Microbial Pathogenesis 46 (2009) 166–170

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Transcript analysis of *nrrF*, a Fur repressed sRNA of *Neisseria gonorrhoeae*

Thomas F. Ducey [a,b,*,1], Lydgia Jackson [a,b], Joshua Orvis [a,b], David W. Dyer [a,b]

[a] Laboratory for Genomics and Bioinformatics, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA
[b] Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104, USA

ARTICLE INFO

Article history:
Received 18 September 2008
Received in revised form
12 December 2008
Accepted 30 December 2008
Available online 6 January 2009

Keywords:
Small RNA
nrrF
Fluorescent primer extension

ABSTRACT

Like most microorganisms, *Neisseria gonorrhoeae* alters gene expression in response to iron availability. The ferric uptake regulator Fur has been shown to be involved in controlling this response, but the extent of this involvement remains unknown. It is known that in addition to working directly to repress gene expression, Fur may also work indirectly by controlling additional regulatory elements. Using *in silico* analysis, we identified a putative small RNA (sRNA) homolog of the meningococcal *nrrF* locus, and demonstrate that this sRNA is iron-repressible, suggesting that this is the gonococcal analog of the *rhyB* locus in *Escherichia coli*. Quantitative real-time RT-PCR analysis indicates that this transcript may also be temporally regulated. Transcript analysis identified the 5′ start of the transcript, using a single reaction, fluorescent-based, primer extension assay. This protocol allows for the rapid identification of transcriptional start sites of RNA transcripts, and could be used for high-throughput transcript mapping.
Published by Elsevier Ltd.

1. Introduction

For almost all organisms, iron (Fe) is an element essential for life, playing a prominent role in a variety of metabolic pathways. Iron serves as a cofactor in a number of proteins, such as catalases, cytochromes, peroxidases, the oxygen transporting proteins hemoglobin and myoglobin, and ribonucleotide reductase [1]. Paradoxically, despite its importance, iron is a difficult nutrient to manage, and given the biological conditions (aqueous, neutral pH, oxygenated), ferrous iron (Fe^{2+}) quickly oxidizes to the ferric state (Fe^{3+}) [2]. Ferric ions are extremely insoluble (Ksp ≈ 10–37 M) and toxic, being responsible for driving the Fenton reaction that results in free radical production, which in turn causes lipid peroxidation, DNA damage and protein degradation [3]. To counter this toxicity, and keep iron soluble, mammals sequester iron in a number of proteins intracellularly (hemoproteins, ferritin) and extracellularly (lactoferrin, transferrin) [1]. In addition to managing Fe successfully for the host, this sequestration results in a non-specific suppression of microbial growth, known as "nutritional immunity" as free Fe concentrations (approximately 10^{-18} M) are far below that necessary to support microbial growth, which typically requires Fe concentrations of 10^{-6} M or greater [4].

To counter this nutritional immunity, pathogens such as *Neisseria gonorrhoeae* have evolved specific strategies to acquire iron in the host. This obligate human pathogen expresses several receptors capable of binding and removing Fe from human hemoglobin (HmbR), hemoglobin–haptoglobin complexes (HpuAB), lactoferrin (LbpAB) and transferrin (TbpAB) [5]. The gonococcus also can use exogenously-produced enterobactin (a siderophore) via the FetA receptor; presumably, this is important for allowing the organism to metabolize catecholate siderophores found in mixed microbial environments such as the female urogenital tract [6]. Expression of these receptors is Fe-repressible, as the genes encoding these proteins are transcriptionally regulated by the ferric uptake regulator (Fur). Fur, with Fe as a cofactor, dimerizes and binds to specific DNA regulatory elements known as Fur boxes [7–9]. Fur boxes have been predicted in the promoters of *fetA*, *hmbR*, *fbpABC*, *hpuAB*, *lbpAB* and *tbpAB* [10]. There also are accounts of Fur serving as an activator [11,12], and other regulators within the Fur superfamily are involved in manganese, nickel, and zinc homeostasis [13]. We have reported that the gonococcal iron regulon is controlled by what appears to be a regulatory cascade, with Fur serving as a primary regulator [14]. In *Escherichia coli*, RyhB, a small RNA (sRNA) has been shown to repress a number of genes by binding to their mRNA in the presence of the RNA chaperone Hfq [15]. These sRNA:mRNA hybrids are then quickly targeted for RNase degradation [16]. In *E. coli*, *rhyB* is repressed under iron-replete conditions [15]. Small RNAs behaving in a similar fashion have been identified in *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Neisseria meningitidis* [17]. Here we report the transcript analysis of the Fe associated sRNA

