

Role of Hfq in iron-dependent and -independent gene regulation in *Neisseria meningitidis*

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In *Neisseria meningitidis*, iron-responsive gene regulation is mediated primarily by the ferric uptake regulator (Fur) protein. When complexed with iron, Fur represses gene expression by preventing transcription initiation. Fur can also indirectly activate gene expression via the repression of regulatory small RNAs (sRNA). One such Fur- and iron-regulated sRNA, NrrF, was previously identified in *N. meningitidis* and shown to repress expression of the *sdhA* and *sdhC* genes encoding subunits of the succinate dehydrogenase complex. In the majority of Gram-negative bacteria, sRNA-mediated regulation requires a cofactor RNA-binding protein (Hfq) for proper gene regulation and stabilization. In this study, we examined the role of Hfq in NrrF-mediated regulation of the succinate dehydrogenase genes in *N. meningitidis* and the effect of an *hfq* mutation on iron-responsive gene regulation more broadly. We first demonstrated that the stability of NrrF, as well as the regulation of *sdhC* and *sdhA* *in vivo*, was unaltered in the *hfq* mutant. Secondly, we established that iron-responsive gene regulation of the Fur-regulated *sodB* gene was dependent on Hfq. Finally, we demonstrated that in *N. meningitidis*, Hfq functions in a global manner to control expression of many ORFs and intergenic regions via iron-independent mechanisms. Collectively these studies demonstrate that in *N. meningitidis*, iron- and NrrF-mediated regulation of *sdhC* and *sdhA* can occur independently of Hfq, although Hfq functions more globally to control regulation of other *N. meningitidis* genes primarily by iron-independent mechanisms.

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INTRODUCTION

In the Gram-negative pathogen *Neisseria meningitidis*, a portion of the genome is regulated in response to iron (~10%) (Grifantini *et al.*, 2003) and the majority of this regulation is mediated by the iron-responsive ferric uptake regulator (Fur) (Delany *et al.*, 2006; Grifantini *et al.*, 2003). In an iron-replete environment, Fur binds to DNA regulatory sequences known as Fur boxes repressing transcription (Desai *et al.*, 1996; Escolar *et al.*, 1998a, b; Stojiljkovic & Hantke, 1995). In *Escherichia coli*, Fur has also been demonstrated to positively regulate gene expression via a Fur-dependent regulatory small RNA (sRNA) (Masse & Gottesman, 2002). Regulatory sRNAs are non-coding RNA molecules that act at the post-transcriptional level

to mediate their effects. While a small number of sRNAs function by modulating protein activity, the most common class of regulatory sRNAs act by base-pairing with target mRNAs over short regions of complementarity. Base-pairing between an sRNA and an mRNA generally leads to repression of mRNA translation (Urban & Vogel, 2007), although in some instances, sRNA:mRNA interactions can increase mRNA translation (Repoila *et al.*, 2003).

In *E. coli*, the Fur-regulated sRNA RyhB is repressed under iron-replete conditions and, when active, functions as a post-transcriptional repressor of genes involved in iron homeostasis (Masse *et al.*, 2003, 2005). Recently, our group and others identified a Fur-regulated sRNA, NrrF, in *N. meningitidis* (Mellin *et al.*, 2007). NrrF regulates components of the succinate dehydrogenase complex as do Fur-regulated sRNAs in other organisms, and fulfils a functionally analogous role to these other Fur-regulated sRNAs.

For nearly all sRNAs studied in *E. coli* and other Gram-negative species, the interaction with a target mRNA *in vivo*

Abbreviations: CDM, chemically defined media; IG, intergenic; qRT-PCR, quantitative RT-PCR; sRNA, small RNA.

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Two supplementary figures and two supplementary tables are available with the online version of this paper.

requires a cofactor RNA chaperone protein named Hfq (Gottesman, 2004; Majdalani *et al.*, 2005; Urban & Vogel, 2007). Hfq is a homohexameric ring-shaped protein first described as a host factor required for replication of the Q β phage in *E. coli* (Franze de Fernandez *et al.*, 1968). It is a member of the Sm family of proteins associated with RNA splicing events in eukaryotes and is widely conserved in bacterial species. Nearly all of the sRNAs examined from *E. coli* and other Gram-negative bacteria were shown to require Hfq as an essential cofactor for regulating mRNA targets, as well as for sRNA stability (Christiansen *et al.*, 2006; Gottesman, 2004; Majdalani *et al.*, 2005). However, recent studies have identified Hfq-independent sRNAs in the Gram-positive organisms *Staphylococcus aureus* and *Bacillus subtilis* (Bohn *et al.*, 2007; Heidrich *et al.*, 2006, 2007). In *B. subtilis*, the Fur-regulated sRNA FsrA requires three proteins hypothesized to be RNA chaperones which act in place of Hfq (Gaballa *et al.*, 2008).

In this report, we examined the requirement for Hfq in *N. meningitidis* iron-responsive gene regulation. We demonstrate that Hfq is dispensable for NrrF-mediated *in vivo* regulation of the succinate dehydrogenase genes. We also established that iron-responsive regulation of the Fur-regulated *sodB* gene was dependent on Hfq. Finally, we demonstrate that in *N. meningitidis*, Hfq influences the expression of several ORFs as well as intergenic (IG) regions via iron-independent mechanisms.

METHODS

Bacterial strains, media and growth conditions. *N. meningitidis* MC58 was the wild-type strain used in this study. An *hfq* mutant was generated by transforming wild-type MC58 with pSLhfq-KO, a derivative of pSLfur-C1 (Delany *et al.*, 2003) constructed with *hfq*-flank primers (Table 1) to generate strain JM23. Complementation of JM23 was by transformation with pSLhfq-C1, a derivative of pSLfur-C1 (Delany *et al.*, 2003) constructed with *hfq*comp primers (Table 1). JM30 carries the *hfq* gene in the IG region between NMB1074 and NMB1075. Strain JM44 was generated by transforming a derivative of the pLES94 plasmid (Silver & Clark, 1995) into wild-type MC58 (Supplementary Fig. S1, available with the online version of this paper). The plasmid contains the *sdhC* promoter region from -277 to +14 with respect to the translational start site. Strains JM45 and JM46 were constructed in the same manner, but using JM23 and JM30 in place of the wild-type, respectively. Strain JM47 was generated by transforming a derivative of the pLES94 plasmid containing the *sodB* promoter into wild-type MC58. This fusion contains the *sodB* promoter region from -166 to +13. Strains JM48 and JM49 were constructed in a similar manner, but using JM23 and JM30 in place of the wild-type, respectively. All DNA manipulations were carried out as described previously (Delany *et al.*, 2003) or by following the manufacturer's instructions; correct insertion of the *lacZ* transcriptional fusion as well as all other constructs was verified by PCR.

