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## The genes that encode the gonococcal transferrin binding proteins, *tbpB* and *tbpA*, are differentially regulated by MisR under iron-replete and iron-depleted conditions

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### Summary

*Neisseria gonorrhoeae* produces two transferrin binding proteins, TbpA and TbpB, which together enable efficient iron transport from human transferrin. We demonstrate that expression of the *tbp* genes is controlled by MisR, a response regulator in the two-component regulatory system that also includes the sensor kinase MisS. The *tbp* genes were up-regulated in the *misR* mutant under iron-replete conditions but were conversely down-regulated in the *misR* mutant under iron-depleted conditions. The *misR* mutant was capable of transferrin-iron uptake at only 50% of wild-type levels, consistent with decreased *tbp* expression. We demonstrate that phosphorylated MisR specifically binds to the *tbpBA* promoter and that MisR interacts with five regions upstream of the *tbpB* start codon. These analyses confirm that MisR directly regulates *tbpBA* expression. The MisR binding sites in the gonococcus are only partially conserved in *Neisseria meningitidis*, which may explain why *tbpBA* was not MisR-regulated in previous studies using this related pathogen. This is the first report of a *trans*-acting protein factor other than Fur that can directly contribute to gonococcal *tbpBA* regulation.

### Graphical Abstract

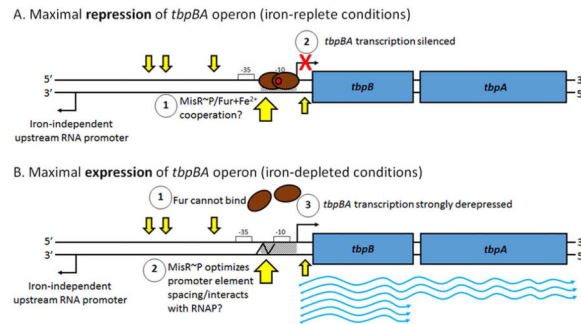
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## Introduction

*Neisseria gonorrhoeae* is the causative agent of the sexually transmitted infection, gonorrhea. This common infection afflicts both genders but causes significant morbidity among women, with post infection sequelae including ectopic pregnancy, pelvic inflammatory disease (Paavonen *et al.*, 2008), infertility (Westrom *et al.*, 1992) and even disseminated infections. The WHO estimated that there were 78 million cases of gonorrhea worldwide in 2012 (Newman *et al.*, 2015). The CDC estimates that the actual incidence of gonococcal infections in the U.S. is 820,000 new cases per year (CDC, 2015). Annual medical costs related to *N. gonorrhoeae* infections and related sequelae exceed \$1 billion in the US alone (Aledort *et al.*, 2005). Infection with *N. gonorrhoeae* does not elicit protective immunity (Hedges *et al.*, 1999, Hedges *et al.*, 1998), so repeat infections are common. Despite decades of research on the pathogenesis of gonococcal disease, no vaccine is available to prevent infections caused by this stealth pathogen. Even more concerning is the evolution of antimicrobial drug resistance among *N. gonorrhoeae* isolates (Barbee, 2014, Unemo & Nicholas, 2012, Hook & van Der Pol, 2013). Penicillin and tetracycline were abandoned years ago as appropriate empiric therapies to treat gonorrhea. More recently, resistance to fluoroquinolones increased to such a degree that this class of drugs was likewise removed from the chemotherapeutic arsenal (CDC, 2007). The currently-recommended therapy to treat uncomplicated gonococcal infections is the combination of ceftriaxone and azithromycin (CDC, 2012). Unfortunately, resistance to both of these drugs has emerged and these resistant strains are becoming increasingly prevalent worldwide (CDC, 2011, Soge *et al.*, 2012, Unemo & Nicholas, 2012).

With increasing prevalence of disease and evolution of antimicrobial resistance, the specter of untreatable gonorrhea is increasingly likely (Barbee, 2014). This situation makes the search for gonococcal vaccine candidates that much more urgent and compelling. The gonococcus has been referred to as “chameleon-like” (Robinson *et al.*, 1989) due to its ability to vary the antigenic character of surface antigens at high frequency. In contrast to most outer membrane proteins produced by *N. gonorrhoeae*, the components of the transferrin iron acquisition system are not subject to high-frequency antigenic or phase variation. The proteins that enable the gonococcus to use human transferrin as the sole source of iron include transferrin binding proteins A and B (TbpA and TbpB). These proteins are ubiquitously expressed by all strains and they are relatively well-conserved in sequence (for reviews see (Cornelissen, 2008, Cornelissen & Sparling, 1994)). Importantly,

we demonstrated that a mutant unable to express the *tbp* genes was unable to elicit signs and symptoms of urethritis in a human male infection model (Cornelissen *et al.*, 1998). These results indicate that expression of the transferrin receptor components is necessary to initiate mucosal infection in human males.

TbpA is an integral outer membrane protein in the TonB-dependent transporter family (Cornelissen *et al.*, 1992), while TbpB is a surface-exposed lipoprotein (DeRocco & Cornelissen, 2007). While TbpA is absolutely essential for iron internalization from human transferrin (Cornelissen *et al.*, 1992), TbpB only enhances the efficiency of iron uptake by approximately 50% (Anderson *et al.*, 1994). While TbpA forms the essential conduit for iron through the outer membrane, TbpB enhances the iron internalization process by specifically recognizing the ferrated form of transferrin (Cornelissen & Sparling, 1996) and by enhancing deferrated transferrin release (DeRocco *et al.*, 2008). Moreover, structural studies indicate that TbpB completes an iron chamber in the three-part protein complex comprised of TbpA, TbpB and transferrin (Noinaj *et al.*, 2012b), thereby preventing iron diffusion after the metal has been extracted from transferrin (for review see (Noinaj *et al.*, 2012a)).

Like most proteins involved in iron acquisition, the genes encoding the transferrin binding proteins are regulated by the internal concentration of iron. The mechanism by which this occurs is quite well understood (Bagg & Neilands, 1987). Ferrous iron, when at high concentration inside the cell, complexes with the Fur protein, which subsequently dimerizes for interaction with target DNA. The Fur repressor protein binds to a specific motif (the Fur box or binding site) within iron-regulated promoters, thereby repressing transcription. When internal iron pools decline, Fur is no longer an effective repressor and it therefore disassociates from promoter regions allowing transcription to ensue (Bagg & Neilands, 1987). Expression of the gonococcal *tbp* genes is further modulated by the expression of an upstream RNA, which is transcribed in the direction opposite to that of the *tbp* genes (Velez Acevedo *et al.*, 2014). We recently demonstrated that inactivation of this upstream RNA alters both transcription and translation of the *tbp* locus (Velez Acevedo *et al.*, 2014).

The transferrin binding proteins, TbpA and TbpB, are encoded by a bicistronic operon, with the *tbpB* gene located upstream of the *tbpA* gene (Ronpirin *et al.*, 2001). We recently mapped the transcription start site and Fur binding site in the *tbpB* promoter region (Velez Acevedo *et al.*, 2014). Furthermore, we demonstrated that under iron stress and at steady-state, the *tbpB* transcripts outnumber *tbpA* transcripts by 2:1, suggesting that processivity throughout the bicistronic transcript is incomplete (Ronpirin *et al.*, 2001). This differential in *tbpB:tbpA* transcripts may be manifested as greater numbers of TbpB proteins relative to TbpA proteins under iron stress as we have also shown that TbpB-specific binding sites outnumber TbpA-specific binding sites on the cell surface by at least 2:1 (Cornelissen & Sparling, 1996).

In the current study, we sought to define additional conditions and regulators that impact expression of the *tbp* genes. Employing RNA-Seq, we characterized the MisR regulon in *N. gonorrhoeae* for the first time. MisR is the response regulator of the MisR-MisS two-component regulatory system, and its cognate sensor kinase is MisS (Tzeng *et al.*, 2004, Tzeng *et al.*, 2006). To date, the signal to which MisS responds in the pathogenic *Neisseria*

species has not been identified. Among the genes regulated by gonococcal MisR, the *tbpB* gene demonstrated greater than five-fold up-regulation in the *misR* mutant. Meningococcal MisR has been demonstrated to impact the expression of iron- and metal-storage and transport genes, including *hmbR*, *bfrAB*, and *tdfH* (Tzeng *et al.*, 2008). However, in contrast with the data shown here for gonococci, the meningococcal MisR regulon does not appear to include the *tbp* genes (Newcombe *et al.*, 2005, Tzeng *et al.*, 2008). In this report, we investigated the phenotype of the gonococcal *misR* mutant with respect to expression of the *tbp* genes, production of the Tbp proteins, and iron uptake from transferrin. Under iron-replete conditions, MisR down-regulates both *tbpA* and *tbpB*. Interestingly, under iron-depleted conditions, MisR appears to up-regulate both *tbp* genes, as evidenced by decreased *tbp* transcript and Tbp protein levels in the *misR* mutant when grown in low iron conditions. Consistent with this observation, the *misR* mutant is impaired for iron uptake from human transferrin relative to the wild-type strain. Furthermore, using DNA-binding experiments, we found that purified, phosphorylated MisR binds to defined sites in the promoter region upstream of the *tbpBA* operon.

## Results

### Expression of *tbp* genes is controlled by MisR

We employed RNA-Seq to evaluate the changes in gene expression in a *misR::kan* mutant compared to the wild-type strain FA19. As shown in Fig. 1 and Supplemental Table S1, *tbpA* and *tbpB* gene expression was increased in the *misR* mutant compared to the wild-type strain, indicating that these genes are MisR-repressed under the conditions tested. Quantitatively, *tbpA* was 3-fold and *tbpB* 5.8-fold down-regulated by MisR in the RNA-Seq analysis (Fig. 1). Also shown in Fig. 1 are the fold-changes in expression for the previously-reported meningococcal MisR target genes, *nadC* (Newcombe *et al.*, 2005) and *dsbD* (Tzeng *et al.*, 2008), which, as expected, were repressed and activated, respectively, by gonococcal MisR. These genes served as internal controls.

