5-12-2011

Roles of Secreted Virulence Factors in Pathogenicity of Haemophilus Influenzae: A Dissertation

Charles V. Rosadini
University of Massachussetts Medical School, Charles.Rosadini@umassmed.edu

ROLES OF SECRETED VIRULENCE FACTORS IN PATHOGENICITY OF *HAEMOPHILUS INFLUENZAE*

A Dissertation Presented

By

Charles Victor Rosadini

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 12th, 2011

Molecular Genetics and Microbiology

ROLES OF SECRETED VIRULENCE FACTORS IN PATHOGENICITY OF *HAEMOPHILUS INFLUENZAE*

A Dissertation Presented
By

Charles Victor Rosadini

The signatures of the Dissertation Defense Committee signifies completion and approval as to style and content of the Dissertation

_____
Dr. Brian J. Akerley, Thesis Advisor

_____
Dr. Jon Goguen, Member of Committee

_____
Dr. Christopher Sassetti, Member of Committee

_____
Dr. Sanjay Ram, Member of Committee

_____
Dr. Michael Koomey, Member of Committee

The signature of the Chair of the Committee signifies that the written dissertation meets the requirements of the Dissertation Committee

_____
Dr. John Leong, Chair of Committee

The signature of the Dean of the Graduate School of Biomedical Sciences signifies that the student has met all graduation requirements of the school

_____
Anthony Carruthers, Ph.D.,
Dean of the Graduate School of Biomedical Sciences

Program
Molecular Genetics and Microbiology

May 12, 2011

This thesis is dedicated to the memory of Donald G. Rosadini Sr. who taught me the values of hard work, perseverance, and determination that were instrumental in completion of this work.

AKNOWLEGMENTS

First, I would like to thank my thesis mentor Dr. Brian Akerley for allowing me to join his lab and participate in relevant research in the area of bacterial pathogenesis. Despite his busy schedule, he has always made time to answer my questions or discuss experiments or data. This thesis would not have been possible without his guidance, patience, and expertise.

I must thank my thesis advisory committee: Dr. John Leong, Dr. Jon Goguen, Dr. Christopher Sassetti, and Dr. Egil Lien. Their support, ideas, and expert knowledge were critical in completion of this thesis. I must also thank Dr. Michael Koomey and Dr. Sanjay Ram for their participation in my dissertation examination.

I would like to thank my collaborators who went above and beyond in helping to turn my thesis into a complete story: Dr. Sanjay Ram, for his contributions to the complement studies performed in this thesis and Dr. José M. Argüello and Dr. Daniel Raimunda for their help in performing the zinc binding experiments.

I would like to thank my lab-mate, Dr. Jeffrey Gawronski, who provided a great deal of encouragement and support throughout my graduate career. His willingness to go out of his way to participate in my thesis project is greatly appreciated.

Finally, I would like to thank my wife Rachel for her support and encouragement throughout my graduate career. Her energy, positive attitude, and sense of humor have been a driving force behind my success at Umass.

ABSTRACT

Haemophilus influenzae is a pathogenic Gram-negative bacterium that colonizes the upper respiratory tract of humans and can cause otitis media, upper and lower respiratory infections, and meningitis. Factors important for *H. influenzae* to colonize humans and cause disease are not fully understood. Different bacterial pathogens are armed with virulence mechanisms unique to their specific strategies for interacting with their hosts. Many of the proteins mediating these interactions are secreted and contain disulfide bonds required for function or stability. I postulated that identifying the set of secreted proteins in *H. influenzae* that require periplasmic disulfide bonds would provide better understanding of this bacterium's pathogenic mechanisms.

In this thesis, the periplasmic disulfide bond oxidoreductase protein, DsbA, was found to be essential for colonization and virulence of *H. influenzae*. Mutants of *dsbA* were also found to be sensitive to the bactericidal effects of serum. However, the DsbA-dependent proteins important for pathogenesis of this organism have not been previously identified. To find them, putative targets of the periplasmic disulfide bond pathway were identified and examined for factors which might be important for mediating critical virulence aspects. By doing so, novel virulence factors were discovered including those important for heme and zinc acquisition, as well as resistance to complement. Overall, the work presented here provides insight into requirements for *H. influenzae* to survive within various host environments.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF THIRD PARTY COPYRIGHTED MATERIAL

CHAPTER I

Introduction and literature review

Haemophilus influenzae

Haemophilus influenzae is a non-motile Gram-negative coccobacillus which belongs to phylum gammaproteobacteria and family *Pasteurellacae*. This exclusive human pathogen normally colonizes the respiratory tract asymptomatically; however, it can cause disease by invading sites such as the inner ear, lungs, or blood. *H. influenzae* has an absolute requirement for supplementation with exogenous heme and NAD to grow aerobically in vitro (72), but does not require heme supplementation for growth as a facultative anaerobe. With proper supplementation of heme and NAD, it can be readily cultured in vitro on Brain Heart Infusion (BHI), chocolate agar, or complex defined media (15, 37). *H. influenzae* grows normally at 35 to 37°C, however, is highly sensitive to greater temperatures. Additionally, it is naturally competent, a quality that allows it to uptake double stranded DNA from its environment and integrate it into its chromosome (15). In the laboratory, this quality makes *H. influenzae* a genetically tractable model organism allowing for examination of aspects of its pathogenesis.

Encapsulated strains

H. influenzae strains are classified into two categories, encapsulated and non-encapsulated strains (225). Encapsulated strains are “typed” based on the antigenicity of their protective outer surface polysaccharide capsule, which comes in six forms: types a through f (225). The most virulent of these, *H. influenzae* type b (Hib) was a major cause

of invasive infections, including septicemia and meningitis until wide spread use of a vaccine in 1990 (33, 271). Although the incidence of meningitis caused by Hib has declined rapidly in vaccinated populations, it remains a major cause of disease in developing countries where the vaccine is not readily available (33).

Nontypeable strains

Non-encapsulated or nontypeable *Haemophilus influenzae* (NTHi) lack an outer surface capsule and are common commensals of the upper respiratory tract. Unlike encapsulated varieties, NTHi are aclonal in population and highly variable in their surface antigens (195). They are currently important causes of sinusitis, conjunctivitis, and pneumonia (201, 203) and are the second most common cause of otitis media, or middle ear infection, behind *Streptococcus pneumoniae* (203). NTHi are also one of the most prevalent organisms found in the lungs of patients with exacerbations of chronic obstructive pulmonary disease (COPD) and may persist in the lungs of these patients for months or even years causing considerable morbidity and mortality (168, 202, 205, 251). Moreover, NTHi strains are commonly isolated from the lungs of patients with cystic fibrosis (CF) (86, 187, 245), where pulmonary infections result in 90% of the deaths due to CF (10, 51, 242).

NTHi strains are not often associated with invasive disease and most instances of bacteremia occur in children with underlying medical issues (203, 215). However, recent studies suggest that healthy individuals are also at risk (31, 34, 47, 129, 211). The Hib vaccine, based on the type b polyribosylribitol phosphate capsule, is not effective against

NTHi strains (226) and currently a vaccine remains elusive, likely due to high variability within surface antigens between strains (251). Therefore, the threat of disease from NTHi remains of importance even in regions where the Hib vaccine is universally available.

Virulence determinants of *H. influenzae*

To cause disease, bacterial pathogens must possess specific virulence mechanisms for mediating interactions with their hosts. These mechanisms are often important for resistance to host defenses, invasion of host tissues, and production of toxins for host cell manipulation. *H. influenzae* is not known to produce protein toxins, and its ability to colonize the human respiratory tract and opportunistically invade privileged host environments, such as the blood, lungs, and middle ear, is thought to be its most important pathogenesis feature. However, our understanding of factors important for *H. influenzae* to survive within its host is limited. Current knowledge suggests that these factors are likely to mediate processes such as adherence to human cells, nutrient acquisition, and defense against host immune functions such as complement, opsonophagocytosis, and oxidative radicals produced by phagocytes. Additionally, gene regulation is thought to be an important feature for transition of the bacteria between sites of infection. Below is a review of known and potential virulence mechanisms of *H. influenzae*:

Polysaccharide capsule

The polysaccharide capsule is an important virulence feature of encapsulated *H. influenzae* strains. It is known to be required for bacteremia and meningitis caused by Hib

(196). Although the mechanism by which the capsule promotes invasive disease is not well understood, the capsule has been shown to protect Hib from host immune functions. This is exemplified by the fact that increases in surface polysaccharide and copy number of the Cap b locus, which encodes genes required for capsule expression, result in increased serum resistance of Hib strains (212, 264). Hib strains containing four copies of Cap b also exhibited reduced C3 deposition on their surface and reduced binding to murine or human macrophages compared with strains containing two copies (212). Furthermore, strains expressing the type-b capsule were protected from complement mediated opsonophagocytosis by macrophages, whereas capsule deficient strains were susceptible to engulfment (213).