For all growth experiments, bacteria were plated from glycerol stocks onto GCB agar plates and grown overnight. Cells were then inoculated into chemically defined media (CDM) (Morse & Bartenstein, 1980) to an OD₆₀₀ of 0.06–0.08 using a Genesys 20 spectrophotometer and cuvettes with a pathlength of 10 mm. For experiments in which RNA was to be isolated, cells were grown to an

OD₆₀₀ of 0.4–0.5 before being divided and inoculated with either 100 μ g ferric nitrate ml⁻¹ or 100 μ g desferal ml⁻¹ (an iron chelator) to constitute iron-replete or iron-depleted conditions, respectively. Cultures were grown for an additional 30 min before RNA was harvested. When appropriate, antibiotics were used at the following concentrations: 100 μ g kanamycin ml⁻¹, 5 μ g erythromycin ml⁻¹, 5 μ g chloramphenicol ml⁻¹.

RNA isolation and quantitative RT-PCR (qRT-PCR). Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA was treated with DNase I and successful removal of DNA contamination was verified by PCR. RNA integrity was confirmed on precast 5% TBE-urea ready polyacrylamide gels (Bio-Rad). For qRT-PCR experiments, 25 μ l reactions were prepared with Quantitect SYBR green RT-PCR reagents (Qiagen). Total RNA (25 ng) was added to each reaction and primers were used at a final concentration of 2 ng μ l⁻¹. Reactions were cycled in an ABI Prism 7000 cyclor and analysed with the SDS 1.2.3 software suite (ABI). Transcript quantities were calculated using a standard curve and normalized to the 16S transcript. Two biological replicates were analysed and fold changes were calculated as an average of the two values.

β -Galactosidase assays. *N. meningitidis* strains harbouring the *sdhC* promoter fusion or *sodB* promoter fusion were grown to an OD₆₀₀ of 0.4–0.5 before the addition of iron or desferal. Cells were then collected by centrifugation and β -galactosidase assays were carried out as described previously (Miller, 1972). The optical density was determined at 420 nm and again at 550 nm to correct for light scattering. Miller units were then calculated using the following equation: Miller units = $1000 \times [OD_{420} - (1.75 \times OD_{550})] / (V \times T \times OD_{600})$, where *V* is the volume in ml and *T* is the time in min.

Half-life experiments. *N. meningitidis* strains were grown in CDM to an OD₆₀₀ of 0.4, inoculated with 100 μ g desferal ml⁻¹ to induce *nrrF* transcription and grown for an additional 15 min. Cells were then treated with rifampicin (500 μ g ml⁻¹) and nalidixic acid (40 μ g ml⁻¹) to halt further transcription and DNA synthesis. Aliquots were removed at subsequent time points and were mixed 1 : 1 with a frozen slurry of stop buffer (10 mM Tris pH 7.2, 5 mM MgCl₂, 0.4 mg chloramphenicol ml⁻¹ and 20 mM sodium azide). Cells were harvested by centrifugation and RNA was isolated using Trizol reagent (Invitrogen). Total RNA (4 μ g) was then run on precast 5% TBE-urea ready polyacrylamide gels (Bio-Rad), transferred to a Hybond-N+ nylon membrane (Amersham Biosciences), UV cross-linked and prehybridized for 1 h at 42 °C in ULTRAHyb Oligo hybridization solution (Ambion). Membranes were subsequently probed overnight at 42 °C with a γ -³²P-labelled DNA oligonucleotide probe (10⁶ c.p.m. ml⁻¹) complementary to *nrrF*, then stripped and re-probed with a probe to the 5S rRNA. The intensity of the bound probe was quantified using a phosphorimager and normalized to 16S rRNA intensity.

Microarray procedures. DNA microarrays were designed by Nimblegen using the *N. meningitidis* MC58 genome as a reference (GenBank accession no. NC_003112.2) (Tettelin *et al.*, 2000). Arrays were designed using the Nimblegen 4-plex format consisting of four arrays on each individual chip. Each array contained 72 000 probes, for a total of 385 000 probes per chip. For interrogation of each target ORF, ten unique corresponding 60-mer synthetic DNA oligonucleotide probes were synthesized directly onto Nimblegen 4-plex arrays, and each probe was printed in duplicate. One hundred per cent of identified ORFs were represented on the arrays. Additionally, 60-mer tiling probes were designed to both strands of the IG regions providing complete coverage of IG regions. Intergenic probes were also printed on each array in duplicate. Total RNA (30 μ g) for each sample was converted to double-stranded cDNA using the

Table 1. Strains, plasmids and oligonucleotides used in this study

All primers are given in 5'–3' orientation.

