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Control by Fur of the nitrate respiration regulators NarP and NarL in *Salmonella enterica*

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Summary. Anaerobic metabolism is controlled by several transcriptional regulators, including ArcA, Fnr, NarP, and NarL, with the Fnr and ArcA proteins sensitive to the cell's redox status. Specifically, the two-component ArcAB system is activated in response to the oxidation state of membrane-bound quinones, which are the central electron carriers of respiration. Fnr, by contrast, directly senses cellular oxidation status through the [4Fe-4S] cluster present in its own structure. In this study, a third additional redox-associated pathway that controls the nitrate respiration regulators NarL and NarP was identified. The results showed that, in *Salmonella enterica*, the expression of these two transcriptional regulators is under the control of Fur, a metalloregulator that senses the presence of Fe^{2+} and regulates the homeostasis of this cation inside the cell. Thus, the Fur-Fe^{2+} complex increases the expression of *narL* and represses that of *narP*. Furthermore, studies of *S. enteric* mutants defective in the Fur-regulated sRNA RfrA and RfrB showed that those sRNA control both *narP* and *narL* expression. These results confirm Fur as a global regulator based on its involvement not only in iron uptake and detoxification but also in the control of nitrate/nitrite respiration by sensing cellular redox status. [**Int Microbiol** 2010; 13(1):33-39]

Keywords: *Salmonella enterica* · ferric uptake regulator · nitrate respiration · sRNA control · iron · anaerobiosis

Introduction

The regulation of electron-transport components is essential for the adaptation of bacteria to environmental conditions [22,44] and is carried out by several global mechanisms. In the absence of oxygen, the ArcA/ArcB system inhibits the expression of genes required for optimal energy production when this element is the final electron acceptor [44]. The Fnr protein plays a major role in bacterial switching from aerobic to anaerobic growth, acting as a positive regulator of a set of genes involved in anaerobic respiration [40]. Fnr is active in the absence of oxygen, in which case it specifically binds to target-DNA sites, enhancing the expression of genes under its regulation [4,24]. Conversely, the presence of oxygen induces a conformational change in the Fnr regulator such that it loses its specific-DNA binding capacity, prompting a decrease in the expression of genes required for anaerobic growth [4].

In facultative anaerobic bacteria, electron acceptors are often engaged in a specific order or hierarchy [44]. Under anoxic conditions, nitrate is the preferred electron acceptor and its presence results in the repression of other anaerobic pathways [44]. In Enterobacteriaceae, the use of nitrate as well as nitrite is under the control of the two-component regulatory systems NarX/L and NarP/Q [37,41]. NarX and NarQ membrane sensory kinases are activated by the pres-

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ence of either nitrate or nitrite and mediate phosphorylation of the cytoplasmic regulators NarL and NarP [37,41]. In the phosphorylated state, these transcriptional regulators control the expression of their target genes [41]. In some cases, the NarL and NarP regulons functionally overlap and the two proteins may have opposing effects [32,44]. For instance, the respiratory nitrite reductase (encoded by the *nrfABCDEFG* genes) and periplasmic nitrate reductase (*napFDAGHBC*) are repressed by NarL and activated by NarP [44]. Other genes, such as nitrate reductase-A (*narGHIJ*) and the cytoplasmic nitrite reductase (*nirBDC*), are positively regulated only by NarL [41,44].

