Identification of the *in vitro* target of an iron-responsive AraC-like protein from *Neisseria meningitidis* that is in a regulatory cascade with Fur

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In this study we characterized a genetic locus that is predicted to encode one of the three AraC-like regulators of *Neisseria meningitidis*, a homologue of MpeR of *Neisseria gonorrhoeae* which is specific to the pathogenic *Neisseria* species. Previous microarray studies have suggested that this gene is a member of the Fur regulon. In strain MC58, it is a pseudogene (annotated as two ORFs, NMB1879 and NMB1878) containing a frameshift mutation which we show is common to all strains tested belonging to the ST-32 hypervirulent clonal complex. Using primer extension and S1 nuclease protection assays, we mapped two promoters in the upstream intergenic region: the *mpeR* promoter and the NMB1880 promoter. The latter promoter drives transcription of the divergent upstream locus, which is predicted to encode a high-affinity iron uptake system. We demonstrated that both promoters are induced during iron limitation and that this regulation is also mediated by the Fur regulator. DNA-binding studies with the purified MpeR protein revealed that it binds to a region directly upstream of the NMB1880 divergent promoter, suggesting a role in its regulation. Mutants of *N. meningitidis* strains lacking MpeR or overexpressing MpeR showed no significant differences in expression of the *P_{NMB1880}* promoter, nor did global transcriptional profiling of an MpeR knockout identify any deregulated genes, suggesting that the MpeR protein is inactive under the conditions used in these experiments. The presence of MpeR in a regulatory cascade downstream of the Fur master iron regulator implicates it as being expressed in the iron-limiting environment of the host, where it may in turn regulate a group of genes, including the divergent iron transport locus, in response to signals important for infection.

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INTRODUCTION

Neisseria meningitidis, the meningococcus, is a Gram-negative diplococcus and an important human pathogen. *N. meningitidis* resides within the nasopharyngeal tract of humans, its only known habitat, and about 5–15% of the human population carries the bacteria without any symptoms of infection (Fowler *et al.*, 2004). A major step in infection is the ability of some strains to cross the nasopharyngeal epithelium into the circulatory system, where the bacteria proliferate and adapt to the host environment, causing septicaemia. Clinical responses to infection are varied, ranging from benign to extreme, fatal forms (meningococemia). In the most severe form of

infection, bacteria in the blood make their way to the brain and cross the blood–brain barrier and induce inflammation of the meninges (meningitis).

Although extensive transcriptional regulation is expected to accompany the infection process of *N. meningitidis*, the roles of only a few transcriptional regulators during infection by pathogenic neisseriae have been identified and investigated to date. *N. meningitidis* and the closely related pathogen *Neisseria gonorrhoeae* (the gonococcus) have many homologous regulators, as predicted from their genome sequences, although their infectious processes and niches are quite distinct. Included in this arsenal are three AraC-like proteins that exhibit a high level of identity (approximately 95% identity). One of these, the MtrA protein, was characterized in *N. gonorrhoeae* (Rouquette *et al.*, 1999) and it was found to activate the expression of *mtrCDE*, which encodes an energy-dependent efflux pump that serves to export diverse antimicrobial and hydrophobic agents (HAs). However, it was subsequently reported that although the meningococcus has a functional *mtrCDE*-encoded efflux pump, its expression is independent of MtrA (Rouquette-Loughlin *et al.*,

The array layout has been submitted to EBI ArrayExpress and it is available with the identifier A-MEXP-1967. The entire set of supporting microarray data has been deposited in the ArrayExpress public database under the accession number E-MTAB-687.

A supplementary table, listing microarray results comparing RNA from cultures of M1239 and M-2Δ strains grown to mid-exponential phase in GC medium and exposed for 15 min to iron-limitation, is available with the online version of this paper.

2004). In another report, a second AraC regulator termed MpeR (for Mtr protein efflux regulator) was suggested to modulate a twofold change in *mtrF* expression that is needed for full HA resistance, but the underlying mechanism is still unknown (Folster & Shafer, 2005). The AraC family of transcriptional regulators is a growing family that contains over 800 members (Egan, 2002). These proteins often regulate virulence by controlling antibiotic and oxidative stress resistance, expression of type III secretion, toxin production and other processes that are important during infection (Finlay & Falkow, 1997; Francis *et al.*, 2002; Childers & Klose, 2007).

AraC-like regulators are often part of complex regulatory cascades, involving hierarchical induction of sequential regulators in response to multiple signals, which ensures tight regulation and induction under the appropriate specific conditions. For example, the AraC-like regulator ToxT activates many virulence factors of *Vibrio cholerae* but is itself under the control of the ToxR and TcpP regulators which respond to environmental stimuli and ensure that ToxT is expressed only under virulence-inducing conditions in the human intestine (reviewed by Childers & Klose, 2007).

N. meningitidis, as with many bacteria, uses the ferric uptake regulator (Fur) to induce gene expression in response to the iron-limiting environment of the host. Iron is an essential micronutrient, present in growth limiting concentrations in the host; however, an excess of iron is also toxic and Fur plays an essential role in regulating iron homeostasis to maintain the intracellular concentration of the metal within appropriate limits (Escobar *et al.*, 1999b). *N. meningitidis* is highly adapted to its exclusive niche within the human host and has evolved outer-membrane receptors that sequester iron directly from transferrin, lactoferrin and haemoglobin host proteins. Although the meningococcus does not produce siderophores, it does express siderophore receptors and can, presumably, scavenge iron-loaded siderophores produced by other bacteria in the naso-pharynx (Carson *et al.*, 1999). Some of these high-affinity iron-uptake systems are important virulence factors in bacteria and have been shown to play a major role in promoting the survival of the meningococcus within the host (reviewed by Perkins-Balding *et al.*, 2004). Given the location of iron receptors on the cell surface, their role in pathogenicity and often their inter-strain sequence conservation, these types of proteins have been under study as possible vaccine candidates for the disease (Banerjee-Bhatnagar & Frasch, 1990).

Analysis of the genes regulated by Fur has revealed that this protein also controls functions that are not directly related to iron metabolism. These include cellular processes as varied as the acid-shock response, defence against oxygen radicals, production of toxins, virulence factors and downstream transcriptional regulators, which may in turn control expression of their regulon (Escobar *et al.*, 1999a). Therefore, transcriptional regulators that are induced by signals similar to those found in the host environment, for

instance under iron limitation in a Fur-mediated way, may be important in controlling gene expression *in vivo*. Study of these regulators may give important information about genes expressed in the host and therefore provide a deeper understanding of the infection cycle of this important pathogen.

