Post-transcriptional regulation of target genes by the sRNA FnrS in Neisseria gonorrhoeae

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Abstract
Small non-coding RNAs (sRNAs) are well-established post-transcriptional regulators of gene expression in bacteria that respond to a variety of environmental stimuli. They usually act by base-pairing with their target mRNAs, which is commonly facilitated by the RNA chaperone Hfq. In this study we initiated the analysis of the sRNA FnrS of Neisseria gonorrhoeae, which is induced under anaerobic conditions. We identified four putative FnrS target genes using bioinformatics approaches and validated these target genes using translational reporter gene fusions in both Escherichia coli and N. gonorrhoeae, thereby demonstrating their downregulation by direct base-pairing between the respective mRNA and FnrS. We demonstrate deregulation of target mRNAs upon deletion of fnrS and provide evidence that the isc gene cluster required for iron–sulfur cluster biosynthesis, which harbours iscS, which is a direct target of FnrS, is coordinately downregulated by the sRNA. By mutational analysis we show that, surprisingly, three distinct regions of FnrS are employed for interaction with different target genes.

INTRODUCTION
Neisseria gonorrhoeae, the gonococcus, is the causative agent of gonorrhea, the second most common bacterial sexually transmitted disease, which is a major health concern due to increasing treatment failure arising from the rapid spread of antibiotic multi-resistant strains [1]. Gonococcal infection in men is typically accompanied by acute urethritis with urethral discharge and dysuria, while most infected women do not develop symptoms. However, undiagnosed persistent infection in women may cause serious sequelae, like pelvic inflammatory disease, ectopic pregnancy and infertility. Gonococci may also spread from local infections, causing systemic disseminated gonococcal disease (DGI) with manifestations like endocarditis, arthritis, dermatitis and sepsis. Rectal and pharyngeal infections with N. gonorrhoeae are also observed but mostly remain asymptomatic [2].

The ability of a pathogen to adapt quickly to changing environments encountered within the host is a prerequisite for successful colonization. N. gonorrhoeae is able to grow in the absence of oxygen via anaerobic respiration by using nitrite or nitric oxide as an electron acceptor in a truncated denitrification pathway [3, 4]. The observation that antibodies against the anaerobically induced outer-membrane protein AniA are present in sera from patients with gonococcal infections strongly suggests that N. gonorrhoeae faces oxygen limitation during host colonization [5]. Transcriptional profiling and proteome analysis of gonococci grown in planktonic culture or forming biofilms on glass surfaces in continuous flow chambers revealed upregulation of genes that belong to the anaerobic respiratory metabolism, i.e. aniA, norB and ccp encoding nitrite reductase, nitric oxide reductase and cytochrome c peroxidase in the biofilm [6, 7]. Since electron microscopic analysis of biopsy specimens from patients suffering from cervicitis [8] revealed the presence of gonococcal biofilms, anaerobic metabolism might play an important role in the colonization and persistence of Neisseria in the female genital tract.

The unexpectedly large anaerobic stimulon of N. gonorrhoeae comprises 198 chromosomal genes, mostly involved in energy metabolism, biosynthesis of small molecules, adaptation and stress response, regulation, transport and binding, as well as genes encoding hypothetical and phage-associated proteins [9]. Only a few members of the anaerobic stimulon have been reported to be controlled by the oxygen-sensing transcriptional regulator FNR [10]. For the
most extensively studied FNR from *Escherichia coli*, it is
known that in the absence of oxygen a [4Fe–4S] cluster
binds to the N-terminal sensory domain of the protein via
four cysteine residues, resulting in dimerization and activa-
tion of the DNA-binding properties of the transcriptional
regulator. Exposure to oxygen causes the disassembly of the
[4Fe–4S] cluster, resulting in dimer disassociation and inac-
tivation of FNR [11]. Interestingly, a small non-coding
RNA (sRNA) transcript was strongly induced in anaerobi-
cally grown gonococci, and this was named FnrS due to the
presence of a perfect consensus FNR-binding site in the
upstream region, with proper spacing to the transcription
start site [9]. Transcriptional regulation by FNR has been
confirmed for the homologous sRNA from *Neisseria menin-
gitidis*, termed AniS [12]. sRNAs have increasingly been rec-
nognized as important regulatory elements in bacteria. They
may interact directly with proteins, thereby modifying their
activity, yet their predominant mode of function is post-
transcriptional regulation exerted via base-pairing to a tar-
get mRNA [13]. *Trans*-acting sRNAs transcribed from
intergenic regions usually have limited complementarity
with their target mRNA and commonly require the RNA
copherone Hfq for efficient base-pairing [14]. Typically
sRNAs bind to the 5′-UTR of the mRNA target and modu-
late translation initiation by either obstructing the ribo-
some-binding site (RBS) or preventing the formation of an
inhibitory secondary structure of the mRNA itself, which
masks the RBS, resulting in the repression or activation of
translation, respectively. Translational repression and activ-
ation is usually coupled to destabilization or stabilization of
the respective mRNA due to the connection between ribosome loading and mRNA stability. However, sRNA
binding may also directly affect mRNA levels by the specific
recruitment of RNaseE or RNaseIII, leading to rapid degra-
dation of the mRNA, or by the masking of intrinsic RNase
cleavage sites, resulting in transcript stabilization [13, 15].
*Cis*-encoded sRNAs are located in the antisense strand of
their target gene and can modulate mRNA stability and
translation, affect transcription termination or cause tran-
scriptional interference [16]. Besides FnrS/AniS, few sRNAs
have been described in the pathogenic neisseriae. The
Fur-regulated sRNA NrrF, which is transcribed in response
to iron depletion, controls the expression of succinate
dehydrogenase by destabilization of the *sdhCDAB* mRNA
[17–20], and global analysis of transcript stability in a *N.
gonorroeae* nrrF mutant suggested that this sRNA has addi-
tional targets [20]. The sRNA BnsI is upregulated upon
culture of *N. meningitidis* in human blood and was recently
shown to be controlled in response to carbon source avail-
ability by a transcriptional regulator of the GntR family [21, 22]. A bnsI mutant exhibits altered transcription of a me-
tabolic operon encoding enzymes of the methylcitrate cycle,
allowing proionic acid utilization [22]. Furthermore,
sRNAs have been described that affect pilin antigenic varia-
tion in both gonococci and meningococci [23, 24]. Here we
describe the identification and validation of four function-
ally unrelated target genes of gonococcal FnrS.