Bacterial nitrate and nitrite respiratory reductases require iron in their reactive center to carry out their biological activity [20]. In many bacterial groups, Fe^{2+} uptake is under the control of Fur (ferric uptake regulator), which normally acts as a negative regulator of several genes involved in this process [21]. In *Escherichia coli*, the *fur* gene product is a 17-kDa protein that has Fe^{2+}-dependent DNA-binding activity [3]. Genes under the direct control of Fur require at least three contiguous NAT(A/T)AT-like hexamers in their promoters. This sequence, known as the Fur box, can appear in either the direct or the inverse orientation. When iron is present in the medium, the Fur-Fe^{2+} protein complex binds to the Fur box and represses genes under Fur control [17,38]. In addition, the *E. coli* Fur protein controls some genes indirectly, through repression of the RyhB small RNA (sRNA) [27]. Usually, RyhB pairs at the ribosome binding site of its mRNA targets and induces degradation by recruiting the RNA degradosome [28]. Thus, Fur exerts a positive effect on RhyB-controlled genes. However, negative indirect control by Fur via RyhB also has been described [36]. In this case, RyhB binds to the upstream region of the *E. coli shiA* mRNA, thereby disrupting an intrinsic inhibitory structure that sequesters the ribosome-binding site and the first translation codon. The RyhB interaction therefore results in a positive effect on *shiA* translation [36].

Despite the Fe^{2+} requirement of bacterial nitrate and nitrite respiratory reductases, to our knowledge the relationship between the Fur regulator and the synthesis of these enzymes has not been studied. Accordingly, and in the context of the significant role played by respiratory nitrate reductases in *Salmonella enterica* nitric oxide homeostasis [19], we examined expression of the genes *narL* and *narP* in this pathogen under conditions of low Fe^{2+} and in a *S. enterica fur*-defective mutant in the presence or absence of O_2.

Table 1. Strains and plasmids used in this work

Strains and plasmids	Relevant features	Source
Salmonella enterica strains		
SV 5015	SL 1344 His^+	[45]
UA 1779	ATCC 14028 Δ*fur*::*cat*; Cam^R	[7]
UA 1880	As SV 5015 but Δ*fur*::*cat*; Cam^R	This work
UA 1875	SL 1344 carring the pKOBEGA TS plasmid; Amp^R	This work
UA 1881	As SV 5015 but Δ*rfrA*::*kan*; Kan^R	This work
UA 1882	As SV 5015 but Δ*rfrB*::*kan*; Kan^R	This work
UA 1883	As SV 5015 but Δ*rfrA*::*kan* Δ*rfrB*; Kan^R	This work
UA 1884	As SV5 015 but Δ*fur*::*cat* Δ*rfrA*::*kan* Δ*rfrB*; Cam^R Kan^R	This work
Escherichia coli strains		
DH5α	*supE4* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi*-1 *relA1*	Clontech
BL21(DE3)pLysE	F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal dcm* (DE3) pLysE (Cam^R)	Stratagene
Plasmids		
pET15b	His_6 tag expression vector; Amp^R	Novagen
pGEM®-T	PCR cloning vector; Amp^R	Promega
pKOBEGA	*bla* P_{BAD} *gam bet exo* pSC101 oriTS	[8]
pKD4	*bla* FRT *Km* FRT PS1 PS2 oriR6K	[12]
pCP20	*bla cat cI*857 λP_R*flp* pSC101 oriTS	[9]

Materials and methods

Bacterial strains, plasmids, and growth conditions. *Escherichia coli* and *S. enterica* strains used in this work are listed in Table 1. In all cases, strains were incubated at 37°C. For oxic conditions, strains were grown in Luria-Bertani broth (LB). For anaerobic growth, media were based on 3-[N-morpholino]propane sulfonic acid (MOPS) medium [33], with several modifications described in Stewart et al. [42], and with the addition of 80 mM glucose and 40 mM $NaNO_3$ as carbon and nitrate sources, respectively. In this case, the cultures were incubated in GasPak (BBL) anoxic jars with the BD GasPak EZ anoxic container system. The maintenance of anoxic conditions was monitored by BD BBL dry anoxic indicator strips. When necessary, ampicillin (100 μg/ml), chloramphenicol (34 μg/ml), kanamycin (150 μg/m), or EDTA (1.5 mM) was added to the culture. DNA techniques were those described elsewhere [39].

