Published in final edited form as:
Mol Microbiol. 2011 March ; 79(6): 1557–1573. doi:10.1111/j.1365-2958.2011.07546.x.

Characterization of DsbD in *Neisseria meningitidis*

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Abstract

Proper periplasmic disulfide bond formation is important for folding and stability of many secreted and membrane proteins, and is catalyzed by three DsbA oxidoreductases in *Neisseria meningitidis*. DsbD provides reducing power to DsbC that shuffles incorrect disulfide bond in misfolded proteins as well as to the periplasmic enzymes that reduce apo-cytochrome *c* (CcsX) or repair oxidative protein damages (MrsAB). The expression of *dsbD*, but not other *dsb* genes, is positively regulated by the MisR/S two-component system. qRT-PCR analyses showed significantly reduced *dsbD* expression in all *misR/S* mutants, which was rescued by genetic complementation. The direct and specific interaction of MisR with the upstream region of the *dsbD* promoter was demonstrated by EMSA, and the MisR-binding sequences were mapped. Further, the expression of *dsbD* was found to be induced by dithiothrietol (DTT), through the MisR/S regulatory system. Surprisingly, we revealed that inactivation of *dsbD* can only be achieved in a strain carrying an ectopically located *dsbD*, in the *dsbA1A2* double mutant or in the *dsbA1A2A3* triple mutant, thus DsbD is indispensable for DsbA-catalyzed oxidative protein folding in *N. meningitidis*. The defects of the meningococcal *dsbA1A2* mutant in transformation and resistance to oxidative stress were more severe in the absence of *dsbD*.

Keywords

Neisseria meningitidis; DsbD; MisRS; two-component regulatory system; DsbA

INTRODUCTION

Neisseria meningitidis, an exclusive pathogen of humans, remains a leading cause of meningitis and sepsis, usually in otherwise healthy individual (Stephens *et al.*, 2007, Stephens, 2007). As a result of successful conjugate vaccines in reducing the incidence of meningitis caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* infections, *N. meningitidis* has become a leading cause of bacterial meningitis in children and young adults in the United States (Schuchat *et al.*, 1997, Whitney *et al.*, 2003). Despite the sensitivity of meningococcus to many antibiotics, meningococcal disease still causes substantial mortality and morbidity (Bilukha & Rosenstein, 2005).

N. meningitidis inhabits the human nasopharynx of approximately 10% of the population and can also rapidly disseminate to cause invasive disease (Stephens & Zimmer, 2002, Tzeng & Stephens, 2000). To successfully colonize and survive as an obligate human pathogen, *N. meningitidis* responds to signals emanating from the human host, a task often

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carried out by environmental 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 and the phosphoryl group is subsequently transferred to the cognate response regulator. The phosphorylation status of the response regulator typically mediates affinity of the protein to the promoters of target genes. In contrast to other gram-negative bacteria such as *E. coli*, which encodes more than 30 two-component regulatory systems (Oshima *et al.*, 2002, Yamamoto *et al.*, 2005), the meningococcus contains only four predicted pairs (Tettelin *et al.*, 2000, Parkhill *et al.*, 2000), a characteristic common to obligate pathogens from restricted environments. Capsular polysaccharides, lipooligosaccharides (LOS), outer membrane receptors for iron acquisition and pili are among the important virulence factors critical for meningococcal colonization and pathogenesis (Tzeng & Stephens, 2000). The meningococcal MisR/MisS two-component system has been shown to influence phosphorylation of the LOS inner core (Tzeng *et al.*, 2004), directly repress expression of the type I secretion proteins (Sannigrahi *et al.*, 2009), and activate expression of the HmbR hemoglobin receptor and the HemO heme oxygenase, both important in heme utilization (Zhao *et al.*, 2010). Inactivation of the response regulator MisR also 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 gram-negative bacteria, many membrane and exported proteins are stabilized by intramolecular disulfide bridges between two cysteine residues which are introduced by the oxidoreductase, DsbA. The lack of disulfide bonds in periplasmic proteins results in protein misfolding and loss of function with ultimate removal due to increased sensitivity to proteolytic degradation in the periplasm. To complete the protein oxidation pathway, DsbA is reoxidized by the inner membrane protein DsbB, which utilizes the respiratory chain as electron acceptors. The protein oxidation pathway is partnered by an isomerization pathway consisting of an inner membrane protein, DsbD, which utilizes the cytoplasmic thioredoxinto reduce the disulfide isomerase DsbC. DsbC catalyses the shuffling of multiple disulfide bonds within a single substrate protein until the correct conformation is obtained (Collet & Bardwell, 2002, Kadokura *et al.*, 2003). Dsb proteins are critical for physiological processes such as cytochrome c maturation (Kranz *et al.*, 1998, Thony-Meyer, 2002), and are necessary for many important pathogenic phenotypes including attachment, invasion and intracellular survival (Yu, 1998, Raivio, 2005, Bringer *et al.*, 2007).

N. meningitidis encodes three *dsbA* genes (Sinha *et al.*, 2004, Tinsley *et al.*, 2004): *dsbA1* is meningococcal specific (Tinsley & Nassif, 1996), while *dsbA2* and *dsbA3* are also present in *Neisseria gonorrhoeae*. Similar to most other bacterial DsbA proteins, DsbA3 is a soluble periplasmic protein; however, DsbA1 and DsbA2 are found to be lipoproteins (Tinsley et al., 2004). Phenotypic analyses have shown that meningococcal *dsbA1A2* double mutants have reduced transformation efficiency and adherence to endothelial cells (Tinsley et al., 2004, Sinha *et al.*, 2008). Further, the defect of the *dsbA1A2* mutant in natural competence has been linked to inefficient folding of PilE and PilQ (Tinsley et al., 2004, Sinha et al., 2008). Single copies of *dsbB*, *dsbC* and *dsbD* have been identified in the meningococcal genomes (Sinha et al., 2004, Tinsley et al., 2004).

We have previously carried out microarray analyses of a *misR* mutant to identify MisR-regulated genes and to define the minimal regulon (Tzeng *et al.*, 2008). Our study has found that expression of *dsbD* is significantly down regulated in the *misR* mutant (Tzeng et al., 2008). As this is the first regulatory network impacting the expression of the isomerization arm of the Dsb system, we further characterized the mechanisms of transcriptional control of meningococcal *dsbD*. Consistent with its role as a transcriptional activator, a direct and

specific interaction of MisR with the upstream region of the *dsbD* promoter was demonstrated.

Surprisingly, we found that DsbD is required for the viability of *N. meningitidis*, and can only be mutated in the presence of a second functional copy or when the major activity of the oxidative Dsb pathway was inactivated.

RESULTS

Expression of *dsbD* is controlled by the MisR/MisS two-component system

We have previously observed a significant down-regulation of *dsbD* in a *misR* mutant by microarray analysis (Tzeng et al., 2008) and this regulation was further characterized in this study. qRT-PCR was conducted to examine *dsbD* expression in the *misR*, *misS* and *misRS* mutants and the complemented *misR* and *misS* mutants. As shown in Fig. 1A, significant decreases in *dsbD* transcription (8–13 fold) were observed in all mutants, while complementation with an ectopically located second copy of *misR* or *misS* rescued the *dsbD* transcriptional defect. DsbD is one of several meningococcal Dsb proteins involved in the periplasmic disulfide formation that include DsbB, DsbC and three DsbAs. Although we did not detect transcriptional changes in these *dsb* genes by microarray, another microarray analysis (Newcombe *et al.*, 2005) reported that expression of *dsbA2*, *dsbA3*, and *dsbC* was altered in a serogroup C *misR* mutant. Thus, we further assess whether MisR might influence expression of other *dsb* genes by qRT-PCR analyses. Transcription in the wild type strain and the *misS* mutant was compared and no significant differences in any other *dsb* genes were detected (Fig. 1B), thus the MisRS system specifically regulates *dsbD* expression.

Mapping of the MisR regulatory region within the *dsbD* promoter

We first determined the transcriptional start site of *dsbD* by primer extension using total RNA isolated from the wild-type strain NMB and a reverse primer complementary to the coding strand of *dsbD*. A single major product was observed corresponding to the -29cytosine nucleotide (Fig. 2), indicating a single promoter. The same start site was verified with a second complementary primer (data not shown). Examination of the sequence upstream of the transcriptional start site revealed appropriate promoter elements of a -10 (TATAAT) and a -35 (ATCCCA) sequence separated by 17-bp (see Fig. 4B).

