Riboregulation in Neisseria meningitidis

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Chapter 4

Molecular characterization and identification of proteins regulated by Hfq in Neisseria meningitidis

Yvonne Pannekoek, Robert Huis in’t Veld, Carla Hopman, Ankie Langerak, Dave Speijer, and Arie van der Ende

FEMS Microbiology Letters, 2009
Chapter 4

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Molecular characterization & identification of Hfq-regulated proteins in *N. meningitidis*

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Abstract

Hfq is a highly conserved pleiotropically acting prokaryotic RNA-binding protein involved in the posttranscriptional regulation of many stress responsive genes by small RNAs. In this study, we show that Hfq of the strictly human pathogen Neisseria meningitidis is involved in regulation of expression of components involved in general metabolic pathways, iron metabolism and virulence. A meningococcal hfq deletion strain (H44/76Δhfq) is impaired in growth in nutrient rich media and does not grow at all in nutrient limiting medium. The growth defect was complemented by expression of hfq in trans. Using proteomics, the expression of 28 proteins was found to be significantly affected upon deletion of hfq. Of these, 20 proteins are involved in general metabolism, among them 7 iron responsive genes. Two proteins (PilE, TspA) are involved in adherence to human cells, a step crucial for the onset of disease. One of the differentially expressed proteins, GdhA, was identified as an essential virulence factor for establishment of sepsis in an animal model, studied earlier. These results show that in N. meningitidis Hfq is involved in the regulation of a variety of components contributing to survival and establishment of meningococcal disease.
Introduction
A prokaryotic riboregulated network, in which small RNAs (sRNAs) in conjunction with specific proteins, regulate the translation and/or decay of mRNAs has recently been discovered. These regulatory events commonly require the action of the Sm-like protein Hfq. Hfq is a strikingly conserved pleiotropically acting RNA-binding protein, facilitating base pairing between sRNA and mRNA, which in general may either decrease ribosome binding or unmask the RNaseE cleavage site leading to mRNA decay, or improve ribosome binding leading to mRNA stability. Recent studies have shown that Hfq extensively impacts bacterial physiology, including control of virulence factors. Null mutants of hfq of a variety of pathogens among are highly attenuated in animal models.

The strictly human pathogen *Neisseria meningitidis* causes septicemia and meningitis, life-threatening disease, especially in childhood, and is a serious public health problem world-wide. This pathogen possesses a variety of genes involved in the adaptation to the different environments encountered in the host, including iron depletion. All four available completely sequenced genomes of *N. meningitidis* contain a gene with significant homology to *hfq*. Hfq of *N. meningitidis* was identified as an essential protein for the onset of septicemia by using signature-tagged transposon-mutated meningococci in an infant rat model. This observation strongly suggests that Hfq and genes under control of Hfq in the meningococcus are of importance for establishing disease. To identify which genes of *N. meningitidis* are regulated by Hfq, we constructed a hfq knock out strain and used a proteomic approach to identify proteins of which the expression is under control of Hfq.

Materials & Methods

Bacterial strains and culture conditions
*N. meningitidis* strain H44/76, B: P1.7,16: F3-3: ST-32 (cc32), is closely related to the sequenced serogroup B strain MC58 and belongs to the same clonal complex (van der Ende *et al.*, 1999). Meningococci were cultured in tryptic soy broth (TSB) (BD), GC broth or on GC plates (Difco) supplemented with 1% (vol/vol) Vitox (Oxoid) at 37°C in a humidified atmosphere of 5% CO₂. If appropriate, plates or broth were supplemented with erythromycin (Erm) (5 μg/mL) and/or chloramphenicol (Cm) (25 μg/mL). Growth was monitored by measuring the
optical density of cultures at 600 nm (OD$_{600}$) at regular time intervals. Growth experiments were repeated 5 times.

