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EFFECTS ON BACTERIAL GROWTH AND BIOGENESIS OF FUNCTIONAL TYPE IV PILI*⑤

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Many proteins, especially membrane and exported proteins, are stabilized by intramolecular disulfide bridges between cysteine residues without which they fail to attain their native functional conformation. The formation of these bonds is catalyzed in Gram-negative bacteria by enzymes of the Dsb system. Thus, the activity of DsbA has been shown to be necessary for many phenotypes dependent on exported proteins, including adhesion, invasion, and intracellular survival of various pathogens. The Dsb system in *Neisseria meningitidis*, the causative agent of cerebrospinal meningitis, has not, however, been studied. In a previous work where genes specific to *N. meningitidis* and not present in the other pathogenic *Neisseria* were isolated, a meningococcus-specific *dsbA* gene was brought to light (Tinsley, C. R., and Nassif, X. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 11109–11114). Inactivation of this gene, however, did not result in deficits in the phenotypes commonly associated with DsbA. A search of available genome data revealed that the meningococcus contains three *dsbA* genes encoding proteins with different predicted subcellular locations, *i.e.* a soluble periplasmic enzyme and two membrane-bound lipoproteins. Cell fractionation experiments confirmed the localization in the inner membrane of the latter two, which include the previously identified meningococcus-specific enzyme. Mutational analysis demonstrated that the deletion of any single enzyme was compensated by the action of the remaining two on bacterial growth, whereas the triple mutant was unable to grow at 37 °C. Remarkably, however, the combined absence of the two membrane-bound enzymes led to a phenotype of sensitivity to reducing agents and loss of functionality of the pili. Although in many species a single periplasmic DsbA is sufficient for the correct folding of various proteins, in the meningococcus a membrane-associated DsbA is required for a wild type DsbA+ phenotype even in the presence of a functional periplasmic DsbA.

Many proteins, especially membrane and exported proteins, are stabilized by intramolecular disulfide bridges between cysteine residues without which they are misfolded, unstable, and often inactive. The correct formation of disulfide linkages on export into the periplasmic space of Gram-negative bacteria is catalyzed by a group of functionally related enzymes called disulfide oxidoreductases (Dsb). The first of these enzymes, DsbA, discovered in a screen for mutants affecting protein export (1), donates its disulfide bond to cysteine-containing proteins, thus stabilizing their mature, correctly folded forms. Subsequent studies have shown that DsbA is part of an extensive system for the catalysis of disulfide bond formation and isomerization in exported proteins (reviewed in Ref. 2).

The Dsb system studied in *Escherichia coli* consists of two separate, non-interacting branches acting in the periplasm. The enzyme DsbC shows disulfide isomerase activity and is involved in the correct folding of proteins containing multiple disulfide bonds (3, 4), thus also demonstrating chaperone activity (5). In contrast, DsbA is thought to be purely oxidative *in vivo*. Though the oxidation of disulfide bonds will occur naturally by the action of dissolved oxygen, an absence of catalysis in DsbA mutants, although not lethal under normal conditions, has considerable effects. Among these effects are a buildup of reduced forms of normally disulfide bond-containing proteins that in some cases decrease enzymatic activity and stability to proteolysis in the periplasm (1) and phenotypes such as the loss of motility due to the incorrect assembly of the flagellar apparatus (6).

Because secreted proteins will be the first to come into contact with the environment, they are of particular importance in pathogenic species for bacteria-host interactions. A major meningococcal virulence attribute is the type IV pilus, which allows the bacteria to interact with the host cells. The pili are filamentous appendages assembled from a protein subunit, pilin, containing an internal disulfide bridge. DsbA enzymes in other pathogenic bacteria have been shown to be necessary for the expression of virulence factors. They are necessary, for example, for pilus-mediated adhesion in enteropathogenic *E. coli* (7) and *Vibrio cholerae* (8) and for the correct folding of proteins responsible for the secretion of invasion proteins by *Yersinia pestis* (9) and the intracellular survival of *Shigella flexneri* (10).

In a previous work that used representational difference analysis to search for genes specific to *Neisseria meningitidis*, the causative agent of cerebrospinal meningitis, a meningococcus-specific DsbA homologue was brought to light. Its gene was part of a genetic island that was absent from the other pathogenic *Neisseria* species, *Neisseria gonorrhoeae* (11, 12). In this work we demonstrate that this meningococcus-specific *dsbA* homologue encodes an enzyme with a disulfide oxidoreductase

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activity. A search of available genome data revealed that the meningococcus contains, in addition to the meningococcus-specific *dsbA*, two other homologues of these genes that are also present in *N. gonorrhoeae*. Two of the DsbA homologues, including the meningococcus-specific enzyme, are apparently inner membrane lipoproteins. In contrast to the case with *E. coli*, the periplasmic enzyme in the meningococcus is not by itself sufficient to confer a wild type DsbA+ phenotype as judged by the growth in reducing conditions and the elaboration of functional type IV pili. We show that the presence of at least one of the membrane-associated DsbA enzymes is required for a wild type DsbA+ phenotype and for pilus-mediated adhesion to human cells.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions—The strain of *N. meningitidis* used was the 8013 variant 2C43 (13). Bacteria were grown on GC agar (GCB; Difco) with the addition of Kellogg's defined supplement (14) for 12–20 h at 37 °C in a moist atmosphere containing 5% CO_2. Liquid media were GC-PO_4 (1.5% proteose peptone number 3 (Difco), 0.5% NaCl, and 30 mM potassium phosphate, pH 7.5) and, for assays of transformation, GC-Hepes (30 mM potassium phosphate replaced by 30 mM Hepes, pH 7.5, plus 1 mM potassium phosphate); both media were supplemented as for the solid medium. *E. coli* were grown on Luria-Bertani agar or in Luria-Bertani liquid medium. The antibiotics used were ampicillin (50 μg/ml), chloramphenicol (5 μg/ml), erythromycin (2.5 μg/ml), kanamycin (60 μg/ml), nalidixic acid (25 μg/ml), and spectinomycin (50 μg/ml).

Molecular Biological Techniques—Standard molecular biological techniques were performed as suggested (15, 16) or as described previously (12). Protein concentrations were determined using the bicinchoninic acid assay system (Pierce).

Transformation of Neisseria meningitidis—Mutants were engineered in *N. meningitidis* by transformation with cloned DNA fragments, which leads to homologous recombination and replacement of the wild type gene. Where the DNA fragment was marked with a cassette encoding resistance to an antibiotic, transformation was carried out essentially as described (17), and transformants were selected by growth in the presence of the appropriate antibiotic. If transformation was to be performed without the use of antibiotic selection, the method was that of Gunn and Stein (18), and colonies were tested individually by PCR and confirmed by sequencing. All mutations were verified by PCR, Southern blot, and, where necessary, sequencing.

For quantitative transformation assays, volumes (500 μl) of bacterial suspension with an optical density at 600 nm of 0.1 in GC-Hepes containing 10 mM $MgCl_2$ were incubated with 1 μg of chromosomal DNA carrying a selectable marker for 30 min at 37 °C before the addition of 40 μg of DNase I. The transformations were diluted 10-fold and allowed to grow with agitation for a further 2 h. Serial dilutions were plated onto normal and selective media, and the ratio of the cells that had incorporated the marker was calculated. Strains were tested for the acquisition of resistance to both nalidixic acid (a spontaneous mutant presumably in the DNA gyrase gene) and chloramphenicol (chloramphenicol acetyl transferase gene, GenBank™ accession number AF031037, replacing bases 600 to 3000 of the gene *hap* (adhesion and penetration protein, NMB1985), GenBank™ accession number NC_003112.1, gene identifier 15675948).

