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Reprogramming of Anaerobic Metabolism by the FnrS Small RNA

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Summary

Small RNAs (sRNA) that act by base pairing with *trans*-encoded mRNAs modulate metabolism in response to a variety of environmental stimuli. Here, we describe an Hfq-binding sRNA (FnrS) whose expression is induced upon a shift from aerobic to anaerobic conditions and which acts to down regulate the levels of a variety of mRNAs encoding metabolic enzymes. Anaerobic induction in minimal medium depends strongly on FNR but is also affected by ArcA and CRP. Whole genome expression analysis showed that the levels of at least 32 mRNAs are down regulated upon FnrS overexpression, many of which are predicted to base pair with FnrS by *TargetRNA*. The sRNA is highly conserved across its entire length in numerous enterobacteria, and mutation analysis revealed that two separate regions of FnrS base pair with different sets of target mRNAs. Many of the target genes previously reported to be down regulated in an FNR-dependent manner lack recognizable FNR binding sites. We thus suggest that FnrS extends the FNR regulon and increases the efficiency of anaerobic metabolism by repressing the synthesis of enzymes that are not needed under these conditions.

Keywords

base pairing; FNR; CRP; ArcA; *sodB*

Introduction

Small RNAs (sRNAs) are now known to be key regulators in all organisms. Approximately 80 sRNAs, generally between 80 and 200 nucleotides in length, have been identified in *Escherichia coli*. The majority of these sRNAs act either by base pairing with target RNAs or by binding proteins and modifying their activities. Base pairing sRNAs can be encoded in *cis* or *trans* with respect to their targets. The *cis*-encoded sRNAs have extensive complementarity with their targets, while the *trans*-encoded sRNAs exhibit more limited complementarity with target mRNAs and require the RNA chaperone protein Hfq for base pairing [reviewed in (Waters and Storz, 2009)].

Most of the *trans*-encoded base pairing sRNAs in *E. coli* are induced in response to a specific environmental condition and modulate metabolism by regulating the expression of enzymes and transporters. For example, the OmrA and OmrB RNAs, whose transcription is induced by the OmpR-EnvZ two-component regulators in response to high osmolarity, and the MicA and RybB sRNAs, whose expression is regulated by σ^E in response to perturbations in the cell envelope, all modulate the synthesis of major outer membrane porins [reviewed in (Guillier *et al*., 2006, Vogel and Papenfort, 2006)]. The Spot42 RNA, whose levels are modulated by CRP (cAMP receptor protein) in response to glucose availability, represses the synthesis of

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enzymes involved in carbon metabolism (Polayes *et al.*, 1988, Moller *et al.*, 2002). Finally, the RyhB RNA, whose levels increase upon iron depletion when Fur repression is relieved, down regulates a variety of iron-containing enzymes thus making iron available for the most critical enzymes under these iron-limiting conditions (Massé and Gottesman, 2002, Massé *et al.*, 2005).

E. coli is able to grow in both aerobic and anaerobic environments and not surprisingly a wide reprogramming in gene expression, with significant effects on cell metabolism, is observed upon shifts between different low and high oxygen conditions (Salmon *et al.*, 2003, Kang *et al.*, 2005, Constantinidou *et al.*, 2006). Two transcriptional regulators, FNR (fumarate and nitrate reduction) and ArcA (aerobic respiratory control), whose activities are modulated by oxygen availability, impact many of the changes in gene expression associated with a transition from aerobic to anaerobic metabolism. FNR is a direct sensor of oxygen availability. This protein is only active under anaerobic conditions because of the requirement for an $[4Fe\text{-}4S]^{2+}$ cluster which permits FNR dimerization. ArcA is the cytosolic response regulator of a two-component pair, in which ArcB is the transmembrane histidine kinase sensor. Oxygen levels are sensed indirectly by the ArcA/B pair (Georgellis *et al.*, 2001). During aerobiosis, oxidized quinones repress autophosphorylation of ArcB. Under anaerobic conditions, when the levels of oxidized quinone decrease, ArcB becomes autophosphorylated and transfers the phosphate group to ArcA to activate the response regulator. FNR has been shown to repress a number of genes with aerobic functions as well as activate even more genes encoding enzymes of anaerobic pathways (Kang et al., 2005, Salmon et al., 2003, Constantinidou et al., 2006). In contrast, ArcA represses many genes encoding enzymes required for aerobic respiration and acts as a positive regulator of a few genes required for anaerobic metabolism (Iuchi and Lin, 1988, Iuchi *et al.*, 1989) [reviewed in (Gunsalus and Park, 1994)]. There is also significant overlap in the genes modulated by the two regulators. While a subset of the genes induced under anaerobic conditions have been found to have FNR and/or ArcA binding sites, other genes whose expression changes in *fnr* and *arcA* mutants do not, raising the question of how these genes are regulated (Constantinidou et al., 2006, Liu and De Wulf, 2004).

In addition to ArcA and FNR, the expression of anaerobic genes is modulated by transcriptional regulators that sense glucose availability or the levels of electron acceptors. For example, CRP activates numerous genes involved in the catabolism of amino acids and sugar when glucose levels are low and cAMP levels are high (Buchet *et al.*, 1999). The amino acid sequence of CRP is similar to that of FNR, and many of the sites recognized by FNR can also be recognized by CRP (Sawers *et al.*, 1997). Furthermore, there are a number of two-component systems that respond to the presence of different electron acceptors. Thus, for example, nitrate, the preferred electron acceptors in the absence of oxygen, and/or nitrite are sensed by the dual two-component systems NarX/L and NarQ/P [reviewed in (Stewart, 2003)].

Here we describe an sRNA, denoted FnrS, whose expression is induced by anaerobic conditions in an FNR- and ArcA-dependent manner. This sRNA down regulates at least 32 mRNAs, many of which encode enzymes directly involved in energy metabolism and previously reported to be part of the FNR regulon. For the five mRNA targets (*sodB*, *maeA*, *gpmA*, *folE* and *folX*) examined in more detail, two separate regions of FnrS were shown to base pair with different sets of targets. We suggest that this allows the FnrS RNA, which is conserved across its entire length, to regulate the expression of a large set of genes, thus extending the FNR regulon.

Results

Novel sRNA in the *ydaN-dbpA* intergenic region

Based on sequence conservation, the 477 base pair intergenic region between *E. coli ydaN* and *dbpA* was predicted to encode a sRNA, but no signal was detected by Northern analysis

(Wassarman *et al.*, 2001, Carter *et al.*, 2001). However, recent tiling array analysis of RNAs that co-immunoprecipitate with the RNA chaperone protein Hfq in *E. coli* (Zhang *et al.*, unpublished data) and deep sequencing of Hfq binding sRNAs in *Salmonella typhimurium* (Sittka *et al.*, 2008) again indicated a sRNA was encoded on the Watson strand of the intergenic region, overlapping the section showing the highest conservation (Fig. 1A). To detect this sRNA by Northern analysis, we used an oligonucleotide probe complementary to the region showing a signal in the tiling array analysis to probe total RNA isolated from wild-type cells as well as RNA that co-immunoprecipated with Hfq (data not shown). A band of slightly longer than 100 nucleotides was barely visible for the Hfq immunoprecipitation sample. The 5′ end of this transcript was mapped to position 1,407,153 by 5′ RACE analysis and a Rho independent terminator was predicted at approximately 100 nt from the +1 (Fig. 1B). An alignment of this region showed that the entire sequence of this sRNA, denoted FnrS as explained below, is conserved among enterobacterial species (Fig. 1C). One flanking gene, *ydaN*, which is predicted to encode a zinc transporter in *Salmonella* (Worlock and Smith, 2002), is always found upstream of *fnrS*. The other flanking gene is more variable. In *Escherichia, Salmonella, Shigella, Citrobacter* and *Klebsiella* species, the downstream gene is *dpbA*, which encodes a 3′ to 5′ RNA helicase (Diges and Uhlenbeck, 2005), while genes encoding a MerR family transcriptional regulator, a tRNA thiolase, a glyoxylase resistance protein and an uncharacterized ORF flank *FnrS* in *Yersinia, Erwinia, Serratia* and *Sodalis*, respectively.

Tjaden et *al.* reported an sRNA microarray signal in the *ydaN-dpbA* region (C0343), also on the Watson strand but downstream of the region probed above (Fig. 1A) (Tjaden *et al.*, 2002). Possibly this signal corresponds to the leader of the *dbpA* transcript. However no signal was detected in this region by the tiling array analysis. In contrast, a tiling array signal was noted on the Crick strand. This region was predicted to encode an sRNA by Carter et al. 2001, but we did not detect a transcript in our Northern analysis (data not shown).

FnrS RNA induction during anaerobic growth

Upon examination of the sequence upstream of the FnrS, we noticed a putative FNR (TTGAT-N_4-ATCAA) and/or CRP (TGTGA-N_6-TCACA) binding site at −41.5 relative to the start of transcription (Eiglmeier *et al.*, 1989). FNR and CRP can activate transcription at two different classes of promoters. Class I promoters have binding sites centered at −61.5, −71.5, −82.5 or −92.5, while class II promoters have binding sites centered at −41.5 relative to the transcriptional start site (Fig. 1B) [reviewed in (Busby and Ebright, 1999)]. In addition, we noticed a putative binding site for ArcA ([A/T]GTTAATTA[A/T]) at approximately −35, overlapping the CRP/FNR site (Lynch and Lin, 1996) and a putative binding site for NarL/NarP (TACYYMT, where Y= C or T and M = A or C) at position −22.5 (Darwin *et al.*, 1997).