Lipooligosaccharide

Lipopolysaccharide (LPS), a major component of the outer leaflet of the Gram-negative bacterial outer membrane, mediates interactions between the bacterium and the host immune system (195). In ***H. influenzae*** and many other respiratory pathogens, LPS typically lacks long polysaccharide repeats and is referred to as lipooligosaccharide (LOS) (197). In NTHi stains, LOS is a critical factor for defense against host complement, which is encountered in the blood (46, 214) and at mucosal surfaces (9, 169, 208, 209, 278). The LOS consists of lipid A attached via phosphorylated Kdo to an inner core of three L-*glycero*-D-*manno*-heptose residues and outer core of short hexose chains of glucose and galactose attached to each heptose residue (175, 224, 248). Several of the enzymes which synthesize LOS have been shown to be important for serum resistance of

NTHi. For example, the galactosyltransferase encoded by *lgtC* was shown to be important for serum resistance of an invasive NTHi isolate (117, 122). Expression of *lgtC* resulted in delayed deposition of C4b on the surface of the strain, thereby inhibiting complement mediated killing (117). Additionally, the glucosyltransferases encoded by the genes *losA, lex2A,* and *lic2B* have been shown to add important sugars to the LOS which are necessary for enhancing serum resistance (69, 96, 303). Thus, LOS plays an important role in defense against host immunity.

H. influenzae strains also incorporate sialic acid into the LOS outer core, and the presence of this amino sugar helps avoid immune clearance. However, *H. influenzae* cannot synthesize sialic acid and must acquire it from the host. Sialic acid is transported by a tripartite ATP-independent periplasmic transporter encoded by *siaPQM* (27, 281). It is then activated by CMP-sialic acid synthetase, encoded by *siaB* (123), and added to certain hexose residues of the LOS outer core by the sialyltransferase, encoded by *lic3A* (120). Genes involved in sialic acid uptake and attachment are required for serum resistance of *H. influenzae* (27, 123). Additionally, strains with disruptions in *lic3A* or *siaB* are defective for survival in a chinchilla model of otitis media (27, 120, 136). Depletion of complement restored the ability of a *siaB* mutant to colonize the chinchilla ear (75), demonstrating that sialylation of the LOS is a critical strategy for complement defense of *H. influenzae* during infection.

Adhesion and invasion

To prevent clearance of the bacterium by the host mucociliary escalator, *H. influenzae* expresses several adhesion proteins and surface fibrils for attachment to host mucosal structures. Many of these have been shown to promote binding of *H. influenzae* to epithelial cells in vitro, including several auto transporter proteins such as the High-molecular-weight proteins 1 and 2 (HMW1 and HMW2) of nontypeable strains (259), *Haemophilus influenzae* adhesin (Hia) of encapsulated strains (260), and Hap which is broadly distributed (76, 261). Additionally, outer membrane proteins play a role in adherence such as P2 which binds respiratory mucin, P5 which binds mucin as well as CEACAM1 (carcinoembryonic antigen-related cell adhesion molecule 1) and ICAM1 (intercellular adhesion molecule 1) receptors (26, 115, 132, 186, 236) and OapA whose target molecule is unknown (228). Furthermore, *Haemophilus* surface fibril (Hsf), expressed by Hib, or protein E (PE), expressed by both NTHi and Hib strains, bind host cell extracellular matrix protein, vitronectin (102, 106). Lastly, some *H. influenzae* strains contain pili, encoded by *hifA-hifE* or *pilA,* and these structures have been shown to promote adherence of NTHi to epithelial cells in tissue culture (74, 137, 165, 235, 261, 274). PilA was also shown to be important for NTHi to form biofilms and colonize the chinchilla middle ear (137) suggesting that adherence is an important virulence property for survival of *H. influenzae* during infection.

Binding of *H. influenzae* to host cells is thought to promote invasion of host tissues allowing for the bacteria to access the basolateral surface and enter the bloodstream. For example, when NTHi are incubated with host cell monolayers, they enter cells or disrupt tight junctions (146, 277). Moreover, addition of phosphorylcholine

(ChoP) to the LOS promoted binding to host cell PAF (platelet activating factor) receptors and invasion of human bronchial epithelial cells in vitro (266). Expression of protein D, a glycerophosphodiester phosphodiesterase important for addition of ChoP to the LOS (73, 198), was also shown to promote invasion of human monocytic cells (2), further demonstrating that ChoP addition promotes adhesion and invasion.

H. influenzae IgA protease has also been implicated in invasiveness of NTHi. IgA protease cleaves immunoglobulin A at the hinge region and its activity is thought to prevent agglutination at the mucosal surface and subsequent mechanical clearance of the pathogen (147, 282). Increased levels of IgA protease activity correlated with increased invasiveness of NTHi strains in humans, similar to data produced for IgA protease produced by *Neisseria meningitidis* (282, 283). Likely increased survival of NTHi at the mucosal surface allows for increased pathogenesis and invasion (282).

Phase variation

Phase variation is thought to be an important immune evasion strategy during infection where the outer surface is modified to adapt to changes in the host environment (195). For example, many phase variable loci encode factors important for production of the outer core of LOS (69, 96, 122, 289, 293, 294). Phase variation allows genes to be turned on or off through the action of slipped strand base mispairing, usually at tetranucleotide repeats within the 5' end of their coding regions (122, 160, 200, 294). By modulating expression of these structures, *H. influenzae* can likely avoid antibody binding and complement-mediated lysis. For instance, LOS decoration via addition of

ChoP is important for *H. influenzae* colonization of the respiratory tract and middle ear (119, 219, 295). However, expression of ChoP has also been shown to reduce the virulence of Hib in an infant rat model of infection (125). Additionally, ChoP is a target for the classical complement pathway activator, CRP (78, 286, 295), and its expression results in sensitivity of Hib to complement-mediated lysis (125). The *lic1* locus, which encodes the machinery for adding ChoP, is phase variable (293). Therefore, ChoP is thought to be modulated allowing for efficient colonization of the respiratory tract and infection of the bloodstream (291). This example highlights the importance of phase variation in protection of *H. influenzae* against host defenses.

Several genes encoding outer surface proteins have also been shown to undergo phase variation. Examples include the hemoglobin or hemoglobin-haptoglobin-binding proteins, HgbA, HgbB, and HgbC (38) and adhesins, such as, OapA, HMW1, HMW2, and outer surface fibrils (56, 276, 290). Additionally, heterogeneity within protein sequences may also contribute to serum and resistance and inhibition of host antibody binding. During the course of persistent infections such as chronic bronchitis, the outer membrane proteins P2 and P5 were found to vary significantly in molecular weight and sequence between isolates (62, 63, 97, 284, 288). Subsequently, variation in outer loops of P2 was shown to contribute to differential antibody binding between strains (217, 273). Variation was proposed to be driven by selective pressure imposed by host antibody and complement encountered by the bacteria during infection (62). Together, variation of surface proteins is thought to prevent recognition of *H. influenzae* by host antibody and therefore reduce complement mediated killing and opsonophagocytosis.

Binding of host complement regulatory factors

Binding of complement regulators is thought to be an important survival strategy for ***H. influenzae*** during colonization and infection (105). Normally, complement regulators benefit the host by protecting tissues from unnecessary damage during complement activation. However, these regulators can also be hijacked by bacterial pathogens and used to subvert host defenses. ***H. influenzae*** utilizes several different complement regulatory factors to block activity at various steps throughout the complement pathway, including the classical pathway inhibitor C4b binding protein (C4BP), the alternative pathway inhibitors factor H (FH) and factor H-like protein-1 (FHL-1), as well the membrane attack complex inhibitor, vitronectin (105). Binding of C4BP appears to be exclusive to NTHi strains (103), whereas FH, FHL-1 (107), and vitronectin are bound by both Hib and NTHi (102, 106). As mentioned above, the adhesins Hsf and PE bind vitronectin (102, 106); however, the outer surface structures important for binding of C4BP and FH and FHL-1 remain undiscovered.

Several reports have demonstrated that binding of regulatory factors protects ***H. influenzae*** from the bactericidal effects of serum complement (102, 103, 107). Additionally, NTHi stains isolated from patients with upper respiratory-tract infections or sepsis exhibited in vitro binding to C4BP, FH, or vitronectin (104). Together, binding of complement regulatory factors is a likely an important mechanism for ***H. influenzae*** to subvert complement activation and cause disease.