Construction of a $hfq$ knock-out mutant of *N. meningitidis*

A *N. meningitidis* H44/76 $hfq$ knock-out mutant (H44/76Δ$hfq$) was constructed using the PCR-ligation-PCR method$^{15,16}$. PCR products were generated with primer pairs ABHfq1/ABHfq2 and ABHfq3/ABHfq4, ligated and the ligation product was reamplified with primer pair ABHfq1/ABHfq4. The resulting PCR product was cloned into pCR2.1 (Invitrogen). The EcoRI digested Erm resistance cassette from pAErmC$^{17}$ was introduced into the created unique MfeI restriction site yielding plasmid pHfq10. H44/76Δ$hfq$ was generated by natural transformation of strain H44/76 with pHfq10 and selection for erythromycin resistance. Replacement of $hfq$ (NMB0748) by the Erm cassette was confirmed by PCR with combinations of primers JP19-20, JP22, ABHfq1 and ABHfq4. Oligonucleotides are listed in Table 1.

**Table 1. Oligonucleotides used in this study.** Genomic localization according to Tettelin et al.$^{13}$, GenBank AE002098.2 and Projan et al.$^{18}$, GenBank M17990.1.

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Complementation of H44/76Δ$hfq$

To complement the $hfq$ deletion, $hfq$ from strain H44/76 was amplified with primer pair ALHfq11/ALHfq12, containing NdeI and RcaI restriction sites respectively. The resulting PCR product and shuttle vector pEN11-pldA$^{19}$ were digested with NdeI and RcaI, and the PCR product was cloned into NdeI-RcaI predigested pEN11-pldA and transformed to *E. coli* TOP10F$'$ (Invitrogen). Cm-resistant colonies were checked by colony PCR and sequencing, using universal M13 primers. Plasmid DNA of a clone containing a complete intact $hfq$ coding region gene (pEN11-$hfq$2) was isolated and used to transform H44/76Δ$hfq$.  

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Expression of *hfq* was induced by addition of IPTG to the culture medium to a final concentration of 1 mM. The DNA sequence of *hfq* of H44/76 was deposited into Genbank (FJ606876).

**RT-PCR**

RNA was isolated using RNeasy Midi Kit (Qiagen). RT-PCR was done using SuperScriptIII (Invitrogen). Primer pairs ALHfq13/ALHfq15, ALHfq13/ALHfq16 and ALHfq14/ALHfq16 were used to investigate whether NMB0747-NMB748-NMB749 is transcribed as a polycistronic operon and primer pair ALNMB0747F/ALNMB0747R to investigate transcription of NMB0747 in H44/76Δhfq.

**Cell fractionation**

Meningococci were grown in broth until OD$_{600}$ = 0.6-0.8, harvested by centrifugation (10 min at 3000 RCF) and resuspended in 50 mM Tris-HCl (pH 7.8). Of the remaining culture medium, blebs were removed by centrifugation (100,000 × *g*, 1 h, 4 °C). The so-obtained supernatant was used as source of secreted proteins. Meningococcal cells were disrupted by sonication (Branson B15 Sonifier, 50 W, 10 min, 50% duty cycle, 4°C), followed by centrifugation (3,000 × *g*, 10 min, 4 °C). The supernatant was centrifuged (28000 × *g*, 30 min, 4°C) and pellets, containing the cell envelops (inner and outer membranes) were resuspended in 2 mM Tris-HCl (pH 6.8) containing 1% Sodium Lauroyl Sarcosinate and incubated overnight at 4 °C to dissolve inner membranes. The outer membrane fraction was then obtained by centrifugation (100,000 × *g*, 2 h, 4 °C) and dissolved overnight in 200 µl 2 mM Tris-HCl (pH 6.8) at 4 °C. All fractions were stored at -20 °C. Protein concentrations were determined by Protein Assay (Bio-Rad).

**One-dimensional SDS-PAGE**

Proteins were resolved by SDS-PAGE$^{20}$ Gels (12% or 25%) were stained with the PageBlue kit (Fermentas), washed in MilliQ water and stored in 1% acetic acid at 4 °C until bands of interest were excised for further analysis.