The predicted binding sites prompted us to examine FnrS levels in cells grown with and without oxygen, with different carbon sources (glucose and glycerol), and with different terminal electron acceptors (nitrate and fumarate) (Fig. 2A). This Northern analysis showed that FnrS is barely detectable in cells grown aerobically regardless of carbon source or final electron acceptor. In contrast, FnrS levels are strongly induced when the cells were shifted to anaerobic conditions. The levels of the sRNA are slightly higher in cells grown in glucose medium and appear to be somewhat repressed by nitrate, especially under aerobic conditions. These results show that regulation of FnrS is relatively complex, with maximal expression during anaerobic growth. The available carbon source and final electron acceptor also impact FnrS levels, but to a lesser extent.

FNR, ArcA and CRP-dependent transcription of FnrS RNA

To determine what transcriptional regulators are required for FnrS induction, we deleted the genes encoding the CRP, FNR and ArcA regulators and examined FnrS levels in these strains in glycerol medium with fumarate during aerobic and anaerobic growth. In the presence of oxygen, the sRNA is barely detectable in all strains (Fig. 2B). After a shift from aerobic to anaerobic conditions, FnrS levels are slightly higher in the Δ*crp* strain than in wild-type cells and barely detectable in the Δ*fnr* strain. The anaerobic induction is also significantly reduced in the Δ*arcA* strain. Thus FNR (hence the name FnrS), and to a lesser extent ArcA, act together to activate FnrS transcription during anaerobic growth. Furthermore, CRP has a negative impact on anaerobic expression of FnrS.

FnrS activation by CRP and ArcA in an *fnr* mutant strain

In the course of this study, we found that one laboratory stock of MG1655 (hereafter referred to as MG1655$_{fnr-}$) had significantly lower levels of FnrS compared to another laboratory stock of MG1655 (Fig. 2C). Since FNR had the strongest effect on FnrS expression, we sequenced the *fnr* gene in the MG1655$_{fnr-}$ strain. The sequencing revealed an insertion of six amino acids between amino acids 21 and 22 of FNR (Fig. S1). The arginine at position 10 and the serine at position 13 were also mutated to glycine and phenylalanine, respectively (Fig. S1). Previous studies showed that the $[4Fe\text{-}4S]^{2+}$ cluster required to form the transcriptionally-active FNR dimer is ligated by cysteines 20, 23, 29, 122 (Sharrocks *et al.*, 1990 1990,Melville and Gunsalus, 1990). Thus the mutations in the MG1655$_{fnr-}$ probably disrupt the binding of the $[4Fe\text{-}4S]^{2+}$ cluster, explaining the lower FnrS expression in this strain.

Nevertheless, FnrS expression is still induced by a shift to anaerobic conditions in MG1655$_{fnr-}$, though the induction is approximately 10 fold higher in glycerol than in glucose (Fig. 2D). In contrast to what was found for MG1655, no effect of the final electron acceptor was observed for MG1655$_{fnr-}$. To identify the transcription factors responsible for FnrS induction, we deleted the *arcA* and *crp* genes in MG1655$_{fnr-}$. Northern analysis showed that ArcA and CRP are both required for full FnrS expression in anaerobic growth (Fig. 2E). We also deleted the *fnr* gene; not surprisingly, the deletion does not affect FnrS expression in the MG1655$_{fnr-}$ strain, showing that the six amino acid insertion and point mutations completely abolish FNR activity. We conclude that FnrS expression during anaerobic growth is completely ArcA- and CRP-dependent in MG1655$_{fnr-}$. Furthermore, in MG1655$_{fnr-}$ grown aerobically in minimal medium containing glycerol, FnrS expression is essentially CRP-dependent.

FnrS secondary structure

Several related secondary structures are predicted by the *Mfold* program (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi) (Zuker, 2003) for the *E. coli*, *Serratia*, *Shigella*, *Yersinia*, *Erwinia* and *Citrobacter* FnrS RNAs. All structures contain the same Rho independent terminator. However, the first seven nucleotides at the 5′ end are alternatively predicted to be unstructured or in a small stem followed by a long stem-loop structure. The most stable predicted structure for *E. coli* FnrS is shown in Fig. 3A. To directly examine the FnrS structure, we carried out *in vivo* probing with 50 mM dimethylsulfate (DMS), which methylates unpaired adenosines and cytidines in RNA, and then carried out primer extension reactions, which terminate at the methylated nucleosides. The results of the primer extension analysis on the DMS treated samples (Fig. 3B) and the positions of the methylated residues (Fig. 3A) are consistent with the structure in Fig. 3A, with some breathing of the bottom of the first stem-loop. This structure with three stem-loops and an extended single-stranded region is typical of other Hfq-binding sRNAs (Wassarman *et al.*, 1999).

FnrS down regulation of mRNAs encoding a variety of metabolic enzymes

Given that the FnrS RNA co-immunoprecipitated with the Hfq protein, we surmised that FnrS would act by base pairing with *trans*-encoded mRNAs as is the case for other Hfq-bound sRNAs. To identify potential base pairing targets of FnrS, we transiently overexpressed FnrS and examined the genome-wide changes in transcript levels by microarray analysis, an approach that has led to the successful identification of other sRNA targets (Tjaden *et al.*, 2006, Guillier and Gottesman, 2006, De Lay and Gottesman, 2009). FnrS was cloned behind the arabinose-inducible P_{BAD} promoter of pAZ3, and expression was induced by the addition of 0.2% arabinose for 15 min. As a control, strains carrying the parental plasmid (pAZ3) were also treated with 0.2% arabinose. Microarray analysis revealed that 32 genes were repressed more than two fold after FnrS over-expression in three independent experiments (Table 1 and Table S1).

The mRNAs whose levels were repressed by FnrS encode proteins required for a variety of processes. Several of the mRNAs whose levels are down regulated encoded dehydrogenases. Two of the dehydrogenases are NAD-dependent; malate dehydrogenase (*maeA*) which catalyzes the conversion of malate to pyruvate (Yamaguchi, 1979), and the ethanol dehydrogenase/reductase (*adhP*), which can convert ethanol to an acetaldehyde or ketone and can also catalyze the reverse reaction of acetaldehyde reduction to ethanol. A third dehydrogenase, D-lactate dehydrogenase (*dld*), which converts lactate to pyruvate and is required for aerobic growth on lactate, is a membrane bound flavoprotein (Haugaard, 1959). Another down regulated mRNA encodes the most abundant phosphoglycerate mutase isozyme (*gpmA*), which converts 3-phosphoglycerate into 2-phosphoglycerate (Fraser *et al.*, 1999). The *sodB* mRNA, already known to be down regulated by the RyhB RNA (Massé and Gottesman, 2002) and encoding one of the three *E. coli* superoxide dismutase enzymes which protect against superoxide radicals generated during aerobic growth, is also repressed (Carlioz and Touati, 1986, Farr *et al.*, 1986). Finally, two mRNAs encoding enzymes involved in folate metabolism, dihydroneopterin triphosphate epimerase (*folX*) and a GTP cyclohydrolase I (*folE*), are down regulated by FnrS overexpression.

Half of the genes whose expression is down regulated by FnrS overproduction were reported to be in the FNR regulon based on microarray analysis of a wild-type MG1655 strain and the corresponding Δ*fnr* mutant (Constantinidou *et al.*, 2006). However, most of these genes do not have a predicted FNR binding site indicating that the effects of FNR could be indirect, possibly mediated by FnrS (Table 1).

To confirm FnrS regulation of putative targets encoding a broad range of enzymes, we specifically examined the levels of the *maeA* and *gpmA* mRNAs (central metabolism), the *sodB* mRNA (oxidative stress) and the *folE* and *folX* mRNAs, (folate metabolism) upon FnrS overexpression. Total RNA was isolated from wild-type cells carrying the pAZ3 control plasmid or pAZ3-FnrS plasmid and treated with 0.2% arabinose to induce the P_{BAD} promoter. After 15 min, cells were washed two times in LB + 0.2% glucose, to repress the P_{BAD} promoter. The cultures were then incubated an additional 15 min. The RNA isolated from the samples at different time points after induction was subjected to Northern analysis with oligonucleotide probes specific to *maeA*, *gpmA*, *sodB*, *folE*, *folX* as well as to FnrS (Fig. 4). We detected transcripts of the size expected for the monocistronic mRNA for all of the genes. The levels of all the mRNAs decreased upon FnrS induction and then increased after the arabinose was removed.

FnrS base pairing with target mRNAs

The *maeA*, *gpmA*, *sodB*, *folE* and *folX* mRNAs are all predicted to be base pairing targets of FnrS by the *TargetRNA* program (http://snowwhite.wellesley.edu/targetRNA) (Tjaden *et al.*,

2006), and given that 59% of the genes identified by the microarray analysis are predicted by *TargetRNA*, we assume that many base pair directly with FnrS (Table 1). Interestingly, both *TargetRNA* and *Mfold* analysis predict that different regions of FnrS base pair with the *maeA* and *gpmA* mRNAs compared to the *sodB*, *folE* and *folX* mRNAs (Fig. 5A). The first region comprises the single-stranded region between the first and second stem-loops and the second region is the 5′ end of the partially open stem of the first stem-loop (Fig. 3A).

