-GGTTGTTTTGCCAGATTCAGCAGAGTCTG-3′).

Other methods. Chromosomal DNA was isolated according to the method of Sambrook et al. (27). Beta-galactosidase enzyme activity was assayed in cultures of exponentially growing cells, and the specific activity was calculated as described previously (23). Values reported are the averages of at least three inde-

pendent experiments performed in triplicate, and the standard error of the values reported was not more than 15%.

Formate dehydrogenase (FDH-N) and nitrate reductase (NR) enzyme activities were performed as described previously (10).

FNR was purified according to the method of Kaiser and Sawers (15). Protein concentrations were determined using bovine serum albumin as the standard by the method of Lowry et al. (21). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins was performed as described previously (19), and Western blotting was carried out according to Towbin et al. (43). Anti-FNR antiserum (a kind gift from G. Unden, Mainz, Germany) was diluted 1,500-fold before use, and the antibody-antigen reaction was visualized using the ECL chemiluminescent method (Amersham Biosciences, Uppsala, Sweden) exactly as recommended by the manufacturer. Densitometric analyses were performed using SynGene GeneTools analysis software.

RESULTS

Strain-dependent differences in anaerobic regulation of *focA-pfl* operon expression by Hfq. Multiple promoters control transcription of the *focA-pfl* operon in *E. coli*. Anaerobic induction of operon expression is regulated by the FNR and ArcA transcription factors (7, 15, 28, 30). During a survey of mutant alleles of *E. coli* MC4100 it was noted that an *hfq* mutation reduced anaerobic induction of *pfl* expression (Fig. 1). The coordinate induction of the multiple *pfl* transcripts normally observed under anaerobic conditions in the wild type was significantly reduced in the MC4100 *hfq* mutant, GS081. In particular, transcripts 4, 5, and 6 were no longer detectable in the *hfq* mutant and the anaerobic levels of transcripts 2 and 3, although reduced compared to the wild-type levels, still afforded some anaerobic induction of *pfl* transcription (Fig. 1). This phenotype is also observed in an *fnr* mutant (28, 30). Transduction of the *hfq*::Ω (Cm^r) allele into a "clean" strain MC4100 genetic background, delivering strain RM1010, yielded results indistinguishable from those found with the GS081 mutant (data not shown). Analysis of *pfl-lacZ* expression confirmed that anaerobic induction of gene expression was reduced approximately eightfold in the *hfq* mutant RM1010 compared to the results seen with its isogenic wild-type parent strain MC4100 (Table 2).

The effects of the *hfq* mutation on both *pfl* operon transcription and expression of the *pfl-lacZ* fusion were reminiscent of the phenotypes observed in a *fnr* mutant (see Table 2) (28, 29, 30). Moreover, the activities of the anaerobically induced, nitrate- and FNR-regulated enzymes formate dehydrogenase N (FDH-N) and nitrate reductase (NR) were undetectable in crude extracts of a MC4100 *hfq* mutant compared to activities in extracts of MC4100 of 0.1 μmol of formate oxidized min^{-1} mg^{-1} of protein for FDH-N and 0.99 μmol of nitrate reduced min^{-1} mg^{-1} of protein for NR. These results confirm that the effect of the *hfq* mutation on *pfl* transcription was indirect and a result of reduced FNR synthesis.

To determine whether the effect of the *hfq* mutation on *pfl* expression was also observed for other *E. coli* strains, the *pfl-lacZ* fusion was introduced into the fnr^+ derivative of strain MG1655 (prototroph) and the *fnr* derivative of MG1655 (NCM3105) (38). Levels of *pfl* expression after aerobic and anaerobic growth in MG1655 were very similar to those observed for strain MC4100 (Table 2). Similarly, the *fnr* mutation resulted in an approximately sixfold reduction in levels of *pfl-lacZ* expression after growth of cultures anaerobically. Introduction of the *hfq*::Ω mutation into wild-type MG1655