In this work we studied and characterized the regulation of a genetic locus of *N. meningitidis* annotated in the MC58 genome as NMB1878/9 that is predicted to encode an AraC-like regulator, the orthologue of MpeR of the gonococcus. We generated null mutant and overexpressing derivatives as well as performing DNA-binding studies with the purified protein, and we identified a direct target of the regulator *in vitro*.

METHODS

Bacterial strains and culture conditions. The *N. meningitidis* strains (Table 1) and their recombinant derivatives (see below) used in the present study were routinely cultured in GC-based (Difco) agar medium supplemented with Kellogg's supplement I at 37 °C in a 5 % CO₂/95 % air atmosphere at 95 % humidity. Strains were stocked in 10 % skimmed milk and stored at –80 °C. Each bacterial manipulation was started from an overnight culture of a frozen stock. For liquid cultures, *N. meningitidis* strains were grown overnight on solid medium, resuspended in PBS to OD₆₀₀ 1 and inoculated at 1:100 dilution into GC broth supplemented with Kellogg's supplement I and 12.5 µM Fe(NO₃)₃; when required, erythromycin, chloramphenicol and IPTG were added to final concentrations of 5 µg ml^{–1} for the antibiotics and 0.03, 0.1, 0.3 or 1 mM for IPTG. *Escherichia coli* strains DH5α (Hanahan, 1983) and BL21(DE3) (Studier & Moffatt, 1986) were cultured in Luria-Bertani medium and, when required, ampicillin or IPTG was added to a final concentration of 100 µg ml^{–1} or 1 mM respectively.

Construction of plasmids and recombinant strains. DNA manipulations were carried out routinely as described for standard laboratory methods (Sambrook *et al.*, 1989). The Fur-knockout mutant derivative of MC58 (MC-Fko, here named Fko) was previously described (Delany *et al.*, 2003). In order to knock out the *mpeR* gene in strain M1239 (which has the full-length *mpeR* gene), by substituting it with an erythromycin cassette, the p1879-ko:Ery plasmid was constructed. The upstream region of the *mpeR* gene, containing the intergenic region between *mpeR* and NMB1880 and 22 bp of the *mpeR* gene, was amplified by PCR with primers 1879-F/1879-R and cloned in the pGEMT vector (Promega) as a *Bam*HI–*Eco*RI fragment, generating the plasmid pGemT1879. Then the downstream region of the *mpeR* gene was amplified by PCR with primers 1879-3/1879-4 and cloned into pGemT1879 as a *Bam*HI–*Nsi*I fragment. An erythromycin cassette was inserted into the *Bam*HI site, between the flanking regions, generating p1879-ko:Ery. The plasmid was then linearized and used for transformation of the M1239 strain to make an *mpeR* knockout mutant, M-2Δ. To generate a strain of MC58 that could overexpress the full-length MpeR protein in an IPTG-inducible manner (MC-P_{ind}MpeR) we generated the pPind-mpeR plasmid, by replacing the *crgA* gene from plasmid pPindcrgA (Ieva *et al.*, 2005) with the *mpeR* gene, amplified from the M1239 genome using the primers 1878-F2/1879-RN (Table 2) and cloned as a *Nde*I–*Nsi*I fragment. The plasmid pPind-mpeR was then used to transform MC58, resulting in the integration of a copy of *mpeR* under the control of the P_{tac} promoter between the NMB1428 and NMB1429 genes.

Table 1. Occurrence of stop mutation in *mpeR* in clinical strains of *N. meningitidis* and *N. gonorrhoeae* isolated from diseased patients over diverse geographical and temporal span

Strain	Sg*	GL†	Year of isolation	Presence of <i>mpeR</i> mutation‡	Clonal complex§
MC58	B	GB	1985	+	ST-32
BZ169	B	NL	1985	+	ST-32
BZ83	B	NL	1984	+	ST-32
H44/76	B	N	1976	+	ST-32
CU385	B	C	1980	+	ST-32
N44/89	B	BRA	1989	+	ST-32
2996	B	GB	1975	—	ST-8
961-5945	B	AUS	1996	—	ST-8
M986	B	USA	1963	—	ST-11
NGP165	B	N	1974	—	ST-11
M01-240185	B	GB	2001	—	ST-11
8047	B	USA	1978	—	ST-11
NGH15	B	N	1988	—	ST-41/44
394/98	B	NZ	1998	—	ST-41/44
M1239	B	USA	1994	—	ST-41/44
M1390	B	USA	1995	—	ST-41/44
M01-240149	B	GB	2001	—	ST-41/44
BZ232	B	NL	1964	—	ST-37
1000	B	CIS	1988	NA	ST-18
F6124	A	CH	1988	—	Sub.III
BZ133	C	NL	1977	—	Sub.VII
F62	Ng	USA	1960	—	ND

*Serogroups A, B and C are all *N. meningitidis*, whereas Ng corresponds to *N. gonorrhoeae*.

†Geographical locations: AUS, Australia; BRA, Brazil; C, Cuba; CH, Chad; CIS, Russia; GB, Great Britain; N, Norway; NL, Netherlands; NZ, New Zealand.

‡NA, Did not result in amplification by PCR.

§Nomenclature of clonal complex according to multi locus sequence typing (Maiden *et al.*, 1998), ND, not determined.

To make a NMB1880 knockout we generated the plasmid pGem1880KO as follows. Upstream and downstream flanking regions of the NMB1880 gene were amplified from the MC58 genome using primer pairs 1880-NS/1879-R and 1880-F/1880-R, respectively. The downstream fragment of 359 bp was cloned via TA cloning into pGEMT and subsequently the upstream fragment of 295 bp was cloned as an *EcoRI*–*SphI* fragment flanking the downstream fragment. Finally a kanamycin cassette from pILL600 (Labigne-Roussel *et al.*, 1988) was cloned between the two flanking regions into the *EcoRI* site, generating the pGem1880KO construct. The plasmid was linearized and used for transforming MC58, to generate the MC-1880KO strain.

Transformants were selected for antibiotic resistance and then tested for the double crossover in the flanking regions by PCR. Induction of the MpeR protein was achieved by growing strain MC-P_{ind}MpeR in GC broth with IPTG to the exponential phase.