* Corresponding author. Coastal Plains Soil, Water, and Plant Research Center, ARS-USDA, 2611 West Lucas Street, Florence, SC 29501, USA. Tel.: +1 843 669 5203; fax: +1 843 669 6970.
E-mail address: Thomas.Ducey@ars.usda.gov (T.F. Ducey).
[1] Current address: Coastal Plains Soil, Water, and Plant Research Center, ARS-USDA, Florence, SC 29501, USA.

0882-4010/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.micpath.2008.12.003

nrrF, originally identified in *N. meningitidis* [17]. We identified the gene for this sRNA using a bioinformatics approach similar to Wilderman et al. [18], and we demonstrate *nrrF* repression by Fe. We hypothesize that NrrF participates in the gonococcal Fe response similarly to the *hfq* and *rhyB* loci in *E. coli*. Quantitative real-time RT-PCR data suggest that NrrF also is temporally regulated. We also report the fluorescent-based primer extension mapping of the 5′ end of *nrrF* and the determination of its transcriptional start.

2. Results

2.1. In silico *identification of a Fur-regulated small RNA in N. gonorrhoeae*

Based on the analysis of a number of sRNAs of *E. coli* [19,20], in particular the Fur-regulated sRNA *rhyB* [15], Wilderman et al. developed an approach to identify Fur-regulated sRNAs in *P. aeruginosa* [18]. This approach resulted in the identification of two Fur-regulated sRNAs in *P. aeruginosa*, *prrF1* and *prrF2* [18]. We adopted a similar bioinformatics approach to identify Fur-regulated sRNA genes in *N. gonorrhoeae*. This *in silico* screening resulted in the identification of one region containing the aforementioned characteristics. This region has a predicted intergenic Fur box and a Rho-independent terminator sequence, separated by a gap of no more than 200 bp, and contains a string of at least three terminal T nucleotides (U in RNA). This region was clustered ≈200 bp downstream of NGO2002 and ≈300 bp upstream of NGO2004 (Fig. 1A). Further analysis of this region identified a strong σ^{70} promoter, with placement of the putative Fur box in the classical position between the −35 and −10 promoter regions (Fig. 1B). From promoter to poly-T tail, the region spans 195 bp, from complemented nucleotides 1,975,968 to 1,976,163 of the annotated FA1090 gonococcal genome. In addition, mFOLD prediction of this RNA species (Fig. 1C) suggests a secondary structure similar to *ryhB* of *E. coli* [15]. The absence of start codons and the presence of multiple stop codons throughout the reading frames of this region indicate that this gene does not encode a protein product. This region likewise shares 94% identity with *nrrF*, a previously identified Fur-regulated sRNA in *N. meningitidis* [17].