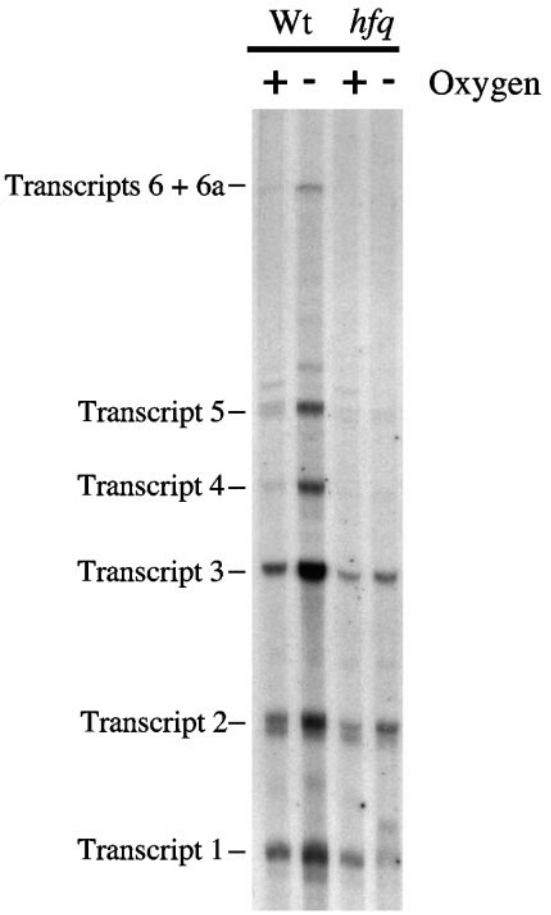

FIG. 1. Impaired anaerobic induction of the multiple *focA-pfl* operon transcripts in strain MC4100 *hfq*. Total RNA was isolated from strains MC4100 (Wt) and GS081 (*hfq*) after aerobic and anaerobic growth in LB plus glucose. After S1 nuclease treatment (see Materials and Methods) the transcripts were separated in a denaturing 4% (wt/vol) polyacrylamide gel. The locations of the transcripts are indicated.

λRM123 (*pfl-lacZ*) resulted in an approximately 20% decrease in expression after anaerobic growth (Table 2). These results indicate that *pfl* expression is not dependent on the presence of Hfq in strain MG1655.

Hfq is required for *fnr* expression in strain MC4100. To determine whether expression of *fnr* was affected by the *hfq*

TABLE 2. Expression of a *pfl-lacZ* fusion is Hfq dependent in strain MC4100 but not in strain MG1655

Strain[a]	Beta-galactosidase enzyme activity (Miller units ± SD)	
	Aerobic	Anaerobic
MC4100	200 ± 28	7,775 ± 310
RM1010 (*hfq*)	150 ± 12	1,020 ± 60
RM135 (*fnr*)	145 ± 13	980 ± 115
MG1655	160 ± 6	7,646 ± 610
RM1011 (*hfq*)	240 ± 35	5,800 ± 690
MG1655 (*fnr*)	230 ± 10	1,265 ± 112

[a] All strains contain a chromosomal copy of λRM123.