Strain, plasmid or oligonucleotide	Genotype, phenotype or sequence	Description/reference
<i>N. meningitidis</i> strains		
MC58	Clinical isolate; sequenced strain	Tettelin <i>et al.</i> (2000)
JM23	Mutant derivative of MC58 carrying a kanamycin insertion/deletion of the <i>hfq</i> locus	This study
JM30	Complemented <i>hfq</i> mutant in which the <i>hfq</i> gene was cloned into the IG region between NMB1074 and NMB1075 under the control of its own promoter	This study
JM44	MC58 with the <i>sdhC:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
JM45	JM23 with the <i>sdhC:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
JM46	JM30 with the <i>sdhC:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
JM47	MC58 with the <i>sodB:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
JM48	JM23 with the <i>sodB:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
JM49	JM30 with the <i>sodB:lacZ</i> transcriptional fusion inserted into the <i>proA/B</i> genes	This study
Plasmids		
pSL-hfqKO	Kan ^R Amp ^R	This study
pSL-hfq-C1	Kan ^R Amp ^R Erm ^R	This study
pLES94	Amp ^R Chlor ^R	Silver & Clark (1995)
Primers (for cloning)*		
hfq-5' flankF	<u>GGA</u> ACTAGTAGCCACAATCCCGTAAACAG	Amplifies a 893 bp <i>SpeI/XhoI</i> fragment
hfq-5' flankR	GACTCGAGGCACGAAGCATGACGTGT	
hfq-3' flankF	<u>GGACG</u> GCCGATTTTTAACTCCGTTATTATGATTGTG	Amplifies a 744 bp <i>EagI/SaII</i> fragment
hfq-3' flankR	<u>GGAGT</u> CGACTGGACATGAACGAAACCGT	
hfqcompF	<u>ACAGG</u> ATCCAAAGATATGACACGTCATGCTTC	Amplifies a 568 bp <i>EagI/BamHI</i> fragment
hfqcompR	<u>ACACG</u> GCCGCAACCCGTTACCATCGTATTG	
tonBflankF	<u>ATGCG</u> GCCGCAACCCGCTAATTC AATTCAA	Amplifies a 901 bp <i>EagI/SaII</i> fragment
tonBflankR	<u>ATGGT</u> CGACTTGTA AAAATAGGTTGCA AATAGGAA	
exbBflankF	<u>ATGACT</u> AGTCAGCTATCCTTTTGATTAAGCAGG	Amplifies a 676 bp <i>SpeI/XhoI</i> fragment
exbBflankR	<u>ATGCT</u> CGAGGTGTTGGAATCATTATGAATTTGAAA	
Oligonucleotides used for EMSA		
SdhC	UUUUUAGUCGAUUAUUAACUACAGAGGAAUUGACUAUGUCUGCC	Cy-5 labelled 45 bp RNA probe
Nrrf	GGAAGCCGUCCGUUCUAUACGAGACAUACAUUCCCUUUUAUUAUCAGAUAC	Cy3-labelled 53 bp RNA probe
Primers (for qRT-PCR)		
NMB0382F	GTTACGATGCTGCGGATTTT	Amplifies a 407 bp fragment of the <i>rmp</i> gene
NMB0382R	GAAACCATTTCCTGTCTGC	
NMrrnaA16SF	CATACCGTGTAAGCGGACT	Amplifies a 236 bp fragment of the 16S rRNA
NMrrnaA16SR	TGGTCGGTACAGAGGGTAGC	
NMB0950F	ATTACCACGGCGAAGTTGTC	Amplifies a 288 bp fragment of the <i>sdhA</i> gene
NMB0950R	ATGCATCAACGTTTTACCA	
NMB0948F	GGGGTCGGGCTGTTATTAT	Amplifies a 151 bp fragment of the <i>sdhC</i> gene
NMB0948R	GGTGCAGATAAGCCACAAT	
NMB0884F	GGCGTTGGAAGTAGAAACCA	Amplifies a 277 bp fragment of the <i>sodB</i> gene
NMB0884R	CCTGCCTTTGGAAGATTG	
Probes for Northern blots		
Nrrf	GTATGTCTCGTATATGCCGACTCCAAGTGTGAAAGTGATGATGGGGAAAT	
5S rRNA	TTTCACGGTCTGTTCCGGGATGGGAAGGCGTGGGACCAACTCGCTATGGC	

*Restriction sites are underlined. F, forward primer, R, reverse primer

SuperScript double-stranded cDNA conversion kit (Invitrogen). Samples were labelled and hybridized according to Nimblegen's protocols. Data were normalized using the method described by Bolstad *et al.* (2003). Expression values were determined as described previously (Irizarry *et al.*, 2003). Data were further analysed using the Arraystar 2.0 software suite (DNASTAR). For each strain and condition, three biological replicates were analysed for a total of 18 arrays. Student's *t*-test was used to calculate *P*-values. The GEO accession no. for the microarray experiments carried out in this paper is GSE20294.

RESULTS

Growth of the *hfq* mutant is attenuated *in vitro*

To examine the role that Hfq plays in general meningococcal physiology, we constructed a *N. meningitidis hfq* mutant (JM23) and a complemented *hfq* (JM30) strain. When grown in CDM, growth of the *N. meningitidis hfq* mutant strain was slightly attenuated when compared with the wild-type or complemented *hfq* strain, as determined by OD₆₀₀ readings, and was more severely attenuated as determined by c.f.u. measurements (Fig. 1). The *N. meningitidis hfq* mutant exhibited approximately a 1 log reduction in c.f.u. compared with the *N. meningitidis* wild-type and complemented *hfq* strains when examined at 3 h, and a 2.5 log reduction when examined at 5 h (Fig. 1b). Additionally, *hfq* mutant colonies were smaller in size compared with wild-type and complemented mutant strains when grown on GCB agar plates overnight (data not shown). The attenuated growth of the *N. meningitidis hfq* mutant was largely corrected in the complemented strain (Fig. 1).

Hfq is dispensable for NrrF-mediated regulation of the succinate dehydrogenase genes

We previously reported that expression of the *sdhA* and *sdhC* genes was increased under iron-replete conditions and was repressed under iron-depleted conditions in an NrrF-dependent manner (Mellin *et al.*, 2007). Therefore,

we next examined the expression of the *sdhA* and *sdhC* genes by qRT-PCR in *N. meningitidis* wild-type (MC58), *hfq* mutant (JM23) and complemented *hfq* mutant (JM30) strains grown in CDM under either iron-replete or -depleted conditions. As expected, in *N. meningitidis* wild-type cultures grown in iron-replete versus -depleted conditions, we observed a 6.13-fold increase in the expression of the *sdhA* transcript (Fig. 2a). In *N. meningitidis*, JM23 and JM30 cultures grown in iron-replete versus -depleted conditions, we observed a 4.68- and 3.83-fold increase, respectively, in the expression of the *sdhA* transcript. Similar results were obtained when we examined the expression of the *sdhC* transcript in *N. meningitidis* wild-type, JM23 and JM30 cultures grown under iron-replete conditions compared with iron-depleted conditions (Fig. 2b). Thus, repression of both the *sdhA* and *sdhC* genes under iron-depleted growth conditions was not altered in the *hfq* mutant.