To test whether pairing was direct or indirect and whether the predicted regions were required, we constructed three mutants of FnrS (I, II, III, Fig. 3A and 5A) by directed mutagenesis of pBR-FnrS. This plasmid contains an IPTG inducible promoter P_{lac} (Guillier and Gottesman, 2006). Upon induction, the levels of FnrS-I are comparable to wild-type FnrS levels expressed from the same vector. FnrS-II levels are about 50% relative to wild type, while FnrS-III levels are somewhat lower (Fig. 5B). Northern analysis showed that none of the mutant forms of FnrS were as effective as the wild-type sRNA in repressing the *sodB*, *gpmA*, *maeA* and *folE* mRNA levels. However, FnrS-I is particularly defective at down regulating *maeA*, FnrS-II is very defective are repressing *gpmA*, and FnrS-III was most defective with respect to *sodB* and *folE*. In general the mutations support the conclusion that different regions of FnrS base pair with different sets of targets.

We also constructed chromosomal fusions between the P_{BAD} promoter, the 5′ untranslated regions and first codons of the *maeA*, *gpmA*, *sodB*, *folE* and *folX* transcripts and *lacZ* such that these translational fusions are inducible by arabinose as described in (Mandin and Gottesman, 2009). We then monitored the effects of FnrS, FnrS-I, FnrS-II and FnrS-III overexpression in these strains. Expression of the translational fusions was first induced by the addition of 0.2% arabinose. After 5 min, expression of the FnrS wild-type or FnrS mutant RNAs from the pBR plasmids was induced with 100 μM IPTG, and the levels of β-galactosidase activity were assayed after 30 min. All fusions were repressed from 2- to 4-fold by wild-type FnrS overexpression and were unaffected by the empty vector (Fig. 6A). The *maeA-lacZ* fusion was also repressed by FnrS-III, despite the decreased levels of this sRNA, but was barely repressed by FnrS-II and not repressed by FnrS-I. Similarly, the *gpmA-lacZ* fusion was repressed by FnrS-III, but was repressed less well by FnrS-I and was unaffected by FnrS-II. The *sodB-lacZ, folE-lacZ and folX-lacZ* fusions were repressed by all mutants except FnrS-III. These assays substantiate the results obtained by Northern analysis and support the conclusion that the central single-stranded region of FnrS is required for base pairing with *maeA* and *gpmA* while the 5′ end of FnrS base pairs with *sodB*, *folE* and *folX* (Fig. 5A).

We further constructed compensatory mutations in three of the target fusions denoted *maeA-I*, *gpmA-II* and *sodB-III* based on the corresponding FnrS mutations (Fig. 6B). Assays of the mutant *lacZ* fusion strains expressing the mutant FnrS RNAs showed that only FnrS-I down regulates *maeA-I*, only FnrS-II down regulates *gpmA-II* and only FnrS-III down regulates *sodB-III*. These assays confirm direct base pairing between FnrS and these targets and again support the conclusion that different regions of FnrS are involved in base pairing with different targets.

Decreased target mRNA repression in an Δ*fnrS* mutant strain

We examined FnrS RNA levels after shifts between aerobic and anaerobic growth. Cells were grown aerobically in M63 medium containing glucose and fumarate until mid-exponential phase. The cultures were then placed into an anaerobic chamber and total RNA was extracted after 10 and 20 min of anaerobic growth. Subsequently, the cultures were shifted back to aerobic conditions, and total RNA was extracted after 10, 20 and 60 min. As shown in Fig. 7, FnrS levels were maximally induced around 20 min after cells were shifted to anaerobic conditions. In addition, the sRNA is almost completely absent 10 min after the culture was re-aerated. These results show that both induction and presumably also degradation of FnrS are rapid.

We also monitored the levels of the *sodB* and *gpmA* mRNAs in the samples taken above as well as in the samples taken from Δ*fnrS* cells exposed to the same treatment (Fig. 7). The levels of the *gpmA* mRNA were stable between aerobic and anaerobic growth in the wild-type strain. In contrast, the *gpmA* RNA levels increased after the anaerobic shift in the Δ*fnrS* mutant strain. These elevated levels persisted upon the shift back to aerobic growth. In contrast to the *gpmA* transcript, the levels of the *sodB* mRNA decreased under anaerobic conditions, particularly in the wild-type strain. These results show that FnrS expressed from the chromosome impacts the levels of the target mRNAs, presumably to fine tune metabolism as cells are shifted between environments with different oxygen levels.

Discussion

An increasing number of Hfq-binding sRNAs are being found to remodel metabolism and transport in response to particular environmental stimuli. Here we describe a newly-identified sRNA that is induced upon a shift to anaerobic conditions and that represses the expression of enzymes that are dispensable during growth in low oxygen.

Regulation of FnrS expression

The finding that the strong anaerobic induction of FnrS depends on FNR is in agreement with the prediction of an FNR binding site at −41.5 and the FNR signal detected by chromatin immunoprecipitation experiments (Grainger *et al.*, 2007). ArcA had a more limited effect on FnrS transcription, but was still required for optimal anaerobic induction. Dual regulation by both FNR and ArcA has been described for other anaerobically-induced genes (reviewed in (Sawers and Nakano, 2004)). These regulators can act in conjunction to either increase or decrease expression of the target gene or they can act in an opposing manner; one regulator activating transcription and the other repressing transcription. Only a few genes whose expression is activated by both FNR and ArcA, as observed for FnrS, have been described. These include *fumB* gene (encoding fumarase) and the *focA-pflB* operon (encoding a formate transporter and pyruvate formate-lyase, respectively) (Tseng, 1997, Sawers, 2006). In general, dual regulation by FNR and ArcA may allow the fine-tuning of gene expression during the shift from aerobic to anaerobic growth. An examination of transcriptomic data shows that FNR responds very rapidly while ArcA responds more slowly to a shift (Partridge *et al.*, 2007).

Interestingly, in an MG1655$_{fnr-}$ strain lacking functional FNR, FnrS levels were still induced during anaerobic growth, though at a 20-fold lower level and more so in glycerol than in glucose medium (Fig. 2D). In the MG1655$_{fnr-}$ background, FnrS expression is dependent on CRP and ArcA. These results indicate that CRP can recognize the FNR binding site as demonstrated previously for the *focA-pflB* operon as well as the *hlyE* gene encoding hemolysin E (Kaiser and Sawers, 1997,Sawers et al., 1997,Green and Baldwin, 1997,Westermark *et al.*, 2000) and can even partially replace FNR under anaerobic conditions. FnrS activation by CRP and ArcA is less efficient than the activation by FNR and ArcA possibly because CRP has a lower affinity than FNR for the binding site and/or is less efficient at stimulating FnrS transcription together with ArcA. The lower activation of FnrS by CRP could explain its negative impact on FnrS anaerobic expression in wild type strain where FNR and CRP compete for the same binding site (Fig. 2A and 2B). The dual regulation at the *hlyE* promoter allows induction of this gene under several different growth conditions, during anaerobic growth and upon glucose starvation, respectively. However, unlike the case for *hlyE*, expression of *focA-pflB* and FnrS is only slightly induced by CRP in a wild type strain growing aerobically. We proposed that at these promoters, CRP acts more as a negative regulator to limit *focA-pfl* and FnrS expression during anaerobic growth (Fig. 2A and 2B).

Finally, we found that nitrate slightly represses FnrS expression during both aerobic and anaerobic growth (Fig. 2A and 2D). The putative NarL/NarQ binding site centered at −22.5 in

the *FnrS* promoter might explain this effect. The NarXL and NarPQ two-component systems program cell metabolism for nitrate respiration when nitrate is available (Unden and Bongaerts, 1997). It is unclear why these regulators might repress *fnrS*, but the expression of other anaerobically-induced genes has also been found to be modulated by a plethora of transcriptional regulators.

Two base pairing domains in FnrS

Microarray analysis indicated that the levels of at least 32 mRNA species are down regulated two-fold or more by FnrS overexpression. Five of these targets (*maeA*, *gpmA*, *sodB*, *folE* and *folX*) were confirmed to be strongly repressed by FnrS by Northern blot analysis and β-galactosidase assays of translational fusions to the 5′ untranslated regions of these mRNAs. The *TargetRNA* program predicts that FnrS base pairs with these targets, as well as most of the other targets identified by microarray experiments, at or near the Shine-Dalgarno sequences of the target mRNAs (Fig. 5A). The program also predicts that two different single stranded regions of FnrS are involved in base pairing with the targets. Mutations introduced into these regions together with the corresponding compensatory mutations into targets confirmed the *TargetRNA* predictions. We observed that pairing with a central single stranded region of FnrS is important for regulation of *maeA* and *gpmA*, while the 5′ end of FnrS is required for *sodB*, *folE* and *folX* repression. Both mutations in the central single stranded region affect *gpmA* and *maeA* repression, probably because base pairing between FnrS and the *maeA* or *gpmA* mRNAs can be extended through the whole region affected by the mutations (Fig. 5A). We also predicted that FnrS could use both the 5′ and the central regions to regulate *sodB* mRNA (Fig. 5A) but only the FnrS-III mutation at the 5′ end strongly affected *sodB* regulation.