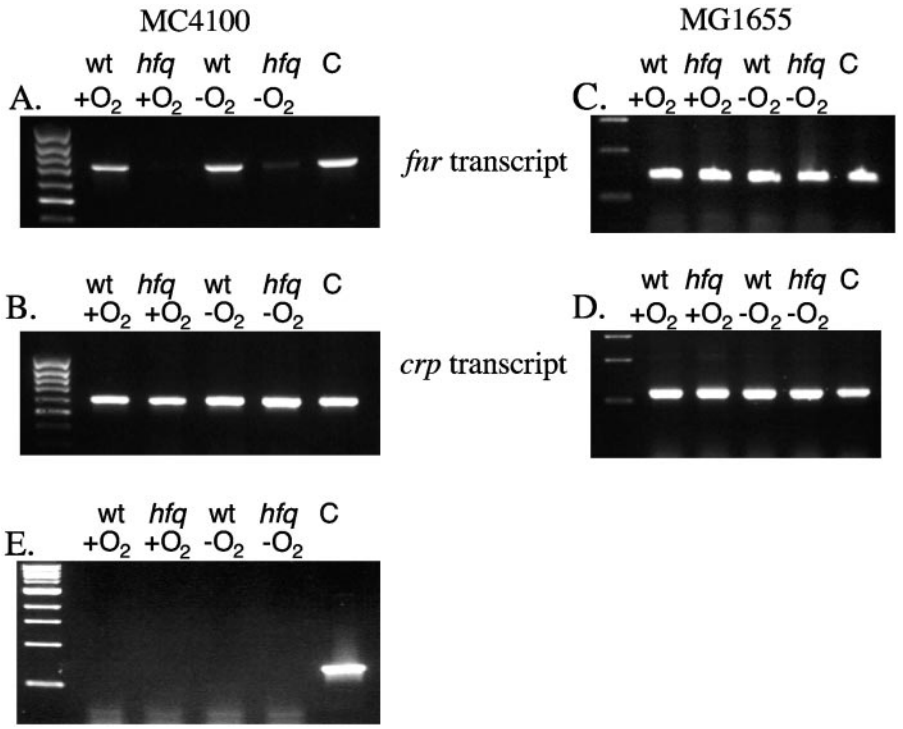

FIG. 2. RT-PCR analysis of *fnr* and *crp* transcripts in strain MC4100 and MG1655 *hfq* mutants. Total RNA was isolated from *E. coli* strains grown aerobically and anaerobically in LB plus glucose, and RT-PCR analysis was performed (see Materials and Methods). Oligonucleotides Fnr-1 and Fnr-2 were used to detect the 712-base *fnr* transcript (panels A, C, and E); oligonucleotides Crp-1 and Crp-2 were used to detect the 590-base *crp* transcript (panels B and D). Panel E represents a control experiment in which the RNA samples were from strain MC4100; the oligonucleotides were Fnr-1 and Fnr-2, but reverse transcriptase was omitted from the reactions. Lanes labeled C were controls in which chromosomal DNA, rather than RNA, was used as a template. wt designates the wild type.

mutation in strain MC4100, total RNA was isolated from MC4100 and its *hfq* derivative strain RM1010 after aerobic and anaerobic growth and the presence of the *fnr* transcript was detected by reverse transcription followed by PCR (RT-PCR). RT-PCR of total RNA samples isolated from wild-type MC4100 grown either aerobically or anaerobically delivered a cDNA product of the expected size (712 bp) and demonstrated that the levels of the *fnr* transcript were similar under these conditions (Fig. 2A). This result is in agreement with previous findings (25, 40). However, when the same amount of total RNA isolated from RM1010 (*hfq*) was used, essentially no PCR product was detected (Fig. 2A). This indicates that the level of the *fnr* transcript in the *hfq* derivative of strain MC4100 is dramatically reduced.

Control experiments using oligonucleotides specific for the *crp* gene delivered the anticipated 590 bp (Fig. 2B), and the levels of transcript were similar, both aerobically and anaerobically and between strains MC4100 and RM1010 (*hfq*). RT-PCR experiments lacking reverse transcriptase showed no specific product for the *fnr* gene (Fig. 2E).

Analysis of *fnr* transcripts by RT-PCR in strain MG1655 revealed that in this strain the *hfq* mutation had no effect on transcript levels (Fig. 2C). This result indicates that Hfq regulates *fnr* transcript levels in strain MC4100 but not in strain MG1655. A control experiment examining *crp* expression revealed that as in strain MC4100, *crp* expression was unaffected by the *hfq* mutation (Fig. 2D).

Determination of the initiation site of *fnr* transcription in strains MC4100 and MG1655 by primer extension demonstrated that the same transcription initiation site was used in both strains (Fig. 3) and that this site is the same as that determined previously (8, 35). However, compared with transcript levels in strain MG1655, the levels were reduced between three- to fourfold in strain MC4100 (Fig. 3), as determined by densitometric quantification. Whereas in strain MG1655 the *hfq* mutation had no effect on *fnr* transcript levels, in agreement with the RT-PCR results, no detectable transcription initiation could be detected in strain RM1010 (MC4100 *hfq*).