To delineate the *dsbD* promoter region required for transcriptional activation by MisR, a series of *dsbD::lacZ* transcriptional fusions with promoter lengths ranging from −277-bp to −8-bp upstream of the transcriptional start site were made (Fig. 3A). These fusions were inserted into a permissive chromosomal locus as a single copy. First, the activity of the YT350 strain with the longest promoter fragment was examined throughout the growth phase and Fig. 3B showed that the *dsbD* expression remained constant during the exponential growth phase and decreased slightly in the stationary phase. No β-galactosidase activity was detected in the D8 strain, thus confirming the location of the promoter(Fig. 3C). A *dsbD* promoter fragment that contains the nucleotide sequence between −67 and +123bp (strain D67) generated an activity similar to those with longer promoter sequences, indicating that the ~70-bp sequence upstream of the transcriptional start site was sufficient for full transcriptional activity. It was expected that in the absence of the sequence at which MisR exerts its regulation, transcription of *dsbD* will not be affected by inactivation of the MisRS system. Thus, the reporter constructs were introduced into the *misS* mutant for comparison. The activities of the D67 reporter and other longer promoter fusions were significantly reduced in the *misS* mutant. In contrast, the strain with only the −35 and −10 promoter elements (strain D37) yielded significantly reduced activity in the wild type

background, and similar activities were observed between the wild type strain and the *misS* mutant. Thus, a minimal of ~ 30-bp sequence upstream of the *dsbD* promoter elements was required for MisR regulation as well as a full transcription activity.

MisR directly interacted with the *dsbD* promoter

Although prior EMSA experiments have not detected direct binding of MisR to the *dsbD* promoter (Tzeng et al., 2008), in light of the strong regulatory effect of MisR on *dsbD* expression, we re-examined whether MisR directly interacts with the *dsbD* promoter. In contrast to our previous observation, we detected a specific binding of MisR protein to the *dsbD* promoter DNA using different MisR preparations (Fig. 3D). The binding of MisR to various promoter fragments used in the reporter constructs were evaluated. As shown in Fig. 3D, reduced binding were seen between the D227 and D137 fragments and between the D137 and D67 fragments. Consistent with the *lacZ* reporter data, no binding was detected with the D37 probe. The specificity of MisR binding was demonstrated with competition EMSA where only excess specific DNA, but not nonspecific DNA fragments, eliminated the shift (Fig. 3E). Thus, MisR directly interacted with the sequence upstream of the *dsbD* promoter, a commonly observed location for transcriptional activators.

To identify the precise location of MisR binding site(s), DNase I protection assays were performed on both strands of a 350-bp ^{32}P-end-labeled *dsbD* probe (−227 to +123, the D227 fragment in Fig. 3), which displayed strong binding in EMSA. In the presence of increasing amounts of MisR, ~24-bp regions were protected from DNase I digestion on both the coding strand and the noncoding strand (Fig. 4A). The upstream DNase I protected region is present in the D227 fragment, truncated in the D137 fragment, and is absent in the D67 fragment, therefore consistent with the apparent gradual reduction in binding strength detected by EMSA (Fig. 3D). The downstream DNase I protected site fell within the *dsbD* promoter sequence deemed important in the reporter assays as the D67 fragment conferred a full MisR-regulated transcriptional activity (D67 vs. D37, Fig. 3C). Two putative MisR binding motifs were found to flank and partially overlap the downstream DNase I protected region (Fig. 4B). The motif at the 3′ end is most likely more important as it has a 10-nucleotide overlap with the protected sequence. A possible explanation for the absence of potential MisR binding motif in the upstream protected sequence is that the MisR binding consensus sequence (Tzeng et al., 2008) might be more degenerate than previously defined. Alternatively, a cooperative binding of MisR at the downstream site may result in an additional interaction with this upstream sequence. Taken together, these *in vitro* data supported a model that MisR directly activated *dsbD* expression.

Exposure to DTT enhanced *dsbD* expression via the induction of the MisRS system

DsbD functions as the redox hub of the Dsb system and is regulated by the environmental sensing MisRS two-component system, thus we tested whether redox changes can affect its expression. The *dsbD::lacZ* reporter strain (YT350) was treated with various redox agents and, interestingly, we discovered that exposure to 1 mM DTT for 30 minutes yielded ~2-fold induction of *dsbD* transcription(Fig. 5A). To determine whether the induction is dependent of the MisR regulation, we first tested whether DTT also induced *misR* expression. The MisRS system is autoregulated (Tzeng *et al.*, 2006), thus its own expression will be up-regulated if the DTT signal is transmitted through the MisRS system. We found that the *misR::lacZ* expression was indeed induced in a similar manner (Fig. 5A). Next, we determined if the DTT induction required a functional MisS kinase. The *dsbD* and *misR* reporters in the *ΔmisS* background was treated with DTT and as shown in Fig. 5A, the induction was abolished by the *misS* mutation, thus supporting the involvement of the sensor kinase in transmitting the inducing signal. The induction of *dsbD* and *misR* by DTT and the dependence on MisS were further confirmed by qRT-PCR analyses (Fig. 5B). As the

expression of *misR* was reduced in the *misS* mutant, we tested whether the lack of DTT response in the *misS* mutant was due to insufficient MisR protein. An IPTG-inducible *misR* construct was introduced into the *misS* mutant to generate strain PKT392. The expression of *misR* was induced by IPTG and then the cultures were similarly treated with DTT. As shown in Fig. 5B, in the presence of MisR over expression (~3-fold and 8-fold increases relative to the wild type strain and the *misS* parental strain, respectively) no DTT induction of *dsbD* expression was detected in strain PKT392, confirming that MisS was indeed required for transmitting the DTT signal. The fact that the *dsbD* expression in strain PKT392 was not significantly different from that of the *misS* strain despite over expression of MisR also indicated that MisR phosphorylation by MisS is critical in *dsbD* regulation. No growth inhibition was detected with the short DTT treatment, and the induction was dose-dependent (Fig. 5C) and time-dependent (data not shown). Subsequently, transcription of the autoregulated *misR* was monitored for specific signaling effect through the MisRS two-component system. No induction by oxidized DTT, N-acetyl cysteine, reduced and oxidized glutathione, *tris*(2-carboxyethyl) phosphine (TCEP), paraquat, copper, hydrogen peroxide (all at 1 mM) or heat shock (45°C, 30 minutes) was detected (107%, 115%, 118%, 103%, 114%, 113%, 107%, 97% and 99% relative to no treatment, respectively), suggesting that induction by DTT is most likely not a general stress response.

DsbD is essential for *N. meningitidis*

In order to better understand the biological consequence of the MisRS regulation of *dsbD* in response to environmental changes and investigate whether DsbD plays a role in meningococcal virulence, we attempted to create a meningococcal *dsbD* mutant. Our data showed that *dsbD* is independently transcribed (Fig. 3A), thus a *dsbD::Ω* mutational construct was created. Despite numerous transformation attempts, we could not identify any correct meningococcal *dsbD* mutant (data not shown). Another *dsbD* deletion-insertion mutational construct utilizing the nonpolar *aphA3* cassette was subsequently generated. Although many kanamycin resistant transformants were obtained, colony PCR analyses indicated that these clones carried a *dsbD::aphA3* mutation as well as a wild type copy of *dsbD*. Transformation with either the purified PCR product or the linearized plasmid construct gave similar results. These results implicated that *dsbD* might be essential for meningococci. Thus, we generated a meningococcal strain with a second copy of *dsbD* at a permissive chromosomal *lctP-aspC* locus (Mehr & Seifert, 1998) under the control of an IPTG inducible promoter. The resulting strain was then transformed with the *dsbD::aphA3* construct, and in contrast to previous attempts, transformants with a correct *dsbD* mutation can be readily recovered. The correct transformants can be obtained without IPTG induction as the P_{lac} control was leaky and yielded a basal level of expression in *N. meningitidis*.

The results of PCR analyses were further confirmed by Southern blots. Detection with a probe that hybridizes with the remaining *dsbD* 3′ coding sequence (Fig. 6A) revealed a doublet in the *dsbD** mutant that was recovered from the wild type strain (Fig. 6A, lane 2): the upper band corresponded to that of the wild type *dsbD* (lane 1), while the lower band was the mutated *dsbD*. In contrast, the strain with a *dsbD* mutation created in the complemented background (lane 3) only contained the lower band (the second smaller fragment is the complemented copy at the *lctP-aspC* locus). Further, a probe that hybridizes within the deleted region of the *dsbD::aphA3* mutation detected the upper band in both the wild type and the *dsbD** mutant (lanes 1 and 2 in Fig. 6B), but this signal was absent in the complemented *dsbD* mutant (lane 3). The probable duplication junctions and the extent of duplication were not determined. Therefore, the fact that the wild type *dsbD* locus can be disrupted only in the presence of a second functional copy of *dsbD* indicated that *dsbD* is essential for *N. meningitidis*.

