Regulation of *Neisseria meningitidis* cytochrome *bc*₁ components by NrrF, a Fur-controlled small noncoding RNA

Yvonne Pannekoek¹,*, Robert Huis in ’t Veld¹,*, Kim Schipper¹, Sandra Bovenkerk¹, Gertjan Kramer²,†, Dave Speijer² and Arie van der Ende¹

1 Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, The Netherlands
2 Department of Medical Biochemistry, Academic Medical Center, Amsterdam, The Netherlands

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**Correspondence**
Y. Pannekoek, Department of Medical Microbiology, Center for Infection and Immunity Amsterdam (CINIMA), Academic Medical Center, Room L1-115, PO Box 22660, 1100 DD Amsterdam, The Netherlands
Fax: +31 20 5669606
Tel: +31 20 5664862
E-mail: y.pannekoek@amc.uva.nl

**Present address**
Genome Biology Unit, EMBL Heidelberg, Heidelberg, Germany

*These authors contributed equally to this work and should be considered co-first authors

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NrrF is a small regulatory RNA of the human pathogen *Neisseria meningitidis*. NrrF was previously shown to repress succinate dehydrogenase (*sdhCDAB*) under control of the ferric uptake regulator (Fur). Here, we provide evidence that cytochrome *bc*₁, encoded by the polycistronic mRNA *petABC*, is a NrrF target as well. We demonstrated differential expression of cytochrome *bc*₁ comparing wild-type meningococci and meningococci expressing NrrF when sufficient iron is available. Using a *gfp*-reporter system monitoring translational control and target recognition of sRNA in *Escherichia coli*, we show that interaction between NrrF and the 5’ untranslated region of the *petABC* mRNA results in its repression. The NrrF region essential for repression of *petABC* was identified by site-directed mutagenesis and is fully conserved among meningococci. Our results provide further insights into the mechanism by which Fur controls essential components of the *N. meningitidis* respiratory chain. Adaptation of cytochrome *bc*₁ complex component levels upon iron limitation is post-transcriptionally regulated via the small regulatory RNA NrrF.

Riboregulated networks in which small RNA (sRNA) regulate both stability and accessibility (and thus, translation) of transcripts (mRNA) abound in prokaryotes. Hundreds of sRNA have been identified by using a wide variety of both computational and experimental approaches in various bacterial species during the last decade. One class of sRNA, typically ranging in size between 50 and 300 nucleotides, is found in intergenic regions distant from the genes encoding their mRNA targets. With few exceptions, they do not contain open reading frames and typically have limited regions of complementarity (10–30

**Abbreviations**
BIGSdb, Bacterial Isolate Genome Sequence database; Fur, ferric uptake regulator; RBS, ribosome binding site; SD, Shine–Dalgarno; Spp, Species; WTA, whole transcriptome analysis; wt, wild-type.
polycistronic operon

Detailed studies of these so-called trans-encoded sRNA have revealed general principles in their mode of action. The majority of these sRNA interact by noncontiguous base pairing with mRNA targets by an antisense mechanism to activate or, more frequently, to repress translation of target mRNA. Duplex formation generally results in sequestering of the Shine–Dalgarno (SD) or translational start codon (AUG) sequences of the ribosome binding site (RBS). This duplex formation will compete with initiating ribosomes and will lead to ribosome occlusion, often followed by rapid RNA decay. This model of sRNA action is supported by many examples of sRNA that pair around the SD or AUG to inhibit translation [4–10]. In numerous, mostly Gram-negative bacteria, the RNA-binding protein Hfq is required for the action and stability of many trans-encoded sRNA [11]. Thus, translational regulation can be achieved by a variety of mechanisms. Translation can be repressed via catalytic degradation of mRNA: through coupled degradation of sRNA–mRNA duplexes or through mRNA sequestration, or activated by opening up the ribosome binding site [2,12,13]. In many cases, the aforementioned RNA chaperone Hfq mediates the interactions between trans-encoded sRNA and their mRNA targets and protects the sRNA from RNase E–mediated degradation [5,11,14,15]. Environmental stimuli, including anaerobic conditions, oxidative stress, glucose availability, osmotic imbalance and iron availability, may affect the expression of sRNA [12,16,17].

In the opportunistic strictly human pathogen Neisseria meningitidis, several trans-encoded sRNA have been described. AniS is anaerobically induced through activation of its promoter by the Fnr global regulator and downregulates the expression of a protein of unknown function [18,19]. Recently, we identified a sRNA that targets genes encoding TCA cycle enzymes specifying their importance in the adaption to changing environments of the host. The riboregulated network of the sibling sRNA is part of the RelA-regulated stringent response [20]. NrrF is synthesized under iron-limiting conditions and controlled by the ferric uptake regulator (Fur) [21,22]. Fur is essential for iron homeostasis in many prokaryotes [23]. Fur inhibits expression of genes essential for iron acquisition by means of binding to a specific consensus target sequence (the so-called Fur box) in their promoters. Fur also acts as a positive regulator, affecting the production of factors storing or containing iron [24]. The sRNA dehydrogenase complex, encoded by the polycistronic operon sdhCDAB, donates electrons to iron-cytochrome $bc_1$ in the respiratory electron transport chain [25]. Its expression is regulated by iron and Fur, but the promoter of sdhCDAB lacks a Fur box [19,26,27]. Instead, sdhCDAB was shown to be regulated by NrrF [21,22], demonstrating crosstalk between the riboregulated network and the Fur-regulated network in meningococci. By using a well-established heterologous (Escherichia coli) reporter system for translational control and target recognition of sRNA in vivo, we showed a direct interaction between NmsR–A and sdhC [20,28]; though, we did not observe regulation in meningococci. The regulation of sdhC by multiple sRNA has been shown before, and sdhCDAB might thus be the first example of a cistronic mRNA in meningococci that is subjected to regulation by two different sRNA species [29]. The cytochrome $bc_1$ complex transfers electrons to c-type cytochromes $c_2$, $c_4$ and $c_5$, which in turn donate electrons to the cytochrome $cbb_3$ complex. Cytochrome $c_5$ is also an important electron donor for the AniA nitrite reductase [30–32]. Expression of the cytochrome $bc_1$ complex, the cytochromes $c_4$, $c_5$ and the cytochrome $cbb_3$ complex also appears to be controlled by Fur and/or iron but the promoter regions of the genes encoding these proteins lack a Fur box as well [19,26,27]. Hence, the mechanism by which the expression of these genes is controlled is unknown.