**METHODS**

**Growth conditions**

All gonococcal strains were grown on Difco GC medium
base (Becton, Dickinson and Co.) plates with 1 % vitamin
mix. Sodium nitrite (5 mM) was added to the plates to be
used for anaerobic cultures. For RNA isolation, overnight
plate-grown bacteria were harvested in PPM medium
(proteose peptone no. 3 (15 g), soluble starch (1 g), KH2
PO4 (4 g), K2HPO4 (1 g) and NaCl (5 g) per litre deionized
H2O). Aerobic plate cultures were grown for 14–16 h in a
37 °C incubator supplying 5 % CO2. Anaerobic cultures
were grown in anaerobic jars using anaerobic gas-genera-
tion kits (Anaerocult A; Merck) at 37 °C for 36–48 h. All
*E. coli* strains were grown in Luria–Bertani (LB) broth or on
LB plates at 37 °C. When required, antibiotics were applied at
the following concentrations: 100 µg ml−1 ampicillin,
50 µg ml−1 kanamycin and 20 µg ml−1 chloramphenicol.
The *E. coli* strains DH5α and TOP 10 were used for cloning.

**Construction of plasmids and *N. gonorrhoeae* mutants**

The plasmids and *N. gonorrhoeae* strains used in this study
are listed in Table S1 (available in the online Supplementary
Material). Unless stated otherwise, chromosomal DNA of
*N. gonorrhoeae* strain MS11 was used as template DNA for
the PCR reactions, which were performed using ReproFast
proofreading DNA polymerase (Genaxxon Bioscience). A
DNA uptake sequence was added to PCR fragments design-
ed for the transformation of *N. gonorrhoeae* via an appro-
priate primer sequence. All PCR fragments used for the
cloning or transformation of *N. gonorrhoeae* were con-
irmed by sequencing. The oligonucleotides used in this
study are listed in Table S2.

To study the FnrS-mediated regulation of target genes, we
used the green fluorescent protein (GFP)-based reporter
system developed by Urban and Vogel [25]. The 5′-UTR
and 30–159 bp of the N-terminal coding region of the gene
of interest were amplified by PCR with appropriate primers
(Table S2) adding an *NsI* site at the 5′-end of the resulting
fragment, which was then cloned into pXG10-SF [26], in frame to GFP. The intrapo-
eronic *iscS* fusion established in pXG30-SF [26] was con-
structed as above, but the sense oligonucleotide was
added to an *NsiI* site at the 3′-end of the resulting
fragment, which was then cloned into pXG10-SF [26], in frame to GFP. The intra-
operonic *iscS* fusion established in pXG30-SF [26] was con-
structed as above, but the sense oligonucleotide was
added to the *NsI* restriction site in frame to a truncat-
ed *lacZ* gene which is part of the vector.

To generate the pHG-fnrS plasmid, PCR was performed
using the primers fnrS FW/pJp RV. The amplified product
containing the *fnrS* gene and 174 nt of its upstream
region was then cloned into pGEM-T as a compatible plas-
mid for cotransformation with the pXG plasmids. The empty
gem-T plasmid was used as a negative control. To
obtain plasmid pJV-152, vector DNA was prepared by the
amplification of plasmid pJV300 [27] with primers pJV
FW/pJV RV and subsequent restriction with XbaI. A PCR
fragment containing sRNA gene NgncR_152 [28] was
amplified with the primer pair NgncR152-1/NgncR152-2 and ligated via blunt end/XbaI cloning. Site-specific mutations were introduced into fnrS by PCR-directed mutagenesis using the plasmid pGEM-fnrS as template DNA. To create the plasmid pGEM-fnrSim1, a PCR fragment generated with the primer pair fnrS FWP/fnrS-ymut1 was ligated directly into pGEM-T vector DNA. DNA fragments harbouring the mutants fnrS-im and fnrS-ym2 were generated via overlap extension PCR. The DNA fragments to be combined, which harboured the desired mutations, were amplified with the primer pairs fnrS FWP/fnrS-imut1 and fnrS-imut2/fnrS RV in the case of fnrS-im, and fnrS FWP/fnrS-ymut2 and fnrS-ymut3/fnrS RV in the case of fnrS-ym2. The combined DNA fragments were finally cloned into pGEM-T vector DNA.