A *dsbD* mutation can be established with compensatory *dsbA* mutations

DsbD together with the oxidoreductase DsbC constitute the reducing branch of the Dsb pathway and its main function is to transfer reducing power to the periplasm for correcting mis-formed disulfide bonds and for reducing apo-cytochrome *c* proteins prior to heme ligation. We postulated that if the oxidative Dsb pathway was inactivated, the requirement for a fully functional reductive branch might be lessened and consequently a *dsbD* mutation could be tolerated. To test this hypothesis, we created a strain with *dsbA1*, *dsbA2* and *dsbA3* triple mutations, and tested whether a clean *dsbD* mutation can be obtained. Interestingly, the correct *dsbD* mutation was indeed created in the *dsbA1A2A3* triple null mutant (Fig. 6, lane 8). Subsequently, we tested combinations of double *dsbA* mutations and found that *dsbA1A2*, but not the *dsbA2A3* and *dsbA1A3* combinations, can sustain the *dsbD* mutation (Fig. 6, lanes 6 and 7 for *dsbA2A3* and *dsbA1A2*, respectively; data not shown for *dsbA1A3*). Finally, an incorrect *dsbD* mutation was also detected in the single *dsbA1*, *dsbA2* and *dsbA3* mutants (Fig. 6, lanes 4 and 5 for *dsbA1* and *dsbA2*, respectively, and data not shown). The correct *dsbD* mutation was stable with repeated passages. We have also attempted to generate the *dsbD* mutation in the wild type background by supplementing the selection media with various concentrations of DTT. How ever, no correct *dsbD* mutants could be recovered (Fig. S2). The fact that the *dsbA1A2* double mutant produces the strongest phenotypes among the double mutational combinations and behaves similarly to the *dsbA* triple null mutant (Sinha et al., 2008, Tinsley et al., 2004) suggested that inactivation of DsbD can only be tolerated in situations with a minimal DsbA oxidative activity.

Characterization of the *dsbD* mutation in the *dsbA1A2* background

We examined the growth rates of the mutants in standard GC broth. Analogous to previous reports (Sinha et al., 2004, Tinsley et al., 2004), no obvious growth defect was detected for the *dsbA1A2* mutant (Fig. 7A), while the triple *dsbA* null mutant exhibited reduced growth (data not shown). The growth rate of the *dsbA1A2D* mutant was also similar to that of the wild type strain (Fig. 7A), thus in subsequent characterization of DsbD contribution in various meningococcal phenotypes we compared the *dsbA1A2D* mutant to its parental *dsbA1A2* strain. In addition, a complemented *dsbD* mutant with an IPTG inducible expression of *dsbD* at the nics locus (Mehr & Seifert, 1998) was also generated in the *dsbA1A2* background for comparison.

First, we assessed whether the cytochrome *c* maturation process was affected by the *dsbD* mutation, as *c*-type cytochromes are important in electron transfer chain and viability of meningococci (Deeudom *et al.*, 2008, Li *et al.*, 2010, Aspholm *et al.*, 2010). The chemilumine scent detection of heme (Feissner *et al.*, 2003) was used as a qualitative assessment of the overall cytochrome *c* profiles. Equal amounts of total proteins were loaded, resolved by SDS-PAGE in the absence of reducing agents and heat denaturing and then transferred to PVDF membranes for heme staining. As shown in Fig. 7B, wild type like cytochrome *c* profiles were detected in the *dsbA1A2* and *dsbA1A2D* strains. Thus, the overall cytochrome *c* profiles appeared to be normal in both mutants, correlating with their growth phenotypes.

It has been previously reported that the meningococcal transformation efficiency was reduced by the *dsbA1A2* mutation (Tinsley et al., 2004, Sinha et al., 2008) due to inefficient folding of the secret in PilQ (Sinha et al., 2008). PilQ is required for type IV pilin biogenesis and DNA binding, both necessary for natural transformation. We thus investigated whether the *dsbD* mutation will further influence meningococcal natural competence. Using the chromosomal DNA of strain NMB with a Cm^R marker at the nics locus as the transforming DNA, we found that the *dsbA1A2D* mutant exhibited ~10% of transformation frequency of its parental *dsbA1A2* strain. Complementation with an IPTG inducible *dsbD* rescued the

transformation defect (Fig. 7C). These results indicated that the folding defect of PilQ might be more profound in the absence of DsbD. However, the total PilQ protein levels of the mutants were not significantly different from that of the wild type strain when examined by Western blots (Fig. 7D). Thus, additional proteins involved in competence might be affected by the absence of DsbD.

The absence of *dsbD* enhanced the sensitivity of the *dsbA1A2* mutant to oxidative stress

DsbC and DsbD of the isomerization pathway were shown to play a key role in protecting *E. coli* against oxidative copper stress (Hiniker *et al.*, 2005). In addition, the methionine sulfoxide reductase MrsA/B, which repairs oxidative damage to proteins, acquires the reducing power from DsbD (Brot *et al.*, 2006). It is likely that the increased numbers of periplasmic proteins carrying free thiol groups in the meningococcal *dsbA1A2* mutant due to a slower oxidation process will render the strain more sensitive to oxidative stresses that cause incorrect disulfide bond formation and protein damage via nonspecific oxidation. We reasoned that this sensitivity will be further augmented in the absence of DsbD. Thus, we examined the sensitivity of the *dsb* mutants to hydrogen peroxide, paraquat and copper by disc diffusion assays. As shown in Table 2, relative to the wild type strain the *dsbA1A2* mutant was more sensitive to paraquat and copper but not to hydrogen peroxide; while the *dsbA1A2D* mutant displayed enhanced sensitivities to all oxidants when compared to the parental *dsbA1A2* strain. Complementation of *dsbD* reversed this sensitivity profile to levels consistent with the parental *dsbA1A2* strain. Thus, we detected a decreased resistance to oxidative stress of the meningococcal *dsbA1A2* strain and this sensitivity was further augmented by the *dsbD* mutation.

DISCUSSION

Many bacterial membrane and exported proteins are stabilized and attain their functional conformation by forming intramolecular disulfide bonds, which are catalyzed by the Dsb system in the periplasm. The activities of the Dsb system are necessary for many important pathogenic phenotypes including attachment, invasion and intracellular survival (Yu, 1998, Raivio, 2005, Bringer et al., 2007). In particular, DsbD plays a role in the translocation of reducing power from cytoplasm to several periplasmic thioredoxin-like proteins that include the disulfide isomerase DsbC, the cytochrome c reductase CcmG/CcsX, the peptide methionine sulfoxide reductase MsrA, a repair enzyme that contributes to the maintenance of functional adhesions (Brot et al., 2006, Wizemann *et al.*, 1996), and additional periplasmic proteins shown to play a role in defense against oxidative stress and intracellular survival (Skaar *et al.*, 2002, Brot et al., 2006, Achard *et al.*, 2009). DsbD or its smaller homolog CcdA is present in all organisms that synthesize *c*-type cytochromes (Kranz *et al.*, 2002) with the exception of *Rickettsia*, which is an obligate intracellular pathogen and likely uses host-derived reducing environment for its extracytoplasmic redox processes (Kranz et al., 2002).

Very little is known about how bacteria regulate the expression of Dsb proteins. The only regulation of the Dsb system illustrated to date is the control of *dsbA*. Expression of *dsbA* is regulated by the CpxRA two-component system in *E. coli* (Danese & Silhavy, 1997, Pogliano *et al.*, 1997); while RtsA and H-NS regulate *dsbA* in *Salmonella* (Gallant *et al.*, 2004, Ellermeier & Slauch, 2004). The response regulator CpxR has been shown to bind to the *dsbA* promoter and directly activate its expression (Pogliano et al., 1997). The MisRS two-component system is the first regulatory mechanism described for *dsbD* and is the first regulation identified for the meningococcal Dsb system. Our study has found that expression of *dsbD*, but not any of the other Dsb proteins, is significantly down regulated in the *misR/S* mutants (Fig. 1B), suggesting that MisR acts as a specific activator of *dsbD* transcription. Our current study further defined that MisR specifically binds to, and likely directly

activates transcription from the *dsbD* promoter. A mutation of *misS* leads to a 5–10 fold decrease in *dsbD* expression. Hence, the MisRS system appears to contribute to the basal level of *dsbD* expression under normal growth conditions, suggesting that a low level of phosphorylated MisR may be present under unstimulated growth conditions. Why *N. meningitidis* evolves to specifically regulate DsbD of the reductive branch of the Dsb system is not clear. However, a coordinated control of three DsbAs in *N. meningitidis* presents a challenge. It seems likely that certain *in vivo* conditions may necessitate DsbD induction by the MisRS system to promote expedited proper disulfide folding as well as repair cell envelope/periplasmic proteins. For example, overexpression of DsbD upon the induction of the MisRS system by host derived environmental signals might combat stresses associated with certain host immune responses or microenvironments that resulted in protein damage within the extracytoplasmic compartments.