RNA extraction, primer extension, S1 nuclease assay and RT-PCR. *N. meningitidis* strains were grown in liquid culture to exponential phase and then split into two samples that were exposed for 15 min to treatment with or without 100 μ M 2,2'-dipyridyl (a specific iron chelator; Sigma). After 15 min, the cultures were added to an equal volume of frozen medium to bring the temperature immediately to 4 °C, and RNA was extracted from the pelleted cells as previously described (Delany *et al.*, 2003).

Primer extension was performed as previously reported (Delany *et al.*, 2003). To ensure correct mapping of the promoter, a sequencing

reaction was carried out with a T7 sequencing kit (USB) using the same primer as in the primer extension reaction (1879-2) and the plasmid consisting of the relevant cloned promoter (pGemT-1879).

A radioactively labelled DNA probe for S1 mapping of the 5' region of the NMB1880 transcript from position +1 was prepared as follows. The probe was amplified from the MC58 chromosome using the 1880-S1/1880-S2 primer pair, the appropriate 360 bp fragment was extracted from an agarose gel and, after purification, 2 pmol of the probe was labelled at both extremities with T4 polynucleotide kinase and 4 pmol [γ -³²P]ATP. One labelled extremity was removed by digestion with *EcoRI*, a site that was incorporated into the upstream primer (1880-S1) and the resultant 337 bp probe, labelled on the 5' complementary strand, was purified using Chroma Spin TE-100 columns (Clontech). Approximately 20 fmol labelled probe was coprecipitated with 30 μ g total RNA and the S1 nuclease assay was performed as previously described (Fantappiè *et al.*, 2009). Quantification of the signals from the digested probes was performed using a Phosphorimager and ImageQuant software (Molecular dynamics). A G-A sequencing reaction (Maxam & Gilbert, 1977) was performed with the probe parallel to the S1 nuclease reactions to provide a molecular mass ladder for mapping the 5' end of the transcript.

RT-PCR on the *mtrF*, NMB1880 and NMB1882/*tdff* genes was performed on cDNAs prepared from RNA extracted from M1239 and M-2Δ strains grown to mid-exponential phase and treated for 15 min with or without 100 μ M 2,2'-dipyridyl as previously described

Table 2. Oligonucleotides used in this study

Name	Sequence*	Restriction sites†
1719-F	AATACCGCTTCTGAATTGGG	–
1719-R	CCGAGGGAATGAAAGATGAT	–
1879-2	CGGAGCGGACATAGGTTTG	–
1879-3	attcaggatCCTTCGGATGTGAAGAAGTGCTGATG	BamHI
1879-4	attcagccatggTTACCACAGCCTGTCCGACGATC	NcoI
1879-F	attcaggatccGGTAGATGGCGGCGGTGTTTC	BamHI
1878-F2	cggatccatATGAACACCGCCGCATCTACCG	NdeI
1878-GEX	attcagctcgagGCACTTCTTCACATCCGAAGGC	XhoI
1879-R	AttcagaattcGCGGTTTCACGGTGTGCGGTTCC	EcoRI
1878-R2	attcagggatccTCAGCACTTCTTCACATCCGAAGG	BamHI
1879-RN	attcagatgcatTCAGCACTTCTTCACATCCGAAGG	NsiI
1880-F	attcagaattcGCAAATCATCGTCATGCCTGCCCG	EcoRI
1880-NS	attcagcatgcatCGGAGCGGACATAGGTTTGG	NsiI/SphI
1880-R	attcagccatggCGGAGCTTCTGAAATCGCAGC	NcoI
1880-RT-F	ATTGAAGGCGCAGATTGAC	–
1880-RT-R	CGTGTATCCAACCTGCCAAC	–
1880-S1	CGGTCGGCACGGTCAGCGTGCC	–
1880-S2	GGAACGAGTTGTCTAACAAATGAATTcAATAGG	EcoRI
1882-RT-F	CAACGACGGCTACACTGTTT	–
1882-RT-R	CATTTGTTGCGATGTGATGA	–
adk-PE1	CGCGCCTAAAAGTAATGC	–
Eri R	atatatggatccgggacaccttttagctcttgg	BamHI

*Capital letters indicate *N. meningitidis*-derived sequences, small letters indicate sequences added for cloning purposes and underlined letters indicate recognition sites.

†Restriction enzyme sites added for cloning purposes.

(Fantappiè *et al.*, 2009) using primers 1719-F and 1719-R, 1880-RT-F and 1880-RT-R, and 1882-RT-F and 1882-RT-R, respectively.

Microarray procedures: design, cDNA labelling and hybridization and data analysis. DNA microarray analysis was performed using an Agilent custom-designed oligonucleotide array as previously described (Fantappiè *et al.*, 2011). For microarray analysis, RNA was extracted from pelleted cultures using the RNeasy Mini-kit (Qiagen). Three hybridizations were performed using cDNA probes from three independent pools, corresponding to RNA extracted from a total of nine independent bacterial cultures for each strain. Differentially expressed genes (exhibiting a fold change of at least 2 with respect to the reference) were assessed by grouping all log₂ ratios of the Cy5 and Cy3 values corresponding to each gene, within experimental replicates and spot replicates, and comparing them against the zero value by Student's *t*-test statistics (one-tailed) with a *P*-value of <0.01. The array layout was submitted to the EBI ArrayExpress and it is available with the identifier A-MEXP-1967. The entire set of supporting microarray data has been deposited in the ArrayExpress public database under the accession number E-MTAB-687.

DNase I footprinting. For footprinting analysis, the pGemT1879 plasmid was 5'-end labelled with [γ -³²P]ATP at its *NcoI* site and separated from the vector by PAGE after digestion with *SpeI*, producing a probe of the *mpeR* promoter region labelled at only one extremity, as previously described (Oriente *et al.*, 2010). DNase I footprinting experiments with the purified glutathione *S*-transferase (GST)-MpeR protein and the radioactively labelled *mpeR* promoter probe were carried out as previously described (Oriente *et al.*, 2010). Binding reactions were performed in binding buffer consisting of 10 mM HEPES pH 7, 50 mM NaCl, 10 mM KCl, 1 mM DTT, 0.01 % NP-40 and 10 % glycerol.

Expression and purification of the MpeR protein, and generation of anti-MpeR serum.