To further support the results obtained by qRT-PCR analysis, we created *N. meningitidis lacZ* translational fusions in strains MC58, JM23 and JM30 with the promoter of *sdhC*, the first gene of the succinate dehydrogenase operon. We observed similar iron-dependent and Hfq-independent regulation of the *sdhC:lacZ* fusion strains (Fig. 3). These results indicate that Hfq is dispensable for iron-mediated regulation of the *sdhC* gene.

NrrF stability is unchanged in the *N. meningitidis hfq* mutant

We next examined the stability of NrrF in the *N. meningitidis* wild-type, *hfq* and complemented *hfq* mutant strains. We observed that the NrrF sRNA was stable in the three *N. meningitidis* strains, as observed at 5 min, and the transcript levels persisted at low levels for 15 min (Fig. 4a–c). The calculated half-life of the NrrF transcript, as determined by densitometry analysis, was 5.0, 4.0 and 4.1 min in the *N. meningitidis* wild-type, *hfq* mutant and complemented *hfq* mutant strains, respectively (Fig. 4d).

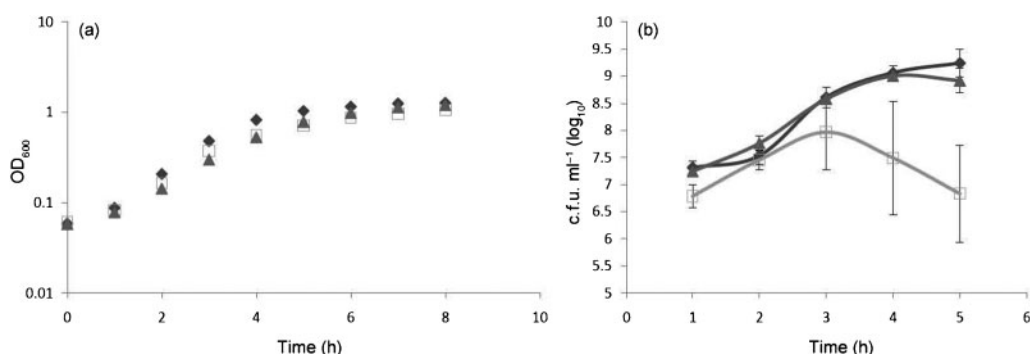


Fig. 1. *N. meningitidis* wild-type, *hfq* deletion mutant and complemented *hfq* strains were grown in CDM (Morse & Bartenstein, 1980), growth was monitored by optical density (OD₆₀₀) (a) and colony forming unit (b) measurements. Results represent the average of three experiments and error bars represent SD. Wild-type, ◆; *hfq* mutant, □; complemented *hfq*, ▲.

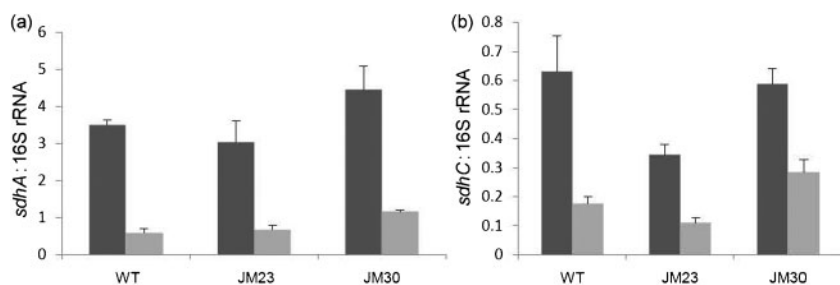


Fig. 2. Effect of the *hfq* mutation on regulation of the succinate dehydrogenase genes. Expression of *sdhA* (a) and *sdhC* (b) transcripts under iron-replete (dark grey bars) versus iron-depleted (light grey bars) conditions was evaluated by qRT-PCR. Transcript levels were normalized to the 16S rRNA transcript which is not regulated in response to iron. Primers amplified a 151 bp fragment of the *sdhC* gene, a 288 bp fragment of the *sdhA* gene, and a 236 bp fragment of the 16S rRNA gene. Results represent the average of two independent experiments and error bars represent SD.

While we did observe an approximately 20% difference in the half-life between the *N. meningitidis* wild-type and *hfq* mutant strains, this was not statistically significant. The small differences observed in the half-life of NrrF may not be directly related to the lack of Hfq, but rather to secondary effects of the mutant and complemented strains, as it was not corrected in the complemented mutant. Thus, while the *hfq* mutation had a minimal effect on NrrF stability, the results overall indicate that NrrF stability was not influenced by Hfq, since nearly all Hfq-dependent sRNAs are significantly less stable in an *hfq* null background (Christiansen *et al.*, 2006; Moller *et al.*, 2002).

Iron-responsive gene regulation of the Fur-regulated *sodB* gene is Hfq-dependent

To determine whether Hfq played a role in the regulation of other iron- and Fur-responsive gene networks in *N. meningitidis*, we examined transcription of the iron- and Fur-activated gene, *sodB*, as it is the target of Fur-regulated sRNAs in other organisms (Večerek *et al.*, 2003). Hfq-dependent regulation of *sodB* was first examined by qRT-PCR and expression was observed to be derepressed under iron-depleted conditions in the *hfq* mutant compared with the wild-type strain (Fig. 5). These results were further

examined by the construction of translational fusions containing the promoter of *sodB* fused to *lacZ*. The *sodB::lacZ* fusion showed similar derepression under iron-depleted conditions (Fig. 6). These results indicate that regulation of the *sodB* promoter was abolished in the *N. meningitidis* *hfq* mutant strain.

Hfq functions globally to control gene expression in *N. meningitidis* via iron-independent mechanisms

To further evaluate the role of Hfq in *N. meningitidis* iron-dependent and -independent gene expression on a global level, we utilized a whole-genome tiling microarray to examine the transcriptional profiles of *N. meningitidis* wild-type, *hfq* and complemented *hfq* mutant strains grown under iron-replete and -depleted conditions. To identify genes that required Hfq for proper iron-responsive regulation, we analysed the microarray data for genes that exhibited iron-dependent regulation in the *N. meningitidis* wild-type and complemented *hfq* mutant strains (>2.5-fold) but little or no change in the *hfq* mutant (<1.5-fold). This analysis identified only one such gene meeting these criteria, *sodB*, further confirming the results presented above.