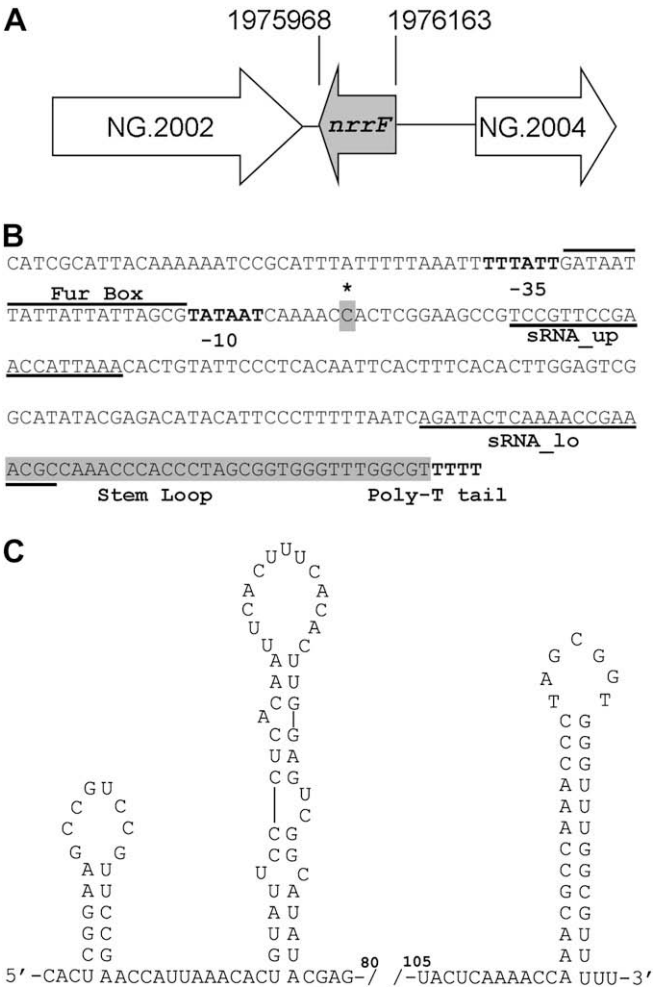

Fig. 1. *N. gonorrhoeae nrrF*. (A) Map of *nrrF*. (B) Sequence of *nrrF* and promoter. The −35 and −10 regions of the promoter are marked. Predicted Fur box is indicated by the overhead line. Transcript start as determined by FPE (see Fig. 2) is identified with an overhead asterisk (*) and shading, stemloop is indicated by shading. qRT-PCR primer binding sites are underlined and labeled. (C) mFOLD prediction of *nrrF*.

2.2. 5′ Transcript mapping of nrrF

To confirm the location of the putative promoter, we used a single reaction, the fluorescent primer extension (FPE) reaction. FPE of cDNA generated from the *nrrF* transcript mapped the 5′ end to complemented base pair 1,976,123, a cytosine, of the gonococcal genome; seven bases from the end of the predicted −10 site of the sRNA promoter (Figs. 1B and 2). Comparison of FPE of cDNA generated from RNA harvested from iron-replete conditions (Fig. 2A) resulted in identically sized FPE of cDNA generated from RNA harvested from iron-deplete conditions (Fig. 2B), but at a lower fluorescent intensity (3.5-fold reduction). These results are consistent with the iron-repressible nature of this transcript (see below).

2.3. Iron-regulated expression of nrrF

The presence of a predicted Fur box in the promoter of *nrrF* suggested that this transcript would be repressed under iron-replete conditions. Fur titration assays (FurTA) performed using *E. coli* expressing gonococcal Fur, confirm that this Fur box is capable of binding the gonococcal Fur protein [21]. In these analyses, a 897 bp fragment corresponding to complemented bases 1,975,608–1,976,505 of the gonococcal genome was found to titrate Fur [21]. This region includes the entirety of *nrrF* and its sole predicted Fur box [21]. RT-PCR analysis of *nrrF* transcripts isolated from gonococcal cells grown to mid-log under iron-deplete and iron-replete conditions confirmed the iron-repressible nature of this transcript. Appreciable differences in expression levels were seen, with lower levels of the *nrrF* transcript being seen under iron-replete conditions, with *porI* transcript, previously identified as a constitutively expressed gene [14], showing no appreciable differences (Fig. 3A). Expression levels of *nrrF* were accurately quantitated by qRT-PCR, through the course of a grown curve (Fig. 3B). NrrF levels increased approximately 170-fold after 3 h of iron starvation, but subsided at 4 h, suggesting that *nrrF* may be regulated by temporal signals in addition to iron availability.

