INFECTION AND IMMUNITY, Apr. 2008, p. 1498–1508 Vol. 76, No. 4
0019-9567/08/$08.00+0 doi:10.1128/IAI.01378-07

The Periplasmic Disulfide Oxidoreductase DsbA Contributes to *Haemophilus influenzae* Pathogenesis[▽]

Charles V. Rosadini, Sandy M. S. Wong, and Brian J. Akerley*

Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01655

Received 13 October 2007/Returned for modification 25 November 2007/Accepted 8 January 2008

Haemophilus influenzae **is an obligate human pathogen that persistently colonizes the nasopharynx and causes disease when it invades the bloodstream, lungs, or middle ear. Proteins that mediate critical interactions with the host during invasive disease are likely to be secreted. Many secreted proteins require addition of disulfide bonds by the DsbA disulfide oxidoreductase for activity or stability. In this study, we evaluated the role in *H. influenzae* pathogenesis of DsbA, as well as HbpA, a substrate of DsbA. Mutants of *H. influenzae* Rd and type b strain Eagan having nonpolar deletions of *dsbA* were attenuated for bacteremia in animal models, and complemented strains exhibited virulence equivalent to that of the parental strains. Comparison of predicted secreted proteins in *H. influenzae* to known DsbA substrates in other species revealed several proteins that could contribute to the role of *dsbA* in virulence. One candidate, the heme transport protein, HbpA, was examined because of the importance of exogenous heme for aerobic growth of *H. influenzae*. The presence of a *dsbA*-dependent disulfide bond in HbpA was verified by an alkylation protection assay, and HbpA was less abundant in a *dsbA* mutant. The *hbpA* mutant exhibited reduced bacteremia in the mouse model, and complementation restored its in vivo phenotype to that of the parental strain. These results indicate that *dsbA* is required in vivo and that HbpA and additional DsbA-dependent factors are likely to participate in *H. influenzae* pathogenesis.**

Haemophilus influenzae efficiently colonizes the human nasopharyngeal mucosa in a primarily asymptomatic manner, and the carriage frequency is ~80% in healthy adults (48). However, it can disseminate to other anatomical sites and cause otitis media, upper and lower respiratory tract infections, septicemia, and meningitis in children (13, 22, 37, 44, 48–50, 63). The incidence of *H. influenzae* meningitis has dramatically declined in populations immunized with a vaccine against the type b capsular polysaccharide. The vaccine has not affected the incidence of infection with nontypeable *H. influenzae* (NTHi) strains, which lack the capsule. NTHi strains predominantly cause respiratory tract infections and otitis media but in rare cases can invade the bloodstream, leading to meningitis. This disease profile raises the possibility that genes promoting intravascular invasion could be present in NTHi strains (15, 18, 53, 54). However, the molecular basis for the invasive properties of *H. influenzae* that promote transmission from the nasopharynx to the bloodstream or middle ear is not fully understood.

Secreted bacterial proteins mediate critical aspects of pathogenesis, including attachment, nutrient utilization, and subversion of host defenses. Many secreted proteins of gram-negative bacteria acquire disulfide bonds in the periplasm that stabilize their mature, folded structures (9). Formation of such linkages has been most extensively studied in *Escherichia coli*, in which a series of disulfide oxidoreductases (Dsb) create and exchange disulfide bonds in periplasmic proteins (for reviews, see references 34 and 51). The soluble periplasmic disulfide oxidoreductase, DsbA, directly catalyzes this process by exchanging its disulfide bond with free thiol groups of cysteine residues in target proteins (21, 75). DsbA is efficiently reoxidized by DsbB, a membrane protein that transfers electrons to quinones for subsequent transfer to electron acceptors of the respiratory chain (8, 51). The soluble periplasmic DsbC and DsbG proteins mediate rearrangement of mispaired disulfides using electrons transferred via the membrane-bound DsbD protein from cytoplasmic thioredoxin (3, 10, 38, 58, 64, 76). Mutants defective in periplasmic disulfide bond formation are viable under standard culture conditions but exhibit a range of phenotypes as a result of defective maturation of secreted proteins. The effects vary depending on the repertoire of periplasmic and secreted substrates of DsbA in different bacteria. The deficiencies can involve single enzymes that require a disulfide bond for activity, such as the periplasmic alkaline phosphatase, PhoA, of *E. coli*, as well as defects in components of transporters, resulting in inappropriate localization of substrates.

DsbA homologs contribute to the pathogenesis of multiple bacterial species, in which they are required for maturation or export of major secreted virulence factors. DsbA activity is required for production of functional type IV pili (also called fimbriae) that mediate adherence to host surfaces in *Vibrio cholerae*, *Neisseria meningitidis*, enteropathogenic *E. coli*, and uropathogenic *E. coli* (33, 55, 66, 77). Toxin production or secretion is defective in many *dsbA* mutants; the toxins affected include cholera toxin in *Vibrio cholerae*, heat-labile and heat-stable *E. coli* enterotoxins, and pertussis toxin in *Bordetella pertussis* (55, 65, 74). Type III secretion systems consist of multisubunit protein conduits that inject effector proteins directly from the bacterial cytoplasm into host cells to subvert diverse host cell functions. Components of the type III secretion apparatus are defective in *dsbA* mutants of *Yersinia pestis*, *Shigella flexneri*, *Pseudomonas* spp., and *Salmonella enterica* serovar Typhimurium (26, 32, 42, 68). Fur-

* Corresponding author. Mailing address: Department of Molecular Genetics and Microbiology, University of Massachusetts Medical School, 55 Lake Ave., N., S6-242, Worcester, MA 01655. Phone: (508) 856-1442. Fax: (508) 856-1422. E-mail: Brian.Akerley@umassmed.edu.

▽ Published ahead of print on 22 January 2008.

thermore, DsbA has been implicated in systemic infection by *E. coli* K1 and prolonged survival of adherent-invasive *E. coli* within macrophages (12, 23).

The *dsbA* gene of *H. influenzae* (HI0846, also called *por*) transcomplements a *dsbA* mutant of *E. coli* (67). Disruption of *dsbA* with a transposon insertion resulted in changes in secreted protein localization in a cellular fractionation experiment and dramatically reduced the natural transformation efficiency (67). The role of DsbA in *H. influenzae* pathogenesis has not been examined. However, a transposon-based "signature-tagged mutagenesis" screen detected the putative *dsbB* homolog as a virulence gene candidate in an infant rat model of bacteremia, suggesting a potential role for periplasmic disulfide bond formation in *H. influenzae* pathogenesis (28). In *H. influenzae*, the protein targets of DsbA and virulence factors dependent on its activity have not been identified. In this study, we demonstrated that *dsbA* is required for *H. influenzae* bacteremia caused by both unencapsulated strain Rd and a virulent encapsulated type b strain. Heme uptake is required for aerobic growth of *H. influenzae*, which cannot synthesize the porphyrin ring (24, 70), and several heme utilization pathways have been implicated in bloodstream infection by *H. influenzae* (47, 61). We demonstrate that the heme transport protein, HbpA, contains a DsbA-dependent disulfide bond. A nonpolar *hbpA* deletion mutant caused reduced bacteremia in mice, yet the defect was not as pronounced as that of the *dsbA* mutant. Based on these results, it is likely that *dsbA* is required in vivo for production of optimal levels of *hbpA* and that additional virulence factors that remain to be identified also participate in the critical role of DsbA in *H. influenzae* pathogenesis.

MATERIALS AND METHODS

Strains and culture conditions. *H. influenzae* Rd, a capsule-deficient serotype d derivative (71), and a virulent streptomycin-resistant derivative of *H. influenzae* type b strain Eagan (Hib) (5) were grown in brain heart infusion broth (BHI) supplemented with 10 μg/ml hemin and 10 μg/ml NAD (sBHI), in MIc, a low-nutrient medium capable of supporting growth of *H. influenzae* (7), or on sBHI agar plates at 35°C. Development of competence for transformation of *H. influenzae* was accomplished as previously described (7). For selection of Rd- and Hib-derived strains, antibiotics were used at the following concentrations: 8 μg/ml tetracycline, 20 μg/ml kanamycin, 10 μg/ml gentamicin, and 100 μg/ml streptomycin.

***dsbA* strain construction.** Plasmids and PCR products were constructed using standard molecular biology techniques (6). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, which does not replicate in *H. influenzae* (71). The pXT10-based plasmids contained upstream (*xylF*) and downstream (*xylB*) homologous regions flanking the SapI cloning sites that allowed precise fusion of genes of interest to the xylose-inducible *xylA* promoter, as previously described (71). Recombination at the xylose catabolic locus replaced the endogenous *xylA* gene with the cloned fragment and the *tetAR* tetracycline resistance cassette. Plasmids were linearized by digestion with PciI and SacI, and tetracycline-resistant (Tet^r) recombinants were selected on sBHI agar plates. Double crossovers within *xylF* and *xylB* were confirmed by performing PCR with primers specific for sequences outside the inserted recombinant region.

To generate a *dsbA* mutant and a complemented strain of *H. influenzae* which requires DsbA for natural transformation, we first generated a strain containing an inducible copy of *dsbA* and sequentially introduced the *dsbA* deletion and the complementation construct or the "empty vector" construct into this background. Initially, an additional copy of *dsbA* under control of the xylose-inducible promoter of *xylA* was introduced into *H. influenzae* Rd to create strain RX. The coding sequence of DsbA lacking the translational termination codon was amplified by PCR with primers F-NTdsb (5′-AAAGATCTGCTCTTCAATGAAAAAAGTATTACTTGC-3′) and 3dsbAHA (5′-AAAGATCTGCTCTTCGTAATGCATAATCTGGCACATCATATGGATATTTTTGCAATAAACCTTTTACGGTT-3′), which introduced SapI sites in the termini of the fragment. The resulting fragment was cloned into pXT10 that had been digested previously with SapI. The resulting plasmid, pXyldsbA1.1, was linearized and used to transform *H. influenzae* to tetracycline resistance to create strain RX.

