Regulation of the type I protein secretion system by the MisR/MisS two-component system in *Neisseria meningitidis*

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Neisseria meningitidis, an obligate human pathogen, remains a leading cause of meningitis and fatal sepsis. Meningococci are known to secrete a family of proteins, such as FrpC, with sequence similarity to the repeat-in-toxin (RTX) proteins via the type I secretion system. The meningococcal type I secretion proteins are encoded at two distant genetic loci, *NMB1400* (*hlyB*) and *NMB1738/1737* (*hlyD/tolC*), and are separated from the RTX toxin-like substrates. We have characterized the promoter elements of both *hlyB* and *hlyD* by primer extension and *lacZ* reporter fusions and revealed the growth phase-dependent upregulation of both genes. In addition, we showed that the MisR/MisS two-component system negatively regulates the expression of *hlyB* and *hlyD/tolC*. Direct binding of MisR to *hlyB* and *hlyD* promoters was demonstrated by electrophoretic mobility shift assay (EMSA), and DNase I protection assays identified MisR binding sites overlapping the promoter elements. Direct repression of *hlyB* transcription by MisR was supported by *in vitro* transcription assays. Mutations in the MisR/S system affected, but did not eliminate, the growth phase-dependent upregulation of *hlyB*, suggesting additional regulatory mechanisms. Increased secretion of RTX toxin-like proteins was detected in the cell-free media from *misS* mutant cultures, indicating that the amounts of extracellular RTX toxin-like proteins are, in part, controlled by the abundance of the type I secretion apparatus. This is, to our knowledge, the first example of a two-component system mediating secretion of cytotoxin family proteins by controlling expression of the type I secretion proteins.

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INTRODUCTION

Neisseria meningitidis inhabits the nasopharynx of approximately 10 % of the population, but remains a leading cause of meningitis and rapidly fatal sepsis, usually in otherwise healthy individuals (Stephens, 2007; Stephens *et al.*, 2007). Capsular polysaccharides, lipooligosaccharides and pili are among the important virulence factors critical for meningococcal colonization and pathogenesis (Tzeng & Stephens, 2000). Meningococci have been shown to secrete a family of iron-regulated proteins, named Frp for Fe-regulated protein and represented by FrpC, with sequence similarity to the cytotoxins of the repeat-in-toxin (RTX) family (Thompson *et al.*, 1993a, b). RTX toxins are characterized by C-terminal domains with varying numbers of tandem glycine-rich nonapeptide repeats that are implicated in

calcium binding and contain the secretion signal (Frey & Kuhnert, 2002; Lally *et al.*, 1999; Welch, 2001). Post-translational modifications are usually required to activate the biological activities of RTX toxins, which include cytolysis and actin cytoskeleton perturbation (Lally *et al.*, 1999). Many RTX toxins play an important role in pathogenesis in a wide range of Gram-negative pathogens (Lally *et al.*, 1999; Welch, 2001); however, no biological function has been elucidated for meningococcal FrpC or FrpC-like proteins.

Many cytotoxins, including the RTX family proteins, are secreted by the type I secretion system. In general, genes encoding the synthesis, activation and secretion of the RTX toxins are organized as an operon. A genetic organization exemplified by the *Escherichia coli* *hlyCABD* operon, encoding gene products responsible for α -haemolysin activation (HlyA/C) and secretion (HlyB/D), is common among many RTX toxin-producing Gram-negative bacteria. HlyB is an ATP-binding cassette (ABC)-type transporter that couples ATP hydrolysis to substrate export, and HlyD belongs to the membrane fusion protein family. Together with the usually unlinked TolC outer membrane porin, these three components form a type I

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Abbreviations: EMSA, electrophoretic mobility shift assay; IHF, integration host factor; qRT-PCR, quantitative real-time RT-PCR; RTX, repeat-in-toxin.

A supplementary table listing primers used in this study is available with the online version of this paper.

secretion system that exports toxins directly from the cytoplasm to the bacterial surface without a periplasmic intermediate (Holland *et al.*, 2005; Wandersman & Deleplaire, 1990). The meningococcal type I protein secretion system, however, is encoded at distant genetic islands that are flanked by ORFs of transposon or phage origin (van Ulsen & Tommassen, 2006). Meningococcal *hlyB* is encoded in the vicinity of a highly polymorphic *frpD/C/A* locus and is separated from *hlyD*. A *tolC* homologue is directly downstream and co-transcribed with *hlyD* (Kamal *et al.*, 2007). In addition, the *frpC* alleles of the RTX toxin-like structural genes are located within three genetic islands that appear to have undergone extensive rearrangements, although their chromosomal locations are conserved (van Ulsen & Tommassen, 2006). The gene products of meningococcal *hlyB*, *hlyD* and *tolC* have been shown to be functional and are responsible for the secretion of FrpC RTX-like proteins (Wooldridge *et al.*, 2005). Interestingly, homology search, microarray comparative genomic hybridization and Southern blotting have not detected genes encoding either the RTX toxin-like proteins or the HlyB/HlyD secretion proteins in *Neisseria gonorrhoeae* and commensal *Neisseria lactamica* (Klee *et al.*, 2000; Stabler *et al.*, 2005; van Ulsen & Tommassen, 2006). *FrpC* alleles with variable RTX repeats have been detected by PCR in all invasive meningococcal strains (Osicka *et al.*, 2001), suggesting that FrpC has a unique role in meningococcal pathogenesis.

The contribution of RTX proteins to meningococcal pathogenesis has not been well defined. High levels of antibodies recognizing a recombinant FrpC protein have been detected in convalescent-phase sera of patients recovering from invasive meningococcal disease and are absent in the sera at hospital admission, indicating expression of FrpC-like proteins during disease (Osicka *et al.*, 2001). However, using an infant rat model of meningococcal infection, no difference in the course of bacteraemia was detected between the wild-type strain and the *frpC* mutant (Forman *et al.*, 2003), and deleting the genes encoding *hlyD* and *tolC* did not affect the ability of the mutant to multiply in the bloodstream (Klee *et al.*, 2000). Most of the RTX toxins require post-translational modification by a specific activator protein to become biologically active (Lally *et al.*, 1999). Interestingly, meningococcal FrpC has been shown to exhibit a novel 'clip-and-link' autocatalytic processing activity resulting in the formation of high-molecular-mass oligomeric species, and this activity has been shown to be calcium-dependent (Osicka *et al.*, 2004). In addition to being secreted, FrpC can also be anchored on the bacterial outer membrane through a high-affinity interaction with an accessory lipoprotein, FrpD, which is upstream of and co-transcribed with FrpC.