For most base pairing sRNAs described thus far, only one region of the sRNA has been found to be involved in base pairing. This single-stranded region can be localized at the 5′ end of the sRNA as described for OmrA and OmrB in *E. coli* and RybB in *Salmonella* (Guillier and Gottesman, 2006, Bouvier *et al.*, 2008) or in a central part such as for *E. coli* RyhB (Massé and Gottesman, 2002. Results presented here show that two regions of FnrS are involved in base pairing, both the 5′ end and a more central region. The results presented here are the first to clearly demonstrate that an *E. coli* sRNA can use two separate regions to target two different sets of targets. DsrA was proposed to use two different stem loop structures to regulate *rpoS* and *hns*, but these regions are adjacent and probably even overlap since some mutations affect both *rpoS* and *hns* regulation (Lease et al., 1998, Majdalani et al., 1998). GcvB has also been predicted to use different regions to base pair with the *cycA*, *oppA* and *dppA* mRNAs, but mutational analyses have not confirmed this prediction (Pulvermacher et al., 2009). In addition, two different regions of the OxyS RNA have been shown to be involved in base pairing, but, in this case, the different regions base pair with the same mRNA target (Argaman and Altuvia, 2000).

The involvement of two different regions of FnrS in base pairing could allow for the regulation of a larger number of targets and could explain the extensive conservation of FnrS through out its entire length. Based on predictions by *TargetRNA* and the results presented above, we propose that different sets of targets are regulated by the two regions, with genes linked to oxidative stress as well as folate and methionine metabolism regulated by the 5′ end and genes of central metabolism regulated by the central region.

Physiological role of FnrS RNA

Many of the mRNAs whose expression is repressed by FnrS encode enzymes involved in central and energy metabolism while a few encode enzymes linked to aerobic respiration, folate and amino acid metabolism and protection against stress (Table 2). Down regulation of these genes by FnrS during anaerobic growth reduces the expression of proteins that are not required

under these conditions, such as enzymes used for aerobic respiration (*dld*) or cytochrome assembly (*cydDC*), or redundant enzymes like the malate dehydrogenase (*mqo*). Mqo, which has the same properties as Mdh, seems to be unnecessary since the TCA cycle is blocked before malate formation during anaerobic growth. Moreover, FnrS repression of *sodB*, which encodes an abundant superoxide dismutase, can also be explained in that oxidative stress is limited under anaerobic conditions.

Several targets down regulated by FnrS encode proteins known or predicted to bind zinc. For example, the FolE and MetE proteins require zinc for their enzymatic activities (Auerbach *et al.*, 2000, Gonzalez *et al.*, 1996), and, based on sequence comparisons, YggG, AdhP and Dcp are inferred to bind zinc (Henrich *et al.*, 1993). It is also noteworthy that FnrS is always encoded adjacent to *zntB* (*ydaN* in *E. coli*), a zinc transporter in *Salmonella.* The ZntB protein is an efflux pump (Caldwell & Smith, 2003) and its expression is decreased in an *fnr* mutant strain (Salmon et al., 2003). Since the anaerobic growth of facultative anaerobes is absolutely dependent on ribonucleotide reductase III, which requires zinc to be active (Luttringer *et al.*, 2009), we propose that FnrS may also contribute to the optimization of zinc utilization under anaerobic conditions, analogous to how RyhB optimizes iron utilization under aerobic conditions.

Like many commensal and pathogenic microorganisms, *E. coli* thrives in the gastrointestinal tract of humans and animals. In this environment, oxygen is limited and the cell must produce energy from anaerobic respiration with alternative electron acceptors or by the fermentation of simple sugars. The cell responds to decreases in oxygen tension by modulating pathways for carbon and energy flow. Thus far, FNR was considered as the main regulator of these adaptations. We now described an FNR-regulated sRNA whose expression is induced by anaerobic conditions and regulates numerous genes. We propose that FnrS expands the FNR regulon since many of the FnrS-repressed genes were reported to show altered expression in an *fnr* mutant, yet did not have upstream FNR binding sites (Constantinidou et al., 2006). This layered regulation allows the cells to precisely adjust energy consumption during anaerobic growth.

Experimental procedures

Bacterial strains

The bacterial strains used in this study are listed in Table 2, and the oligonucleotides used to generate the strains are listed in Table S2. The mini-λ-Red recombination system was used to create the *arcA* and *fnr* deletion strains (Datsenko and Wanner, 2000,Yu *et al.*, 2000,Court *et al.*, 2003). In all cases, pKD13 (Datsenko and Wanner, 2000), which encodes kanamycin resistance, was used as a template in PCR reactions together with oligonucleotide primers containing 20 bases of pKD13 sequence and approximately 40 nucleotides of homology to the chromosomal region being replaced. Subsequently, the kanamycin cassette was removed using pCP20 (Cherepanov and Wackernagel, 1995). The Δ*fnrS* strain was constructed by replacing *fnrS* with a barcoded sequence as described in (Hobbs *et al.*, 2009). Each deletion mutation was moved into MG1655 by P1 transduction and was confirmed by PCR. The Δ*crp::cat* allele from CV600 (De Lay and Gottesman, 2009) was also moved into MG1655 by P1 transduction.

Translational fusions between the 5′ ends of the *maeA*, *gpmA*, *sodB*, *folE* and *folX* genes and *lacZ* were generated in PM1205 as previously described (Mandin and Gottesman, 2009). Briefly, the products from 5′ RACE-PCR were used to transform PM1205 to place each fusion under the arabinose-inducible P_{BAD} promoter. Directed mutagenesis on *maeA*, *gpmA* and *sodB* was carried out by PCR in using the parental strain as the template. PCR products were used to transform PM1205. The *lacZ* fusions were all confirmed by sequencing.

Plasmid construction

The plasmids used in this study are listed in Table 2, and the oligonucleotides used for cloning are listed in Table S2. Plasmid DNA was always isolated using the Qiagen Mini Plasmid Kit. Plasmid pAZ3 (Kawano *et al.*, 2007), a derivative of pBAD18 with an EcoRI site at +1, was used to overproduce FnrS from the P_{BAD} promoter. For cloning into pAZ3, the *fnrS* gene along with 20 bases pairs downstream of the 3′ end were amplified from MG1655 genomic DNA by PCR. The products were purified using a Qiagen PCR Purification Kit, digested with EcoRI and HindIII and cloned into the corresponding sites of pAZ3. Wild-type *fnrS* was cloned into pBR-lac (Guillier and Gottesman, 2006) using the same strategy except both the plasmid and PCR fragment were digested with AatII and EcoRI. The *fnrS* mutants were generated by PCR-directed mutagenesis using pBRlac-FnrS as a template. For each mutation, two oligonucleotides of approximately 40 bases complementary to each other and carrying the mutation in the middle of the oligonucleotide were used in PCR reaction with the primers used to clone the wild-type fragment. The fragments again were digested with AatII and EcoRI and cloned into pBR-lac. The sequences of all inserts were confirmed by sequencing.

Growth conditions

E. coli K-12 MG1655 was grown in Luria–Bertani (LB, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per l) at 37°C. When needed kanamycin (30 μg/ml) and chloramphenicol (25 μg/ml) were added. To overexpress FnrS from pAZ3-FnrS, arabinose was added at $OD_{600} \approx 0.4$ at a final concentration of 0.2%. Wild-type FnrS and the FnrS mutants were induced from the pBR-lac plasmid by the addition of 100 μM IPTG for 30 min. For anaerobic experiments, cells were first grown aerobically in M63 minimal medium (KD Medical) with 0.001% vitamin B1, and 0.2% glucose. At $OD_{600} \approx 0.4$, 5 ml of cells were collected by centrifugation at 4°C and then resuspended in M63 medium pre-incubated in anaerobic chamber (Coy Laboratory) and containing 0.001% vitamin B1, 10 μM ammonium molybdate, 0.2% glucose or 0.4% glycerol and 40 mM fumarate or 20 mM nitrate as indicated. When anaerobically-growing cells were shifted back to aerobic conditions, the cells again were collected by centrifugation and then resuspended in aerobic M63 glucose fumarate medium.

RNA extraction

For cultures grown in LB, total RNA was extracted by using a modified version of the hot phenol technique (Massé *et al.*, 2003). Briefly, 750 μl of cell culture were mixed with 102 μl of lysis solution (320 mM Na acetate at pH 4.6, 8% SDS, 16 mM EDTA). The lysed cells were then mixed with 500 μl of acid phenol (Ambion) at 65°C for 10 min. After centrifugation, the supernatant was extracted twice with acidic phenol and precipitated with 700 μl of 100% ethanol. For cultures grown in M63 medium, total RNA was extracted by using a modified version of the hot phenol technique as described in (Kawano *et al.*, 2002). Briefly, 5 ml of cells were collected in 50 ml Falcon tubes by centrifugation at 5000 *g* for 5 min at 4°C. Cells were then resuspended in 500 μl of solution A (0.5% SDS, 20 mM sodium acetate, 10 mM EDTA, pH 5.5) and mixed thoroughly. Thereafter the mixture was transferred to a 1.5 ml eppendorf tube containing 500 μl of acid phenol and incubated at 65°C for 10 min. After vortexing, cells were extracted exactly as described for the LB samples. In all cases, the resulting RNA pellets were resuspended in H_2O treated with diethyl pyrocarbonate (DEPC) and stored at −80°C. RNA concentrations were determined based on OD_{260}.