Next, the native copy of *dsbA* was deleted from RX by replacement with the *aacC1* gentamicin resistance gene to create strain RdsbAX by PCR "stitching" as follows. Overlapping PCR fragments generated with primers representing the 951-bp region immediately 5′ of the *dsbA* translational start codon (primers 5844H [5′-TTTAAGCTTTTAGATGACTGTTTTCTTTAAATC-3′] and 3Dsbout [5′-TTCTTTCCTCTTATTTAATGATACCGCGAG-3′]), the 569-bp *aacC1* gene encoding gentamicin resistance (primers 5GentD [5′-TAAATAAGAGGAAAGAAATGTTACGCAGCAGCAACGATGTT-3′] and 3GentD [5′-CATTAAACCAATTTTTCGTTAGGTGGCGGTACTTGGGTCGAT-3′]), and the 1,641-bp 3′ region starting at the *dsbA* termination codon (primers 5Dsbout [5′-CGAAAAATTGGTTTAATGCCAGCCC-3′] and 3848H [5′-TTTAAGCTTCTACTTGCGAATGAGCCATAGGC-3′]) were combined by overlap extension PCR with primers 5844H and 3848H to precisely replace the *dsbA* coding sequence with the coding sequence of *aacC1*. The resulting 3,126-bp DNA fragment was used to transform strain RX, and gentamicin-resistant (Gm^r) recombinants were isolated to create strain RdsbAX, which contained a single copy of *dsbA* under control of the xylose-inducible *xylA* promoter.

To complement the *dsbA* knockout with a wild-type copy of *dsbA* under control of its own promoter, overlap extension PCR was performed as follows. Primers pXT10thyA-F (5′-AGGGCTTGAATCGCACCTCCA-3′) and 3dsbkan1 (5′-CATCAGAGATTTTGAGACACGGGCCTCTTATTTTTGCAATAAACCTTTTACGGT-3′) were used in PCR to amplify a 1,983-bp fragment containing *dsbA* from a pXT10-based plasmid carrying *dsbA* coding sequences, pDsbA1.2. A 2,716-bp PCR product was amplified from a kanamycin-marked derivative of pXT10 with primers 5pkan1 (5′-GAGGCCCGTGTCTCAAAATCTCTGATG-3′) and 3revRfaD1 (5′-AACAGGCTACGATAAACCATTCAAAACAGT-3′). The 1,983- and 2,716-bp fragments were joined via the 27 bp of overlapping sequence by PCR performed with primers pXT10thyA-F and 3revRfaD1, the resultant 4,672-bp PCR product was transformed into strain RdsbAX (grown in the presence of 1 mM D-xylose to induce expression of *dsbA*), and kanamycin-resistant (Km^r) transformants were isolated to create strain RdsbAC.

To control for effects of modification of the *xyl* locus, a *dsbA* mutant containing the integrated "empty vector" sequences was generated by transforming RdsbAX grown in the presence of 1 mM D-xylose with a 4,334-bp PCR product having a precise deletion of the *dsbA* coding sequences of the 4,672-bp construct described above in RdsbAX, except that primers 3xylF1 (5′-ACGTTTATCAACAGCGATAGGATCAAGT-3′) and 3pDsbAsapKan (5′-CATCAGAGATTTTGAGACACGGGCCTCTTACGAAGAGCGGCGCGCCGCTCTTCCCATTTCTTTCCTCTTATTTAATGATACCGCGA-3′) were used instead of primers pXT10thyA-F and 3dsbAkan. Selection for Km^r transformants resulted in isolation of strain RdsbAV. To construct a strain that contained the "empty vector" in a wild-type background, the same 4,334-bp PCR product was transformed into *H. influenzae* Rd, and Km^r transformants were isolated to create strain RXV.

Similarly, the same set of constructs was used to generate the *dsbA* mutant HdsbAV, a vector-only strain (HXV), and a complemented strain (HdsbAC) in the *H. influenzae* type b strain Eagan background. The wild-type and *dsbA* mutant phenotypes of all strains were verified using a dithiothreitol (DTT) sensitivity assay (described below), and all mutations were confirmed by sequence analysis of the recombinant loci.

HbpA strain construction. *hbpA* mutant strain RhbpA was constructed by replacement of the coding sequence of *hbpA* with the kanamycin resistance gene, *aphI*. The exchange fragment was synthesized by overlap extension PCR between three regions: a 1,083-bp PCR product containing the 5′ flanking region of *hbpA* generated using primers 5hbp1 (5′-AGTCATTCACGCCAGTTGGCACTGGAT-3′) and 3hbp1 (5′-TTCCCGTTGAATATGGCTCATACCTCAATGTTAGGCAGGGAATGCCCTA-3′), an 816-bp PCR product containing the coding region for the kanamycin resistance gene generated with primers 5kan1.1 (5′-ATGAGCCATATTCAACGGGAA-3′) and 3kan1.1 (5′-TTAGAAAAACTCATCGAGCATCAAATG-3′), and a 1,020-bp PCR product containing the 3′ flanking region of *hbpA* generated with primers 5hbp3 (5′-CATTTGATGCTCGATGAGTTTTTCTAATTCATATTGATTTACTTATTTTAAGCCCT-3′) and 3hbp3 (5′-CAAAAGGGGTGAGTATAAATTTACACTCAA-3′). The 1,083-, 1,020, and 816-bp fragments were joined in a PCR via their complementary ends using primers 5hbp1 and 3hbp3. The resulting 2,871-bp fragment was introduced into *H. influenzae* Rd, and Km^r transformants were selected on sBHI containing kanamycin to create strain RhbpA. To construct an *hbpA* knockout mutant carrying the integrated empty exchange vector, strain RhbpA was transformed

with linearized vector pXT10, and Tet^r transformants were isolated to create strain RhbpAV.

To complement the *hbpA* mutation with a copy of *hbpA* expressed from the *hbpA* promoter, a 1,842-bp fragment containing the *hbpA* coding region and including 142 bp upstream of *hbpA* was amplified from Rd using primers 5hbpha (5′-AAAGCT CTTCAATGATTAATTTGTTATAATCCATAGA-3′) and 3hbpha (5′-TTTGCT CTTCTTTATGCATAATCTGGCACATCATATGGATATTTACCATCAACA CTCACACCATA-3′). This set of primers also added a C-terminal hemagglutinin (HA) epitope tag to *hbpA*. The PCR product was cloned between the two SapI sites of pXT10 to generate plasmid pXhbp1.5, which was then introduced into strain RhbpA with selection for Tet^r to create strain RhbpAC. To introduce a nonpolar, in-frame deletion of *dsbA* into strain RhbpAC, this strain was transformed with the 3,126-bp *dsbA* replacement fragment described above, and Gm^r transformants were selected to create strain RhbpACΔ*dsbA*.

Other strains. Strain RdV carrying pXT10 "empty vector" sequences in the *xyl* locus and strain RdlacZ (*H. influenzae* Rd carrying *lacZ* at the *xyl* locus) were constructed as previously described (72). Strain RdgalU was constructed by replacement of *galU* with the *aphI* Km^r cassette. For all mutant strains, replacement of endogenous loci by double-crossover homologous recombination with mutant constructs was confirmed by PCR performed with primers specific for sequences flanking the inserted recombinant region.

DTT sensitivity assay. To evaluate sensitivity to DTT, strains were inoculated in triplicate using inocula from overnight cultures into 25 ml of sBHI in 50-ml Erlenmeyer flasks to obtain an optical density at 600 nm (OD_{600}) of 0.01 and incubated at 35°C with shaking at 250 rpm. When cultures reached the log phase, they were diluted in sBHI to obtain an OD_{600} of 0.02, and 100 μl was transferred to a 96-well flat-bottom dish. Each well in the dish was then treated with 100 μl of sBHI containing 10 mM DTT at a final concentration of 5 mM or with sBHI alone (control wells). The dish was then incubated at 35°C for 16 h in a $Versa_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA) set to read the absorbance at 600 nm every 10 min. Sensitivity was assessed using the relative OD_{600} values at the end of the incubation period.

Hydrogen peroxide sensitivity. To determine the sensitivity of the *dsbA* deletion mutant to H_2O_2, strains Rd, RXV, RdsbAV, and RdsbAC were inoculated using inocula from overnight cultures in triplicate into 25 ml of sBHI in 50-ml Erlenmeyer flasks or into 5 ml of sBHI in culture tubes to obtain an OD_{600} of 0.01. The resulting cultures were incubated aerobically at 35°C with shaking at 250 rpm (flasks) or in an anaerobic chamber (culture tubes) with BBL GasPak Plus generators (Becton, Dickinson and Company, Sparks, MD). When cultures reached the log phase, they were diluted in sBHI to obtain an OD_{600} of 0.02, and 100 μl of each culture was seeded into a 96-well flat-bottom dish. Hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) diluted in 100 μl of sBHI was then added to cultures grown in 25-ml flasks at final concentrations of 0, 62.5, 125, and 250 μM in sBHI and to anaerobically grown cultures at final concentrations of 0, 62.5, 125, and 500 μM. The dishes were then incubated at 35°C for 16 h in a microplate reader, and the absorbance at 600 nm was determined every 10 min to evaluate the growth rates and final culture densities.

Growth of *dsbA* strains. To determine the growth rates in rich media and in defined media, strains were inoculated in triplicate to obtain an OD_{600} of 0.01 by using inocula from standing overnight cultures into 25-ml Erlenmeyer flasks containing 15 ml of sBHI and MIc, respectively. The resulting cultures were incubated at 35°C with shaking at 250 rpm, and aliquots were removed to determine the absorbance at 600 nm every 30 min for 6.5 h. Growth rates were calculated by nonlinear regression analysis.

To evaluate the growth of the *dsbA* mutant in comparison to the growth of the *hbpA* mutant under heme limitation conditions, strains RXV, RdsbAV, RhbpAV, and RhbpAC were grown in standing overnight cultures, washed once in sterile Hanks' balanced salt solution (HBSS) (Invitrogen, Carlsbad, CA), and diluted to obtain an OD_{600} of 0.01 in BHI broth supplemented with NAD and different concentrations (5, 0.5, 0.25, and 0.025 μg/ml) of heme (Sigma-Aldrich, St. Louis, MO) or hemoglobin (Becton, Dickinson and Company) in a 96-well microplate (final volume, 200 μl). The cultures were then incubated at 35°C in the microplate reader, and the absorbance at 600 nm was determined every 10 min for 16 h.