To successfully colonize and survive as an obligate human pathogen, *N. meningitidis* responds to signals emanating from the human host, a task often carried out in bacteria by environment-sensing two-component systems that

generally consist of a sensor histidine kinase and a response regulator (Hoch, 2000). Upon sensing specific signals, the histidine kinase autophosphorylates the conserved histidine residue, and the phosphoryl group is subsequently transferred to the cognate response regulator. The phosphorylation status of the response regulator mediates its affinity for DNA motifs present in the promoter region of target genes. The meningococcal genomes encode only four predicted pairs of two-component systems (Parkhill *et al.*, 2000; Tettelin *et al.*, 2000), most likely due to the restricted human host environment encountered. The MisR/MisS two-component system has been reported to influence phosphorylation of the inner core of lipooligosaccharide (LOS) (Tzeng *et al.*, 2004). LOS is a major virulence factor of *N. meningitidis*, and its structural changes are important in meningococcal pathogenesis. The *misR* mutation results in sensitivity to antimicrobial peptides (Johnson *et al.*, 2001; Tzeng *et al.*, 2004) and virulence attenuation in a murine model of infection (Newcombe *et al.*, 2004). In this study, we established that the MisR/S two-component system regulates secretion of the RTX cytotoxin family proteins by directly repressing expression of the type I secretion proteins in *N. meningitidis*.

METHODS

Bacterial strains, medium and reagents. Strains and plasmids used in this study are listed in Table 1, while primers are listed in Supplementary Table S1. Meningococcal strains were grown with 3.5% CO₂ at 37 °C and gonococcus (GC) base agar (Difco), supplemented with 0.4% glucose and 0.68 mM Fe(NO₃)₃, or GC broth with the same supplements and 0.043% NaHCO₃. BHI medium (37 g brain heart infusion l⁻¹) with 1.25% fetal bovine serum was used when kanamycin selection was required. Antibiotic concentrations (in µg ml⁻¹) used for *E. coli* strains were ampicillin, 100; kanamycin, 50; spectinomycin, 60; and erythromycin, 300; and for *N. meningitidis* were kanamycin, 80; spectinomycin, 60; and erythromycin, 3. *E. coli* strains DH5α and TOP-10 cultured on Luria-Bertani (LB) medium were used for cloning and propagation of plasmids. Meningococci were transformed by the procedure of Janik *et al.* (1976). *E. coli* strains were transformed by electroporation with a GenePulser (Bio-Rad) according to the manufacturer's protocol.

Construction of the $\Delta hlyB$ mutant and complementation of the $\Delta misS$ mutant. Using chromosomal DNA of a serogroup B strain, NMB, as template, a 664 bp PCR product containing the 5' region of *hlyB* was amplified using primer pair hlyB-F and hlyB-2R. Another 837 bp PCR product containing the 3' region of *hlyB* was obtained using primers hlyB-3F and hlyB-4R. The two PCR products were digested with *Sma*I, purified through Qiaquick columns (Qiagen) and then ligated using T4 ligase. The ligation mixtures were used as templates for the second round of PCR with primers hlyB-F and hlyB-4R. The expected ~1.5 kb PCR product was purified using a gel extraction kit (Qiagen) and then cloned into the pCR2.1 vector using the TOPO-TA cloning kit (Invitrogen). The correct plasmid carrying the newly created *Sma*I site was confirmed by restriction digestion and sequencing analysis, and designated pYT357. The Ω (sp) cassette released from pHP45 Ω by *Sma*I digestion was inserted into the *Sma*I site of pYT357, yielding pYT358. Removal of the *hlyB* internal sequence and the presence of the Ω cassette in the resulting pYT358 plasmid were confirmed by sequencing analysis. The plasmid was cut

Table 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference
<i>N. meningitidis</i> strains		
NMB	B:2b:P1.2,5:L2 (CDC8201085)	Stephens <i>et al.</i> (1991)
NMB <i>misR</i>	NMB with <i>misR</i> :: <i>erm</i> mutation	Tzeng <i>et al.</i> (2004)
YT310	NMB with Δ <i>misS</i> :: <i>aphA</i> -3 mutation	Tzeng <i>et al.</i> (2006)
YT322	NMB with a chromosomal <i>misR</i> :: <i>lacZ</i> fusion	Tzeng <i>et al.</i> (2006)
YT356	NMB with a chromosomal <i>hlyB</i> (−348/+62):: <i>lacZ</i> fusion	Tzeng <i>et al.</i> (2008)
YT356s	YT356 with a Δ <i>misS</i> :: <i>aphA</i> -3 mutation	Tzeng <i>et al.</i> (2008)
YT356rs	YT356 with a Δ <i>misRS</i> :: <i>aphA</i> -3 mutation	This study
YT359	NMB with a chromosomal <i>hlyB</i> (−728/+62):: <i>lacZ</i> fusion	This study
YT360	NMB with a chromosomal <i>hlyB</i> (−518/+62):: <i>lacZ</i> fusion	This study
YT361	NMB with a chromosomal <i>hlyB</i> (−133/+62):: <i>lacZ</i> fusion	This study
YT347	NMB with a chromosomal <i>hlyD</i> (−299/+199):: <i>lacZ</i> fusion	Tzeng <i>et al.</i> (2008)
YT347s	YT347 with a Δ <i>misS</i> :: <i>aphA</i> -3 mutation	Tzeng <i>et al.</i> (2008)
YT347rs	YT347 with a Δ <i>misRS</i> :: <i>aphA</i> -3 mutation	Tzeng <i>et al.</i> (2008)
YT362	NMB with <i>hlyD</i> (−182/+103):: <i>lacZ</i> fusion in the chromosomal 988 locus	This study
YT363	NMB with <i>hlyD</i> (−639/+103):: <i>lacZ</i> fusion in the chromosomal 988 locus	This study
YT364	NMB with <i>hlyD</i> (−299/+103):: <i>lacZ</i> fusion in the chromosomal 988 locus	This study
YT358	NMB with Δ <i>hlyB</i> :: Ω mutation	This study
YT391	NMB310 complemented with <i>misS</i> at the <i>lctP</i> and <i>aspC</i> locus.	This study
<i>E. coli</i> strains		
DH5 α	Cloning strain	New England Biolabs
TOP10	Cloning strain	Invitrogen
Plasmids		
pFlag-CTC	Cloning vector for Flag tag fusion	Sigma
pCR2.1	TA cloning vector, Ap ^R , Km ^R	Invitrogen
pHP45 Ω	Source of Ω (sp) cassette	Prentki & Krisch (1984)
pGCC4	Meningococcal complementation plasmid with <i>lac</i> promoter	Skaar <i>et al.</i> (2002)
pYT328	Cloning vector for <i>lacZ</i> fusion in a chromosomal intergenic region	Tzeng <i>et al.</i> (2006)
pYT347	<i>hlyD</i> :: <i>lacZ</i> carrying 498 bp (−299/+199) promoter region	Tzeng <i>et al.</i> (2008)
pYT356	<i>hlyB</i> :: <i>lacZ</i> carrying 410 bp (−348/+62) promoter region	Tzeng <i>et al.</i> (2008)
pYT357	<i>hlyB</i> -F/ <i>hlyB</i> -2R and <i>hlyB</i> -3F/ <i>hlyB</i> -4R fragments cloned into pCR2.1 with a new <i>Sma</i> I site within <i>hlyB</i>	This study
pYT358	Ω cassette from pHP45 Ω into <i>Sma</i> I of pYT357	This study
pYT359	<i>hlyB</i> :: <i>lacZ</i> carrying 790 bp (−728/+62) promoter region	This study
pYT360	<i>hlyB</i> :: <i>lacZ</i> carrying 580 bp (−518/+62) promoter region	This study
pYT361	<i>hlyB</i> :: <i>lacZ</i> carrying 195 bp (−133/+62) promoter region	This study
pYT362	<i>hlyD</i> :: <i>lacZ</i> carrying 285 bp (−182/+103) promoter region	This study
pYT363	<i>hlyD</i> :: <i>lacZ</i> carrying 742 bp (−639/+103) promoter region	This study
pYT364	<i>hlyD</i> :: <i>lacZ</i> carrying 402 bp (−299/+103) promoter region	This study
pYT391	<i>misS</i> -Flag in pGCC4	This study