Real-time quantitative RT-PCR assays. RNA was isolated as previously described [2]. Real-time quantitative RT-PCR analysis of gene expression was carried out for all bacterial species as previously reported [6] and using suitable oligonucleotide primer pairs for each gene (Table 2). In

Table 2. Oligonucleotides used in this work

Name	Sequence (5′→ 3′)[a]	Position[b]	Application
*fur*_NdeI	CATATGACTGACAACAATACCGC	1	Upper primer used to obtain the *S.enterica fur* gene
*fur*_BamHI	GGATCCTTATTTAGTCGCGTCATCGTGC	453	Lower primer used to obtain the *S.enterica fur* gene
P*narX*_up	GGAAAAGTCGTCATCACC	–266	Upper primer used to obtain the promoter region of *narX*
P*narX*_dw	GCCAGTTGGTTAACCAGC	47	Lower primer used to obtain the promoter region of *narX*
*rfrA*_P1	AGGGCCCGGAGCGTACTAAATGTACGTGAGGA GCACGAGCACTTCCCGGGGACAAAATGACAA GTAAGCCAGGCTGAAACgtgtaggctggagctgcttc	3715315[c]	P1 primer to construct *S. enterica rfrA* mutant by one step inactivation
rfrA_P2	CACTATTTCACTCATTCCTTATCTCCTGCAGG GTTAATTGTGTATTTACACTCGCTGAGAAAGA AAATTCCGTCAACCGCatgggaattagccatggtcc	3715632[c]	P2 primer to construct *S. enterica rfrA* mutant by one step inactivation
*rfrB*_P1	CGGCGCTGGAGATGACCCCGTATCACGCAAAA TAGCGCGGCTGAAAAAAGACCATGAATTCGAC ATGGGATAGATAGCGGgtgtaggctggagctgcttc	1352791[c]	P1 primer to construct *S. enterica rfrB* mutant by one step inactivation
*rfrB*_P2	GGAAAGTGAAGTTTGTGATGTCCATCACCTT TTAGCGTCGTGGATAAAAGCGCATAAATATCA GGGTTGCAATCATTAATatgggaattagccatggtcc	1353024[c]	P2 primer to construct *S. enterica rfrB* mutant by one step inactivation
*rfrA*_up	TCAGTTTGTTCACGGCAAGC	3715291[c]	Upper primer for *S. enterica rfrA* mutant confirmation
*rfrA*_dw	CGTAATCTTTCGGTTCAGCG	3715660[c]	Lower primer for *S. enterica rfrA* mutant confirmation
*rfrB*_up	TGGGGTTTATGCAGCAGG	1352755[c]	Upper primer for *S. enterica rfrB* mutant confirmation
*rfrB*_dw	CGTTAGCGGTTTATTTGCCG	1353117[c]	Lower primer for *S. enterica rfrB* mutant confirmation
narP RT up	CGCTACGCCGGGATGGTG	218	Upper primer used to analyze *narP* gene expression by RT-qPCR[d]
narP RT dw	ACTCTCGTTCGGTCAGGATACTAA	475	Lower primer used to analyze *narP* gene expression by RT-qPCR[d]
narL RT up	GAATAATCAGGAACCGGCAACCATC	3	Upper primer used to analyze *narL* gene expression by RT-qPCR[d]
narL RT dw	CTTTAGCGCCGTGACGACATCTTCT	300	Lower primer used to analyze narL gene expression by RT-qPCR[d]
sodB RT up	AAGGCACGGCGTTTGAAGG	131	Upper primer used to analyze *sodB* gene expression by RT-qPCR[d]
sodB RT dw	GGCGTACCGGCATTTGAGG	434	Lower primer used to analyze *sodB* gene expression by RT-qPCR[d]
foxA RT up	TAGCGCCGCCGTGTATCGTA	1572	Upper primer used to analyze *foxA* gene expression by RT-qPCR[d]
foxA RT dw	ATGCCGGAGCCCAAAGTCAG	1871	Lower primer used to analyze *foxA* gene expression by RT-qPCR[d]