Heme utilization

Because *H. influenzae* lacks the biosynthetic pathways required for producing heme, it must procure this essential molecule from its host (91). Heme is an important prosthetic group for proteins related to *H. influenzae* pathogenesis, including those involved in oxygen sensing, electron transport, detoxification, and responding to oxidative stress (218). For example, catalase, a heme containing protein encoded by ***hktE***, was shown to be necessary for Hib to colonize the rat nasopharynx and resist hydrogen peroxide (24). Although *H. influenzae* cannot synthesize heme *de novo* from 5-aminolevulinic acid (218), it has the capability of producing heme from its immediate precursor, protoporphyrin IX (PPIX). This process is achieved by a ferrochelatase encoded by ***hemH***, which joins Fe^{2+} to PPIX to produce heme (91, 247). However, heme is likely to be the only source of porphyrin available to *H. influenzae* during infection.

Most heme sources in vivo are sequestered in host cells (157). Additionally, heme released from necrotic host cells is rapidly bound by host-carrier proteins such as albumin or hemopexin, which bind it avidly (39). To overcome these limitations, *H. influenzae* has evolved outer membrane heme binding proteins to scavenge heme from host molecules including heme-hemopexin, hemoglobin, hemoglobin-haptoglobin, and heme-albumin (42, 193). Many of these outer surface proteins are required for pathogenesis of *H. influenzae* such as the hemoglobin/hemoglobin-haptoglobin binding proteins, HgpABC, which were shown to be required for virulence of NTHi in a chinchilla middle ear infection model (133, 188, 238) and the heme-hemopexin binding proteins, HxuCBA, which are required for Hib infection of infant rats (40-42, 191). Moreover, ***tehB*** and ***hel***, which encode factors important for growth of *H. influenzae* on multiple heme sources,

were also required for pathogenesis of Hib in the infant rat (194, 237, 296). The multitude of outer membrane heme binding proteins highlights the importance of heme acquisition in colonization and virulence of *H. influenzae*.

Oxygen responsive gene regulation

Virulence factors of *H. influenzae* have been shown to be modulated in response to environmental oxygen concentrations. One way genes can be regulated in response to oxygen is through the ArcA/ArcB two-component system (98). In this signal transduction pathway, the membrane associated sensor kinase, ArcB, detects low oxygen and phosphorylates the soluble response regulator ArcA, which can then bind to DNA and regulate genes (98). Mutants of ***arcA*** are defective for serum resistance and survival in a mouse bacteremia model (57, 302). It was discovered that ***lic2B,*** which encodes a glycosyltransferase that adds a galactose to the LOS outer core, is positively regulated by ArcA during low oxygen conditions (303). NTHi ***lic2B*** mutants are defective for serum resistance and survival during blood stream infection (303). Likely, serum resistance defects of an ***arcA*** mutant after growth in low oxygen can be attributed to lack of expression of *lic2B*.

A second response regulator, FNR, has been implicated in pathogenesis of ***H. influenzae***. FNR directly senses low oxygen environments via reduction of its iron-sulfur center allowing for it to maintain an active dimer form that can bind to DNA to either repress genes required for aerobic growth or activate those needed for growth in low oxygen (156). When oxygen is present the iron-sulfur center is oxidized and FNR

assumes an inactive monomeric form (45, 61). During low oxygen conditions FNR positively regulated the *nrfA* and *ytfE* genes, which encode factors critical for resistance to nitric oxide (110). Furthermore, in low oxygen, *ytfE* was shown to be essential for NTHi resistance to nitric oxide produced by IFN-γ stimulated macrophages (110). These results suggested that nitric oxide is likely to be encountered at cites of infection that contain low oxygen, such as high bacterial density on mucosal surfaces or during invasion of submucosal layers (110).

Modification of the LOS with ChoP has also been shown to be regulated by oxygen. Addition of ChoP is increased during in vitro culture with high aeration conditions and inhibited during low oxygen conditions (300). However, the mechanism behind this regulation is not known (300). Taken together, regulation of genes in response to oxygen may serve as an important strategy for modulating factors important for evading host immune functions at various sites of infection.

Bacterial virulence factors and disulfide bonds

Little is known about factors important for *H. influenzae* to survive within its host. However, many of these factors such as the adhesins and heme uptake factors described above are secreted into the extracytoplasmic spaces including the periplasm, outer membrane, and extracellular space. Unlike the cytoplasm, which is a reducing environment, the extracytoplasmic spaces expose secreted proteins to oxidizing conditions (230). Thus, many secreted proteins contain disulfide bonds that likely enhance stability and provide proper folding allowing for function. This process has been

extensively studied in ***E. coli*** and shown to be catalyzed by the periplasmic Dsb proteins (Figure 1.1). The following sections will review the mechanism of disulfide bond formation and discuss the known roles for disulfide-dependent proteins in virulence of Gram-negative bacteria and the potential role for disulfide formation in pathogenesis of *H. influenzae.*

Disulfide bond formation

DsbA, the major disulfide forming protein of ***E. coli***, catalyzes disulfide bond formation by donating a disulfide from its active site cysteines to pairs of reduced cysteines on newly secreted proteins (17, 93, 143, 312). DsbA's active site, Cys^{30}-Pro^{31}-His^{32}-Cys^{33}, possesses a high oxidizing potential of -122 mV due to the extremely low pKa of its N-terminal cysteine (Cys^{30}) (124, 306). At physiological pH, the oxidized form of DsbA is less stable than its reduced form, thus DsbA is likely to donate its disulfide to achieve a lower energy state (312). Disulfide exchange occurs when Cys^{30} is attacked by a deprotonated cysteine on a newly secreted protein, resulting in formation of an intermolecular disulfide between DsbA and the protein (Figure 1.2) (52, 140, 252, 312). A second deprotonated cysteine on the secreted protein can then attack the mixed-disulfide resulting in disulfide formation between cysteines on the substrate. The end products of this reaction are an oxidized substrate and reduced DsbA.

After disulfide exchange with a substrate, DsbA is recycled by reoxidation of its active site cysteines by DsbB (Figure 1.1). DsbB is an inner membrane protein with four trans-membrane helices and two periplasimc loops which contain essential pairs of

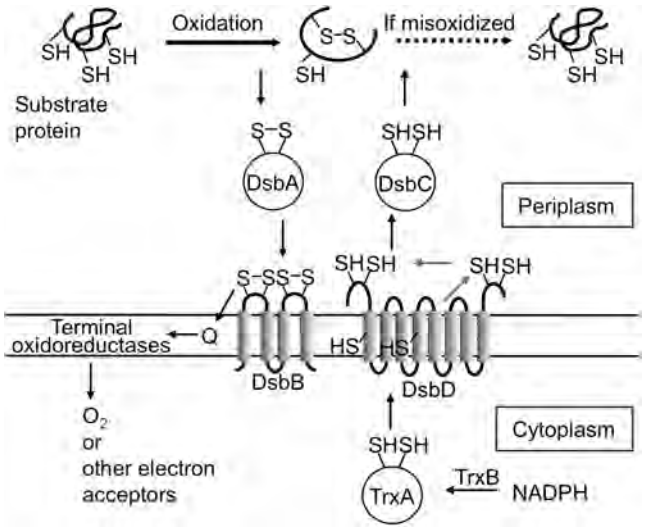

Figure 1.1. Models for disulfide-bond formation in the periplasm of *E. coli*. The *solid black rightward arrow* indicates the oxidative folding reaction catalyzed by DsbA. In the instance in which the first folding reaction resulted in a misoxidized protein, DsbC may repair it by acting as a reductase (*dotted arrow*) or an isomerase (not shown in this figure) of the incorrect disulfide bond. The *thinner arrows* indicate the flow of reducing equivalents. Q, quinones (ubiquinone or menaquinone). For simplicity, DsbC, a dimeric molecule, is depicted as a monomer. This figure was reproduced from (139) with permission from Mary Ann Liebert Publishers.

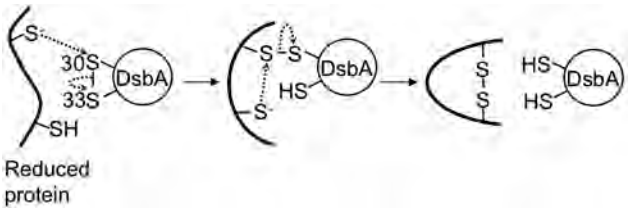

Figure 1.2. Disulfide-bond formation by DsbA. Substrate oxidation by DsbA likely proceeds through two steps. First, a deprotonated cysteine of a substrate attacks the sulfur atom of Cys30 of the oxidized DsbA, leading to the formation of a disulfide-linked complex between DsbA and the substrate. In the next step, one of the remaining cysteines of the substrate is deprotonated and attacks the sulfur atom of the substrate cysteine that is disulfide bonded with Cys30 of DsbA. This reaction results in the formation of a disulfide bond in the substrate and reduction of DsbA. This figure was reproduced from (139) with permission from Mary Ann Liebert Publishers.

cysteines for catalyzing disulfide formation (127, 138). Reoxidation of DsbA begins when deprotonated Cys^{30} on DsbA attacks Cys^{104} in the second periplasmic loop of DsbB (99, 128, 151). Subsequently, the mixed disulfide between DsbA and DsbB is resolved by attack of the disulfide by DsbA Cys^{33}, resulting in reoxidized DsbA and reduced Cys^{104} and Cys^{130} of DsbB (150). Cys^{104} and Cys^{130} are then reoxidized by the disulfide on first loop of DsbB (92, 150). The first loop Cys are maintained in an oxidized state by quinones that shuttle electrons from DsbB to the electron transport chain, thus driving the oxidizing potential of the system (13, 92, 154). Additionally, DsbB is highly specific for DsbA as a substrate and cannot exchange disulfides with other periplasmic substrates (126).