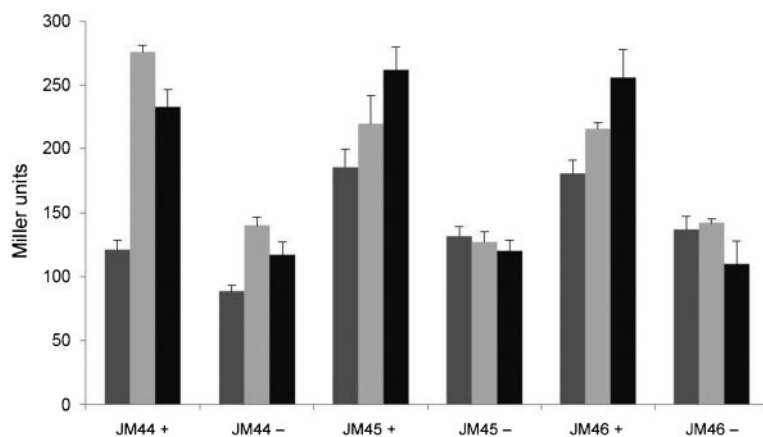


Fig. 3. Effect of the *hfq* mutant on iron regulation of the *sdhC* promoter. JM44 (wild-type *N. meningitidis* with the *lacZ* fusion), JM45 (*hfq* mutant strain with the *lacZ* fusion) and JM46 (complemented *hfq* mutant strain with the *lacZ* fusion) cultures were grown under iron-replete or iron-depleted conditions for 30 (dark grey bars), 60 (light grey bars) or 120 (black bars) min. β -Galactosidase assays were carried out as described in Methods. Cultures grown under iron-replete and iron-depleted conditions are indicated by + and -, respectively. Assays were carried out in duplicate and results represent the average of three independent experiments. Error bars, SD.

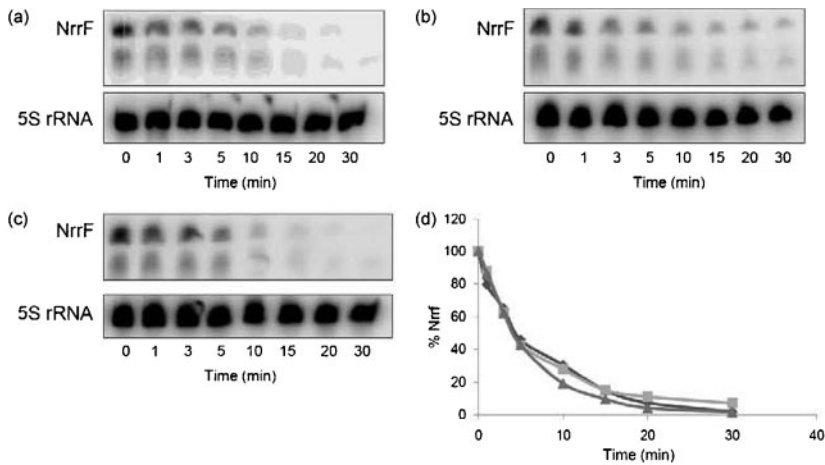


Fig. 4. Half-life of NrrF in the *hfq* mutant and *hfq* complemented strains. Cultures of wild-type (a), *hfq* (b) and complemented *hfq* (c) strains were grown in CDM to an OD₆₀₀ of 0.4–0.5. Aliquots were taken at the time points indicated and RNA was isolated. Levels of bound probe were quantified using a phosphoimager. All blots were stripped and reprobated with a probe to the 16S rRNA to ensure equal levels of RNA were loaded onto each lane; levels of NrrF were then normalized to levels of 5S rRNA. (d) The decay of the probe over time. Wild-type, ◆; *hfq* mutant, ■; complemented *hfq*, ▲.

The observation that the *N. meningitidis* *hfq* mutant had an attenuated growth phenotype suggested that Hfq might serve a broad role in meningococcal physiology and gene regulation. Thus, we next analysed the microarray data to identify genes that required Hfq independently of iron (Table 2 and Supplementary Table S1). We identified 45 genes that were Hfq-regulated: 27 exhibited increased expression and 18 exhibited decreased expression in the *hfq* mutant relative to the wild-type strain. The majority of genes that exhibited increased expression in the *N. meningitidis* *hfq* mutant were classified as either hypothetical or uncharacterized genes. Of the known genes that exhibited increased expression in the *N. meningitidis* *hfq* mutant, several are involved in metabolism, including *ackA-1*, *adhP*, *acnB*, *prpB*, *prpC* and *prpF*.

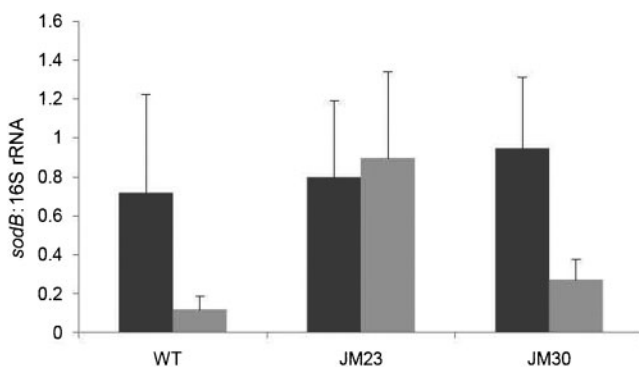


Fig. 5. Effect of the *hfq* mutation on regulation of *sodB*. The expression of *sodB* under iron-replete conditions versus iron-depleted conditions was evaluated by qRT-PCR. RNA was isolated from wild-type (WT), *hfq* mutant (JM23) and complemented *hfq* mutant (JM30) strains grown under iron-replete (dark grey bars) and iron-depleted (light grey bars) conditions in CDM. Transcript levels were normalized to the 16S rRNA transcript which is not regulated in response to iron. Results represent the average of three independent experiments; error bars represent SD.