Taken together, these findings indicate that the level of *fnr* transcripts is dependent on the presence of Hfq in strain MC4100 but not in strain MG1655. Furthermore, *fnr* transcripts are less abundant in strain MC4100 than in strain MG1655.

IS*5* is inserted within the *fnr* regulatory region in MC4100. To determine how Hfq influences *fnr* expression in MC4100, the promoter-regulatory region of the *fnr* gene was amplified from chromosomal DNA by PCR to determine the DNA sequence in both MC4100 strain and strain MG1655. Oligonucleotide Fnr-Tr1 hybridized in the extreme 3′ end of the *ogt* gene, which is located immediately upstream of *fnr* (35), and Fnr-Tr2 hybridized within the 5′ portion of the *fnr* gene. The amplified DNA product was 246 bp when strain MG1655 chromosomal DNA was used as the template (Fig. 4A, lane 2).

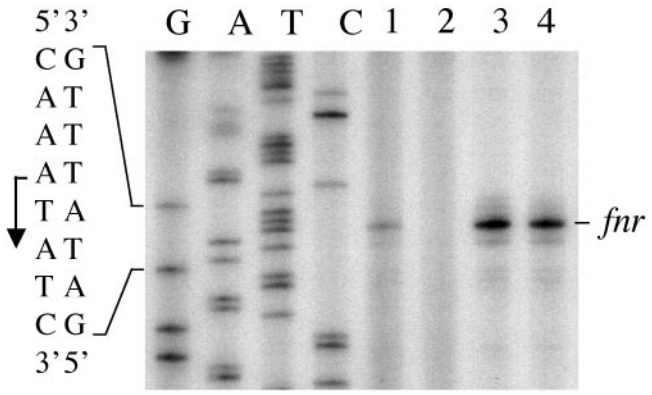

FIG. 3. Transcription initiation of the *fnr* gene is silenced in an MC4100 *hfq* mutant. Total RNA was isolated from anaerobically grown strains. Primer extension reactions with oligonucleotide Fnr-F2 were performed as described in Materials and Methods. Identical amounts of each reaction mixture were separated on a denaturing 6% polyacrylamide gel. Lane 1, strain MC4100 (wild type); lane 2, strain RM1010 (MC4100 *hfq*); lane 3, strain MG1655 (wild type); lane 4, strain RM1011 (MG1655 *hfq*). The precise location of the transcription initiation site is shown on the left side of the panel.

DNA sequence analysis of the fragment amplified from strain MG1655 yielded a sequence identical to that published by Blattner et al. (1). Surprisingly, however, strain MC4100 chromosomal DNA delivered a product of ~1.4 kb (Fig. 4A, lane 1). Sequence analysis of this DNA fragment revealed that the first 67 bp of sequence upstream of the *fnr* gene AUG codon were identical to those of strain MG1655. The next 1,195 bp were identical to the insertion element IS*5* (9), with the gene *ins5A* having the opposite orientation to the *fnr* gene (Fig. 4B). *E. coli* strains bear various numbers of copies of IS*5*, with a maximum of 23 copies identified in strain W3110 (45). The locations of the IS*5* elements have been determined in the chromosome of W3110, and they have been named *is5A* through *is5W*. The IS*5* element located upstream of *fnr* is *is5F*. PCR analysis of the *fnr* regulatory region of strain W3110 determined that like that of strain MC4100, it also has an IS*5* element insertion (data not shown).

IS*5* inserts into the chromosome nonrandomly and recognizes the target sequence C/T T A/T G (9). The sequence TTAG in the *fnr* regulatory sequence (Fig. 5) is the recognition sequence of *is5F* and is duplicated by the insertion. The *fnr* sequence upstream of the insertion element in the chromosome of strain MC4100 is identical to that of the published sequence for strain MG1655 (1).