Occasionally, incorrect disulfide bonds are formed and these errors can be repaired by the periplasmic disulfide isomerase DsbC (Figure 1.1), an enzyme which possess both isomerization and chaperone activities (20, 35, 116, 239, 254, 314). DsbC contains an active site CXXC motif similar to DsbA, but is maintained in a reduced form allowing it to bind to misfolded proteins and to catalyze isomerization or reduction of misoxidized disulfides (139). It exists as a V-shaped homodimer with its active sites facing a hydrophobic cleft which is necessary for substrate specificity (139, 176). This configuration is also thought to inhibit interaction with DsbB and therefore prevent oxidation of the active site (11).

A second protein, DsbG, is similar in structure and amino acid sequence to DsbC and also forms V-shaped homodimers (112). Similar to DsbC, DsbG possesses in vitro

isomerase and chaperone activity (23, 253); however, its primary role appears to be interaction with the reduced forms of three enzymes of the peptidoglycan cross-linking pathway, YbiS, ErfK, and YnhG (58). These enzymes all contain single cysteine residues which are essential for their functions (167). By interacting with DsbG, these cysteines are effectively protected from the oxidizing environment of the periplasm.

Both DsbC and DsbG are maintained in a reduced form by the inner membrane protein DsbD (23, 185, 239). DsbD is reduced by cytoplasmic thioredoxin, which in turn is reduced by thioredoxin reductase, which is reduced by NADPH (239) (Figure 1.1). In this way, DsbD utilizes an electron transfer cascade to generate the reducing potential of the system.

DsbA-dependent proteins and their role in virulence

Mutation of ***dsb*** genes results in loss of stability and function of secreted enzymes. For example, mutation of ***E.coli dsbC*** causes instability within proteins that contain disulfides on non-consecutive cysteines such as periplasmic endoribonuclease (RNase I), penicillin-insensitive murine endopeptidase (MepA), acid phosphatase (AppA), and endonuclease-1 (End1) (20, 116, 178, 280). However, DsbA affects a much wider range of proteins, which according to informatics-based estimates may number ~300 in ***E. coli*** (64, 206). These include periplasmic alkaline phosphatase (PhoA), the P-ring of flagella (FlgI) and outer membrane protein A (OmpA) of ***E. coli*** (49, 141, 158). More importantly, mutation of ***dsbA*** results in defects in maturation or export of major secreted virulence factors in a variety of bacterial pathogens, including biogenesis and

stability of pili in *Vibrio cholerae, Proteus mirabilus, Neisseria meningitidis, enteropathogenic E. coli, and uropathogenic E. coli* (30, 131, 222, 268, 315), toxin production in *V. cholerae*, *E. coli*, and *Bordetella pertussis* (216, 222, 263, 308, 311), and type III secretion in *Yersinia pestis*, *Shigella flexneri*, *Pseudomonas* spp., and *Salmonella enterica* (101, 130, 181, 287) (Table 1.1). Consequently, *dsbA* mutants exhibit defects for virulence properties such as the ability to adhere and invade host cells (29, 101, 268, 287) and cause disease in animal models (30, 89, 181).

Due to the large number of virulence-related defects of *dsbA* mutants, identification of DsbA substrates may lead to discovery of additional bacterial virulence factors. The full range of DsbA-dependent proteins has not been elucidated in any organism; however, attempts at identifying DsbA-substrates of *E. coli* using biochemical techniques have been described. These studies utilized 2-D gel based analyses in which substrates were identified by comparing wild-type *E.coli* with *dsbA* mutants for either decrease in substrate concentration on the gel or change in binding of thiol reactive probes (differential thiol trapping) (116, 158). Additionally, Kadokura and co-workers identified substrates using a DsbA P151T mutant, which accumulates DsbA-substrate complexes in its periplasm allowing them to be affinity purified and sequenced (141). Combined, biochemical techniques identified only 25 substrates, with little overlap between studies (Table 1.2). This suggests that a large number of DsbA-dependent proteins have yet to be found and other techniques will have to be employed to identify them.

Table 1.1. Virulence defects of *dsbA* mutants

Organism	Affected proteins	Defective virulence properties in *dsbA* mutants
E. coli	STI, STII	Secretion of heat stable and heat labile enterotoxin, systemic infection in infant rats (89, 216, 308)
Adherent-invasive	unknown	Survival in macrophages (29)
Enteropathogenic	EscC, BfpA	Type III secretion, bundle forming pilus, (182, 315)
Uropathogenic	PapD	P. fimbriae (131)
Salmonella enterica	SpiA, Pef	Spi-2 type III secretion, virulence in mice (181)
Shigella flexneri	Spa32	Type III secretion, invasion of epithelial cells (287)
Proteus mirabilus	unknown	MR/P fimbriae, virulence in mice (30)
Bordetella pertussis	S1, S2	Stability of pertussis toxin (263)
Vibro cholerae	unknown	Pili, cholera toxin, heat-labile enterotoxin (222, 311)
Neisseria meningitidis	unknown	Type IV pili, adhesion to cells (268)
Pseudomonas aruginosa	PilA	Type III secretion, intracellular survival (101)
Yersinia pestis	YscC	Type III secretion of Yops (130)

Table 1.2. List of *E. coli* DsbA substrates discovered by biochemical techniques

Substrate	Function	Reference
ArtJ	Arginine ABC transporter periplasmic-binding protein	(158)
DppA	Dipeptide binding lipoprotein	(141, 158)
Imp	Organic solvent tolerance protein precursor	(141, 158)
MepA	Penicillin-insensitive murein endopeptidase	(116)
OmpA	Outer membrane protein A	(141, 158)
OppA	Oligopeptide transporter periplasmic-binding protein	(141, 158)
ZnuA	Zinc transporter subunit: periplasmic-binding component of ABC superfamily	(141)
DegP	Periplasmic serine protease	(116)
HisJ	Amino acid ABC transporter periplasmic-binding protein	(141, 158)
RcsF	Dat1 predicted outer membrane protein	(135)
GltX	Glutamyl-tRNA synthetase	(158)
SspA	Stringent starvation protein A	(158)
FlgI	Flagellar hook-filament junction protein	(116)
GltI	Glutamate and aspartate transporter subunit	(116, 141)
LivJ	Leucine/isoleucine/valine transporter subunit	(116, 141)
LivK	Leucine transporter subunit	(141)
Rna	RNAse I	(116)
PhoA	Alkaline phosphatase	(116)
UgpB	Glycerol-3-phosphate transporter subunit	(158)
YbjP	Predicted lipoprotein	(141)
YcdO	Conserved protein	(141)
YedD	Predicted protein	(141)
YggN	Predicted protein	(116)
YodA	Conserved metal-binding protein	(141)
YibQ	Predicted polysaccharide de-acetylase	(141)

DsbA substrates may be virulence factors of H. influenzae

The components of the *E. coli* disulfide bond pathway are conserved in *H. influenzae* (270). However, the role of the Dsb pathway in pathogenesis of this organism has not been previously investigated. Evidence from high-throughput screening techniques indicated that DsbB is required for Hib infection in infant rats (114) and is induced in a chinchilla model of NTHi otitis media (173), suggesting that periplasmic disulfide bond formation may be required for virulence of *H. influenzae*. Additionally, some *H. influenzae* virulence-associated proteins have DsbA-dependent homologs in *E. coli*. Examples include the cytochrome C-type nitrite reductase NrfA, which is important for *H. influenzae* defense against nitric oxide (110, 179), and outer membrane protein P5, which has been shown to be important for virulence of *H. influenzae* (256). Together, this suggests that DsbA-dependent factors are virulence determinants of *H. influenzae*.

Thesis overview

Many virulence factors of *H. influenzae* are not known but are likely to be secreted and contain disulfide bonds for function and stability. Therefore, I hypothesized that the periplasmic disulfide oxidoreductase, DsbA is important for pathogenesis in *H. influenzae* and furthermore, identification of the substrates of the disulfide pathway will lead to discovery of virulence determinants of this organism.