Different strategies were used in order to overexpress and obtain purified recombinant MpeR protein, including an N-terminally histidine-tagged MpeR (His-MpeR) protein which was purified under denaturing conditions in order to raise antibodies and a natively purified GST fusion (GST-MpeR) protein to use in DNA-binding studies. In order to purify the His-MpeR protein the *mpeR* gene was amplified from the M1239 genome by PCR using 1878-F2/1878-R2 primers and cloned into pET15b (Stratagene), generating plasmid pET15-1878, which was transformed into *E. coli* strain BL21(DE3). From an overnight culture of the BL21(DE3)(pET15-1878) strain, 200 ml Luria-Bertani medium was inoculated and the strain grown to OD₆₀₀ 0.5 at 37 °C and expression of the recombinant His-MpeR protein was induced by the addition of 1 mM IPTG and further incubation for 3 h. Attempts to purify the His-MpeR protein under native conditions failed; therefore it was purified by Ni-NTA (Qiagen) affinity chromatography under denaturing conditions, following the manufacturer's instructions. A concentration of approximately 0.8 mg His-MpeR ml⁻¹ was obtained. The concentration of urea in the eluted protein was reduced to 2 M by dialysis and this preparation was used to immunize mice. All attempts to renature the protein into an active DNA-binding form failed. To prepare anti-MpeR serum, 20 µg denatured purified protein was used to immunize 6-week-old CD1 female mice; four mice were used. The protein was given intraperitoneally, together with complete Freund's adjuvant for the first dose and incomplete Freund's adjuvant for the second (day 21) and third (day 35) booster doses. Bleed-out samples were taken on day 49 and used in Western blot analysis.

In order to obtain an active form, *mpeR* was expressed as a GST-MpeR fusion. The *mpeR* gene was amplified by PCR using primers

1878-F2/1878-GEX and cloned into pGexNN (Amersham) generating pGex-1878NN. To increase the quantity and folding of the overexpressed MpeR soluble protein, the heat-shock response in the *E. coli* BL21 expression culture was induced by growing for 90 min at 42 °C, as previously described (Roncarati *et al.*, 2007). Cells were harvested after 4 h induction with IPTG and resuspended in 1 × PBS. The protein was purified by affinity chromatography with Glutathione Sepharose 4B (Pharmacia Biotech) using 1 × PBS as washing buffer and 10 mM reduced glutathione in 50 mM Tris/HCl, pH 8, as elution buffer, according to the manufacturer's instructions and dialysed for 12 h against binding buffer (10 mM HEPES pH 7, 50 mM NaCl, 10 mM KCl, 1 mM DTT, 0.01 % NP-40 and 10 % glycerol) at 4 °C. A final concentration of 1 mg ml⁻¹ was obtained and used for DNA-binding assays.

Western blot analysis. Liquid cultures were grown in 7 ml GC medium in 15 ml tubes to OD₆₀₀ 0.5. For iron chelation 25 µM desferal (Sigma) was added to the GC medium. For induction of the MpeR protein in strain MC-P_{ind}MpeR, IPTG was added to 0.03, 0.1, 0.3 or 1 mM final concentration. A 1 ml volume of 7 ml liquid culture was pelleted in a benchtop centrifuge and resuspended in 100 µl SDS-PAGE loading buffer. A 10 µl volume of each total protein sample was separated by SDS-PAGE and transferred onto nitrocellulose filter by standard methods (Sambrook *et al.*, 1989). The filters were blocked for 1 h at room temperature by agitation in blocking solution (3 % skimmed milk and 0.1 % Triton X-100 in PBS), followed by incubation for a further 1 h with a 1 : 1000 dilution

of the MpeR antibody, 1:500 anti-NMB1880 and 1:500 anti-TdF sera in blocking solution. After a washing step, the filters were incubated in a 1:2000 dilution of peroxidase-conjugated anti-mouse immunoglobulin (Dako) in blocking solution for 1 h, and the resulting signal was detected using the SuperSignal West Pico chemiluminescent substrate (Pierce).

RESULTS

The AraC-like locus: *mpeR*

In the MC58 genome, the locus encoding an AraC-like regulator consists of a pseudogene, which has been annotated as two ORFs, NMB1879 and NMB1878, due to a stop codon at 309 bp from the predicted translational start (Fig. 1a). In other available meningococcal genomes, a full-length gene of 960 bp encodes a protein of the AraC family with a tryptophan codon (TGG) in place of the stop codon (TGA) of the MC58 sequence. Previous microarray studies suggested that the NMB1879/8 locus is regulated by Fe and by Fur (Delany *et al.*, 2006), and footprint studies showed that Fur binds two sites in the upstream intergenic region (Delany *et al.*, 2006; Fig. 1b). These data suggest that

Fig. 1. (a) Structural organization of the *N. meningitidis* MC58 *mpeR* locus. The annotated genes are indicated by the NMB number or gene name. The black boxes represent the two Fur-binding sites previously identified (Delany *et al.*, 2006). The genes divergently oriented from *mpeR* appear to be in an operon, as they are all in the same orientation, with no terminator or promoter-like sequences in the intergenic regions. A putative rho-independent terminator is located immediately downstream of *tdfF* and is indicated by a stem-loop. (b) Nucleotide sequence of the *mpeR*-NMB1880 intergenic sequence. The bent arrows indicate transcriptional start sites, including the transcriptional start site of NMB1880 under iron-limitation (*P'*). Underlined letters indicate -10 and -35 promoter consensus sequences of the *P_{mpeR}* and *P_{NMB1880}* promoters. Bold type indicates Fur-binding sites as identified previously (Delany *et al.*, 2006). The rectangle indicates the MpeR-binding site.

Fur directly binds to the promoter region of this gene and may regulate its transcription. From now on, we refer to NMB1879/8 as a single gene that we call *mpeR* according to the name of its orthologue in *N. gonorrhoeae* (Folster & Shafer, 2005).