[a] Restriction endonuclease recognition sites are underlined. P1 and P2 sequences, homolog to the pKD4 plasmid are represented in lower case.
[b] Position of the 5′ end of the oligonucleotide with respect to the translation start point of the corresponding gene.
[c] Position of the 5′ end of the oligonucleotide with respect to the *S. enterica* serovar Typhimurium LT2 genome sequence in the National Center for Biotechnology Information.
[d] RT-qPCR, real-time quantitative RT-PCR.

all cases, the results were normalized with respect to the *hisG* gene. This housekeeping gene is not affected either by iron concentration or the presence or absence of O_2 [5,10]. The induction factor (IF) of each gene under each growing condition was defined as the ratio between its expression in either the *fur* or *rfrA rfrB* mutant derivatives or in the presence of EDTA and in the wild-type strain.

Construction of *Salmonella enterica* mutant derivatives. The *S. enterica* SL1344 *fur* mutant derivative was constructed by transduction, as previously reported [7], using the P22int7(HT) bacteriophage and UA1779 as donor strain [7]. The absence of the prophage in chloramphenicol-resistant transductants was determined by streaking them onto green plates, as described previously [13]. *Salmonella enterica rfrA* and *rfrB* mutants were constructed using the one-step PCR-based gene replacement method [12]. The kanamycin antibiotic resistance cassette was amplified from the pKD4 plasmid using suitable 100-nt-long oligonucleotides containing an 80-nt region homologous to the target gene (Table 2). All PCR products were transformed in UA1875 (Table 1) carrying the pKOBEGA plasmid [8]. If necessary, the resistance cassette was excised using the pCP20 plasmid [9]. When needed, the genetic constructs were transferred to the suitable derivative strain by transduction, carried out as described above [7]. All mutant constructs were verified by PCR using the appropriate oligonucleotides (Table 2) as well as by sequencing.

Fur purification and electrophoretic mobility shift assays. Fur protein was purified using the pET15b overexpression vector (Novagen), as previously described [30] but with modifications. Briefly, the *S. enterica fur* gene was PCR-amplified and cloned into the pET15b expression vector, which was transformed into *E. coli* strain BL21(DE3)pLysE to overexpress the encoded protein. The Fur protein was purified using the Talon Metal Affinity Resin Kit (Clontech), as reported [30]. The protein was eluted from the affinity column by thrombin cleavage and using the appropriate elution buffer (10 mM Bis-Tris/borate buffer, pH 7.5, 10% glycerol, 1 mM $MgCl_2$, 40 mM KCl, 100 μM $MnCl_2$). The activity of the purified Fur protein was confirmed based on its ability to bind the *foxA* promoter [43]. PCR using a DIG-labeled oligonucleotide was carried out to obtain promoter DNA probes (Table 2), as described [1]. Electrophoretic mobility shift assays (EMSAs) were done as previously described using the reported buffers [14]. The binding mixture was incubated for 10 min at 37°C, after which the samples were loaded onto a 5.5% polyacrylamide gel. DIG-labeled DNA-protein complexes were detected following the manufacturer's (Roche) protocol.

Results and Discussion

To determine the putative relationship between anaerobic metabolism and iron, the expression levels of the genes *arcA, fnr, narP*, and *narL* were determined by quantitative real-time RT-PCR analysis assays conducted in the presence of the chelator EDTA or in a *S. enterica fur*-defective genetic background. Addition of the chelator to the culture increased the expression of *narL* and decreased that of *narP* (Fig. 1A). Note that the transcriptional behavior of *narL* and *narP* in the *fur* mutant was the same as in the wild-type strain grown in the presence of EDTA (Fig. 1A). However, *arcA* expression was not affected by the presence of either the *fur* mutation or EDTA (data not shown). Likewise, and as expected, the transcriptional expression pattern of *fnr* did not change (data not shown), since in *E. coli* this gene is not under Fur control [34]. By contrast, the genes *foxA* and *sodB* were, respectively, over- and under-expressed in the *S. enterica fur* mutant, in concordance with previous data [15,25].