In the knock-out mutant MS11 ΔfnrS the fnrS gene was substituted by the kanamycin resistance cassette of transposon EZ-Tn5 <KAN-2> (Epicentre Biotechnologies). To create this mutant strain, MS11 was transformed with a PCR fragment comprising approximately 500 bp of the upstream and downstream regions of fnrS, which flank the kanamycin resistance cassette. This DNA fragment was created by two consecutive steps of overlap extension PCR performed with individual PCR fragments amplified with the primer pairs fnrS UP FW/fnrS UP RV, kan FW/kan RV and fnrS DN FW/fnrS DN RV, respectively.

For complementation studies, a DNA fragment comprising fnrS and 137 bp of its upstream region (PCR-amplified with primer pair fnrS PVW/fnrS PRV) was cloned into Ndel- and SalI-digested pMR68 vector DNA [29], thereby replacing the P<sub>ig</sub> promoter and the tet repressor gene. The resulting plasmid, pMR68-fnrS, was then transformed into MS11 ΔfnrS, and erythromycin-resistant clones carrying an insertion of fnrS in the iga-trpB locus were selected, yielding strain MS11 ΔfnrS: fnrS. The mutated derivatives of fnrS were amplified from the plasmids pGEM-fnrSim, pGEM-fnrSym1 and pGEM-fnrSym2, with appropriate primers introducing SalI and Ndel sites at the 5′- and 3′-termini. The resulting DNA fragments were cloned into pMR68 and the respective plasmids were transformed into MS11 ΔfnrS. All N. gonorrhoeae mutants were checked by PCR with appropriate primers and sequencing of the resulting DNA fragments.

To study post-transcriptional regulation of FnrS targets in N. gonorrhoeae, yhhF was replaced by a C-terminally FLAG-tagged yhhF derivative in MS11 and MS11 ΔfnrS. For this purpose, the PCR fragments comprising yhhF, including the FLAG-tag and the erythromycin resistance cassette from pMR68, were amplified using the primer pairs yhhF-5/yhhFF-3 and ermC-F5/ermC-3(PstI), respectively, and then assembled via overlap extension PCR. The resulting PCR product was subsequently cloned into the plasmid pSL1180 [30], together with a DNA fragment comprising the downstream region of yhhF, which was PCR-amplified with the primers 1774-5/1774-3. This plasmid was then used for transformation of MS11 and MS11 ΔfnrS to replace yhhF with yhhF: FLAG by homologous recombination.

To prove that prlC and NGFG_02339 are targets of FnrS, GFP fusions were created. To this end, the upstream region, including the promoter and 5′-UTR, as well as 30–90 bp of the N-terminal coding region of the respective genes, was amplified (using the primer pairs prlCgfp FW/prlCgfp RV and 2339gfp FW/2339gfp RV), and the resulting fragments were fused to gfp using overlap extension PCR. gfp was amplified using plasmid pXG10-SF as template DNA with the primer pair Lgfp-5/PFcsiSgfp3. The overlap amplification product was then digested with SalI/XbaI and cloned into pMR68 vector. The resulting plasmids were transformed in MS11 and MS11 ΔfnrS strains.

To prove that iscS is one of the targets of FnrS, iscS was PCR-amplified using the primers iscS-5/iscS-3 and cloned into SacI/PstI-digested pQE30 (Qiagen) vector DNA. The resulting plasmid pQE-iscS was transformed in E. coli M15. Protein expression was induced by the addition of 1 mM IPTG and the recombinant protein was purified using a Ni-NTA column (Akta; GE Healthcare). Antibodies against the purified protein (riscS) were raised in rabbits (Davids Biotechnologie).

**RNA isolation and qRT-PCR**

Total RNA was isolated from N. gonorrhoeae strains using the RNeasy kit (Qiagen), and traces of DNA were removed by an additional on-column digestion with DNase I, according to the manufacturer’s instructions (Qiagen). RNA concentration and quality were measured using a Nano-Drop 1000 instrument (Thermo Fisher Scientific). All RNA samples were checked for DNA contamination in PCR reactions with appropriate primers.

For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using random hexamer primers (Promega) as recommended by the manufacturer. All quantitative reverse transcription PCRs (qRT-PCRs) were performed in triplicate using 1× Brilliant SYBR Green QPCR Master Mix (Stratagene) and appropriate qRT-PCR primers (Table S2). The amplification and detection of specific products were performed with an ABI real-time PCR system. The 5S rRNA gene was used as the endogenous reference control and the relative gene expression was determined using the 2<sup>-ΔΔCT</sup> relative quantification method [31].

**Northern blot analysis**

For the detection of the FnrS transcript, total RNA (30 µg) was separated on a denaturing 15 % polyacrylamide/8 M urea gel and transferred to a nylon membrane (GE Healthcare Life Sciences) for 3 h at 50 V in 0.5× TBE in a wet blotting chamber. An FnrS-specific oligonucleotide probe was labelled with [γ-<sup>32</sup>P]ATP using T4 polynucleotide kinase (Thermo Fisher Scientific). After crosslinking of the nylon membrane, hybridization and wash steps were performed essentially as described previously [28]. 5S RNA was used as a loading control. Size was determined by RNA decade marker (Ambion).