The regulatory mechanism(s) of the disulfide-folding process likely senses either cell envelope and/or periplasmic protein folding defects or senses the conditions that lead to folding defects. These signals must be transmitted across the inner membrane, a signal transduction mechanism that the two-component regulatory system is well suited for. We have found that exposure to the reducing agent DTT further induced expression of DsbD and its regulator MisR. DTT *per se* is not likely the actual signal, but rather its reducing effect on other physiological conditions or processes may signal the induction of the MisRS system. As DTT is freely diffusible through the inner membrane, it is also not clear the precise subcellular location for its effects. No induction was seen with a charged reducing agent, *tris*(2-carboxyethyl) phosphine (TCEP), which is a more powerful reducing agent and is commonly used to substitute DTT in many biochemical assays (Getz *et al.*, 1999). TCEP is most likely unable to penetrate the inner membrane due to its charged nature, thus the induction of the MisRS system might require the reducing agent to diffuse through or act within the cytoplasmic membrane. Alternatively, TCEP has been found to be selective toward disulfide bonds, while DTT can react with Fe^{+3} or Ni^{+2} (Getz et al., 1999), implying that DTT might introduce a complicated effect on the cellular environment. Additional analyses are needed to clearly delineate the signal transduction mechanism initiated by DTT treatment that leads to the activation of the MisRS system. Further, it remains to be determined whether the perturbed redox balance of a particular subcellular environment or general or specific misfolding of proteins induces the global MisRS regulatory cascade.

In this study, we demonstrated that DsbD is essential for *N. meningitidis*. In the presence of an ectopically located *dsbD*, a correct mutation at the wild type *dsbD* locus can be achieved. On the other hand, numerous attempts to replace the *dsbD* gene by two independent mutational constructs in the wild type background were unsuccessful, and yielded clones carrying a duplicated wild type copy of *dsbD* elsewhere on the chromosome (Fig. 6). Similar duplication phenomenon has been described in *N. gonorrhoeae*. Attempts to inactivate the essential *imp* gene, which encoding an outer membrane protein important for lipooligosaccharide transport, in *N. gonorrhoeae* have resulted in clones carrying a wild type copy and a mutated copy of *imp*, representing merodiploids that were attributed to a rare recombination event (Bos *et al.*, 2004, Tobiason & Seifert, 2010). Tobiason et al. have recently showed that both *N. meningitidis* and *N. gonorrhoeae* are polyploid and genetically haploid (Tobiason & Seifert, 2010). It is not clear whether the meningococcal polyploid genotype contributes to the duplication event.

Interestingly, a correct *dsbD* mutation can be attained in either the *dsbA1A2* or *dsbA1A2A3* genetic backgrounds, but not any other combinations of *dsbA* mutations. It has been shown that among the three double mutant combinations, the *dsbA1A2* mutation yielded the most significant phenotypic changes that are similar to those of the triple *dsbA* null mutant (Tinsley et al., 2004, Sinha et al., 2008). Lafaye et al. showed that meningococcal DsbAs

exhibit similar *in vitro* redox properties and are the most oxidizing thioredoxin-like enzymes known to date (Lafaye *et al.*, 2009). The expression levels of *dsbA* genes normalized to 16s in the wild type strain NMB were assessed by qRT-PCR using $2^{-\Delta\Delta Ct}$ comparison relative to *dsbA1*. The results indicated that expression levels among the *dsbA* genes are in the following order: *dsbA1* ≥ *dsbA2* >dsbA3 (1, 0.7 ± 0.3, 0.5 ± 0.1 for *dsbA1*, *dsbA2* and *dsbA3*, respectively). Thus, in addition to the likely different substrate specificity of each DsbA, the amounts of DsbA1 and DsbA2 relative to DsbA3 may also contribute to the variations in phenotypes of the double mutants. A possible explanation for the compensatory mutation effect of *dsbA1A2* and *dsbD* might be the redox requirement of cytochrome *c* maturation. *Neisseria* species produce several *c*-type cytochromes that participate in electron transfer chain (Deeudom et al., 2008) and the cytochrome *c* oxidase cbb_3 is essential for aerobic growth (Aspholm et al., 2010). The survival of *N. gonorrhoeae* was also shown to depend upon the presence of either cytochrome c_4 or c_5 (Li et al., 2010). All *c*-type cytochromes are located in the periplasm and the maturation process involves the covalent attachment of heme to two cysteine residues of a CXXCH motif of the apo-protein. Two bacterial cytochrome *c* biogenesis pathways termed system I and II have been revealed (Kranz et al., 2002, Kranz et al., 1998), and *N. meningitidis* utilizes the System II pathway. The system II biogenesis pathway has been well characterized in *Bordetella pertussis* to require at least four specific assembly proteins, CcsA (ResC), CcsB (ResB), CcsX and DsbD (or its smaller homologue, CcdA) (Beckett *et al.*, 2000). The CcsB and CcsA proteins form a cytochrome *c* synthetase complex that also functions in heme transport (Frawley & Kranz, 2009), while the thioredoxin like protein CcsX in cooperation with DsbD mediates the reduction of apo cytochrome *c*. A plausible model to explain the *dsbA*/*dsbD* dependence in meningococci is that the cysteine residues in the CXXCH motifs of apo *c*-type cytochrome proteins are oxidized by DsbAupon entering the periplasm and require re-reduction prior to heme ligation. This re-reduction involves DsbD as a donor of reducing power. Thus, the diminished reducing power within the periplasm caused by *dsbD* inactivation is compensated by the absence of both DsbA1 and DsbA2 and, therefore, allows for continued *c*-type cytochrome biosynthesis. Interestingly, Kranz et al. has reported failed attempts to make deletions in genes required for System II cytochrome *c* biogenesis in *N. meningitidis*, although the specific gene targeted was not described (Kranz et al., 2002). Analogous to our observation in *N. meningitidis*, the lack of DsbA in *Bacillus subtilis* or *Rhodobacter capsulatus* suppresses the cytochrome *c* deficiency of the *ccdA* (*dsbD*) mutants, indicating that the reductive branch becomes dispensable in the absence of the oxidative pathway (Erlendsson & Hederstedt, 2002, Deshmukh *et al.*, 2003). In addition, the defect in *c*-type cytochrome biosynthesis of *dsbD* mutants in *E. coli* can be restored by the cytoplasmic membrane-impermeable thiol, mercaptoethane sulfonic acid, supplemented in the growth medium (Sambongi & Ferguson, 1994), again suggesting that a balance of oxidative and reductive reactions is required for cytochrome *c* maturation. However, an exogenous supplementation of DTT could not rescue the *dsbD* defect in *N. meningitidis*.

Alternatively, the more error prone oxidation by other periplasmic oxidants that are oxidized by DsbAs could potentially damage essential enzymatic functions within periplasm. In a study designed to identify periplasmic substrates of DsbG, Depuydt et al. showed that the single cysteines of several proteins belonging to the family of L, D-transpeptidases, which catalyze the crosslinking of peptidoglycan for cell wall synthesis, are oxidized to a sulfenic acid (Cys-SOH) by oxidants present in the periplasm and are protected by the function of DsbG and DsbC (Depuydt *et al.*, 2009). DsbC and DsbG (absent in meningococci) that are kept reduced by DsbD were shown to control the level of cysteine sulfenylation in the periplasm and provides reducing equivalents to rescue oxidatively damaged proteins (Depuydt et al., 2009). Thus, the electron flux mediated by DsbD provides the reducing power required for not only the correction of incorrect disulfides but also the rescue of sulfenylated orphan cysteines (Depuydt et al., 2009)in critical periplasmic enzymes.

Consequently, together with the role of DsbD in cytochrome *c* maturation discussed above, only a less oxidative periplasmic environment lacking DsbAs can sustain a *dsbD* null mutation in *N. meningitidis*.

Due to the necessity of *dsbA1* and *dsbA2* mutations in maintaining the *dsbD* mutation, we have examined the contribution of DsbD to several phenotypes with respect to that of the parental *dsbA1A2* background. In general, the *dsbD* mutation further enhanced the defect of the *dsbA1A2* mutant. This was seen in the transformation efficiency (Fig. 7C). The transformation deficiency of the *dsbA1A2* mutant has been attributed to folding defects of PilQ and PilE that reduce the PilQ protein level to cause deficiency in DNA binding and uptake but allow the formation of non-functional pilus fibers on cell surface (Tinsley et al., 2004, Sinha et al., 2008). Similar to these earlier studies, we did not detect differences in levels of the pilin subunits (PilE) between the *dsbA1A2D* mutant and the wild type strains by immunoblots (data not shown); while in contrast to the earlier report (Sinha et al., 2008), no clear reduction in PilQ protein levels can be detected in the *dsbA1A2* and *dsbA1A2D* mutants. Our results suggested that inactivation of *dsbA1A2* and *dsbD* in the meningococcal strain NMB did not appear to severely impair the disulfide folding and assembly of PilQ multimers but likely affected other competence-related proteins with multiple cysteine residues. For example, the DprA protein that is essential for natural competence (Smeets *et al.*, 2000, Takata *et al.*, 2005) has four cysteines, thus may requires a functional DsbD for proper expression.