We hypothesized that more components of the respiratory electron transport chain of meningococci that are under indirect control of Fur could be subjected to regulation by sRNA and studied the potential role of NrrF in this regulation. Here, we focused on regulation of the cytochrome $bc_1$ complex, encoded by the polycistronic operon petABC. Differential protein profiling of a nrrF mutant vs. meningococci overexpressing nrrF showed downregulation of PetA. This downregulation was confirmed by western blotting. Direct interaction between NrrF and the 5′ untranslated region (5′UTR) of petABC was again confirmed in a heterologous gfp-reporter system [28].

Thus, we first show that NrrF is involved in the regulation of proteins involved in iron uptake, iron-dependent metabolic processes and the oxidative stress response. We then further characterize a novel target of NrrF, functionally involved in respiration in N. meningitidis, extending the experimentally validated NrrF-regulated network of N. meningitidis. In addition, we provide important insights into the mechanism by which an essential component of the respiratory chain is indirectly controlled by Fur. Adaptation of expression of components of the cytochrome $bc_1$ complex to iron limitation is mediated at the posttranscriptional level through the action of the small regulatory RNA NrrF.
### Materials and methods

#### Bacterial strains and culture conditions

All strains used in this study are listed in Table 1. *Neisseria meningitidis* strain H44/76, B: P1.7,16: F3-3: ST-32 (cc32), is closely related to the serogroup B strain MC58, belonging to the same clonal complex [33,34]. Meningococci were cultured in GC broth or on GC plates supplemented with 1% (v/v) Vitox (Difco/Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C in a humidified atmosphere of 5% CO₂. Plates or broth were supplemented with kanamycin (Km) (100 μg·mL⁻¹), erythromycin (Erm) (5 μg·mL⁻¹) and/or chloramphenicol (Cm) (5 μg·mL⁻¹). Overexpression of nrrF in meningococci was induced after 4 h of growth in liquid culture of strains containing 3XFLAG-tagged genes by the addition of 0.5 mM IPTG to the culture medium for 1 h. Growth in broth was monitored by measuring optical density of cultures at 530 nm (OD₅₃₀) at regular time intervals. *Escherichia coli* strain Top10 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was used to clone *gfp* fusions, and in experiments that involved co-expression of *gfp* fusions and sRNA. *Escherichia coli* strain Top10F⁺ (Invitrogen, Thermo Fisher Scientific) was used to clone sRNA expression plasmids. *Escherichia coli* strains were grown in lysogeny broth (LB) or on LB plates at 37 °C. Antibiotics were applied at the following concentrations: 100 μg·mL⁻¹ ampicillin and 20 μg·mL⁻¹ chloramphenicol. *Neisseria meningitidis* H44/76 *hfg*-knockout mutant (Δ*hfg*) was constructed as described previously [35].

#### Plasmids and oligonucleotides

All plasmids used in this study are listed in Table 1 and oligonucleotides in Table S1. Plasmid pCR®2.1 (Invitrogen) was used for cloning and sequencing of PCR products. Plasmids pXG-0 (control for autofluorescence), pXG-1 (control for sRNA effect on *gfp* expression) and pXG-10 (standard plasmid for *gfp* fusion cloning) were kindly provided by J. Vogel (Würzburg) and have been described previously [28]. The 5’UTR of *petA* was fused with *gfp* in pXG-10, thereby creating petA::gfp using primer pair ALpetAF/ALpetAR. The nrrF gene was inserted into the sRNA-expressing plasmid based on pZE12-luc, containing a ColE1 replicon and a strong rrnB terminator, thereby

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**Table 1.** Plasmids and strains used in this study.

| Name          | Plasmid backbone | Genotype or characteristics                              | References       |
|---------------|------------------|---------------------------------------------------------|-----------------|
| pXG-0         | pZA31-luc        | Control for cellular autofluorescence                    | [28]            |
| pXG-1         | pZA31-luc/pXG-10 | Control for sRNA effect on *gfp* expression              | [28]            |
| pXG-10        | pXG-0/pWH601     | for construction of *gfp* fusions                       | [28]            |
| pPetA::gfp    | pXG-10           | *petA* (NMB2053)                                        | This study; [28]|
| pNmNrrF       | pZE12-luc        | *nrrF* (IGR NMB2073-NMB2074)                            | This study; [28]|
| pJV300        | pZE12-luc        | Control nonsense RNA                                     | [28]            |
| nrrF          | pNmNrrF          | *nrrF* (IGR NMB2073-NMB2074)                            | This study; [75]|
| pPetA::gfp    | pXG-10           | *petA* (NMB2053)                                        | This study; [75]|
| pEN11_NrrF    | pEN11_pldA       | *nrrF* (IGR NMB2073-NMB2074)                            | This study; [75]|
| pEN11_Empty   | pEN11_pldA       | Vector control                                          | [39]            |
| pDOC-F        | pEX100T          | 3XFLAG::KmR                                             | Invitrogen      |
| pCR®2.1       |                  |                                                         |                 |

**Plasmids**

**Strains**

| Name          | Relevant marker/Genotype                                      | References       |
|---------------|--------------------------------------------------------------|-----------------|
| H44/76        | Parental strain                                              | [33,34]         |
| H44/76Δ*nrrF* | *nrrF* knockout                                               | This study       |
| YPS1004       | *petA::FLAG::KmR*                                             | This study       |
| YPS1006       | Δ*hfg::ErmR* petA::FLAG::KmR                                  | This study       |
| Escherichia coli Top10 | *mcra Δ (mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG* | Invitrogen      |
| Top10F⁺       | F[lacIq Tn10 (TetR)] mcra Δ (mrr-hsdRMS-mcrBC) Δ80lacZΔM15 ΔlacX74 deoR recA1 araD139 Δ (ara-leu)7697 galU galK rpsL endA1 nupG* | Invitrogen      |
| JVS-2001      | Δ*hfg::KmR*                                                  | [28]            |