Northern analysis

For the detection of FnrS transcript, total RNA (5 μg) was separated on a denaturing 6% polyacrylamide-8 M urea gel and transferred to a Zeta-Probe Membrane (Bio-Rad) for 5 h at 55 V in 0.5X TBE. Oligonucleotide probes, specific for the FnrS RNA, were labelled with ^{32}P using T4 polynucleotide kinase (New England Biolab). Hybridization and wash steps

were as described previously (Opdyke et al., 2004). For the detection of the *sodB, maeA, gpmA, folE* and *folX* mRNAs, total RNA (5 μg) was separated on a 1X TBE-1% agarose gel, transferred to a Zeta-Probe Membrane (Bio-Rad) by a gravity blotting for 3 h in a 0.01 N NaOH, 5X SSC buffer. Membranes were hybridized and washed as described previously (Opdyke et al., 2004). The RNA century marker (Ambion) and RNA millennium marker (Ambion) are used with acrylamide and agarose northerns, respectively, and visualized on the membrane by U.V. (254 nm).

5′ RACE

5′ RACE analysis was carried out as described (Argaman *et al.*, 2001). The sequences of the oligonucleotides used to generate FnrS or the mRNA target cDNAs are given in Table S1. The amplified cDNA fragments were then cloned into vector pCRII Topo (Invitrogen) for FnrS or used to transform PM1205 (Mandin and Gottesman, 2009) to generate the target fusions. All 5′ ends were mapped by sequencing.

In vivo RNA structure probing

To probe the FnrS structure in vivo, a culture of MG1655 grown aerobically in M63 with glucose to $OD_{600} \approx 0.4$ was split, the cells were harvested and then resuspended in M63 medium containing glucose, fumarate, vitamin B1 and ammonium molybdate (pre-incubated in anaerobic chamber). One of the cultures was left untreated while the other was treated immediately with 50 mM dimethylsulfate (DMS). After 4 min, total RNA was extracted as described above, and primer extension reactions using end-labeled oligo FnrS-ext were carried out with AMV reverse transcriptase (Life Sciences). The extension products together with sequencing reactions primed with the same end-labeled FnrS-ext primer were separated on an 8% sequencing gel.

Microarray analysis

MG1655 cells harboring pAZ3 or pAZ3-FnrS were grown to $OD_{600} \approx 0.5$ in LB and were induced with arabinose at a final concentration of 0.2%. Cells were harvested after 15 min and total RNA was prepared as described previously (Kawano *et al.*, 2002). The preparation of the cDNA and hybridization to the Affymetrix *E. coli* Genome 2.0 array were performed as described in Affymetrix manual Section 3: Prokaryotic Sample and array Processing (www.affymetrix.com/support/downloads/manuals/expression_s3_manual.pdf).

β-galactosidase assays

All strains were grown until $OD_{600} \approx 0.3$ and induced with 0.2% arabinose. After 5 min of induction, 100 μM IPTG was added to induce expression from the pBR-lac plasmid, pBR-FnrS, pBR-FnrS-I, pBR-FnrS-II and pBR-FnrS-III. After 30 min of induction with IPTG, cells were lysed in 800 μl of Z-buffer with 5 μl of SDS 0.1% and 10 μl of chloroform. β-galactosidase assays were carried out as described by Miller (1972).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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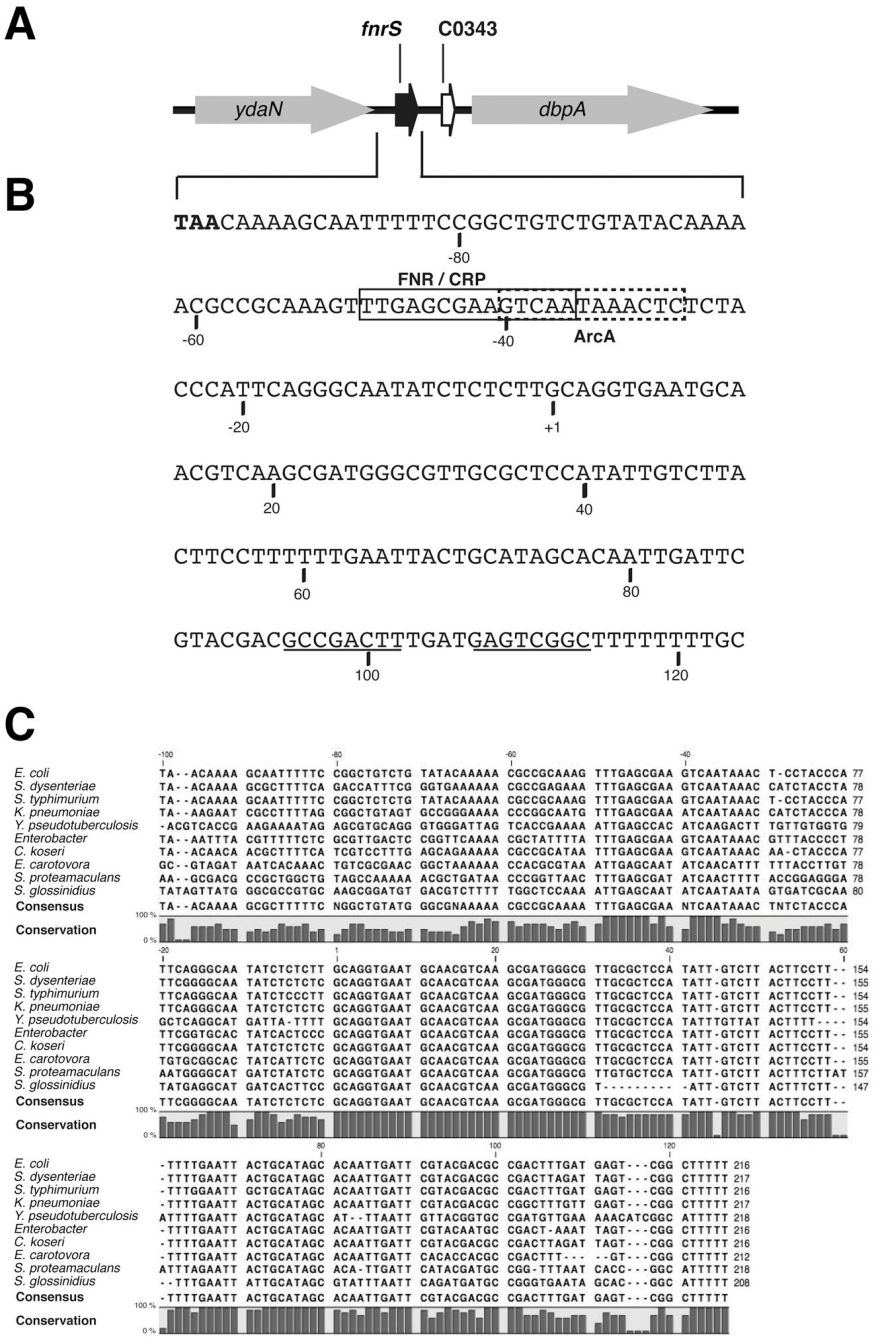

Fig. 1. FnrS RNA encoded in the *ydaN-dbpA* intergenic region

A. Map of *ydaN-dbpA* region.

B. Sequence of the first 217 nucleotides of the *ydaN-dbpA* intergenic region. +1 indicates the mapped start of the FnrS RNA, the stop codon of *ydaN* is in bold, the probable FnrS Rho-independent terminator is underlined, and putative binding sites for FNR/CRP and ArcA are boxed in continuous and dotted lines, respectively.

C. Alignment of the region encompassing FnrS created using the *CLC sequence viewer* (www.clcbio.com). Sequences used for this alignment are from *Escherichia coli K12, Shigella dysenteriae Sd197, Salmonella thyphimurium LT2, Klebsiella pneumoniae subsp. pneumoniae MGH 78578, Yersinia pseudotuberculosis PB1/+, Enterobacter sp. 368, Citrobacter koseri*

ATCC BAA-895, Erwinia carotovora subsp. atroseptica SCRI1043, Serratia proteamaculans 568, Sodalis glossinidius str. 'morsitans'.