Mutation in *dsbA1A2* increased the meningococcal sensitivity to oxidative stresses elicited by paraquat, and copper, but not hydrogen peroxide, whereas the *dsbD* mutation further enhanced the sensitivity of *N. meningitidis* to all three stresses (Table 2). Hydrogen peroxide is known to oxidize critical cysteine or methionine residues in folded proteins, but it likely elicits only modest effect on DsbA-catalyzed oxidative protein folding. Most of the copper-mediated hydroxyl radical formation occurs in the periplasm (Macomber *et al.*, 2007). In *E. coli*, the *dsbA* mutant is not sensitive to oxidative copper stress comparing to wild type, but the *dsbA* mutation does increase copper sensitivity of a *dsbD*$^{-}$ strain (Hiniker et al., 2005). Mutation in meningococcal *dsbA1A2*, however, increased the sensitivity to oxidative copper stress. It is possible that due to the need of three DsbA proteins, *N. meningitidis* relies more on DsbA for proper disulfide folding than does *E. coli*, and thus displays a stronger copper phenotype when DsbAs are inactivated. The further augmentation of oxidative stress sensitivity of the meningococcal *dsbA* mutant by the *dsbD* mutation may due to the lack of reducing power from DsbD to the methionine sulfoxide reductase MrsAB, which is involved in survival against oxidative stress (Brot et al., 2006, Skaar et al., 2002).

DsbD has not been shown to be essential for any other bacteria, likely because 1) the presence of other types of cytochrome oxidases allows the lack of *c*-type cytochrome biosynthesis or 2) other mechanisms or proteins capable of delivering reducing power to the periplasm can compensate for the absence of DsbD. For example, a heterodimeric ABC transport system, CydDC, which exports glutathione and cysteine from cytoplasm into the periplasm, represents a redundant pathway of DsbD and has been shown to be required for assembly of the cytochrome *bd*-type terminal oxidase in *E. coli* (Pittman *et al.*, 2005). As overexpression of *cydDC* restores defects of a *dsbD* mutant, it appears that in *E. coli* the CydDC exporter can compensate the inadequate periplasmic reducing capability of a *dsbD* mutant for cytochrome assembly (Pittman et al., 2005). However, no clear *cydDC* homolog can be found in *N. meningitidis*. Taken together, our data suggest that the meningococcal Dsb system plays an important role in combating periplasmic oxidative stress and that the MisRS system is critical in controlling the function of the Dsb system via the regulation of *dsbD* expression.

Experimental procedures

Plasmids, strains and media

The strains and plasmids used in this study are listed in Table 1. *Neisseria meningitidis* strain NMB (CDC 8201085) is a serogroup B meningococcal strain originally isolated in 1982 from the cerebrospinal fluid of a patient with meningococcal meningitis in Pennsylvania. Meningococcal strains were grown with 5% CO_2 at 37°C on GC base (GCB; Difco) agar containing supplements of 0.4% glucose and 0.68 mM $Fe(NO_3)_3$, or GC broth with the same supplements and 0.043% $NaHCO_3$. Brain heart infusion (BHI) medium with 1.25% fetal bovine serum was used when kanamycin selection was required. *Escherichia coli* DH5α or Top 10 strains were routinely grown in Luria Bertani broth for cloning and propagation of plasmids. *N. meningitidis* was transformed by the procedure of Janik et al. (Janik *et al.*, 1976). *E. coli* strains were transformed by chemical competence (Top 10) or by electroporation (DH5α) with a GenePulser (Bio-Rad) according to the manufacturer's protocol. Antibiotic concentrations (μg/ml) for *N. meningitidis* were: kanamycin 80, chloramphenicol, 5 (Neil & Apicella, 2009), tetracycline, 5, spectinomycin, 60 and erythromycin 3; and for *E. coli* strains were: ampicillin 100, kanamycin 50, chloramphenicol 34, spectinomycin 100, and erythromycin 300.

The *ΔdsbD* constructs and the *dsbD* complementation plasmid

A nonpolar *aphA3* insertional mutation with a 1296-bp deletion of *dsbD* (1803 bp) was generated by overlapping PCR. A 562-bp PCR product containing the upstream and 5′ *dsbD* coding sequence (94-bp) was generated using primer pair 5Dkan-F and 5Dkan-R. Another 779-bp PCR product containing the 416-bp 3′ sequence of *dsbD* was amplified using primer pair 3Dkan-F and 3Dkan-R. The *aphA3* cassette from pUC18K (Menard *et al.*, 1993) was amplified with primers aphA3-cla-f2 and aphA3-cla-r2 and digested with SmaI. The first overlapping PCR was performed with the *aphA3* and 3′ *dsbD* fragments, and the resulting product was purified, digested with SmaI, and used for the second round of overlapping PCR with the 5′ fragment. The final PCR product with the expected size was purified and cloned into pCR2.1. Removal of the *dsbD* sequence and the presence of a correctly oriented *aphA-3* cassette in the resulting pPK101 plasmid were confirmed by sequencing analysis. To generate the meningococcal *dsbD* mutant, pPK101 was linearized with ScaI to disrupt the Amp^R gene and the digestion mixture was used to transform meningococcal strains. Alternatively, the PCR product was used directly for transformation of meningococci. Colonies were selected on BHI agar plates with kanamycin.

A *dsbD::Ω(Sp)* construct was also made. A 2745-bp DNA containing the entire *dsbD* and the flanking sequence was obtained with primers ΔdsbD-F and ΔdsbD-R and cloned into pCR2.1 by TOPO cloning method (Invitrogen). The resulting plasmid was digested with HincII to remove ~ 1.3 kb insert, gel purified and then ligated with the *Ω(Sp)* fragment released from pHP45Ω (Prentki & Krisch, 1984) with SmaI digestion. Deletion of the *dsbD* sequence and insertion of Ω in the resulting pYT382 plasmid was confirmed by sequencing analysis.

For complementation, the *dsbD* coding sequence was PCR amplified with dsbD-PacI and dsbD-PmeI primers from strain NMB. The PCR product was digested with PacI and PmeI and then cloned into pGCC4 (Mehr & Seifert, 1998) cut with the same enzymes to yield pPK102. The presence of correct insert was confirmed by PCR and sequencing analyses.

The *dsbA* mutants

dsbA1—A 2.5 Kb fragment encompassing the *dsbA1* gene was amplified using DAP275 and DAP263 primers and cloned into the HincII site of pHSG576 by blunt end ligation. The

resulting plasmid was digested with BssHII, blunt-ended and ligated with the *Ω* cassette released from pHP45Ω to yield pJKD2639. *N. meningitidis* strain NMB was transformed with pJKD2639 and Spec^R colonies selected. The correct transformants were confirmed by PCR with the chromosomal specific primer DAP418 and a *Ω* cassette specific primer DAP140.

dsbA2—The *dsbA2* locus of strain NMB was amplified by PCR using primers DAP292 and DAP201. The 3.5 kb PCR product was cloned into the HincII site of pHSG576. The resulting plasmid was cut with the unique NotI site within the *dsbA2* coding sequence, ligated with the EcoRV fragment of *tetM* obtained from pJKD2410 to produce pJKD2641. The pJKD2641 plasmid was used to transform strain NMB and Tet^R transformants screened with primers DAP285 and DAP420, which anneal outside of the cloned region. An increase of 4 Kb in PCR product size compared to that obtained from strain NMB confirmed the allelic exchange.

dsbA3—Similarly, the *dsbA3* fragment (1.2 Kb using primers DAP268 and DAP269) was cloned into the HincII site of pHSG576 and a HincII-BssHII fragment was deleted prior to the insertion of the *ermC* cassette. The Erm^R colonies were screened for correct *ermC* orientation relative to *dsbA3*, and the resulting pJKD2643 plasmid was then used for meningococcal transformation. Transformants were examined with primers DAP267 and DAP265 flanking the cloned fragment and clones with a 0.8 Kb increase in PCR product size were saved.