**Materials and methods**

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creating pNmNrrF using primer pair ALsRNA3F/ALsRNA3R2. Cloning strategies used are described in [28]. The nrrF gene of *N. meningitidis* strain H44/76 is 100% identical to the sequence of the gene as present in strain MC58 [34,36]. It was cloned at the transcriptional start and end points, as determined by whole transcriptome analysis (WTA) [37]. Mutations were generated using Quickchange site-directed mutagenesis (Stratagene, La Jolla, CA, USA). A nrrF-knockout mutant (ΔnrrF) was created by homologous recombination, replacing nrrF with an Erm resistance cassette ligated between PCR products of primer pairs YPsRNA3FWKO1/YPsRNA3RPKO2 and YPsRNA3FWKO3/YPsRNA3RPKO4 as described before [35]. Correct insertion and orientation of Ern was confirmed with Sanger sequencing using primer pairs YPsRNA3FWKO1/JP22 and YPsRNA3FWKO4/JP19. Overexpression of NrrF in meningococci was achieved using the cloning strategy as described previously [38]. In brief, to construct a vector allowing overexpression of nrrF in meningococci (designated pEN11_NrrF), the region from the unique MauBI through one nucleotide upstream from the transcriptional start of shuttle vector pEN11_pldA was PCR-amplified using primer set YPpen11MauBI/YPpen11plus1. The nrrF gene was amplified using primer set RH_NrrF_C_FW1/RH_NrrF_C_RV1. Both fragments were ligated and PCR-amplified using YPpen11MauBI/RH_NrrF_C_RV1 as primer combination. The resulting PCR product was digested with MauBI and BspHI and ligated into the ~8600-bp gel-purified MauBI and BspHI-predigested pEN11 core plasmid. Appropriate clones were selected using primer pair pen11FW2/pen11R. Expression of nrrF upon induction was verified by RT-qPCR. Plasmid pDOC-F was used as template to generate a DNA fragment by PCR coding for 3xFLAG tag and the Km resistance cassette [39]. A vector control strain was created by cloning pEN11 with pldA removed in the nrrF-knockout strain (ΔnrrF+pEN11_Empty). All constructs were verified by Sanger sequencing.

### Differential protein profiling using LC-MS/E

*Neisseria meningitidis* H44/76ΔnrrF+pEN11_Empty and H44/76ΔnrrF+pEN11_nrrF (three biological replicates each) were grown in iron-rich broth (see above) to logarithmic phase (OD\sub{530}=0.5), centrifuged and frozen at −80 °C. Subsequently, reverse-phase liquid chromatography followed by data-independent alternate scanning mass spectrometry (LC-MS/E) was performed as described [40,41]. Differential expression was analysed by Student’s *t*-test (two-tailed distribution, equal variances assumed) at *P* ≤ 0.01 with false discovery rate (FDR) control according to Benjamini–Hochberg (BH) at q ≤ 0.05 [42]. When appropriate, phase-variable genes were discarded [36,43–49]. To allow for statistical analysis of proteins detected in only one condition, proteins in other samples were assumed to be quantified at least 10% lower than the lowest detected protein (0.08). Up to two samples were given the value 0.07 to minimize overestimation of significance and fold regulation. Previously, this approach led to results that could be satisfactorily confirmed by independent experiments [41]. Gene identification was taken from the original *N. meningitidis* MC58 annotation and updated using the KEGG and UniProt databases [36,50,51].

### Construction of a meningococcal strain expressing a C-terminal 3XFLAG-tagged cytochrome bc₁

A meningococcal strain coding for 3XFLAG-tagged cytochrome bc₁ was constructed as follows. First, a fragment of ~500 bp, ending three nucleotides upstream of the translational STOP codon of petABC, was amplified using the primer pair KSpetC1/KSpetC2 and genomic DNA of strain H44/76 as template to generate fragment A. Next, the 3XFLAG tag and the Km resistance cassette were amplified using plasmid pDOC-F as template and primer set pDOCF1 and pDOCF2 to generate fragment B. Last, a ~500-bp fragment downstream of the STOP codon of the gene to be 3XFLAG-tagged was amplified using primer pair KSpetC3/KSpetC4 and genomic DNA of strain H44/76 as template to generate fragment C. Fragment A was ligated to fragment B and the ligation product was amplified using primer KSpetC1/KSpDOCF2 to generate fragment AB. Next fragment C was ligated to fragment AB and amplified using primer KSpetC1/KSpetC4 to generate fragment ABC. The 3XFLAG tag at the C terminus of petABC was introduced via homologous recombination into the chromosome of strain H44/76 or Δhfq by transformation of the DNA fragment and selection of transformants for Km resistance. *Neisseria meningitidis* was transformed as described previously [52]. Resistant transformants were checked by PCR and Sanger sequencing for integration and orientation of the FLAG epitope and the Km resistance cassette.

### Western blotting

SDS/PAGE and western blotting were performed as previously described [53]. Briefly, culture samples of 1 mL were taken and centrifuged for 3 min at 16 000 g. Pellets were resuspended in 1x SDS/PAGE loading buffer (83 mM Tris/HCl pH 6.8, 13% β-mercaptoethanol, 2% SDS, 3% glycerol, 0.01% bromophenol blue) in such a volume that all samples contain an equal number of cells that is approximately equivalent to an OD\sub{600} of 0.01 per μL. Then, samples were heated for 10 min at 99 °C and an equivalent of an OD\sub{600} of 0.1 was loaded onto an 11% SDS/PAGE. Proteins of the whole-cell fractions were separated at 30 mA for 4 to 5 h. Gels were transferred overnight to nitrocellulose (Schleicher & Schuell BioScience, Dassel, Germany) at 50 mA in a wet tank using a bicarbonate buffer (10 mM
RNA was extracted from meningococci grown to log phase (OD_{600} 0.2–0.5) using the miRNeasy mini kit (Qiagen, Hilden, Germany) followed by Turbo DNase TURBO DNA-free™ kit (Life Technologies, Thermo Fisher Scientific) treatment. Then, cDNA was synthesized from 1.5 μg of RNA and random oligonucleotide hexamers using ThermoScript™ RT (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s recommendations. Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master in the LightCycler® 480 System (Roche, Basel, Switzerland). Identities of the resulting amplicons were checked by melting curve analysis using the LightCycler 480. Reaction mixtures containing no template were included in each real-time PCR experiment to control for contamination. Transcripts of target and reference genes included in each real-time PCR experiment to control for the autofluorescence measured in strains harbouring the NrrF expression plasmid (pNmNrrF) or control nonsense sRNA (pJV300) in combination with the negative control plasmid pXG-0 [expressing luciferase (luc), i.e. no gfp]. The regulatory effect of NrrF on the petA::gfp fusion was expressed as fold regulation (mean of the triplicate values). This is calculated by dividing the unregulated petA::gfp-specific fluorescence (negative control sRNA (pJV300) by the regulated specific fluorescence petA::gfp [sRNA of interest (pNmNrrF)].