Growth of *hbpA* strains. To compare the generation times obtained with different heme concentrations under anaerobic and aerobic conditions, overnight cultures of strains Rd, RdV, RhbpAV, and RhbpAC (pelleted and resuspended in HBSS) were used to inoculate 10 ml of BHI containing different concentrations of free heme (10, 0.5, 0.05, and 0 μg/ml). The cultures were then aliquoted into the wells of 11 96-well flat-bottom dishes. One dish was incubated at 35°C for 14 h in the microplate reader, and the absorbance at 600 nm was determined every 10 min (no aerobic growth was detected in wells not supplemented with heme). The other 10 dishes were sealed in individual BD GasPak EZ Anaerobe gas-generating pouches (Becton, Dickinson and Company) and incubated at 35°C. Dishes were removed from the pouches at appropriate intervals, and the absorbance at 600 nm was recorded. Growth rates were determined as described above.

Competence assay. Cultures were grown in triplicate as described above for the DTT sensitivity assay, and competent cells were prepared from these cultures as previously described (7). The competence of mutant and parental strains was measured by assessing the transformation frequencies with chromosomal DNA from a streptomycin-resistant (Sm^r) *H. influenzae* strain (1 μg) and selection on sBHI agar plates containing 100 μg/ml streptomycin. Transformation efficiencies were calculated by dividing the number of Sm^r colonies by the number of colonies on sBHI agar plates without antibiotic. Transformation frequencies were normalized by $\log_{10}$ transformation and analyzed with Prism 4.0c (GraphPad Software, San Diego, CA) using analysis of variance (ANOVA) with Bonferroni's multiple-comparison test to evaluate differences in frequency between RdsbAV and all other strains.

Murine bacteremia model. Standing overnight cultures of strains having an OD_{600} of 0.01 were inoculated into 10 ml of sBHI in culture tubes. The resulting cultures were incubated in an anaerobic chamber with shaking at 120 rpm and 35°C for 5 h, conditions that were permissive for growth of the *hbpA* mutant. For coinfection, each experimental strain was mixed with the RdlacZ reference strain at a 1:1 ratio. Prior to inoculation, bacteria were washed and diluted in HBSS to obtain a final concentration of 2×10^9 bacteria per ml. Female 6.5-week-old C57BL/6J mice (four or five mice per strain; The Jackson Laboratory, Bar Harbor, ME) were inoculated by intraperitoneal (i.p.) injection of 200 μl of a bacterial suspension. Twenty-four hours after inoculation, 5 μl of blood was recovered aseptically from each mouse via tail bleeding. The blood was diluted into BHI broth, plated on sBHI agar plates for single-strain infections or on sBHI agar plates containing S-Gal (3,4-cyclohexenoesculetin β-D-galactopyranoside; Sigma-Aldrich) for coinfections, and incubated overnight in an anaerobic chamber at 35°C to determine the number of CFU. For statistical analysis, the numbers of CFU/ml for single-strain infections were normalized by $\log_{10}$ transformation for ANOVA using Prism 4.0c. The coinfection CFU data were $\log_{10}$ transformed, and the ratio of each experimental strain to RdlacZ was calculated and analyzed using Prism 4.0c. Comparisons of two data sets were performed using the *t* test, and comparisons of more than two data sets were performed using ANOVA with Bonferroni's multiple-comparison test. All procedures with animals were conducted in accordance with NIH guidelines and with prior approval by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Infant rat infections. *H. influenzae* type b-derived strains were inoculated using inocula from standing overnight cultures having an OD_{600} of 0.01 into 50 ml of sBHI in 50-ml Erlenmeyer flasks. Cultures were incubated with shaking at 120 rpm at 35°C to obtain an OD_{600} of 0.4. Cells were washed once and diluted in sterile HBSS to obtain a final concentration of 2×10^3 bacteria per ml. Five-day-old Sprague-Dawley rat pups (Charles River Laboratories, Boston, MA) were inoculated i.p. with 100 μl of strains HXV ($n = 11$) and HdsbAV ($n = 11$) or with HdsbAC ($n = 12$). Infants inoculated i.p. with each strain were returned to their mothers, and each group was housed separately. Blood (5 μl) was collected aseptically via tail bleeding at 12, 36, and 120 h postinoculation, diluted into BHI, and plated on sBHI agar plates for to determine the number of CFU as described above. For statistical analysis, ANOVA with Bonferroni's multiple-comparison test was used as described above.

HbpA Western blotting. For analysis of HbpA, strains were inoculated using inocula from standing overnight cultures into duplicate 50-ml sBHI cultures in 50-ml flasks to obtain a starting density of 0.01 OD_{600} and were incubated at 35°C with shaking at 250 rpm. When cultures reached the log phase, 1 ml was removed and pelleted by centrifugation (18,000 × *g* for 5 min) for immunoblot analysis, and the remaining culture was used for RNA isolation as described below. After removal of the supernatant, the pellets were normalized by resuspension in an appropriate volume of HBSS. Cells (0.3 OD_{600} equivalents per lane) were then boiled in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer, and proteins were separated by 8% SDS-PAGE, followed by electrotransfer to Immobilon-P (Millipore, Billerica, MA). HbpA-HA was visualized by Western blotting using the primary antibody anti-HA1.1 (1:1000; Covance, Berkeley, CA) and the secondary antibody goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (1:5,000; Upstate, Lake Placid, NY). Equal sample concentrations were verified by Coomassie blue staining. HbpA-HA was quantified by generating a 10% dilution series of each protein sample and resolving proteins by 8% SDS-PAGE. HbpA-HA was then visualized by Western blotting as described above. HbpA levels were quantified by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

qRT-PCR. To quantify *hbpA* mRNA, we isolated total RNA in parallel from the 50-ml cultures that were used for the HbpA Western blot analysis using the TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was then treated with DNase

TABLE 1. Strains and plasmids used in this work

Plasmid or strain	Relevant features, genotype, and/or description	Reference
Plasmids		
pXT10	Delivery vector for chromosomal expression at the xylose locus of *H. influenzae* containing *xylF*, *xylB*, $xylA_{\Delta 4\text{-}804}$, and the *tetAR* tetracycline resistance cassette	71
pXyldsbA1.1	pXT10 carrying *dsbA* expressed from the *xylA* promoter	This study
pDsbA1.2	pXT10 carrying *dsbA* expressed from the *dsbA* promoter	This study
pXhbp1.5	pXT10 carrying an HA-tagged *hbpA* gene expressed from the *hbpA* promoter	This study
Strains		
Rd	Wild type; *H. influenzae* capsule-deficient type d	72
RXT10	Rd $xylA_{\Delta 4\text{-}804}$::*tetAR*; Rd carrying empty Tet^r vector sequence from pXT10 inserted at the *xyl* locus in place of *xylA*	71
RX	Rd $xylA_{\Delta 4\text{-}804}$::*dsbA*; Rd carrying *dsbA* expressed via the *xylA* promoter from pXyldsbA1.1 replacing *xylA*	This study
RdsbAX	RX *dsbA*::*aacC1*; *dsbA* deletion mutant carrying *dsbA* expressed via the *xylA* promoter from pXyldsbA1.1 replacing *xylA*	This study
RXV	Rd $xylA_{\Delta 4\text{-}804}$::*aphI*; Rd carrying the Km^r cassette replacing *xylA*	This study
RdsbAV	RdsbAX $xylA_{\Delta 4\text{-}804}$::*aphI*; *dsbA* deletion mutant carrying the Km^r cassette replacing *xylA*	This study
RdsbAC	RdsbAX $xylA_{\Delta 4\text{-}804}$::*dsbA*; *dsbA* mutant complemented with *dsbA* expressed via the *dsbA* promoter from pDsbA1.2 replacing *xylA*	This study
RhbpA	*hbpA*::*aphI*; *hbpA* deletion mutant	This study
RhbpAV	RhbpA $xylA_{\Delta 4\text{-}804}$::*tetAR*; *hbpA* deletion mutant carrying empty Tet^r vector sequence from pXT10 replacing *xylA*	This study
RhbpAC	RhbpAV $xylA_{\Delta 4\text{-}804}$::*hbpA*; *hbpA* mutant carrying *hbpA* expressed via the *hbpA* promoter from pXhbp1.5 replacing *xylA*	This study
RhbpACΔ*dsbA*	RhbpAC *dsbA*::*aacC1*; *hbpA dsbA* double mutant carrying *hbpA* expressed via the *hbpA* promoter from pXhbpA1.5 replacing *xylA*	This study
RdlacZ	Rd $xylA_{\Delta 4\text{-}804}$::*lacZ*; Rd carrying a copy of *lacZ* replacing *xylA*	72
Hib	Wild type; *H. influenzae* type b Eagan	
HXV	Hib $xylA_{\Delta 4\text{-}804}$::*aphI*; Hib carrying the Km^r cassette replacing *xylA*	This study
HdsbAV	Hib *dsbA*::*aacC1* $xylA_{\Delta 4\text{-}804}$::*aphI*; *dsbA* deletion mutant carrying the Km^r cassette replacing *xylA*	This study
HdsbAC	Hib *dsbA*::*aacC1* $xylA_{\Delta 4\text{-}804}$::*dsbA*; *dsbA* mutant complemented with *dsbA* expressed via the *dsbA* promoter from pDsbA1.2 replacing *xylA*	This study

I (Ambion, Austin, TX), extracted with acid phenol, extracted with chloroform, and concentrated by ethanol precipitation. The RNA samples (total amount, 5 μg) were used as templates for cDNA synthesis with random primers (New England Biolabs, Beverley, MA) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). Quantitative reverse transcription PCR (qRT-PCR) was performed with iQ SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA), and fluorescence was measured using the DNA Engine Opticon II system (MJ Research, Waltham, MA). One-tenth of each cDNA reaction mixture was used as a template for qRT-PCR performed with primers 5′hbpART (5′-ATG ATTAATTTGTTATAATCCATAGA-3′) and 3′hbpART (5′-CAAGCTGCCA AAACAAGAGT-3′), which amplified the first 200 bp of *hbpA*. Primers RpoA5′ (5′-GTAGAAATTGATGGCGTATTG-3′) and RpoA3′ (5′-TCACCATCATA GGTAATGTCC-3′) were used to amplify the RNA polymerase alpha subunit gene, *rpoA*, as an internal reference. The real-time cycler conditions used have been described previously (72).