3. Discussion

The identification of the gonococcal *nrrF* expands to six the number of regulatory elements we hypothesize to be involved in the gonococcal response to iron availability. In addition to Fur, our previous study identified two iron repressed AraC-like regulators (NGO0025 and NGO2115), one putative phage-like repressor (NGO1013), and a MerR-like regulator (NG0602) [14]. Additional work by Jackson et al. has identified an additional eight regulators

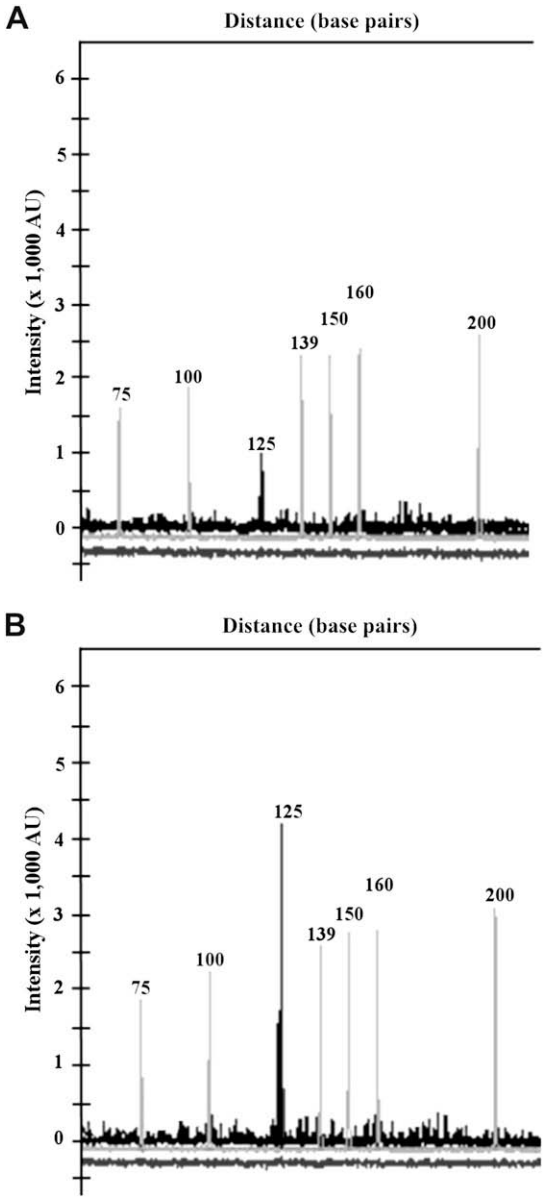

Fig. 2. *nrrF* primer extension. Fragment analysis of fluorescent primer extension. The 125 bp peak is 6-FAM fluorescein labeled primer extension products of cDNA generated from RNA harvested from *N. gonorrhoeae* grown in iron-replete (A) or iron-deplete (B) conditions. Other labeled peaks correspond to GeneScan 500-LIZ ladder.