**Two-dimensional gel electrophoresis and MALDI-TOF Mass Spectrometry**

Samples were dissolved in 2D-sample buffer (7.7 M urea, 2.2 M thiourea, 30 mM Tris-HCl pH 8.5, 4% CHAPS and a trace bromophenol blue), 1.25 µL DeStreak
solution (GE Healthcare) and 2.5 µL IPG-buffer pH 4-7 (GE Healthcare) were added. First dimension IPG-strips (Immobiline DryStrip pH 4-7, GE Healthcare) were applied on top of the sample solution, covered with oil and incubated overnight at RT. Isoelectric focusing (IEF) was performed on the Protean IEF cell (Bio-Rad) basically with gradually increasing voltage up to 3,500V according to the standard GE Healthcare protocol. After IEF, strips were incubated in 1 mL equilibration buffer (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromophenol blue) with 10 mg dithiothreitol (DTT) added, for 20 minutes on a shaker at RT. Strips were transferred to 1 mL equilibration buffer with 25 mg iodoacetamide and again incubated for 20 minutes. The IPG strip was transferred from the tube to the top of a 12% SDS-PAGE gel and the gel was covered with a warm solution of 1% low-melting-point agarose, 15% glycerol and a trace of bromophenol blue in Laemmli buffer. Electrophoresis was conducted at 200 V. Gels were stained with PageBlue, washed in MilliQ water and stored in 1% acetic acid at 4 °C until spots of interest were excised for further analysis. MALDI-TOF mass spectrometry was carried out as described previously. All included protein assignments are unambiguous. Monoisotopic peaks in the peptide mass fingerprint spectrum were searched against complete non-redundant database of all organisms (MSDB at MASCOT). Only in case of a significant MOWSE score of a meningococcal protein as top hit was the identification considered reliable (pI and MW were also not restricted in the search and were found to match). We only analyzed proteins the expression of which was reproducibly and markedly altered (‘on-off’ proteins) when comparing wt, H44/76Δhfq and pEN11-hfq2 complemented cells (in both one-dimensional and two-dimensional gels). This approach guarantees that the protein identified is indeed the one under Hfq control.

Results

Hfq is conserved among neisserial species and part of a polycistronic operon

The complete genome sequences of four meningococcal, two strains of the closely related human pathogen N. gonorrhoeae and one commensal neisserial sp. (N. lactamica) are known. The amino acid sequence of Hfq of the meningococcal strain used in this study (H44/76) is identical to the Hfq sequence of meningococcal strain FAM18 and 98% identical to the sequences of the other three meningococcal strains. In addition, the sequence of H44/76 is 98% identical.
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to gonococcal Hfq and 95% identical to the Hfq sequence of *N. lactamica*. The H44/76 *neisserial* Hfq amino acid sequence shows 63 and 60% identities to Hfq proteins of *E. coli* and *Vibrio* spp., respectively (Figure 1). Of importance, hardly any amino acid substitutions in the meningococcal *hfq* sequence, compared to those of *E. coli* and *Vibrio* spp. are observed in highly conserved regions of the protein shown to be important for functionality (Figure 1)23.

**Figure 1. Amino acid sequence alignment of Hfq proteins of E. coli serotypes, Vibrio species, N. meningitidis, N. gonorrhoeae strains and N. lactamica.** The secondary structural elements of Hfq protein are shown above the alignment with the N-terminal helix α1 in cyan. The Sm1 and Sm2 motif regions are boxed. The sole conserved residue Gly34 is indicated in red. Highly conserved hydrophobic residues within the Sm1 region are indicated by a lower-case h, and the two highly conserved glycine and aspartic acid residues within the Sm1 motif are indicated by G and D, respectively. The absolutely conserved glutamine of helix α1 that is important for base recognition and the highly-conserved tyrosine (or phenylalanine) are indicated in green. Within the Sm2 region, the ‘Hfq Sm2 motif YKH’ is colored yellow. Other conserved residues are indicated by an asterisk (*). Note the minimal sequence variation between Hfq of *E. coli* serotypes (lines 1-5), *Vibrio vulnificus* (line 6), *V. harveyi* (line 7), *V. parahaemolyticus* (line 8), *V. fischeri* (line 9), *N. gonorrhoeae* strains (line 10 and 11), *N. meningitidis* strains (lines 12-15) and *N. lactamica* (line 16) (adapted from23).