with *Sca*I and used to transform meningococcal strain NMB. Spectinomycin-resistant colonies were screened by PCR to confirm the insertion of the Ω cassette through allelic exchange.

The *misS* coding sequence was PCR-amplified using primers *misS*-5'-ERI and *misS*-3'-*Sma*I and chromosomal DNA to incorporate the *Eco*RI and *Sma*I sites at 5' and 3' end of the PCR product, respectively. The digested PCR fragment was cloned into pFlag-CTC that had been sequentially cut with *Sma*I and *Eco*RI. Correct transformants were identified by PCR and sequencing analysis confirmed the incorporation of the Flag tag at the C terminus of *misS*. Subsequently, the *misS*-Flag sequence was amplified from the plasmid using primers YT191-PacI and Flag-PmeI and Phusion polymerase (New England Biolabs). After *Pac*I and *Pme*I double digestion, the fragment was cloned into pGCC4 (Kamal *et al.*, 2007) that had been cut with the same enzymes, and erythromycin-resistant colonies were selected. The correct construct, named pYT391, was

confirmed by PCR. The meningococcal *misS* mutant YT310 was transformed with pYT391 and selected for kanamycin and erythromycin resistance. The presence of the original *misS* mutation and the integration of *misS*-flag into the *lctP* and *aspC* loci were verified by a panel of PCR analyses.

Construction of reporter strains and β -galactosidase reporter assays. Promoter regions were obtained by PCR amplification using the wild-type chromosomal DNA as a template and cloned into pCR2.1. The inserts were released with *Eco*RI, purified with a Qiaquick gel extraction kit (Qiagen), and then subcloned into the pYT328 vector (Tzeng *et al.*, 2006). The ligation reactions were transformed into *E. coli* DH5 α , and kanamycin-resistant colonies were selected. Correct orientation of the promoter relative to the *lacZ* gene was confirmed by PCR using an outward primer at the 5' end of *lacZ*, YT168, and a forward primer within the cloned promoter. For *hlyB*

reporters, primer pairs hlyB-pF1/hlyB-R, hlyB-pF2/hlyB-R, hlyB-pF/hlyB-R and hlyB-pF3/hlyB-R, were used to generate the pYT359, pYT360, pYT356 and pYT361 constructs, respectively. For *hlyD*, primer pairs hlyD-pF3/hlyD-pR2, hlyD-pF/hlyD-pR2, hlyD-pF2/hlyD-pR2 and hlyD-pF/hlyD-pR were used to construct pYT363, pYT364, pYT362 and pYT347, respectively. The resulting plasmids were linearized by *NcoI* digestion and used directly to transform meningococcal strains with erythromycin selection. The integration of the *lacZ* fusion via homologous recombination into an irrelevant intergenic region (NMB0428 and NMB0430) was confirmed by PCR. β -Galactosidase assays were performed in triplicate using a 96-well plate-based procedure (Tzeng *et al.*, 2001). Samples for growth-dependent transcription were collected, frozen at -80°C and assayed the next day.

RNA extraction and quantitative real-time RT-PCR (qRT-PCR).

Meningococcal strains grown in supplemented GC broth to mid-exponential phase were treated with RNAProtect bacteria reagent (Qiagen) to stabilize RNA prior to sample collection, and total RNA was extracted using an RNeasy mini kit (Qiagen). The RNA samples were further treated with DNase to remove contaminating chromosomal DNA, and then purified by phenol/chloroform extraction and ethanol precipitation. The final RNA preparations were used as templates in standard PCR amplification to ensure no DNA contamination.

The dsDNA binding dye SYBR Green detection method was employed for qRT-PCR. A 1 μg sample of total RNA was reverse-transcribed using random hexamers and a Gene Amp kit (Applied Biosystems). Specific primer pairs are listed in Supplementary Table S1 and have been confirmed to have similar amplification efficiencies. Reactions (25 μl) consisting of 0.2 μM of each of the 5' and 3' primers, 1.6 μl reverse-transcription reaction mixture and 1 $\times$ SYBR Green Supermix (Bio-Rad) were performed in triplicate, and the cycling conditions have been described previously (Tzeng *et al.*, 2004). Melting-curve analyses were performed following each RT-PCR experiment to ensure that each reaction contained only a single specific product. *rpsE*, encoding the constitutively and highly expressed meningococcal 30S ribosomal protein, was used in each experiment as an endogenous reference gene for normalization. Control reactions without reverse transcription were also analysed to confirm the absence of contaminating chromosomal DNA. The relative fold changes were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen, 2001). Each qRT-PCR was repeated with at least two independent RNA preparations. Student's *t* test with a two-tailed hypothesis was used to detect significant differences ($P < 0.01$) between two variables.

Primer extension. Total RNA (30 μg) was used in primer extension reactions with ^{32}P -labelled *hlyB* (hlyB-PE2 and hlyB-PE3)- and *hlyD* (hlyD-PE1 and hlyD-PE2)-specific primers. The RNA and primer mixtures (25 μl) were heated at 85°C for 10 min, cooled on ice for 10 min, and then annealed at 65°C for 20 min, before slowly cooling to room temperature for 15 min. Aliquots of reaction mixtures (15 μl) were added to give final concentrations of 1 $\times$ RT buffer (Roche), 1 mM dNTP, 20 U RNasin inhibitor (Promega), and 20 U Transcriptor reverse transcriptase (Roche). The reactions were incubated at 50°C for 1 h, and then the reverse-transcription enzyme was inactivated at 95°C for 5 min and held at 4°C for 5 min. Excess RNA was degraded by incubating with 1 μl (10 ng) RNase A at 37°C for 30 min. The extension products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research). The corresponding DNA sequencing reactions were carried out using the same labelled oligonucleotides and PCR fragments of the promoter regions with a Thermo Sequenase dye primer manual cycle sequencing kit (USB). The extension products and sequencing ladders were resolved on an 8 % sequencing gel.