Complement binding. Western blotting for assessment of binding of complement C3 and C4 activation products was performed as previously described (19, 56). Briefly, cultures of strains RXV, RdsbAV, and RdsbAC were grown as described above for HbpA Western blotting and then washed and suspended in HBSS containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ (final reaction mixture volume, 0.5 ml). Normal human serum (NHS) pooled from 12 healthy individuals was added to a final concentration of 2% and incubated for 30 min at 37°C, which was followed by differential treatment with 1 M methylamine (pH 11), which dissociates complement ester linkages but not amide-linked complement from target structures (19, 56). Bacteria were lysed in 1× SDS-PAGE sample buffer and analyzed by immunoblotting using primary antibodies to human C3 (Sigma-Aldrich, St. Louis, MO) and C4 (Biodesign, Saco, ME) and alkaline phosphatase-conjugated secondary anti-human antibodies as described previously (19). No differences in the binding profiles of the strains to C3 or C4 subunits with and without methylamine treatment were observed.

Serum bactericidal assay. The sensitivity of *dsbA* mutants to serum was determined as previously described (57). Briefly, triplicate cultures of strains RXV, RdsbAV, RdsbAC, and RdgalU were grown as described above for the DTT sensitivity assay. At log phase, 2,000 CFU from each culture was diluted in HBSS and incubated at 37°C for 30 min with or without 2% (final concentration) NHS in a 150-μl reaction mixture. To determine the number of CFU, 15 μl was plated on sBHI agar. Bacteria were also incubated in parallel with serum that had been previously inactivated by incubation at 56°C for 30 min.

Thiol modification. Ten optical density units of cells grown as described above for anti-HA immunoblotting was harvested at log phase by centrifugation at 5,000 × *g* for 5 min. Before thiol modification of periplasmic proteins, the outer membrane was disrupted using the methods described in a PeriPreps periplasting kit (Epicenter, Madison, WI). Briefly, the cell pellets were resuspended in 2 ml of 200 mM Tris (pH 7.4), 1 mM EDTA, 20% sucrose, and 30 U of lysozyme (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 5 min. After incubation, 3 ml of cold water was added, which was followed by 10 min of incubation on ice. Each 5-ml preparation was then divided in half; one half was treated with 5 mM EZ-Link maleimide-(ethylene oxide)$_2$-biotin (MPB) (which added 525.23 Da per bond) (Pierce, Rockford, IL), and the other half was not treated. After incubation for 50 min at room temperature, the resulting spheroplasts and associated membranes were collected by centrifugation at 4,000 × *g* for 15 min and resuspended in 375 μl of Peripreps lysis buffer (10 mM Tris-HCl [pH 7.5], 50 mM KCl, 1 mM EDTA, 0.1% deoxycholate). After lysis, equivalent 0.30 OD_{600} of each sample was boiled for 5 min in SDS loading buffer, and proteins were separated by nonreducing 8% SDS-PAGE. HbpA-HA was then visualized by Western blotting as described above. The apparent levels of HbpA-HA in the spheroplasts were similar to those in whole-cell lysates of the same number of cells (data not shown), suggesting that HbpA-HA is localized primarily in this fraction, which is consistent with membrane localization of the predicted HbpA lipoprotein.

RESULTS

Phenotypic properties of a nonpolar *dsbA* deletion mutant. A series of strains were constructed to evaluate the potential role of DsbA in *H. influenzae* pathogenesis (Table 1). We first

verified that the *dsbA* mutant exhibited the DTT sensitivity phenotype previously seen with *dsbA* mutants of other species (43), and we determined its growth properties. Wild-type parent strain Rd, Rd carrying the "empty vector" (RXV), a *dsbA* deletion mutant carrying the "empty vector" (RdsbAV), and the complemented strain (RdsbAC) were evaluated to determine their growth under a range of conditions and to determine defects in DTT resistance and transformation. The generation times under aerobic conditions in rich medium (sBHI) for RXV, RdsbAV, and RdsbAC were 32 ± 2, 32 ± 3, and 36 ± 4 min, respectively, and the generation times in defined medium (MIc) were 48 ± 4, 41 ± 2, and 38 ± 3 min, respectively. Similarly, the growth yields of these strains after 6.5 h of anaerobic growth in sBHI were indistinguishable. The growth yields of all $DsbA^+$ strains (Rd, RXV, and RdsbAC) were equivalent after 16 h in the presence of 5 mM DTT, with final average densities of ~0.5 OD_{600}, whereas the growth of the $DsbA^-$ strain, RdsbAV, was dramatically attenuated under these conditions and the density did not exceed 0.1 OD_{600}, similar to results obtained with *dsbA* mutants of *E. coli* (43). Strain RdsbAX, which contains a D-xylose-inducible copy of *dsbA* and was used to construct strains RdsbAV and RdsbAC, was resistant to DTT in the presence of 1 mM D-xylose and sensitive to DTT in the absence of D-xylose.

H. influenzae dsbA (*por*) was previously implicated in natural transformation. Therefore, we evaluated the transformation efficiencies of our strains using *H. influenzae* DNA carrying a streptomycin resistance allele. The transformation efficiencies relative to the wild-type parental strain Rd for strains RXV, RdsbAV, and RdsbAC, were 1.12, 1.33×10^{-6}, and 1.04, respectively, and the 6-log-lower transformation frequency of the *dsbA* mutant (RdsbAV) relative to the other strains was statistically significant ($P < 0.0001$). Therefore, previously reported phenotypes associated with *dsbA* mutants were observed with our in-frame *H. influenzae dsbA* deletion mutants, and complemented strains exhibited the wild-type phenotypes.

DsbA is required during *H. influenzae* infection in mice. The strains generated as described above provided a well-defined set of mutants for investigation of the role of *dsbA* during infection. Although not a recent clinical isolate, Rd has virulence properties similar to those of clinically important NTHi strains in several models of infection and has provided a useful system for studies of *H. influenzae* biology and pathogenesis (16, 40). The mouse model was used to evaluate bloodstream survival of the *dsbA* mutant, RdsbAV, compared to that of the vector-only control strain, RXV. At 24 h postinoculation, 48-fold-fewer bacterial CFU were recovered from mice inoculated with the *dsbA* mutant than from mice inoculated with the control strain (Fig. 1A). The level of bacteria recovered from most of the mice inoculated with RdsbAV was close to the limit of detection. An additional experiment was conducted to confirm this result with a complemented strain, RdsbAC. To further assess the level of attenuation, this experiment was performed as a competition between each strain and strain RdlacZ, which expresses *E. coli lacZ* at the *xyl* locus, using mixed infections. Consistent with results obtained from single-strain inoculations, the competitive index of RdsbAV was 100- to 170-fold less than that of Rd, RXV, or RdsbAC (Fig. 1B). Therefore, infection with a mutant containing a nonpolar *dsbA* deletion resulted in reduced levels of bacteremia in mice, and

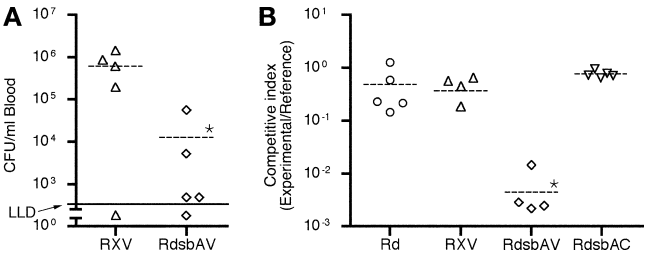

FIG. 1. Effect of *dsbA* mutation on survival of *H. influenzae* in the mouse model of bacteremia. Strains were inoculated i.p. into mice, and bacteremia was assessed after 24 h. The symbols indicate data for individual animals, and the dashed lines indicate the averages. The lower limit of detection (LLD) was 500 CFU/ml. Values less than the lower limit of detection indicate that bacteremia was not detected. (A) Single-strain infection. The asterisk indicates that the *P* value is 0.039 (*t* test). (B) Coinfections with the experimental strains indicated and reference strain RdlacZ. The competitive index is the ratio of the $LacZ^-$ experimental strain to $LacZ^+$ reference strain RdlacZ. The asterisk indicates that the *P* value is <0.001 (ANOVA with Bonferroni's multiple-comparison test).

complementation verified that this effect was specific to the *dsbA* mutation.

Pathogenesis-associated phenotypes of the *dsbA* mutant. *H. influenzae* lacks previously implicated *dsbA*-dependent virulence factors found in other species, including exotoxins and type III secretion structures. Production of the type IV pilus found in some NTHi strains is likely to require *dsbA*; however, the pilus gene cluster is absent in Rd (20). Therefore, we examined several major virulence-associated phenotypes of *H. influenzae* to determine whether a defect in a known pathogenic mechanism could account for the survival defect of the *dsbA* mutant in vivo.

Resistance to oxidative stress generated by hydrogen peroxide exposure has been correlated with *H. influenzae* pathogenesis in several studies (17, 72). Therefore, we addressed the possibility that loss of DsbA confers sensitivity to this oxidant. After exposure to either anaerobic or aerobic pregrowth conditions, the mutant and the wild type exhibited equal levels of growth inhibition during exposure to hydrogen peroxide at a range of doses (data not shown).