The genetic organization of the chromosomal locus of *hfq* among all except one (*N. gonorrhoeae* FA1090) *neisserial* strains investigated is also conserved. In *neisserial* spp. the *hfq* gene is preceded by a gene encoding D-alanyl-D-alanine endopeptidase (NMB0749, *pbp7*). Eighty-four bp downstream of *neisserial* *hfq*, an ORF encoding a conserved hypothetical protein is found (NMB0747, Figure 2a). To determine whether *neisserial* *hfq* was transcriptionally linked to either of the two flanking genes, RT-PCR analysis was performed, templated by total RNA from meningococcal strain H44/76 using primers annealing to *pbp7* and the
downstream gene. This RT-PCR yielded a product of ~800 bp, indicating that hfq is transcriptionally linked to pbp7 and NMB0747 (Figure 2b).

Figure 2. Transcriptional analysis of the hfq operon.
A. Schematic representation of the hfq polycistronic operon. Primers used in RT-PCR are indicated in black arrows (→). The size of calculated RT-PCR products is indicated below the black lines (\_). A

B. RT-PCR results. Products obtained by RT-PCR were separated on agarose gel. Numbers on the left represent marker sizes, primer-pairs used in the RT-PCR are indicated above the lanes. Reactions in which the addition of Reverse Transcriptase was omitted did not yield any products (not shown). B
Hfq is required for optimal growth of *N. meningitidis*

A H44/76Δhfq strain of *N. meningitidis* was created by complete gene replacement of *hfq* with an Erm resistance cassette. Upon examination of the growth characteristics of the H44/76Δhfq strain, it was observed that this strain formed very tiny colonies after overnight growth on rich solid media, compared to wild type (wt) strain. In addition, the strain did not grow in TSB, a relatively nutrient poor broth, and exhibited a growth deficiency in GC broth, compared to the wt strain (Figure 3). As the Erm resistance cassette used here does not contain a terminator, transcription of NMB0747 should be unaffected. This was confirmed by RT-PCR (not shown). In addition, expression of *hfq* in *trans* in GC broth clearly restored growth (Figure 3).

Figure 3. Growth kinetics of H44/76Δhfq and complementation of the growth defect by expression of neisserial hfq in *trans*. Growth of the strains at 37°C was followed by measuring the density of the cultures at intervals. +: H44/76 wild type strain; Δ = H44/76Δhfq; □ = H44/76Δhfq + pEN11-hfq2 (induced by IPTG). Shown is a representative experiment.
Identification of proteins differentially expressed in H44/76 wt as compared to H44/76∆hfq strain

To identify genes of which the expression is controlled by Hfq, protein patterns of the WT strain, the H44/76∆hfq strain and the complemented strain were compared by one- and two-dimensional gels. Only those proteins, of which the differential regulation was confirmed in at least two independent experiments and of which the expression was turned to wt strain levels in the complemented strain were considered truly differentially regulated. Proteomic analyses of patterns of whole cell lysates, cytoplasm, cell envelopes, outer membranes and growth medium (secreted proteins) showed that the expression of at least 28 proteins in *N. meningitidis* was affected by Hfq (Figure 4). Of these 28 proteins, 23 were upregulated in the H44/76∆hfq strain while the other 5 proteins (PilE, RplE, GdhA, AtpA and AtpD) were downregulated in the absence of Hfq (Figure 4, Table 2). The majority (n= 19) of the differentially regulated proteins were identified in the whole cell lysates and/or cytoplasmic fractions, 8 proteins were identified in membrane fractions and 1 protein (isocitrate dehydrogenase: icd; NMB0920) was identified in both the whole cell lysate as well as in the secreted protein fraction of H44/76∆hfq strain. (Figure 4, Table 2).