The *dsbD::lacZ* transcriptional fusion and β-galactosidase assays

Promoter fragments were obtained using the following primer pairs: dsbD-f2/dsbD-r1 (350-bp), dsbD-f3/dsbD-r1 (260-bp), dsbD-f4/dsbD-r1 (190-bp), dsbD-f6/dsbD-r1 (160-bp) and dsbD-f5/dsbD-r1 (131-bp). Each promoter fragment with flanking EcoRI sites was PCR amplified with chromosomal DNA of strain NMB as template using Phusion polymerase (New England Biolab). After EcoRI digestion, the fragments were purified and cloned into the EcoRI site of pYT328 (Tzeng et al., 2006). The resulting plasmids were linearized with NcoI and used to transform meningococcal strain NMB. Correct transformants were identified by colony PCR as described and named D227, D137, D67, D37, and D8, respectively. The reporters in the *misS* mutant background were generated by transformation of strain YT310, and designated similarly to those of the wild type strain with an S extension. β-Galactosidase activity was assayed by the Miller method (Miller, 1972) performed in triplicates. The reporter strains were grown in GC broth with supplements at 37°C with 200 rpm aeration to mid-log phase (OD_{600} ~ 0.5).

qRT-PCR

Cultures were grown in standard GC broth and cells were collected at mid-log phase. Two volume of RNA protect Bacteria Reagent was added into cultures and RNA was isolated using RNeasy mini kit (Qiagen) following the manufacturer's recommendations with on-column DNase digestion. PCR amplification of the RNA samples using primers confirmed no genomic DNA contamination. cDNA was obtained by reverse transcription of total RNA (1 μg) using GeneAmp RNA PCR core kit (Applied Biosystems) and reactions without the reverse transcriptase were used as a negative control. The transcription of genes of interest was measured using the SYBR green detection method (SYBR Green Supermix, BioRad) and the reaction conditions have been described previously (Tzeng et al., 2004). The 16s rRNA was used as an internal control in each experiment for normalization. The primers designed with Primer 3 software (http://fokker.wi.mit.edu/primer3/; (Rozen & Skaletsky, 2000)) were listed in Table S1 and have been confirmed to yield similar amplification efficiencies and thus were suitable for the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001)to

determine the relative transcriptional changes between the mutant strains and the wild type strain. The values of the wild type strain were used as the calibrator for comparison. RT negative control reactions were also analyzed to measure whether there was contaminating chromosomal DNA and melting curve analyses were performed following each qRT-PCR experiment to ensure that each reaction contained only a single specific product. Each qRT-PCR was performed in triplicates. Student's *t* test with a two-tailed hypothesis was used to determine the significant difference ($p<0.01$) between two variables in this study.

Electrophoretic mobility shift assay (EMSA) and DNase I protection assay

Both EMSA and DNase I protection assays were performed following previously reported procedures (Tzeng et al., 2006). The promoter fragments used in EMSA were obtained by PCR amplification and end-labeled using T4 kinase, while the probes for DNase I protection assay was prepared by PCR using ^{32}P-labeled primers, dsbD-pf2 and dsbD-pr1. Competition with excess specific (unlabeled probes) and nonspecific competitors, a 237-bp internal coding sequence of *misS* obtained by PCR amplification using primers YT150 and YT141 was performed to assess the specificity of the interaction.

Primer extension

Total RNA (30 μg) was used in primer extension reactions with ^{32}P-labeled primers (YT144 and dsbD-PE1) following previously described procedure (Sannigrahi et al., 2009). The corresponding DNA sequencing reactions were carried out using the same labeled oligonucleotides and PCR fragments of the promoter regions with Thermo Sequenase dye primer manual cycle sequencing kit (USB). The extension products and the sequencing ladders were resolved on an 8% sequencing gel.

Chromosomal DNA isolation and Southern blots

Meningococcal chromosomal DNA was prepared according to the method of Nath (Nath, 1990). The PCR DIG probe synthesis and detection kit (Roche) was used to perform DNA hybridization. The digoxigenin labeled probe for detecting the deleted *dsbD* sequence was generated by with dsbD-For and dsbD-Rev, while the probe for the remaining *dsbD* sequence was made with 3Dkan-F and dsbD-PmeI. Chromosomal DNA was digested with SspI overnight and resolved on a 0.8 % agarose gel. DNA was transferred to a nylon membrane and hybridization and development of the blots were performed following the manufacturer's protocol.

Heme staining

The previously described procedure (Feissner et al., 2003, Feissner *et al.*, 2005) was followed. Plate grown cultures was collected and suspended in PBS. One ml of cell suspensions at an OD_{600} of 1.5 was pelleted and the supernatant removed. Pellets frozen at −80°C for a minimum of 15 min to aid in cell disruption were thawed on ice and resuspended in 100 μl of B-PER extraction reagent (Pierce) by pipetting and vortexing. The supernatant obtained after centrifugation at 15,000 × g for 5 minutes was saved and protein concentration determined by BCA assay using BSA as a standard (Pierce). Equal amounts of proteins were mixed with DTT-free loading buffer and loaded directly without heat denaturing. Samples were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were washed twice in PBS and SuperSignal West Pico chemiluminescent substrates (Pierce) prepared according to manufacturer's instruction were applied to the membranes for 5 minutes, followed by exposure to X-ray film.

Transformation efficiency

Plate-grown meningococcal strains were suspended in GC broth supplemented with 5 mM $MgCl_2$. One μg chromosomal DNA was added to aliquots (100 μl) of cell suspension at an OD_{550} of 0.1 and then incubated for 1 hr at 37°C. Pre-warmed GC broth with complete supplements (500 μl) and DNase I (2 units) was added and the incubation continued for another hour. Serial dilutions were plated onto both selective and non-selective plates and the colony forming units (cfu) determined after overnight growth. The efficiencies were calculated as the ratio of cfu from the selection plate to the cfu of non-selective plates. The transforming DNA was chromosomal DNA prepared from strain NMB carrying a chloramphenicol cassette at the *aspC-lctP* locus.

Western blot

The meningococcal strains were grown on GC plates overnight and resuspended in PBS. Whole cell lysates were prepared by sonication and equal amounts of proteins (8 μg) were resolved by 10% SDS-PAGE, and transferred to nitrocellulose membranes. The antisera against PilQ (Tonjum *et al.*, 1998) and Opa (Takahashi *et al.*, 2008) were used at 1:2000 dilutions and anti-rabbit IgG-HRP conjugate secondary antibody was used at 1:15,000 dilution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants R01 AI061031 to Y. T. from the National Institutes of Health. We thank Dr. Sarah Satola for providing Southern hybridization reagents. We are grateful to Dr. H. Takahashi for the generous gift of Opa and PilE antisera and to Dr. Ashild Vik for the PilQ antisera.

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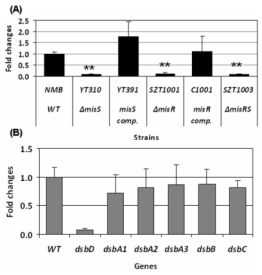

Figure 1.

(A) qRT-PCR determinations of relative transcriptional changes of *dsbD* in the *misR/S* mutants and complemented mutants. The relative changes in transcriptional level were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001)with the wild type strain as the calibrator. The expression level of the wild type strain was set as 1 for normalizing the relative transcription levels of mutants and complemented mutants. **(B)** qRT-PCR analyses of all *dsb* genes comparing the wild type strain and the *misS* mutant. The expression of individual *dsb* genes in the wild type is set as 1. Each qRT-PCR was examined in triplicate and was repeated with at least four independent RNA preparations. The asterisks indicate statistically significant difference between the wild type strain and the mutant as determined by the Student's t test with a 2-tailed distribution ($P < 0.01$).

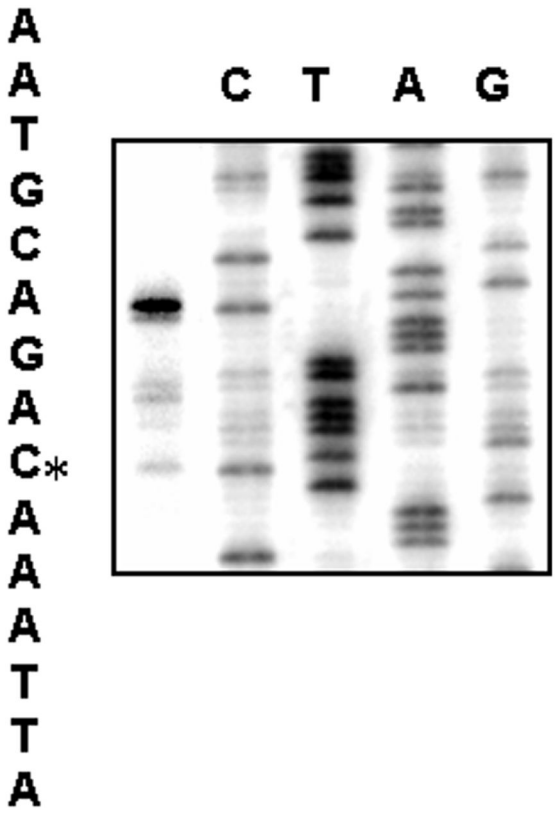

Figure 2.
Primer extension of *dsbD*. The YT144 primer was labeled and used to generate the sequencing ladder. Lanes C, T, A, G indicate the dideoxy sequencing reactions. The asterisk indicates the transcriptional start site.