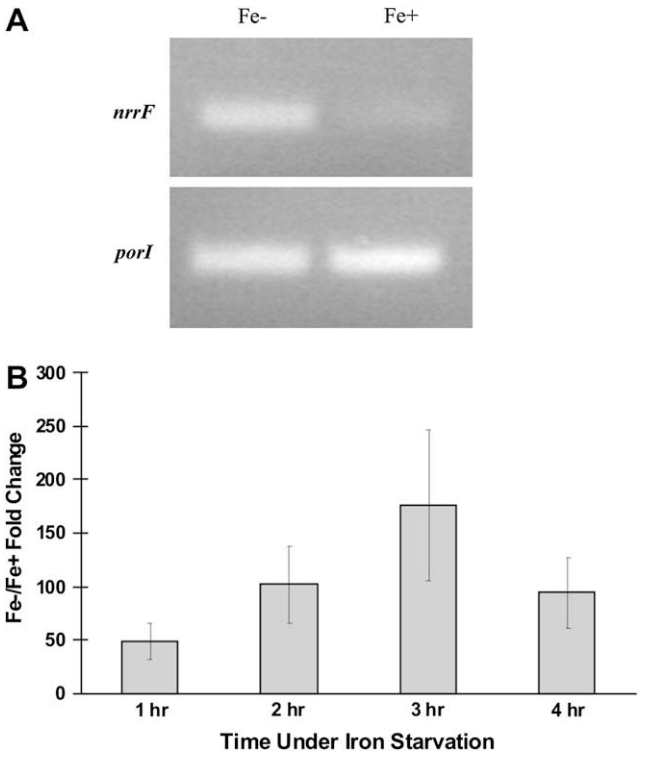

Fig. 3. Expression of *nrrF* under iron-deplete and iron-replete conditions. (A) rtPCR analysis of *nrrF* and *porI* transcript levels. (B) qRT-PCR analysis of *nrrF* transcript levels. Each column corresponds to the mean and SD of three independent assays.

that appear to be members of the Fur regulon, bringing the total to 14 distinct regulatory genes involved in the gonococcal response to iron availability [21]. In addition, microarray data suggest that the MerR-like regulator may function independently in the gonococcal iron response regulon; this situation may be analogous to the multiple Fur homologs found in *Staphylococcus aureus* and *Bacillus subtilis* [22–24]. The addition of NrrF to this cascade may further explain some of the gonococcal responses to iron availability which cannot be directly attributed to Fur. Further work is needed to extensively map the regulon boundaries of each of these gonococcal regulators.

Like *ryhB* of *E. coli*, the transcription of *nrrF* is iron-repressible, and has a similar secondary structure as predicted by mFOLD. RyhB has been shown to be involved in the regulation of two genes in the tricarboxylic acid cycle, two ferritin genes and *sodB* [15]. In the case of *sodB*, RyhB functions by coordinating with Hfq, an RNA chaperone, to target message for RNase E degradation in low iron conditions [25]. When insertionally inactivated, a similar pair of sRNAs in *P. aeruginosa*, PrrF1 and PrrF2, results in an increase in the expression of several genes involved in iron storage, defense against oxidative stress and intermediary metabolism [18]. The gonococcus has homologs for both Hfq (NGO0326) and RNase E (NGO1785), suggesting that in conjunction with these loci, NrrF may enhance degradation of mRNA encoding for iron containing proteins during times of iron depletion.

We also have utilized a single reaction, fluorescent primer extension protocol that is ideally suited to capillary electrophoresis systems such as the ABI 3730*xl*. This protocol is an improvement in cost and labor intensiveness over standard primer extension assays which rely on multiple reactions per promoter and the incorporation of radionucleotides [26], and could be adapted easily as a high-throughput strategy for genome-wide identification of transcriptional start sites. Such data would be an extremely valuable adjunct for a variety of purposes, including genome annotation.

4. Materials and methods

4.1. Strains, plasmids, and growth conditions

N. gonorrhoeae FA1090 was stored as −80 °C freezer stocks GC (Difco) media supplemented with 20% glycerol. *N. gonorrhoeae* was routinely plated on GCB agar (Becton Dickinson) supplemented with 2% IsoVitaleX (Becton Dickinson). Incubation of organisms on solid media was carried out at 37 °C in a 5% CO_2 atmosphere. For iron-regulation analyses, broth cultures of *N. gonorrhoeae* FA1090 were grown at 37 °C, in the Chelex-treated (Bio-Rad), chemically defined medium (CDM) of Morse and Bartenstein [27] as modified by Dyer et al. [28]. CDM was either supplemented with 100 μM $FeNO_3$ (CDM-100), 10 μM $FeNO_3$ (CDM-10) or not (CDM-0).