Inactivation of the dsbA Genes—Oligonucleotides were designed to amplify DNA fragments from the chromosome of *N. meningitidis* 8013–2C43 extending ~1-kb pair on either side of the first cysteine codon in each of the three *dsbA* genes. Oligonucleotides were designed such that a ligation of the two fragments creates a restriction endonuclease recognition site in place of the DNA sequence coding for the predicted signal peptidase recognition site, and the translational frame of the downstream part of the gene, which would code for the mature protein in the wild type, is shifted by one base. Oligonucleotides used to amplify the genes were as follows: dsbA1a, 5′-GAACAT**GGATCC**CGTCCACA-CACTTTACG-3′; dsbA1b, 5′-GCGGCC**GAATTC**CAACAGGGTCAAT-GAAGT-3′; dsbA1c, 5′-CTGTTG**GAATTC**GGCCGCTTGTAGCAAACA-GGCT-3′; dsbA1d, 5′-TAGTAC**GGTACC**GATTCACTTGGTGCTT-3′; dsbA2a, 5′-TTGTCG**AGATCT**TTGGCAAAACCGGGTGCGGA-3′; dsb-A2b, 5′-CGGCAAGTGCG**TGATCA**GGCGGCAACGCCGAGGGCGA-3′; dsbA2c, 5′-GCGTTGCCGCC**TGATCA**CGCACTTGCCGCGTGCGACA-3′; dsbA2d, 5′-TTACCTG**CGGCCG**CGTATCGCGCCGTTAT-3′; dsbA-3a, 5′-AGCAGCC**TCTAGA**AACGGAAATCCTGAAACCGAA-3′; dsbA-3b, 5′-AGGG**CATATG**CCTGCAGGCGGACAACACTGCCGACAGCA-

3′; dsbA3c, 5′-GCAGTGTTGTCCGCCTGCAGG**CATATG**CCCTGACG-GAA-3′; dsbA3d, 5′-AAATTC**GGTACC**TTCTACCAAGCCCATCATCA-3′. Boldface characters represent the restriction endonuclease recognition sites artificially introduced into the oligonucleotide sequence. Two fragments were created by PCR amplification for each gene using primer pairs "a" plus "b" and "c" plus "d" and then joined by PCR ligation. The resulting PCR products were digested with the restriction enzymes corresponding to the sites introduced near the 5′ ends of oligonucleotides "a" and "d" and cloned into pBluescriptII KS(−) (Stratagene). Where the gene was to be interrupted with a resistance cassette, the plasmid containing the cloned insert was cleaved with the enzyme corresponding to the restriction enzyme recognition site introduced at the junction between the two primary PCR products. An antibiotic-resistance cassette prepared from the plasmids pT1Omega1, pT1K1, and pT1Cm1 (12),[1] having suitable cohesive ends and flanked by the neisserial uptake sequences (17) necessary for transformation in the meningococcus, was ligated into the cloned gene at the point of cleavage.

Because the meningococcus undergoes frequent phase changes in genes encoding surface structures important in pathogenesis, chromosomal DNA from the verified mutants (of *dsbA1* and *dsbA2*) was used to transform wild type bacteria to antibiotic resistance, and a pool of 50–100 transformant colonies was taken for subsequent study to achieve a statistically homogeneous population of bacteria.

The gene *dsbB* was inactivated by the cloning of the meningococcal gene from strain 8013–2C43 using oligonucleotides dsbBa (5′-CCTC-CGGCATATGTATTTTTTTGTGGGCATT-3′) and dsbBb (5′-CGGATC-CTTATTTAGCCCTTGCCCACGCCA-3′), based on the genomic sequence of strain Z2491 (the genomic sequence of strain 8013–2C43 being incomplete in this region), into plasmid pCR2.1 TOPO (Invitrogen) and then into pUC19 modified to remove all but the EcoRI site from the polylinker. The chloramphenicol resistance cassette from plasmid pT1Cm1 (as above) was inserted into the PstI site at position 378 of the gene, and the plasmid was used to transform *N. meningitidis* to chloramphenicol resistance.

Cloning of the Meningococcus-specific dsbA Gene (dsbA1) and Production of Recombinant DsbA—The portion of the gene coding for the predicted mature protein from *N. meningitidis* was amplified using oligonucleotides dsbA1e (5′-GCTTGTGGTAC**CATATG**AGCAAACAG-GCTGAAACCAGT-3′) and dsbA1f (5′-TCAATC**CTCGAG**TTGCG-GCTTTTTCTGCTCTT-3′) and cloned into the expression vector pET20b(+) (Novagen, R&D Systems) between the restriction endonuclease sites NdeI and XhoI (boldface in the oligonucleotide sequence). The recombinant gene does not encode a lipoprotein signal sequence but starts with a codon for methionine, followed by the natural gene sequence. An XhoI site, replacing the natural stop codon, allows an in-frame link to the expression vector's hexahistidine-encoding sequence. The resulting plasmid (pDsbA1) was propagated in *E. coli* BL21(DE3)

Bacteria were grown in liquid culture in Luria-Bertani medium to an OD_{600} of 0.1. Production of the protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM and, after a further 2 h, the bacteria were harvested and disrupted by sonication. The soluble fraction was passed through a column made from 1 ml of "poly His protein purification resin" (Roche Applied Science). Unbound protein was eluted with 10 ml of phosphate-buffered saline containing 10 mM imidazole. The recombinant DsbA1 protein was eluted in phosphate-buffered saline and 50 mM imidazole. Fractions containing pure recombinant DsbA1 (as evaluated by SDS-PAGE analysis) were pooled, dialyzed against phosphate-buffered saline, and stored at −80 °C. About 20 mg of protein was obtained from 200 ml of culture.

Immunization with Recombinant DsbA for the Production of Antisera—Rabbits were immunized four times at intervals of 15 days with the recombinant C-terminal His-tagged DsbA1 (100 μg of antigen in Freund's complete adjuvant for the first immunization and in Freund's incomplete adjuvant for subsequent immunizations). Immune serum was taken by cardiac puncture 12 days after the last immunization. Monoclonal antibodies were prepared after the immunization of mice with the same antigen and were provided by GlaxoSmithKline, Rixensaart, Belgium.

Purification of the Inner and Outer Membranes of N. meningitidis—Separation and analysis of the membrane fractions was performed as described (19). Briefly, bacteria were harvested by centrifugation, and pellets were washed three times with phosphate-buffered saline. Pellets were resuspended in 50 mM Tris-HCl, pH 8, and 50 μg/ml RNase (Roche

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[1] C. R. Tinsley, unpublished constructions.

Applied Science) and DNase (Roche Applied Science) were added. Bacteria were passed twice through a French pressure cell at 15,000 p.s.i. Unbroken cells were removed by centrifugation, and the supernatant was loaded onto a discontinuous sucrose gradient consisting of a 6-ml, 55% (w/w) sucrose cushion and a 9-ml, 15% sucrose top layer, both in 3 mM EDTA pH 8. After centrifugation for 2 h at 50,000 r.p.m. in a Beckman Ti60 rotor at 4 °C, the crude membrane fraction was collected from the top of the cushion with a J-shaped Pasteur pipette. The sucrose concentration of this crude membrane fraction was lowered to 30% sucrose with 3 mM EDTA, pH 8, followed by separation on a second discontinuous sucrose gradient consisting of 3-ml layers of 45, 40, and 35% sucrose on top of a 2-ml 50% sucrose cushion. The gradient was centrifuged for 36 h at 33,200 r.p.m. in an SW41 Beckman rotor at 4 °C, and 1-ml fractions were collected from the top to the bottom of the gradient and stored at −20 °C. Lactate dehydrogenase activity in the fractions was measured as described (20).

Assay for Disulfide Oxidoreductase Activity—Assays of the disulfide oxidoreductase activity of purified proteins were performed essentially as described (1). Bovine insulin was dissolved in Tris/HCl to a concentration of 10 mg/ml (1.67 mM) and adjusted to pH 8 as described (21). Reaction mixtures (0.8 ml) contained 150 μM insulin in 100 mM potassium phosphate, pH 7, and 2 mM EDTA containing 0.33 mM dithiothreitol.

Inhibition of Lipidation by Globomycin—Meningococci were inoculated at an OD_{600} of 0.1 into GC-PO_4 medium and allowed to grow for 2 h at 37 °C in the presence of various concentrations of globomycin. The cells were sedimented by centrifugation and then resuspended in 0.1% SDS. Samples containing equal quantities of protein were subjected to SDS-PAGE and Western blotting using an anti-DsbA1 monoclonal antibody. Globomycin was the kind gift of Dr. Y. Akiyama of the Sanko Chemical Corporation.

