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/toIC), 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/toIC. 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.

INTRODUCTION

Neisseria meningitidis inhabitsthe 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, lipo polysaccharides 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 z-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 & Delepelaire, 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

intermediate (Holland cytoplasm to the bacterial surface without a periplasmic secretion system that exports toxins directly from the

frpD/C/A locus and is separated from hlyB origin (van Ulsen & Tommassen, 2006). Meningococcal islands that are flanked by ORFs of transposon or phage secretion system, however, is encoded at distant genetic

Delepelaire, 1990). The meningococcal type I protein 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. BH 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 GenePulsor (Bio-Rad) according to the manufacturer’s protocol.

Construction of the ΔhlyB mutant and complementation of the ΔmisS 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 SmaI, 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 SmaI site was confirmed by restriction digestion and sequencing analysis, and designated pYT357. The D(s) cassette released from pH4572 by SmaI digestion was inserted into the SmaI site of pYT357, yielding pYT358. Removal of the hlyB internal sequence and the presence of the D cassette in the resulting pYT358 plasmid were confirmed by sequencing analysis. The plasmid was cut
with ScaI 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’-SmaI and chromosomal DNA to incorporate the EcoRI and SmaI 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 the same enzymes, and erythromycin-resistant transformants were identified by PCR and sequencing analysis 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 lacZ 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 EcoRI, purified with a Qiagen gel extraction kit (Qiagen), and then subcloned into the pYT328 vector (Tzeng et al., 2006). The ligation reactions were transformed into E. coli DH5α, 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::lacZ::aphA2 mutation This study

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<tr>
<th>Strain or plasmid</th>
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<td>pYT391</td>
<td>misS-Flag in pGCC4</td>
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Digestion and used directly to transform meningococcal strains with homologous recombination into an irrelevant intergenic region transcription were collected, frozen at between two variables.


tailed hypothesis was used to detect significant differences (http://mic.sgmjournals.org 1591 protection assay.

Electrophoretic mobility shift assay (EMSA) and DNase I precipitation. The final RNA preparations were used as templates in 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 µ reverse-transcription reaction mixture and 1 × 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^(-ΔΔCt) 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 32P-labelled hlyB (hlyB-PF2 and hlyB-PF3)- and hlyD (hlyD-PF1 and hlyD-PF2)-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 × 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-Xhol and gel-purified. The promoter for the aphb-3 kanamycin-resistance gene of plasmid pRS462 (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 × transcription buffer (40 mM Tris, pH 7.4, 150 mM KCl, 10 mM MgCl2, 10 mM DTT, 0.01 % Triton X-100), 0.5 µl [α-32P]UTP [10 µCi (370 kBq)] µl-1, 800 Ci (29.6 Tba) 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 PhlyB reaction and 300 ng for the Pflan 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 µ; 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 × 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 × 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 × 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 using a 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/toIC loci

Encoded at two distant chromosomal loci, meningococcal hlyB and hlyD/toIC 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 (TTAAAT) 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 greater in YT356 than in YT347 and over 100-fold greater than in YT356. In contrast, hlyD expression was lower in YT347 than in YT356 and was not significantly different from levels in YT356 or YT347. The expression of hlyD was consistently substantially lower than hlyB throughout the growth phase. The hlyB and hlyD promoters were also transcribed in early stationary phase (OD600 ~0.4), but no hlyD transcription was detected in exponential phase (OD600 ~0.4) or late stationary phase (OD600 ~3.0).

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 ± 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 (OD550 ~0.2) and at mid-exponential phase (OD550 ~0.6)
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 $2^{-\Delta\Delta CT}$ 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 ($3.07 \pm 1.09$ and $3.45 \pm 1.51$, respectively), while the expression of misR and dsBD remained constant ($0.87 \pm 0.22$ and $1.18 \pm 0.41$, respectively). Although the absolute level of expression [cycle threshold (Ct) 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
putative integration host factor (IHF) binding site within the untranslated region of \textit{hlyB}, and the promoter fragment of the \textit{hlyD} reporter strain YT362 contains three putative IHF sites using the \textit{E. coli} consensus sequence (Craig & Nash, 1984). Whether IHF contributes to growth-dependent regulation remains to be determined.

**Direct interaction of MisR with the \textit{hlyB} and \textit{hlyD} promoters**

\textit{hlyB} and \textit{hlyD} have been shown to be members of the meningococcal MisR/MisS two-component regulon (Tzeng \textit{et al.}, 2008). The expression of \textit{hlyB}, \textit{hlyD} and \textit{tolC} in the \textit{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 \textit{misS} mutation and the \textit{misRS} double mutation were introduced into the \textit{hlyB} (YT356s and YT356rs, respectively) and \textit{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 \textit{hlyB} and \textit{hlyD} activity, respectively. The \textit{in vivo} transcriptional fusion data indicate that nonphosphorylated MisR does not play a critical role in repressing \textit{hlyB} and \textit{hlyD} expression, since no significant additional derepression occurred without MisR (comparing the activity of the \textit{misRS} mutant with that of the \textit{misS} mutant). Thus, our results are consistent with a model in which the phosphorylated form of MisR is the major transcriptional repressor of \textit{hlyB} and the \textit{hlyD/tolC} operon \textit{in vivo}.

Both nonphosphorylated and phosphorylated MisR, which was generated by treatment with acetyl phosphate, directly interact with the promoters of \textit{hlyB} and \textit{hlyD/tolC} loci, as shown previously by EMSA using purified MisR-(His)$_6$ protein (Tzeng \textit{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 ΔmisS::aphA-3 mutant (dotted bars) and the ΔmisRS::aphA-3 mutant (grey bars). The mean and so 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-32P]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 μg specific DNA; 4, with addition of 4 μg 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 μM) as described in Methods. An RNA size marker (M) (Ambion) is shown with the size in nucleotides indicated to the right.

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 WWTGTAAVG 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
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 (OD600 ~3.0) versus exponential phase (OD600 ~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 (OD550 ~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 OD550 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
level in the non-secreting ΔhlyB strain YT358 (lane 1) and the hyper-secreting ΔmisS 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 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

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**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 [x, YT356 and YT347 in (a) and (b), respectively] and the misS::aphA-3 [■, 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.

**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 ΔhlyB mutant YT358 (lane 1), wild-type strain NMB (lane 2), ΔmisS mutant YT310 (lane 3) and complemented ΔmisS 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 antiserum against MisR. A non-specific reactive band is marked with an asterisk. Samples for lanes 1 and 5 were prepared from strain YT358 (ΔhlyB), lanes 2 and 6 from strain NMB (wild type), lanes 3 and 7 from strain YT310 (ΔmisS), and lanes 4 and 8 from strain YT391 (complemented ΔmisS). 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 ΔmisS 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 ΔmisS strain YT391 (lane 4).
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 ΔmisS 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 MisRS 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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