### Quantitative RT-PCR (qRT-PCR) confirms that *tbp* transcription is influenced by MisR

To validate the RNA-Seq results, we utilized qRT-PCR with gene specific primers for detection of *tbpA* and *tbpB*. We assessed gene expression with 10 mM Mg<sup>2+</sup> (as was employed for the RNA-Seq analysis; Fig. S1) and without additional Mg<sup>2+</sup> (Fig. 2), under both iron-replete and iron-depleted conditions. Expression of *tbpBA* expression is maximized when grown in iron-depleted media (Anderson *et al.*, 1994, Cornelissen *et al.*, 1992). Consistent with the RNA-Seq data, *tbpA* and *tbpB* expression under iron-replete conditions was up-regulated in the *misR* mutant (Fig. 2A and B). Expression of *tbpA* was five-fold upregulated in the mutant compared to the wild-type strain (Fig. 2A) while *tbpB* expression was upregulated approximately three-fold in the *misR* mutant relative to the wild-type strain (Fig. 2B). However, when iron was chelated by the addition of Desferal, both *tbpB* and *tbpA* were down-regulated in the *misR* mutant. These observations are supported whether the expression data are presented as relative gene expression compared to an internal standard (Fig. 2A) or as fold change in expression, mutant compared to wild-type (Fig. 2B). All pairwise comparisons with the *misR* mutant were statistically different ( $p < 0.05$ ). The reversal in regulatory trend was important, since expression of *tbpBA* is nearly

undetectable in the presence of iron (Velez Acevedo *et al.*, 2014) and transcriptional differences under iron-depleted conditions are far more biologically relevant.

To test the impact of MisR on the derepression of *tbpBA*, we next compared the transcription of *tbpB* and *tbpA* under iron-depleted vs. iron-replete conditions for all three strains. As shown in Fig. 2C, the fold repression of *tbpBA* was decreased from 40–60 fold in WT FA19 to less than 10 fold in the *misR* mutant. These results indicate that the degree to which the *tbp* genes can be derepressed under iron-restricted conditions is dramatically reduced by loss of *misR*. Complementation with the wild-type copy of *misR* reversed the effect on regulation of the *tbp* genes. All pairwise comparisons with the *misR* mutant were statistically different ( $p < 0.01$ ). Taken together, these data indicate that MisR differentially regulates transcription of *tbpBA* depending on the availability of iron, and is required for maximal derepression of *tbpBA* transcription during iron stress.

### Production of the Tbp proteins is also influenced by MisR

We next evaluated TbpA and TbpB protein production under iron-depleted and iron-replete conditions by Western blot. Protein levels under each condition were quantified using ImageJ (Schneider *et al.*, 2012). All lanes were loaded equivalently as assessed by solubilizing the strains grown to the same culture density, loading equivalent protein amounts, and finally staining the nitrocellulose membrane with Ponceau S to confirm equivalent protein loading (data not shown). TbpA and TbpB proteins were upregulated 2.0 to 4.8 fold in the *misR* mutant under iron-replete conditions (Fig. 3), consistent with the RNA-Seq data. This suggested that efficient Fur+Fe<sup>2+</sup>-mediated repression of *tbpBA* requires MisR input. In contrast, under iron-depleted conditions, protein levels were down-regulated by approximately 1.5 fold in the *misR* mutant (Fig. 3). Complementation reversed the effects on both proteins. Fold iron repression of TbpA was decreased from 13 fold in the wild-type to 1.8 fold in the *misR* mutant. Fold iron repression of TbpB was similarly reduced from 40 fold in the wild-type to 13.7 fold in the *misR* mutant.

### Growth of *misR* mutant and complemented strain on media containing ferric nitrate or human transferrin

Since MisR clearly impacted *tbpBA* expression, we next evaluated the ability of the mutant strains to grow on low-iron, solid media with defined iron sources in order to test whether MisR regulation was physiologically relevant. Regardless of the iron source [ferric nitrate or partially iron-saturated human transferrin], the *misR* mutant grew normally on solid GC medium, when provided either ferric nitrate (Fig. 4A) or human transferrin (Fig. 4B) as the sole iron source. As a control, a *tbpA* mutant strain (FA6747; (Cornelissen *et al.*, 1992)) grew on GCB with ferric nitrate but could not grow when transferrin was provided as the sole iron source. We have previously demonstrated that even very low functionality of the TbpA transporter still enables growth on transferrin as a sole iron source (Cash *et al.*, 2015). This observation is consistent with our data showing that down-regulation of *tbp* genes by MisR apparently has no effect on growth when transferrin is the only source of iron. Thus, we quantified the functionality of the transferrin transporter in the *misR* mutant employing a more sensitive measure of iron internalization from transferrin.

### The *misR* mutant internalizes less iron from transferrin than the wild-type strain

We grew the wild-type strain, *misR* mutant and *misRc* strain under iron-depleted conditions and then measured the amount of transferrin-iron internalized by each strain in a 30-min iron internalization assay (Cash *et al.*, 2015, DeRocco & Cornelissen, 2007, Anderson *et al.*, 1994). We determined that the *misR* mutant only internalized about 50% of the iron that can be internalized by the wild-type strain in the 30 min assay (Fig. 5). The *tbpB* deletion strain (FA6905; (Cornelissen & Sparling, 1996)) is similarly able to internalize only about 50% of wild-type levels of transferrin-derived iron, by the function of TbpA alone, consistent with our previous results (Anderson *et al.*, 1994). The negative control strain (FA6815), has a polar  $\Omega$  cassette in *tbpB* and cannot express *tbpA*, which is absolutely required for iron uptake from transferrin, while expression of *tbpB* is not (Anderson *et al.*, 1994). As expected, the negative control showed negligible iron uptake from transferrin. The results shown in Fig. 5 are consistent with a decrease in *tbp* gene expression in the *misR* mutant under iron-depleted growth conditions, leading to a decrease in the ability of the TbpBA proteins to accomplish iron uptake. Transferrin-iron internalization was restored to wild-type levels in the *misR* complemented strain.

### Phosphorylated MisR (MisR~P) specifically binds to the promoter region upstream of *tbpBA*

In order to determine if MisR directly regulates expression of *tbpBA*, we performed electrophoretic mobility shift assays (EMSA) using purified MisR-His<sub>6x</sub> and a PCR-generated DNA probe from upstream of the *tbpBA* operon through the 5' end of the *tbpB* gene. In Fig. 6A, a titration EMSA demonstrated that MisR~P can efficiently shift the labeled *tbpBA* promoter probe. In contrast, non-phosphorylated MisR did not shift the *tbpBA* promoter probe (data not shown). As shown in Fig. 6B, we next performed a competitive EMSA, which demonstrated that MisR~P binding to *tbpBA* promoter probe was significantly diminished in the presence of unlabeled specific competitor (Fig. 7B, lane 3). In contrast, MisR~P binding was not impacted in the presence of unlabeled nonspecific competitor *rmpB* (Fig. 7B, lane 4). These results demonstrate that MisR~P binds specifically to the *tbpBA* promoter region.

### Footprint analysis demonstrates that MisR~P binds to distinct regions of the *tbpBA* promoter

Using DNase I footprinting, we next sought to determine where in the *tbpBA* upstream region MisR~P bound. As shown in Fig. 7, footprint analysis revealed that MisR~P protected five regions (brackets labeled A through E). One region of the *tbpBA* upstream region (labeled bracket D) demonstrated a DNase I hypersensitivity site (asterisk). Region D also corresponds to a stretch of 27 nucleotides on the antisense strand that overlaps both the *tbpBA* -10 promoter element and the Fur binding site (Fig. 8).

### The *tbpBA* promoter and MisR binding sites are partially conserved between *N. gonorrhoeae* and *N. meningitidis*

To explore whether MisR binding site differences might contribute to the lack of apparent MisR regulation of meningococcal *tbpBA* (Tzeng *et al.*, 2008; Newcombe *et al.*, 2005), we



aligned the upstream *tbpBA* promoter regions of four gonococcal strains with those of four meningococcal strains. While the sequences upstream of *tbpB* in the gonococcal strains are nearly identical, the upstream sequences from the *N. meningitidis* strains diverge significantly from the gonococcal sequences (Fig. 9). Conservation between *N. gonorrhoeae* and *N. meningitidis* sequences was considerably higher near the start of the *tbpB* structural gene (green shading, Fig. 9) compared to sequences near the 5' end of the aligned region. All 5 MisR binding sites were well-conserved among these *N. gonorrhoeae* strains but only partial conservation was detected in sites A, D and E in the *N. meningitidis* sequences. These observations suggest that MisR binding site conservation is maintained among gonococcal *tbpBA* promoters but the sequence diverges significantly among the *N. meningitidis* promoters.

## Discussion

MisR is the OmpR-family response regulator in a gonococcal two-component regulatory system which also includes the sensor kinase, MisS (Tzeng *et al.*, 2008). In the meningococcus, loss of MisR prevents the addition of phosphoethanolamine (PEA) to the inner core structure of lipooligosaccharide, thus leading to the gene acronym *mis* (meningococcal inner core structure) (Tzeng *et al.*, 2004). The pathogenic *Neisseriae* encode far fewer (4 have been described) two-component regulatory systems than other Proteobacteria (which encode between 10 and 35 on average; (Qi *et al.*, 2010)), and thus MisR/S may have evolved to “multitask” to some degree. Johnson *et al.* (Johnson *et al.*, 2001) proposed that the meningococcal *misR/S* genes instead be named *phoP/Q* due to their similarity to the homologous genes in *Salmonella*. This group also suggested that MisR/MisS (termed “PhoP/PhoQ” therein) responds to Mg<sup>2+</sup> as a signal, similar to the *Salmonella* PhoP/Q system (Johnson *et al.*, 2001) (Newcombe *et al.*, 2005). However, subsequent direct testing of this hypothesis (Tzeng *et al.*, 2006) demonstrated that meningococcal MisR/MisS is unresponsive to changing Mg<sup>2+</sup> concentrations. In another study, Jamet *et al.* (Jamet *et al.*, 2009) suggested that the meningococcal MisR/MisS combine the functions of both the PhoP/Q and CpxR/A systems in other bacteria. Regardless of the genetic heritage of *misR/misS*, we demonstrate here, for the first time, that the gonococcal transferrin binding genes *tbpB* and *tbpA* are regulated by MisR at the transcriptional level, and that this regulation scheme is associated with a 50% decrease in iron uptake efficiency from human transferrin during iron starvation.

As mentioned, Fur is the primary, known regulator of *tbpB* and *tbpA*. Accessory *cis*-elements, such as the hairpin loop between *tbpB* and *tbpA* (Ronpirin *et al.*, 2001) and an upstream region encoding various repeat units and a long, non-coding RNA (Velez Acevedo *et al.*, 2014) can also modulate expression of the *tbp* genes between 2 to 7 fold. However, until now no *trans*-acting transcriptional regulatory protein other than Fur has been shown to directly regulate *tbpBA* in gonococci. After initial observations that *tbpB* and *tbpA* were controlled by MisR in the RNA-Seq experiment, we confirmed MisR regulation of this locus using qRT-PCR. However, since the media used in the RNA-Seq experiment were iron-replete, *tbpBA* expression would be very low under those conditions due to Fur+Fe<sup>2+</sup>-mediated repression (Velez Acevedo *et al.*, 2014). Thus, we also tested transcription of the *tbp* genes under iron-restricted conditions and found that MisR again had a profound effect,

but surprisingly in the opposite direction. Subsequent Western blot experiments recapitulated the results of our transcription assays, and together these expression studies demonstrate that MisR is necessary for wild-type repression under iron replete conditions but MisR is also important for wild-type de-repression during iron starvation. Consistent with these results, despite normal growth on plates containing partially iron-saturated transferrin as the only iron source, *misR* mutant gonococci displayed reduced uptake of iron from human transferrin similar to a gonococcal mutant completely lacking TbpB.