In Chapter II, the role of DsbA in pathogenesis of *H. influenzae* was investigated; *dsbA* mutants were generated and evaluated for growth in vitro and virulence in several infection models. A comparison of known DsbA substrates in other species to predicted

secreted proteins in *H. influenzae* revealed several proteins that could contribute to virulence of this organism. One of these proteins, the heme utilization factor, HbpA, was examined due to the importance of exogenous heme for aerobic growth of *H. influenzae*.

In Chapter III, informatics-based techniques were used to identify *H. influenzae* proteins that may require DsbA-dependent disulfide bonds for function and stability. This list was cross-referenced with data from a whole genome transposon mutagenesis screen that identified factors important for *H. influenzae* to colonize the lung. Through this method, a system important for zinc utilization during infection was discovered and characterized.

In Chapter IV, I investigated the role of DsbA-substrates in serum resistance. This project was based on the observation that a *dsbA* mutant was sensitive to serum complement. The list of potential DsbA substrates was examined for proteins that have been implicated in serum resistance of other organisms. By doing so, the outer membrane protein P5 was identified and its role in serum resistance was investigated.

Chapter V provides a summary of this thesis, conclusions, and future research directions.

PREFACE TO CHAPTER II

This Chapter has been published

Charles V. Rosadini, Sandy M.S. Wong, and Brain J. Akerley. The periplasmic disulfide oxidoreductase DsbA participates in *Haemophilus influenzae* pathogenesis. Infection and Immunity. 76(4): 1498-508. 2008

Charles V. Rosadini performed all experiments.

Sandy M.S. Wong assisted with animal experiments and RNA purification.

Brain J. Akerley and Charles V. Rosadini designed the experiments and wrote the manuscript.

CHAPTER II

The periplasmic disulfide oxidoreductase DsbA contributes to *Haemophilus influenzae* pathogenesis

ABSTRACT

H. 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 their 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 (Hib) Eagan containing nonpolar deletions of *dsbA* were attenuated for bacteremia in animal models, and complemented strains exhibited equivalent virulence 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 in 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.

INTRODUCTION

Haemophilus influenzae efficiently colonizes the human nasopharyngeal mucosa in a primarily asymptomatic manner with a carriage frequency of ~80% in healthy adults (197). However, it can disseminate to other anatomical sites and cause otitis media, upper and lower respiratory tract infections, septicemia, and meningitis in children (86, 152, 187, 197, 202, 205, 251). The incidence of *H. influenzae* meningitis has dramatically declined in populations immunized with a vaccine against the type b capsular polysaccharide (33). The vaccine has not affected the incidence of infection with nontypeable strains (NTHi), which lack the capsule. NTHi 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 among NTHi strains (47, 71, 211, 215). However, the molecular basis for the invasive properties of *H. influenzae* that promote transmission from the nasopharynx to the bloodstream or middle ear are 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 (17). Formation of such linkages has been most extensively studied in *E. coli*, in which a series of disulfide oxidoreductases (Dsb) creates and exchanges disulfide bonds in periplasmic proteins (reviewed in (140, 206)). 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 (79, 313). DsbA is efficiently re-oxidized by DsbB, a membrane protein that transfers electrons to quinones for subsequent transfer to electron acceptors of the respiratory chain (16, 206). The soluble periplasmic DsbC and DsbG proteins mediate rearrangement of mispaired disulfides using electrons transferred via the membrane bound DsbD protein from cytoplasmic thioredoxin (6, 23, 154, 155, 239, 314). 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. These effects vary depending on the repertoire of periplasmic and secreted substrates of DsbA among different bacteria. These 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* (131, 222, 268, 315). Toxin production or secretion is defective in many *dsbA* mutants including cholera toxin in *Vibrio cholerae*, heat-labile and heat-stable *E. coli* enterotoxins, and pertussis toxin in *Bordetella pertussis* (222, 263, 311). 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 *Y. pestis, S. flexneri, Pseudomonas spp.,* and *Salmonella enterica sv. typhimurium* (101, 130, 181, 287). Furthermore, DsbA has been implicated in systemic infection by *E. coli* K1, and prolonged survival of adherent-invasive *E. coli* within macrophages (29, 89).

The *dsbA* gene of *H. influenzae* (*HI0846*, also called *por*) transcomplements a *dsbA* mutant of *E. coli* (270). Disruption of *dsbA* with a transposon insertion resulted in changes in secreted protein localization in a cellular fractionation experiment and dramatically reduced natural transformation efficiency (270). 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 (114). In *H. influenzae*, the protein targets of DsbA and virulence factors dependent on its activity have not been identified. In this study, we demonstrate that *dsbA* is required for *H. influenzae* bacteremia with 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 (91, 297), and several heme utilization pathways have been implicated in bloodstream infection by *H. influenzae* (194, 249). We demonstrate that the heme transport protein, HbpA, contains a DsbA-dependent disulfide bond. A nonpolar *hbpA* deletion mutant exhibited a reduced magnitude of bacteremia in mice, yet this 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 (301), and a virulent streptomycin resistant derivative of *H. influenzae* type b strain Eagan (Hib) (8) were grown in Brain Heart Infusion broth supplemented with 10 µg/ml hemin and 10 µg/ml β-nicotinamide adenine dinucleotide (sBHI) or on sBHI agar plates at 35°C. Competence development for transformation of *H. influenzae* was as previously described (15). For the selection of Rd and Hib derived strains, antibiotics were used at concentrations of 8 µg/ml tetracycline (Tet), 20 µg/ml kanamycin (Km), 10 µg/ml gentamicin (Gm), or 100 µg/ml streptomycin (Sm).

DsbA strain construction. Plasmids and PCR products were constructed using standard molecular biology techniques (12). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent *Sap*I restriction sites of the chromosomal delivery vector pXT10, which does not replicate in *H. influenzae* (301). The pXT10-based plasmids contain upstream (*xylF*) and downstream (*xylB*) homologous regions flanking the *Sap*I cloning sites that allow precise fusions of genes of interest to the xylose inducible *xylA* promoter as previously described (301). Recombination at the xylose catabolic locus replaces the endogenous *xylA* gene with the cloned fragment and the *tetAR* tetracycline resistance cassette. Plasmids were linearized by digestion with *Pci*I and *Sac*I and tetracycline resistant (Tet^R) recombinants were selected on sBHI agar plates. Double crossovers within *xylF* and *xylB* were confirmed by PCR with primers specific to sequences outside of the inserted recombinant region.

To generate a *dsbA* mutant and a complemented strain in *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 the 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 introduce *Sap*I sites in the termini of the fragment. The resulting fragment was cloned into pXT10 that was digested previously with *Sap*I. 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 the indicated 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

1641 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 that of *aacC1*. The resulting 3126 bp DNA fragment was used to transform strain RX and Gm^R recombinants isolated to create strain RdsbAX, which contains a single copy of *dsbA* under the control of the xylose-inducible *xylA* promoter.

To complement the *dsbA* knockout with a wild type copy of *dsbA* under 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 1983 bp fragment containing *dsbA* from a pXT10-based plasmid carrying *dsbA* coding sequences. A 2716 bp PCR product was amplified from a kanamycin-marked derivative of pXT10 with primers 5pkan1 (5'-GAGGCCCGTGTCTCAAAATCTCTGATG-3') and 3revRfaD1 (5'-AACAGGCTACGATAAACCATTCAAAACAGT-3'). The 1983 bp and 2716 bp fragments were joined via their 27-bp of overlapping sequence by PCR with primers pXT10thyA-F and 3revRfaD1, and the resultant 4672 bp PCR product was transformed into strain RdsbAX (grown in 1mM D-xylose to induce expression of *dsbA*) and Km^R transformants isolated to create strain RdsbAC.

To control for effects of the insertion at 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 4334 bp PCR product containing a precise deletion of the *dsbA* coding sequences of the 4672 bp construct described above in RdsbAX except that primers 3xylF1 (5'-ACGTTTATCAACAGCGATAGGATCAAGT-3') and 3pDsbAsapKan (5'-CATCAGAGATTTTGAGACACGGGCCTCTTACGAAGAGCGGCGCGCCGCTCTTCCCATTTCTTTCCTCTTATTTAATGATACCGCGA-3') were used in place of primers pXT10thyA-F and 3dsbAkan. Selection for Km^R transformants resulted in isolation of strain RdsbAV. To construct a strain that contains the 'empty vector' in a wild type background, the same 4334 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), vector only strain (HXV), and a complemented strain (HdsbAC) in the Hib strain Eagan background. All strains were verified for wild type and *dsbA* mutant phenotypes in the DTT sensitivity assay and evaluated by sequence analysis to contain the desired recombinant loci as described for the corresponding Rd derivatives above.