**Immunoblot analysis**

One millilitre of bacterial culture of OD<sub>600</sub> 1.0 was used for sample preparation. Cell suspensions were spun for 10 min
RESULTS

In silico prediction of putative FnrS targets

Deep-sequencing-based analysis of the differential gene expression of *N. gonorrhoeae* F62 under aerobic and anaerobic growth conditions revealed the presence of the 108 nt long sRNA FnrS, which was strongly induced in the absence of oxygen [9]. FnrS was also detected in an RNA-seq transcriptome analysis of *N. gonorrhoeae* strain MS11 cultivated at standard conditions and this was confirmed by Northern blot analysis [28]. To analyse the anaerobic regulation of FnrS expression in strain MS11, Northern blot analysis was performed on RNA from bacteria grown on GC agar plates in the presence or absence of oxygen. As expected, FnrS was also induced anaerobically in *N. gonorrhoeae* MS11, and it was found to be threefold more abundant in the RNA extracted from anaerobically grown bacteria (Fig. 1b). According to Mfold minimum free energy secondary structure predictions [33], FnrS folds into a long 5′-stem loop, followed by a single-stranded region and a hairpin structure at the 3′-end representing the transcription terminator (Fig. 1a). Genome-wide in silico target prediction was performed using the online tools CopraRNA and targetRNA2 [34–36]. To increase the stringency of the prediction, seven bases with perfect complementarity were set as the minimum seed length in the sRNA–mRNA interaction. The putative targets that were predicted by both tools were considered for further analysis, i.e. *yhhF* (NGFG_01775) encoding a putative RsmD family RNA methyltransferase and *iscS* (NGFG_01164) encoding cysteine desulfurase. In Fig. 2 the respective sRNA–mRNA interactions according to the IntaRNA prediction are depicted [36]. The large tripartite interaction region of FnrS with the *yhhF* mRNA extends from the translation start codon (position +2) to position +46 on the mRNA, and covers parts of the single-stranded region and terminator hairpin in the proposed secondary structure of FnrS (positions 62 to 96). In the case of the *iscS* mRNA, the region of complementarity is located in the 5′-UTR immediately upstream of the start codon (positions −2 to −13), including the putative ribosome-binding site (RBS), which, however, shows only limited homology to the Shine–Dalgarno consensus sequence. Binding of the *iscS* mRNA is predicted to be mediated by the single-stranded loop of the 5′-stem–loop of FnrS (positions 19 to 30). RNA-seq analysis indicated that *yhhF* is transcribed monocistronically [28]. *iscS* was suggested to be part of an operon comprising five genes (NGFG_01163 to NGFG_01167 [28]), two of which – *iscS* itself and NGFG_01167 encoding the iron–sulfur (Fe–S) cluster assembly scaffold protein IscU – are involved in Fe–S cluster synthesis, while the first gene of the operon, NGFG_01163, encodes the corresponding transcriptional regulator IscR [28]. In the case of the open reading frames (ORFs) NGFG_01165 and NGFG_01166, annotated as hypothetical and cell filamentation proteins, respectively, a functional relationship to the

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**Fig. 1.** sRNA FnrS is induced under anaerobic conditions. (a) Secondary structure of FnrS. The lowest free-energy structure of FnrS was predicted using Mfold (http://unafold.rna.albany.edu). Regions that are involved in target mRNA binding are highlighted. *yhhF*, solid line; *prIC*, dashed line; NGFG_02339, dotted line; *iscS*, hyphenated line. Sequence motifs that were mutated in FnrS-im (position 24–30). FnrS-ym1 (position 90–96) and FnrS-ym2 (position 73–79) are indicated by bold letters. (b) Northern blot analysis of RNA extracted from *N. gonorrhoeae* MS11 grown under aerobic (+O₂) and anaerobic conditions (−O₂) using a radiolabelled oligonucleotide probe specific for FnrS. Probing for 5S RNA was used as the loading control. For each condition, 5S levels were set to 1, and the relative change in the FnrS level was determined using Image J [32].
Fe–S cluster assembly is not apparent. Interestingly, in *N. meningitidis* these ORFs are not part of the *isc* cluster, indicating a gene acquisition event after the divergence of the pathogenic *Neisseria* from a common ancestor. A closer inspection of the operon structure of the *isc* gene cluster by RT-PCR revealed that the downstream gene NGFG_01168 encoding the Fe–S cluster assembly protein IscA also belongs to the respective transcriptional unit (Fig. S2).

Fantappiè *et al.* [12] suggested two targets for the *N. meningitidis* homologue of FnrS, AniS, namely NMB1468 and NMB0214, due to the observation that compared to the wild-type, the respective mRNAs were downregulated in a mutant of *N. meningitidis* MC58 constitutively expressing FNR, but upregulated when AniS was deleted in the constitutive FNR mutant. In the case of NMB1468, a direct interaction between AniS and the target mRNA could be demonstrated [12]. The MS11 homologue of NMB1468, NGFG_02339, encoding a hypothetical protein, was also detected in our targetRNA2 analysis, but did not score in CopraRNA. The NMB0214 homologue NGFG_01681 (*prlC*) encoding oligopeptidase A was not among the hits in either of our target predictions. Nevertheless, both NGFG_02339 and *prlC* were considered for further experiments for target validation. The regions of complementarity between FnrS and the NGFG_02339 and *prlC* mRNAs as predicted by IntaRNA are shown in Fig. 2. In both cases FnrS is predicted to bind to the 5′-UTR of the respective mRNA, thereby blocking the RBS. In the case of NGFG_02339 and *prlC*, the sRNA–mRNA interactions mainly (NGFG_02339, positions 45 to 69) or exclusively (*prlC*, positions 54 to 79) engage the predicted single-stranded region of FnrS. Both NGFG_02339 and *prlC* are monocistronic genes in *N. gonorrhoeae* MS11 [28].