4.2. Bioinformatics

A perl script was written to examine the potential presence of sRNA in *N. gonorrhoeae* FA1090 using criteria established by Wilderman et al. [18]. This script searched intergenic space for stem-loop structures containing 7–12 complementary bases, with a loop of 5–7 bases, followed by at least three T nucleotides (U in RNA) [18]. mFOLD was used to examine the folding of the putative sRNA [29]. The sRNA region was examined for a σ^{70} promoter by utilizing the sequence alignment kernel (SAK) [30].

4.3. RNA extraction, reverse transcriptase PCR (rtPCR) and quantitative real-time PCR (qRT-PCR)

RNA extraction from *N. gonorrhoeae* FA1090 was performed as previously described [14]. Briefly, an aliquot of bacterial culture was immediately added to an ice-cold phenol/ethanol (EtOH) (95:5) mixture at a 1:10 (vol/vol) ratio. Cells were pelleted by centrifugation at 5000 × *g* for 10 min, resuspended in TE (10 mM Tris–HCl, 1 mM EDTA) pH 8.0, lysed by the addition of a 1/10th volume of 10% sodium dodecyl sulfate (SDS), and incubated at 64 °C for 2 min. A 1/10th volume of 1 M sodium acetate, pH 5.2, was added and RNA was extracted by the addition of an equal volume of prewarmed water-saturated phenol followed by extraction with an equal volume of chloroform. Bulk RNA was then precipitated using a standard EtOH precipitation. Prior to rtPCR and qRT-PCR, 1 μg or 2 μg of RNA respectively, was treated with 5 U of RNase-free DNase according to manufacturer specifications (Invitrogen). For rtPCR, after heat inactivation at 75 °C for 15 min, the DNase-treated RNA was used directly in a reverse transcription reaction at 42 °C for 60 min using 200 U Superscript III reverse transcriptase, 40 U RNase inhibitor, and 1 ng of gene specific primer in a 20 μl volume according to manufacturer specifications (Invitrogen). For rtPCR, a 1/10th volume of the reverse transcription reaction was used in a standard Taq PCR reaction (Roche) with 100 ng of both forward and reverse primers. Complete removal of contaminating DNA from the RNA preparations was ensured by performing parallel reverse transcription reactions *sans* Superscript III. DNA products were analyzed by 2% agarose gel electrophoresis with Tris-acetate-EDTA (TAE) buffer. For qRT-PCR reactions, the DNase-treated RNA was utilized as previously described [14]. RNA was isolated from FA1090 grown in iron-deplete and iron-replete conditions as previously described [14], at 1, 2, 3 and 4 h time points. First strand cDNA synthesis was generated from 2 μg total RNA with random hexamers according to the TaqMan® Reverse Transcription Reagents Kit (PE Applied Biosystems). A negative reverse transcription reaction (minus the reverse transcriptase) was included for each sample to detect the presence of genomic DNA. Primers are listed in Table 1. Real-time PCR amplification was performed on the ABI PRISM™ 7500 Sequence Detection System (PE Applied Biosystems) using SYBR® Green PCR Master Mix (PE Applied Biosystems). Relative gene expression was quantified using the comparative threshold cycle $2^{-\Delta\Delta CT}$ method (PE Applied Biosystems User Bulletin #2) with *porI* (a constitutively expressed major outer membrane porin) as the endogenous reference. Validation experiments for all experimental primer pairs with the endogenous control primers were done to ensure that the efficiency of the target amplification and the efficiency of the endogenous control were equal (PE Applied Biosystems User Bulletin #2). Each gene was assayed in duplicate and the mean C_T value from three separate growth curves was used for comparisons.

Table 1
Oligonucleotides used in this study.