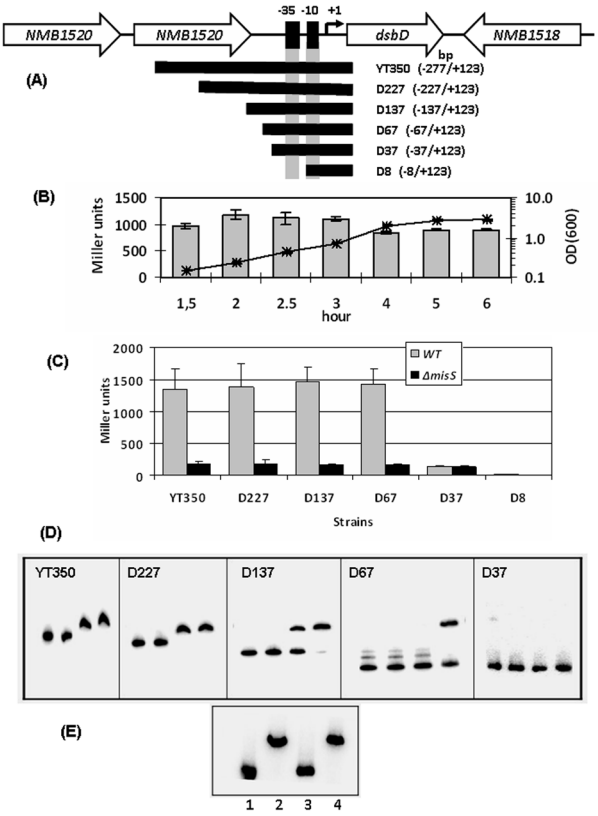

Figure 3.

(A) Schematic of the *dsbD* locus. The cloned region with respect to the transcriptional start site (+1) is shown in parentheses. The schematic is not to scale. **(B)** The expression of *dsbD* was not growth phase dependent. The β-galactosidase activity of strain YT350 were monitored over the growth curve, which is shown as a line graph with a semi-log scale on the secondary axis. Triplicate measurements were performed for each time points and the average of two independent experiments is shown. Error bars indicate the standard deviation. **(C)** Activities of various *dsbD::lacZ* transcriptional fusions. β-Galactosidase activities of strains grown to mid-log phase are expressed as mean values ± standard deviations of at least four independent assays done in triplicate measurements. **(D)** The interaction of MisR with the *dsbD* promoter examined by EMSA. The PCR products of various *dsbD* promoter fragments were ^{32}P-labeled by T4 kinase, mixed with increasing amounts of MisR for 20 minutes at 30°C and then subjected to gel electrophoresis. In each panel, the left lane contains DNA probe only and the remaining lanes contain 10, 20 and 40 pmol of MisR, respectively. **(D)** Competition EMSA. The ^{32}P-labeled D227 *dsbD* probe (lane 1) was mixed with 40 pmol of MisR alone (lane 2) or with either specific (1 μg, lane 3) or nonspecific DNA (1 μg, lane 4), and analyzed as described above.

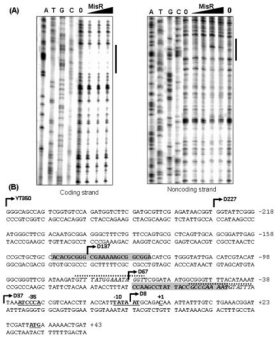

Figure 4.

MisR binding sites located upstream of the *dsbD* promoter. **(A)** DNase I protection. The coding (left) or noncoding (right) strand of ^{32}P-end labeled *dsbD* promoter fragment between −227 to +123 with respect to the *dsbD* transcriptional start site was incubated with increasing amounts of MisR for 20 minutes at 30°C and then subjected to DNase I digestion. The amounts of MisR used in the left panel are 0, 40, 80, and 120 pmol, while in the right panel are 0, 40, 80, 120, 200 and 0 pmol. The dideoxy chain-termination sequencing ladders were generated by extension of ^{32}p-labeled pF2 primer for the coding strand or pR1 primer for the noncoding strand using the corresponding PCR product as template. Black bars indicate protected regions. **(B)** The *dsbD* promoter sequence. The protected regions are indicated as shaded boxes in both strands. The transcriptional start site +1, −10 and −35 promoter elements are bolded and underlined. The predicted MisR binding motifs are italicized and marked with dashed lines above the sequence. Bent arrows indicate the 5′ ends of various promoter fragments cloned in reporter strains.

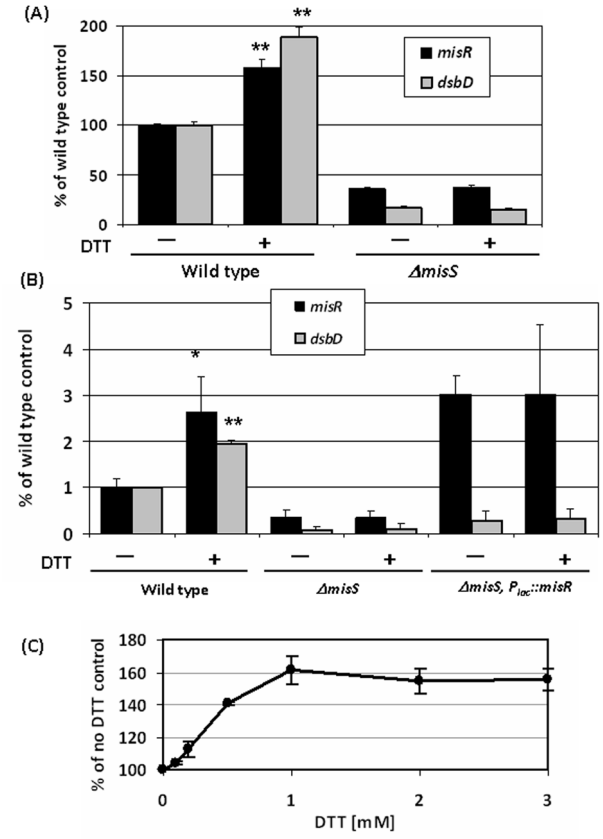

Figure 5.

Induction of *dsbD* and *misR* transcription by DTT. **(A)** The YT322 (*misR*) and YT350 (*dsbD*) reporter strains in the wild type and *ΔmisS* backgrounds were grown to mid-log phase and the cultures were divided into two: one treated with 1 mM DTT for 30 minutes and the other serves as non-induced control. The activities were normalized to those of the wild type backgrounds without DTT treatment, which were set as 100%. The data represent the means and standard deviation of at least four and two independent assays for the wild type and the *misS* backgrounds, respectively. The asterisks indicate statistically significant difference between the induced and non-induced samples as determined by the Student's t test with a 2-tailed distribution (**, $p < 0.001$). **(B)** qRT-PCR analysis. The wild type strain NMB, the *misS* mutant YT310 and the PKY392 strain (YT310, P_{lac}*::misR*) induced with 1 mM IPTG were treated with or without 1mM DTT for 30 minutes and total RNA isolated. The data are the mean and standard deviations of RT-PCR experiments done in triplicate using at least two independent RNA preparations. (*, $p < 0.01$; **, $p < 0.001$) **(C)** Dose-dependent induction by DTT. The YT322 strain was treated with various DTT concentrations for 30 minutes prior to activity assay. The sample without DTT treatment was set as 100%. The data are the means and standard deviations of two independent experiments done in triplicate.

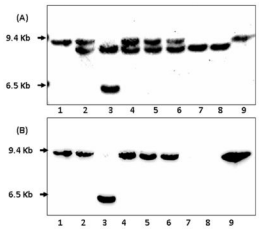

Figure 6.