To further confirm the relationship between Fur and *narP* and *narL* gene expression, quantitative real time RT-PCR experiments under anoxic conditions and in the presence of nitrate were also carried out. As shown in Fig. 1, the expression levels of both genes were in concordance with those observed under oxic conditions. The Fnr protein is known to repress expression of the *narL* gene [35]. This negative control must interfere in the IF levels observed under anoxic conditions. For this reason, *narL* induction was lower than that observed in the presence of oxygen, where Fnr was not active and control by the Fur protein of *narL* was maximized.

Moreover, and in accordance with the above-mentioned results, Fur-mediated regulation of *narP* and *narL* may also affect the expression patterns of genes encoding enzymes directly involved in nitrate respiration. For this reason, the behavior of *S. enterica narG, napC, nirB*, and *nrfA* was analyzed in the presence of EDTA and in the *fur*-defective strain (Fig. 1B). Note that, in *E. coli*, NarL positively regulates nitrate reductase A (encoded by *narGHIJ*) and the cytoplasmic nitrite reductase (*nirBDC*) whereas *nap* (encoding periplasmic nitrate reductase) and *nrf* (respiratory nitrite reductase) are regulated by NarL and NarP, which act as repressor and activator, respectively [41,44]. Figure 1B shows that basal expression of *napC* and *nrfA* decreased in the *S. enterica fur* mutant whereas the transcription of *narG* and *nirB* increased dramatically. These results are in concordance with the transcription behavior of *narL* and *narP* in the presence of EDTA and in *S. enterica fur* cells (Fig. 1A). Moreover, the *napC* and *nirB* results are in agreement with data obtained in arrays performed using Fur-defective *E. coli* strain [31]. These data unequivocally show that in *Salmonella enterica* the nitrate and nitrite respiration processes are associated with the Fe^{2+} concentration and under Fur control, through the transcriptional regulation of NarP and NarL.

As described above, Fur can act directly as a repressor by binding to the Fur box, located in the promoter region of its target genes [17]. Since *narL* expression was increased in the *fur* mutant strain, in silico searches were done to determine whether the *narL* promoter included a putative Fur box, but none was identified. Likewise, EMSAs using this promoter region and the purified Fur protein revealed no shift in promoter mobility (data not shown). Together, these data provide evidence that negative regulation of *narL* by Fur must be indirect.

It has been widely stated that, in *E. coli*, the positive, indirect regulatory action of Fur is associated with the RhyB