The binary nature of MisR's regulatory role at the *tbpBA* locus may be explained by the location of its binding sites as determined by EMSA and DNase I footprinting analyses. We note that one MisR binding site (site "D") occurred in the same place as the Fur box and -10 promoter element, but on the opposite strand. Recent structural studies have shown that metal-bound Fur protein can complex with dsDNA at recognition sequences either as a dimer on one side of the double-helix, or as two dimers on opposite sides (Deng *et al.*, 2015). If this behavior holds true for neisserial Fur+Fe<sup>2+</sup>, we hypothesize that MisR might improve the affinity of Fur+Fe<sup>2+</sup> for the *tbpBA* promoter without causing steric hindrance (Fig. 10), although we have no direct evidence of a MisR-Fur interaction. On the other hand, positioning of a transcriptional regulator between the -10 and -35 promoter elements can compensate for impaired RNA polymerase binding and open complex formation at promoters with non-optimal spacing between these two *cis*-elements (Lee *et al.*, 2012). We note that the spacing between the -35 and -10 elements of the gonococcal *tbpBA* promoter is indeed a sub-optimal 16 bp (Velez Acevedo *et al.*, 2014), and as was shown for a mutated *mtrR* promoter (Hagman & Shafer, 1995), small spacing differences can greatly alter transcription in gonococci. Thus, MisR may help to improve *tbpBA* transcription during iron-starvation by compensating for the spacing between the -10 and -35 promoter elements (Fig. 10). Additionally, the nearby, undefined promoter driving divergent transcription of the long noncoding RNA is ~220 bp upstream of the *tbpBA* transcriptional start site (Velez Acevedo *et al.*, 2014), and MisR-protected sites A-C were located between these two promoters (Fig. 10). While we did not detect any impact of MisR on expression of the upstream RNA in the RNA-Seq data, we have not yet explored the possibility that MisR might modulate expression of this RNA species under iron-depleted conditions, which could in turn affect *tbpBA* expression. Future work is needed to determine if any interplay between Fur, iron, MisR, the long non-coding upstream RNA, and the *tbpBA* transcript exist.

MisR regulates a large number of genes in both *Neisseria* species; however, reports of the MisR regulons are not completely consistent with one another (this study; (Newcombe *et al.*, 2005, Tzeng *et al.*, 2008)). This inconsistency may be due to differences between the growth conditions used for culturing prior to microarray and RNA-Seq analysis. Diverse conditions included growth on blood agar plates, growth to mid- vs. late-log phase in liquid GC medium, and differences in MgCl<sub>2</sub> concentration ((Newcombe *et al.*, 2005, Tzeng *et al.*, 2008) and the current study). Nevertheless, some findings were consistently reported and were likewise noted in the current *N. gonorrhoeae* study. For example, expression of bacterioferritin, encoded by *bfrA* and *bfrB*, and *dsbD* encoding a membrane bound thiol:disulfide interchange protein, was clearly activated by MisR in all three studies. With respect to the gonococcal MisR regulon, genes encoding chaperones, heat shock proteins,



protein quality control factors, redox factors, and envelope-localized proteins were prominently represented.

Sequence divergence between *N. meningitidis* and *N. gonorrhoeae* in the region upstream of *tbpB* may explain why the *tbp* genes were not identified as being *misR* regulated in previous studies of the meningococcal MisR regulon (Tzeng *et al.*, 2008; Newcombe *et al.*, 2005). Other factors that could explain the inconsistent results between the current study and the previous study in *N. meningitidis* include the fact that our study used a more sensitive RNA-Seq approach to identify the gonococcal MisR regulon, as compared to employing microarray analysis in the previous studies (Tzeng *et al.*, 2008; Newcombe *et al.*, 2005). In addition, cultures were harvested for RNA isolation at different time points in the growth curve in these two studies, perhaps leading to the disparate results. Regardless of the methodologies and potential species and sequence differences, our cumulative results clearly indicate that the *tbp* genes are part of the gonococcal MisR regulon and that MisR-P binds specifically to the gonococcal *tbpBA* promoter region.

Analysis of the RNA-Seq data indicates that MisR regulates other metal-homeostasis genes in addition to *bfrAB* and *tbpBA* in the gonococcus. For example, MisR repressed the AraC-family regulator, MpeR, which is an activator of the xenosiderophore uptake system encoded by the *fet* operon (Hollander *et al.*, 2011). Furthermore, MisR activated the *laz* (azurin) and NGO1215 copper-binding proteins; *laz* mutants are more sensitive to copper and H<sub>2</sub>O<sub>2</sub> (Wu *et al.*, 2005). While both gonococci and meningococci clearly use MisR to regulate metal-related genes, as we noted, meningococcal *tbpBA* was not a MisR target, even during iron starvation (Tzeng *et al.*, 2008, Zhao *et al.*, 2010). We propose that this regulatory divergence between gonococci and meningococci is related to the sequence variations between MisR binding sites upstream of *tbpBA*.

While this is the first report characterizing a functional interplay between iron stress and MisR-MisS in gonococci, others have found that Fur and CpxR-CpxA co-regulate expression of the EfeUOB ferrous iron transport system in *E. coli* (Cao *et al.*, 2007), and recent work on *V. cholerae* demonstrates that CpxR-CpxA regulates several iron-responsive genes and helps this pathogen adapt to iron-scarce conditions (Acosta *et al.*, 2015). Additionally, MisR regulation at the gonococcal *tbpBA* promoter described in this report is reminiscent of MisR regulation of the *hemO-hmbR* hemoglobin receptor locus in meningococci (Zhao *et al.*, 2010). While gonococci are thought to primarily use the HpuBA hemoglobin receptor (Cornelissen & Hollander, 2011) and seem to encode only pseudogenes for *hmbR* (Harrison *et al.*, 2013) the striking parallels between these two regulatory schemes suggest that pathogenic *Neisseriae* may deploy both Fur and MisR to balance their nutritional requirements for iron and their need to limit oxidative damage and the presentation of conserved antigens. In agreement with this hypothesis, *misR* mutant meningococci were more sensitive to oxidative stress (Tzeng *et al.*, 2008) and generated broad, cross-reactive immunity in an intraperitoneal mouse model of infection (Newcombe *et al.*, 2004). Future work is needed to assess the importance of gonococcal MisR for resistance to oxidative stress and modulation of the host immune response.

The *N. gonorrhoeae* MisR regulon also includes genes that encode adhesins (e.g., *opcA*, *ompA*), an efflux pump, permeases (e.g., *macAB*, *pitB*, *putP*), proteins involved in basic metabolism (e.g., *ppk2*, GAPDH, *glnA*, *nrdAB*), DNA repair enzymes (e.g., *exoIII*, *recN*), and cell division factors (e.g., *scpA*, *nlpC*, *pbp2*, *maf*). The MisR regulons from both *Neisseria* species also include many genes encoding conserved hypothetical proteins of unknown function, but it is important to note that most of these regulatory targets remain unconfirmed by secondary methods. Additionally, the activating signal for MisR/MisS remains undefined (Kumar *et al.*, 2011). Irrespective of these experimental caveats, we propose that MisR control of gonococcal gene expression is important for the survival of gonococci during infection as it would help this human pathogen to respond to a number of stresses and changing environmental conditions.

In summary, this is the first report to characterize the MisR regulon in *N. gonorrhoeae*, which differs significantly from that published for *N. meningitidis*. This is also the first demonstration that the *tbp* genes are regulated through direct interaction with any other protein regulatory factor besides Fur. We defined the molecular parameters for MisR interaction with the *tbp* promoter region, demonstrating that MisR~P specifically binds to five regions in the DNA upstream of the *tbpB* start codon, and quantitatively showed that MisR is required for optimal iron uptake from transferrin. Finally, differences between the *Neisseria* species in the promoter regions upstream of *tbpB* may explain why the *tbp* operon was not previously recognized as a target of MisR-dependent regulation in *N. meningitidis*. The data presented in this study suggest that *N. gonorrhoeae* fine-tunes *tbpBA* expression depending upon both iron status and the activation state of the two-component regulatory system comprised of MisR and MisS. The many inputs for *tbpBA* regulation may allow the gonococcus to rapidly adapt to *in vivo* signals and remodel the bacterial cell surface for optimum growth and survival.

## Experimental Procedures

### Bacterial strains and growth conditions

*Neisseria gonorrhoeae* strains were maintained on GC medium base (Difco) containing Kellogg's supplement I (Kellogg *et al.*, 1963) and 12  $\mu$ M ferric nitrate. Plates and liquid cultures were incubated at 37°C in an atmosphere supplemented with 5% CO<sub>2</sub>. Liquid cultures of the wild type and mutant strains were grown in GC broth (GCB) in acid-washed glassware. Iron-replete conditions were generated by the addition of ferric nitrate at inoculation and after one mass doubling. Iron-depleted conditions were generated by addition of 10  $\mu$ M Desferal (deferoxamine) after one doubling of the population of cells. To induce expression of *misR* in the *misRc* strain, 1 mM IPTG was added to liquid growth cultures. Addition of IPTG to the wild-type and mutant strains had no effects on growth or assay outcomes. For some experiments, cultures were additionally supplemented with 10 mM MgCl<sub>2</sub> as noted in the results.

### Construction of *misR* and *misRc* strains

The *misR* gene was inactivated using the non-polar *aphA3* kanamycin resistance cassette (Menard *et al.*, 1993). FA19 *misR::kan* was constructed by transforming FA19 with

meningococcal genomic DNA from the *misR*-deficient *N. meningitidis* mutant SZT1001 constructed previously (Zhao *et al.*, 2010). Plate transformations were performed as described previously (Gunn & Stein, 1996). In general, completely *misR*-deficient transformants could be obtained with 3–4 days of incubation on GC agar containing kanamycin at 50 µg/mL, and we often found that kanamycin resistant transformants arising 1–2 days after plating on selection were merodiploid (data not shown). The FA19 *misR::kan* mutant was complemented with a wild-type (WT) copy of *misR* cloned into pGCC4 using primers *misR3*PacI (5'-CAAACAATTAATTAACGCCACGCCCG-3') and *misR4*PmeI (5'-GTTGGAACAGTTTAAACTGCCCG-3') and methods described previously (Lewis *et al.*, 2009). Loss of *misR* was confirmed by PCR across the *misRS* operon using primers *misR1* (5'-CCATGAGCCGCGTATTACTC-3') and *MisSR* (5'-GCGGACGGTATGTGCGTGA-3'). Complementation in pGCC4 was confirmed by PCR using primers *lctP* (5'-GCGCGATCGGTGCGTTCT-3') and *aspC1* (5'-GCCGGATGCGTCTTTGTAC-3'). Mutant and complement strains were further verified by Western blot (anti-MisR antisera kindly provided by Yih-Ling Tzeng, Emory University), which demonstrated the complete loss of MisR protein in true *misR::kan* mutants and recovery of MisR protein production in the complemented strain upon induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

### RNA-Seq experiments

RNA-Seq fold change (2-fold was the reporting cutoff) was calculated by averaging the normalized RPKM (reads-per-kilobase-per-million-reads) values from three independent experiments. RNA-Seq raw data (and methods) for the results shown herein have been published (Velez Acevedo *et al.*, 2014) (GEO accession number GSE50184; SRA accession number SRP029218). All average fold changes satisfied a statistical significance cutoff value of <0.05 as determined using the Bonferroni correction.