Electrophoretic mobility shift assay (EMSA) and DNase I protection assay. Procedures for both these experiments have been reported previously (Tzeng *et al.*, 2006).

In vitro transcription. To generate the *hlyB* template for the run-off transcription reactions, a 481 bp PCR product containing the *hlyB* promoter region was amplified with primers hlyB-pF3 and hlyB-R-*XhoI* and gel-purified. The promoter for the *aphA-3* kanamycin-resistance gene of plasmid pJRS462 (Roberts *et al.*, 2007) was used as the transcription control template. The plasmid was cut with *XcmI* to generate a 510 nt transcription product control, while the expected *hlyB* transcription product is 347 nt. Each 25 μl of reaction mixture contained 1 $\times$ transcription buffer (40 mM Tris, pH 7.4, 150 mM KCl, 10 mM MgCl_2 , 10 mM DTT, 0.01 % Triton X-100), 0.5 μl [α - ^{32}P]UTP [10 μCi (370 kBq) μl^{-1} , 800 Ci (29.6 TBq) mmol^{-1} ; Perkin Elmer], 0.5 μl RNasin, 0.5 mM each of CTP, GTP and ATP, and 0.05 mM UTP. The DNA templates (50 ng for the P_{hlyB} reaction and 300 ng for the P_{kan} control reactions) were incubated with or without the MisR protein at 37°C for 10 min prior to the addition of *E. coli* RNA polymerase holoenzyme (0.5 U; Epicentre) and the reactions were incubated for another 5 min. The reactions were initiated by adding the NTP mix and were allowed to proceed for 10 min before addition of an additional 2 μl 10 mM UTP. The reactions were quenched with 290 μl stop solution containing 10 μl 3 M sodium acetate, 200 μl ethanol, 2 μl 11 mg ml^{-1} yeast tRNA (Sigma) and 75 μl water. The products were precipitated, washed with 75 % ethanol, resuspended in 10 μl loading dye and analysed on an 8 % sequencing gel.

Preparation of secreted protein and whole-cell lysates, and Western blotting.

Strains were grown in GC broth with standard supplements to OD₆₀₀ 1.0, and bacteria were pelleted from 2 ml cultures by centrifugation at 4000 *g* for 15 min. The cell-free supernatants were obtained by filtering through 0.45 μm pore-size syringe filters (Millipore). The supernatants were further concentrated using a Microcon filtration apparatus with a 50 kDa cutoff membrane (Amicon) according to the manufacturer's procedure, and the recovered concentrates were mixed with equal volumes of 2 $\times$ SDS-PAGE loading buffer (0.125 M Tris, pH 6.8, 4 % SDS, 0.2 M DTT, 20 % glycerol, 0.02 % Bromophenol Blue). For whole-cell lysates, the same numbers of cells were collected based on OD₆₀₀ measurements, and then suspended in 20 μl 1 $\times$ SDS-PAGE loading buffer. All samples were heated at 95°C for 5 min before loading onto a 7.5 % mini SDS-PAGE gel. Resolved proteins were transferred to nitrocellulose membranes using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked in blocking buffer (5 % non-fat dried milk in 1 $\times$ Tris-buffered saline) at room temperature for 2 h, and then incubated at 4°C overnight with the primary antibody, 9D4, raised against the RTX repeat of *Bordetella pertussis* adenylate cyclase toxin, CyaA (Santa Cruz Biotechnology; 1:5000 dilution in 1 $\times$ TBS with 0.1 % Tween 20). After two 10 min washes, membranes were incubated with anti-mouse IgG-horseradish peroxidase conjugate (Pierce) (diluted 1:25 000) for 1 h at room temperature. After three washes, the membranes were developed with the ECL immunoblot system (Pierce). For MisR Western blots, the membranes were incubated with polyclonal sera against the recombinant MisR protein (Tzeng *et al.*, 2006) (1:10 000 dilution) for 1 h at room temperature, and then treated with goat anti-rabbit IgG-HRP conjugate (1:20 000 dilution) for 1 h at room temperature.

RESULTS

Characterization of the promoter regions of the *hlyB* and *hlyD/tolC* loci

Encoded at two distant chromosomal loci, meningococcal *hlyB* and *hlyD/tolC* have been shown to be functional and responsible for the secretion of the RTX cytotoxin family

proteins FrpC and FrpC2 (Wooldridge *et al.*, 2005). As depicted in Fig. 1, an IS1106 element is located 812 bp upstream of *hlyB*, whereas *hlyD* is transcribed divergently from NMB1739, a hypothetical protein and the downstream *tolC* is separated from *hlyD* by 68 bp. Although *hlyD* and *tolC* were originally reported to be transcribed independently (Wooldridge *et al.*, 2005), a recent study has shown that these two genes form an operon (Kamal *et al.*, 2007). To determine the necessary promoter length and possible regulatory sequences, *lacZ* reporter fusions with various promoter sequence lengths were generated in *N. meningitidis* and their activities measured. As shown in Fig. 1, promoter activities of *hlyB* fusions varying from 790 to 195 bp in length are very similar, indicating that the 195 bp sequence provides full promoter activity for *hlyB* expression and that no additional upstream regulatory elements are required. Likewise, all *hlyD* reporters displayed similar activities. Interestingly, the *hlyD* promoter was approximately fivefold weaker than that of *hlyB*.

Determination of the *hlyB* and *hlyD* promoter elements

To understand the difference in promoter activities between *hlyB* and *hlyD*, the promoter elements were further characterized. Primer extension experiments were performed to map the transcriptional start sites. It has been noted elsewhere that the annotated meningococcal *hlyB* ATG start codon in the MC58 genome yields an additional 32 amino acid residues compared with its *E. coli* counterpart (Wooldridge *et al.*, 2005). However, a single transcriptional start site of *hlyB* was mapped to locate 88 bp upstream of the second ATG start codon (Fig. 2a, b) and is downstream of the annotated ATG start codon for *hlyB*. Additional primer extension experiments using a different primer also detected the same +1 site (data not shown), thus confirming that only the short form of *hlyB* is transcribed. A promoter element with near consensus –10 (TACAAT) and –35 (TTTACA) sequences separated by the optimal 17 bp space can be derived from the mapped +1 site (Fig. 2b). The annotated upstream ATG codon overlaps with this derived –10 sequence. Interestingly,

Wooldridge *et al.* (2005) showed that the FrpC secretion defect of a *hlyB* mutant is complemented by the long form of *hlyB*, but only weakly complemented by the short form with methionine at position 33 as the first residue. The short fragment cloned in their study contained 20 bp of upstream sequence from the correct ATG start without the *hlyB* promoter elements, while the long construct contained the mapped –10 element. It is possible that including the *hlyB* promoter element downstream of the vector-encoded *porB* promoter in the long construct enhanced the transcription of *hlyB* and consequently resulted in a high level of secreted FrpC proteins, whereas *hlyB* was not efficiently transcribed and/or translated from the short construct. As demonstrated below, the secretion of FrpC proteins was limited by the level of the type I secretion apparatus.