Oligonucleotide	Sequence	Reference
rt-*porI*-fwd	TGTCCGTACGCTACGATTCTC	Ducey et al. [14]
rt-*porI*-rev	AGCCAACGTGGTAAGATTCGC	Ducey et al. [14]
sRNA_up	TCCGTTCCGAACCATTAAA	This study
sRNA_lo	GCGTTTCGGTTTTGAGTATCT	This study
sRNA_lo_mod	6-FAM-GCGTTTCGGTTTTGAGTATCT	This study

4.4. Fluorescent primer extension (FPE)

Determination of the transcriptional start of the gonococcal *nrrF* gene was performed in a manner similar to that of Fekete et al. [31]. Thirty micrograms of both iron-deplete and iron-replete RNA were treated with 30 U RNase-free DNase (Invitrogen), ethanol precipitated and resuspended at a concentration of 1 μg/μl. Ten micrograms of each RNA were mixed with 40 μM of a 5′-labeled 6-fluorescein phosphoramidite (6-FAM) primer (Table 1), denatured at 90 °C, and cooled to 30 °C over 30 min. Then 2000 U of Superscript III, 20 U of RNase inhibitor, and 200 μM of each dNTP (Roche) were added to the annealed RNA/primer mixtures in an rtPCR reaction according to manufacturer specifications (Invitrogen). The reactions were run at 42 °C for 120 min. Unincorporated dNTPs and primers were removed using a Sephadex G50 (Sigma) packed spin column (Millipore). The purified DNA was EtOH precipitated, and subsequently resuspended in 10 μl ABI Hi-Di. To this suspension GeneScan 500-LIZ ladder was added, and the mix was electrophoresed on an ABI 3730*xl* according to manufacturer specifications for genotyping (Applied Biosystems). After capillary electrophoresis, a standard curve of the GeneScan 500-LIZ ladder was generated using the GeneMapper software (Applied Biosystems), which was used to calculate the length of the 6-FAM labeled cDNA products.

4.5. Nucleotide sequence accession number

The annotated sequence of *nrrF* is available at GenBank under accession number EU366283.

Acknowledgements

Joshua Orvis is currently located at the Institute for Genome Sciences, Baltimore, MD. We thank B. Todd Campbell and Jeff Novak for a critical review of this manuscript. This study was supported in part by a grant from the University of Oklahoma Health Sciences Center, College of Medicine Graduate Student Association.

References

[1] Litwin CM, Calderwood SB. Role of iron in regulation of virulence genes. Clin Microbiol Rev 1993;6:137–49.
[2] Bagg A, Neilands JB. Molecular mechanism of regulation of siderophore-mediated iron assimilation. Microbiol Rev 1987;51:509–18.
[3] Touati D. Iron and oxidative stress in bacteria. Arch Biochem Biophys 2000;373:1–6.
[4] Weinberg ED. Iron and infection. Microbiol Rev 1978;42:45–66.
[5] Rohde KH, Dyer DW. Mechanisms of iron acquisition by the human pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Front Biosci 2003;8: d1186–218.
[6] Carson SD, Klebba PE, Newton SM, Sparling PF. Ferric enterobactin binding and utilization by *Neisseria gonorrhoeae*. J Bacteriol 1999;181:2895–901.
[7] Calderwood SB, Mekalanos JJ. Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J Bacteriol 1988;170:1015–7.
[8] de Lorenzo V, Wee S, Herrero M, Neilands JB. Operator sequences of the aerobactin operon of plasmid ColV-K30 binding the ferric uptake regulation (*fur*) repressor. J Bacteriol 1987;169:2624–30.
[9] Escolar L, Perez-Martin J, de Lorenzo V. Opening the iron box: transcriptional metalloregulation by the Fur protein. J Bacteriol 1999;181:6223–9.
[10] Genco CA, Desai PJ. Iron acquisition in the pathogenic *Neisseria*. Trends Microbiol 1996;4:179–84.