**Validation of FnrS–mRNA interactions in *E. coli***

To validate the *in silico* predicted FnrS targets, we investigated their sRNA-mediated post-transcriptional regulation in *E. coli* using the GFP-based reporter system developed by Urban and Vogel [25]. The 5′-UTR and region comprising up to 30 codons of the monocistronic putative targets *yhhF*, NGFG_02339 and *prlC*, were fused in frame to *gfp* in vector

![Fig. 2. Predicted regions of complementarity between FnrS and its target mRNAs yhhF (a), iscS (b), prlC (c) and NGFG_02339 (d). Numbers refer to the nucleotide positions with respect to the translational start site (+1) in the case of mRNAs and the transcription initiation site in the case of FnrS. Putative ribosomal binding sites are marked in bold. Nucleotides that were mutated to disrupt the region of complementarity between sRNA and mRNA targets are underlined.](image-url)
pXG10-SF [26]. In the case of iscS, the target gfp fusion was generated in the operon fusion vector pXG30-SF [26]. E. coli DH5α cells harbouring the various pXG plasmids were transformed with either pGEM-fnrS expressing FnrS under the control of its native promoter or the empty pGEM-T plasmid as a negative control. The plasmid pJV-152 expressing the gonococcal Bns1 homologue, NgncR_152, under the control of the P1lacO promoter, served as an additional control. GFP expression from the translational fusions in the various E. coli strains was monitored by Western blot analysis (Fig. 3). As expected, compared to the empty vector control, GFP levels were unaffected by the presence of pJV-152 expressing NgncR_152, which is not supposed to interact with the putative FnrS targets (Fig. 3a, b, c, lane 2 versus lane 1). However, protein expression from the yhhF-, iscS- and NGFG_02339–gfp translational fusions was downregulated to various extents by the presence of pGEM-fnrS, suggesting post-transcriptional regulation of the respective targets (Fig. 3a, b, c, lane 3 versus lane 1). Whereas downregulation was moderate in the case of YhhF-gfp and IscS-gfp (1.7-fold and 2.6-fold, respectively), GFP was no longer detectable in the case of NGFG_02339–gfp. GFP expression from the prIC–gfp fusion was not altered in the presence of pGEM-fnrS (data not shown).

To study FnrS–target mRNA interactions in more detail, mutated derivatives of the fnrS gene were generated and cloned into pGEM-T. In pGEM-fnrSym1, nucleotides 90 to 96 of fnrS were changed from GCCGCCA to TAATATT, abolishing complementarity to the yhhF mRNA in close proximity to the translation start codon (positions 2 to 8 of yhhF mRNA). In a second fnrS mutant, fnrS-ym2 present in pGEM-fnrSym2, the sequence corresponding to nucleotides 73 to 79 (TGTTGTTT), was altered to CCAACCC, disrupting complementarity in the middle part of the tripartite FnrS-binding region of the yhhF mRNA (positions 22 to 27) (Fig. 2a). To abolish complementarity between FnrS and the 5′-UTR of the iscS mRNA, the sequence from positions 24 to 30 of fnrS (CTGTTTC) was changed to AGGCCCG (Fig. 2b) in the plasmid pGEM-fnrSim. The FnrS secondary structure was not affected by mutations ym1 and ym2 according to Mfold analysis, whereas a marginally altered loop structure was predicted for the FnrS 5′-stem loop in the case of FnrS-im (Fig. S3). The mutated sRNA FnrS-ym1 was unable to downregulate GFP expression from the yhhF–gfp translational fusion (Fig. 3a, lane 4 versus lane 3), while only a slight decrease in the amount of GFP was observed in the case of FnrS-ym2, indicating that nucleotides 73 to 96 of FnrS indeed participate in the binding to yhhF mRNA. In fact, our data suggest a larger contribution of nucleotides 90 to 96 exhibiting complementarity to the very 5′-end of the yhhF coding region. sRNA FnrS-im being mutated in a region that is not predicted to interact with yhhF mRNA downregulated GFP levels in E. coli harbouring pXG-yhhF to the same extent as wild-type FnrS. On the other hand, post-transcriptional regulation of the iscS–gfp fusion was abolished in the presence of pGEM-fnrSim (Fig. 3b, lane 4 versus lane 3), confirming the predicted interaction site between FnrS and the iscS mRNA, while both FnrS-ym1 and FnrS-ym2 caused GFP downregulation that was as efficient as that from wild-type FnrS (Fig. 3b, lanes 5 and 6 versus lane 3). These observations indicate that the fnrS mutations we introduced do not result in severe changes of the secondary structure of the molecule, since it remains fully functional as long as the sites required for the particular sRNA–mRNA interaction are not affected. Accordingly, GFP could not be detected when pGEM-fnrSym1, pGEM-fnrSym2 or pGEM-fnrSim was cotransformed with pXG-2339 harbouring the NGFG_02339–gfp translational fusion, as was observed with pGEM-fnrS encoding the wild-type sRNA (Fig. 3c, lanes 4, 5 and 6 versus lane 3). Taken together, target validation in the E. coli background demonstrates that FnrS employs different interaction sites to bind to its yhhF, iscS and NGFG_02339 mRNA targets.