Results

Proteomic analysis of Neisseria meningitidis nrrF mutant vs. overexpression strains shows changes in proteins involved in iron homeostasis and oxidative stress response

A total of 559 proteins were reliably detected in the nrrF mutant and overexpression mutants, representing a quarter of the predicted protein-coding content of N. meningitidis strain MC58 [36] (Table S2). Twenty-three proteins were differentially regulated at P ≤ 0.01, 13 being upregulated and 10 downregulated (Table 2). NrrF is a Fur-induced sRNA and is typically repressed in an iron-rich environment. We hypothesized that artificially overexpressing nrrF in an iron-rich medium would upregulate the translation of proteins normally involved in iron uptake and downregulate proteins involved in iron-dependent processes. Indeed, three proteins involved in iron transport (ExbB and FbpA) and (host-acquired) haeme oxidation (HemO) are upregulated. Among the downregulated proteins are CyaY, a protein involved in iron-sulfur-cluster protein assembly and the oxidative stress response protein glutathione peroxidase (GpxA). Of special interest, two other proteins involved in iron-dependent energy generating respiration are downregulated as well. They are the previously identified target SdhA and a novel putative target of NrrF, PetA.
Table 2. Overview of genes differentially regulated in ΔnrrF::pEN11 Empty vs. ΔnrrF::pEN11 _nrrf (P ≤ 0.01).

| Gene ID  | Name          | Function                                      | Pathway or Biological role | Fold change | In silico target |
|---------|---------------|----------------------------------------------|----------------------------|-------------|-----------------|
| Upregulated                      |               |                                              |                        |             |                 |
| NMB2096 | mgo           | Malate:quinone oxidoreductase                | TCA cycle                 | 10.1        |                 |
| NMB2086 | obg           | GTPase                                       | GTPase                    | 8.7         |                 |
| NMB0378 | cysP          | Putative phosphate permease                  | Ion transporter           | 8.6         |                 |
| NMB1839 | fhs           | Formate-tetrahydrofuranate ligase            | Carbon metabolism         | 7.2         |                 |
| NMB0477 | hscB          | GTP-binding protein                          | Unknown                   | 5.1         | Yes             |
| NMB1524 | hscB          | FAD-binding oxidoreductase                   | Unknown                   | 3.4         |                 |
| NMB1383 | fhs           | Formate-tetrahydrofuranate ligase            | Carbon metabolism         | 7.2         |                 |
| NMB0479 | Uncharacterized protein |                | Protein folding           | 3.2         |                 |
| NMB1428 | Putative metalloamineopeptidase             | Aminopeptidase              | 2.6         |             |
| NMB1729 | exbB          | Bipolymer transport protein                  | Iron transporter          | 2.0         | Yes             |
| NMB1445 | recA          | Protein RecA                                 | DNA replication & repair  | 1.9         |                 |
| NMB1946 | Outer membrane lipoprotein                  | Unknown                     | 1.2         |             |
| NMB0634 | fbpA          | Major ferric iron binding protein            | Iron transporter          | 1.2         |                 |
| NMB1669 | hemO          | Heme oxygenase                              | Iron oxidation            | 1.2         |                 |
| Downregulated                     |               |                                              |                  |             |                 |
| NMB1710 | gdhA          | Glutamate dehydrogenase                     | Amino acid metabolism     | –1.6        |                 |
| NMB0479 | Uncharacterized protein |                | Unknown                   | –2.1        | Yes             |
| NMB0171 | minD          | Septum site-determining protein              | Cell division             | –2.6        |                 |
| NMB1978 | cyaY          | Fe-S cluster assembly                        | Oxidative phosphorylation | –4.8        | Yes             |
| NMB2053 | petA          | Ubiquinol-cytochrome c reductase iron-sulfur subunit | Pentose phosphate pathway | –7.3        |                 |
| NMB0815 | gnd           | 6-phosphogluconate dehydrogenase            | TCA cycle                 | –8.2        | Yes             |
| NMB0960 | stdA          | Succinate dehydrogenase flavoprotein subunit | TCA cycle                 | –9.3        |                 |
| NMB1347 | tshB          | Extragenic suppressor protein                | Carbohydrate metabolism   | –13.3       |                 |
| NMB0933 | tada           | tRNA-specific adenosine deaminase            | Transcription/translation | –13.3       |                 |
| NMB1621 | gpxA          | Glutathione peroxidase                      | Oxidative stress          | –13.7       | Yes             |

*a Gene identification and name according to original MC58 annotation, updated based on KEGG and Uniprot databases [36,50,51].
*b Function, pathway or biological role according to the KEGG and Uniprot databases [50,51].
*c LC-MS results, fold change, all genes P ≤ 0.01, boldfaced reached FDR control (BH) q ≤ 0.05.
*d Predicted in silico by TargetRNA, TargetRNA2 or CopraRNA [56,76,77].

Cytochrome bc1 levels are repressed upon expression of _nrrF_.

To confirm the mass proteomic result that expression of _petABC_ was repressed upon expression of _nrrF_, we assessed protein levels of cytochrome _bc1_ before and after overexpression of _nrrF_ in _N. meningitidis_ H44/76. We engineered a _petABC::3XFLAG_ construct and introduced it, by homologous recombination, in the _N. meningitidis_ chromosome at the _petABC_ locus. The recombinant was then transformed with a plasmid containing _nrrF_ under control of an inducible promoter (pEN11_NrrF) to establish overexpression of _nrrF_ independent of the iron concentration in the growth medium. Cytochrome _bc1::3XFLAG_ levels were assessed before and after overexpression of _nrrF_ by western blotting using anti-FLAG antibodies. Relative transcript levels assessed by RT-qPCR of NrrF were ~150-fold higher after overexpression in meningococci, compared to levels in the wild-type (wt) strain (relative transcript levels ~0.1) (not shown). Overexpression of _nrrF_ reduced protein levels of cytochrome _bc1::3xFLAG_ compared to corresponding levels in noninduced cells (~2.5-fold; see Fig. 1). These results indicate that in meningococci, cytochrome _bc1_ levels are repressed upon expression of _nrrF_. This was confirmed by RT-qPCR assessment of transcript levels of _petC_. Overexpression of _nrrF_ reduced transcript levels of _petC_ ~2-fold (P < 0.0001) compared to levels in noninduced cells (not shown).

_In silico_ target interaction analysis of _petA_, a novel putative target of NrrF.