In order to investigate if other strains of *N. meningitidis* contain a mutation in the *mpeR* gene similar to strain MC58, we selected 19 clinical isolates from serogroup B including various clonal complexes. We also included isolates from serogroups A and C and an isolate of the closely related pathogenic bacterium *N. gonorrhoeae*. We amplified the corresponding *mpeR* region from their chromosomal DNA and sequenced these regions. The results we obtained are shown in Table 1. The majority of strains have the full-length gene, including *N. gonorrhoeae* and *N. meningitidis* A, C and most of serogroup B. Six of the 19 serogroup B strains contain the stop mutation, and surprisingly these all correspond to members of the ST32 clonal complex (ST-32cc), while 13 strains from the other hypervirulent clonal complexes of serogroup B do not contain the mutation, nor do the strains from serogroups A and C, nor the *N. gonorrhoeae* tested. Furthermore, the strains used were isolated from different parts of the world from 1963 to 2001; thus this panel represents a good geographical distribution of *N. meningitidis* strains isolated over a significant period of time. We conclude that *mpeR* is a pseudogene in all the ST-32cc strains tested, and in none of the other 16 ST types tested, indicating a significant association of the mutation with the ST32cc (Fisher's exact P -value = 1.3×10^{-5}). As the ST-32cc complex is one of the most virulent complexes of *N. meningitidis*, *mpeR* might have a redundant function, and its inactivation has not hindered the ST-32 complex during the process of infection. Alternatively, it is also possible that the mutation gives an advantage to the ST-32cc strains, and deregulation of its target gene(s) renders the complex more successful in its transmission.

The divergent ORF (NMB1880) directly upstream of *mpeR* is annotated as encoding a putative periplasmic solute-binding protein that has significant homology to a family of periplasmic lipoproteins involved in transporting siderophores through the periplasm (49% identity to FetB, thought to transport enterobactin across the periplasm) (Carson *et al.*, 1999). Downstream of and in the same orientation as NMB1880, there are two ORFs encoding a hypothetical protein and TdFf (TonB-dependent family protein F). Most members of this family of outer-membrane TonB-dependent receptors are thought to function as channels that, opening in presence of a ligand, allow the access of the ligand into the periplasmic space. TdFf shows approximately 30% identity to hydroxamate siderophore receptor proteins such as FhuE and FpvA, which are involved in high-affinity binding of the coproduct (produced by fungi) and ferripyoverdine (produced by pseudomonads) siderophores by other bacteria. The *mpeR* locus including these divergently oriented NMB1880-TdFf genes is only present in the genomes of the pathogens *N. meningitidis* and *N. gonorrhoeae* and is absent in the other genome sequences

of the genus *Neisseria*, including the commensal species. Since footprinting experiments previously identified a Fur-binding site proximal to this divergent locus, we decided to further characterize the transcriptional regulation of the *mpeR* and NMB1880 promoters.

Mapping the P_{mpeR} and $P_{NMB1880}$ promoters

Due to the organization of the locus, within the 234 bp of DNA upstream of the *mpeR* gene there is the possibility of at least two promoter elements controlling the transcription of the divergent genes *mpeR* and NMB1880 (Fig. 1). To identify the position of these promoters with respect to the two Fur-binding sites identified by footprinting studies, primer extension was performed on total RNA from *N. meningitidis* with multiple primers complementary to their transcripts. We decided to perform the analysis on total RNA from the MC58 Fur mutant strain (Fko) because in previous studies of Fur-regulated genes (Delany *et al.*, 2003, 2006), no bands corresponding to elongation products were visible in the MC58 wild-type strain due to the Fur-dependent repression of the promoters.

A clear elongation product was generated using an *mpeR*-specific primer on total RNA from the Fko strain (Fig. 2a, lane Fko). This band maps to 26 bp upstream of the ATG translational start site of the *mpeR* gene and probably defines the *mpeR* promoter start site of transcription. Analysis of the DNA sequence revealed the presence of -10 TTTAAT and -35 TTGATT regions upstream of the *mpeR* promoter (Fig. 1b), which show conservation with the *E. coli* sigma⁷⁰ -10 TATAAT and -35 TTGACA-recognized promoters.

We were unable to obtain reproducible results by primer extension with NMB1880-specific primers; therefore in order to map the NMB1880 promoter we set up an S1 nuclease protection assay with the radioactively labelled DNA probe. The results show an S1-resistant band migrating at a position corresponding to 149 nt of protected probe (Fig. 2b). This corresponds to 25 bp upstream of the GTG translational start site of NMB1880 and probably defines the NMB1880 promoter start site of transcription. Analysis of the DNA sequence revealed the presence of poorly conserved -10 TAAACA and -35 TCTGCA regions upstream of the NMB1880 promoter (Fig. 1b).

Using these two techniques we identified two divergent promoters in the intergenic region between *mpeR* and NMB1880 which we called P_{mpeR} and $P_{NMB1880}$. It is interesting to note that all promoters are overlapped, in part, by the Fur-binding sites (Fig. 1b).

Iron and Fur regulation of *mpeR* and NMB1880 transcription

To understand the effects of Fur and iron on the transcription level of *mpeR* and NMB1880, we monitored the accumulation of specific transcripts in total RNA

Fig. 2. (a) Mapping of the 5' end of the *mpeR* gene by primer extension. A 20 µg sample of total RNA prepared from culture of the MC58 Fur knockout mutant (Fko) strain grown to mid-exponential phase was hybridized with an *mpeR*-specific primer (1879-2) and elongated with reverse transcriptase. Sequencing reactions (G, A, T and C) were performed with the same primer on the cloned promoter region (pGemT-1879) and run in parallel. The elongated primer band mapping the 5' end of the *mpeR* gene transcript is indicated. The corresponding +1 nt of transcriptional initiation and the upstream promoter sequences are indicated on the left. The 5' end of the *mpeR* transcript was confirmed using a second *mpeR*-specific primer (data not shown). (b) Mapping of the 5' end of the NMB1880 gene by S1 nuclease protection assay. The DNA probe was radioactively labelled at one end, hybridized to 30 µg total RNA prepared from a culture of the Fur mutant strain (Fko) grown to mid-exponential phase and digested with S1 nuclease for mapping of the 5' end of the NMB1880 transcript. Two control samples with *E. coli* tRNA instead of total RNA were processed in parallel with (lane +) and without (lane -) addition of S1 nuclease. The position of the RNA-specific S1 nuclease-protected band corresponding to the 5' end of the NMB1880 transcript is indicated. Lane G+A contained a G-A sequence reaction of the DNA probe used as a size marker (Maxam & Gilbert, 1977). Lane Fko is from a longer exposure of the same gel, as the RNA-protected signal was very low with respect to the other samples. The corresponding +1 nt of transcriptional initiation and the upstream promoter sequences are indicated on the left.