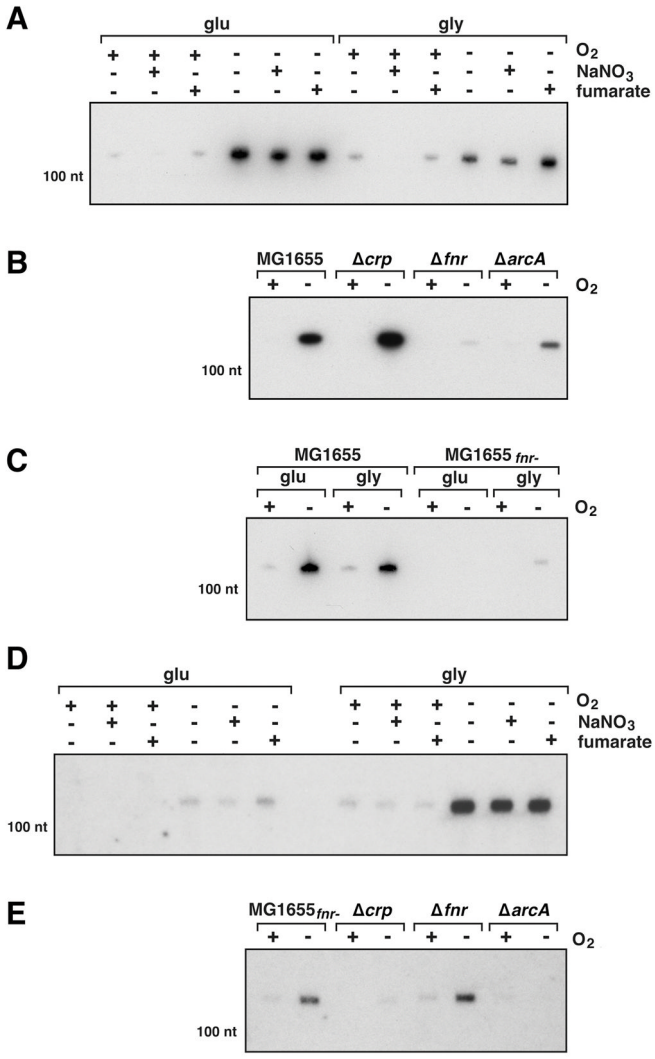

Fig. 2. FnrS expression under different growth conditions and in various mutant strains

A. MG1655 cells were grown in M63 with 0.2% glucose to $OD_{600} \approx 0.4$ under aerobic conditions, the culture was split into multiple aliquots, the cells were collected and resuspended in the indicated medium (M63 with 0.2% glucose or 0.4% glycerol and 20 mM nitrate or 40 mM fumarate) and incubated aerobically or anaerobically for 20 min.

B. MG1655, MG1655Δ*crp*, MG1655 Δ*fnr* and MG1655 Δ*arcA* were grown in M63 with 0.2% glucose to $OD_{600} \approx 0.4$ under aerobic conditions, the cultures were split into two aliquots, the cells were collected and resuspended in M63 with 0.4% glycerol and 40 mM fumarate and incubated aerobically or anaerobically for 20 min.

C. MG1655 and MG1655$_{fnr-}$ strain were grown in M63 with 0.2% glucose to $OD_{600} \approx 0.4$ under aerobic conditions, the cultures were split into four aliquots, cells were collected and resuspended in M63 with either 0.2% glucose or 0.4% glycerol and incubated aerobically or anaerobically for 20 min.

D. MG1655$_{fnr-}$ cells were grown and treated as for (A).

E. MG1655$_{fnr-}$, MG1655$_{fnr-}$ Δ*crp*, MG1655$_{fnr-}$ Δ*fnr* or MG1655$_{fnr-}$ Δ*arcA* were grown and treated as for (B). For all samples, total RNA (5 μg) was separated on an acrylamide gels, transferred to nitrocellulose and probed with a ^{32}P-labelled oligonucleotide specific to FnrS. For all panels the position of the band corresponding to a 100-nucleotide marker RNA is

indicated on the left. The Northern blots in (A), (B) and (C) were exposed overnight, while the Northern blots in (D) and (E) were exposed for one week.

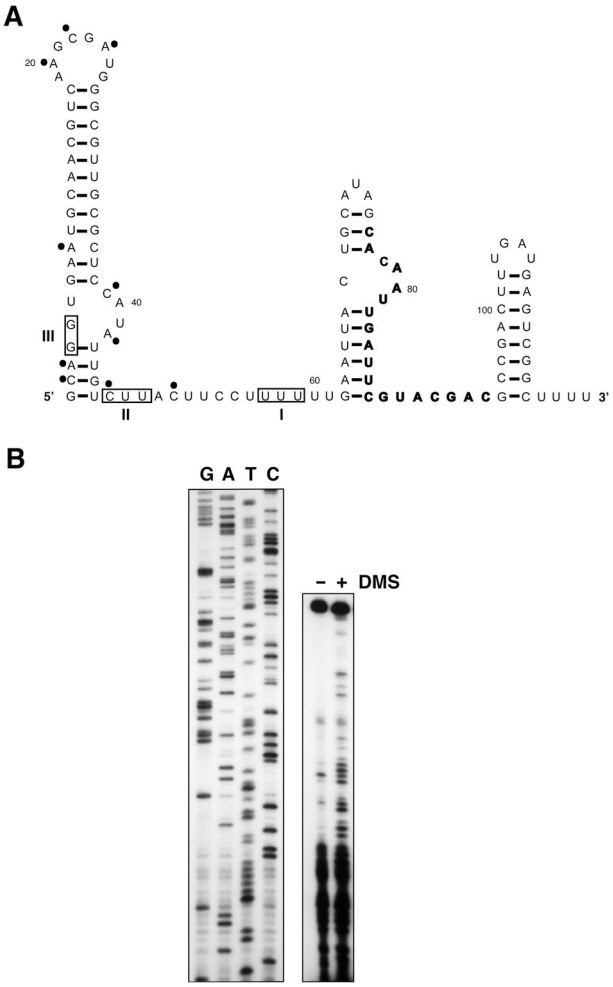

Fig. 3. FnrS structure

A. FnrS structure predicted by Mfold and supported by dimethylsulfate (DMS) modification data. The sequence complementary to the oligonucleotide used in the reverse transcription reaction is in bold. Dots indicate residues that reacted with DMS and boxes denote residues modified in mutants I, II and III (see Fig. 5A).

B. *In vivo* probing of the FnrS RNA structure. Cells were grown anaerobically in M63 with 0.2% glucose and 40 mM fumarate and half of the culture was treated with dimethylsulfate for four min. Total RNA extracted from these cultures was analyzed by primer extension reactions.