HbpA strain construction. The *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 1083 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 1020 bp PCR product containing the 3' flanking region of ***hbpA*** generated with primers 5hbp3 (5'-CATTTGATGCTCGATGAGTTTTTCTAATTCATATTGATTTACTTATTTTAAGCCCT-3') and 3hbp3 (5'-CAAAAGGGGTGAGTATAAATTTACACTCAA-3'). The 1083 bp, 1020 bp, and 816 bp fragments were joined in a PCR reaction via their complementary ends in combination with primers 5hbp1 and 3hbp3. The resulting 2871 bp fragment was introduced into ***H. influenzae*** Rd and Km^R transformants selected on sBHI containing Km to create strain RhbpA. To construct an ***hbpA*** knockout 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 1842 bp fragment containing the ***hbpA*** coding region and including 142 bp upstream of ***hbpA*** was amplified from Rd using primers 5hbpha (5'-AAAGCTCTTCAATGATTAATTTGTTATAATCCATAGA-3') and 3hbpha (5'-TTTGCTCTTCTTTATGCATAATCTGGCACATCATATGGATATTTACCATCAACACTCACACCATA-3'). This set of primers also adds a C-terminal HA epitope tag to ***hbpA***. This PCR product was cloned between the two ***Sap***I sites of pXT10 to generate the plasmid pXhbp1.5, which was then introduced into strain RhbpA with selection for Tet^R,

to create strain RhbpAC. To introduce a non-polar, in-frame deletion of *dsbA* into strain RhbpAC, this strain was transformed with the 3126 bp *dsbA* replacement fragment described above and Gm^R transformants selected to create strain RhbpAC*ΔdsbA*.

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 (302). 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 with primers specific to sequences flanking the inserted recombinant region.

DTT sensitivity assay. To determine the sensitivity of *dsbA* knockouts to dithiothreitol (DTT), strains were inoculated in triplicate from overnight cultures into 25 ml of sBHI in a 50-ml Erlenmeyer flask at an optical density at 600 nm (OD_{600}) of 0.01 and incubated at 35°C with shaking at 250 RPM. When cultures reached log phase, they were diluted in sBHI to 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 to a final concentration of 5 mM, or with sBHI alone in control wells. The plate was then incubated at 35°C for 16 hours in a $Versa_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA) set to read absorbance at 600 nm every 10 min. Sensitivity was scored as a relative growth yield as assessed by OD_{600} 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 from overnight

cultures in triplicate into 25 mls of sBHI in 50-ml Erlenmeyer flasks or 5 mls in culture tubes at an OD_{600} of 0.01. The resulting cultures were incubated aerobically at 35°C, 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 log phase, they were diluted in sBHI to 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 µM, 62.5 µM, 125 µM and 250 µM in sBHI and for anaerobically grown cultures, 0 µM, 62.5 µM, 125 µM and 500 µM. The plate was then incubated at 35°C for 16 hours in the microplate reader and absorbance read at 600 nm every 10 min for evaluation of growth rates and final culture densities.

Growth of *dsbA* strains. To determine growth rates in rich media and in defined media, strains were inoculated in triplicate at 0.01 OD_{600} from standing overnight cultures into 25-ml Erlenmeyer flasks containing 15 ml of sBHI or MIc, a low nutrient media capable of supporting growth of *H. influenzae* (15). The resulting cultures were incubated at 35°C, shaking at 250 RPM and aliquots removed for absorbance measurements at 600 nm every 30 min for 6.5 hours. Growth rates were calculated by nonlinear regression analysis.

To evaluate growth of the *dsbA* mutant in comparison to the *hbpA* mutant under heme limiting conditions, strains (RXV, RdsbAV, RhbpAV, and RhbpAC) were grown as standing overnight cultures, washed once in sterile Hanks' Balanced Salt Solution

(HBSS) (Invitrogen, Carlsbad, CA), and diluted to an OD_{600} of 0.01 in BHI broth supplemented with NAD and different concentrations (5 µg/ml, 0.5 µg/ml, 0.25 µg/ml, and 0.025 µg/ml) of heme (Sigma-Aldrich, St. Louis, MO) or hemoglobin (Becton, Dickinson and Company) in a 96-well microplate at a final volume of 200 µl. Cultures were then incubated at 35°C in the microplate reader, and absorbance at 600 nm measured every 10 min for 16 hours.

Growth of *hbpA* strains. To compare the generation times at 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 mls of BHI containing different concentrations of free heme (10 µg/ml, 0.5 µg/ml, 0.05 µg/ml, and 0 µg/ml). These cultures were then aliquoted into the wells of eleven 96 well flat bottom dishes. One dish was incubated at 35° for 14 hours in the microplate reader and absorbance read at 600 nm every 10 min (no aerobic growth was detected in wells not supplemented with heme). The other ten dishes were sealed in individual BD GasPak EZ Anaerobe Gas Generating Pouches (Becton, Dickinson and Company) and incubated at 35° C. Plates were removed from bags at appropriate intervals and absorbance at 600 nm was recorded. Growth rates were determined as described above.

Competence assay. Cultures were grown in triplicate as described for DTT sensitivity assays above, and competent cells were prepared from these cultures as previously described (15). Competence in mutant and parental strains was measured by assessing transformation frequencies with chromosomal DNA from a streptomycin resistant *H.*

influenzae strain (1 µg) and selection on sBHI agar plates containing 100 µg/ml streptomycin. Transformation efficiencies were calculated as the number of streptomycin resistant colonies divided 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 ANOVA with Bonferroni's multiple comparison test to evaluate frequency differences between RdsbAV and all other strains.

Murine bacteremia model. Strains were inoculated from standing overnight cultures at an OD_{600} of 0.01 into 10 mls of sBHI in a culture tube. The resulting cultures were incubated in an anaerobic chamber shaking at 120 RPM and 35°C for 5 hours, a condition that was permissive for growth of the *hbpA* mutant (Table 3.2). For co-infections, strain RdlacZ (reference strain) was mixed with each experimental strain at a 1:1 ratio. For inoculation, bacteria were washed and diluted in HBSS to a final concentration of 2×10^9 bacteria per ml. Female 6.5 week old C57BL/6J mice (4 or 5 per strain) (The Jackson Laboratory, Bar Harbor, ME) were inoculated by intraperitoneal (IP) injection of 200 µl of bacterial suspension. Twenty-four hours after inoculation, 5 µl of blood was recovered aseptically from each mouse via tail bleeds. The blood was diluted into BHI broth, plated on sBHI agar plates (for single strain infections) or sBHI agar plates containing S-Gal (Sigma-Aldrich) (for co-infections), and incubated overnight in an anaerobic chamber at 35°C for CFU determination. For statistical analysis, CFU/ml for single strain infections were normalized by Log_{10} transformation for ANOVA using Prism 4.0c. CFU for co-infection data were Log_{10} transformed and the ratio of

experimental strains to RdlacZ was calculated and analyzed using Prism 4.0c. Comparisons of two data sets utilized the *t*-test and comparisons of greater than two data sets were performed using ANOVA with Bonferroni's multiple comparison test. All procedures 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 from standing overnight cultures at an OD_{600} of 0.01 into 50 mls of sBHI in a 50-ml Erlenmeyer flask. Cultures were incubated with shaking at 120 RPM at 35°C until they achieved a density of 0.4 OD_{600}. Cells were washed once and diluted in sterile HBSS to a final concentration of 2×10^3 bacteria per ml. Five-day-old Sprague-Dawley rat pups (Charles River Laboratories, Boston, MA) were inoculated IP with 100 µl of strains HXV (n = 11) and HdsbAV (n = 11), or HdsbAC (n = 12). Infants inoculated with each strain were returned to mothers and each group was housed separately. Blood (5 µl) was collected aseptically via tail bleeds at 12, 36, and 120 hours post inoculation, diluted into BHI, and plated on sBHI agar plates for enumeration of CFU's as described above. For statistical analysis, ANOVA with Bonferroni's multiple comparison test was used as described above.

HbpA western blot. For analysis of HbpA, strains were inoculated from standing overnight cultures into duplicate 50 ml sBHI cultures in 50-ml flasks at a starting density of 0.01 OD_{600} and incubated at 35°C with shaking at 250 RPM. When cultures achieved log phase, 1 ml was removed and pelleted by centrifugation (18000 x 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 SDS-PAGE sample buffer and proteins separated on 8% SDS-PAGE followed by electrotransfer to Immobilon-P (Millipore, Billerica, MA). HbpA-HA was visualized via Western blot using primary antibody anti-HA1.1, 1:1000 (Covance, Berkeley, CA) and secondary antibody goat anti-mouse IgG HRP conjugate, 1:5000 (Upstate, Lake Placid, NY). Equal sample concentration was verified by Coommassie Blue staining. HbpA-HA was quantified by generating a 10% dilution series of each protein sample and separating them on 8% SDS-PAGE. HbpA-HA was then visualized by Western blot 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 same 50 ml cultures used for the HbpA western blot using TRIzol Reagent (Invitrogen, Carlsbad, CA). RNA was then treated with DNaseI (Ambion, Austin, TX), extracted with acid phenol, chloroform extracted, and concentrated by ethanol precipitation. The RNA (5µg total) samples served as a template for cDNA synthesis with random primers (New England Biolabs, Beverley, MA) and Super Script II reverse transcriptase (Invitrogen, Carlsbad, CA). The qRT-PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) and fluorescence measured using the DNA Engine Opticon II system (MJ Research, Waltham, MA). One tenth of each cDNA reaction was used as a template for qRT-PCR with primers 5'hbpART (5'-

ATGATTAATTTGTTATAATCCATAGA-3') and 3'hbpART (5'-CAAGCTGCCAAAACAAGAGT-3') that amplify the first 200 bp of *hbpA*. Primers RpoA5' (5'-GTAGAAATTGATGGCGTATTG-3') and RpoA3' (5'-TCACCATCATAGGTAATGTCC-3') were used to amplify the RNA polymerase alpha subunit gene, *rpoA* as an internal reference. Real time cycler conditions were as described previously (302).