### Assessment of mutant growth on solid medium containing transferrin, lactoferrin and ferric nitrate

Wild-type and mutant strains were grown on GC medium or chemically defined, chelexed medium (CDM) (West & Sparling, 1987) supplemented with human transferrin (2.5 µM, 20% saturated with iron), human lactoferrin (2.5 µM, 30% saturated with iron) or ferric nitrate (12 µM). Plates were incubated for 48 hours at 37°C in an atmosphere supplemented with 5% CO<sub>2</sub> before imaging (Alpha Innotech Imager).

### Iron internalization assays

Transferrin-iron internalization assays were performed as described previously (DeRocco & Cornelissen, 2007). Human transferrin was saturated to 20% with <sup>55</sup>Fe (Perkin-Elmer). Gonococcal strains, grown overnight on GC medium plates containing 10 µM Desferal, were standardized to the same optical density and then mixed with 3 µM <sup>55</sup>Fe labeled transferrin in a final volume of 150 µl of CDM plus BSA (bovine serum albumin) as a nonspecific protein blocker. Duplicate samples contained 215 µM KCN to metabolically poison *N. gonorrhoeae* cells, allowing for the assessment of the amount of <sup>55</sup>Fe bound to but not internalized into cells. Following a 30 min incubation period, Millipore multiscreen microtiter dishes containing the labeled gonococcal cells were filtered, washed and the

nitrocellulose filters at the bottom of the dishes were allowed to air dry. The filters were removed from each plate and radioactive iron associated with or internalized by the gonococcal cells was detected using a Beckman LS6500 scintillation counter. Triplicate counts were averaged and surface-associated counts were subtracted from total counts to determine the counts internalized during the 30 min experiment. The data are presented as values normalized to the positive control (wild-type FA19).

### Western blotting to detect TbpA and TbpB (including ImageJ analysis)

*N. gonorrhoeae* strains were grown under iron-depleted and iron-replete conditions (as described above) in GCB. Strains were grown for 4 hours after one doubling, standardized to cell density by Klett reading, and pelleted by centrifugation. Standardized cell pellets were resuspended in Laemmli solubilizing buffer (Laemmli, 1970) followed by protein assay by BCA. Standardized amounts of protein from each strain were supplemented with 5% beta-mercaptoethanol and then loaded into lanes of an SDS polyacrylamide gel (Laemmli, 1970). Separated proteins were transferred to nitrocellulose membrane in a submerged transfer apparatus (BioRad) by the method of Towbin (Towbin *et al.*, 1979). Nitrocellulose membranes were stained with Ponceau S to ensure that each lane was loaded equivalently with whole-cell lysate proteins from each strain. To detect TbpA, nitrocellulose blots were blocked with 5% BSA in high salt tris-buffered saline (TBS). Subsequently a polyclonal anti-TbpA antibody (Cornelissen *et al.*, 1992) was used to probe the blots, followed by washing with high-salt TBS, addition of the secondary goat-anti-rabbit alkaline phosphatase conjugate, additional washing, and finally development with the NBT/BCIP system (Sigma). To detect TbpB, nitrocellulose filters were blocked with 5% non-fat skim milk in low salt TBS, followed by the addition of a polyclonal anti-TbpB serum (Thomas *et al.*, 2006), washing and then addition of secondary antibody and detection system as described above for TbpA. Blots were imaged using ImageJ software (Schneider *et al.*, 2012).

### qRT-PCR

*N. gonorrhoeae* was grown in GCB under iron-depleted and iron-replete conditions as described above. Four hours after addition of the iron chelator (Desferal), supplemental iron or IPTG, cells were mixed with 10 ml of RNAProtect (Qiagen), vortexed for 5 sec and incubated at RT for 5 min. The cell suspension was centrifuged, the supernatant decanted and the pellets stored at  $-20^{\circ}\text{C}$  until further processing. Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. Purified RNA was treated with RNase-free DNase twice, followed by addition of Superase-In (Ambion) and finally storage at  $-80^{\circ}\text{C}$ . Reverse transcription of RNA into cDNA was accomplished using the Accuscript high-fidelity 1<sup>st</sup> strand cDNA synthesis kit (Agilent Technologies) and random hexamers as primers. cDNA was amplified with gene specific primers and the SensiMix SYBR no-ROX kit (Bioline) employing the CFX96 real-time system (Bio-Rad). Expression of target genes (*tbpA* and *tbpB*) was normalized to *porB1A* expression as an internal control. Each assay was performed at least in triplicate. Relative expression values for each gene were calculated using the  $2^{-\text{Ct}}$  method (Livak & Schmittgen, 2001).

## Purification and phosphorylation of MisR-His<sub>6x</sub> protein

His-tagged MisR protein was purified using methods adapted from (Tzeng *et al.*, 2006). Two 5 mL cultures of *E. coli* BL21 (DE3) cells harboring the pYT298 plasmid were grown overnight in LB broth with kanamycin selection (50 µg/mL) in a 37°C water bath, shaking at 200 rpm. These cultures were used as an inoculum for a 1 L culture grown under the same conditions as above. At mid-log (OD<sub>600</sub> of ~0.35) the culture was induced with 1 mM IPTG. IPTG-induced expression of MisR-His<sub>6x</sub> was allowed to continue for approximately 3 hours. These stationary phase cells were harvested by centrifugation at 7,500 rpm for 10 min at 4°C, then pellets were stored at -70°C. Frozen pellets were thawed on ice and resuspended in a total of 30 mL of lysis buffer + 1 mM PMSF (Tzeng *et al.*, 2006). BL21 (DE3) lysates were obtained using 3 rounds of disruption by a French Press Cell Disruptor (Thermo Electron Corporation) at 600 psi with 10 min cooling intervals on ice. Cell debris was removed by centrifugation at 10,000 rpm for 15 min at 4°C. Lysate supernatant was passed through 0.2 µm sterile filters to remove any remaining intact cells. 3 mL of packed Ni-NTA agarose (Qiagen) were equilibrated on a 20 mL Econo-Pac<sup>®</sup> chromatography column (Biorad) using two 10 mL washes with lysis buffer. MisR-His<sub>6x</sub> filtrate was added to the equilibrated Ni-NTA agarose and rotated on column overnight at 4°C. Washes, elution, and validation by SDS-PAGE/Coomassie stain were performed as described previously (Tzeng *et al.*, 2006). The MisR-containing fractions were pooled, loaded into pre-equilibrated dialysis tubing (Spectra/Por Dialysis Membrane, MWCO: 12–14 kDa, cat. # 132 676) and dialyzed in dialysis buffer + 1 mM dithiothreitol (DTT) for 2 hours with 2 buffer changes at 4°C. Dialyzed protein MisR-His<sub>6x</sub> (molecular weight ~25 kDa) was concentrated to ~0.9 µg/µL at 4°C using an Amicon Ultra-4 filter and stored in 10% (v/v) glycerol at -70°C. Purified MisR-His<sub>6x</sub> protein was phosphorylated by incubation with 50 mM acetyl phosphate for 30 min at 37°C.

## Electrophoretic mobility shift assay

Primers *tbpBA*\_1090F (5'-CTTGTGTTTTAGAAAGACTCAGGG-3') and *tbpBA*\_1090R (5'-CACAGGCAACACCATAGCAGC-3') and chromosomal DNA were used to amplify a 297 bp product corresponding to the promoter region (-221 to +75) of the *tbpBA* operon from *N. gonorrhoeae* strain FA1090, which was subsequently purified by QIAquick Gel Extraction Kit (Qiagen). Nonspecific competitor *mpB* DNA was similarly prepared using primers RnpB1F (5'-CGGGACGGGCAGACAGTCGC-3') and RnpB1R (5'-GGACAGGCGTAAGCCGGGTTC-3'). Electrophoretic mobility shift assays (EMSA) were performed as described previously (Tzeng *et al.*, 2006).

## DNase I footprint analysis

DNase I footprinting assays were performed as described previously (Tzeng *et al.*, 2006). Briefly, the location of MisR-His<sub>6x</sub> binding sites was assessed by incubating dsDNA (labeled on either the sense or antisense strand with  $\gamma$ -<sup>32</sup>P-ATP) with MisR-His<sub>6x</sub> protein as described above for EMSAs. Note that the binding buffer for DNase I footprinting does not contain salmon sperm DNA. Bound DNA was digested with 0.0625 ng/µL DNase I for 50 seconds, then stopped using a phenol:chloroform:isoamyl alcohol (25:24:1) and ethanol extraction. Pelleted, digested, labeled DNA was resuspended in TE (pH 8.0) and loading

dye, then normalized by counts per min on a scintillation counter. Sanger sequencing reactions (Epicentre, Madison, WI) were run alongside the sense and antisense footprinting reactions for all four bases. All samples were heated to 95°C for 2 min then iced prior to loading on an 8% denaturing polyacrylamide gel, which was run and imaged using autoradiography as described previously (Johnson *et al.*, 2011).

### Sequence alignment

The extended promoter regions upstream of the *tbpB* genes from *N. gonorrhoeae* and *N. meningitidis* strains were aligned using T-Coffee (<http://tcoffee.org.cat/apps/tcoffee/all.html>). Sequence data was aligned from the following sources: *N. gonorrhoeae* FA 1090 (<http://www.ncbi.nlm.nih.gov/nuccore/AE004969>); *N. gonorrhoeae* FA19 (<http://www.ncbi.nlm.nih.gov/nuccore/KJ579425>); *N. gonorrhoeae* F62 (<http://www.ncbi.nlm.nih.gov/nuccore/KJ579423>); *N. gonorrhoeae* MS11 (<http://www.ncbi.nlm.nih.gov/nuccore/KJ579424>); *N. meningitidis* MC58 (<http://www.ncbi.nlm.nih.gov/nuccore/AE002098>); *N. meningitidis* L91543 (<http://www.ncbi.nlm.nih.gov/nuccore/JTJE01000016>); *N. meningitidis* H44/76 (<http://www.ncbi.nlm.nih.gov/nuccore/CP002420>); *N. meningitidis* 053442 (<http://www.ncbi.nlm.nih.gov/nuccore/CP000381>).