Assay for Meningococcal Adhesion to Human Endothelial Cells—Techniques were as described (22). Bacteria grown in RPMI medium (Invitrogen) containing 10% fetal calf serum were diluted to give $\sim10^6$ bacteria per milliliter, and 1 ml of the suspension was added to confluent monolayers of human umbilical vein endothelial cells growing in tissue culture wells of ~2 cm^2. After 1 h, the suspension was removed for the counting of colony forming units. At this time and every hour thereafter, the cells were washed to remove unbound bacteria. After 3 h, the human cell membranes were solubilized with saponin. Cell-associated bacteria were counted and compared with the bacteria present at 1 h.

Two-dimensional Gel Electrophoresis and Western Blotting of Pilin—Two-dimensional electrophoresis was performed according to the manufacturer's (Bio-Rad) recommendations using a protean isoelectric focusing cell for the first dimension and a protean II electrophoresis cell for the second dimension. Bacteria from agar plates were suspended in sample buffer (8 M urea, 2 M thiourea, 4% CHAPS[2] detergent, and 100 mM dithiothreitol containing 0.5% ampholyte, pH 3–10), left at room temperature for 30 min, and then centrifuged to remove undissolved particles. The supernatant was applied to the "ReadyStrip-immobilized pH gradient strips" and left overnight to rehydrate the strips. First dimension electrophoresis was performed at 150 V/cm for 1 h. The strips were re-equilibrated by soaking for 20 min, first in 6 M urea, 50 mM Tris-HCl, pH 8.8, 20% glycerol, and 2% SDS containing 100 mM dithiothreitol and then in the same buffer containing 65% iodoacetamide. They were then subjected to SDS-PAGE on 13% polyacrylamide gels. Proteins separated in the gels were transferred to nitrocellulose sheets, and the migration of the pilin was revealed after reaction with a monoclonal antibody developed against meningococcal pilin.

RESULTS

The Meningococcus-specific dsbA Homologue Encodes a Lipoprotein with a Disulfide Oxidoreductase Activity—A previous work that used representational difference analysis (11, 12) to search for genes specific to *N. meningitidis*, the causative agent of cerebrospinal meningitis, brought to light a gene encoding a meningococcus-specific homologue of the periplasmic disulfide oxidoreductase DsbA. In this work the gene was seen to be conserved and intact in a variety of strains. The predicted amino acid sequence shows a 19-amino acid hydrophobic lipoprotein signal sequence (23) that would be cleaved by the
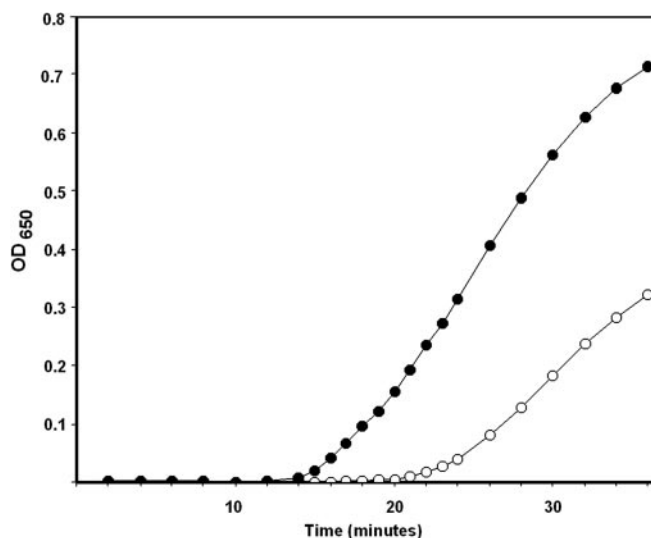
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[2] The abbreviation used is: CHAPS, 3-[3-(cholamidopropyl)dimethyamino]-1-propanesulfonic acid.

FIG. 1. **Assay for the disulfide oxidoreductase activity of the meningococcus-specific DsbA homologue.** Precipitation of insulin was measured after the addition of the protein catalysts thioredoxin (1 μM; *filled circles*) or recombinant meningococcal DsbA homologue (5 μM; *empty circles*). The absorbance due to background precipitation of insulin by non-enzymatic reduction, which is measured in control tubes containing dithiothreitol but no added protein catalyst, is subtracted. Lag times varied between 10 and 15 min for thioredoxin and between 20 and 30 min for recombinant meningococcal DsbA. A typical experiment is shown.

lipoprotein signal peptidase to leave a mature protein in which the N-terminal cysteine is modified by lipidation. The two other cysteines in this protein are part of the "CXXC" motif (amino acids 75 to 79) typical of thioredoxin and disulfide oxidoreductases (24). Although DsbA homologues have been implicated in the virulence of some other pathogenic bacteria, nothing is known concerning the system of disulfide oxidoreductases in the meningococcus.

To demonstrate that this meningococcal *dsbA* gene codes for a disulfide oxidoreductase, the gene was cloned from *N. meningitidis* strain 8013–2C43 and expressed in *E. coli* as a C-terminal hexahistidine-tagged protein. The purified recombinant protein was assayed for its ability to catalyze the reduction by dithiothreitol of insulin disulfide bonds, thus causing the protein to become insoluble (21). In this assay, the meningococcus-specific DsbA protein, caused an increase in the OD_{650} of 0.003 ± 0.0008 ($n = 3$) per minute per micromolar protein (Fig. 1), 7% of the value obtained with thioredoxin and similar to the activity obtained in previous studies of the DsbA of *E. coli* (1). Hence, the biochemical results were in accordance with the bioinformatic data, demonstrating that the meningococcus-specific *dsbA* gene does indeed encode a disulfide oxidoreductase.

A peculiarity of the meningococcus-specific DsbA is that, according to the nucleotide sequence, the enzyme is predicted to be a lipoprotein (see first sequence in Fig. 3) in contrast to the majority of investigated DsbAs, which are periplasmic. The signal peptide, relatively short and hydrophobic, ends with the consensus LAA(S)C recognized by the lipoprotein-specific signal peptidase II (reviewed in Ref. 25). To verify that the meningococcus-specific *dsbA* was indeed a lipoprotein, bacteria were grown in the presence and absence of the lipoprotein signal peptidase II inhibitor globomycin. Proteins of strain 8013–2C43 separated by SDS-PAGE were reacted on Western blot with an antibody directed against the meningococcus-specific DsbA. At higher concentrations of the antibiotic, a band of higher molecular weight was seen corresponding to the unprocessed precursor protein (Fig. 2), thus suggesting that DsbA1 is
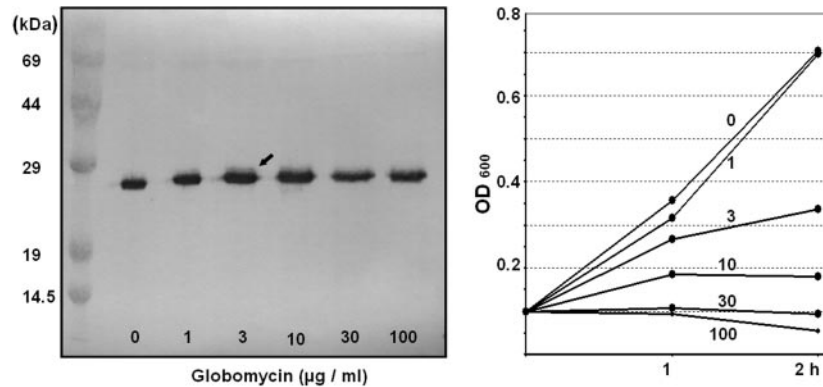
Fɪɢ. 2. **Identification of the meningococcus-specific DsbA as a lipoprotein.** Inhibition of maturation of the meningococcal DsbA by globomycin is shown. Meningococci were grown in the presence of different concentrations of globomycin. After 2 h, cells were harvested and subjected to SDS-PAGE electrophoresis and Western blotting with a monospecific monoclonal antibody directed against the meningococcus-specific DsbA. Note the appearance of a band of higher molecular mass (*arrow*) at higher concentrations of the antibiotic. Growth of the bacteria was followed at the same time (*right-hand panel*). The numbers on the graph refer to the concentrations of globomycin present in the medium, as noted on the Western blot.

a lipoprotein. In Gram-negative bacteria, lipoprotein processing occurs in three successive stages: (i) a lipidation of the cysteine that follows the signal sequence via its sulfur atom; (ii) a cleavage of the signal peptide, which is a prerequisite for the third stage; (iii) the attachment of an acyl chain to the exposed amino group. In the presence of globomycin, the difference in apparent molecular weight is due to the absence of cleavage of the signal peptide and the nonlipidation of the amino group of the cysteine.