The *hlyD* transcriptional start site was mapped to 49 bp upstream of the ATG start codon with two different primers and agrees with the proposed promoter element (Kamal *et al.*, 2007) containing near consensus (five out of six matches) –10 (TAAAAT) and –35 (TTAACA) hexamer sequences, separated by 17 bp (Fig. 2c). Comparing the mapped *hlyB* and the *hlyD* promoter sequences, both promoters likely yield similar activities, and it is not evident why *hlyB* transcripts are detected at a higher level than are *hlyD* transcripts by both reporter assays (Fig. 1) and qRT-PCR analysis (described below).

Growth phase-dependent expression of *hlyB* and *hlyD/tolC*

The expression of *hlyB* and *hlyD* was monitored throughout the growth phase using the β -galactosidase reporter constructs (YT356 and YT347 for *hlyB* and *hlyD*, respectively), and the β -galactosidase activities of both fusions increased in a cell density-dependent manner (Fig. 3a). The maximal activities were obtained when cells entered stationary phase. The induction ratio between cells at exponential phase (OD₆₀₀ ~0.4) versus stationary phase (OD₆₀₀ ~3.0) was ~3 for both *hlyB* and *hlyD*. In addition, *hlyB* expression was consistently approximately fivefold

Fig. 1. Transcriptional activities of various *hlyB*::*lacZ* and *hlyD*::*lacZ* reporter fusions. The genetic organizations of both loci are depicted at the top, with the length of the intergenic region indicated. β -Galactosidase activities (Miller units) expressed by strains grown in GC broth to mid-exponential phase are shown as mean $\pm$ SD of three independent experiments with triplicate measurements. No statistically significant differences were observed among the four *hlyB* and *hlyD* reporter constructs.

higher than that of *hlyD* throughout the growth phase. As a control, the *lacZ* reporter fusion of the response regulator *misR* was also examined, and no significant changes in expression at different growth phases were observed (Fig. 3b).

To further confirm that the increases in reporter activity at stationary phase are not due to the accumulation of β -galactosidase during growth, total RNAs extracted from the wild-type meningococcal strain at early exponential phase ($OD_{550} \sim 0.2$) and at mid-exponential phase ($OD_{550} \sim 0.6$)

Fig. 2. Primer extension analyses of *hlyB* and *hlyD* expression. (a) Transcriptional start sites of *hlyB* (left panel) and *hlyD* (right panel) were determined by primer extension using primers hlyB-PE2 and hlyD-PE1, respectively, with RNA isolated from strain YT310 during mid-exponential growth in standard GC broth. Lanes G, A, T and C indicate the dideoxy sequencing reactions performed with PCR fragments corresponding to the *hlyB* (hlyB-F/hlyB-R1) and *hlyD* (hlyD-PF/hlyD-PR) upstream sequences. The asterisks indicate the transcriptional start sites. hlyB-PE3 and hlyD-PE2 yielded the same transcriptional start sites (data not shown). (b) The nucleotide sequence of the *hlyB* promoter region. The 5' end of each *lacZ* reporter fusion is indicated by a bent arrow. The 3' end of all *lacZ* reporter fusions is indicated by the primer hlyB-R. Putative IHF binding sites are shown as dotted arrows below the sequences. The correct ATG start codon, the transcriptional start site (+1) and the -10 and -35 elements are boxed, while the annotated ATG start is in bold type with a dotted line above. The MisR binding sites mapped by DNase I protection assays (A and B, Fig. 5) are shaded, with sequences matching the MisR consensus motif in bold type and double-underlined. (c) The nucleotide sequence of the *hlyD* promoter region. Primer hlyD-pR is the 3' end of the reporter strain YT347, while the other reporter strains ended with primer hlyD-pR2.

were examined by qRT-PCR. The expression of *misR* and *dsbD*, the disulfide bond isomerase, was included as a control. The expression at early exponential phase was used for normalization and fold-changes at mid-exponential phase were determined. Relative fold-changes between the mid-exponential phase ($OD_{550}=0.59 \pm 0.07$) and the early exponential phase ($OD_{550}=0.16 \pm 0.02$) were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001). The mean values and standard deviations were determined from four qRT-PCR experiments with each gene measured in triplicate using three independent RNA preparations. Both *hlyB* and *hlyD* showed approximately threefold increases in transcription at the later growth phase (3.07 ± 1.09 and 3.45 ± 1.51 , respectively), while the expression of *misR* and *dsbD* remained constant (0.87 ± 0.22 and 1.18 ± 0.41 , respectively). Although the absolute level of expression [cycle threshold (C_t) values] for

hlyB was higher than that for *hlyD*, the magnitude of growth-dependent increases appeared to be similar. Thus, these data verified that the expression of *hlyB* and *hlyD* is regulated by growth phase.

To identify the relevant promoter sequence for growth phase regulation, the expression profile of the *hlyB* reporter strain YT361, which contains the shortest promoter sequence (Fig. 2b), was determined at different time points throughout the growth curve. As shown in Fig. 3(c), YT361 still exhibits a growth-dependent increase in transcription, indicating that the further upstream sequences do not contribute to the growth phase-dependent regulation. Similarly, the shortest *hlyD* reporter construct (YT362, Fig. 2c) retains growth phase-dependent increases in transcriptional activity (data not shown). Examining the promoter sequences of YT361 revealed the presence of a

Fig. 3. (a) Growth phase-dependent activation of *hlyB* and *hlyD* expression. β -Galactosidase activities of strains YT356 (*hlyB::lacZ*, black bars) and YT347 (*hlyD::lacZ*, grey bars) were monitored during the growth phase. The growth curves are shown as line graphs with a semi-logarithmic scale on the secondary axis (×, YT347; ●, YT356). (b) The expression of the *misR::lacZ* fusion strain YT322 was not growth phase-dependent. (c) Growth phase-dependent regulation of the *hlyB::lacZ* fusion strain YT361. Strain 361 carries a 195 bp promoter fragment (Fig. 2b). Triplicate measurements were performed for each time point and a representative of four independent experiments is shown. Error bars, SD.

putative integration host factor (IHF) binding site within the untranslated region of *hlyB*, and the promoter fragment of the *hlyD* reporter strain YT362 contains three putative IHF sites using the *E. coli* consensus sequence (Craig & Nash, 1984). Whether IHF contributes to growth-dependent regulation remains to be determined.