Of the genes that exhibited decreased expression in the *hfq* mutant relative to the wild-type strain, most were involved in transport. These included four ABC transport genes as well as genes encoding three proteins involved in the transport of lactate, phosphate and uracil. Such a global lack of proper metabolism and transport control in the *hfq* mutant strain could explain the attenuated growth phenotype we observed. Additionally, NMB0024, a member of the unexpressed *pilS* cassette was demonstrated to exhibit decreased expression in the *hfq* mutant relative to the wild-type strain. In agreement with the results obtained above, we did not observe differences in the expression of the *sdhC* and *sdhA* genes in the *hfq* mutant relative to the wild-type strain (Supplementary Table S2). Our tiling array was also designed to analyse IG regions and it detected several which showed reduced abundance in the *hfq* mutant strain compared with the wild-type or complemented *hfq* mutant strains (Table 3). Expression from IG regions is consistent with known sRNA transcriptional sites and most Hfq-dependent sRNAs exhibit reduced stability in the absence of Hfq (Christiansen *et al.*, 2006; Moller *et al.*, 2002). Therefore, it is plausible that IG regions that were identified as Hfq-regulated in our study had the potential to function as Hfq-dependent sRNAs in *N. meningitidis*. Consistent with previous data, the abundance of the NrrF IG transcript was not lowered in the *hfq* mutant strain, in fact it increased. In the wild-type strain the expression was 203 units, in the *hfq* mutant the expression was 537 units and in the complemented *hfq* strain, the expression was 182 units. The higher expression level in the *hfq* mutant is most likely the result of unknown downstream effects of the mutation.

DISCUSSION

In this study, we determined that Hfq does not play a major role in iron- and NrrF-mediated regulation of the *sdhA* and *sdhC* genes in *N. meningitidis*. We demonstrated that the regulation of *sdhC* and *sdhA* *in vivo* was unaltered in the *hfq* mutant. These results were confirmed using a

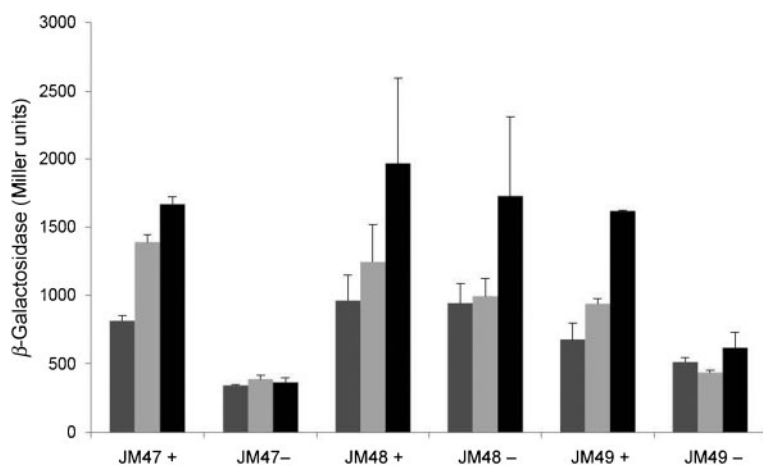


Fig. 6. Effect of the *hfq* mutant on the iron regulation of the *sodB* promoter. JM47 (wild-type *N. meningitidis* with the *lacZ* fusion), JM48 (*hfq* mutant strain with the *lacZ* fusion) and JM49 (complemented *hfq* mutant strain with the *lacZ* fusion) cultures were grown under iron-replete or iron-depleted conditions for 30 (dark grey bars), 60 (light grey bars) or 120 (black bars) min. β -Galactosidase assays were carried out as described in Methods. Cultures grown under iron-replete and iron-depleted conditions are indicated by + and -, respectively. Assays were carried out in duplicate and results represent the average of three independent experiments. Error bars, SD.

translational *lacZ* fusion. We also demonstrated that the stability of NrrF was unaltered in the *hfq* mutant in contrast with the half-lives of Hfq-dependent sRNAs reported for *hfq* mutants constructed in other organisms (Christiansen *et al.*, 2006; Moller *et al.*, 2002). The contribution of Hfq to iron-responsive regulation in *N. meningitidis* was limited to the regulation of the *sodB* gene. While the *sodB* gene has been shown to be regulated by an sRNA in *E. coli* (Masse & Gottesman, 2002), we previously reported that *sodB* is regulated independently of NrrF in *N. meningitidis* (Mellin *et al.* 2007). How Hfq mediates *sodB* regulation via other sRNA-mediated mechanisms in *N. meningitidis* remains to be defined.

The majority of sRNA-mediated regulation systems in Gram-negative bacteria described to date have been shown to require Hfq as a cofactor (Gottesman, 2004; Majdalani *et al.*, 2005; Moller *et al.*, 2002; Murphy & Payne, 2007). Fur-regulated sRNAs of *E. coli* and *Vibrio cholera*, including RyhB, also require Hfq (Davis *et al.*, 2005; Masse & Gottesman, 2002). Since *sdhC* is regulated in the absence of Hfq, it is possible that either another protein can function as a cofactor with NrrF or NrrF functions without a cofactor. The recent identification of an Hfq-independent, Fur-regulated sRNA in *B. subtilis* which is proposed to work in conjunction with three Fur-regulated chaperone proteins supports the idea that other sRNA cofactor proteins may exist in *N. meningitidis*. Our laboratory has previously identified a number of uncharacterized, Fur-regulated ORFs, and an effort to reexamine these proteins for potential protein cofactors of NrrF-mediated regulation is underway.