Expression of *fnr-lacZ* fusions. To determine the level of *fnr* expression that occurs with only the 41 bp of regulatory DNA sequence upstream of the transcription initiation site, a 94-bp DNA fragment generated from the point of IS*5* insertion (including the IS*5* recognition sequence but lacking any IS*5* DNA sequence) (Fig. 5) to the start of the *fnr* gene was fused to *lacZ* and expression levels were determined for single-copy fusions in different *E. coli* strains (Table 3). Expression was approximately 20% higher anaerobically than the level seen with aerobically grown cultures. It was noted that after anaerobic growth, expression in an *fnr* mutant was increased twofold compared with the level in aerobic cultures, indicating that this short construct still exhibits negative autoregulation (25, 40). Expression levels were similar in strains MC4100 and MG1655. Notably, an *hfq* mutation had essentially no effect on expression of the truncated *fnr-lacZ* derivative for either strain (Table 3 and data not shown). This indicates that in accord with the transcript analyses, the *fnr* gene is expressed in the truncated promoter-regulatory region derivative and suggests that the consequence of the *hfq* mutation on *fnr* transcription is due to the insertion of IS*5*. Attempts to construct a *lacZ* fusion derivative including the IS*5* element proved unsuccessful due to the high instability of the derivative.

Construction and analysis of an *fnr-lacZ* fusion including the complete *ogt-fnr* intergenic region revealed an expression pattern similar to that seen with the truncated *fnr-lacZ* derivative (Table 3). The main difference was that expression levels were between three- to fourfold higher in the full-length derivative. This observation correlates well with the three- to fourfold difference in *fnr* transcription levels between strains MC4100 and MG1655. Expression levels were generally higher in strain MG1655 than in strain MC4100, and the *hfq* mutation did not affect *fnr* expression. These data indicate that *fnr* expression is not dependent upon the presence of Hfq in strain MG1655. Moreover, they suggest that DNA sequences upstream of position −41 bp in the *fnr* regulatory region are important for maximal *fnr* expression. This latter observation is in accord with previous findings (25, 40, 42).

Taken together, the transcriptional fusion data corroborate

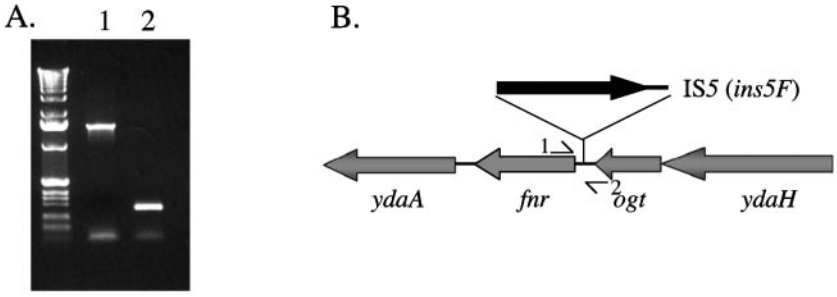

FIG. 4. Analysis of the IS*5* insertion element in the *fnr* regulatory region of MC4100. (A) PCR amplification of the *fnr* regulatory region from strains MC4100 (lane 1) and MG1655 (lane 2) was performed using chromosomal DNA as the template. The oligonucleotides used were Fnr-Tr1 and Fnr-Tr2. (B) Schematic representation of the *fnr* locus on the chromosome of strain MC4100. The numerals represent the locations of oligonucleotides used in the PCR experiment shown in panel A. 1, Fnr-Tr1; 2, Fnr-Tr-2. The location of the *ins5A* gene, encoding the transposase of IS*5*, is represented by the thick black arrow.