Complement binding. Western blotting for assessment of binding of complement C3 and C4 activation products was performed as previously described (75, 231). Briefly, cultures of strains RXV, RdsbAV, and RdsbAC were grown as described for HbpA westerns above, then washed and suspended in HBSS containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ in a final reaction volume of 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^{o}C followed by differential treatment with 1M methylamine (pH 11), which dissociates complement ester-linkages but not amide linked complement from target structures (75, 231). Bacteria were lysed in 1X 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 (75). No differences were observed in binding profiles of these strains to C3 or C4 subunits with or without methylamine treatment.

Serum bactericidal assay. Sensitivity of *dsbA* mutants to serum was determined as previously described (233). Briefly, triplicate cultures of strains RXV, RdsbAV,

RdsbAC, and RdgalU were grown as described for DTT sensitivity assays above. At log phase, 2,000 CFU from each culture were diluted in HBSS and incubated at 37^{o}C for 30 min with or without a final concentration of 2% NHS in a 150 µl reaction volume. For CFU determination, 15µl was plated on sBHI agar. Bacteria were also incubated in parallel with serum that had been previously inactivated by incubation at 56^{o}C for 30 min.

Thiol modification. 10 OD units of cells grown as described above for the anti-HA immunblot were harvested at log phase, by centrifugation at 5000 x G for 5 min. Before thiol modification of periplasmic proteins, the outer membrane was disrupted using the methods provided in the PeriPreps periplasting kit (Epicentre, Madison, Wisconsin). Briefly, the cell pellets were resuspended in 2 mls of 200 mM Tris (pH7.4), 1mM EDTA, 20% sucrose and 30 units of lysozyme (Sigma-Aldrich, St. Louis, MO) and incubated at room temperature for 5 min. After incubation, 3 ml of cold water was added followed by 10 min of incubation on ice. Each 5 ml preparation was then divided in half, with one half receiving treatment with 5 mM EZ-Link Maleimide-PEO_2 Biotin (which adds 525.23 daltons per bond) (Pierce, Rockford, IL) and the other half receiving no treatment. After incubation for 50 min at room temperature the resulting spheroplasts and associated membranes were collected by centrifugation at 4000 x g for 15 min and resuspended in 375 µl Peripreps lysis buffer (10mM Tris-HCl pH 7.5, 50 mM KCl, 1mM EDTA and 0.1% deoxycholate). After lysis, 0.30 OD_{600} equivalents of each sample were boiled for 5 min in SDS loading buffer, and proteins were separated by non-reducing 8% SDS-PAGE. HbpA-HA was then visualized by Western blot as described above. 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, 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 2.1). To verify that the *dsbA* mutant exhibits the DTT sensitivity phenotype seen with *dsbA* mutants of other species (184), and to determine its growth properties, wild type parent (Rd), Rd carrying the 'empty vector' (RXV), *dsbA* deletion mutant carrying the 'empty vector' (RdsbAV), and the complemented strain (RdsbAC) were evaluated for growth phenotypes under a range of conditions and for defects in DTT resistance and transformation. Generation times under aerobic conditions in rich medium (sBHI) for RXV, RdsbAV, and RdsbAC were 32 ± 2 min, 32 ± 3 min, and 36 ± 4, and in defined medium (MIc) 48 ± 4 min, 41 ± 2 min, and 38 ± 3 min, respectively. Similarly, growth yields of these strains after 6.5 hours of anaerobic culture in sBHI were indistinguishable. All DsbA+ strains (Rd, RXV, and RdsbAC) exhibited equivalent growth yields after 16 hrs in the presence of 5 mM DTT, reaching final average densities of ~0.5 OD_{600}, whereas growth of the DsbA- strain, RdsbAV, was dramatically attenuated under this condition and did not exceed a density of 0.1 OD_{600} similar to results with *dsbA* mutants in *E. coli* (184). 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 1mM 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 transformation efficiencies of our strains using *H. influenzae*

Table 2.1. Strains and plasmids used in this chapter

Strains and Plasmids	Relevant features		Source
Plasmids			
pXT10	Delivery vector for chromosomal expression at the xylose locus of *H. influenzae*, containing *xylF*, *xylB*, *xylA*$^{\Delta 4\text{-}804}$ and tetracycline resistance cassette, *tetAR*		(301)
pXylHA1.1	pXT10 carrying a 614 bp fragment immediately 3' of P*xylA* containing ATG to 1 codon before TAA of *dsbA* from RdAW with a C- terminal HA epitope tag		This study
pDsbAHA1.2	pXT10 carrying a 1060 bp fragment immediately 3' of P*xylA* containing P*dsbA* to 1 codon before TAA of *dsbA* from RdAW with a C- terminal HA epitope tag		This study
pXhbp1.5	pXT10 carrying a 1842 bp fragment immediately 3' of PxylA containing p*HbpA* to 1 codon before TAA of *hbpA* from RdAW with a C-terminal HA epitope tag		This study
Strains	*Genotype*	*Phenotype*	
RdAW	*H. influenzae* capsule deficient type D	WT	(302)
RXT10	*xylA*::pXT10, tet^R	tetracycline resistant	(301)
RX	*xylA*::*dsbA*, tet^R	*dsbA*$^+$, xylose inducible *dsbA*	This study
RdsbAX	RdxyD2, *dsbA*::gent^R	*dsbA*$^-$, xylose inducible *dsbA*	This study
RXV	*xylA*::kan^R	kanamycin resistant	This study
RdsbAV	RdxyG2.1, *xylA*::kan^R	*dsbA*$^-$	This study
RdsbAC	RdxyG2.1, *xylA*::*dsbA*, kan^R	*dsbA*$^+$, complemented	This study
RhbpA	*hbpA*:: kan^R	*hbpA*$^-$	This study
RhbpAV	RdhbpA6, *xylA*::pXT10, tet^R	*hbpA*$^-$	This study
RhbpAC	RdhbpV2.1, *xylA*::*hbpA*, tet^R	*hbpA*$^+$, complemented	This study
RhbpACD	RdhbpX6.2, *dsbA*::gent^R	*dsbA*$^-$, *hbpA*$^+$	This study
RdlacZ	*xylA*::*lacZ*, tet^R	*lacZ*$^+$	(302)

DNA carrying a streptomycin resistance allele (Materials and Methods). Transformation efficiencies relative to the wild type parental Rd strain for strains RXV, RdsbAV, and RdsbAC, were 1.12, 1.33 x 10^{-6}, and 1.04, respectively, and this 6-log decrease in the 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 are observed with our in-frame *H. influenzae dsbA* deletion mutants and complemented strains exhibit the wild type phenotypes.