Multiple structures of the lipooligosaccharide (LOS) outer core have been implicated in animal models of *H. influenzae* bacteremia (25, 31, 60), and resistance to complement has emerged as an important virulence mechanism mediated by these structures (19, 30). Therefore, we investigated whether the *dsbA* mutant exhibits major LOS structural alterations or increased susceptibility to killing by serum complement. We detected no apparent differences in LOS mobility on SDS-PAGE gels for the *dsbA* mutant and the wild type. Wild-type, *dsbA* mutant, and complemented strains were compared using a serum bactericidal assay with 2% pooled NHS. The levels of survival for strains RdV, RdsbAV, and RdsbAC were 12, 2.9, and 11.6%, respectively ($P < 0.05$). For comparison, a *galU* mutant deficient in synthesis of the LOS outer core, a structure predicted to be essential for complement resistance, was tested in parallel and exhibited 0% survival. No killing of *H. influenzae* was observed with heat-inactivated serum, consistent with an essential role for complement in this assay. Differences in levels of complement binding to strains RdV, RdsbAV, and

TABLE 2. Potential DsbA targets

Putative DsbA target in *E. coli*	Known or proposed function in *E. coli*	Potential *H. influenzae* homolog	BLAST identity (%)	Expect value
ArtJ	Arginine ABC transporter periplasmic binding protein	ArtI (HI1179)	115/244 (47)	7e-57
DppA	Dipeptide/heme binding protein	HbpA (HI0853)	282/517 (54)	3e-168
Imp/OstA	Organic solvent tolerance protein	OstA (HI0730)	372/791 (47)	0
MepA	Penicillin-insensitive murein endopeptidase	MepA (HI0197)	113/271 (49)	2e-75
OmpA	Outer membrane porin	P5 (HI1164)	169/371 (45)	2e-67
OppA	Oligopeptide transporter periplasmic binding protein	OppA (HI0213)	281/531 (52)	5e-157
ZnuA	Zinc uptake system periplasmic binding protein	ZnuA (HI0119)	140/341 (41)	6e-70

RdsbAC were not detected, as assessed on anti-C3 and anti-C4 immunoblots containing lysates of cells that had been incubated with 2% pooled NHS (data not shown), although it is possible that a small difference in C3 or C4 binding not detected by immunoblotting could have mediated the moderate increase in serum sensitivity observed for the mutant.

We concluded that the hydrogen peroxide resistance and LOS production of the *dsbA* mutant were not markedly impaired under the conditions tested. An effect on serum resistance that could play a role was observed. However, this effect was moderate, and it seems likely that DsbA influences additional factors required for virulence. To address this hypothesis, we sought the identities of potential DsbA substrates in *H. influenzae*. Proteins containing DsbA-dependent disulfide bonds have been identified in *E. coli* (29, 35, 39). These proteins were compared by BLASTP (1) (http://www.ncbi.nlm.nih.gov) to the predicted proteins in the *H. influenzae* genome to derive a list of potential DsbA targets in *H. influenzae* (Table 2). The *H. influenzae* proteins identified by this search include a predicted periplasmic lipoprotein, HbpA, which is required for utilization of multiple heme sources (27, 45). Multiple systems participate in scavenging heme from sources in the host that include heme-hemopexin, hemoglobin, hemoglobin-haptoglobin, heme-albumin, and free heme (14, 46). HbpA appears to be required for scavenging low levels of heme regardless of the source or carrier protein, suggesting that it could be critical for growth in vivo. The link between DsbA and HbpA suggested a potential mechanism of attenuation of the *dsbA* mutant in the mouse model.

Heme uptake protein HbpA is a target of disulfide oxidoreductase. Based on comparison to the crystal structure of the highly related *E. coli* DppA protein, which has an intramolecular disulfide bond (52), HbpA has a predicted disulfide bond between cysteine residues Cys27 and Cys255 of the mature protein. A third cysteine located at the N terminus constitutes the predicted lipoprotein acylation site. Many proteins containing DsbA-dependent disulfide bonds are less stable in DsbA-deficient cells. Therefore, we examined the effect of the *dsbA* deletion mutation on levels of HbpA. To address this question, we developed a functional derivative of HbpA fused to an epitope tag from the influenza virus HA (HbpA-HA). We first constructed a nonpolar *hbpA* deletion mutant (RhbpAV) of *H. influenzae*. The mutant was defective for aerobic growth on medium containing low levels of heme, as previously reported for an independently derived *hbpA* insertional mutant (45). Furthermore, the *hbpA* mutant exhibited anaerobic growth equivalent to that of wild-type strain Rd and the isogenic "vector-only" strain (RdV), regardless of heme availability (Table 3). When expressed in the *hbpA* mutant background, HbpA-HA fully complemented the mutant for aerobic growth at all heme concentrations tested (strain RhbpAC), suggesting that the epitope tag does not impair its function (Table 3).

The resulting strains were used to assess the effect of a *dsbA* mutation on levels of HbpA-HA on Western blots, and transcript levels were assessed in parallel by qRT-PCR. The levels of HbpA-HA in the *dsbA* deletion mutant RhbpACΔ*dsbA* were approximately 50% of the levels detected in the DsbA$^+$

TABLE 3. Growth phenotypes of *hbpA* mutants

Strain	Generation time (min)						
	Aerobic conditions with free heme supplement at a concn of[a]:			Anaerobic conditions with free heme supplement at a concn of[b]:			
	10 μg/ml	0.5 μg/ml	0.05 μg/ml	10 μg/ml	0.5 μg/ml	0.05 μg/ml	0 μg/ml
Rd	46 ± 3	47 ± 3	57 ± 4	57 ± 8	56 ± 1	61 ± 2	64 ± 3
RdV	45 ± 1	45 ± 2	50 ± 3	65 ± 13	62 ± 7	65 ± 6	73 ± 3
RhbpAV	41 ± 3	55 ± 7	NG[c]	49 ± 4	54 ± 2	55 ± 4	56 ± 5
RhbpAC	46 ± 3	48 ± 2	53 ± 1	61 ± 4	60 ± 2	66 ± 1	66 ± 2

[a] Growth was determined with a microplate reader.
[b] Growth was determined in an anaerobic chamber.
[c] NG, no growth.

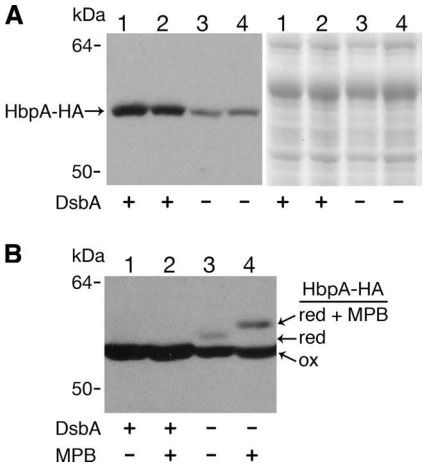

FIG. 2. Effects of *dsbA* mutation on HbpA protein levels and thiol redox state. (A) Detection of HbpA levels in DsbA$^+$ and DsbA$^-$ strains. Whole-cell lysates of duplicate cultures of RhbpAC (complemented *hbpA* deletion mutant carrying *hbpA*-HA in the *xyl* locus) (lanes 1 and 2) and RhbpACΔ*dsbA* (RhbpAC with *dsbA* deletion) (lanes 3 and 4) were resolved by 8% SDS-PAGE under reducing conditions and detected by anti-HA Western blotting (left panel). Equal sample concentrations were verified by Coomassie blue staining (right panel). (B) Differential modification of thiols on HbpA in the *dsbA* mutant compared to the wild type. Spheroplasts were prepared from log-phase cultures, resolved by 8% SDS-PAGE under nonreducing conditions, and detected by anti-HA Western blotting. The strains were the same as those used for panel A and were treated (+) or not treated (−) with 5 mM MPB as indicated. The arrows indicate the oxidized form of HbpA (ox), the reduced form of HbpA (red), and the reduced form of HbpA with thiols modified with MPB (red + MPB).

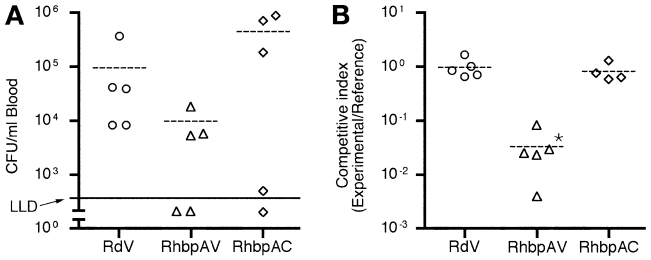

FIG. 3. Effect of *hbpA* mutation on survival of *H. influenzae* in the mouse model of bacteremia. Strains were inoculated i.p. into mice, and bacteremia was assessed after 24 h. The symbols indicate data for individual animals, and the dashed lines indicate the averages. The lower limit of detection (LLD) was 500 CFU/ml. Values less than the lower limit of detection indicate that bacteremia was not detected. (A) Single-strain infection. (B) Coinfections with the experimental strains indicated and reference strain RdlacZ. The competitive index is the ratio of the LacZ$^-$ experimental strain to LacZ$^+$ reference strain RdlacZ. The asterisk indicates that the *P* value is <0.001.

control strain, RhbpAC, as determined by densitometry (Fig. 2A). qRT-PCR detected no differences in the levels of *hbpA*-specific transcripts in these cultures (data not shown). Together, these results suggest that the effect of DsbA on HbpA abundance is mediated at a posttranscriptional level, consistent with its role as a disulfide oxidoreductase.

To more directly assess the role of DsbA in formation of disulfide bonds in HbpA, the HbpA-HA protein was analyzed by nonreducing SDS-PAGE after isolation from the DsbA$^-$ and DsbA$^+$ *H. influenzae* strains (Fig. 2B). Whereas HbpA-HA from DsbA$^+$ cells appeared as a single band, samples from DsbA$^-$ cells yielded an additional HbpA-HA band with lower electrophoretic mobility. Treatment of cells prior to protein isolation with a thiol reactive ligand, MPB, resulted in no change in HbpA-HA in the parental strain (Fig. 2B, lanes 1 and 2), as expected if the two nonacylated cysteine residues were in the oxidized state as a disulfide bond. In contrast, the more slowly migrating species in the *dsbA* mutant (Fig. 2B, lane 3) exhibited an additional decrease in mobility in samples from MPB-treated cells (Fig. 2B, lane 4), consistent with addition of MPB to free thiols on cysteine residues of this protein. Relatively low levels of the reduced form of HbpA were detected, consistent with decreased stability of the reduced form relative to the oxidized form in the *dsbA* mutant, a characteristic property of many DsbA-dependent proteins (9). Therefore, a longer exposure time was used to clearly visualize the reduced form in Fig. 2B, masking the decrease in total HbpA levels that was detected in the *dsbA* mutant in the quantitative studies described above (Fig. 2A).