Fig. 3. Iron and Fur regulation of *mpeR* and NMB1880. Total RNA was prepared from the wild-type (MC58) and the Fur-null mutant (Fko) grown to mid-exponential phase under iron-replete conditions before (+) and after (-) 15 min treatment with iron chelator (2,2'-dipyridyl), and then primer extension analysis on the *P_{mpeR}* promoter (a) or S1 nuclease protection assay on the *P_{NMB1880}* promoter (c) was performed. The level of *adk* housekeeping gene transcript was also quantified by primer extension using the *adk*-PE1 primer as a negative control in total RNA samples from each tested strain (b). The figure shows a representative experiment of triplicate RNA preparations from independent bacterial cultures.

extracted from MC58 wild-type cells and Fko cells exposed to iron-replete or iron-limiting conditions. The strains were grown under iron-replete conditions to exponential phase and treated for 15 min with 100 µM 2,2'-dipyridyl, and total RNA was extracted from the cells before and after treatment. These RNAs were used in primer extension experiments to measure the relative accumulation of mRNA at the *P_{mpeR}* promoter (Fig. 3a). In the wild-type MC58 background, treatment with the iron chelator resulted in an increase of the *mpeR* transcript, indicating that this promoter is induced during iron limitation. The amount of transcript initiating at the *P_{mpeR}* is high in Fko cells exposed to iron-replete or iron-limiting conditions, indicating that transcription from this promoter no longer responds to iron in the *fur* mutant, and is equivalent to that in the iron-depleted treatment from the MC58 wild-type strain. This indicates that the absence either of iron or of the Fur protein has the same effect in derepressing the *P_{mpeR}*. As expected we obtained similar levels of *adk* transcript in all RNA preparations (negative control) (Fig. 3b).

We carried out S1 nuclease protection assays with an NMB1880-specific probe hybridized to an equal amount of total RNA from MC58 and Fko cells grown under iron-replete and iron-limiting conditions (Fig. 3c). Only very weak bands were obtained, possibly indicating that NMB1880 is not highly expressed. However, there is a slight

increase of the NMB1880 transcript under iron-limiting conditions in the MC58 wild-type, as well as in Fko cells exposed to iron-replete or iron-limiting conditions. Interestingly, a second, higher, band is seen under iron-limiting conditions, migrating at a position corresponding to 154 nt of protected probe. This corresponds to 30 bp upstream of the GTG translational start site of NMB1880. Analysis of the DNA sequence revealed the presence of -10 AATAAT and -35 TGGTTT regions upstream of this putative promoter that we called $P'_{NMB1880}$ (Fig. 1b). This could represent another promoter or it could arise from a repositioning of the RNA polymerase.

We conclude that transcription from the P_{mpeR} and $P_{NMB1880}$ promoters is regulated during iron limitation and is also mediated by the Fur regulator, which represses transcription in response to iron.

Analysis of MpeR expression in different strains of *N. meningitidis*

In an attempt to understand the role of MpeR in the possible regulation of the upstream genes from the $P_{NMB1880}$ promoter, we generated a strain of MC58 that could overexpress the full-length protein in an IPTG-inducible manner (namely MC- P_{ind} MpeR). Furthermore, we made a knockout of the *mpeR* gene (named M-2 Δ) in the M1239 strain, which is predicted to express a full-length protein (Table 1). In order to evaluate the expression of the MpeR protein in the different *N. meningitidis* strains generated, we cloned and expressed a His-tagged form of the protein and purified it under denaturing conditions (as described in Methods) and raised antibodies against it in mice.

We grew parallel cultures of MC58, M1239 and its *mpeR* knockout derivative strain M-2 Δ under iron-replete and iron-limiting conditions. Fig. 4(a) shows the Western blot results obtained. A band of approximately 34 kDa, which should correspond to MpeR protein, is seen when the M1239 strain was grown with iron chelator while in iron-replete conditions no band is visible. This confirms the iron-regulated production of this regulatory protein. As expected, in MC58 cells grown under iron-replete and -limiting conditions no band corresponding to MpeR was detected.

The MC- P_{ind} MpeR strain was grown in liquid culture with increasing amounts of IPTG, to induce expression of the *mpeR* gene and aliquots of each sample were collected and tested by Western blotting (Fig. 4b). The antibodies recognized a protein band migrating at approximately 34 kDa in the MC- P_{ind} MpeR strain when cells were grown in the presence of 0.03 mM IPTG (Fig. 4b, lane 3) and the amount increased with higher amounts of IPTG in the culture medium.

We performed Western blot analysis with polyclonal anti-NMB1880 and anti-NMB1882 antiserum on the extracts of the wild-type, the *mpeR* mutant or overexpressing strains (as well as a NMB1880 knockout for anti-NMB1880) grown under iron-replete and iron-limiting conditions, but

Fig. 4. Western blot analysis of MpeR expression. (a) Western blot analysis showing iron-regulated MpeR expression. Wild-type MC58 and M1239 and the *mpeR* knockout strain M-2 Δ were grown under iron-replete (+) (supplemented GC medium) and iron-limiting (-) (supplemented GC medium with 25 μ M desferal) conditions and bacteria were harvested at OD₆₀₀ 0.5. Equal amounts of total protein from each culture were fractionated by SDS-PAGE, blotted onto nitrocellulose filters, and stained with antiserum raised against the MpeR protein (34 kDa). A faint band of approximately 34 kDa was detected at a very low level in the *mpeR* knockout mutant of M1239 (lane 6) that was treated with iron chelator. This weak band may represent a cross-reactive band of a similar-sized protein which may also respond to iron. The figure shows a representative experiment from duplicate independent bacterial cultures. (b) Western blot analysis showing MpeR expression in the MC- P_{ind} MpeR complemented strain. For induction of MpeR, IPTG was added to the growth medium (lanes 3–6 correspond to 0.03, 0.1, 0.3 and 1 mM, respectively) and the figure shows a representative experiment.

no band corresponding to the predicted molecular mass of NMB1880 or NMB1882 was detectable in any of the cell extracts (results not shown), suggesting that these proteins are not expressed in any of these strains or that the antisera did not recognize the native proteins. Furthermore, we prepared RNA from the M-2 Δ and the MC- P_{ind} MpeR strains to assess the accumulation of the transcript from the divergent $P_{NMB1880}$ promoter; however, by S1 nuclease protection assays no significant differential regulation could be detected between M1239 and the M-2 Δ knockout nor the MC58 and MC- P_{ind} MpeR strains (data not shown). Taken together, these experiments suggest either that MpeR is not involved in the regulation of this putative operon or that under the conditions of the experiment the MpeR protein was not active as a transcriptional regulator.