Direct interaction of MisR with the *hlyB* and *hlyD* promoters

hlyB and *hlyD* have been shown to be members of the meningococcal MisR/MisS two-component regulon (Tzeng *et al.*, 2008). The expression of *hlyB*, *hlyD* and *tolC* in the *misR* mutant was shown by qRT-PCR to be higher than that of the wild-type strain (4.4 ± 0.9 , 4.1 ± 1.0 and 2.6 ± 0.7 , respectively), suggesting that MisR acts as a repressor of these two promoters. To provide additional independent confirmation, the *misS* mutation and the *misRS* double mutation were introduced into the *hlyB* (YT356s and YT356rs, respectively) and *hlyD* (YT347s and YT347rs, respectively) reporter fusions and their corresponding promoter activities assessed. Significant increases

in promoter activity were observed in both mutants when compared with the wild-type parental strain (Fig. 4a). Mutations in the MisR/S two-component system resulted in approximately fourfold and approximately threefold increases in *hlyB* and *hlyD* activity, respectively. The *in vivo* transcriptional fusion data indicate that nonphosphorylated MisR does not play a critical role in repressing *hlyB* and *hlyD* expression, since no significant additional derepression occurred without MisR (comparing the activity of the *misRS* mutant with that of the *misS* mutant). Thus, our results are consistent with a model in which the phosphorylated form of MisR is the major transcriptional repressor of *hlyB* and the *hlyD/tolC* operon *in vivo*.

Both nonphosphorylated and phosphorylated MisR, which was generated by treatment with acetyl phosphate, directly interact with the promoters of *hlyB* and *hlyD/tolC* loci, as shown previously by EMSA using purified MisR-(His)₆ protein (Tzeng *et al.*, 2008). To confirm the specificity of MisR binding, competition assays were performed. Excess amounts of specific DNA nearly completely abolished the binding of MisR~P, while the presence of a non-specific

Fig. 4. (a) β -Galactosidase assays of the *hlyB::lacZ* (YT356) and *hlyD::lacZ* (YT347) reporter strains in the wild-type strain NMB (black bars), the $\Delta misS::aphA-3$ mutant (dotted bars) and the $\Delta misRS::aphA-3$ mutant (grey bars). The mean and SD of three independent measurements are presented. Asterisks indicate significant differences ($P < 0.01$) between the mutants and the corresponding wild-type parental strain. (b) Competition EMSA experiments demonstrating specific interaction of MisR proteins with the *hlyB* and *hlyD* promoter sequences. A 410 bp *hlyB* fragment (*hlyB*-PF/*hlyB*-PR) and a 498 bp *hlyD* fragment (*hlyD*-PF/*hlyD*-PR) were end-labelled with [γ - 32 P]ATP using T4 kinase. Lanes: 1, probe only; 2, probe with MisR~P protein (102 pmol for *hlyB* and 170 pmol for *hlyD*); 3, with addition of 4 μ g specific DNA; 4, with addition of 4 μ g non-specific DNA. Prior to the binding reactions, MisR protein was reacted with 50 mM acetyl phosphate for 30 min at 37 °C, and ~50% of MisR protein was phosphorylated as estimated by native gel electrophoresis (data not shown). (c) Repression of the *hlyB* promoter by MisR *in vitro*. *In vitro* run-off transcription initiated from the *hlyB* promoter (lanes 1 and 2) and from the *aphA-3* promoter (lanes 3 and 4) yielded 347 and 510 nt products, respectively. Reactions were performed in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of MisR protein (4.6 μ M) as described in Methods. An RNA size marker (M) (Ambion) is shown with the size in nucleotides indicated to the right.

DNA did not affect the binding of MisR~P to the *hlyB* and *hlyD* promoter fragments (Fig. 4b). These results thus confirm that the binding of MisR to the *hlyB* and *hlyD* promoters is sequence-specific. Phosphorylation of response regulators often leads to an oligomeric status that provides cooperative binding to their target DNA sequences, resulting in enhanced affinity to regulatory targets. Phosphorylated MisR showed higher binding affinity (Tzeng *et al.*, 2008), and this difference in binding affinity may account for the phosphorylation requirement for its regulatory effect *in vivo* as discussed above.

Identification of the MisR binding sites within *hlyB* and *hlyD* promoters

The binding sites of MisR within the *hlyB* and *hlyD* promoter regions were further defined by DNase I protection assays. The footprinting data for the non-coding strand of *hlyB* and the coding strand of *hlyD* are shown in Fig. 5(a) and Fig. 5(b), respectively (additional footprinting data not shown), and the protected sequences are indicated in Fig. 2(b, c). MisR protects two regions within the *hlyB* promoter element (Fig. 5a). Similarly, MisR protects two sites within the *hlyD* promoter, and the downstream site overlaps with both the -10 and -35 elements. Putative MisR recognition motifs that match the WWTGTA AVG consensus sequence (Tzeng *et al.*, 2008) can be detected within both protected regions (Fig. 2). The overlapping of the MisR binding sites with promoter elements correlates with the repressor role of MisR.

Because it remains possible that MisR indirectly regulates the expression of *hlyB* and *hlyD*, the ability of MisR to repress transcription from the *hlyB* promoter was examined *in vitro*. *In vitro* run-off transcription was performed with *E. coli* RNA polymerase holoenzyme, which has been shown to transcribe meningococcal promoters (Delany *et al.*, 2004). As shown in Fig. 4(c), the presence of MisR reduced the transcript levels initiated from the *hlyB* promoter but did not inhibit transcription from the *aphA-3* promoter control. These results thus confirmed that MisR directly represses transcription from the *hlyB* promoter and likely coordinates the expression of both type I secretion proteins, HlyB and HlyD, through interaction with two distant promoters.