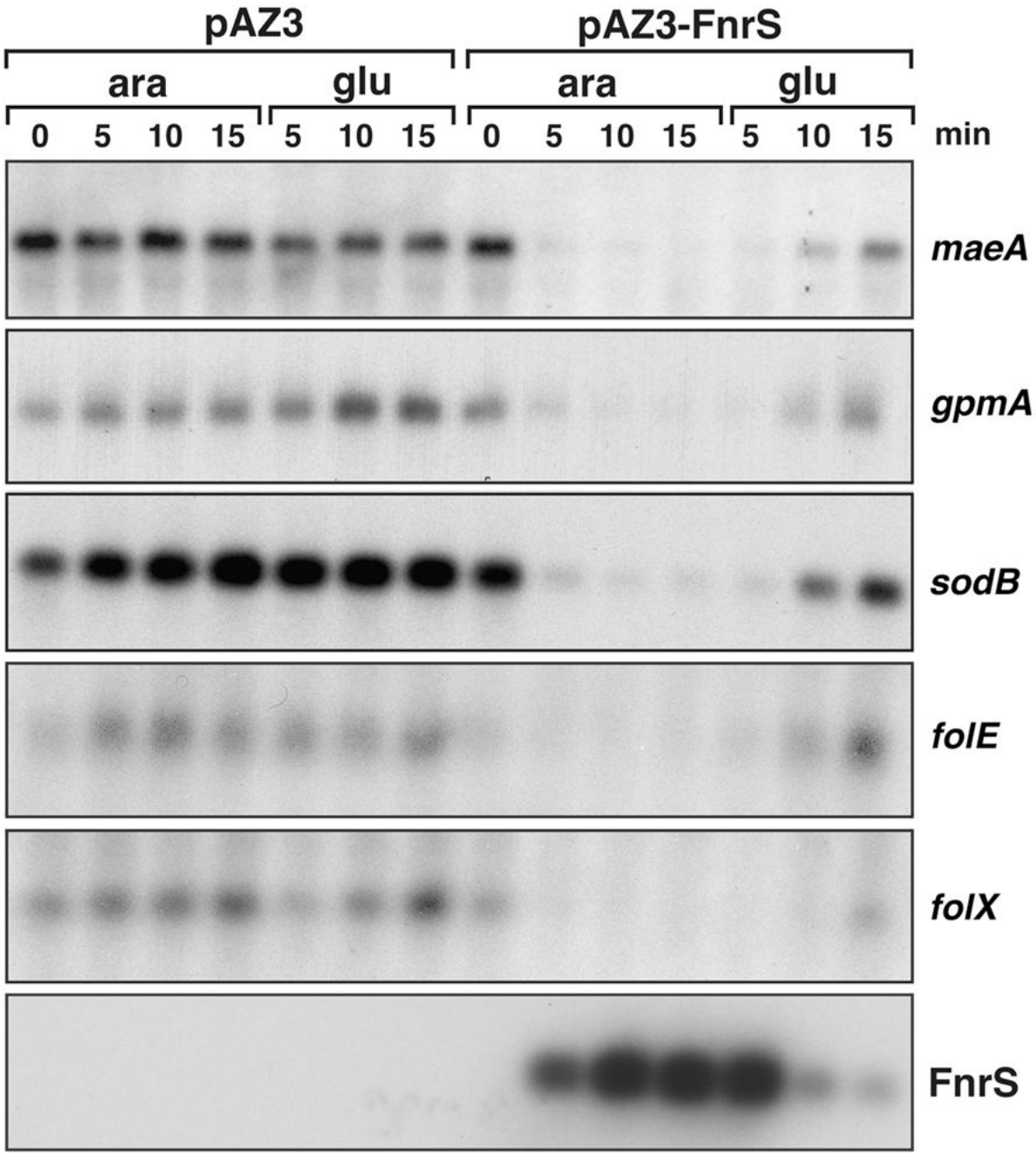

Fig. 4. FnrS repression of *maeA*, *gpmA*, *sodB*, *folE* and *folX*

Cultures of MG1655 carrying pAZ3 or pAZ3-FnrS were grown in LB to $OD_{600} \approx 0.4$ and treated with 0.2% arabinose. After 15 min, cells were washed two times in LB + 0.2% glucose and grown an additional 15 min. The time of incubation (min) with arabinose (ara) and glucose (glu) before RNA extraction are indicated on top. For all samples, total RNA (5 μg) was separated on an agarose gels, transferred to nitrocellulose and probed with a ^{32}P-labelled oligonucleotides specific the genes indicated on the right.

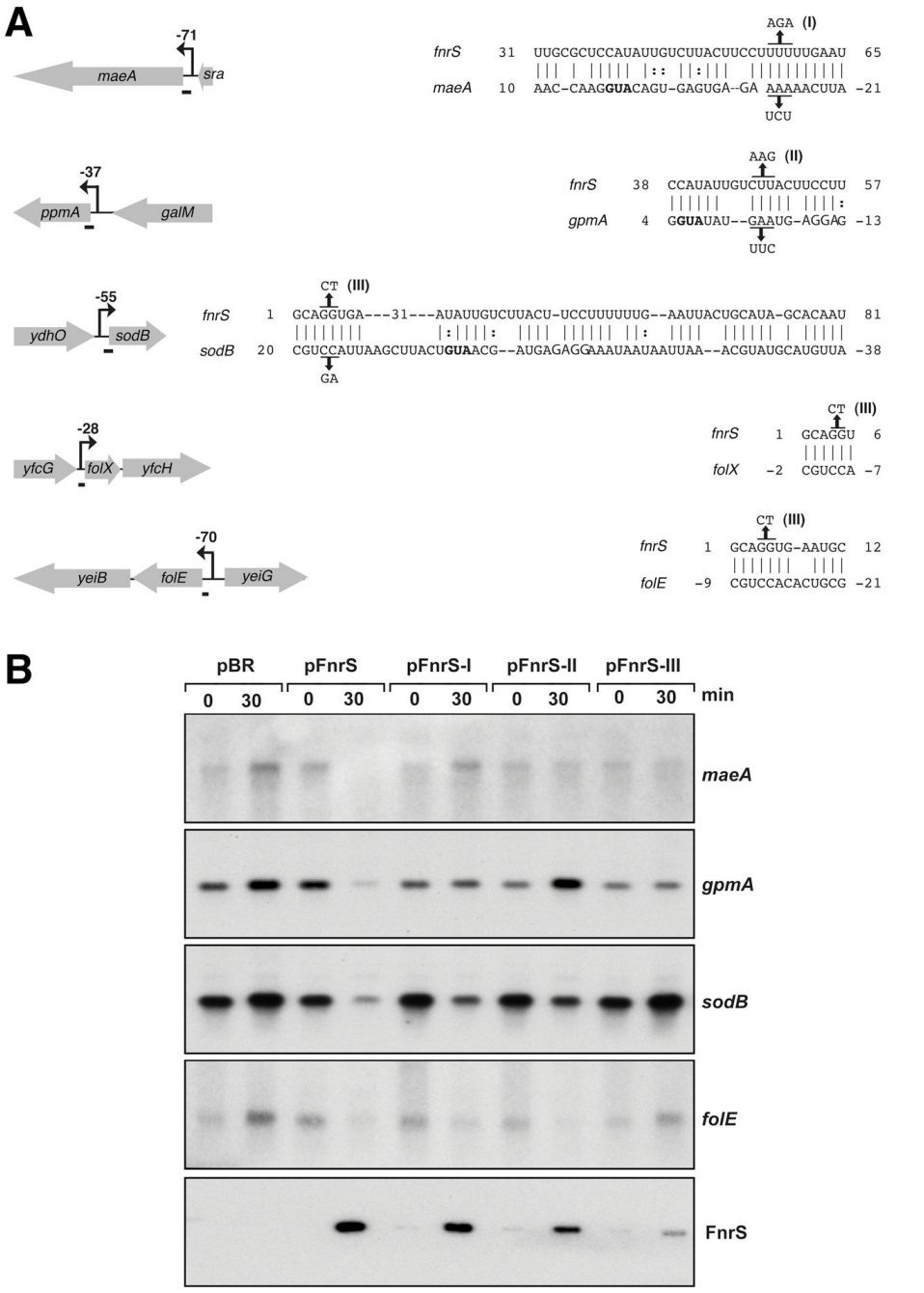

Fig. 5. Base pairing between FnrS and target mRNAs

A. Predicted base pairing interactions. Black arrows indicate the promoters mapped by 5′ RACE PCR, and the numbers correspond to the number of nucleotides between the transcriptional and the translational start sites. The regions of base pairing between FnrS and its targets as predicted by the *TargetRNA* program are symbolized by short bars on the left and are given on the right. FnrS mutations I, II and III are also indicated. The ribosome binding sites are italicized and the start codons are in bold. The sequences of the compensatory mutations are also given.

B. Repression of *maeA*, *gpmA*, *sodB*, *folE* expression by FnrS and FnrS mutants. Total RNA was extracted from MG1655 before and 30 min after the induction of FnrS (from pBR-FnrS)

or FnrS mutant I, II or III (pBR-FnrS-I, II or III) with 100 μM IPTG. Genes probed are indicated on the left. The last panel shows the levels of the wild-type and mutant FnrS transcripts. The Northern blots were carried out as in Fig. 2 and 4.

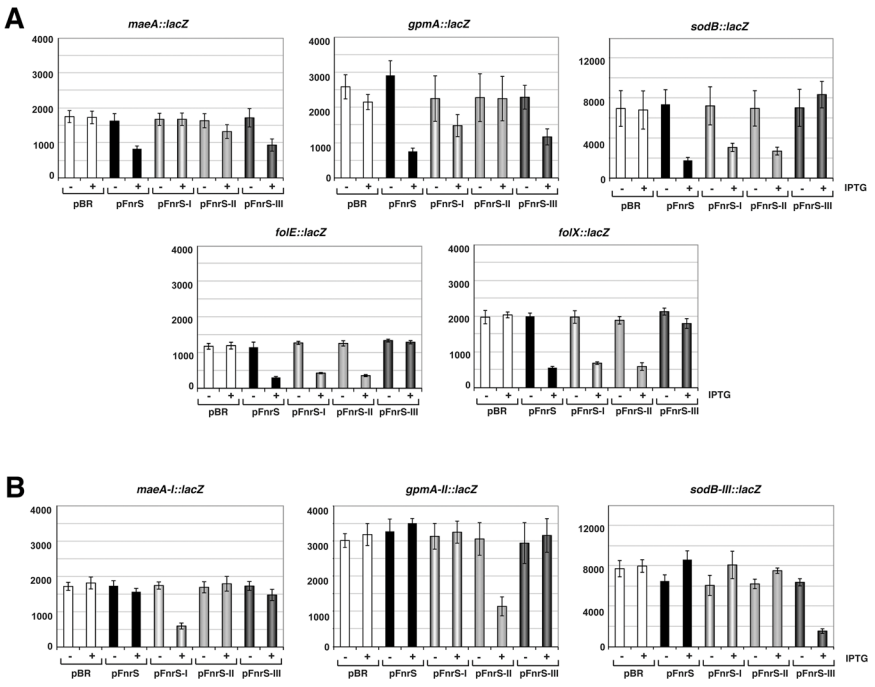

Fig. 6. Mutational analysis of FnrS base pairing

A. β-galactosidase assays of FnrS target mRNA-*lacZ* fusions in presence of pBR (empty plasmid), pBR-FnrS, pBR-FnrS-I, pBR-FnrS-II and pBR-FnrS-III.

B. β-galactosidase assays of FnrS target mRNA-*lacZ* fusions carrying complementary mutations. For both (A) and (B), expression of the *lacZ* fusions was pre-induced for 5 min by the addition of 0.2% arabinose, after which cells were treated with 100 μM IPTG to induce the P_{lac} promoter on the pBR plasmids. The levels of β-galactosidase activity were assayed 30 min later. The averages for the activity in Miller units determined in three independent experiments are shown together with the standard deviation.