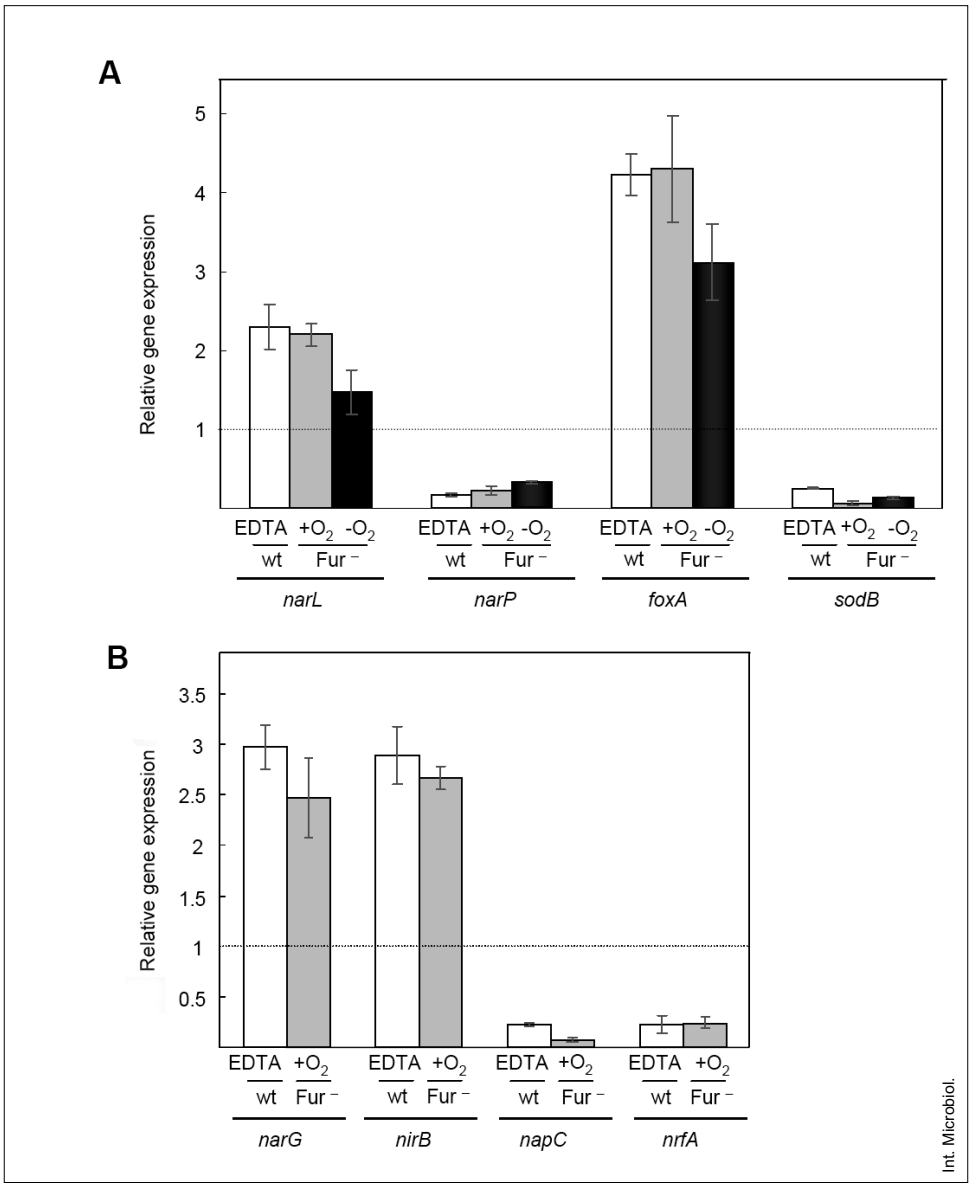

Fig. 1. (**A**) Expression of *Salmonella enterica narL* and *narP* genes under different growth conditions. Gene expression levels were determined in cells treated with 1.5 mM EDTA and in a *S. enterica fur* mutant derivative, under oxic or anoxic conditions. As a control, the *foxA* and *sodB* genes were also tested, serving, respectively, as Fur repressed or activated genes. (**B**) Expression of the NarP- and NarL-regulated genes *narG*, *napC*, *nirB*, and *nrfA* in *S. enterica* EDTA-treated cells and in the *fur* mutant strain. The expression level, measured by quantitative real time RT-PCR, is shown as the ratio between the relative mRNA concentration of each gene in either EDTA-treated cells or in the *fur* mutant and that of the *S. enterica* wild-type strain. mRNA concentrations were normalized to *S. enterica hisG* gene expression. Dotted line indicate an IF = 1, in which expression under the two conditions does not differ. In each case, the mean value from three different biological replicates is shown. (Error bars, SD.)

small RNA (sRNA) [29]. Moreover, and as noted above, it has been shown that some genes indirectly repressed by Fur are also associated with the RhyB sRNA regulon [36]. *Salmonella enterica* contains two sRNA, RfrA and RfrB, homologous to the product of the *E. coli ryhB* gene and which also participate in the Fur-mediated positive control of several iron-related genes [16]. In this context, and to determine the role of *S. enterica* RfrA and RfrB sRNA in the control of *narP* and *narL* expression, strains defective in either one or in both were constructed. The results, shown in Fig. 2, clearly indicated that the pattern of *narP* and *narL* expression was altered by the absence of both sRNA either in the pres-