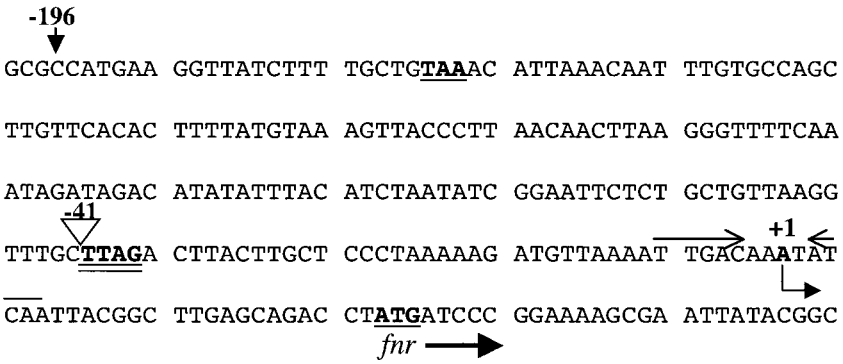

FIG. 5. Nucleotide sequence of the *fnr* regulatory region. The stop codon of the upstream *ogt* gene and translation initiation codon of the *fnr* gene are shown in bold with a single underline. The insertion site of IS*5* is shown by the inverted triangle, and the TTAG target site sequence is shown in bold and is double underlined. Transcription of the *fnr* gene initiates at an A residue, which is shown in bold and is identified with an angled arrow. The locations of the 5′ end of the DNA fragments used to construct the *fnr-lacZ* fusions λRM900 and λRM901 are designated −196 and −41, respectively. The two converging arrows above the DNA sequence and bordering the transcription initiation site signify a FNR-binding site.

the transcript analyses and demonstrate that the *fnr* promoter is active in strain MC4100. The data also indicate that the reduced level of expression of the *fnr* gene in MC4100 is due to insertion of the IS*5* element in the regulatory region. Furthermore, the findings suggest that the *hfq* mutation causes "silencing" of *fnr* transcription and that this is a consequence of the IS*5* insertion. Hfq has also recently been shown to function as an antisilencer in *bgl* operon expression (5).

Strain MC4100 has reduced levels of FNR protein compared with strain MG1655. Immunological analysis of FNR in extracts of strain MC4100 revealed that essentially equivalent amounts of protein were present in cells grown aerobically and anaerobically (Fig. 6A). This finding is consistent with previous observations (46). Extracts of strain MG1655 had threefold higher levels of FNR than strain MC4100 extracts. This finding is consistent with the transcript analyses (Fig. 3).

A derivative of strain MC4100 was constructed that lacked *is5F* and which had an *fnr* regulatory region identical to that in strain MG1655 (see Materials and Methods). PCR analysis of chromosomal DNA from the resulting strain, RM4100, confirmed that the IS*5* element had been removed (data not shown). Western blot analysis of extracts derived from aerobically and anaerobically grown RM4100 revealed that levels of FNR were restored to those observed in MG1655 (Fig. 6B). This result confirms that in strain MC4100, the insertion of IS*5* in the *fnr* regulatory region is solely responsible for the reduced levels of FNR protein observed.

Effects of reduced FNR levels on anaerobic gene expression. The reduced level of FNR protein could have significant effects on anaerobic gene expression in strain MC4100. To test this, chromosomal *lacZ* fusion constructs of five FNR-regulated

TABLE 3. Expression of *fnr-lacZ* fusions in different genetic backgrounds

Strain	Beta-galactosidase enzyme activity (Miller units ± SD)			
	λRM900 (Φ*fnr246′-′lacZ*)		λRM901 (Φ*fnr94′-′lacZ*)	
	Aerobic	Anaerobic	Aerobic	Anaerobic
MC4100	1,170 ± 87	2,870 ± 344	430 ± 60	620 ± 65
RM101 (MC4100 *fnr*)	1,090 ± 62	4,220 ± 180	470 ± 50	900 ± 54
MG1655	1,960 ± 254	2,440 ± 219	550 ± 80	460 ± 39
MG1655 (*fnr*)	2,410 ± 156	5,500 ± 577	560 ± 67	1,050 ± 84
RM1011 (MG1655 *hfq*)	1,180 ± 88	1,560 ± 132	750 ± 82	560 ± 61