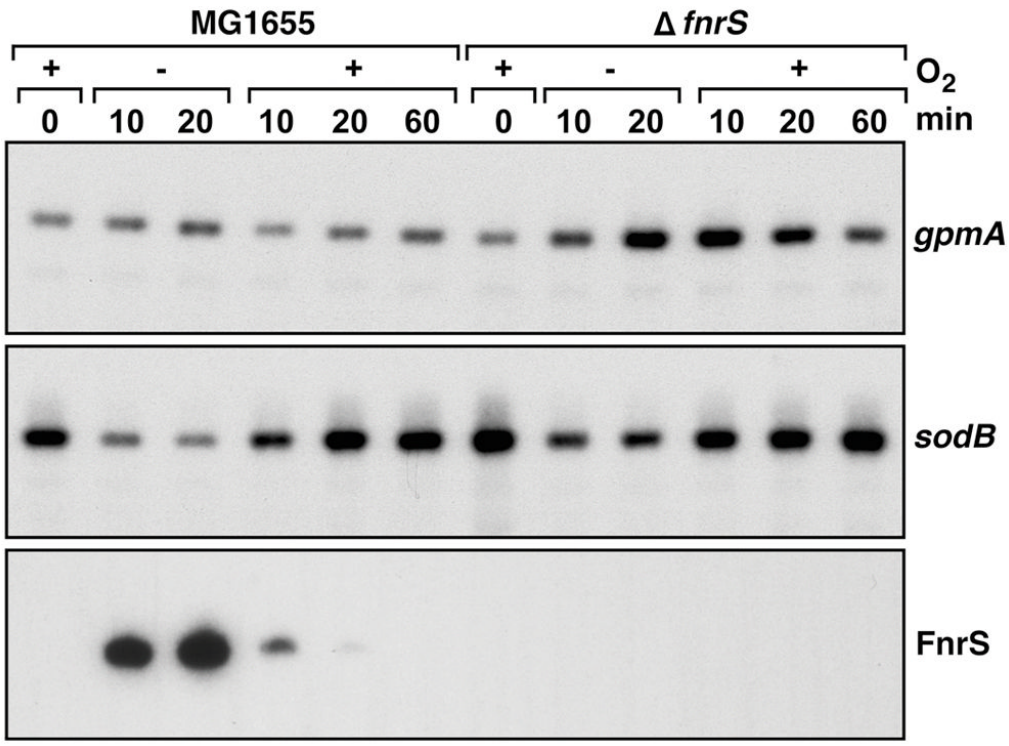

Fig. 7. Effect of RydD on *gpmA* and *sodB* expression upon shifts between aerobic and anaerobic conditions

MG1655 and MG1655 Δ*fnrS* strains were grown in M63 with 0.2% glucose and 40 mM fumarate to $OD_{600} \approx 0.4$ under aerobic conditions. Cells were collected and resuspended in the same medium and grown anaerobically. After 20 min, cells were again harvested, resuspended in the same medium and incubated aerobically for 60 min. Total RNA was extracted at several times (min) during this cycle. Northern blots were probed as in Fig. 2 and 4 for the genes indicated on the right.

Table 1

Genes repressed by FnrS overexpression.

gene/ORF	Description[a]	Ratio[b]	FnrS base-pairing core[c]	FNR regulon[d]	FNR site[e]
	Cytochrome				
cydD	ATP-binding component of cytochrome-related transport, Zn sensitive; NarL and ArcA regulated	3.8	SS	N	
cydC	ATP-binding component of cytochrome-related transport; NarL and ArcA regulated	6.3		N	
yceI	hypothetical protein, homology with cytochrome b561 of *Caulobacter*	4.5	SS	Y	
	Central intermediary metabolism/energy metabolism				
sfcA/maeA	NAD-linked malate dehydrogenase (malic enzyme)	7.3	SS	N	
mqo	malate:quinone oxidoreductase	4.3	SS	Y	11.5
adhP[f]	alcohol dehydrogenase, propanol-preferring	3.7	SS	Y	
gpmA	phosphoglycerolmutase 1; Fur regulated	3.4	SS	Y	
dld	D-lactate dehydrogenase, FAD protein, NADH independent	2.5		Y	
nfsA	modulator of drug activity; SoxS regulated	2.3	SS	N	
	Folate biosynthesis				
folX	D-erythro-7,8-dihydroneopterin tri-phosphate epimerase	3.7	5′	Y	
folE[f]	GTP cyclohydrolase I	2.8	5′	Y	
	Amino acid biosynthesis				
metE[f]	tetrahydropteroyltriglutamate methyltransferase; MetR, MetJ regulated	2.4	SS/5′		
tyrB	tyrosine aminotransferase, tyrosine repressible; TyrR regulated	2.3		Y	
	Metalloprotease				
dcp[f]	dipeptidyl carboxypeptidase II	3.6		N	
yggG[f]	hypothetical protein, Putative metalloprotease lipoprotein	2.6		N	
ybjC	hypothetical protein, predicted metal-dependent membrane protease; SoxS regulated	2.5		N	
	Stress resistance proteins				

gene/ORF	Description[a]	Ratio[b]	FnrS base-pairing core[c]	FNR regulon[d]	FNR site[e]
sodB	superoxide dismutase; Fur, CRP, HNS and IHF regulated	4.8	5′	Y	
ydhD/grxD	glutaredoxin 4	3.4	SS	N	
marA	multiple antibiotic resistance, transcriptional activator of defense system; CRP and Fis regulated	2.1	SS/5′	N	
	Transporters/outer membrane proteins				
ygiW	hypothetical outer membrane protein	5.2		Y	
yobA	hypothetical protein, homolog to copper resistance protein, putative cation transporter	4.2	SS	Y	−26.5
yebZ	putative resistance protein, copper ion homeostasis	4.3		Y	−26.5
yebY	hypothetical protein	3.8		Y	−26.5
bhsA/ycfR	hypothetical protein involved in stress resistance and biofilm formation, putative outer membrane	3.8	5′	N	
chaA	calcium protein antiporter	3.1		N	
	Others				
azuC	28 aa ORF; CRP regulated	4.4		N	
yncE	putative receptor, possible ATP-binding protein	3.9		N	
yfcL	hypothetical protein	2.7		N	
eco	serine protease inhibitor convergent to *mqo*	2.5		N	
yoaB	hypothetical protein, putative translation initiation inhibitor	2.4		N	
ycaO	hypothetical protein	2.3		N	

[a] Categories of gene function based on http://www.ecocyc.org/.

[b] Average ratio of signal for pAZ3 control:pAZ3-FnrS for three experiments.

[c] Region of FnrS predicted to base pair with target mRNA by *TargetRNA* (SS = single strand region between the first and second stem loops, 5′ = 5′ end).

[d] Genes suggested to part of FNR regulon based on microarray data from (Constantinidou et al., 2006) (Y = yes, N = No).

[e] Position of known or predicted FNR binding sites relative to transcriptional start sites based on (Constantinidou et al., 2006).

[f] Targets known or predicted to bind zinc.

Table 2

Strains and plasmids used in this study

Strains	Relevant features	References
MG1655	MG1655 *mal::lacIq* (NM525)	Laboratory stock
CV600	MG1655 lacX74 *crp::cat*	De Lay and Gottesman, 2009
GSO388	MG1655 Δ*crp::cat*	This study
GSO389	MG1655 Δ*fnr::kan*	This study
GSO390	MG1655 Δ*arcA::kan*	This study
$MG1655_{fnr-}$	MG1655 with duplication of amino acids 22 to 27 and mutations R10G and S13F in *fnr* gene	Laboratory stock
GSO391	$MG1655_{fnr-}$ Δ*crp::cat*	This study
GSO392	$MG1655_{fnr-}$ Δ*fnr::kan*	This study
GSO393	$MG1655_{fnr-}$ Δ*arcA::kan*	This study
PM1205	*lacI::PBAD-cat-sacB-lacZ*, mini lambda tet^R	Mandin *et al.*, 2009
GSO394	PM1205 *lacI′::PBAD-gpmA–lacZ*	This study
GSO395	PM1205 *lacI′::PBAD-sodB–lacZ*	This study
GSO396	PM1205 *lacI′::PBAD-maeA–lacZ*	This study
GSO397	PM1205 *lacI′::PBAD-folE–lacZ*	This study
GSO398	PM1205 *lacI′::PBAD-folX–lacZ*	This study
GSO399	PM1205 *lacI′::PBAD-maeA-I–lacZ*	This study
GSO400	PM1205 *lacI′::PBAD-gpmAII–lacZ*	This study
GSO401	PM1205 l*acI′::PBAD-sodBIII–lacZ*	This study
GSO402	MG1655 Δ*fnrS::kan*	This study
Plasmids		
pAZ3	pBAD promoter based expression, kan^R	Kawano *et al.*, 2007
pAZ3-FnrS	EcoRI-HindIII FnrS containing fragment cloned into pBR-Plac	This study
pBR-lac	Plac promoter based expression vector, amp^R	Guillier *et al.*, 2006
pBRlac-FnrS	AatII-EcoRI FnrS containing fragment cloned into pBR-Plac	This study
pBRlac-FnrSI	U57A U58G U59A site-directed mutation in pBRlac-FnrS	This study
pBRlac-FnrSII	C47A U48A U49G site-directed mutation in pBRlac-FnrS	This study
pBRlac-FnrSIII	G4C G5T site-directed mutation in pBRlac-FnrS	This study