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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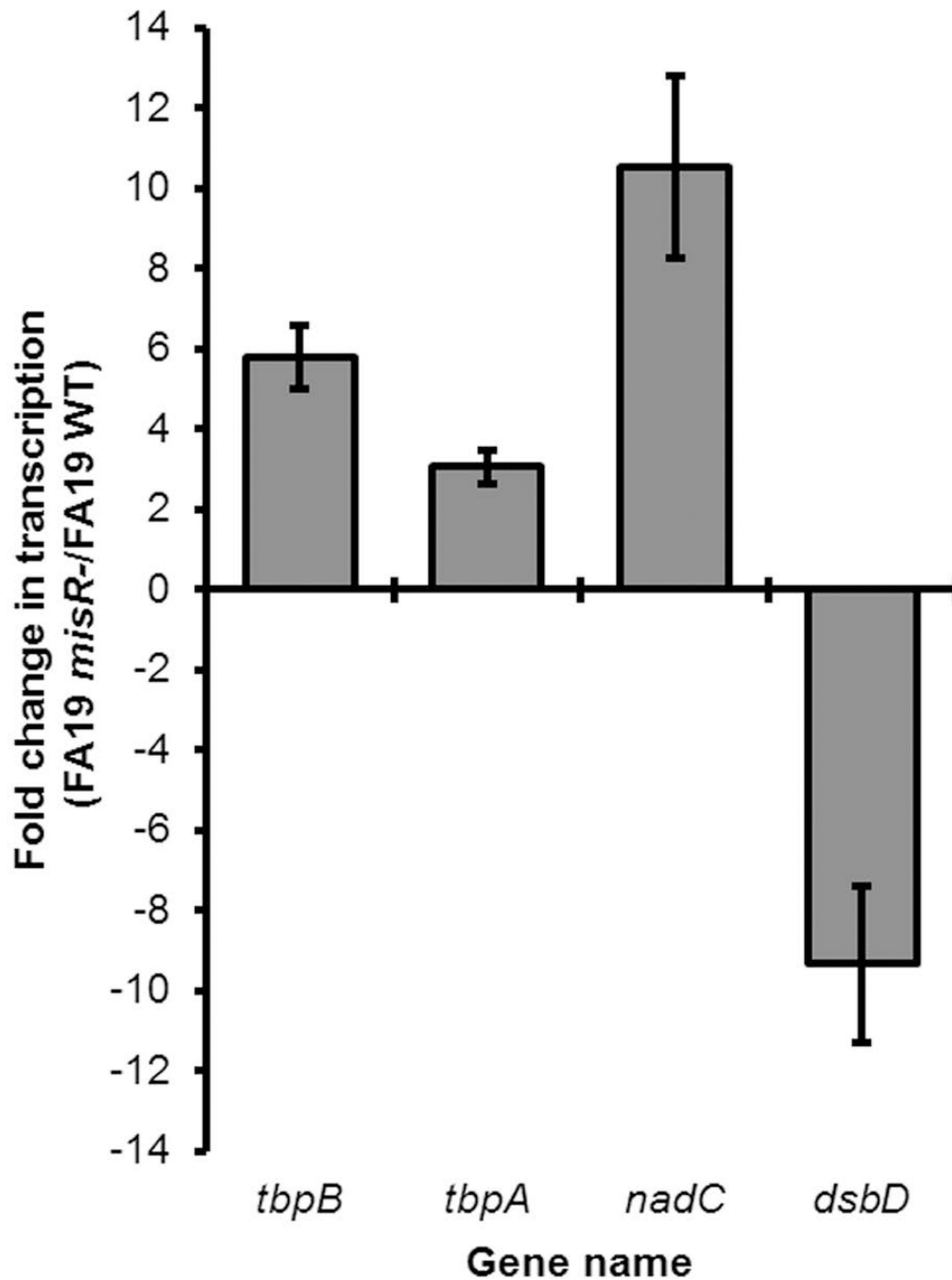
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**Fig. 1. RNA-Seq demonstrated that the *tbp* genes are among those regulated by MisR**  
 Fold change in gene expression (FA19 *misR*<sup>-/-</sup>/FA19 WT) is shown on the Y-axis. Shown are the average fold changes in transcription of *tbpB*, *tbpA*, and 2 control genes *nadC* and *dsbD*, in the presence or absence of MisR for three independent experiments. Error bars represent the standard deviation from the mean for each gene. Similar to our results shown here, control genes *nadC* and *dsbD* were repressed and activated, respectively, by MisR in *N. meningitidis* (see Table S1 in this work and also Table S1 in (Newcombe *et al.*, 2005) and Table S1 in (Tzeng *et al.*, 2008)). These genes serve to validate our findings in the

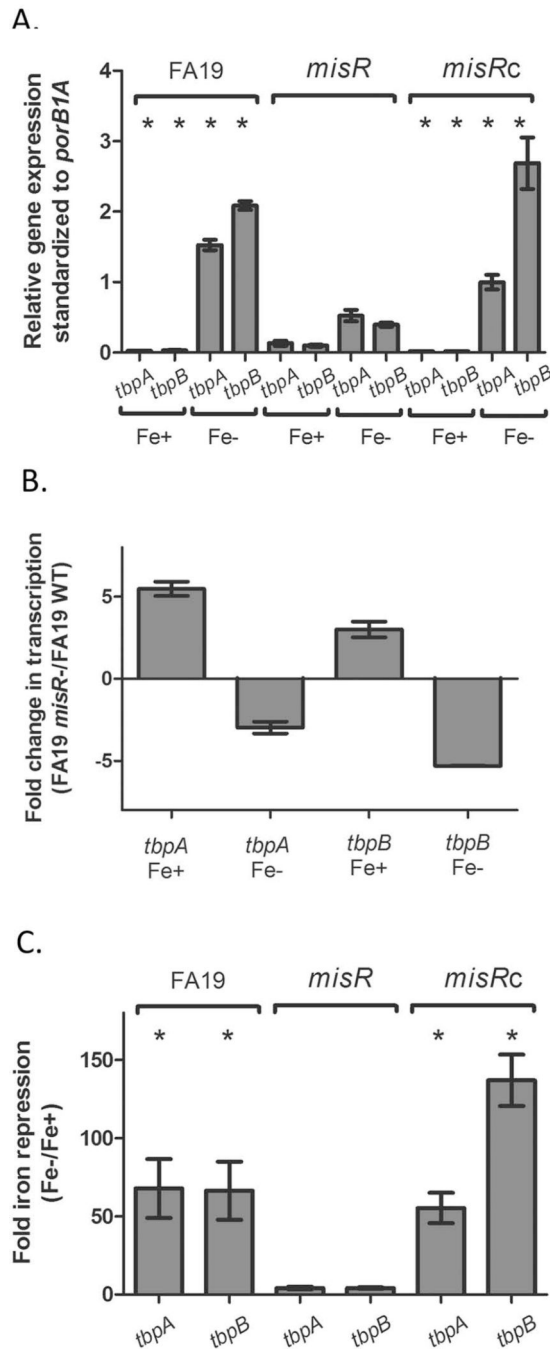
gonococcus. See Table S1 for Bonferroni corrected values demonstrating statistical significance.

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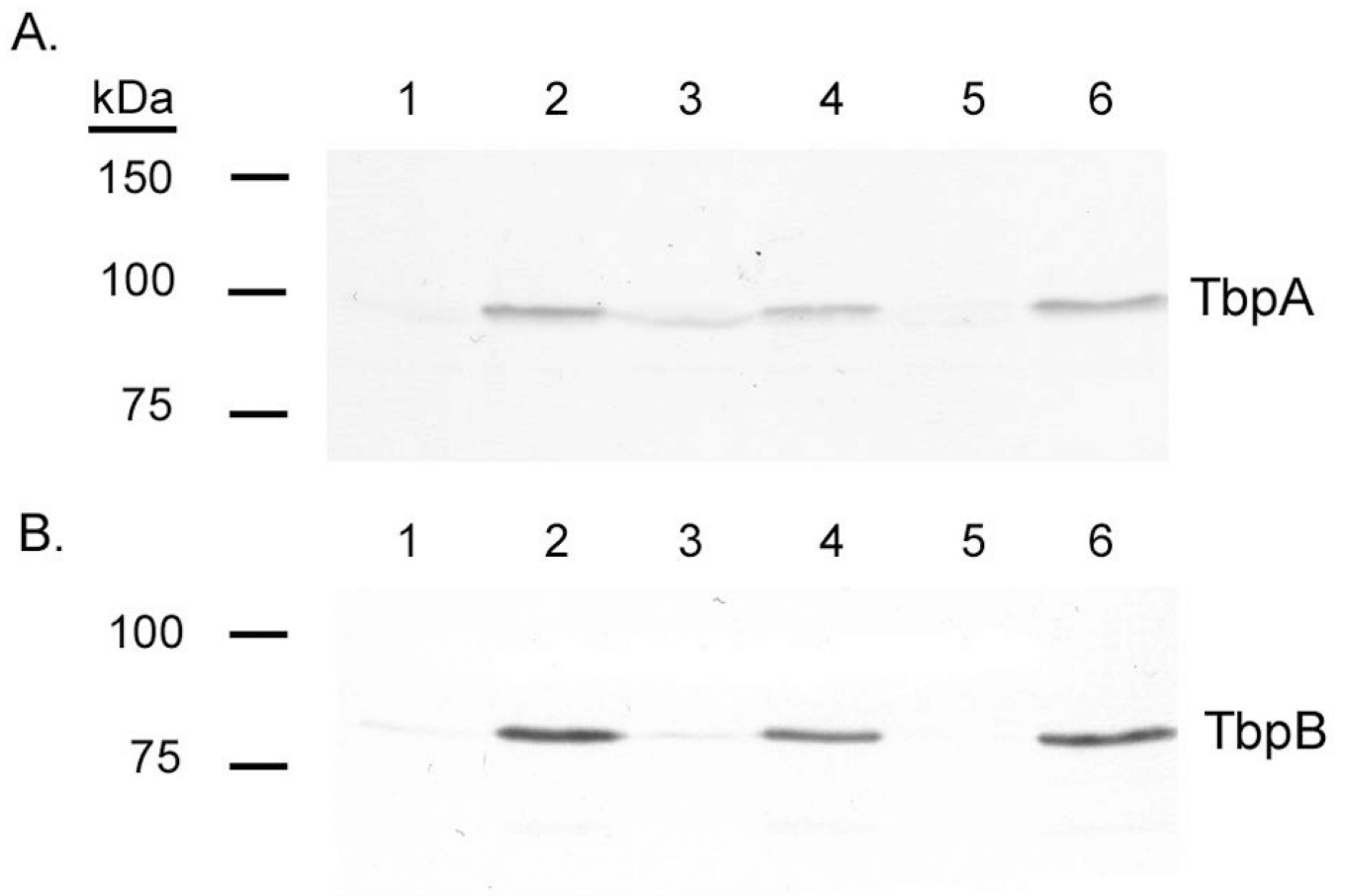
**Fig. 2. qRT-PCR demonstrates differential expression of *tbp* genes depending on the iron status of the growth conditions**

Cultures were grown in GCB medium under iron replete (plus ferric nitrate, Fe<sup>+</sup>) or iron depleted (plus Desferal, Fe<sup>-</sup>) conditions. qRT-PCR experiments were conducted in triplicate.