To investigate the biological role of the meningococcus-specific *dsbA*, the gene was inactivated by insertion of an antibiotic-resistance cassette near its 5′-end. Preliminary tests were performed to investigate phenotypes associated with lack of DsbA in other bacteria. No differences were seen between the wild type and this mutant in their degree of piliation as evidenced by immunofluorescence microscopy or biochemical tests such as resistance to reducing agents. There was no apparent change in the phenotypes associated with virulence such as piliation-dependent adhesion to human cells or capsulation (measured by agglutination with commercial antiserum), the latter being a phenotype associated specifically with the meningococcus and absent from the gonococcus. Hence, we were unable to demonstrate a phenotype associated with the absence of this meningococcus-specific disulfide oxidoreductase.

N. meningitidis Has Three dsbA Genes—The above data regarding the lack of a phenotype associated with a mutation in the *dsbA* specific to the meningococcus prompted us to perform a search of the meningococcal genomes for homologous proteins by BLAST (26) in the three completed and one partially assembled meningococcal genomes. This search revealed that each strain possesses three genes at separate chromosomal loci that show homology to the *dsbA* of *E. coli*. The three genes were designated *dsbA1*, *dsbA2*, and *dsbA3* (Fig. 3). DsbA1 corresponds to the above meningococcus-specific DsbA and shows significant homology to DsbA2 and, to a lesser extent, DsbA3. Homology to the DsbA of *E. coli* is limited to the active site region. Although *N. meningitidis* apparently has three *dsbA* genes with different predicted subcellular localizations, we found only one gene with homology to *dsbB* encoding the enzyme that reoxidizes DsbA.

To perform a thorough mutational analysis of the meningococcal *dsbA* homologues, mutations in the other two genes were engineered. Antibiotic-resistance cassettes were introduced into *dsbA2*. Because the insertion of a resistance cassette into *dsbA3* might potentially have a polar effect on a downstream undecaprenol kinase gene, a point mutation that caused a frameshift and the creation of a termination codon at position

23 was introduced into the frame. Mutations were then combined to produce doubly and triply mutant bacteria. Interestingly, a strain carrying a mutation in all three genes was able to grow at 30 °C on agar plates but grew very poorly at 37 °C. Total proteins of these various mutants were tested by Western blotting with antiserum prepared against recombinant DsbA1, the meningococcus-specific DsbA (Fig. 4). This antiserum reacted not only with the mutant in *dsbA2/dsbA3* (expressing only DsbA1) but also with the mutants in *dsbA1/dsbA3* (expressing only DsbA2) and *dsbA1/dsbA2* (expressing only DsbA3). Thus, these data demonstrate that all three DsbA homologues of *N. meningitidis* are expressed.

DsbA1 and DsbA2 Are Located in the Inner Membrane—As shown above, DsbA1, the meningococcus-specific DsbA protein, is a lipoprotein. The predicted amino acid sequence of DsbA2 showed a similar amino acid hydrophobic lipoprotein signal sequence (23), which would be cleaved by the lipoprotein signal peptidase to leave a mature protein in which the N-terminal cysteine is modified by lipidation. This suggested that DsbA2, like DsbA1, should be membrane-bound, whereas DsbA3 is predicted to be periplasmic. The sorting of lipoproteins (demonstrated in *E. coli*) depends on the amino acid following the cysteine, which is the site of cleavage (27). Thus, the DsbA1 lipoprotein that has a serine at position +2 (of the mature protein) would be expected to be in the outer membrane, whereas the presence in DsbA2 of an aspartate at 2 should result in retention of the protein in the inner membrane. To confirm the localization of these proteins, we separated the outer from the inner membrane by sucrose density gradient centrifugation from a *dsbA2* and a *dsbA1* mutant to determine the localization of DsbA1 and DsbA2, respectively. The results are shown in Fig. 5. A comparison with the distribution of markers for the inner membrane (lactate dehydrogenase activity) and outer membrane (the porin protein PorA and the outer membrane protein OMP85) (28) demonstrated that, despite its serine residue at position +2, the DsbA1 protein is attached to the inner membrane (Fig. 5, *panel A*), casting doubt on the universality of lipoprotein targeting in *N. meningitidis*. On the other hand, as expected, DsbA2 is located in the inner membrane. Hence, both DsbA1 and DsbA2 are inner membrane-localized lipoproteins.

The Expression of a Membrane-bound DsbA Protein (DsbA1 and/or DsbA2) but Not DsbA3 Is Sufficient for Growth under Reducing Conditions—In preliminary observations of growth on agar plates, no differences were observed between the wild type and any of the single or double *dsbA* mutants. However, at
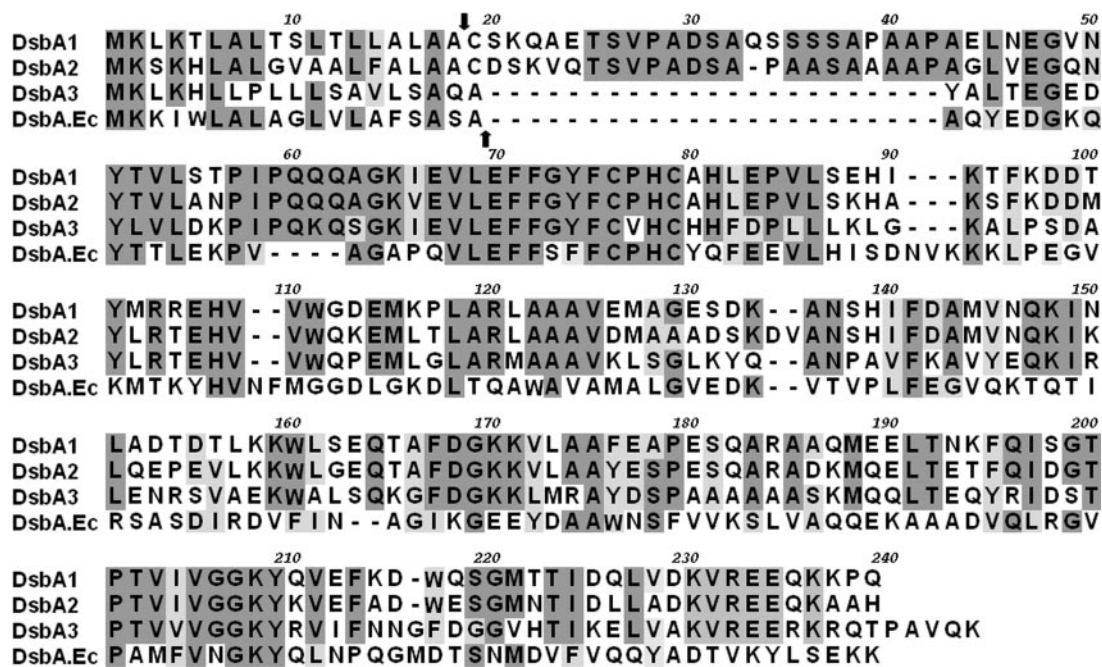
FIG. 3. **Clustal alignment of DsbA sequences from *N. meningitidis* compared with that of DsbA from *E. coli.*** Regions of homology are *shaded*. Amino acids identical in three of the four sequences are *dark gray*, and functionally similar amino acids are *light gray*. The characteristic active site of disulfide oxidoreductases (C*XX*C) comprises amino acids 76 to 79. The conserved proline "151" (corresponding to position 151 of the mature DsbA from *E. coli*) (41) is *underlined*. The predicted signal peptidase cleavage sites are indicated by *arrows*. Amino acid sequences are predicted from the published genome sequence of Nm Z2491 (www.sanger.ac.uk/Projects/N_meningitidis/). Almost identical proteins are found in the genome sequences of three other meningococcal strains, namely MC58 (www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?data base = gnm), FAM18 (www.sanger.ac.uk/Projects/N_meningitidis/seroC.shtml), and 8013-2C43 (Institut Pasteur, Paris, France; sequence not yet in the public domain). DsbA1 is the meningococcus-specific DsbA that is on a genomic island not found in *N. gonorrhoeae*. DsbA2 also has a cleavage site for lipoprotein signal peptidase and is found in both *N. meningitidis* and *N. gonorrhoeae*. DsbA3, like DsbA from *E. coli*, possesses a typical leader peptide, suggesting a periplasmic location.