By using the computational tools TargetRNA and CopraRNA, a region of the 5’UTR of _petABC_ (nucleotides ~43 to ~30; relative to the +1 AUG initiation codon of _petA_; A is +1) was predicted to be base pair with nucleotide region 21 to 34 of NrrF [55,56]. The RNAhybrid algorithm confirmed this predicted NrrF-petA duplex [57]. The lowest free energy prediction (ΔG = −21.8 kcal mol⁻¹) is schematically represented in Fig. 2A. Predicted base pairing of NrrF involves a 4 + 9-bp duplex with the 5’UTR of the _petA_ transcript. Nucleotides 21 to 34 of NrrF, except for
Translational repression of NrrF in Neisseria meningitidis

Translational repression of a petA 5’UTR containing region fused to gfp upon expression of nrrF in Escherichia coli

To investigate target recognition and translational control of petABC by NrrF, the direct interaction between the 5’UTR of petABC and NrrF was assessed in vivo using a well-established green fluorescent protein (GFP) reporter system in which target mRNA fusions with GFP are co-expressed with sRNA [28]. Thus, the 5’UTR and AUG initiation codon region of petA (region −122 to +56) were fused in frame to gfp (petA::gfp fusion) in low copy vector pXG10 (recombinant plasmids are listed in Table 1); the resulting petA::gfp is constitutively transcribed from a P\textsubscript{LacO-1} promoter (P\textsubscript{LacO-1}-gfp expression) to specifically assay post-transcriptional regulation. Escherichia coli cells transformed with the petA::gfp fusion were co-transformed either with a plasmid expressing a nonsense (control) sRNA (pJV300) or with plasmid pNmNrrF expressing nrrF. Both these RNAs are expressed from a constitutive P\textsubscript{LacO-1} promoter [28,59]. Escherichia coli harbouring the plasmid pXG-0 (luciferase, no gfp) transformed with pNmNrrF served as control for the effect of NrrF expression on autofluorescence of E. coli. In addition, cells containing the plasmid pXG-1, expressing full-length gfp, carrying a strong SD sequence and an artificial 5’UTR, were also co-transformed with pJV300 and pNmNrrF to serve as controls for the aspecific effect of sRNA expression on PL\textsubscript{LacO-1} expression. Upon transformation, the level of expression of the petA::gfp fusion was assessed by quantifying GFP fluorescence activity of transformants in liquid culture. Autofluorescence of cells harbouring pXG-0 and pNmNrrF or pJV300 was similar. In addition, fluorescence of cells carrying gfp (pXG-1) with or without pNmNrrF or pJV300 was comparable, indicating that pNmNrrF expression has negligible effect on GFP fluorescence. However, cells co-expressing petA::gfp with NrrF expressed significantly lower fluorescence compared to cells in which the petA::gfp fusion was co-expressed with nonsense sRNA. Levels of petA::gfp were ~4-fold repressed (P < 0.0001) upon co-expressing NrrF compared to unregulated fluorescence levels [in the presence of a control nonsense RNA (pJV300)] (Fig. 2B.C). Reduced fluorescence of petA::gfp in the context of nrrF expression but not in the context of expression of a control nonsense sRNA confirms that NrrF represses the petA mRNA by a direct interaction between the 5’UTR of petA and NrrF, most likely via an antisense mechanism.

NrrF represses petABC by a direct interaction between NrrF and the 5’UTR of petABC

To prove that predicted regions of NrrF and petA are indeed involved in base pairing and regulating expression, we engineered NrrF variants and introduced compensatory base pair exchanges in the target region of petA (depicted in Fig. 2A). The effect of the mutations on repression of petA in vivo was assessed using the gfp-reporter system in E. coli. Nucleotides C\textsubscript{21}, C\textsubscript{22}...
and G23 of nrrF were exchanged for G21, G22 and C23, respectively, to generate NrrFM1. Nucleotides C26 and C27 were exchanged for G26 and G27, generating NrrFM2, and lastly, nucleotide C34 was exchanged for G34 to generate NrrFM3. Of note, transcript levels of NrrF variants were transcribed from the same constitutive promoter (PLlacO-1) as in pNmNrrF and did not differ from levels of wt NrrF (not shown). Both NrrFM1 and NrrFM3 expression in the presence of wt target petA::gfp diminished repression (Fig. 2B). However, restoration of the predicted duplex by co-expression of NrrFM1 and NrrFM3 with petA(M1)::gfp and petA(M3)::gfp, respectively (the latter containing compensatory base pair changes for the corresponding mutant NrrFs), restored repression to wt levels (Fig. 2B). These observations support the model of the predicted base pairing of NrrF to petA. Further proof for the direct interaction between NrrF and petA was obtained by the following experiments: Co-expression of NrrFM2 in the presence of wt petA::gfp completely suppressed repression of petA::gfp (Fig. 2B). However, restoration of the predicted duplex by co-expression of petA(M2)::gfp, containing the compensatory base pair mutations C-35 and C-36, completely restored
repression to wt levels (Fig. 2B). Of note, transcripts of petA variants were transcribed from the same promoter (PLtetO-1) as used for wt petA and were found at levels not different from those of wt petA (not shown). Mutations (C12C13G14→G12G13C14) or downstream (C93C94C95→G93G94G95) of NrrF predicted to interact with petA had no effect on regulation (not shown). Taken together, these results provide experimental validation for the predicted RNA duplex of NrrF with the 5′UTR of petA and confirm that NrrF regulates petA expression by an antisense mechanism resulting in repression of translation.

Contribution of Hfq to post-transcriptional regulation of petA by NrrF

The highly conserved protein Hfq has emerged as a key modulator of riboregulation [11,60]. Recently, we reappraised the meningococcal Hfq regulon, showing direct or indirect regulation of a large variety of cellular processes, including OMPs, the methylcitrate and TCA cycles, iron and zinc homeostasis and the assembly of ribosomal proteins [41]. In another recent study, co-immunoprecipitation combined with RNA sequencing of a FLAG-tagged Hfq revealed an unexpectedly large Hfq-RNA interactome, identifying 23 sRNA and 401 mRNA candidate targets [61].

To investigate the potential role of Hfq in regulation of petA, NrrF was first co-expressed with petA::gfp in the E. coli Hfq-knockout strain JVS-2001. The amount of nrrF transcripts in JVS-2001 was similar to that in wt cells (not shown). Of interest, specific fluorescence levels of cells expressing petA::gfp in the absence of NrrF expression were significantly lower (~1.4-fold; \( P < 0.0001 \)) in JVS-2001 compared to those of wt (Hfq-containing) cells expressing the petA::gfp fusion (Fig. 2B). Still, fluorescence of cells expressing petA::gfp was significantly downregulated (~3-fold; \( P < 0.0001 \)) upon NrrF expression (Fig. 2B). These observations indicate that Hfq is apparently not involved in the interaction between NrrF and petA mRNA in the cellular context of E. coli. Next, we tried to investigate whether repression of petA would also be found to be independent of Hfq in the genetic background of the meningococcus. Here, we used the previously engineered petABC::3xFLAG construct and introduced it in the chromosome at the petABC locus of Δhfq [35]. The amount of nrrF transcript levels in Δhfq petABC::3xFLAG was similar to that in wt cells (not shown). Unfortunately, expression of petABC::3xFLAG in Δhfq was below levels allowing quantification (data not shown).