**fnrS knock-out causes deregulation of yhhF, iscS, NGFG_02339 and prIC mRNAs**

To investigate FnrS-mediated regulation in *N. gonorrhoeae*, a knock-out mutant was created by replacing the fnrS gene with a kanamycin resistance cassette in strain MS11. Target validation experiments in *E. coli* suggested an inhibitory effect of FnrS on the translation of yhhF–, iscS- and NGFG_02339–specific mRNAs. Therefore, we reasoned that protection of the respective mRNAs by the actively translating ribosome should lead to an increase of transcript levels in MS11 ΔfnrS compared to the MS11 wild-type. In fact, transcript quantification by qRT-PCR analysis demonstrated a significant increase of yhhF– (−5-fold), iscS– (−3-fold) and NGFG_02339 (−20-fold)–specific mRNAs. When MS11 ΔfnrS was complemented by integration of fnrS and its upstream region in the iga–trpB locus, the detected amount of yhhF and iscS transcript was similar to the wild-type levels (Fig. 4a). The amount of NGFG_02339 transcript decreased substantially in the complemented mutant compared to MS11 ΔfnrS; but remained higher than in wild-type MS11 (Fig. 4a). When RNA extracted from anaerobically grown MS11 wild-type, MS11 ΔfnrS and MS11 Δfnrs: fnrS was analysed for yhhF–, iscS- and NGFG_02339–specific mRNAs. Therefore, we reasoned that protection of the respective mRNAs by the actively translating ribosome should lead to an increase of transcript levels in MS11 ΔfnS compared to the MS11 wild-type. In fact, transcript quantification by qRT-PCR analysis demonstrated a significant increase of yhhF– (−5-fold), iscS– (−3-fold) and NGFG_02339 (−20-fold)–specific mRNAs. When MS11 ΔfnrS was complemented by integration of fnrS and its upstream region in the iga–trpB locus, the detected amount of yhhF and iscS transcript was similar to the wild-type levels (Fig. 4a). The amount of NGFG_02339 transcript decreased substantially in the complemented mutant compared to MS11 ΔfnrS, but remained higher than in wild-type MS11 (Fig. 4a). When RNA extracted from anaerobically grown MS11 wild-type, MS11 ΔfnrS and MS11 Δfnrs: fnrS was analysed for yhhF– and iscS transcript levels, similar results were obtained; however, upregulation of target RNAs was more pronounced than in aerobically grown bacteria (Fig. S5). We also complemented MS11 ΔfnrS with the mutated fnrS genes, fnrS-ym1, fnrS-ym2 and fnrS-im, and analysed yhhF or iscS mRNA levels in the respective mutants. Complementation with both fnrS-ym1 and fnrS-im resulted in an approximately twofold increase of yhhF mRNA; however, yhhF upregulation was clearly less pronounced than in MS11 ΔfnrS (Fig. 4b). This observation suggests that abolishing complementarity in a single motif of the proposed tripartite FnrS–yhhF interaction region does not completely impair the binding of FnrS to the yhhF mRNA. In contrast, FnrS-im seems to be unable to bind the iscS mRNA, since complementation of MS11 ΔfnrS with fnrS-im resulted in similar iscS mRNA levels to those detected in the knock-out mutant (Fig. 4c). Although FnrS did not affect translation of the prIC–gfp fusion in *E. coli*, we compared the prIC transcript levels in the wild-type, MS11
ΔfnrS and complemented mutant MS11 ΔfnrS:fnrS. Surprisingly, prlC was also found to be deregulated in MS11 ΔfnrS (~10-fold), while complementation rescued the wild-type phenotype, suggesting that prlC is among the target genes controlled by FnrS (Fig. 4a).

Since iscS belongs to an operon, we investigated whether FnrS binding to the polycistronic mRNA affects expression of the other members of the operon. Therefore, the transcript amount of iscR, NGFG_01165 and iscU was compared in MS11 wild-type, MS11 ΔfnrS and MS11

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**Fig. 3.** Validation of FnrS targets in E. coli using translational target–GFP fusions. E. coli DH5α was cotransformed with plasmids expressing either a translational yhhF–gfp fusion (a), a translational iscS–gfp fusion (b) or a translational NGFG_02339–gfp fusion (c) and pGEM-T-derived plasmids expressing FnrS (lane 3) or mutated derivatives (lanes 4, 5 and 6) under the control of its native promoter. The empty pGEM-T plasmid (lane 1) and a plasmid expressing the gonococcal sRNA NgncR_152 (lane 2) were included as negative controls. Bacteria from 1 ml of liquid culture were harvested at an OD₆₀₀=1.0 and used for lysate preparation. Western blot analysis using a monoclonal antibody directed against GFP was performed on samples separated on a 12 % polyacrylamide–SDS gel (upper panel). Hybridization of the same membrane with anti-HSP60 antibodies was used as loading control. One representative experiment from three independent replicates is shown. The relative expression given below (a) and (b) is the mean result from the three independent replicates. A vertical bar chart showing protein quantification and including statistical analysis is presented in Fig. S4.
ΔfnrS: fnrS by qRT-PCR. As observed for iscS, fnrS knock-out resulted in an upregulation of the three transcripts, while complementation restored wild-type mRNA levels (Fig. 5), suggesting destabilization of the complete iscS operon as a consequence of FnrS binding.