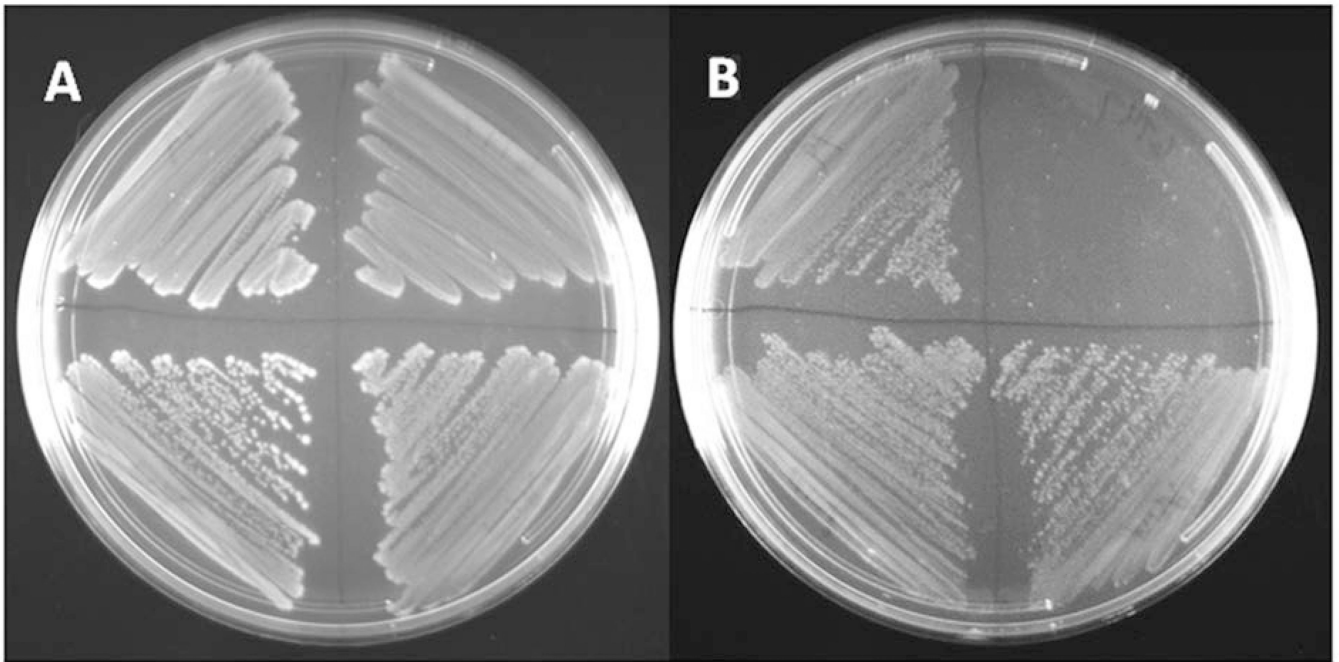
**Panel A.** Relative gene expression for *tbpA* and *tbpB* genes under iron-replete and iron-depleted conditions for WT, *misR* and *misRc* strains. Shown are the average relative gene expression values (standardized to the *porB1A* gene); error bars represent the standard deviation from the mean. Asterisks demonstrate those values that were significantly different



( $P < 0.05$ ) as compared to the *misR* mutant in pairwise comparisons using an unpaired Student's *t*-test. **Panel B.** The average fold change in expression of the *tbp* genes is shown on the Y-axis and represents the ratio of FA19 *misR*-/FA19 WT gene expression (as shown in Fig. 1). Error bars represent standard deviation from the mean for each gene and condition. **Panel C.** Fold iron repression is shown on the Y-axis and represents the relative expression of *tbp* genes under iron-depleted/iron-replete conditions. The error bars represent the standard deviation from the mean. Asterisks demonstrate those values that were significantly different ( $P < 0.01$ ) as compared to the *misR* mutant in pairwise comparisons using an unpaired Student's *t*-test.

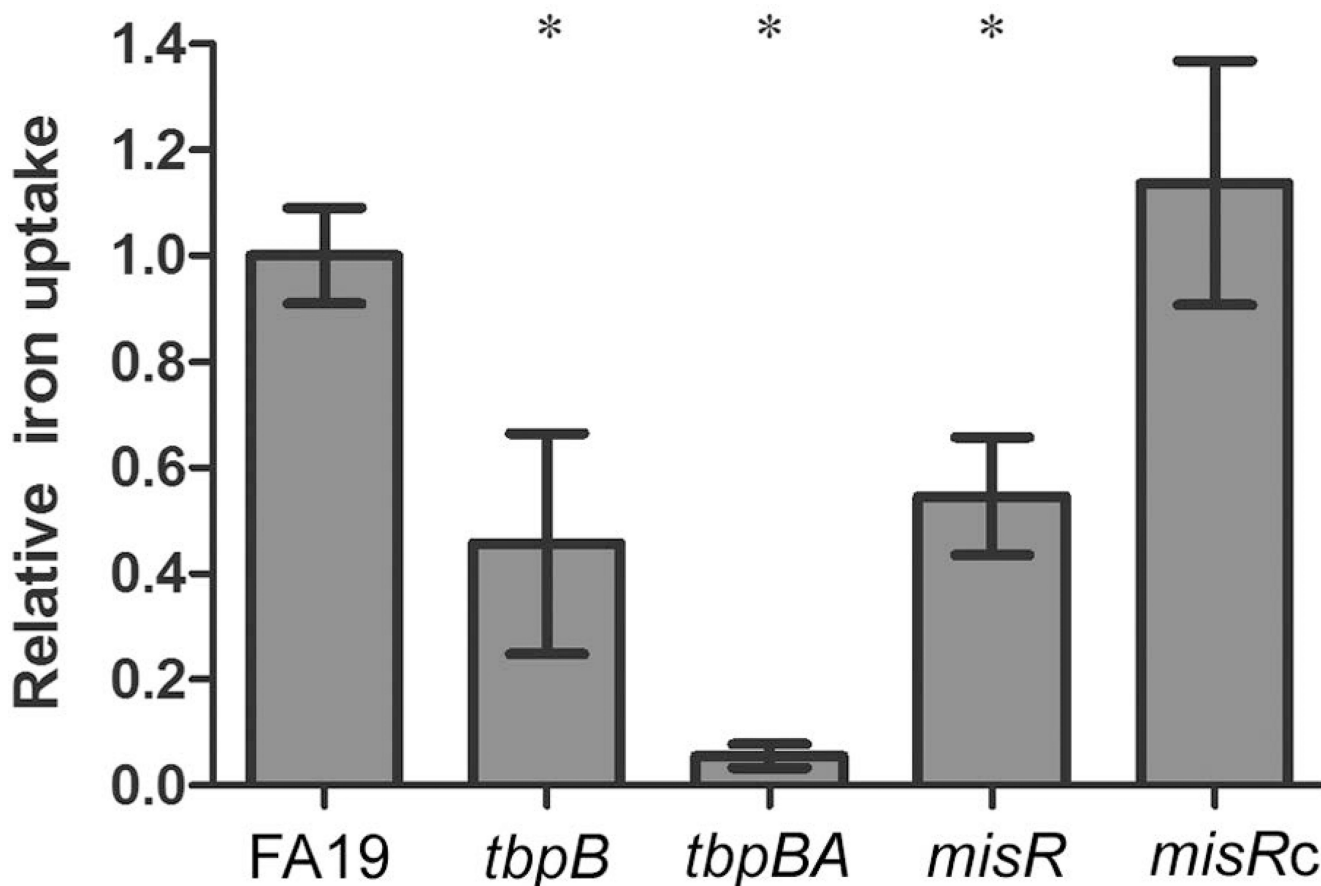


**Fig. 3. Western blotting demonstrates that the Tbp proteins are influenced by expression of *misR***  
**Panel A.** Western blot probed for TbpA. **Panel B.** Western blot probed for TbpB. Lanes contain whole cell lysates, standardized by culture density, of the following strains grown under the described conditions: 1: wild-type FA19 +Fe; 2: wild-type FA19 -Fe; 3: *misR* mutant +Fe; 4: *misR* mutant -Fe; 5: *misRc* strain +Fe; 6: *misRc* strain -Fe. Approximate positions of molecular mass standards are shown to the left of the blots. The blots are representative of at least 10 independent experiments.