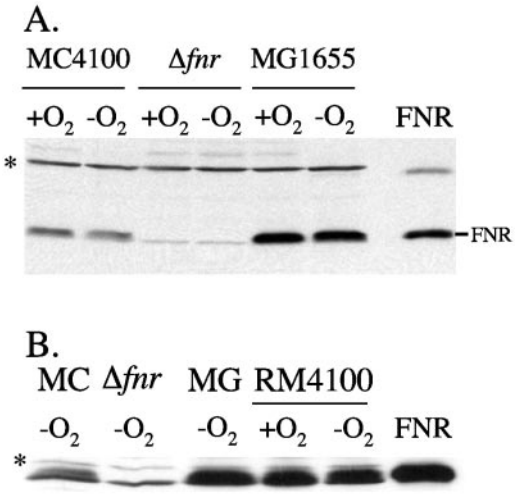

FIG. 6. Reduced levels of FNR protein in strains containing the IS*5* element *is5F*. (A) Polypeptides in crude cell extracts of strains MC4100 (wild type), RM101 (Δ*fnr*), and MG1655 (wild type), prepared after aerobic and anaerobic growth of cells in LB plus glucose, were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12.5% polyacrylamide gels) and probed with anti-FNR antiserum. Aliquots of crude extracts (100 μg of protein) were loaded on the gel, and 50 ng of purified FNR was applied. The location of the FNR polypeptide is indicated. (B) Western blot with anti-FNR antiserum. For each experiment, 100 μg of protein in the case of crude extracts and 100 ng of purified FNR were applied (see panel A above). MC indicates a crude extract derived from strain MC4100 (wild type); Δ*fnr* indicates a crude extract derived from strain RM101 (Δ*fnr*); MG indicates a crude extract derived from strain MG1655 (wild type). The cross-reacting polypeptides (*) have not been identified but served as loading controls.

TABLE 4. Effect of cellular FNR levels on expression of FNR-dependent genes

Strain	Beta-galactosidase enzyme activity (Miller units ± SD)											
	Φ(*pfl-lacZ*)		Φ(*fdn-lacZ*)			Φ(*aspA-lacZ*)		Φ(*ndh-lacZ*)		Φ(*nikA-lacZ*)	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Anaerobic + nitrate	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
MC4100 (*is5F*)	180 ± 20	7,870 ± 630	15	470 ± 61	2,980 ± 295	210 ± 28	1,075 ± 134	990 ± 89	655 ± 78	4	380 ± 45
RM4100	210 ± 10	7,525 ± 715	35	570 ± 48	3,560 ± 425	150 ± 12	850 ± 125	320 ± 44	145 ± 7	4	810 ± 120

genes were chosen for analysis of their expression in strains RM4100 and MC4100 (Table 4). The FNR-regulated genes, including positively as well as negatively regulated genes, were chosen to include a range of expression levels together with one, *fdn*, that exhibits dual regulation in response to anaerobiosis and nitrate.

Essentially no significant differences in *pfl-lacZ* or *aspA-lacZ* expression levels between strains MC4100 and RM4100 were observed (Table 4). A minor but reproducible increase of 15% in *fdn-lacZ* expression in strain RM4100 was seen when cells were grown anaerobically with nitrate. The largest effect of low FNR levels on gene expression was observed with *ndh-lacZ*, where expression levels were reduced ~5-fold in strain MC4100 compared with strain RM4100 results. Finally, anaerobic expression levels of a *nikA-lacZ* fusion were increased twofold in strain RM4100 relative to strain MC4100.

DISCUSSION

The major findings of this study are as follows: first, that in certain *E. coli* strains *fnr* expression is significantly reduced due to insertion of IS*5* (*is5F*) in the regulatory region of the gene; second, that in such strains Hfq determines *fnr* expression, probably by preventing IS*5*-dependent silencing of the *fnr* promoter; and third, that a threefold reduction in the level of FNR has measurable effects on anaerobic expression of certain operons in strain MC4100.