HbpA in the oxidized form was detected in the *dsbA* mutant, and it is likely that some HbpA activity was retained in this mutant. Consistent with this observation, we could not detect a growth defect of the *dsbA* mutant on low-heme media. The growth rates of DsbA$^+$ and DsbA$^-$ strains (RXV and RdsbAV) were compared to those of the *hbpA* mutant and complemented strains (RhbpAV and RhbpAC) with 5, 0.5, 0.25, and 0.025 μg/ml of either heme or heme-hemoglobin (data not shown). RhbpAV exhibited progressively lower growth rates as the heme or hemoglobin concentration was decreased, and RhbpAC grew at the same rates as the wild type, similar to results shown in Table 3. Conversely, no differences between RXV and RdsbAV were observed, suggesting that the residual levels of active HbpA in the *dsbA* mutant were sufficient for acquisition of these heme sources in vitro. Together, these data indicate that the DsbA disulfide oxidoreductase is required to maintain the complete oxidation of free thiols on HbpA and for wild-type levels of this protein in *H. influenzae*.

HbpA is required during bloodstream infection. Heme is required for aerobic growth and is obtained by *H. influenzae* from sources within the host. The decreased levels of HbpA observed in the *dsbA* mutant could have contributed to decreased survival of this strain in the bloodstream by interfering with heme acquisition in vivo, where heme is efficiently sequestered by multiple systems of the host. To evaluate this hypothesis, we assessed the role of *hbpA* in the mouse model using the *hbpA* mutant RhbpAV, the isogenic HpbA$^+$ parent strain RdV, and the complemented strain RhbpAC. Inocula were prepared from cultures grown anaerobically, conditions which were permissive for growth of the *hbpA* mutant (Table 3), and mice were inoculated by the i.p. route (Fig. 3). In single-strain infections there was a decrease in the number of bacterial CFU recovered from mice inoculated with RhbpAV compared to mice inoculated with RdV (17-fold) or RhbpAC, (~60-fold); however, the trend was not statistically significant (Fig. 3A). To control for variation between animals, we repeated the experiment using the competition format. Each strain was coinoculated with an equal number of cells of strain RdlacZ, and competitive indices were evaluated. The mutant exhibited a ~27-fold defect in competition relative to the "vector-only"

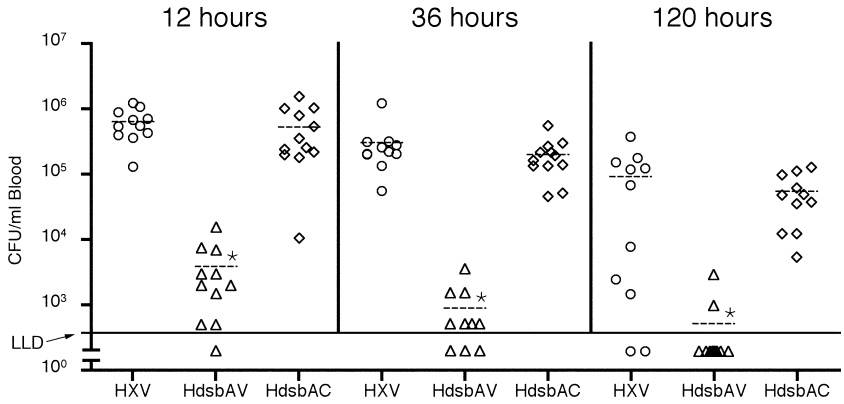

FIG. 4. Effect of *dsbA* mutation on the virulence of *H. influenzae* type b in infant rats. Strains were inoculated i.p. into 5-day-old infant rats. The symbols indicate data for individual animals, and the dashed lines indicate the averages. An asterisks indicates that the *P* value is <0.001. The lower limit of detection (LLD) was 500 CFU/ml. Values less than the lower limit of detection indicate that bacteremia was not detected.

and complemented strains, and the differences were statistically significant (Fig. 3B). We concluded that survival of the *hbpA* mutant is attenuated in the bacteremia model, but to a lesser extent than survival of the *dsbA* mutant. Therefore, a decreased level of HbpA could contribute to the defect in *dsbA* mutants during infection, yet additional factors, such as serum sensitivity and other mechanisms that remain to be identified, are likely involved.

DsbA is required for growth and persistence of virulent *H. influenzae* type b in the bloodstream. We next addressed whether *dsbA* is required during infection by the highly virulent organism *H. influenzae* type b strain Eagan. The infant rat bacteremia model provides a well-characterized system for examining factors required for *H. influenzae* type b pathogenesis. Therefore, the mutations used to evaluate the role of *dsbA* in Rd were moved into the Hib background. Infant rats that were 5 days old were inoculated i.p. with wild-type "vector-only" strain HXV, *dsbA* mutant HdsbAV, and complemented strain HdsbAC, and bloodstream infection was monitored at 12, 36, and 120 h postinoculation (Fig. 4). At all sampling times, the number of *H. influenzae* CFU recovered from animals inoculated with the *dsbA* mutant was at least 100-fold lower than the number of *H. influenzae* CFU recovered from animals inoculated with the parental or complemented strain, and the level of attenuation was statistically significant in all cases. Furthermore, by 120 h postinoculation only 2 of 11 animals inoculated with the *dsbA* mutant had detectable bacteremia, whereas most of the animals infected with the wild-type strain (9/11) or the complemented strain (11/11) remained infected, with mean bacterial levels of 9.5×10^4 and 5.6×10^4 CFU/ml, respectively. These results indicate that *dsbA* is required for efficient production and persistence of a high level of bacteremia in the infant rat model with a virulent clinical isolate of *H. influenzae* type b.

DISCUSSION

We report a role for the *H. influenzae* disulfide oxidoreductase, DsbA, in bloodstream infection. Nonpolar *dsbA* deletion mutations in either the strain Rd or *H. influenzae* type b strain Eagan background resulted in equal levels of attenuation in animal models, and the virulence of complemented strains was equivalent to that of the parental strains. Because the in vivo defect was observed with *dsbA* mutants of both nonencapsulated strain Rd and an encapsulated *H. influenzae* type b strain, an effect on production of capsule would be unlikely to account for these observations. Therefore, we investigated several other potential mechanisms. The primary set of factors implicated in pathogenesis of nonencapsulated *H. influenzae* in animal models includes genes involved in LOS synthesis, evasion of complement deposition, and oxidative stress resistance. We detected no apparent role for *dsbA* in LOS synthesis or hydrogen peroxide resistance, although we cannot exclude the possibility that these phenotypes are influenced in a subtle way that our assays were not sufficiently sensitive to detect. A decrease in serum resistance was observed in the *dsbA* mutant, and it will be of interest to establish the mechanism by which DsbA contributes to this virulence-related trait. However, the effect on serum resistance was only moderate and does not seem to be sufficient to account for the full defect of the *dsbA* mutant in pathogenesis. The results suggest that an unrecognized factor(s) may account for the observed virulence defect of the *dsbA* mutants.

To expand our search to other factors that could participate in the defect of the *dsbA* mutant in vivo, we considered a set of potential secreted substrates of *H. influenzae* DsbA identified by comparison of amino acid sequences to reported DsbA targets in other species. The resulting list of potential DsbA substrates in *H. influenzae* includes a number of known or suspected nutrient transport proteins. Therefore, a nutritional deficiency could contribute to the defect of the *dsbA* mutant in the blood. We examined growth of the *H. influenzae dsbA* mutant under a range of in vitro conditions. The *H. influenzae dsbA* mutant grew normally under aerobic and anaerobic conditions and in a low-nutrient medium. The only in vitro condition under which we could detect a growth defect for the *dsbA* mutant involved the presence of a high concentration (5 mM) of DTT, a condition that *H. influenzae* is unlikely to encounter in vivo. Reducing agents are present in plasma and include glutathione, which occurs as a mixture of reduced and oxidized forms with a total concentration estimated to be ~5 to 30 μM (4). Growth in the presence of glutathione was tested

using concentrations ranging from 0.005 to 1 mM, and growth of the *dsbA* mutant and growth of the parental strain were equivalent under each of these conditions (data not shown). A general growth defect or sensitivity to physiological levels of reducing agents does not appear to account for the decreased virulence of our *dsbA* deletion mutants. If the effect of *dsbA* on pathogenesis involves a defect in nutrient uptake or utilization, then it is likely to involve a nutrient that is selectively limiting in *H. influenzae*'s environment within the host.

One essential factor that *H. influenzae* cannot synthesize and must obtain from the host is the porphyrin ring of heme. The results of an amino acid sequence comparison of *H. influenzae* proteins with known or probable DsbA substrates in other species identified the *H. influenzae* heme binding protein, HbpA, as a potential substrate of DsbA. Deletion of *hbpA* results in an aerobic growth defect on media containing low levels of exogenous heme sources (27, 45) and normal growth under anaerobic conditions (Table 3). We identified the presence of a DsbA-dependent disulfide bond in HbpA and decreased abundance of HbpA in the *dsbA* mutant. Therefore, we evaluated the phenotype of an *hbpA* mutant during infection. The *hbpA* mutant exhibited a defect in the murine bacteremia model, although it was not as pronounced as the defect of the *dsbA* mutant. Complementation restored the ability of the *hbpA* mutant to cause bacteremia at a level similar to that observed with the parental strain. These results suggest that decreased levels of HbpA could contribute to the in vivo defect of the *dsbA* mutants.