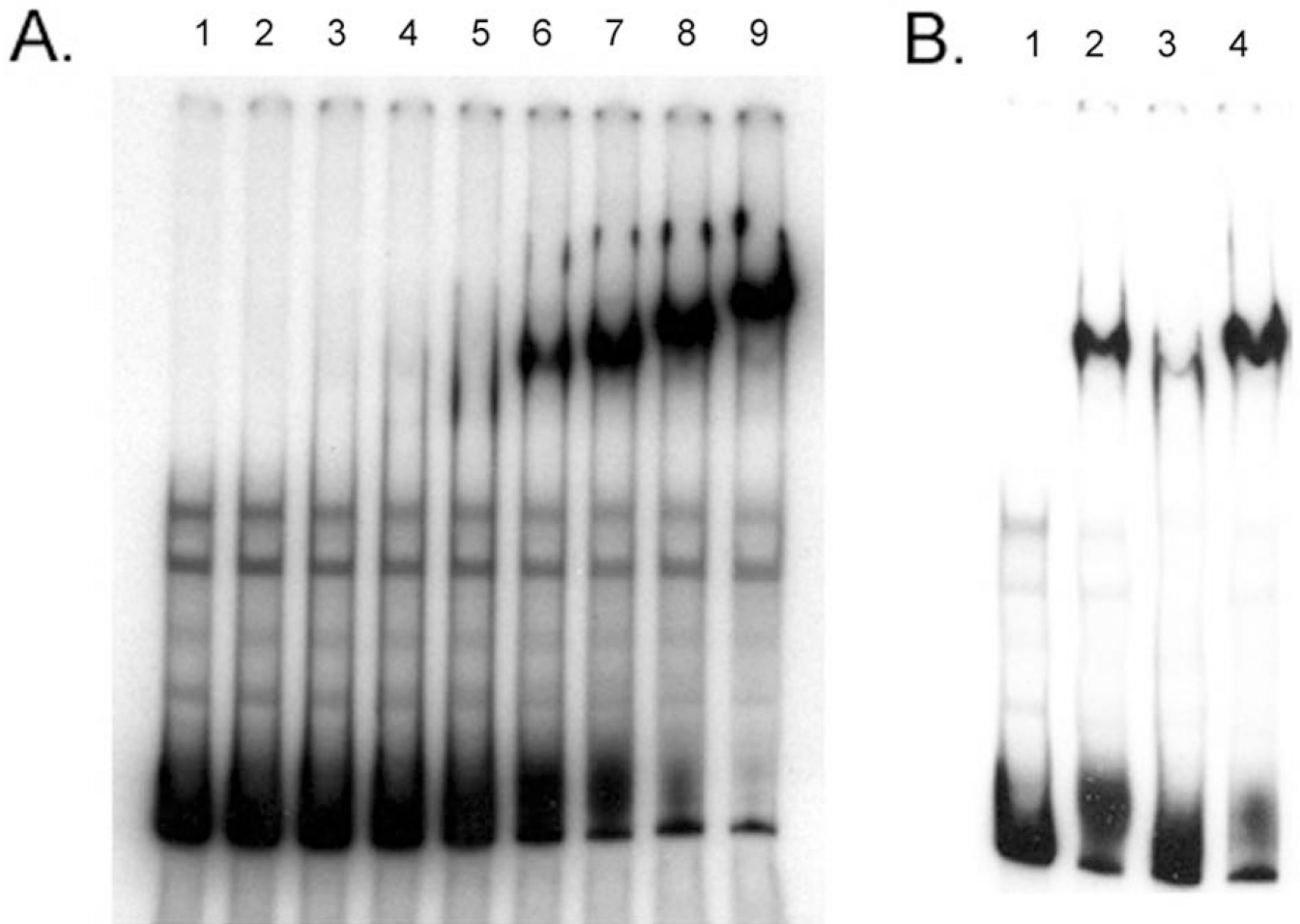
**Fig. 4. Growth of the *misR* mutant and complemented strain on solid medium containing transferrin or ferric nitrate**

Gonococcal strains were streaked in quadrants on solid medium in the following orientation: upper left, WT FA19; upper right, *tbpA* mutant; lower left: *misR* mutant; lower right: *misRc*. Plate A contains ferric nitrate and plate B contains transferrin as the sole iron source.

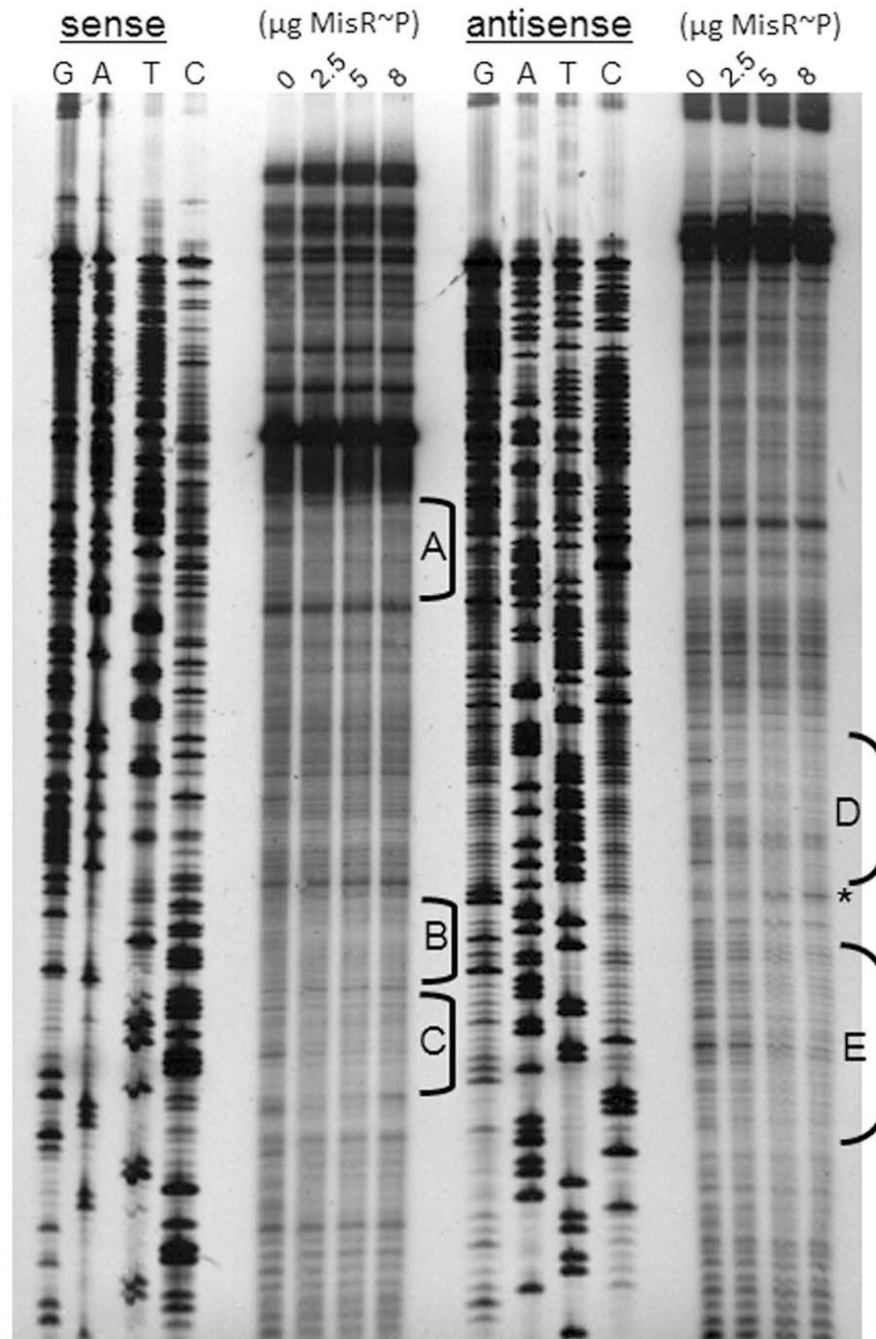


**Fig. 5. Transferrin-iron uptake by the wild-type and mutant gonococcal strains**

Gonococcal strains were grown in iron-depleted conditions, standardized to culture density and then incubated with transferrin- $^{55}\text{Fe}$  for 30 min. Unincorporated  $^{55}\text{Fe}$  was removed by filtration, followed by scintillation counting of extracted filters containing gonococcal strains. The specific amount of iron internalized by each strain was standardized to the wild-type strain, resulting in the relative iron uptake values shown on the y-axis. The each bar represents the mean and standard deviation from the mean generated from three independent assays. Asterisks represent those comparisons in which statistical significance ( $P$ -value  $< 0.05$ ) was reached in pairwise comparisons between wild-type and mutant strains using an unpaired student's  $t$ -test.



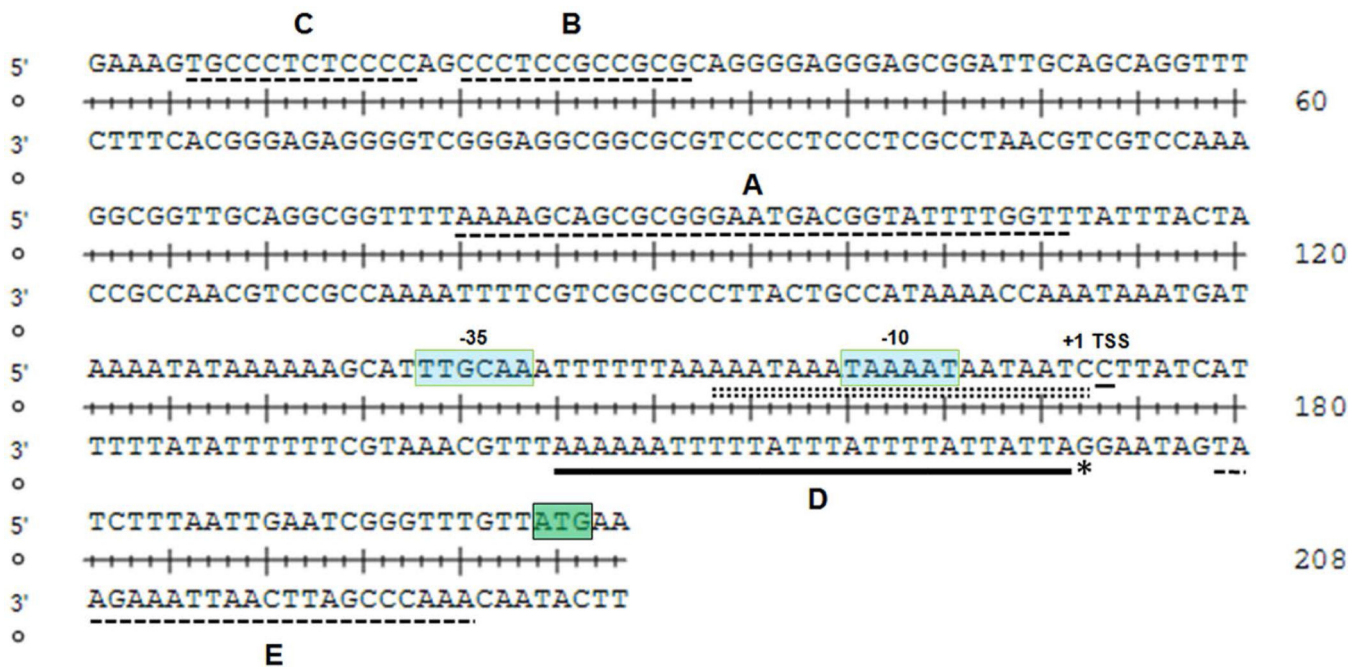
**Fig. 6. Mobility shift assays demonstrate that MisR binds directly to the *tbpA* promoter region**  
**Panel A.** Titration EMSA showing binding of MisR~P to the *tbpA* promoter region. Lane 1: 2 ng labeled probe alone; lane 2: 2 ng labeled probe + 0.5  $\mu$ g MisR~P; lane 3: 2 ng labeled probe + 0.75  $\mu$ g MisR~P; lane 4: 2 ng labeled probe + 1.0  $\mu$ g MisR~P; lane 5: 2 ng labeled probe + 1.5  $\mu$ g MisR~P; lane 6: 2 ng labeled probe + 2.0  $\mu$ g MisR~P; lane 7: 2 ng labeled probe + 2.5  $\mu$ g MisR~P; lane 8: 2 ng labeled probe + 3.0  $\mu$ g MisR~P; lane 9: 2 ng labeled probe + 4.0  $\mu$ g MisR~P. **Panel B.** Competitive EMSA showing specificity of MisR~P binding to the *tbpA* promoter region. Lane 1: 2 ng labeled probe alone; lane 2: 2 ng labeled probe + 2.5  $\mu$ g MisR~P; lane 3: 2 ng labeled probe + 1  $\mu$ g unlabeled specific competitor (unlabeled probe) + 2.5  $\mu$ g MisR~P; lane 4: 2 ng labeled probe + 1  $\mu$ g unlabeled nonspecific competitor (*tnpB*) + 2.5  $\mu$ g MisR~P. Note that the addition of unlabeled specific competitor (unlabeled probe), but not unlabeled nonspecific competitor (*tnpB*) greatly reduces the amount of MisR~P shifted labeled probe.