[11] Shaik YB, Grogan S, Davey M, Sebastian S, Goswami S, Szmigielski B, et al. Expression of the iron-activated *nspA* and *secY* genes in *Neisseria meningitidis* group B by Fur-dependent and -independent mechanisms. J Bacteriol 2007;189:663–9.
[12] Delany I, Rappuoli R, Scarlato V. Fur functions as an activator and as a repressor of putative virulence genes in *Neisseria meningitidis*. Mol Microbiol 2004;52:1081–90.
[13] Lee JW, Helmann JD. Functional specialization within the fur family of metalloregulators. Biometals 2007;20:485–99.
[14] Ducey TF, Carson MB, Orvis J, Stintzi AP, Dyer DW. Identification of the iron-responsive genes of *Neisseria gonorrhoeae* by microarray analysis in defined medium. J Bacteriol 2005;187:4865–74.
[15] Masse E, Gottesman S. A small RNA regulates the expression of genes involved in iron metabolism in *Escherichia coli*. Proc Natl Acad Sci U S A 2002;99:4620–5.
[16] Masse E, Escorcia FE, Gottesman S. Coupled degradation of a small regulatory RNA and its mRNA targets in *Escherichia coli*. Genes Dev 2003;17:2374–83.
[17] Mellin JR, Goswami S, Grogan S, Tjaden B, Genco CA. A novel fur- and iron-regulated small RNA, NrrF, is required for indirect fur-mediated regulation of the *sdhA* and *sdhC* genes in *Neisseria meningitidis*. J Bacteriol 2007;189:3686–94.
[18] Wilderman PJ, Sowa NA, FitzGerald DJ, FitzGerald PC, Gottesman S, Ochsner UA, et al. Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci U S A 2004;101:9792–7.
[19] Hershberg R, Altuvia S, Margalit H. A survey of small RNA-encoding genes in *Escherichia coli*. Nucleic Acids Res 2003;31:1813–20.
[20] Wassarman KM, Repoila F, Rosenow C, Storz G, Gottesman S. Identification of novel small RNAs using comparative genomics and microarrays. Genes Dev 2001;15:1637–51.
[21] Jackson LA, Ducey TF, Zaitshik JB, Orvis J, Dyer DW. Transcriptional and functional analysis of the iron regulated *Neisseria gonorrhoeae* fur regulon, submitted for publication.
[22] Horsburgh MJ, Clements MO, Crossley H, Ingham E, Foster SJ. PerR controls oxidative stress resistance and iron storage proteins and is required for virulence in *Staphylococcus aureus*. Infect Immun 2001;69:3744–54.
[23] Horsburgh MJ, Ingham E, Foster SJ. In *Staphylococcus aureus, fur* is an interactive regulator with PerR, contributes to virulence, and is necessary for oxidative stress resistance through positive regulation of catalase and iron homeostasis. J Bacteriol 2001;183:468–75.
[24] Moore CM, Helmann JD. Metal ion homeostasis in *Bacillus subtilis*. Curr Opin Microbiol 2005;8:188–95.
[25] Geissmann TA, Touati D. Hfq, a new chaperoning role: binding to messenger RNA determines access for small RNA regulator. EMBO J 2004;23:396–405.
[26] Sambrook J, Russell DW. Molecular cloning: a laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2001. p. 7.75–7.81.
[27] Morse SA, Bartenstein L. Purine metabolism in *Neisseria gonorrhoeae*: the requirement for hypoxanthine. Can J Microbiol 1980;26:13–20.
[28] Dyer DW, McKenna W, Woods JP, Sparling PF. Isolation by streptonigrin enrichment and characterization of a transferrin-specific iron uptake mutant of *Neisseria meningitidis*. Microb Pathog 1987;3:351–63.
[29] Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res 2003;31:3406–15.
[30] Gordon L, Chervonenkis AY, Gammerman AJ, Shahmuradov IA, Solovyev VV. Sequence alignment kernel for recognition of promoter regions. Bioinformatics 2003;19:1964–71.
[31] Fekete RA, Miller MJ, Chattoraj DK. Fluorescently labeled oligonucleotide extension: a rapid and quantitative protocol for primer extension. Biotechniques 2003;35:90–4, 97–8.