Of the 28 proteins differentially expressed between the wt and H44/76∆hfq strain, 12 are functionally involved in general metabolism and 8 are involved in ribosomal protein synthesis/modification, amino acid biosynthesis, protein translation and modification. Two proteins involved in detoxification (SodB and SodC), one iron(III) ABC transporter (FbpA), and one molecular chaperone (DnaK) were also identified. In addition, two proteins with largely unknown function (encoded by NMB2091 and NMB0946) were also differentially expressed in the H44/76∆hfq strain (Table 2). Of interest, in cell envelopes of the H44/76∆hfq strain, PilE, the structural subunit of the Type IV pili (Tfp) and a well-characterized virulence factor involved in adherence to cells and cell motility of meningococci24,25 was no longer detectable (Figure 4B). In addition, a protein designated T-cell stimulating protein A (TspA) also implicated in adherence of meningococci to cells25, was found to be upregulated in H44/76∆hfq.
Figure 4. Proteins differentially regulated in H44/76Δhfq (continues next page)
A. One-dimensional analysis of protein patterns of a whole cell lysate of wt and H44/76Δhfq cells. B. One-dimensional analysis of cell envelopes of wt and H44/76Δhfq cells. Differentially expressed proteins comparing wt and H44/76Δhfq cells are indicated. Whole cell lysates were run on 12% gels, cell envelopes on 25% gels.
C. Two-dimensional analysis of protein patterns of whole cell lysates of wt (left panel) and H44/76Δhfq cells (right panel)
### Table 2. Differential regulated proteins in H44/76Δhfq.

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<td>NM08198</td>
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<td>W</td>
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<td>Cu-Zn superoxide dismutase</td>
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<td>NM08172</td>
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<td>+</td>
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<td>NM08158</td>
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<td>3-Hydroxyacyl dehydrogenase</td>
<td>Amino acid metabolism</td>
<td>30378</td>
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<td>21745</td>
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<td>49664</td>
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</table>

*) Gene identification according to Tettelin et al. 13
†) Protein name according to Tettelin et al. 13
‡) Fraction in which the protein was identified: W: whole cell lysate; C: cytoplasm; CE: cell envelop; IM: inner membrane; OM: outer membrane; SP: secreted protein fraction
§) Up or down regulation in hfq knockout
¶) Protein function according to KEGG (http://www.genome.jp/kegg)
‖) Functional classification according to Parkhill et al. 11
**) Protein molecular weight according to JCVI-CMR (http://cmr.jcvi.org/cgi-bin/CMR/shared/Menu.cgi?menu=genome)
††) Isoelectric point according to JCVI-CMR (http://cmr.jcvi.org/cgi-bin/CMR/shared/Menu.cgi?menu=genome)
‡‡) Figure in which spot and/or band is identified. NS; not shown.
§§) Protein name according to Oldfield et al. 25
¶¶) Iron-responsive gene 7-9

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Discussion

The Hfq protein is recognized as a major post transcriptional regulator of bacterial gene expression participating as a RNA chaperone in numerous regulatory pathways\(^2\). In this study, we explored the Hfq regulon of the strictly human pathogen \(N. meningitidis\).

Loss of Hfq function in the meningococcus (coding capacity \(\sim 2200\) genes)\(^1\) resulted in clear deregulation of approximately 28 proteins (>1 % of total coding capacity) as found by comparative proteomics. Using approaches comparable to ours, it was found that loss of Hfq function in \(Salmonella enterica\) serovar Typhimurium and \(Pseudomonas aeruginosa\) leads to deregulation of approximately 2% and 5% of the total coding capacity respectively\(^5,26\) and is thus comparable to what we found in meningococci.

The genetic organization of \(hfq\) of \(N. meningitidis\), however, is different from that found in most other pathogens studied so far. For example, in \(E. coli\), \(S. Typhimurium\) and \(Listeria monocytogenes\), \(hfq\) is located downstream from \(miaA\), which encodes a protein similar to tRNA isopentenylpyrophosphate transferase and upstream from \(hflx\), encoding the putative GTP-binding protein\(^5,27,28\). In these pathogens, \(hfq\) is usually co-transcribed with \(miaA\), and transcription is terminated directly after \(hfq\). In \(N. meningitidis\) \(hfq\) is located downstream from \(pbp7\), encoding D-alanyl-D-alanine-endopeptidase (penicillin binding protein 7) and upstream of a hypothetical gene encoding a predicted rRNA methylase. We demonstrated by RT-PCR that meningococcal \(hfq\) is part of a polycistronic operon and co-transcribed with both of the flanking genes, a situation similar to what is found in \(Moraxella catarrhalis\). In this pathogen, \(hfq\) is also co-transcribed with both of the flanking genes (\(miaA\) and \(kpsF\), the latter encoding a predicted arabinose-5-phosphate isomerase)\(^5,28,29\). By using an Erm cassette without a terminator, we avoided interfering with transcription of NMB0747, part of the polycistron and directly downstream of Hfq. The growth defect observed in meningococci upon deletion of \(hfq\) was substantially reversed upon expression of \(hfq\) in \(\text{trans}\). This observation strongly suggests that Hfq plays an important role in the physiology of the meningococcus and that upon deletion metabolic processes are disturbed resulting in a phenotype with impaired growth.