**Fig. 7. Footprint analysis demonstrates the specific binding sites occupied by MisR~P in the *tbpB* promoter region**

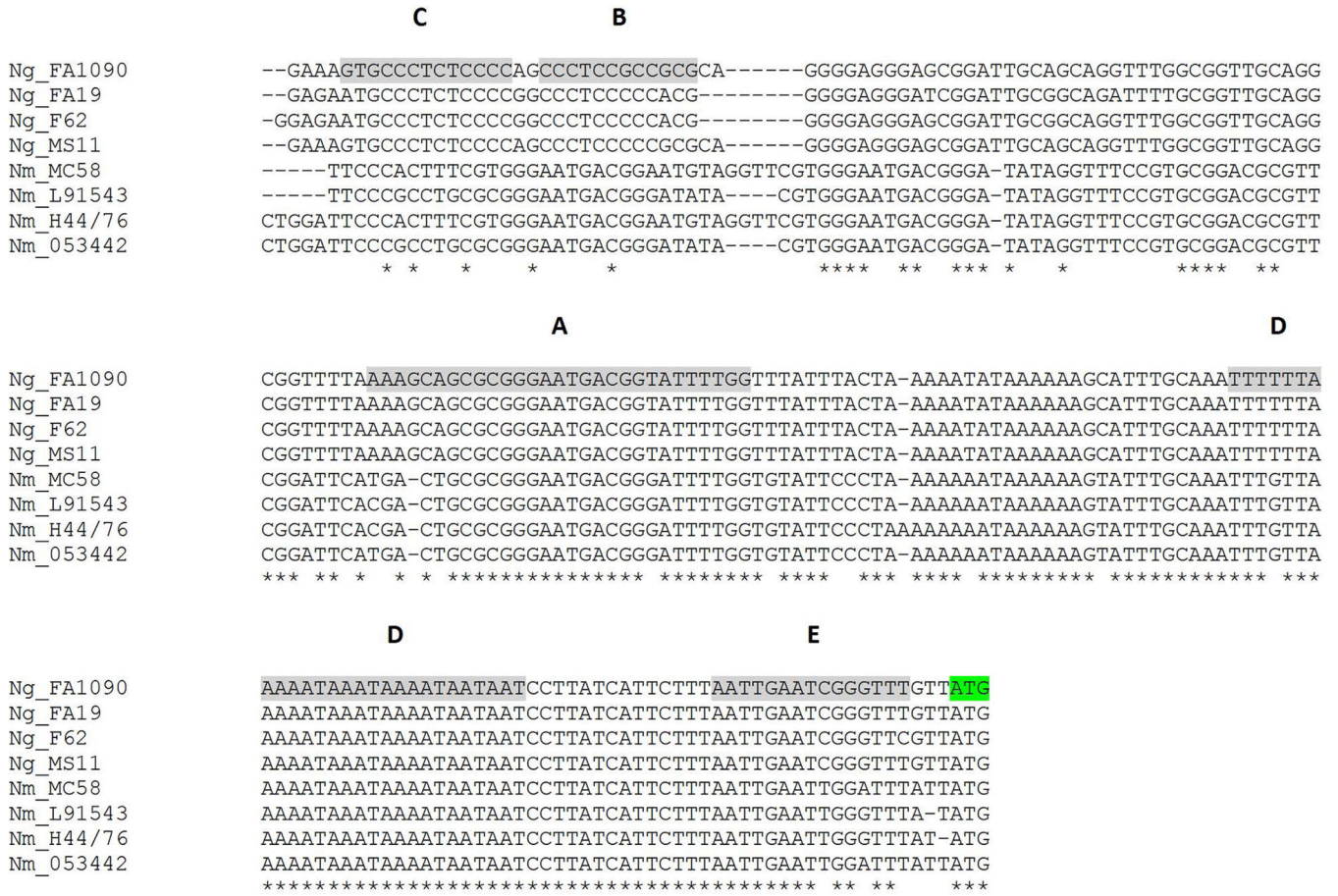
DNase I footprinting used EMSA components described in Figure 6. Areas of protection are highlighted by black brackets (brackets A, B, and C correspond to protection on the sense strand, and brackets D and E correspond to protection on the antisense strand). A DNase I hypersensitivity site is shown by the asterisk.





**Fig. 8. The promoter region upstream of *tbpB* with MisR~P binding sites identified relative to known promoter elements**

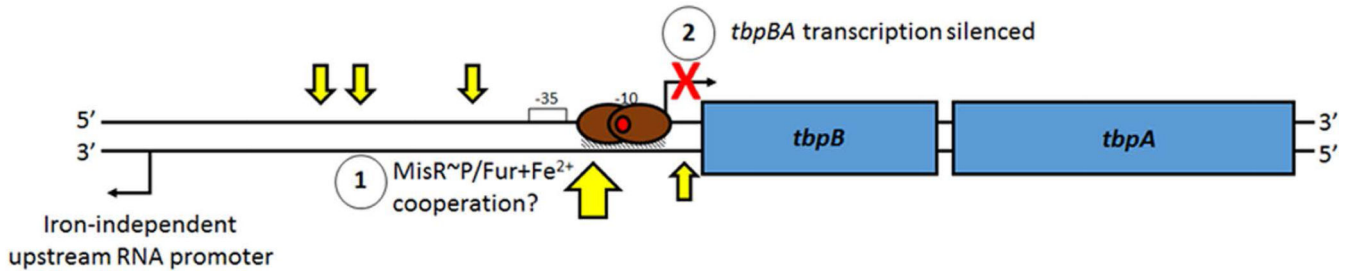
The sequence shown corresponds to the region of the *tbpBA* promoter analyzed by footprint assay in Figure 7. MisR~P protected sites are indicated on the appropriate strand by dashed lines or a black bar and are labeled corresponding to brackets in Figure 7. A DNase I hypersensitivity site is shown by the asterisk. The Fur binding site (Velez Acevedo *et al.*, 2014) is shown as a double dashed line. Promoter elements and the transcriptional start site are indicated with text. The ATG start codon for *tbpB* is highlighted in green.



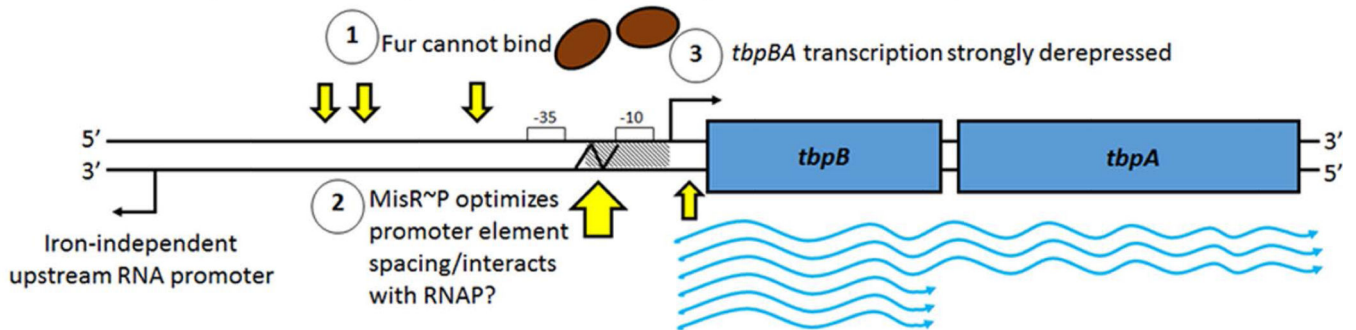
**Fig. 9. Sequence alignment of *tbpBA* promoter regions comparing *N. meningitidis* with *N. gonorrhoeae* strains**

The promoter regions of *tbpBA* from *N. gonorrhoeae* strains FA1090, FA19, F62 and MS11 were aligned with those of *N. meningitidis* strains MC58 (serogroup B), L91543 (serogroup C), H44/76 (serogroup B) and 053442 (serogroup C). Gray shading represents the MisR binding sites identified in Fig. 8. The *tbpB* start codon is highlighted in green.

### A. Maximal repression of *tbpBA* operon (iron-replete conditions)



### B. Maximal expression of *tbpBA* operon (iron-depleted conditions)



**Fig. 10. Iron and MisR~P coordinately regulate expression of the *tbpBA* operon**

**A)** Under iron-replete conditions, the ferric uptake regulator, Fur (brown ovals), binds  $\text{Fe}^{2+}$  (red circle) and dimerizes, allowing strong binding of  $\text{Fur}+\text{Fe}^{2+}$  to the Fur box (hatched) at the *tbpBA* promoter, occluding the  $-10$  element and silencing transcription. We hypothesize that MisR~P might cooperate with  $\text{Fur}+\text{Fe}^{2+}$  to ensure *tbpBA* silencing. **B)** Under iron-depleted conditions, Fur does not bind to iron, cannot dimerize, and releases the *tbpBA* promoter. MisR~P binding may enhance RNA polymerase binding or transcription initiation either by optimizing spacing between the  $-35$  and  $-10$  promoter elements (indicated by the jagged black line) or by making upstream contacts with the holoenzyme. *tbpBA* transcription is strongly de-repressed. *tbpB* transcription is approximately twice that of *tbpA* at steady state (Ronpirin *et al.*, 2001). Un-phosphorylated MisR cannot bind the *tbpBA* promoter region. Four MisR~P binding sites are shown as small yellow arrows, and a fifth MisR~P binding site, associated with a DNase I hypersensitivity site, is shown as a large yellow arrow. RNA polymerase holoenzyme and MisR~P are omitted for clarity. The impact of MisR~P binding on the long, noncoding upstream RNA, which is unresponsive to iron and transcribed divergently from *tbpBA* (Velez Acevedo *et al.*, 2014), is currently unknown.