Discussion

Using proteomic analysis, we have shown that NrrF plays a role in the regulation of proteins involved in iron uptake, iron-dependent metabolic processes and oxidative stress. Consequently, we analysed, to our knowledge for the first time, the detailed regulation of expression of N. meningitidis respiratory chain components by a sRNA. In addition, our results provide an explanation for the previously observed iron and Fur-dependent expression of cytochrome bc1. Grifantini and colleagues showed higher transcript levels of petC in serogroup B meningococci grown in the presence of sufficient iron as compared to meningococci grown in the absence of iron [27]. This was confirmed by Basler and colleagues [26] in serogroup C meningococci. In addition, transcript levels of petA were found to be induced by Fur [19]. Here, we show that the iron and Fur-regulated sRNA NrrF is ‘the missing link’, involved in the regulation of cytochrome bc1 by repressing petABC expression.

NrrF was the first meningococcal sRNA to be identified [21]. It was shown that expression of nrrF is controlled by Fur and under iron-depleted conditions, transcript levels of its putative target mRNA sdhC and sdhA were higher in a nrrF-knockout mutant than in wt meningococci [21]. It was further shown that NrrF forms a complex in vitro with a region of complementarity of the sdhCDAB transcript and this duplex formation most likely results in rapid turnover of the transcript [22]. However, until now, sdhCDAB was the only target for NrrF which was experimentally validated.

We provide experimental evidence that cytochrome bc1, encoded by the polycistronic mRNA petABC, represents a novel target of NrrF. We showed that petABC expression is repressed by NrrF in meningococci. Next, we used a heterologous gfp-reporter system to show that repression of petABC is the result of a direct interaction between NrrF and the 5′UTR of petABC, which was confirmed by (complementary) site-directed mutagenesis of the regions of NrrF and petABC predicted to form a duplex. Surprisingly, we showed that this interaction seems to be independent of Hfq.

The region of NrrF interacting with petABC (nucleotides 21–34), as experimentally validated by site-directed mutagenesis in this study, is of interest for several reasons. First, this region is part of the region of NrrF (nucleotides 19–86) that is suggested to also interact with the intergenic region between sdhD and sdhA of the polycistronic mRNA sdhCDAB [21,22]. Unfortunately, in none of these studies, this in silico observation was further experimentally validated or
delineated. Our data suggest that a region of NrrF as short as 14 nucleotides, composed of two stretches of 4 and 9 Watson–Crick pairs, is sufficient to control regulation of petA. Relative short regions of interactions have been described before. For example, only six bps are critical in SgrS-PtsG [5] and RybB duplexes with OMP mRNA range from 15 to 8 bp [62]. Our observation that regulation of petA is completely suppressed in NrrFM2 (having 2 C→G mutations at positions 26 and 27) but completely restored by compensatory substitutions in the 5′UTR of petA(M2) (having 2 G→C mutations at positions −35 and −36) provides ultimate proof for the direct interaction of NrrF and the 5′UTR of petA and for the fact that this interaction is essential for regulation.

We found the experimentally validated region of interaction, contrary to the flanking nucleotides upstream and downstream of it, to be extremely well conserved among the pathogenic meningococci and gonococci, and in the opportunistic pathogen N. lactamica. In commensal Neisseria spp., this region of interaction is largely truncated or absent. Differences in the site of interaction in nrrF between Neisseria spp. may reflect differences in abilities to fine-tune responses to fluctuating iron availability during the specific environmental challenges they encounter.

Another interesting feature of this interaction is the localization of the duplex. It should be noted that many of the sRNA analysed to date inhibit translational initiation of mRNA targets by sequestering the SD and/or AUG sequence of the RBS and thus act close to these sequences [63]. Duplex formation leading to translational interference was proposed to serve as the primary mechanism of target repression, irrespective of concomitant mRNA degradation [64]. However, some sRNA regulate their targets by alternative mechanisms that involve base pairing more upstream of the SD or more downstream of the AUG sequences. This mode of action might exclude a mechanism that relies on direct competition with initiating ribosomes and suggests that the mRNA window for target repression could be broader than the RBS. Here, we showed that duplex formation between NrrF and petA encompasses the region −43 to −30 of petABC, apparently not on top of the SD and/or AUG sequence of the RBS. However, the physical boundaries of the RBS extend from nucleotides −35 to +19 relative to AUG (A is +1) [65,66]. This means that duplex formation between NrrF and petA could take place just inside the physical boundaries of the RBS, possibly resulting in translational interference.

In numerous Gram-negative bacteria, the RNA-binding protein Hfq assists in duplex formations between sRNA and target mRNA by enhancing local RNA concentrations, changing RNA structures and accelerating strand exchange and annealing [11]. Although many trans-acting sRNA characterized require Hfq for base pairing, there are exceptions, among which sRNA in Vibrio cholerae as well as in E. coli [67,68]. When we investigated the involvement of Hfq in the regulation of petA, we found that in the E. coli reporter system, NrrF-mediated repression of petA is independent of Hfq. It should be noted that this might be because E. coli Hfq cannot efficiently interact with N. meningitidis NrrF due to structural differences between E. coli and meningococcal Hfq. However, amino acid homologies of E. coli Hfq and N. meningitidis Hfq are comparable to those of Hfq of E. coli, V. cholerae and Salmonella enterica serovar Typhimurium [35]. Furthermore, N. meningitidis Hfq has been shown to target endogenous Salmonella sRNA, providing evidence of a conserved inherent sRNA-binding property of Hfq [69]. Small RNA-mRNA interactions of the latter two spp. have been successfully assessed in the gfp-reporter system used here and confirmed in the genetic background of the spp. themselves [28]. Previously, the interaction between NrrF and sdhC has been shown to be Hfq independent as well, and there is no in vivo experimental evidence showing SdhC to be part of the Hfq regulon [41,70]. An alternative explanation for the observed lack of Hfq dependency is illustrated by the behaviour of sRNA DsrA. In an E. coli hfq mutant, chromosome-expressed DsrA was unstable. When expressed from a multicopy plasmid, DsrA was stable in both wt and hfq mutant strains, but it had only partial activity in the hfq mutant strain [71]. This might also explain the lacking Hfq dependency observed for NrrF-mediated regulation in E. coli, as NrrF is expressed from a constitutive promoter in a multicopy plasmid. In the E. coli system, NrrF-mediated repression of petA was the same in wt and in Δhfq cells, although we noted that petA expression in the E. coli hfq mutant was significantly lower (P < 0.001) compared to petA expression levels in wt cells. Taking this into account, these results suggest that Hfq itself is involved in the stability of this transcript, irrespective of NrrF levels or duplex formation between NrrF and petA. The exact mechanism by which the duplex formation between NrrF and the 5′UTR of petABC leads to translational repression awaits further experimentation. Our transcript analyses and target protein determinations in meningococci are compatible with a mechanism in which NrrF allows degradation of petABC. Such sRNA-mediated degradation of targets has been described for other sRNA such as MicM in concert with its target yfbM and for Qrr3 in concert with luxO [13,72,73].
The importance of concomitant regulation of the Krebs cycle and the respiratory chain cannot be overstated. They are linked both directly (succinate dehydrogenase being part of both the Krebs cycle and the respiratory chain) and indirectly (the respiratory chain oxidizing, and thus recycling, the electron-rich compounds NADH and FADH₂ formed by the Krebs cycle). Previously, we have shown that the sibling sRNA NmsRs target genes encoding TCA cycle enzymes including SdhC, which is a target of NrrF as well. It is therefore plausible that regulation on the level of translation of proteins involved in both pathways is tightly coordinated by at least two sRNA.