**FnrS downregulates target gene expression on the protein level**

The regulatory role of FnrS in *N. gonorrhoeae* was also investigated on the protein level. For quantification of iscS expression, the recombinant protein was produced in *E. coli* and a polyclonal antiserum was raised in rabbit. In accordance with the proposed role of FnrS in post-translational regulation of iscS, immunoblot analysis demonstrated a slight (1.5-fold) but significant upregulation in MS11 ΔfnrS, while in the complemented mutant the amount of iscS was similar to wild-type MS11 (Fig. 6a). In order to monitor yhhF, NGFG_02339 and prlC expression, either the respective gene was FLAG-tagged at the 3′-end (yhhF) or translational fusions to gfp under the control of the target gene’s native promoter were constructed (NGFG_02339, prlC) and integrated into the chromosome of wild-type MS11 and MS11 ΔfnrS. Immunoblot analysis using monoclonal anti-FLAG or anti-GFP antibodies revealed an increase in the protein amount upon deletion of fnrS, which was marginal (1.6-fold) in the case of YhhF-FLAG, but clearly evident in the case of PrlC-GFP (2.5-fold) and NGFG_02339-GFP (4.3-fold) (Fig. 6b, c, d). Therefore, these experiments confirm the role of FnrS as a post-transcriptional regulator of iscS, yhhF, NGFG_02339 and prlC.

**DISCUSSION**

Using genome-wide approaches, many sRNAs have been identified in the pathogenic neisseriae [21, 22, 28, 37–39]; however, so far only a few of these putative regulators have been functionally characterized. Here we describe the in silico prediction and validation of target genes of the anaerobically induced sRNA FnrS of *N. gonorrhoeae*. FnrS as well as its meningococcal homologue AniS belong to the FNR...
Deregulation of the transcript and protein levels in the transcript and increased expression of a PrlC and NGFG_02339 was detected in a microarray analysis of gonococcal homologues of the FnrS targets unpublished data). However, no deregulation of the menin-gonorrhoeae and NGFG_02339 are FnrS targets (Figs 4 and 6a, b, d) in N. gonorrhoeae. The changes in protein expression in the absence of FnrS were only marginal (IscS and YhfF) or moderate (PrlC and NGFG_02339); however, it was observed previously that sRNA deletion caused little or no effect on the protein level of target genes, while induced overexpression resulted in a clear downregulation [44].

There is no apparent contribution to a common metabolic process in the case of the four genes controlled by FnrS, and nor is (except for with iscS) the need for anaerobic regulation of the individual genes apparent. NGFG_02339 and prlC encode a lipoprotein and a zinc-dependent metallo-protease whose function is still unclear. yhhF encodes a ribosomal RNA guanine-(N2)-methyltransferase that modifies G966 of the 16S rRNA. The role of this modification is unclear and deletion of yhhF in E. coli caused only a very moderate growth disadvantage [45], yhhF transcription was upregulated upon anaerobic growth, yet yhhF is not part of the FNR regulon in N. gonorrhoeae [9, 10]. Thus, post-transcriptional regulation of yhhF by anaerobically induced FnrS would counteract transcriptional upregulation by an as yet unidentified transcription factor. iscS, as well as other genes from the Fe–S cluster synthesis operon, was found to be downregulated in the absence of oxygen [9]. In E. coli this regulation is mediated by the transcription factor IscR encoded by the first gene of the isc operon, which represses transcription when a [2Fe–2S] cluster is bound to the protein, thereby adjusting Fe–S cluster biogenesis to the cell’s particular demand [11]. Under anaerobic conditions repression is more pronounced due to increased [2Fe–2S] cluster occupancy of IscR as a consequence of less competition between IscR and other Fe–S cluster-containing proteins, since lack of oxygen and reactive oxygen species increases the stability of oxygen-labile Fe–S clusters and, therefore, decreases their turnover [46]. However, the regulatory activity of FNR requires de novo synthesis of [4Fe-4S] clusters via the isc machinery [47]. Post-transcriptional regulation by FnrS might therefore fine-tune Fe–S homeo-stasis under anaerobic conditions, since further downregulation of the isc operon would eventually lead to relief of repression by the negative feedback loop operating via IscR. In fact, our data suggest that binding of FnrS to the S' UTR of the iscS mRNA not only impedes translation (Fig. 3b), but rather downregulates the complete isc operon (Fig. 5), possibly by destabilization of the polycistronic transcript. Such coordinate regulation of an operon by a sRNA has been observed in the case of manXYZ of E. coli encoding a broad spectrum sugar transporter of the phosphoenolpyruvate phosphotransferase system. The sRNA SrgS binds to sequences within the coding region of manX and within the manX–manY intergenic region, and thereby represses translation of manX and the translationally coupled manY and manZ by independent mechanisms. Intriguingly, concomitant sRNA-mediated manXYZ mRNA degradation requires base-pairing of SrgS to both binding sites [48, 49]. Moreover, coordinate regulation was suggested in the case of sRNA SraG of Yersinia tuberculosis, which controls a