A recent study reported that NrrF-mediated regulation of *sdhA* and *sdhC* required Hfq (Metruccio *et al.*, 2009). This study, as well as our laboratory's work, also examined the potential binding of Hfq to *sdhC* and NrrF riboprobes. Hfq has been shown to bind to both *sdhC* and NrrF *in vitro* and aid in the formation of an *sdhC*:NrrF duplex (Metruccio *et al.*, 2009; Supplementary Fig. S2). However, this should not be taken as evidence that Hfq is the required cofactor of NrrF *in vivo*, as Hfq has been shown to bind to many

species of RNA and DNA (Takada *et al.*, 1997; Windbichler *et al.*, 2008; Zhang *et al.*, 1998), and thus it is difficult to find nucleic acids to act as non-specific controls in Hfq binding experiments. The study by Metruccio *et al.* (2009) also utilized an *N. meningitidis hfq fur* double mutant. Since Fur and Hfq are central regulatory proteins which cause significant growth defects when deleted individually, it is possible that the double knockout could have widespread and unknown effects on gene regulation and transcription. Finally, in contrast with Metruccio *et al.* (2009), our studies directly examined the role of Hfq in iron-dependent expression of the *sdhA* and *sdhC* genes. Furthermore, two recent proteomic studies did not identify *sdhC* as an Hfq-regulated gene (Fantappie *et al.*, 2009; Pannekoek *et al.*, 2009). Collectively, these results indicate that NrrF-mediated iron regulation of *sdhA* and *sdhC* can occur independently of Hfq.

While NrrF-mediated iron regulation was not dependent on Hfq, we demonstrated that Hfq has a broad role in the cellular physiology of *N. meningitidis*. First, the *hfq* mutant had significant effects on bacterial growth. Second, when gene expression profiles of wild-type bacteria were compared with those of the *hfq* mutant, numerous genes showed aberrant regulation irrespective of iron conditions, and these defects were largely corrected in the complemented *hfq* mutant. Over 40 genes were dysregulated in the *hfq* mutant compared with the wild-type and the complemented *hfq* mutant strains. Of these, 27 genes were upregulated. This was expected as it is known that Hfq mediates the binding of sRNAs to their mRNA targets, which in most cases leads to degradation of the mRNA. Therefore, the loss of Hfq would disrupt these sRNA circuits and stabilize their mRNA targets. Several genes encoding proteins of central metabolism pathways were upregulated in the absence of Hfq, as were genes encoding hypothetical uncharacterized proteins. Dysregulation of these pathways would be expected to have deleterious effects on growth. Microarray analysis also identified 18 genes that were downregulated in the *hfq* mutant, 53% of which were classified as playing a role in transport. Thus, in

Table 2. Hfq-regulated genes identified by microarray analysis

All genes showing at least 2.5-fold change in regulation between the wild-type strain and *hfq* mutant strains are listed. Fold change is defined as the average expression from three microarray replicates of the wild-type strain divided by the average expression of three microarray replicates of the *hfq* mutant strain or *hfq* complemented mutant strain. If the quotients were less than 1, the reciprocal was taken and a minus sign was added for ease of reading. All *P*-values of a fold change are below 0.02, and fold changes are complemented to between 60 and 140 % of wild-type expression. Because there were several genes with large changes, we also included those that showed a fourfold difference between the *hfq* mutant and complemented *hfq* mutant. WT, *N. meningitidis* MC58; JM23, *hfq* mutant; JM30, complemented *hfq* mutant. Hi, culture was grown under iron-replete conditions; Low, culture was grown under iron-depleted conditions. Genes in bold were also found in the study by either Pannekoek *et al.* (2009) or Fantappie *et al.* (2009).

Gene	Name	Description	Fold change			
			WT vs JM23	WT vs JM23	WT vs JM30	WT vs JM30
			Hi	Low	Hi	Low
Upregulated genes						
Metabolism:						
NMB0430	<i>prpB</i>	2-Methylisocitrate lyase	-20.35	-15.32	-1.18	-1.85
NMB0431	<i>prpC</i>	Methylcitrate synthase	-23.87	-19.69	-1.19	-1.82
NMB0435	<i>ackA-1</i>	Acetate kinase	-13.65	-10.26	-1.75	-2.43
NMB0546	<i>adhP</i>	Alcohol dehydrogenase	-1.71	-3.09	-1.15	-1.09
NMB1572	<i>acnB</i>	Aconitate hydratase	-2.71	-2.97	1.11	1.19
Transport:						
NMB0177		Sodium/alanine symporter	-13.07	-10.65	1.14	1.14
NMB0402	<i>putp</i>	Sodium/proline symporter	-2.94	-1.58	-1.27	-1.70
NMB2136		Peptide transporter	-3.65	-2.54	1.51	1.51
Unclassified:						
NMB0432		Putative membrane protein	-5.86	-4.11	-1.36	-1.65
NMB0752		Putative ferredoxin	-1.20	-2.88	1.32	-1.33
NMB1472	<i>clpB</i>	Heat stress response	-2.66	-1.45	1.03	1.58
NMB1552	<i>pivNM-1A</i>	Pilin gene inverting protein	-2.42	-2.59	-1.04	1.00
Hypothetical:						
NMB0227		Hypothetical protein	-3.28	-2.63	1.22	1.14
NMB0625		Hypothetical protein	-2.02	-2.52	1.08	1.02
NMB0648		Hypothetical protein	-4.24	-2.07	-1.83	-1.07
NMB0649		Hypothetical protein	-2.76	-2.04	-1.38	-1.21
NMB0859		Hypothetical protein	-3.21	-8.85	-1.16	-2.04
NMB0861		Hypothetical protein	-2.14	-2.84	-1.34	-1.44
NMB0865		Hypothetical protein	-5.89	-3.10	-1.15	-1.40
NMB0866		Hypothetical protein	-4.79	-10.85	-1.08	-2.53
NMB1350		Hypothetical protein	-3.28	-2.96	-1.06	-1.81
NMB1406		Hypothetical protein	-3.37	-1.68	-1.30	-3.37
NMB1599		Hypothetical protein	-17.04	-10.64	-2.89	-2.11
NMB1600		Hypothetical protein	-3.82	-4.49	-1.38	-2.28
NMB1764		Hypothetical protein	-3.35	-1.93	-1.34	-1.23
NMB1766		Hypothetical protein	-4.54	-3.16	-1.39	-1.19
NMB1767		Hypothetical protein	-2.59	-2.54	1.27	-1.18
Downregulated genes						
Metabolism:						
NMB0994		Acetyl-CoA dehydrogenase	8.22	2.97	2.59	-2.41
NMB1044	<i>fpr-1</i>	Ferredoxin	2.70	1.79	1.58	1.23
Transport:						
NMB0378		Putative phosphate transporter	1.70	3.18	1.29	1.36
NMB0543		Putative L-lactate permease	2.15	3.82	1.31	1.49
NMB0607	<i>secD</i>	Type II protein secretion	1.97	2.55	1.57	1.39
NMB0787		Amino acid ABC transporter	2.16	3.01	1.22	-1.17
NMB0788		Amino acid ABC transporter	1.90	2.64	1.30	-1.10
NMB0881	<i>cysT</i>	Sulfate ABC transporter	7.29	4.69	1.93	-3.05
NMB1017	<i>sbp</i>	Sulfate ABC transporter	8.12	6.30	2.59	-1.27

Table 2. cont.