In *N. gonorrhoeae*, this homologous regulator has been called MpeR as its inactivation resulted in a twofold increase in the β -galactosidase activity from an *mtrF*

fusion (Folster & Shafer, 2005). The *mtrF* gene encodes a membrane protein required for high-level HA resistance associated with the *mtrCDE* efflux pump. We measured, by RT-PCR, the level of *mtrF* transcript in the wild-type M1239 and *mpeR* mutant M-2Δ strains in high- or low-iron conditions from mid-exponential-phase cultures in GC and also from overnight plate cultures similar to the conditions under which gonococcal MpeR was reported to modulate the *mtrF* reporter (Folster & Shafer, 2005). While we could detect a reproducible increase in steady-state levels of *mtrF* (2.8 ± 0.8 -fold) under conditions of iron chelation, we were unable to detect differences due to the *mpeR* mutation in M-2Δ (data not shown), suggesting that *mtrF* is not affected by the *mpeR* gene in *N. meningitidis*.

In a final attempt to detect active regulation of any gene by MpeR, we compared the global transcriptional profile of the M1239 wild-type and M-2Δ mutant strains by microarray analysis. Three independent two-colour microarray experiments were performed comparing pooled RNA from triplicate cultures of each strain grown to mid-exponential phase in GC medium and exposed for 15 min to iron-limitation for the induction of MpeR expression. The results obtained from these three experiments were averaged after slide normalization, and differentially expressed genes were identified with a threshold of twofold transcriptional change and a *t*-test statistics *P*-value ≤ 0.01 (see Supplementary Table S1, available with the online version of this paper). The only genes that are significantly deregulated by the *mpeR* mutation appear in the *mpeR* locus, i.e. NMB1878 (*mpeR*) which is 9.6-fold down-regulated and the divergent NMB1880 (4.9-fold), NMB1881 (4-fold) and NMB1882/*tdfF* (2.5-fold) which appear upregulated. While RT-PCR analysis confirmed the increase in transcript levels of these genes in the M-2Δ mutant (data not shown), these data are in contrast to the results of our S1 nuclease analysis of transcript levels initiating at the *P*_{NMB1880} promoter. This is likely to be due to polar effects of the insertion of the antibiotic cassette, which is orientated in the same direction as these genes. Complementation of the *mpeR* mutation in the MC58 background (MC-PindMpeR) resulted in no significant effect on transcript levels of either NMB1880 or *tdfF* as measured by RT-PCR (data not shown), confirming the suggested polar effects of the mutation in M-2Δ. Therefore we conclude that from transcriptional profiling we are unable to identify a gene regulated by MpeR activity, although the experiments were performed in conditions maximizing MpeR expression. This strongly suggests that the MpeR protein is not actively regulating gene expression under the conditions of these experiments.

Binding of MpeR to the NMB1880 promoter

AraC-like proteins are known to be very difficult to express and purify in their native forms (Schleif, 2003). Expressing MpeR with an N-terminal or C-terminal histidine tag gave rise to insoluble proteins (results not shown). In order to

obtain an active form of the MpeR protein for use in DNA-binding studies, we expressed it with a GST tag on the N-terminus in *E. coli*. To increase the quantity of soluble protein being expressed we induced the heat-shock response in the *E. coli* expression culture by growth for 90 min at 42 °C as previously described (Roncarati *et al.*, 2007). Although no detectable band of the GST-MpeR protein was visible in the soluble fraction we succeeded in purifying the protein under native conditions (Fig. 5a). We used this protein in DNA-binding assays with the upstream intergenic region. We prepared a radioactively labelled probe, containing *P*_{*mpeR*} and *P*_{NMB1880} promoter regions, which was incubated with increasing amounts of GST-MpeR protein, and a gel shift analysis was performed. When 0.8 μg GST-MpeR protein was added, a slower-migrating band appeared (results not shown), which may be due to the formation of a DNA-protein complex. This result suggests that the MpeR protein is able to bind to the DNA intergenic region. In order to identify where this binding occurs we performed DNase I footprinting experiments. With the addition of increasing amounts of MpeR, a region of protection and DNase I hypersensitivity is clearly seen (Fig. 5c) that corresponds to the region spanning from nucleotides -36 to -55 with respect to the +1 transcription site of the *P*_{NMB1880} promoter. This strongly suggests that the MpeR protein could regulate NMB1880 expression by binding to a region proximal to its promoter.

DISCUSSION

In this study we begin the characterization of a regulator from the AraC family in the meningococcus, previously named MpeR in the gonococcus, which may be important for expression of proteins specifically *in vivo*. We have identified a single promoter, *P*_{*mpeR*}, which is regulated in response to iron due to direct repression by the Fur regulator. Therefore the MpeR protein will be induced in the iron-limiting environment of the host. In the gonococcus, the MpeR regulator has also been reported to be induced under iron limitation (Ducey *et al.*, 2005; Jackson *et al.*, 2010) and therefore the regulation of these homologues appears to be conserved. Interestingly, in contrast to the other two AraC regulators of the meningococcus which are also encoded by the commensal species, the MpeR protein is only present in the pathogenic *Neisseria* species *N. gonorrhoeae* and *N. meningitidis*.

Several regulators within the AraC family control the expression of genes known or thought to be required for virulence of bacterial pathogens (Gallegos *et al.*, 1997; Munson *et al.*, 2001). A small group of AraC-like regulators is involved in regulating the expression of specialized iron uptake systems where the substrate of the uptake system functions as the inducer signal. These include the *Pseudomonas aeruginosa* PchR protein (Heinrichs & Poole, 1996), the YbtA regulator of *Yersinia pestis* (Fetherston *et al.*, 1996) and the AlcR and BfeR proteins of *Bordetella* (Pradel *et al.*, 1998; Anderson & Armstrong, 2004), all of which