FIG. 4. **Reactivity of the anti-DsbA1 antiserum with the DsbA proteins in *dsbA1, 2,* and *3* mutant backgrounds.** Bacterial lysates containing equal quantities of protein prepared from the wild type (*WT*) meningococcus and the single, double, and triple mutant bacteria were subjected to SDS-PAGE and Western blotting with anti-DsbA1 antiserum raised against recombinant DsbA1. Δ*A1* designates the mutant having a deletion of the gene *dsbA1*, and Δ*A1,3* designates the mutant having a deletion of the gene *dsbA1* and a point mutation in the gene *dsbA3* leading to the absence of the protein. The constructions of other mutant designations follow these examples. The sizes of molecular mass standards are shown in kDa.
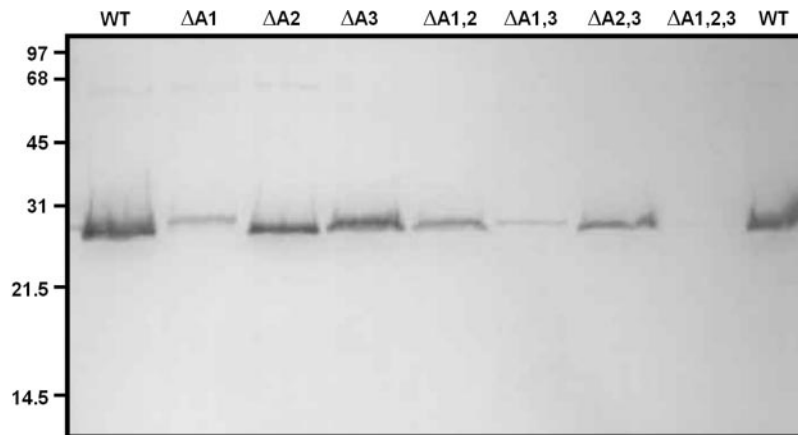
37 °C the triple mutant (DsbA1⁻,DsbA2⁻,DsbA3⁻) was defective for growth. It produced smaller colonies than did the strains carrying any of the double mutations, which had wild type growths on GCB-agar. In addition, these small colonies of the triple mutant were often non-viable after 18 h of growth. On the other hand, this mutant could be successfully grown on agar plates at 30 °C. It was initially considered possible that the combined effects of the mutations were lethal and that those bacteria that survived had undergone secondary mutations permitting growth. This explanation was ruled out by the efficiency of transformation of the *dsbA1* mutation into a *dsbA2,3* background and the efficiency of transformation of the *dsbA2* mutation into a *dsbA1,3* background, which was ~10⁻⁴, a value usual for meningococci (29).

We then tested sensitivity to reducing agents, a phenotype typically associated with mutations in *dsbA*, which leads to an inability to counteract the buildup of reduced, incorrectly folded proteins in the periplasm. Results of these experiments are shown in Fig. 6. Surprisingly, the absence of the two membrane-bound enzymes (or of all three enzymes) resulted in an increased sensitivity to reducing agents, whereas the presence of DsbA1 or DsbA2 permitted a wild type growth. These data suggest that the activity of DsbA3 is lower than that of DsbA1 or DsbA2.

The Expression of a Membrane-bound DsbA Protein (DsbA1 and/or DsbA2) but Not DsbA3 Is Sufficient for the Biogenesis of Functional Type IV Pili—The most abundant extracellular protein in meningococci is pilin, the subunit of the type IV pili necessary for interaction of the bacteria with their human host, which contains a single disulfide bond important for the maintenance of its three-dimensional structure (30, 31). These *dsbA* mutants were therefore tested for pilus-associated phenotypes, *i.e.* natural competence for DNA uptake and interaction with human cells. Results are shown in Fig. 6. The natural compe-

FIG. 5. **Subcellular localization of DsbA1 and DsbA2.** The *dsbA2* mutant strain (detection of DsbA1) (*A*) and the *dsbA1* mutant strain (detection of DsbA2) (*B*) are depicted. Membrane preparations were subjected to centrifugation on sucrose density gradients, the Gram-negative outer membrane being denser than the inner membrane. Fractions (*8–12*) enriched in the inner membrane were identified by lactate dehydrogenase activity (*graphs*). Outer membrane fractions (*3* or *4–9*) were enriched in the major outer membrane porin (*top panels beneath graphs*), corresponding to Coomassie Blue-stained SDS-PAGE gels. The localization of the DsbA proteins was revealed by Western blotting (*bottom panels beneath graphs*) and in both cases coincides with the lactate dehydrogenase activity (hence, the inner membrane).
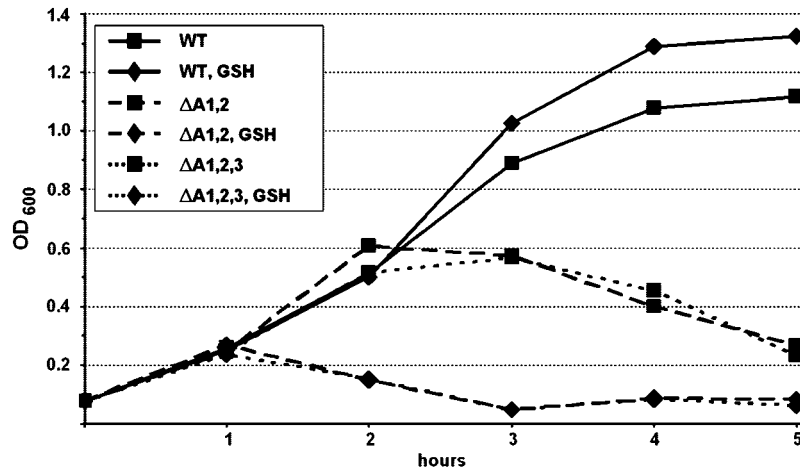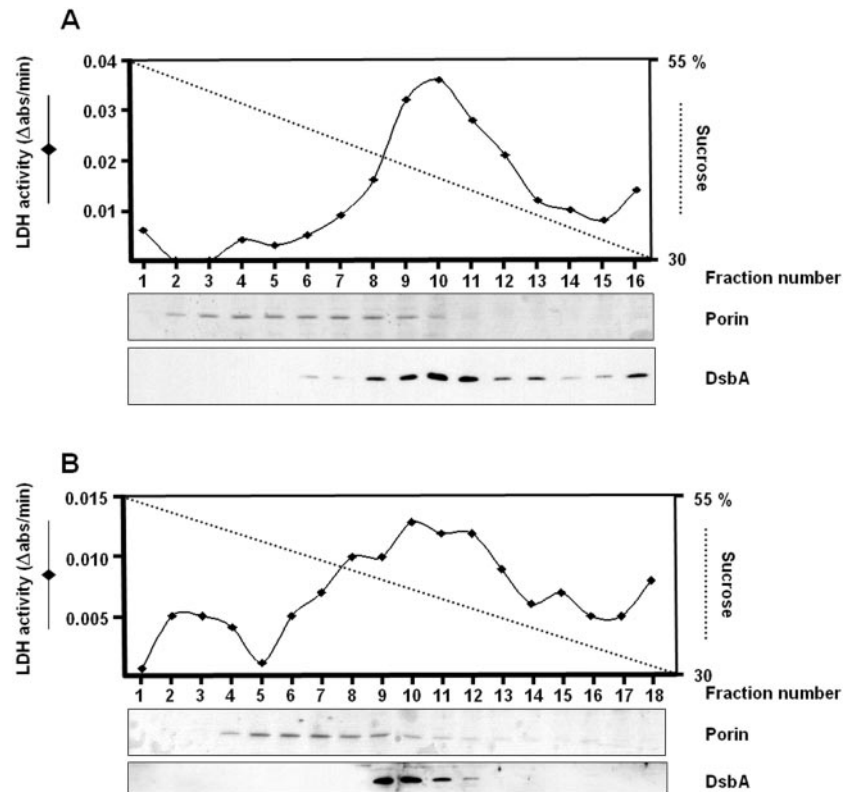