Gene	Name	Description	Fold change			
			WT vs JM23		WT vs JM30	
			Hi	Low	Hi	Low
NMB1315	<i>uraA</i>	Uracil permease	2.20	2.77	-1.03	-1.39
NMB1362		Putative transport protein	1.98	2.70	1.55	1.63
Unclassified:						
NMB0024		<i>pilS</i> cassette	2.83	2.85	1.34	1.00
NMB0748	<i>hfq</i>	Host factor for Q β phage	289.26	837.89	-1.10	1.63
NMB0763	<i>cysK</i>	Cysteine synthase	2.72	2.65	1.66	1.15
NMB0993	<i>Rubredoxin</i>	ETC protein	9.13	5.25	2.46	-2.10
NMB1617	<i>TehB</i>	Methyltransferase	2.98	2.65	1.65	1.56
NMB1934	<i>atpD</i>	ATP synthase	1.79	3.11	1.04	1.11
Hypothetical:						
NMB0986		Hypothetical protein	2.3	2.5	1.4	1.2

Table 3. Hfq-regulated IG regions identified by microarray analysis

All IG regions showing at least 2.5-fold downregulation in the *hfq* mutant strain compared with the wild-type strain are listed. Fold change is defined as the average expression from two microarray replicates of the wild-type strain divided by the average expression of two microarray replicates of the *hfq* mutant strain or *hfq* complemented mutant strain. If the quotients were less than 1, the reciprocal was taken and a minus sign was added for ease of reading. For comparison, the fold change under iron-replete conditions versus iron-depleted conditions is also shown for each IG region. Because there was some variability between the two microarray replicates, IG regions were considered to be Hfq-regulated if SD was less than 0.33 of the fold change under either iron-replete or iron-depleted conditions. In addition, the fold change between the wild-type strain versus the complemented *hfq* mutant strain had to be between 0.6 and 1.4. Because some regions were very highly regulated, we also included IG regions where the fold change between the wild-type strain versus the *hfq* mutant strain was at least three times higher than the fold change between the wild-type strain versus the complemented *hfq* mutant strain. Hi, culture was grown under iron-replete conditions; Low, culture was grown under iron-depleted conditions.

	Fold change				
	WT	WT vs JM23	WT vs JM23	WT vs JM30	WT vs JM30
	Hi vs low	Hi	Low	Hi	Low
IG region (plus strand)					
1 009 467–1 010 148	2.22	3.78	3.14	1.12	-1.56
601 650–602 078	-1.77	3.24	3.02	1.21	1.59
864 734–865 029*	1.48	2.98	1.57	-1.14	-1.79
901 874–902 124	1.69	2.93	2.12	1.26	-2.94
1 099 662–1 101 163	-1.15	2.80	2.03	1.27	1.54
779 574–779 701	-1.41	7.92	10.65	2.82	2.24
2 185 273–2 185 835*	-1.25	4.18	6.60	2.49	1.94
1 486 778–1 488 547	-4.13	-1.14	4.00	2.02	1.35
1 529 930–1 530 289*	-3.63	1.05	3.19	1.14	1.35
327 435–3 279 36*	-3.61	-1.10	2.79	2.73	1.19
1 009 467–1 010 148	1.22	4.13	2.75	1.21	-1.69
IG region (minus strand)					
1 009 467–1 010 148	1.26	4.72	4.06	1.25	-1.86
601 650–602 078	-1.62	3.31	2.52	1.22	1.45
2 091 429–2 092 651	-40.09	1.42	3.24	1.32	1.23
639 542–639 763	1.14	2.71	2.88	2.01	1.38
601 650–602 078	-1.56	2.65	2.64	1.24	1.29
1 599 520–1 600 541	-1.94	1.66	2.57	1.31	1.19

*The IG region has a Rho-independent terminator.

addition to properly regulating central metabolism pathways, Hfq may also be involved in the regulation of genes required for efficient transport of proteins and molecules in and out of the cell.

In examining the gene expression profile of the *hfq* mutant, two general observations can be made. The first is that since the main function of Hfq is to act as a cofactor of sRNAs which downregulate their targets, Hfq/sRNA circuits directly affecting gene expression are most likely to be found when examining genes that are upregulated in an *hfq* mutant. Any of these transcripts has the potential of being directly controlled by an Hfq-dependent sRNA. Indirect regulation is also possible, as Hfq may negatively regulate an activator of these upregulated genes. The second observation is that genes which were downregulated in the *hfq* mutant are likely to be indirectly affected by Hfq and could result from Hfq negatively regulating a repressor protein. Since many of the downregulated genes identified are of only one type (transport) there may be a surprisingly small number of repressors under Hfq control. Several of the genes identified in our microarray analysis were also identified in two recent proteomic studies (Fantappie *et al.*, 2009; Pannekoek *et al.*, 2009) although our study identified additional genes under Hfq control including the *secD*, *cysT*, *sbp*, *uraA*, *putP*, *clpB* and *pivNM-1A* genes.

One important consideration regarding the widespread defects in the *hfq* mutant is that these were the result of disruption of other as-yet-unidentified Hfq-dependent sRNA regulatory circuits. The challenge lies in searching for these sRNAs and determining their mRNA targets. The tiling microarray used in our study examined not only *N. meningitidis* ORFs but also IG regions where most sRNA coding regions are found. To this end, our study proved useful in identifying not only iron-regulated intergenic transcripts but also numerous previously unknown transcripts which likely represent a plethora of novel sRNAs in *N. meningitidis* (Table 3). Work is currently underway in our laboratory to characterize these intergenic transcripts and determine if they are true non-coding sRNAs which have regulatory roles in *N. meningitidis*.

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