Characterization of *dsbD* mutants by Southern blots. SspI-digested chromosomal DNAs were resolved by 0.8% agarose gel. Lanes: 1) wild type strain NMB, 2) the incorrect mutant (*dsbD**) obtained from the wild type strain, 3) the correct *dsbD* mutant created in the complemented strain, 4) *dsbD** mutant of the *dsbA1* strain, 5) *dsbD** mutant of the *dsbA2* strain, 6) *dsbD** mutant of the *dsbA2A3* strain, 7) *dsbD* mutant of the *dsbA2A1* strain, 8) *dsbD* mutant of the *dsbA2A1A3* strain. Removal of an 1.3-kb *dsbD* coding sequence and insertion of the 800-bp *aphA3* cassette gave rise to a smaller fragment that is recognized by the probe made of the remaining 3′ *dsbD* sequence (A), but not by the probe hybridized to the deleted region (B). The *dsbD** designation indicates incorrect *dsbD* clones that also carry a wild type copy of *dsbD.* An additional band in lane 3 of both panels represents the second copy of *dsbD* at the *aspC-lctP* locus.

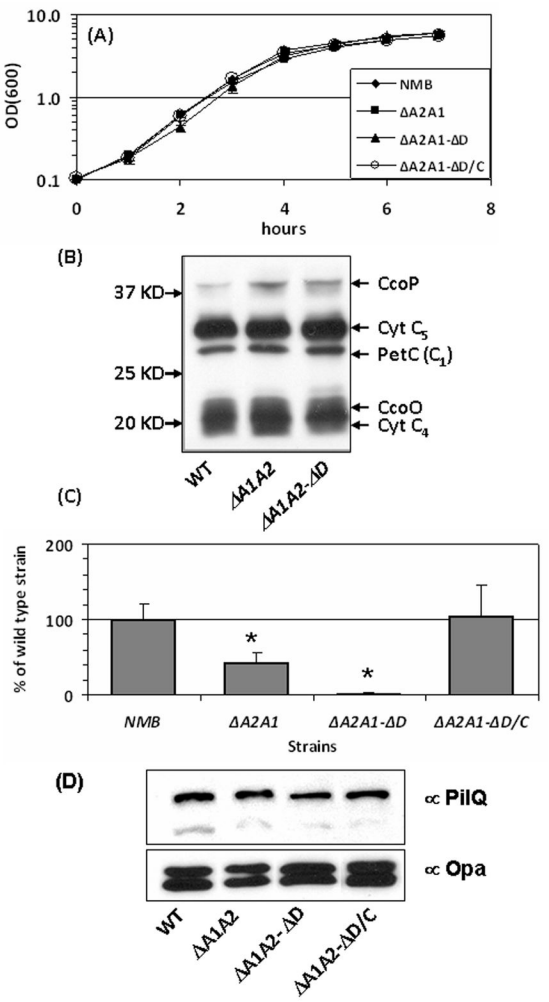

Figure 7.

(A) Growth curves of the meningococcal strains in standard GC broth. **(B)** Heme staining of the wild type, *dsbA2A1* and *dsbA2A1D* strains. The assignment of each band is based on the previous report (Li et al., 2010). **(C)** Natural competence of the wild-type NMB and its mutants. Bacteria were transformed with chromosomal DNA from a Cm^R derivative of NMB, and cfu counts were determined on selective and non-selective plates. Transformation frequencies were calculated by dividing the cfu numbers of selective plates by the cfu numbers of non-selective plates. The frequency of the mutant was normalized to that of the wild type, which was set as 100%. Data are the means ± standard deviations of two assays done in duplicate. **(D)** PilQ levels determined by Western blots. Equal amounts of whole cell lysates (8 μg) were resolved on 10% SDS-PAGE gels and transferred to PVDF membranes. The top half of the membrane was probed with PilQ antisera (Tonjum et al., 1998), while the lower half was probed with Opa antisera (Takahashi et al., 2008) for loading controls.

Table 1

Strains and plasmids used in this study

strain	genotype	reference
N. meningitidis		
NMB	B:2b:P1.2, 5:L2(CDC8201085)	(Stephens *et al.*, 1991)
YT310	NMB/*misS::aphA-3*	(Tzeng et al., 2006)
YT391	NMB310 complemented with P_{lac}*::misS* at the *lctP- aspC* locus	(Sannigrahi et al., 2009)
SZT1001/YT0336	NMB/*misR::aphA-3*	(Tzeng et al., 2008)
C1001	NMB/*misR::aphA-3* complemented with P_{trc}*::misR* in the *iga* locus	(Tzeng et al., 2008)
SZT1003	NMB/*misRS::aphA-3*	(Tzeng et al., 2008)
PKT101	*dsbD::aphA3*	This study
PKT102	*dsbD::aphA3*, P_{lac}*::dsbD*	This study
PKT103	*dsbA1::Ω(Sp)*	This study
PKT104	*dsbA1::Ω(Sp), dsbD::aphA3*	This study
PKT105	*dsbA2::tetM*	This study
PKT106	*dsbA2:: tetM, dsbD::aphA3*	This study
PKT107	*dsbA1::Ω(Sp), dsbA2:: tetM*	This study
PKT108	*dsbA1::Ω(Sp), dsbA2:: tetM, dsbD::aphA3*	This study
PKT109	*dsbA2:: tetM, dsbA3::ermC*	This study
PKT110	*dsbA2:: tetM, dsbA3:: ermC, dsbD::aphA3*	This study
PKT111	*dsbA1::Ω(Sp), dsbA2:: tetM, dsbA3:: ermC*	This study
PKT112	*dsbA1::Ω(Sp), dsbA2:: tetM, dsbA3:: ermC, dsbD::aphA3*	This study
PKT113	*dsbA1::Ω(Sp), dsbA2:: tetM, dsbD::aphA3,* P_{lac}*::dsbD*	This study
PKT392	P_{lac}*::misR* in YT310	This study
YT350	*dsbD::lacZ* (−277/+123)	(Tzeng et al., 2008)
YT350s	*dsbD* (−277/+123)*::lacZ* in YT310	(Tzeng et al., 2008)
YT322	*misR::lacZ* in NMB	(Tzeng et al., 2006)
YT322s	*misR::lacZ* in YT310	(Tzeng et al., 2006)
D227	*dsbD::lacZ* (−227/+123)*::lacZ*	This study
D227s	*dsbD* (−227/+123)*::lacZ* in YT310	This study
D137	*dsbD* (−137/+123)*::lacZ*	This study
D137s	*dsbD* (−137/+123)*::lacZ* in YT310	This study
D67	*dsbD* (−67/+123)*::lacZ*	This study
D67s	*dsbD* (−67/+123)*::lacZ* in YT310	This study
D37	*dsbD* (−37/+123)*::lacZ*	This study
D37s	*dsbD* (−37/+123)*::lacZ* in YT310	This study
D8	*dsbD* (−8/+123)*::lacZ*	This study
E. coli		
DH5α	Cloning strain	New England Biolab

strain	genotype	reference
TOP10	Cloning strain	Invitrogen
Plasmid		
pUC18k	Source of *aphA-3* cassette	(Menard et al., 1993)
pHP45Ω	Source of Ω(Sp) cassette	(Prentki & Krisch, 1984)
pCR2.1	TOPO cloning vector	Invitrogen
pSmartLCKan	Blunt-end cloning vector	Lucigen
pYT328	Cloning vector for chromosomal *lacZ* fusion	(Tzeng et al., 2006)
pGCC4	Complementation vector for integration into *lctP- aspC* locus	(Mehr & Seifert, 1998)
pJKD2639	*dsbA1::Ω(Sp)*	This study
pJKD2641	*dsbA2::tetM*	This study
pJKD2643	*dsbA3:: ermC*	This study
pPK101	*dsbD::aphA3*	This study
pPK102	P_{lac}*::dsbD* in pGCC4	This study
pYT382	*dsbD::Ω(Sp)*	This study
pYT392	P_{lac}*::misR* in pGCC4	This study

Table 2

The *dsbA* and *dsbD* mutations increased sensitivity to oxidative stress

Strain	Genotype	Zone of growth inhibition (mm) ± SD[a] with:		
		H_2O_2 (1%)	Paraquat (100 mM)	Copper (100 mM)
NMB	wild type	35.2 ± 1.1	30.0 ± 1.4	20.4 ± 0.9
PKT107	*dsbA1⁻dsbA2⁻*	36.6 ± 1.3	34.0 ± 2.5*	31.2 ± 1.1**
PKT108	*dsbA1⁻dsbA2⁻dsbD⁻*	42.8 ± 1.1**(**)	39.2 ± 3.0**(*)	36.0 ± 0.0**(**)
PKT113	*dsbA1⁻dsbA2⁻dsbD⁻; P_{lac}::dsbD*	37.2 ± 1.79	34.0 ± 2.5*	30.4 ± 0.9**

[a]Data are the means ± standard deviations growth inhibition zones around at least four 6-mm filter discs obtained with the indicated concentration of reagents after overnight incubation. A Student's t test showed significant differences when compared to the wild type strain (*, $p < 0.05$; **, $p < 0.01$), while the statistically significant differences when compared to the *dsbA1A2* strain are shown in parentheses.