In some bacterial species Hfq is also required for efficient translation of \(rpoS\) mRNA, encoding the general stress sigma factor and/or loss of Hfq results in activation of RpoE, the alternative sigma factor mediating the response to
envelope stress. Analyses of protein patterns on one- and two-dimensional gels showed that the expression of 28 genes of N. meningitidis is affected by Hfq, either directly or indirectly. The identified genes are most likely not under control of RpoS, since N. meningitidis does not possess an RpoS-like sigma factor. Involvement of RpoE however cannot be ruled out, since the genome of N. meningitidis does contain a gene (NMB2144) encoding this alternative sigma factor. The functionality of RpoE and its possible connection to the Hfq regulon in meningococci remains to be addressed.

Classification of the genes identified by comparative analyses shows that the majority of the encoded proteins belong to the functional classes of metabolism (n=12), ribosomal protein synthesis and modification, amino acid biosynthesis and protein translation and modification (n=8). Among these, the NADP-specific glutamate dehydrogenase (NADP-GDH, encoded by gdhA) was found to be downregulated in the absence of Hfq. This is an interesting observation, because gdhA has been shown to be an essential gene for systemic meningococcal infection in an infant rat model and its expression levels are highest in strains belonging to the hypervirulent lineages ET-5 (serogroup B) and IV-1 (serogroup A). Downregulation of GdhA expression in the absence of Hfq suggests that Hfq might promote translation, for instance by stabilization of gdhA mRNA, and thus contribute to the virulence potential of N. meningitidis. Whether this occurs in conjunction with a sRNA remains to be addressed.

Of the 28 proteins identified, 7 are encoded by genes of the expression of which is under control of iron and/or Fur. Recently, an iron and Fur-regulated sRNA (NrrF) was identified in N. meningitidis. This sRNA has been shown to repress expression of succinate dehydrogenase upon iron depletion and its interaction with sdhCDAB transcript in vitro is enhanced in the presence of Hfq. Although none of the identified proteins belong to the succinate dehydrogenase complex, seven other iron-responsive were differentially expressed in the H44/76Δhfq strain. This suggests that in N. meningitidis Hfq, possibly in conjunction with sRNAs, might also contribute to the fine-tuning of factors involved in iron homeostasis. One of the iron-responsive proteins that is differentially expressed between wt and H44/76Δhfq is superoxide dismutase (SodB). In E. coli, SodB is known to be regulated by RyhB, a Fur-regulated Hfq-dependent sRNA. Also, in other pathogens, SodB expression is controlled by Hfq and a sRNA. It is tempting to speculate that in meningococci, SodB expression is also regulated by Hfq in conjunction with a sRNA. This sRNA remains to be identified.
We detected two proteins (PilE and TspA) known to be involved in adherence of meningococci to human cells. Tfp-mediated adherence of meningococci to human cells leads to clumps of bacteria associated with microvillus-like structures on the surface of the cells\textsuperscript{24,38}. This is followed by contact-dependent downregulation of pili and intimate adhesion, mediated by adhesins including TspA\textsuperscript{25}. The absence of detectable PilE, the structural subunit of Tfp and the upregulation of TspA expression in H44/76Δhfq suggests a role of Hfq in the meningococcal adherence strategies. The mechanisms responsible for these observations remain to be elucidated, but riboregulatory processes have previously been shown to be involved in Tfp assembly and cell motility of other bacterial pathogens\textsuperscript{5,26,39}.

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References


Molecular characterization & identification of Hfq-regulated proteins in *N. meningitidis*