All known cytochrome bc₁ complexes are integral membrane modules, varying wildly in subunit composition. In meningococci, the cytochrome bc₁ complex consists of only three subunits, cyt b (PetB), cyt c₁ (PetC) and the iron-sulfur protein (ISP) (PetA) [31,74], all of them essential in coupling electron transfer to proton translocation over the plasma membrane. In this way, chemical energy is converted into a membrane potential (a proton gradient) which can be used for ATP synthesis. The three subunits all use iron as an essential cofactor in their prosthetic groups. Cyt b has two b-type haemes (low potential b haeme, b₄₁, as well as high potential b haeme, b₄II), cyt c₁ has a single c-type haeme, and ISP contains a two-iron–two-sulfur (2Fe-2S) cluster. The complex thus nicely illustrates the vital role of iron in bacterial metabolism. At the same time, free iron is toxic (e.g. contributing to ROS formation) and the cellular response to iron levels in the environment should be fine-tuned. We show NrrF to be exquisitely involved in this regulation.

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Author contributions

YP, RHV, DS and AvdE conceived and designed the project. RHV, KS, SB and GK acquired the data. YP, RHV, KS, SB, GK, DS and AvdE analysed and interpreted the data. YP, RHV, DS and AvdE wrote the manuscript.

**References**

1 Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215–233.
2 Waters LS and Storz G (2009) Regulatory RNAs in bacteria. *Cell* **136**, 615–628.
3 Papenfort K and Vogel J (2010) Regulatory RNA in bacterial pathogens. *Cell Host Microbe* **8**, 116–127.
4 Altuvia S, Zhang A, Argaman L, Tiwari A and Storz G (1998) The *Escherichia coli* OxyS regulatory RNA represses fhlA translation by blocking ribosome binding. *EMBO J* **17**, 6069–6075.
5 Kawamoto H, Koido Y, Morita T and Aiba H (2006) Base-pairing requirement for RNA silencing by a bacterial small RNA and acceleration of duplex formation by Hfq. *Mol Microbiol* **61**, 1013–1022.
6 Bouvier M, Sharma CM, Mika F, Nierhaus KH and Vogel J (2008) Small RNA binding to 5’ mRNA coding region inhibits translational initiation. *Mol Cell* **32**, 827–837.
7 Chen S, Zhang A, Blyn LB and Storz G (2004) MicC, a second small-RNA regulator of Omp protein expression in *Escherichia coli*. *J Bacteriol* **186**, 6689–6697.
8 Udekwu KI, Darfeuille F, Vogel J, Reimegard J, Holmqvist E and Wagner EG (2005) Hfq-dependent regulation of OmpA synthesis is mediated by an antisense RNA. *Genes Dev* **19**, 2355–2366.
9 Argaman L, Hershberg R, Vogel J, Bejerano G, Wagner EG, Margalit H and Altuvia S (2001) Novel small RNA-encoding genes in the intergenic regions of *Escherichia coli*. *Curr Biol* **11**, 941–950.
10 Maki K, Uno K, Morita T and Aiba H (2008) RNA, but not protein partners, is directly responsible for translational silencing by a bacterial Hfq-binding small RNA. *Proc Natl Acad Sci USA* **105**, 10332–10337.
11 Vogel J and Luisi BF (2011) Hfq and its constellation of RNA. *Nat Rev Microbiol* **9**, 578–589.
12 Gottesman S and Storz G (2011) Bacterial small RNA regulators: versatile roles and rapidly evolving variations. *Cold Spring Harb Perspect Biol* **3**, 1–6.
13 Feng L, Rutherford ST, Papenfort K, Bagert JD, van Kessel JC, Tirrell DA, Wingreen NS and Bassler BL (2015) A qrr noncoding RNA deploys four different regulatory mechanisms to optimize quorum-sensing dynamics. *Cell* **160**, 228–240.
14 De Lay N, Schu DJ and Gottesman S (2013) Bacterial Small RNA-based Negative Regulation: Hfq and Its Accomplices. *J Biol Chem* **288**, 7996–8003.
15 Sauer E (2013) Structure and RNA-binding properties of the bacterial LSm protein Hfq. *RNA Biol* **10**, 610–618.
16 Hoe CH, Raabe CA, Rozhdestvensky TS and Tang TH (2013) Bacterial sRNAs: regulation in stress. *Int J Med Microbiol* **303**, 217–229.
24 Troxell B and Hassan HM (2013) Transcriptional...
22 Metruccio MM, Fantappie L, Serruto D, Muzzi A, Mellin JR, Goswami S, Grogan S, Tjaden B and Genco... 20 Pannekoek Y, Huis In ’t Veld RAG, Schipper K, Delany I, Grifantini R, Bartolini E, Rappuoli R and... 19 Delany I, Grifantini R, Bartolini E, Rappuoli R and... 17 Masse E, Salvail H, Desnoyers G and Arguin M (2007) Small RNAs controlling iron metabolism. "Curr Opin Microbiol" 10, 140–145.
18 Fantappie L, Metruccio MM, Seib KL, Oriente F, Cartocci E, Ferlica F, Giuliani MM, Scarlato V and Delany I (2009) The RNA chaperone Hfq is involved in stress response and virulence in Neisseria meningitidis and is a pleiotropic regulator of protein expression. "Infect Immun" 77, 1842–1853.
19 Delany I, Grifantini R, Bartolini E, Rappuoli R and Scarlato V (2006) Effect of N. meningitidis fur mutations on global control of gene transcription. "J Bacteriol" 188, 2483–2492.
20 Pannekoek Y, Huis In ’t Veld RAG, Schipper K, Bovenkerk S, Kramer G, Brouwer MC, van de Beek D, Speijer D and van der Ende A (2017) Neisseria meningitidis Uses Sibling Small Regulatory RNAs To Switch from Cataplerotic to Anaplerotic Metabolism. "mBio" 8, e02293-16.
21 Mellin JR, Goswami S, Grogan S, Tjaden B and Genco CA (2007) 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" 189, 3686–3694.
22 Metruccio MM, Fantappie L, Serruto D, Muzzi A, Roncarati D, Donati C, Scarlato V and Delany I (2008) The hfq-dependent small non-coding (s) RNA NrrF directly mediates fur-dependent positive regulation of succinate dehydrogenase in Neisseria meningitidis. "J Bacteriol" 4, 1330–1342.
23 Hantke K (2001) Iron and transcriptional regulation in bacteria. "Curr Opin Microbiol" 4, 172–177.
24 Troxell B and Hassan HM (2013) Transcriptional regulation by Ferric Uptake Regulator (Fur) in pathogenic bacteria. "Front Cell Infect Microbiol" 3, 59.
25 Cecchini G (2003) Function and structure of complex II of the respiratory chain. "Annu Rev Biochem" 72, 77–109.
26 Basler M, Linhartova I, Halada P, Novotna J, Bezouskova S, Osiika R, Weiser J, Vohradsky J and Sebo P (2006) The iron-regulated transcriptome and proteome of Neisseria meningitidis serogroup C. "Proteomics" 6, 6194–6206.
27 Grifantini R, Sebastian S, Frigimelica E, Draghi M, Bartolini E, Muzzi A, Rappuoli R, Grandi G and Genco CA (2003) Identification of iron-activated and - repressed Fur-dependent genes by transcriptome analysis of Neisseria meningitidis group B. "Proc Natl Acad Sci USA" 100, 9542–9547.
28 Urban JH and Vogel J (2007) Translational control and target recognition by Escherichia coli small RNAs in vivo. "Nucleic Acids Res" 35, 1018–1037.
29 Tzeng YL, Kahler CM, Zhang X and Stephens DS (2008) MisR/MisS two-component regulon in Neisseria meningitidis. "Infect Immun" 76, 704–716.
43 Cahoon LA and Seifert HS (2011) Focusing homologous recombination: pilin antigenic variation in the pathogenic Neisseria. Mol Microbiol 81, 1136–1143.