Fig. 5. MpeR binding to the *mpeR* and NMB1880 promoter regions. (a) Expression and purification of the GST-MpeR protein under native conditions. SDS-PAGE was performed with protein extracts from uninduced (lane -) and IPTG-induced (lane +) cultures of *E. coli* containing the pGex-1879NN expression plasmid, the cleared soluble fraction before (lane SF) and after (lane FT: flow-through) binding to the Glutathione Sepharose 4B resin, the wash fraction (lane W) and the elution fraction (lane E). The GST-MpeR fusion protein has an estimated molecular mass of 60 kDa. (b) DNase I footprinting analysis with purified meningococcal Fur protein and a radioactively labelled DNA probe, 5'-end labelled at the *NcoI* site, corresponding to the *mpeR* and NMB1880 promoter regions. The arrows to the left show the direction of the *mpeR* and NMB1880 genes. The probe was incubated with increasing concentrations of Fur protein and exposed to DNase I digestion for 1 min (lanes 2–4 correspond to concentrations of 0, 137 nM and 550 nM, respectively). The Fur-protected regions are indicated to the right as vertical black bars, and the numbers indicate the boundaries of the binding sites with respect to the +1 transcriptional initiation site of NMB1880. The region of the MpeR-binding site is indicated to the right as a square bracket. Lane 1 corresponds to the G+A sequence reaction run as the molecular mass marker in parallel to the footprinting reactions. (c) DNase I footprinting analysis with purified meningococcal GST-MpeR protein and the same radioactively labelled DNA probe used in (b). The probe was incubated with increasing concentrations of GST-MpeR protein and exposed to DNase I digestion for 1 min (lanes 1–4 correspond to concentrations of 0, 180 nM, 720 nM and 2.9 μ M, respectively); the figure shows results from a representative experiment. The MpeR-protected region is indicated to the right as a vertical black bar, and the numbers indicate the boundaries of the binding site with respect to the +1 transcriptional initiation site of NMB1880. The asterisks indicate DNase I-hypersensitive sites.

regulate siderophore synthesis and uptake genes. Fur and iron regulate both the AraC regulators and their targets in these circuits (Ochsner *et al.*, 1995; Gao *et al.*, 2008;

Brickman & Armstrong, 2009). Therefore, iron limitation and the presence of the cognate siderophore inducer are required for maximal expression.

Divergently upstream of *mpeR* we mapped the $P_{NMB1880}$ promoter, which overlaps the second Fur-binding site within the intergenic region. This promoter weakly responds to iron limitation and is slightly derepressed in the Fur mutant strain, although transcription from this promoter appears to be very low. Footprinting analysis showed that MpeR binds to the $P_{NMB1880}$ promoter region directly upstream of the -35 hexamer, which is a position reflective of the operators of transcriptional activators (Busby & Ebright, 1994) and therefore strongly suggests the role of MpeR as an activator of the $P_{NMB1880}$ promoter. Furthermore, to the best of our knowledge, it is the first time that the target binding site of an AraC regulator of a *Neisseria* species has been determined. This promoter drives transcription of a likely operon, which encodes proteins predicted to be involved in high-affinity iron uptake across the outer membrane and through the periplasmic space, Tdff and NMB1880, respectively. Although the meningococcus and gonococcus do not secrete siderophores they are able to scavenge iron-loaded siderophores synthesized by other micro-organisms. The FetA/FetB system encodes an analogous receptor and periplasmic transporter system, which allows neisseriae to utilize exogenous enterobactin as an iron source (Carson *et al.*, 1999). Furthermore, pathogenic neisseriae have evolved TonB-dependent receptors that sequester host iron-binding proteins including transferrin-binding protein, lactoferrin-binding protein and two haemoglobin receptors (Rohde & Dyer, 2003; Perkins-Balding *et al.*, 2004), as well as many other genes encoding TonB-dependent receptors (Turner *et al.*, 2001) whose roles remain elusive. Fur is known to regulate all of the characterized iron uptake systems, and Fur-binding sites have been described overlapping their promoter regions in the meningococcus (Grifantini *et al.*, 2003; Delany *et al.*, 2006).

We were unable to observe detectable amounts of either the NMB1880 or Tdff proteins by Western blotting, suggesting that derepression of the promoter did not lead to detectable expression. This is in agreement with previous studies, in which Tdff expression could not be detected in the gonococcus grown *in vitro* (Hagen & Cornelissen, 2006) nor in a broad panel of iron-stressed neisseriae including pathogenic and commensal species (Turner *et al.*, 2001). While the MpeR protein could be detected in the M1239 wild-type strain, neither its deletion nor its overexpression affected the transcription of the upstream $P_{NMB1880}$ promoter, suggesting that although MpeR clearly targets the $P_{NMB1880}$ promoter it is not transcriptionally active under *in vitro* growth conditions. Indeed through transcriptional profiling of the M1239 wild-type and the M-2Δ null mutant we were unable to detect any regulation of genes due to MpeR-dependent activity, suggesting that, as with many AraC like proteins, a signal may be needed to induce the regulatory activity of this protein.

Nevertheless, transcription of the *tdff* gene has been reported, by two separate groups, to be induced when the gonococcus infects human cervical epithelial cells (Agarwal *et al.*, 2008; Hagen & Cornelissen, 2006), suggesting that the

regulation of this gene responds to a signal provided by the eukaryotic cell. The Tdff receptor has been implicated in the ability of the gonococcus to acquire iron intracellularly, and mutants in Tdff are incapable of intracellular replication (Hagen & Cornelissen, 2006). It has also been shown that intracellular replication of the meningococcus requires TonB-dependent uptake of a host-derived iron source (Larson *et al.*, 2002). Intracellular meningococci trigger rapid degradation of ferritin within infected epithelial cells (Larson *et al.*, 2004) and iron derived from ferritin degradation supports survival. It is tempting to speculate that this novel host iron source may be the inducer for MpeR and the substrate of the high-affinity iron uptake system encoded by the NMB1880/*tdff* genes. In this model, under iron limitation the iron-uptake operon is expressed to a minimal basal level to allow for the sensing of the iron source and MpeR expression is induced and, on interaction with the iron source, activates the $P_{NMB1880}$ promoter driving maximal expression of the NMB1880/Tdff system allowing active uptake and survival within niches with limited iron sources such as the eukaryotic intracellular environment. However, this remains to be investigated.

The importance of iron acquisition in the development of meningococcal disease has been established in animal models, where the inability to acquire iron corresponds with a strong attenuation of virulence (Calver *et al.*, 1976; Schryvers & Gonzalez, 1989; Stojiljkovic *et al.*, 1995; Klee *et al.*, 2000). We believe that the iron regulation of MpeR, its restricted presence in pathogenic *Neisseria* species, and its targeting of iron-uptake genes that have been associated with survival in the host, implicate this regulator as having an important role in host adaptation.

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