In contrast to *Salmonella* species, which generally do not contain insertion elements, *E. coli* strains have variable numbers of copies of IS*5* (45) and in an analysis of several strains in my laboratory I have determined that *is5F* is present in MC4100, W3110, and RK4353 derivatives whereas it is absent from MG1655 and MC1000 derivatives. This does not mean, however, that over the years of storage of strains that IS*5* might not insert in this hotspot (see reference 24). Indeed, the *is5F* insertion sequence probably has been previously identified (2). A selection for mutants exhibiting aerobic up-regulation of the otherwise anaerobically regulated and nitrate-responsive *narGHJI* operon resulted in identification of a mutant with IS*5* inserted in a location and orientation identical to that reported here; however, the authors did not report whether the original strain also possessed this insertion sequence. In contrast to what was observed here, Bonnefoy et al. (2) observed a 10-fold increase in *fnr* transcription in the mutant. It is conceivable that this phenotype resulted from an IS*5* promoter "up" mutation which caused increased transcriptional polarity with *fnr*.

It has been reported (34) that IS*5* normally exhibits only weak transcriptional polarity in the particular orientation observed upstream of *fnr*. This is consistent with the finding that in strain MC4100 the *fnr* promoter is still active, albeit with reduced activity compared with that of strain MG1655. This result was corroborated by transcript analysis. Remarkably, in the *hfq* mutant this *fnr* transcript was no longer detectable, suggesting that *fnr* was transcriptionally silent in the absence of Hfq.

The best-characterized example of transcriptional silencing in prokaryotes is provided by the *bgl* operon of *E. coli* (34). The mechanism is complex, but the consequence is that the operon is almost completely switched off in wild-type cells (33). Recent evidence indicates that Hfq has an antisilencing function that operates by reducing H-NS-dependent silencing of the cryptic *bgl* operon (5). In the case of *fnr* expression in strain MC4100 it is also only in the absence of Hfq that transcriptional silencing occurs. Nevertheless, this adds a further example of transcriptional silencing to the small list of those discovered so far in prokaryotes (48).

The precise mechanism by which Hfq prevents silencing of *fnr* transcription in strain MC4100 requires further investigation. One possible mechanism could involve the transposase encoded by the *ins5A* gene of IS*5* binding to the terminal inverted repeat of the element (see reference 34) and thus occluding the *fnr* promoter. Hfq could prevent or reduce this occurring by stabilizing a transcript that traverses the end of the IS*5* element. This can be tested by analyzing transcription across the boundary between the *fnr* regulatory region and IS*5*, as well as by determining whether the *ins5A* gene product can occlude *fnr* transcription in vitro.

Notwithstanding the effects of the *hfq* mutation on *fnr* expression in MC4100, truncation of the *fnr* regulatory region due to the insertion element has also revealed that DNA sequences important in controlling *fnr* gene expression extend beyond −64 bp. Although in the present study the IS*5* insertion element foreshortened the *fnr* regulatory region to −41 bp, in an earlier study Spiro and Guest (40) fused the *fnr* regulatory region from the EcoRI site at position −64 bp to the *lacZ* gene and obtained β-galactosidase enzyme activities in the range found here for the truncated fusion derivative. Thus, in the ~100 bp between the EcoRI site and the next gene *ogt* there are sequences important for the approximately threefold difference in expression observed with the full-length *fnr-lacZ* fusion and with *fnr* transcript levels (see also reference 42). It is presently unclear whether both *cis* and *trans* regulation is involved.

Reduced *fnr* expression is also reflected in the lower cellular level of FNR in strain MC4100, and this has measurable effects on gene expression. Despite examination of the expression of only five genes, it is evident that there was a range of responses to the lower level of the regulator. Some genes were unaffected in their expression, while for *ndh* the effect was

more pronounced. Repression of *ndh* expression by FNR requires occupation of two sites, one a high-affinity and one a low-affinity site (22). The low-affinity site might be particularly sensitive to changes in FNR concentration. These differential effects perhaps reflect differences in binding site location and/or affinity of FNR for the respective binding site. It will be of interest to determine the overall consequences of reduced FNR levels on global anaerobic gene expression in strain MC4100 (26) compared with strain RM4100 and with strain MG1655.

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