FIG. 6. **Meningococci lacking the two membrane-associated DsbAs are sensitive to reducing agents.** Bacteria were grown in liquid culture with agitation at 30 °C to an OD_{600} of between 0.4 and 0.6 (mid-logarithmic phase). The cultures were then diluted into the same medium adjusted to the required growth conditions. The presence of a reducing agent (reduced glutathione) potentiated the inhibition of growth of the DsbA mutants. Symbols in *inset*: solid lines, wild type bacteria (*WT*); *dashed lines, dsbA1,2* double mutant; *dotted lines, dsbA1,2,3* triple mutant. *Squares,* growth at 37 °C in normal medium; *lozenges,* growth in medium containing 4 mM glutathione (*GSH*). The addition of oxidized glutathione equimolar to reduced GSH, as a control, increased the growth rate by 10–30%, ruling out the possibility that a metabolite of glutathione is toxic. Results similar to those shown on this graph were obtained when using cysteine as a reducing agent. Note that, unlike what was observed on agar plates, the growth of the *dsbA1/dsbA2* double mutant in broth was reduced to that of the level of the triple mutant.

tence of meningococci for the uptake of transforming DNA is dependent on the presence of pili and ancillary proteins (32). Both the *dsbA1,2* double mutant and the *dsbA1,2,3* triple mutant showed marked decreases in their transformation efficiency (Fig. 7*A*). Pilus-mediated adhesion was tested using human umbilical vein endothelial cells; the levels of adhesion of the DsbA1,2 double mutant and triple mutant were dramatically reduced to similar levels (Fig. 7*B*), underlining the inefficacy of the DsbA3 in this regard.

Alterations in the efficacy of transformation and adhesion to cells are typically the results of mutations affecting the piliation of meningococci. However, in the *dsbA* mutants neither the amount of pilin detected in whole cell lysates on Western blots nor the level of piliation visualized on bacterial cells by immunofluorescence microscopy was changed (data not shown). The above results on transformation and adhesion could be explained by a misfolding of the pilin subunits such that the formation of pilus fibers was still possible but that they were non-functional. This latter hypothesis is supported by two-dimensional PAGE and Western blotting of whole cell extracts of the bacteria that showed that the pilin from the DsbA triple mutant grown at 37 °C did indeed demonstrate differences in its migration with respect to its isoelectric point, probably reflecting abnormalities in its three-dimensional structure or

FIG. 7. **Effects of mutations in the DsbA enzymes on pilus-mediated phenotypes.** *A*, meningococci lacking the two membrane-associated DsbAs show a reduction in the level of transformability by exogenous DNA. Bacteria were transformed with chromosomal DNA carrying a selectable marker and plated onto normal and selective media. The ratio of the cells that had incorporated the marker was calculated. Results are shown relative to the wild type bacteria and are averages of two experiments except for those of the *dsbA1,2* double and *dsbA1,2,3* triple mutants, which are the average of four. Strain designations are the same as those in Fig. 4. In the absence of transforming DNA (−*ve*), no resistant colonies were seen. *B*, possession of one of the two membrane-associated DsbAs is necessary for pilus-mediated adhesion. Adhesion is represented as a percentage relative to the wild type. Values for the *dsbA1,2* double mutant (3.6 ± 2.9) and the *dsbA1,2,3* triple mutant (1.1 ± 0.9) are the averages of three and four experiments, respectively. Strain designations are as in Fig. 4.
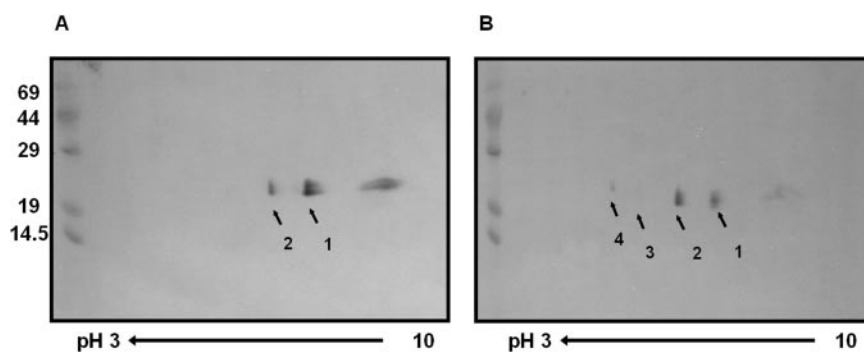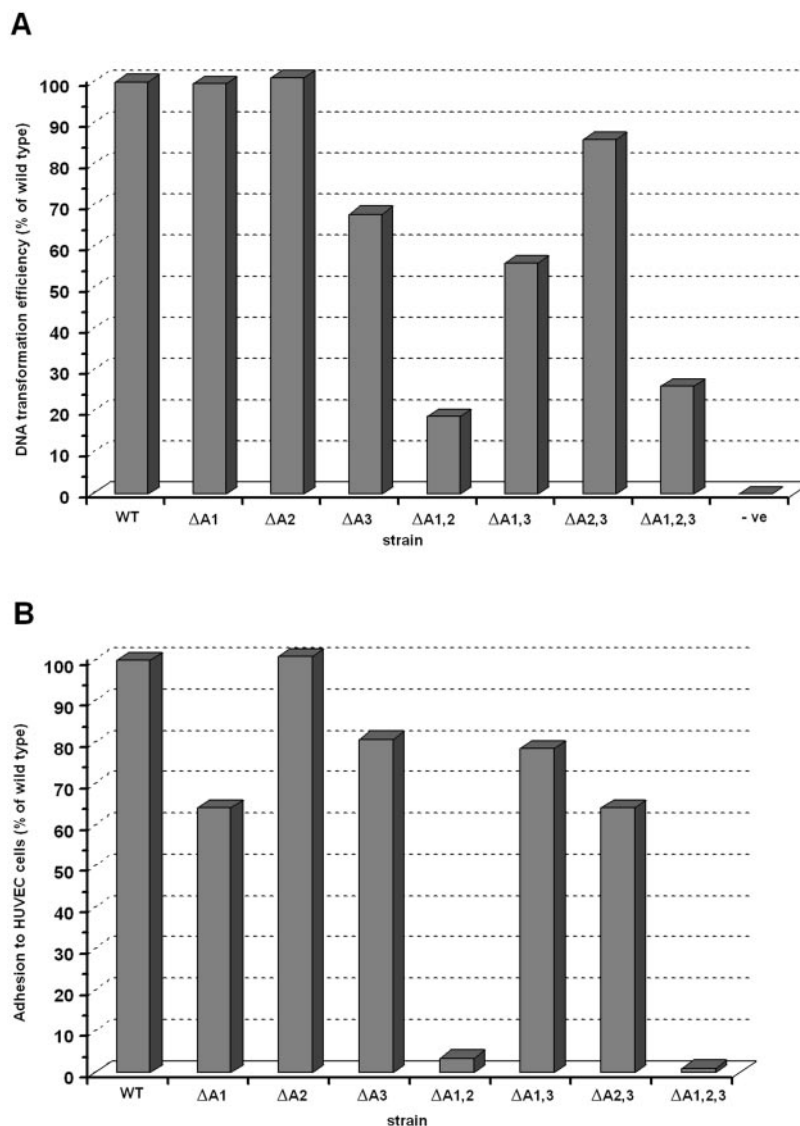