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**Fig. 5.** Transcriptional analysis of the isc operon. The amount of iscR, NGFG_01165- and iscU-specific mRNAs in N. gonorrhoeae MS11, MS11 ΔfnrS and MS11 ΔfnrS:fnrS was detected by qRT-PCR. The ratios of the transcript amounts relative to the wild-type are depicted. The 5S RNA gene was used for data normalization. The indicated ratios represent the means of the results of three qRT-PCR experiments performed in triplicate on cDNAs obtained from independent RNA preparations. Error bars indicate the standard deviation. Statistical significance was determined by using one-way ANOVA and Tukey's post hoc analysis in comparison to wild-type. *P<0.05, **P<0.01, ***P<0.001.
bicistronic mRNA derived from the YPK_1206-1205 operon encoding proteins of unknown function. This regulation was shown to depend on sequences within the coding region of YPK_1206 [50]. Interestingly, in E. coli Fe–S cluster biosynthesis is controlled by the Fur-regulated sRNA RhyB, which is induced upon iron depletion [51, 52]. RhyB base-pairs to the 5¢-UTR of the iscS mRNA and thereby promotes the degradation of the 3¢-region of the polycistronic mRNA encoding iscSUA. The iscR mRNA is protected from degradation by a strong secondary structure, which is formed by the extended intergenic region between iscR and iscS, resulting in accumulation of iscR mRNA when RhyB is induced [53]. Apo-IscR lacking the [2Fe–2S] cluster is a transcriptional activator of the Fur-repressed suf operon encoding another Fe–S cluster synthesis machinery that is operating under iron depletion and oxidative stress [54, 55]. Thus, discordant regulation of the isc operon by RhyB ensures maximal expression of the suf cluster under iron-limiting conditions.

It is assumed that most sRNAs interact with their target mRNAs via a single, highly conserved domain, which is usually located at the 5¢-end [56]. Our data indicate that FnrS employs different regions to base-pair with its target mRNAs. Binding to the yhhF mRNA occurs via sequences covering the terminator hairpin and part of the predicted single-stranded region of FnrS. Expression analysis of a yhhF–gfp translational fusion in E. coli in the presence of wild-type and mutated FnrS suggested that base-pairing of a sequence motif at the 3¢-end of FnrS close to position +1 of the mRNA is most crucial for yhhF downregulation (Fig. 3a). It should be emphasized that sRNA binding only occurs within the coding region in the case of yhhF, and the other three validated targets base-pair within the 5¢-UTR. Sequestering of the RBS is considered to be the prevailing mechanism for translational repression by sRNAs; however, it has been demonstrated that binding to the 5¢-mRNA coding region can also inhibit translational initiation [57]. Mutation analysis confirmed that binding of FnrS to the iscS mRNA is mediated by the loop sequence of the

![Figure 6](image-url)

**Fig. 6.** Protein expression of FnrS targets in N. gonorrhoeae. Equal amounts of proteins extracted from the respective N. gonorrhoeae strains were analysed by immunoblotting using appropriate antibodies, as indicated on the right side of the panel (upper panel). Hybridization of the same membrane with anti-HSP60 antibodies served as the loading control (lower panel). (a) Detection of IscS in MS11 (lane 1), MS11 ΔfnrS (lane 2) and MS11 ΔfnrS : fnrS (lane 3). (b) Detection of fusion protein YhhF-FLAG in MS11 yhhF-F (lane 1) and MS11 yhhF-F, ΔfnrS (lane 2). (c) Detection of fusion protein PrlC-GFP in MS11 prlC-gfp (lane 1) and MS11 prlC-gfp, ΔfnrS (lane 2). (d) Detection of fusion protein NGFG_02339-GFP in MS11 2339-gfp (lane 1) and MS11 2339-gfp, ΔfnrS (lane 2). The relative expression (fold change) depicted below (a), (b), (c) and (d) is the mean result from the three independent replicates. Statistical significance was determined by using Student’s t-test analysis in comparison to wild-type. *P<0.05, **P<0.01, ***P<0.001.
predicted 5'-stem-loop (Fig. 3b), whereas base-pairing with the prlC- and NGFG_02339 mRNAs is predicted to occur via the single-stranded region of FnrS (Fig. 2c, d). Fantappié et al. [12] previously confirmed the binding of AniS to NMB1468 via the single-stranded region of the sRNA. Accordingly, translational repression of an NGFG_02339–gfp fusion in E. coli was not affected by FnrS mutations specifically targeting the regions involved in base-pairing to yhhF and iscS mRNAs (Fig. 3c). Interaction with different sets of target genes via separate regions of the sRNA has previously been reported, for instance in the case of E. coli FnrS [41], spot 42 [58] and Salmonella SdsR [44]. The enterobacterial sRNA GcvB, as a regulator of amino acid metabolism, controls the majority of its target genes via base-pairing of the same single-stranded region (denoted R1) to the mRNAs. However, translational repression of the glycine transporter CycA was shown to involve multiple redundant regions of GcvB [59], while repression of the response regulator PhoP, which was observed in E. coli, requires yet another defined interaction site (R3) located in the 3′-region of GcvB [60]. VqmR of Vibrio cholerae employs a common interaction region (R2) for the control of most of the confirmed directly regulated mRNAs, while one target base-pairs to a different region (R1) of VqmR [61].

In conclusion, we reported the interaction of gonococcal FnrS with four functionally unrelated target mRNAs via base-pairing of three different regions of the sRNA molecule. For the moment, the question of how FnrS-mediated down regulation of the identified target genes contributes to the metabolic adaptation to anaerobic growth remains open. However, a characterization of the complete neisserial FnrS regulon might contribute to the clarification of this issue.

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Conflicts of interest
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