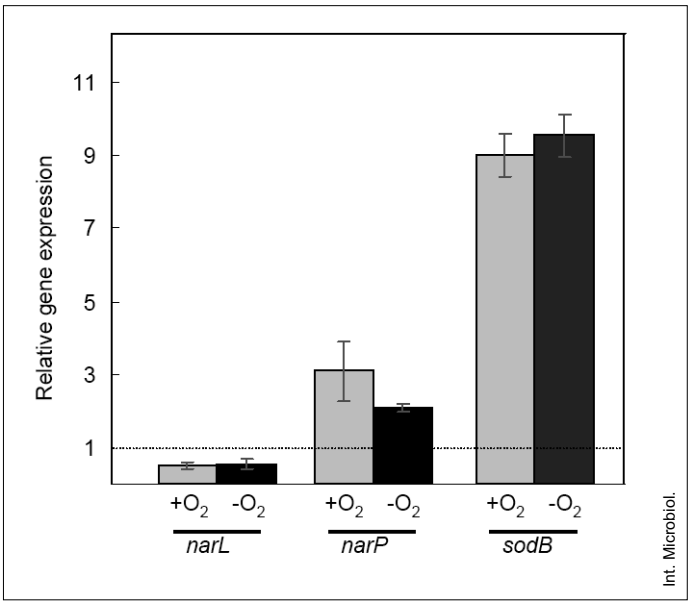

Fig. 2. Expression of the genes *narP* and *narL* in the absence of RfrA and RfrB sRNA under oxic or anoxic conditions. The expression level was calculated as the ratio between *narP* and *narL* gene expression in *Salmonella enterica rfrA rfrB fur* strain and that observed in the *fur* mutant. As a control to confirm the ability of sRNA to repress gene expression, the IF of *sodB* gene is shown. The experiments were otherwise carried out and the results analyzed as described in Fig. 1.

ence or in the absence of oxygen whereas in the single-gene mutants it was the same as in the wild-type strain (data not shown). Thus, in *S. enterica*, both sRNA are necessary for Fur regulation of *narP* and *narL*. Similar results were described for the *S. enterica sodB* gene regarding its regulation by sRNA [16]. Moreover, in *S. typhimurium fur rfrA rfrB* mutants, the level of *narP* mRNA increased whereas that of *narL* decreased (Fig. 2), indicating that sRNA regulation must be different for these genes.

The expression of most genes involved in the anaerobic growth of facultative bacteria is linked to the presence or absence of O_2. Until now, two different pathways to control anaerobic metabolism were known, both associated with the cellular oxidation or reduction (redox) status. One such pathway is through the Fnr regulator, which directly senses oxygen levels. Under conditions of very low oxygen tension, Fnr is in a dimeric state, contains a [4Fe-4S] cluster, and is able to bind DNA site-specifically. As oxygen becomes available, a conversion from the dimeric to the monomeric [2Fe-2S] state is produced through oxidation, generating a non-DNA-binding form of the regulator [4,11,23,24]. The ArcAB two-component system is the second pathway regulating cellular conversion from aerobic to anaerobic growth. In this case, the redox status of membrane-bound quinones, the central electron carriers of respiration, mediates auto-phosphorylation of the sensor kinase ArcB activator [18,26]. It has been shown that most nitrite- or nitrate-dependent respiratory genes are under Fnr regulation but their expression is also controlled by the regulators NarQP or NarXL, which are sensitive to the presence of nitrite and nitrate, respectively, as final electron acceptors under anoxic conditions [37,44]. Our data provide evidence of a third, as yet undescribed, pathway of anaerobic respiratory transcriptional control associated with cellular redox status, involving the NarL and NarP nitrate response regulators. Expression of these genes was clearly shown to depend on the intracellular Fe^{2+} concentration and on Fur control. Thus, the Fur protein seems to act as a sensor of anoxic conditions since free Fe^{2+} is present in the absence of O_2 and only the Fur-Fe^{2+} complex is able to bind to the Fur-box. Our findings support a role for Fur as a global regulator, due to its involvement not only in iron uptake and detoxification but also in sensing the cellular redox status.

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