FIG. 8. **Two-dimensional polyacrylamide gel electrophoresis of pilin from wild type and *dsbA1,2,3* mutant bacteria.** Meningococci were resuspended to an OD_{600} of 0.1 and grown for 2 h at 37 °C. Tween 20 extracts of the sonicated cells were separated first by isoelectric focusing and then by SDS-PAGE electrophoresis. Western blots were probed with antibody directed against whole pilin. Samples were loaded at pH 10; the pH gradient is indicated below the gels. Sizes of molecular mass markers are shown in kilodaltons. Wild type (*A*) and the *dsbA* triple mutant (*B*) are depicted. Note the appearance of two new bands (*3* and *4*) and the inversion of relative intensities of bands *1* and *2* in the mutant bacteria. As is usual in *Neisseria*, there is only one transcribed pilin gene. However, the multiple bands in the wild type may be due to one or more of the numerous posttranslational modifications demonstrated for neisserial pilin, namely cleavage of the signal sequence and *N*-methylation, glycosylation (38), phosphorylation (39), and the addition of α-glycerophosphate (40).

posttranslational processing (Fig. 8). This finding is consistent with the hypothesis that the triple and double *dsbA1/dsbA2* mutants are unable to stabilize the conformation of pilin necessary for its correct processing.

DISCUSSION

Our investigations of a meningococcus-specific protein with homology to the disulfide oxidoreductase DsbA of *E. coli* led us to note the presence of three homologous genes in the menin-
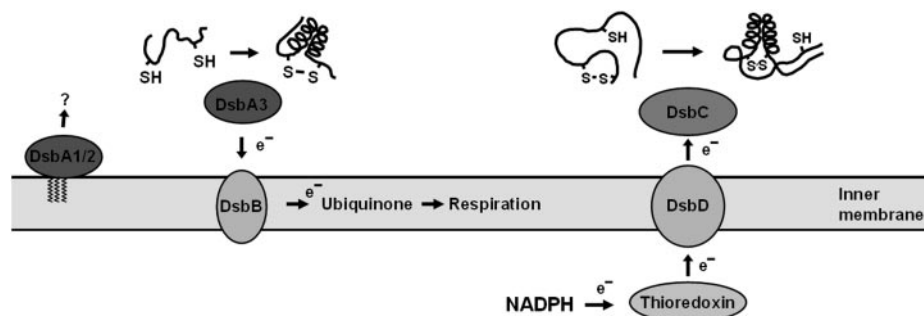
FIG. 9. **Homologues of *E. coli* enzymes of the Dsb system.** A putative schema of the folding of exported proteins in the periplasm of *N. meningitidis*. The model is based on that of *E. coli* where, in a simplified form, the system consists of two separate chains. DsbA is a relatively nonspecific oxidant that takes its oxidizing power, via DsbB, from the electron transport chain. DsbC has a disulfide isomerase activity and is maintained in a reduced state, despite the oxidizing periplasmic environment, by DsbD, which, in turn, obtains its reducing power from cytoplasmic thioredoxin. The genome sequence of *N. meningitidis* predicts homologues to all of these enzymes, although apparently there are no homologues to DsbG and DsbE, disulfide isomerases involved in more specific reactions. In contrast there are three DsbA homologues, two of which are associated with the inner membrane. The electron acceptor for these latter two remains to be determined.

gococcus. Two of the enzymes were membrane-associated, whereas the third was homologous to the periplasmic DsbA of *E. coli*. To elucidate this unusual situation, we undertook a mutational analysis of the three genes, which led to the surprising result that the presence of at least one of the two membrane-bound enzymes is necessary to obtain a DsbA+ phenotype, the third enzyme (the periplasmic enzyme) being incapable of supporting the required disulfide oxidoreductase activities.

In Gram-negative bacteria, the correct formation of disulfide bonds during protein folding in the periplasm is, in general, the result of a combination of the oxidative DsbA disulfide bond formation and the DsbC disulfide isomerization systems. The genome of *N. meningitidis* contains genes homologous to each of the components of these two systems but, unlike *E. coli*, contains three *dsbA* genes, *dsbA1*, *dsbA2*, and *dsbA3* (Fig. 9 and Table I). Furthermore, the measured activity of the gene product of the cloned *dsbA1* gene, combined with its expression as evidenced on the Western blots and the ability of each enzyme to complement the others (to a greater or lesser extent), demonstrates the existence of three DsbA enzymes in the meningococcus. A search of available genome sequences demonstrated that the possession of multiple *dsbA* genes is not restricted to *N. meningitidis*. Although most of the bacteria studied have a single *dsbA* gene, examples of two genes are not uncommon (Table II and supplementary material, which is available in the on-line version of this article). *N. meningitidis* is unusual in having three enzymes, but a more extreme case is that of *Shewanella oneidensis* in which (at least in the sequenced strain) there are four genes.

Although DsbA, as first discovered in *E. coli*, is a periplasmic enzyme in most bacteria, a review of available genome sequences reveals that several DsbAs are predicted to be membrane-bound lipoproteins on the basis of their signal sequences (Table II and supplementary material). Homologues of DsbA with BLAST similarity more significant than 10^{-3} were found to be restricted to Gram-negative proteobacteria, although lipoprotein Dsb enzymes (more similar to DsbG) were also found in several Gram-positive organisms. In this work we present biochemical evidence that DsbA1 is a lipoprotein. DsbA2 may also be expected to be a lipoprotein in consideration of its similarity to DsbA1 and because the signal sequence is also predicted (*e.g.* LipoP; cbs.dtu.dk/services/LipoP/) to be cleaved by lipoprotein signal peptidase. This finding, together with the fact that the hydrophilic protein (which has no membrane-spanning regions apart from the signal peptide) remains associated with the inner membrane, strongly suggests that DsbA2, like DsbA1, is a lipoprotein. In the case of the meningococcus,

TABLE I
Protein homologies between enzymes of the Dsb systems from E. coli and N. meningitidis

Homologies between proteins were calculated by the BLAST algorithm hosted by National Centers for Biological Information. Protein matrix was Blosum 62. Note that the best hit for *E. coli* DsbG is to the *N. meningitidis* DsbC gene. Genes encoding Dsb proteins are not significantly different between the different strains of meningococcus. GI, gene identifier.

E. coli (K12, MG1655)	*N. meningitidis* (Z2491)	*E* value
DsbA (GI:16131701)	DsbA1 (NMA2191)	1.5e-17
	DsbA2 (NMA2209)	7.2e-10
	DsbA3 (NMA2078)	1.4e-12
DsbB (GI:33347526)	DsbB (NMA1903)	8.9e-08
DsbC (GI:16130795)	DsbC (NMA0730)	7.6e-21
DsbD (GI:16131961)	DsbD (NMA1719)	8.0e-63

part of the explanation for the requirement of a membrane-bound DsbA protein may be due to a peculiarity of meningococci, which is to release outer membrane vesicles, or blebs, during normal growth. These blebs contain large amounts of endotoxin, which is particularly important in the fever and cytokine deregulation characteristic of invasive meningococcal disease. This blebbing will also result in the loss of soluble periplasmic enzymes, hence favoring the anchoring of these enzymes to the inner membrane so that the oxidizing power of DsbA would not be lost, as might be the case with that of a soluble disulfide oxidoreductase It is interesting to note in this context that genome sequence data predict that the enzyme DsbC is also a lipoprotein in the meningococcus.