The decreased level of HbpA in the *dsbA* mutant would be expected to influence growth under heme limitation conditions; however, we were unable to detect such an effect in vitro. It is likely that residual HbpA activity in the *dsbA* mutant is capable of supporting in vitro growth with low levels of heme. Nevertheless, both DsbA and HbpA participate in bloodstream infection. For survival in vivo, where diverse host factors efficiently sequester free heme, there may be a more stringent requirement for wild-type levels of HbpA than there were the in vitro conditions tested here. Alternatively, it is possible that the in vivo growth defect of the *dsbA* mutant resulted from effects on a DsbA-dependent protein whose role in virulence remains to be identified. The partial attenuation of the *hbpA* mutant compared to the more dramatic virulence defect of the *dsbA* mutant supports the hypothesis that other factors are involved. In this regard, two potential DsbA targets in *H. influenzae*, Pzp1 (ZnuA) and outer membrane protein P5 (Table 2), are required for growth under zinc-limiting conditions in vitro (41) and adhesion to mucosal epithelium during colonization of the chinchilla nasopharynx (11), respectively. In addition, homologs of Pzp1 and P5 in several other bacterial species have been implicated in pathogenesis (2, 36, 59, 62, 69, 73). We did not observe a requirement for zinc supplementation for in vitro growth of the *dsbA* mutant (data not shown), potentially due to residual activity of Pzp1, yet is possible that the zinc levels available to bacteria within the mammalian host are lower than those in vitro. A defect in the level or activity of Pzp1 in the *H. influenzae dsbA* mutant could contribute to its virulence defect, and additional studies are required to evaluate this hypothesis. In addition, a role for P5 during *H. influenzae* bacteremia has not been reported, and it will be interesting to investigate this possibility. Related outer membrane proteins in other pathogens have been implicated in diverse aspects of pathogenesis, including complement resistance (59, 62, 69), and changes in the outer membrane protein profile of the *dsbA* mutant could account for similar effects in *H. influenzae*. The roles of the other potential DsbA substrates in *H. influenzae* (Table 2) have not been defined, and their putative homologs in *E. coli* do not appear to mediate virulence-related functions. Furthermore, the complete set of *H. influenzae* DsbA substrates remains to be determined experimentally. Investigation of the virulence properties conferred by proteins that contain DsbA-dependent disulfide bonds in *H. influenzae* will likely uncover important aspects of the pathogenesis of this bacterium and may lead to novel approaches to treatment or prevention of invasive disease.

ACKNOWLEDGMENTS

We thank Sanjay Ram for assistance and advice and Jeffrey Gawronski for helpful comments.

This work was supported in part by a grant from the American Heart Association and by NIH NIAID grant 1RO1-AI49437 to B.J.A.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ammendola, S., P. Pasquali, C. Pistoia, P. Petrucci, P. Petrarca, G. Rotilio, and A. Battistoni.** 2007. The high affinity Zn^{2+} uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to virulence of *Salmonella enterica*. Infect. Immun. **75:**5867–5876.
3. **Andersen, C. L., A. Matthey-Dupraz, D. Missiakas, and S. Raina.** 1997. A new *Escherichia coli* gene, dsbG, encodes a periplasmic protein involved in disulphide bond formation, required for recycling DsbA/DsbB and DsbC redox proteins. Mol. Microbiol. **26:**121–132.
4. **Anderson, M. E., and A. Meister.** 1980. Dynamic state of glutathione in blood plasma. J. Biol. Chem. **255:**9530–9533.
5. **Anderson, P., R. B. Johnston, Jr., and D. H. Smith.** 1972. Human serum activities against *Haemophilus influenzae*, type b. J. Clin. Investig. **51:** 31–38.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. E. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1995. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, NY.
7. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol. **204:**321–342.
8. **Bardwell, J. C., J. O. Lee, G. Jander, N. Martin, D. Belin, and J. Beckwith.** 1993. A pathway for disulfide bond formation in vivo. Proc. Natl. Acad. Sci. USA **90:**1038–1042.
9. **Bardwell, J. C. A., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation in vivo. Cell **76:**581–589.
10. **Bessette, P. H., J. J. Cotto, H. F. Gilbert, and G. Georgiou.** 1999. In vivo and in vitro function of the *Escherichia coli* periplasmic cysteine oxidoreductase DsbG. J. Biol. Chem. **274:**7784–7792.
11. **Bookwalter, J. E., J. A. Jurcisek, S. D. Gray-Owen, S. Fernandez, G. McGillivary, and L. O. Bakaletz.** 2008. A CEACAM1 homologue plays a pivotal role in nontypeable *Haemophilus influenzae* colonization of the chinchilla nasopharynx via the OMP P5-homologous adhesin. Infect. Immun. **76:**48–55.
12. **Bringer, M. A., N. Rolhion, A. L. Glasser, and A. Darfeuille-Michaud.** 2007. The oxidoreductase DsbA plays a key role in the ability of the Crohn's disease-associated adherent-invasive *Escherichia coli* strain LF82 to resist macrophage killing. J. Bacteriol. **189:**4860–4871.
13. **CDC.** 2002. Progress toward elimination of *Haemophilus influenzae* type b invasive disease among infants and children—United States, 1998–2000. MMWR Morb. Mortal. Wkly. Rep. **51:**234–237.
14. **Cope, L. D., R. Yogev, U. Muller-Eberhard, and E. J. Hansen.** 1995. A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J. Bacteriol. **177:**2644–2653.
15. **Cuthill, S. L., M. M. Farley, and L. G. Donowitz.** 1999. Nontypable *Haemophilus influenzae* meningitis. Pediatr. Infect. Dis. J. **18:**660–662.
16. **Daines, D. A., L. A. Cohn, H. N. Coleman, K. S. Kim, and A. L. Smith.** 2003. *Haemophilus influenzae* Rd KW20 has virulence properties. J. Med. Microbiol. **52:**277–282.
17. **D'Mello, R. A., P. R. Langford, and J. S. Kroll.** 1997. Role of bacterial Mn-cofactored superoxide dismutase in oxidative stress responses, nasopharyngeal colonization, and sustained bacteremia caused by *Haemophilus influenzae* type b. Infect. Immun. **65:**2700–2706.
18. **Erwin, A. L., K. L. Nelson, T. Mhlanga-Mutangadura, P. J. Bonthuis, J. L.**

Geelhood, G. Morlin, W. C. Unrath, J. Campos, D. W. Crook, M. M. Farley, F. W. Henderson, R. F. Jacobs, K. Muhlemann, S. W. Satola, L. van Alphen, M. Golomb, and A. L. Smith. 2005. Characterization of genetic and phenotypic diversity of invasive nontypeable *Haemophilus influenzae*. Infect. Immun. **73:**5853–5863.