44 Saunders NJ, Jeffries AC, Peden JF, Hood DW, Tettelin H, Rappuoli R and Moxon ER (2000) Repeat-associated phase variable genes in the complete genome sequence of Neisseria meningitidis strain MC58. Mol Microbiol 37, 207–215.

45 Snyder LA, Butcher SA and Saunders NJ (2001) Comparative whole-genome analyses reveal over 100 putative phase-variable genes in the pathogenic Neisseria spp. Microbiology 147, 2321–2332.

46 Alamro M, Bidmos FA, Chan H, Oldfield NJ, Newton E, Bai X, Aidley J, Care R, Mattick C, Turner DP et al. (2014) Phase variation mediates reductions in expression of surface proteins during persistent meningococcal carriage. Infect Immun 82, 2472–2484.

47 Anderson MT and Seifert HS (2013) Phase variation leads to the misidentification of a Neisseria gonorrhoeae virulence gene. PLoS ONE 8, e72183.

48 Lucidarme J, Findlow J, Chan H, Feavers IM, Gray SJ, Kaczmarski EB, Parkhill J, Bai X, Borrow R and Bayliss CD (2013) The distribution and ‘in vivo’ phase variation status of haemoglobin receptors in invasive meningococcal serogroup B disease: genotypic and phenotypic analysis. PLoS ONE 8, e76932.

49 Siena E, D’Aurizio R, Riley D, Tettelin H, Guidotti S, Torricelli G, Moxon ER and Medini D (2016) In-silico prediction and deep-DNA sequencing validation indicate phase variation in 115 Neisseria meningitidis genes. BMC Genom 17, 843.

50 Kanehisa M, Sato Y, Kawashima M, Furumichi M and Tanabe M (2016) KEGG as a reference resource for small, noncoding RNAs in bacteria. Nucleic Acids Res 44, D457–D462.

51 UniProt, C (2015) UniProt: a hub for protein information. Nucleic Acids Res 43, D204–D212.

52 van der Ley P, van der Biezen J, Hohenstein P, Peeters Pannekoek Y, Dankert J and van Putten JP (1995) UniProt, C (2015) UniProt: a hub for protein information. Nucleic Acids Res 44, D204–D212.

53 Pannekoek Y, Dankert J and van Putten JP (1995) Construction of recombinant neisserial Hsp60 proteins and mapping of antigenic domains. Mol Microbiol 15, 277–285.

54 Ruitjer JM, Pfaffl MW, Zhao S, Spies AN, Boggy G, Blom J, Rutledge RG, Sisti D, Lievens A, De Preter K et al. (2013) Evaluation of qPCR curve analysis methods for reliable biomarker discovery: bias, resolution, precision, and implications. Methods 59, 32–46.

55 Tjaden B, Goodwin SS, Opdyke JA, Guillier M, Fu DX, Gottesman S and Storz G (2006) Target prediction for small, noncoding RNAs in bacteria. Nucleic Acids Res 34, 2791–2802.
71 Sledjeski DD, Whitman C and Zhang A (2001) Hfq is necessary for regulation by the untranslated RNA DsrA. *J Bacteriol* **183**, 1997–2005.

72 Overgaard M, Johansen J, Møller-Jensen J and Valentin-Hansen P (2009) Switching off small RNA regulation with trap-mRNA. *Mol Microbiol* **73**, 790–800.

73 Figueroa-Bossi N, Valentini M, Malleret L, Fiorini F and Bossi L (2009) Caught at its own game: regulatory small RNA inactivated by an inducible transcript mimicking its target. *Genes Dev* **23**, 2004–2015.

74 Xia D, Esser L, Tang WK, Zhou F, Zhou Y, Yu L and Yu CA (2013) Structural analysis of cytochrome bc1 complexes: implications to the mechanism of function. *Biochim Biophys Acta* **1827**, 1278–1294.

75 Bos MP, Tefsén B, Voet P, Weynants V, van Putten JP and Tommassen J (2005) Function of neisserial outer membrane phospholipase a in autolysis and assessment of its vaccine potential. *Infect Immun* **73**, 2222–2231.

76 Tjaden B (2008) TargetRNA: a tool for predicting targets of small RNA action in bacteria. *Nucleic Acids Res* **36**, W109–W113.

77 Kery MB, Feldman M, Livny J and Tjaden B (2014) TargetRNA2: identifying targets of small regulatory RNAs in bacteria. *Nucleic Acids Res* **42**, W124–W129.

### Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Oligonucleotides used in this study.

**Table S2.** Differential protein profiling (LC-MS²) results of *N. meningitidis H44/76ΔnrrF+pEN11_Empty* and *H44/76ΔnrrF+pEN11_nrrF*.