A more general explanation might be that the presence of the enzyme in the inner membrane increases the likelihood of interaction of the protein to be oxidized with DsbA1 and 2, but this might also lead to steric restrictions of the availability of the active site of the membrane-bound DsbAs for reoxidation by DsbB. In this regard, it is conceivable that the relative flexibility of lipoproteins that are tethered to the membrane by a lipid anchor (as compared with integral membrane proteins) and the additional amino acids at the N terminus of the protein (as compared with DsbA from *E. coli*; Fig. 3) might allow interaction with the active site of DsbB. However, in preliminary experiments with strains in which *dsbB* was inactivated, none of the four strains tested showed the same phenotype as was seen for the *dsbA1⁻,dsbA2⁻,dsbA3⁻* triple mutants. Further investigation of these mutants is necessary to determine the additional means of reoxidation of DsbA in *N. meningitidis*.

According to the paradigm of *E. coli* (the "+2 rule"), lipoproteins will be retained in the inner membrane if the amino acid following the N-terminal cysteine is an aspartate (27). It has

TABLE II
E. coli DsbA homologues in Gram-negative bacteria

For each *E. coli* DsbA homolog the presence of the C*XX*C active site and the highly conserved proline 151 (41) was checked. The gene identifier and the *E* value are indicated for each homolog. For lipoprotein homologs, the putative lipobox and the position of the conserved cysteine (in parentheses) are indicated. BLAST searches and gene alignments were performed in a set of fully sequenced Gram-negative bacterial genomes using the PEDANT web site (pedant.gsf.de). Representative and medically or commercially important species are shown; a full list is given in the supplementary material available in the on-line version of this article.

Species (strain)	Class of proteobacteria	Gene identifier	*E* value	Lipobox of the putative lipoprotein signal sequence
Campylobacter jejuni (NCTC 11168)	ϵ	6968312	3×10^{-8}	
Caulobacter crescentus (CB15)	α	16124630	4×10^{-4}	LAA-C$_{(21)}$
Escherichia coli (K12)	γ	1790291		
Geobacter metallireducens	δ	23053477	6×10^{-8}	
Haemophilus influenzae (Rd KW20)	γ	1573860	2×10^{-47}	
Neisseria meningitidis (MC58)	β	7225518	5×10^{-15}	LAA-C$_{(19)}$
		7225503	3×10^{-9}	LAA-C$_{(19)}$
		7225629	8×10^{-11}	
Nitrosomonas (ATCC 19718)	β	30248383	7×10^{-8}	
Pasteurella multocida (PM70)	γ	12722222	8×10^{-45}	
Pseudomonas aeruginosa (PAO1)	γ	15600682	3×10^{-18}	
Pseudomonas putida (KT2440)	γ	26986872	2×10^{-17}	
Pseudomonas syringae (pv. tomato str. DC3000)	γ	28867572	5×10^{-19}	
Ralstonia solanacearum	β	17427294	3×10^{-11}	
		17431370	9×10^{-10}	
Rickettsia conorii (M7)	α	15619063	6×10^{-4}	LSS-C$_{(17)}$
Salmonella typhimurium (LT2)	γ	16422558	1×10^{-103}	
		16421749	11×0^{-14}	
Shewanella oneidensis MR1	γ	24371931	3×10^{-34}	
		24375215	6×10^{-20}	
		24374389	2×10^{-17}	
		24375360	8×10^{-11}	
Shigella flexneri (2a_301)	γ	24115149	1×10^{-118}	
Vibrio cholerae (N16961)	γ	9654427	2×10^{-39}	
Xanthomonas campestris (pv. campestris str. ATCC 33913)	γ	21232829	2×10^{-10}	LVA-C$_{(19)}$
		21232828	3×10^{-10}	LAA-C$_{(20)}$
Xylella fastidiosa (Temecula1)	γ	28198567	4×10^{-13}	LVA-C$_{(19)}$
		28198666	3×10^{-8}	
Yersinia pestis (CO92)	γ	15978131	7×10^{-84}	

since been demonstrated that phenylalanine, tyrosine, glycine, proline, or tryptophan at the $+2$ position also cause inner membrane retention (33); other amino acids at this position lead to insertion of the protein in the outer membrane. Hence, the meningococcus-specific DsbA1 (-CS-) would be expected to be an outer membrane protein, whereas the aspartate at position $+2$ of the mature protein DsbA2 (-CD-) should result in its retention in the inner membrane. However, it was seen that both of these proteins were associated with the inner membrane, which calls into doubt the applicability of this rule to the case of *N. meningitidis* and suggests that either different amino acids lead to retention in the inner membrane or that the signal is context-dependent.

A reason for the lesser efficacy of the soluble periplasmic DsbA3 might lie in its primary amino acid sequence. This protein differs from the membrane-bound DsbAs not only in its lipoprotein signal sequence but also at the active site. A search of available protein sequences shows that the amino acid following the first of the cysteine residues at the active site is generally proline, which is small and, because of its secondary amino group, is likely to lead to a particular conformation at the C*XX*C motif. In contrast to the enzyme DsbA3 of *N. meningitidis*, the amino acid at this position is a valine, which, because of its effect on the conformation of the active site, may be expected to alter the redox potential of the enzyme and, hence, its activity (34). It is therefore possible that DsbA3 is badly adapted to assure the disulfide oxidoreductase functions fulfilled by DsbA in other bacteria, these functions being performed by DsbA1 and/or DsbA2. DsbA3 may be a remnant of the periplasmic disulfide oxidoreductase system of *N. meningitidis* of which the functions have been taken over by the more effective DsbA1 and DsbA2 enzymes. Another possibility, also

compatible with the above, is that the functions of the enzymes have diverged, each recognizing one or a group of preferred substrates. This may be a general explanation of the presence of families of DsbAs in a bacterium, and, indeed, a similar case has been described for the pathogen *Salmonella enterica* var. *typhimurium*, where a third DsbA enzyme, the plasmid-encoded SrgA, is necessary to stabilize the correctly folded form of the plasmid-encoded fimbrial adhesin PefA (35).

In the case of the meningococcus, one or both of the membrane-bound DsbA enzymes were found to be essential for the formation of functional pili at the surface of the bacteria because the mutants showed defects in competence for transformation and in binding to human cells, two important bacterial functions dependent on pili. Indeed pilus-mediated adhesion to human cells is essential to the meningococcus, whose only natural habitat is the human nasopharynx, and it is equally necessary for the interaction with the blood-brain barrier in the pathogenesis of meningococcal meningitis. Two cases (in addition to that above of *S. typhimurium*) have been described in which a *dsbA* mutant leads to defects in pilus-associated phenotypes. In enteropathogenic *E. coli*, levels of pilin are reduced because of a decreased half-life of the protein (36). In contrast, *dsbA* mutants of *Vibrio cholerae* show normal levels of pilin but lack pilus function (8). Because the meningococcal mutants investigated here show no significant differences in the levels of pilin or in piliation, it is likely that a folding defect leads to the secretion of pilin molecules that are capable of polymerizing into pili but not of performing their normal functions within the fiber. In support of this idea, the pilin molecules are altered in their behavior in isoelectric focusing. A similar situation has been described recently (37) where pilin variants containing single base pair changes retained their ability to form pilus

fibers, but the resulting fibers were not functional. A scheme for the folding of pilin that would be compatible with the observed effects of the *dsbA* mutants would involve the intervention of the disulfide bond formation at a critical point in the protein folding. In the presence of DsbA, a correct conformation is stabilized that promotes subsequent folding to a functionally active structure. In its absence, a slower heuristic folding process eventually stabilizes the pilin in a conformation suitable for secretion and polymerization but not for pilus functionality. We cannot rule out the possibility that loss of function is a secondary effect of the misfolding of ancillary proteins involved in competence or pilus-mediated adhesion or in pilus secretion, whose function depends on correct disulfide bonding. In this regard, it is interesting to note that the predicted sequences of many of the meningococcal proteins involved in pilus formation contain potential disulfide bonds.

In conclusion, pilus-mediated adhesion in *N. meningitidis* is dependent on one of two novel DsbA proteins regardless of the presence of a periplasmic DsbA, which is in contrast to many other species where a single periplasmic DsbA is sufficient. It is also interesting to note that the very closely related gonococcus, *N. gonorrhoeae*, contains genes essentially identical to those encoding the inner membrane DsbA2 and the periplasmic DsbA3 and relies on pilus-mediated adhesion in a way similar to that of the meningococcus.

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