The MisRS two-component system influences growth phase-dependent regulation

To examine whether MisR regulation leads to the growth phase-dependent transcription, the β -galactosidase activities of the parental *hlyB* (YT356) and *hlyD* (YT347) reporter strains and the corresponding *misS* mutants (YT356s and YT347s, respectively) were measured at different time points throughout the growth curve. As shown in Fig. 6, removal of the repression by MisR significantly increased the promoter activities throughout the growth curve, with a higher fold-increase during the

Fig. 5. DNase I protection assays of the *hlyB* (a) and *hlyD* (b) promoters. The ^{32}P -end-labelled promoter fragment was incubated with increasing amounts of MisR (*hlyB*) or MisR~P (*hlyD*) for 20 min at 30 °C and then subjected to DNase I digestion. The amounts of MisR~P used were 0, 85, 170, 340, 680, 1360 and 2720 pmol, while the amounts of MisR were 0, 170, 340, 1360 and 2720 pmol. Dideoxy chain-termination sequencing ladders corresponding to the probes are shown in the order G, A, T and C. Black bars indicate regions protected by MisR, while grey bars in (b) indicate regions not consistently protected by MisR. Data for the non-coding strand of *hlyB* and the coding strand of *hlyD* are shown.

early growth phase. However, the *misS* mutation diminished, but did not completely abolish, the growth phase-dependent regulation. The gradual transcriptional increases at higher cell densities were still evident for both promoters. The induction ratios for *hlyB* between cells at stationary phase ($\text{OD}_{600} \sim 3.0$) versus exponential phase ($\text{OD}_{600} \sim 0.4$) were 3.6-fold and 1.2-fold for the wild-type and the *misS* mutant, respectively. In the case of *hlyD*, the induction ratios between cells at stationary phase versus early exponential phase were 2.1-fold for both the wild-type and the *misS* mutant. These data suggest that the MisR/S system has an effect on the growth phase-dependent regulation of *hlyB*, but appears not to significantly influence *hlyD* expression. Thus, additional mechanism(s) that control the growth phase-dependent regulation are present.

Mutation in the MisRS system enhances secretion of RTX repeat-containing proteins

To test whether the secreted amount of RTX toxin-like proteins was affected by mutation in the *misRS* two-component system, the levels of secreted RTX repeat-containing proteins in the wild-type parental strain and the *misS* mutant (YT310) were compared. In addition, the complemented ΔmisS mutant (YT391) and the ΔhlyB mutant (YT358) were also examined. The ΔhlyB mutant was constructed as a control for the quality of secreted protein samples, as RTX toxin-like proteins should be absent in the supernatant of this strain if no cell lysis occurs. Secreted proteins were collected from cell-free supernatant of cultures at late-exponential growth phase ($\text{OD}_{550} \sim 1$) and concentrated by ultrafiltration with a

molecular mass cut-off of 50 kDa, since the predicted molecular masses of both meningococcal RTX toxin family proteins, FrpC and FrpC2, are greater than 100 kDa. Whole-cell lysates were also prepared from the same cultures to examine the total amount of RTX toxin family proteins produced. Total secreted proteins standardized by cell numbers (2 ml cultures at $\text{OD}_{550} 1.0$) were concentrated and resolved by SDS-PAGE, transferred to nitrocellulose membranes and then probed with a monoclonal antibody, 9D4, raised against the RTX repeats of *B. pertussis* adenylate cyclase toxin CyaA. This antibody has been shown to cross-react with meningococcal RTX toxin-like proteins (Osicka *et al.*, 2004). Two major 9D4-reactive bands with molecular masses consistent with FrpC and FrpC2 were detected (Fig. 7). Additional minor bands of either higher or lower molecular mass were also detected, as reported elsewhere (Forman *et al.*, 2003; Osicka *et al.*, 2004; Wooldridge *et al.*, 2005), and are consistent with the autocatalytic cleavage and cross-linking activities of FrpC proteins (Osicka *et al.*, 2004).

The signal intensities of FrpC proteins of the *misS* mutant are significantly higher than those of the wild-type strain (Fig. 7a, lane 3 versus lane 2), and this change reverted to the wild-type level in the complemented ΔmisS mutant (Fig. 7a, lane 4 versus lane 2). RTX repeat-containing proteins were not detectable in the cell-free supernatant of the ΔhlyB strain YT358 (Fig. 7a, lane 1), confirming that the secreted protein samples were not contaminated with lysed cells. The total cell-associated RTX toxin-like proteins from whole-cell lysates were examined and similar levels of RTX repeat-containing proteins were detected in all strains (Fig. 7b), although the total intensity of 9D4-reactive bands appeared to accumulate to a slightly higher and a lower

Fig. 6. Growth phase-dependent expression of the *hlyB* (a) and *hlyD* (b) reporter strains in the *misS* mutant. The β -galactosidase activities of the fusions in the wild-type [$\times$, YT356 and YT347 in (a) and (b), respectively] and the *misS*::*aphA*-3 [$\blacksquare$, YT356s and YT347s in (a) and (b), respectively] mutant background were monitored throughout the growth phase. Triplicate measurements were performed for each time point and the corresponding SDs are indicated. Representative results from three independent experiments are shown.

level in the non-secreting $\Delta hlyB$ strain YT358 (lane 1) and the hyper-secreting $\Delta misS$ strain YT310 (lane 3), respectively, when compared with the wild-type parental strain (lane 2). The transcription of *frpC* was not changed in the *misS* mutant (data not shown). Thus, these results indicate that the *misS* mutation does not affect toxin-like protein production (Fig. 7b). To further confirm that the higher amount of secreted FrpC proteins in the *misS* mutant is not due to enhanced lysis of this strain, we repeated immunoblots of both secreted and whole-cell protein samples using antisera against MisR. As shown in Fig. 7(c), no signals corresponding to MisR were detected in the secreted protein samples, while the whole-cell lysate samples showed strong signals with intensities that correlated with the expected patterns of MisR expression (reduced in the *misS* mutant and increased in the

Fig. 7. Immunodetection of RTX repeat-containing proteins. Secreted proteins in culture supernatants (a) and whole-cell lysates (b) were collected as described in Methods. Strains used were $\Delta hlyB$ mutant YT358 (lane 1), wild-type strain NMB (lane 2), $\Delta misS$ mutant YT310 (lane 3) and complemented $\Delta misS$ strain YT391 (lane 4). The RTX repeat-containing proteins were detected using the RTX repeat-specific mAb 9D4. (c) Whole-cell lysate samples as in (b) (lanes 1–4) and secreted protein samples as in (a) (lanes 5–8) were probed with rabbit antisera against MisR. A non-specific reactive band is marked with an asterisk. Samples for lanes 1 and 5 were prepared from strain YT358 ($\Delta hlyB$), lanes 2 and 6 from strain NMB (wild type), lanes 3 and 7 from strain YT310 ($\Delta misS$), and lanes 4 and 8 from strain YT391 (complemented $\Delta misS$). No signals for MisR protein were detected in the secreted protein samples (lanes 5–8) after prolonged exposure. Due to the autoactivation mechanism of the *misRS* operon, the $\Delta misS$ mutation reduced the MisR level in strain YT310 (lane 3) and the overexpression of MisS with IPTG induction increased expression of MisR in the complemented $\Delta misS$ strain YT391 (lane 4).

complemented strain that overexpresses MisS upon IPTG induction). No signal was detected in the secreted samples after prolonged exposure of the films (data not shown). These data demonstrate that a *misS* mutation in the MisRS two-component system leads to enhanced secretion of RTX toxin-like proteins.