19. **Figueira, M. A., S. Ram, R. Goldstein, D. W. Hood, E. R. Moxon, and S. I. Pelton.** 2007. Role of complement in defense of the middle ear revealed by restoring the virulence of nontypeable *Haemophilus influenzae siaB* mutants. Infect. Immun. **75:**325–333.
20. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
21. **Frech, C., M. Wunderlich, R. Glockshuber, and F. X. Schmid.** 1996. Preferential binding of an unfolded protein to DsbA. EMBO J. **15:**392–398.
22. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35–51.
23. **Gonzalez, M. D., C. A. Lichtensteiger, and E. R. Vimr.** 2001. Adaptation of signature-tagged mutagenesis to *Escherichia coli* K1 and the infant-rat model of invasive disease. FEMS Microbiol. Lett. **198:**125–128.
24. **Granick, S., and H. Gilder.** 1946. The porphyrin requirements of *Haemophilus influenzae* and some functions of the vinyl and propionic acid side chains of heme. J. Gen. Physiol. **30:**1–13.
25. **Griffin, R., C. D. Bayliss, M. A. Herbert, A. D. Cox, K. Makepeace, J. C. Richards, D. W. Hood, and E. R. Moxon.** 2005. Digalactoside expression in the lipopolysaccharide of *Haemophilus influenzae* and its role in intravascular survival. Infect. Immun. **73:**7022–7026.
26. **Ha, U. H., Y. Wang, and S. Jin.** 2003. DsbA of *Pseudomonas aeruginosa* is essential for multiple virulence factors. Infect. Immun. **71:**1590–1595.
27. **Hanson, M. S., C. Slaughter, and E. J. Hansen.** 1992. The *hbpA* gene of *Haemophilus influenzae* type b encodes a heme-binding lipoprotein conserved among heme-dependent *Haemophilus* species. Infect. Immun. **60:**2257–2266.
28. **Herbert, M. A., S. Hayes, M. E. Deadman, C. M. Tang, D. W. Hood, and E. R. Moxon.** 2002. Signature tagged mutagenesis of *Haemophilus influenzae* identifies genes required for *in vivo* survival. Microb. Pathog. **33:**211–223.
29. **Hiniker, A., and J. C. Bardwell.** 2004. In vivo substrate specificity of periplasmic disulfide oxidoreductases. J. Biol. Chem. **279:**12967–12973.
30. **Ho, D. K., S. Ram, K. L. Nelson, P. J. Bonthuis, and A. L. Smith.** 2007. lgtC expression modulates resistance to C4b deposition on an invasive nontypeable *Haemophilus influenzae*. J. Immunol. **178:**1002–1012.
31. **Hood, D. W., M. E. Deadman, A. D. Cox, K. Makepeace, A. Martin, J. C. Richards, and E. R. Moxon.** 2004. Three genes, *lgtF*, *lic2C* and *lpsA*, have a primary role in determining the pattern of oligosaccharide extension from the inner core of *Haemophilus influenzae* LPS. Microbiology **150:**2089–2097.
32. **Jackson, M. W., and G. V. Plano.** 1999. DsbA is required for stable expression of outer membrane protein YscC and for efficient Yop secretion in *Yersinia pestis*. J. Bacteriol. **181:**5126–5130.
33. **Jacob-Dubuisson, F., J. Pinkner, Z. Xu, R. Striker, A. Padmanhaban, and S. J. Hultgren.** 1994. PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA. Proc. Natl. Acad. Sci. USA **91:**11552–11556.
34. **Kadokura, H., F. Katzen, and J. Beckwith.** 2003. Protein disulfide bond formation in prokaryotes. Annu. Rev. Biochem. **72:**111–135.
35. **Kadokura, H., H. Tian, T. Zander, J. C. Bardwell, and J. Beckwith.** 2004. Snapshots of DsbA in action: detection of proteins in the process of oxidative folding. Science **303:**534–537.
36. **Kim, S., K. Watanabe, T. Shirahata, and M. Watarai.** 2004. Zinc uptake system (*znuA* locus) of *Brucella abortus* is essential for intracellular survival and virulence in mice. J. Vet. Med. Sci. **66:**1059–1063.
37. **Klein, J. O.** 1997. Role of nontypeable *Haemophilus influenzae* in pediatric respiratory tract infections. Pediatr. Infect. Dis. J. **16:**S5–8.
38. **Krupp, R., C. Chan, and D. Missiakas.** 2001. DsbD-catalyzed transport of electrons across the membrane of *Escherichia coli*. J. Biol. Chem. **276:**3696–3701.
39. **Leichert, L. I., and U. Jakob.** 2004. Protein thiol modifications visualized in vivo. PLoS Biol. **2:**e333.
40. **Leroy, M., H. Cabral, M. Figueira, V. Bouchet, H. Huot, S. Ram, S. I. Pelton, and R. Goldstein.** 2007. Multiple consecutive lavage samplings reveal greater burden of disease and provide direct access to the nontypeable *Haemophilus influenzae* biofilm in experimental otitis media. Infect. Immun. **75:**4158–4172.
41. **Lu, D., B. Boyd, and C. A. Lingwood.** 1997. Identification of the key protein for zinc uptake in *Hemophilus influenzae*. J. Biol. Chem. **272:**29033–29038.
42. **Miki, T., N. Okada, and H. Danbara.** 2004. Two periplasmic disulfide oxidoreductases, DsbA and SrgA, target outer membrane protein SpiA, a component of the *Salmonella* pathogenicity island 2 type III secretion system. J. Biol. Chem. **279:**34631–34642.
43. **Missiakas, D., C. Georgopoulos, and S. Raina.** 1993. Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds in vivo. Proc. Natl. Acad. Sci. USA **90:**7084–7088.
44. **Moller, L. V., A. G. Regelink, H. Grasselier, J. E. Dankert-Roelse, J. Dankert, and L. van Alphen.** 1995. Multiple *Haemophilus influenzae* strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. J. Infect. Dis. **172:**1388–1392.
45. **Morton, D. J., L. L. Madore, A. Smith, T. M. Vanwagoner, T. W. Seale, P. W. Whitby, and T. L. Stull.** 2005. The heme-binding lipoprotein (HbpA) of *Haemophilus influenzae*: role in heme utilization. FEMS Microbiol. Lett. **253:**193–199.
46. **Morton, D. J., A. Smith, Z. Ren, L. L. Madore, T. M. VanWagoner, T. W. Seale, P. W. Whitby, and T. L. Stull.** 2004. Identification of a haem-utilization protein (Hup) in *Haemophilus influenzae*. Microbiology **150:**3923–3933.
47. **Morton, D. J., A. Smith, T. M. VanWagoner, T. W. Seale, P. W. Whitby, and T. L. Stull.** 2007. Lipoprotein e (P4) of *Haemophilus influenzae*: role in heme utilization and pathogenesis. Microbes Infect. **9:**932–939.
48. **Moxon, E. R., and T. F. Murphy.** 2000. *Haemophilus influenzae*, p. 2369–2378. *In* G. L. Mandell, J. R. Bennett, and R. Dolin (ed.), Mandell, Douglas, and Bennett's principles and practices of infectious diseases, 5th ed., vol. 2. Churchill Livingstone, New York, NY.
49. **Murphy, T. F., A. L. Brauer, A. T. Schiffmacher, and S. Sethi.** 2004. Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **170:**266–272.
50. **Murphy, T. F., and S. Sethi.** 2002. Chronic obstructive pulmonary disease: role of bacteria and guide to antibacterial selection in the older patient. Drugs Aging **19:**761–775.
51. **Nakamoto, H., and J. C. Bardwell.** 2004. Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm. Biochim. Biophys. Acta **1694:**111–119.
52. **Nickitenko, A. V., S. Trakhanov, and F. A. Quiocho.** 1995. 2 A resolution structure of DppA, a periplasmic dipeptide transport/chemosensory receptor. Biochemistry **34:**16585–16595.
53. **Nizet, V., K. F. Colina, J. R. Almquist, C. E. Rubens, and A. L. Smith.** 1996. A virulent nonencapsulated *Haemophilus influenzae*. J. Infect. Dis. **173:**180–186.
54. **O'Neill, J. M., J. W. St. Geme III, D. Cutter, E. E. Adderson, J. Anyanwu, R. F. Jacobs, and G. E. Schutze.** 2003. Invasive disease due to nontypeable *Haemophilus influenzae* among children in Arkansas. J. Clin. Microbiol. **41:**3064–3069.
55. **Peek, J. A., and R. K. Taylor.** 1992. Characterization of a periplasmic thiol: disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **89:**6210–6214.
56. **Ram, S., A. D. Cox, J. C. Wright, U. Vogel, S. Getzlaff, R. Boden, J. Li, J. S. Plested, S. Meri, S. Gulati, D. C. Stein, J. C. Richards, E. R. Moxon, and P. A. Rice.** 2003. Neisserial lipooligosaccharide is a target for complement component C4b. Inner core phosphoethanolamine residues define C4b linkage specificity. J. Biol. Chem. **278:**50853–50862.
57. **Ram, S., J. Ngampasutadol, A. D. Cox, A. M. Blom, L. A. Lewis, F. St. Michael, J. Stupak, S. Gulati, and P. A. Rice.** 2007. Heptose I glycan substitutions on *Neisseria gonorrhoeae* lipooligosaccharide influence C4b-binding protein binding and serum resistance. Infect. Immun. **75:**4071–4081.
58. **Rietsch, A., P. Bessette, G. Georgiou, and J. Beckwith.** 1997. Reduction of the periplasmic disulfide bond isomerase, DsbC, occurs by passage of electrons from cytoplasmic thioredoxin. J. Bacteriol. **179:**6602–6608.
59. **Ristow, P., P. Bourhy, F. W. McBride, C. P. Figueira, M. Huerre, P. Ave, I. S. Girons, A. I. Ko, and M. Picardeau.** 2007. The OmpA-like protein Loa22 is essential for leptospiral virulence. PLoS Pathog. **3:**e97.
60. **Rubin, L. G., and J. W. St. Geme III.** 1993. Role of lipooligosaccharide in virulence of the Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius for infant rats. Infect. Immun. **61:**650–655.
61. **Seale, T. W., D. J. Morton, P. W. Whitby, R. Wolf, S. D. Kosanke, T. M. VanWagoner, and T. L. Stull.** 2006. Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus influenzae* virulence in the infant rat model of invasive infection. Infect. Immun. **74:**6213–6225.
62. **Serino, L., B. Nesta, R. Leuzzi, M. R. Fontana, E. Monaci, B. T. Mocca, E. Cartocci, V. Masignani, A. E. Jerse, R. Rappuoli, and M. Pizza.** 2007. Identification of a new OmpA-like protein in *Neisseria gonorrhoeae* involved in the binding to human epithelial cells and in vivo colonization. Mol. Microbiol. **64:**1391–1403.
63. **Sethi, S., and T. F. Murphy.** 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin. Microbiol. Rev. **14:**336–363.
64. **Sone, M., Y. Akiyama, and K. Ito.** 1997. Differential in vivo roles played by DsbA and DsbC in the formation of protein disulfide bonds. J. Biol. Chem. **272:**10349–10352.
65. **Stenson, T. H., and A. A. Weiss.** 2002. DsbA and DsbC are required for secretion of pertussis toxin by *Bordetella pertussis*. Infect. Immun. **70:**2297–2303.
66. **Tinsley, C. R., R. Voulhoux, J. L. Beretti, J. Tommassen, and X. Nassif.** 2004. Three homologues, including two membrane-bound proteins, of the disulfide oxidoreductase DsbA in *Neisseria meningitidis*: effects on bacterial growth and biogenesis of functional type IV pili. J. Biol. Chem. **279:**27078–27087.

67. **Tomb, J. F.** 1992. A periplasmic protein disulfide oxidoreductase is required for transformation of *Haemophilus influenzae* Rd. Proc. Natl. Acad. Sci. USA **89:**10252–10256.
68. **Watarai, M., T. Tobe, M. Yoshikawa, and C. Sasakawa.** 1995. Disulfide oxidoreductase activity of *Shigella flexneri* is required for release of Ipa proteins and invasion of epithelial cells. Proc. Natl. Acad. Sci. USA **92:**4927–4931.
69. **Weiser, J. N., and E. C. Gotschlich.** 1991. Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. Infect. Immun. **59:**2252–2258.
70. **White, D. C., and S. Granick.** 1963. Hemin Biosynthesis in *Haemophilus*. J. Bacteriol. **85:**842–850.
71. **Wong, S. M., and B. J. Akerley.** 2003. Inducible expression system and marker-linked mutagenesis approach for functional genomics of *Haemophilus influenzae*. Gene **316:**177–186.
72. **Wong, S. M., K. R. Alugupalli, S. Ram, and B. J. Akerley.** 2007. The ArcA regulon and oxidative stress resistance in *Haemophilus influenzae*. Mol. Microbiol. **64:**1375–1390.
73. **Yang, X., T. Becker, N. Walters, and D. W. Pascual.** 2006. Deletion of *znuA* virulence factor attenuates *Brucella abortus* and confers protection against wild-type challenge. Infect. Immun. **74:**3874–3879.
74. **Yu, J., H. Webb, and T. R. Hirst.** 1992. A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae*. Mol. Microbiol. **6:**1949–1958.
75. **Zapun, A., and T. E. Creighton.** 1994. Effects of DsbA on the disulfide folding of bovine pancreatic trypsin inhibitor and alpha-lactalbumin. Biochemistry **33:**5202–5211.
76. **Zapun, A., D. Missiakas, S. Raina, and T. E. Creighton.** 1995. Structural and functional characterization of DsbC, a protein involved in disulfide bond formation in *Escherichia coli*. Biochemistry **34:**5075–5089.
77. **Zhang, H. Z., and M. S. Donnenberg.** 1996. DsbA is required for stability of the type IV pilin of enteropathogenic *Escherichia coli*. Mol. Microbiol. **21:** 787–797.

Editor: J. B. Bliska