DISCUSSION

N. meningitidis is a pathogen which inhabits the human nasopharynx but can rapidly disseminate throughout the body during invasive infection and is a worldwide cause of epidemic meningitis and fatal sepsis. A family of meningococcal RTX repeat-containing proteins, represented by FrpC, has been shown to be induced under iron limitation (Thompson *et al.*, 1993a, b) and secreted via a type I

secretion system composed of HlyB, HlyD and TolC (Wooldridge *et al.*, 2005). High levels of antibodies recognizing FrpC have been detected in convalescent-phase sera of most patients recovering from invasive meningococcal disease, strongly indicating that FrpC-like proteins are produced and play a role during invasive meningococcal infection (Osicka *et al.*, 2001). The MisR/S system is one of the four pairs of predicted environmental-sensing two-component systems in *N. meningitidis* that regulate genes important not only for maintaining the organism's ecological niche but also possibly for virulence (Newcombe *et al.*, 2004; Overton *et al.*, 2006; Tzeng *et al.*, 2004). In this study, we established that the expression of distantly encoded *hlyB* and *hlyD/tolC* is coordinately repressed by the MisR/S two-component system.

To date, transcriptional antitermination is the only demonstrated regulatory mechanism of the type I secretion system (Bailey *et al.*, 1997). The expression of the *hlyCABD* operon in *E. coli* is regulated by the RfaH protein through an antitermination mechanism that involves a 5' *cis*-acting element termed the JUMPstart sequence (Bailey *et al.*, 1996, 1997; Leeds & Welch, 1997). Mutation of *rfaH* increases transcriptional polarity along the operon, resulting in a modest decrease in the transcription of *hlyCA* but almost completely abolishing transcription of the distal *hlyBD* genes. As the meningococcal *hlyB* and *hlyD/tolC* are encoded on distant genetic islands and are also separated from their substrates, it is unlikely that RfaH is involved in the regulation of the meningococcal type I secretion system. In addition, no sequence homologous to the consensus sequence of the JUMPstart element (Hobbs & Reeves, 1994) was detected in upstream regions of *hlyB* and *hlyD*.

The levels of exported HlyA in *E. coli* decline rapidly as the bacterial cultures enter stationary phase, and this expression pattern is dependent on growth phase-specific transcription antitermination of the *hlyCABD* operon (Koronakis *et al.*, 1989). In contrast, the expression of meningococcal *hlyB* and *hlyD/tolC* genes increases gradually throughout growth and reaches a maximal level when entering stationary phase. Despite multiple potential IHF binding sites being detected in the promoter regions of *hlyB* and *hlyD* using the *E. coli* consensus sequence (Fig. 2), ~130 bp of sequence upstream of the *hlyB* transcriptional start site that retains a single putative IHF binding site (strain YT361) is sufficient to elicit the maximal activity and retain the growth phase-dependent regulation. The involvement of IHF in the growth phase-dependent regulation of *hlyB* and *hlyD* was not further investigated. Another common regulatory mechanism that modulates transcription in a cell density- and growth phase-dependent manner is quorum sensing (Lazazzera, 2000; Whitehead *et al.*, 2001). However, the presence of conditioned medium (Baca-DeLancey *et al.*, 1999) prepared from a stationary-phase culture of the meningococcal wild-type strain did not result in premature activation of *hlyB* and *hlyD* reporter fusions at early to mid-

exponential phase (data not shown), which is consistent with the report that AI-2 has no effect on meningococcal transcription (Dove *et al.*, 2003). The MisR/S two-component system appears to influence the growth phase-dependent regulation of *hlyB*, but this effect is not due to different levels of the MisR protein during growth since the expression of *misR* is constant throughout the growth phase (Fig. 3b). Furthermore, because the *misRS* operon is positively autoregulated (Tzeng *et al.*, 2006), the constant level of *misR* expression throughout the growth phase suggests that the MisRS system does not respond to changes in cell density. Although the fold-increases during later growth were affected by the *misS* mutation, the transcriptional upregulation at higher cell densities was still apparent for both promoters in the *misS* mutant. Thus, MisR-mediated regulation might work in concert with additional regulatory mechanism(s), which remain to be defined.

The amounts of secreted RTX toxin-like proteins depend on two factors, the level of protein production and the abundance of the type I secretion machinery. Based on the scenario that the numbers of assembled type I secretion systems are the limiting factor in toxin secretion, one would expect the amounts of secreted RTX toxin-like proteins to be higher in the *misRS* mutants due to the increased transcription levels of the type I secretion protein components *hlyB*, *hlyD* and *tolC* in these strains. To test this hypothesis, the levels of secreted RTX toxin-like proteins in the wild-type parental strain and the *misS* mutant (YT310) were compared. Using Western blot analyses that detected RTX repeat-containing proteins in total secreted protein samples standardized by cell numbers, we observed significantly higher signal intensities of RTX proteins in the *misS* mutant than in the wild-type strain, and these changes reverted to the wild-type level in the complemented $\Delta misS$ mutant. Thus, these data suggest that the secreted level of RTX toxin-like proteins is, in part, determined by the available secretion protein complexes. While iron-limiting conditions have been shown to induce the synthesis of FrpC proteins, the expression of the type I secretion proteins is not regulated by iron availability (Wooldridge *et al.*, 2005). Microarray studies have shown that FrpC expression is significantly induced when meningococci are exposed to human serum (Kurz *et al.*, 2003) and when they adhere to HEp-2 human epithelial cells (Dietrich *et al.*, 2003). However, it is not clear whether the extracellular level of secreted FrpC protein is also increased under these conditions. We observed enhanced secretion of RTX proteins in the *misS* mutant grown in iron-replete media, and this observation suggests that despite the presumably lower level of FrpC production (without induction by iron starvation or other inducing conditions), the secreted amount is still limited by the capacity and abundance of the secretion machinery. Thus, without a concomitant increase in the level of the type I secretion apparatus through relieving the repression imposed by the MisR/MisS system, the increase in

extracellular RTX toxin-like proteins is likely to be limited when meningococci encounter the iron-restricted host environment.

In summary, we showed that two separate *hlyB* and *hlyD/tolC* genetic loci encoding the type I secretion system are coordinately repressed by the MisR/MisS two-component system in *N. meningitidis*. In addition, the meningococcal type I secretion system exhibits growth phase-dependent regulation that is influenced by the MisR/S system. Eliminating repression by MisR results in augmented production of the type I secretion proteins and consequently enhances secretion of RTX toxin-like proteins. It is at present unclear whether FrpC proteins are the only substrates exported by the type I secretion machinery. This system appears to be meningococcal-specific and universally present in major clonal lineages of pathogenic meningococci (Stabler *et al.*, 2005). A better understanding of its contribution to meningococcal pathogenesis awaits the identification of the biological roles of its substrates.

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