DsbA is required during *H. influenzae* infection in mice. The strains generated above provided a well-defined set of mutants for investigation of the role of *dsbA* during infection. Though 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 (50, 159). The mouse model was used to evaluate bloodstream survival of the *dsbA* mutant, RdsbAV, versus the vector only control strain, RXV. At 24 hours post-inoculation, 48-fold fewer bacterial CFU were recovered from mice inoculated with the *dsbA* mutant compared to the control strain (Figure 2.1A). Recovery of bacteria 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 more accurately assess the level of attenuation, this experiment was performed as a competition between each strain in mixed infections with strain RdlacZ, which expresses *E. coli lacZ* at the *xyl* locus. Consistent with results from single strain inoculations, the competitive index of RdsbAV was 100 to 170-fold less than that of Rd, RXV, or RdsbAC (Figure

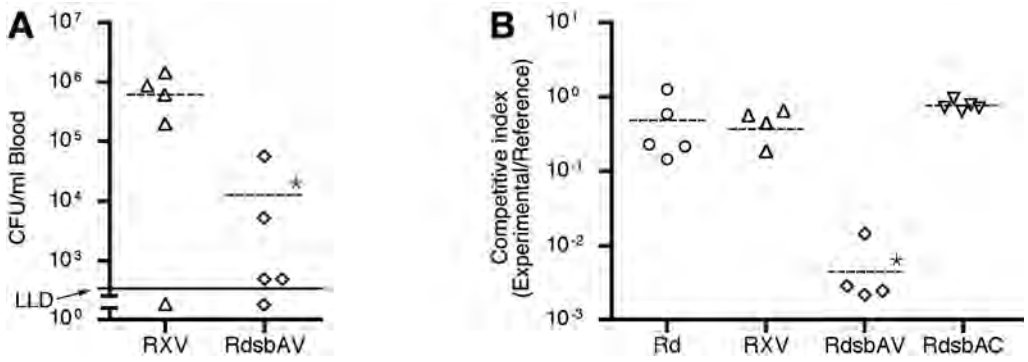

Figure. 2.1. Effect of *dsbA* mutation on survival of *H. influenzae* in the mouse model. Strains were inoculated IP into mice and bacteremia assessed after 24 hrs. Individual animals are represented by symbols and the dashed line represents the average. (A) Single strain infection. Asterisk indicates $p = 0.039$ (*t*-test). (B) Co-infections with the experimental strains indicated and reference strain (RdlacZ). Competitive indices are the ratios of $LacZ^-$ (experimental strain) to $LacZ^+$ (reference strain, RdlacZ). Asterisk indicates $p < 0.001$ (ANOVA with Bonferroni's multiple comparison test). Lower limit of detection (LLD) was 500 CFU/ml. Data points below the LLD indicate bacteremia was not detected.

2.1B). Therefore, infection with a mutant containing a nonpolar *dsbA* deletion resulted in reduced levels of bacteremia in mice, and complementation verified that this effect was specific to the *dsbA* mutation.

Pathogenesis associated phenotypes in 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 is likely to require *dsbA*, however the pilus gene cluster is absent in Rd (77). Therefore, we examined several major virulence-associated phenotypes in *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 (48, 302). Therefore we addressed the possibility that loss of DsbA confers sensitivity to this oxidant. After either anaerobic or aerobic pre-growth conditions, the mutant and 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 outer core have been implicated in animal models of *H. influenzae* bacteremia (95, 121, 243), and resistance to complement has emerged as an important virulence mechanism mediated by these structures (75, 117). Therefore, we investigated whether the *dsbA* mutant exhibits major LOS structural alterations or increased susceptibility to killing by serum complement. We detected no

apparent changes in LOS mobility on SDS-PAGE for the *dsbA* mutant versus wild type. Wild-type, *dsbA* mutant, and complemented strains were compared in a serum bactericidal assay with 2% pooled NHS. Percent survival for strains RdV, RdsbAV, and RdsbAC was 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 of complement in this assay. Differences were not detected in levels of complement binding to strains RdV, RdsbAV, and RdsbAC as assessed on anti-C3 and anti-C4 immunoblots containing lysates of cells that had been incubated with 2% pooled NHS (data not shown), though it is possible that a small difference in C3 or C4 binding not detected by immunoblotting could mediate the moderate increase in serum sensitivity observed in the mutant.

We conclude that the *dsbA* mutant is not markedly impaired under the conditions tested for hydrogen peroxide resistance or LOS production. An effect on serum resistance was observed that could play a role. 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* (116, 141, 158). These proteins were compared by BLASTP (4)(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 2.2). *H. influenzae* proteins identified by this search

Table 2.2 Potential DsbA Targets

Putative DsbA targets in *E.coli*	Proposed function in *E. coli*	Potential *H. influenzae* homologue	Blast identities (%)	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

include a predicted periplasmic lipoprotein, HbpA, which is required for utilization of multiple heme sources (108, 189). Multiple systems participate in scavenging heme from sources in the host that include heme-hemopexin, hemoglobin, hemoglobin-haptoglobin, heme-albumin, and free heme (42, 193). 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.

The 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 (210), 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 hemagglutinin (HbpA-HA). We first constructed a nonpolar *hbpA* deletion mutant (RhbpAV) in *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 (189). Furthermore, the *hbpA* mutant exhibited equivalent anaerobic growth to that of wild type Rd and the isogenic 'vector only' strain (RdV), regardless of heme availability (Table 2.3). When expressed in the *hbpA* mutant background, HbpA-HA fully complemented the mutant for aerobic growth at all heme

Table 2.3. Growth phenotype of *hbpA* mutant

Strain	Generation time in minutes						
	Aerobic conditions with free heme supplement at a concn of[a]			Anaerobic conditions with free heme supplement at a concn of[b]			
	[c]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 in microplate reader
[b]Growth in anaerobic bag system
[c]No growth

concentrations tested (strain RhbpAC) suggesting that the epitope tag does not impair its function (Table 2.3).

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. Levels of HbpA-HA in the ***dsbA*** deletion mutant RhbpAC***DdsbA*** were approximately 50% of the amount detected in the DsbA+ control strain, RhbpAC as determined by densitometry (Figure 2.2A). QRT-PCR detected no differences in 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 post-transcriptional 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 non-reducing SDS-PAGE after isolation from the DsbA- and DsbA+ ***H. influenzae*** strains (Figure 2.2B). Whereas HbpA-HA from DsbA+ cells appeared as a single band, samples from DsbA- cells yielded an additional HbpA-HA band of slower electrophoretic mobility. Treatment of cells prior to protein isolation with a thiol reactive ligand, maleimide PEO_2 biotin (MPB), resulted in no change in HbpA-HA in the parental strain (Figure 2.2B, lanes 1 and 2) as expected if the two non-acylated cysteine residues are in the oxidized state as a disulfide bond. In contrast, the slower migrating species in the ***dsbA*** mutant (Figure 2.2B, lane 3) exhibited an additional decrease in mobility in samples from MPB treated cells (Figure 2.2B, lane 4), consistent with addition of MPB to free thiols on cysteine residues of this protein.

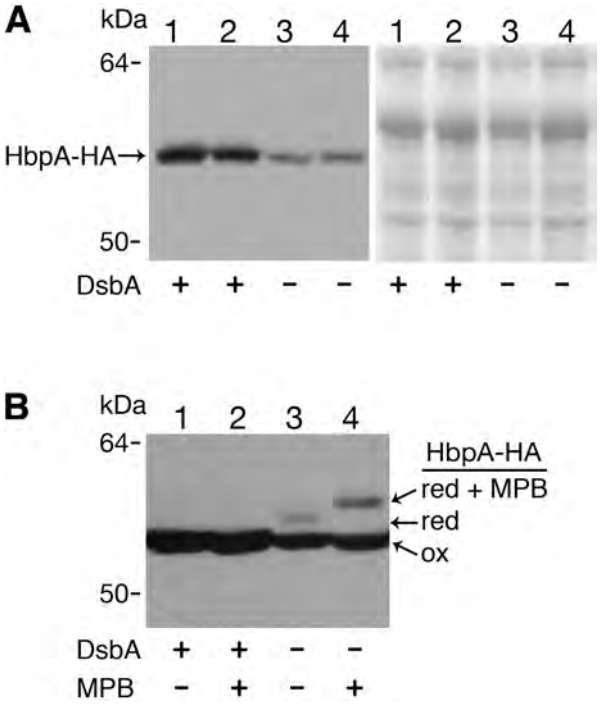

Figure. 2.2. Effects of *dsbA* mutation on HbpA protein levels and thiol redox state. (A) Detection of HbpA levels in DsbA+ versus DsbA- strains. Whole-cell lysates of duplicate cultures of RhbpAC (complemented ***hbpA*** deletion mutant carrying ***hbpA***-HA in the ***xyl*** locus)(lanes 1 and 2) and RhbpACΔ***dsbA*** (RhbpAC with ***dsbA*** deletion)(lanes 3 and 4) were resolved by 8% SDS-PAGE under reducing conditions and detected via anti-HA Western blot (left panel). Equal sample concentration was verified by Coomassie Blue stain (right panel). (B) Differential modification of thiols on HbpA in ***dsbA*** mutant compared to wild-type. Spheroplasts were prepared from log phase cultures, resolved by 8% SDS-PAGE under non-reducing conditions, and detected via anti-HA Western blot. Strains were the same as in panel A, and treated (+) or untreated (-) with 5 mM MPB where indicated. Arrows indicate the oxidized (ox) form of HbpA, the reduced (red) form of HbpA, and the reduced form with thiols modified with MPB (red + MPB).

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 (17). Therefore, a longer exposure time was used to clearly visualize the reduced form in Figure 2B, masking the decrease in total HbpA levels that was detected in the ***dsbA*** mutant in the quantitative studies above (Figure 2.2A).

HbpA in the oxidized form was detected in the ***dsbA*** mutant, and it is likely that some HbpA activity is 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) at 5 µg/ml, 0.5 µg/ml, 0.25 µg/ml, and 0.025 µg/ml of either heme or heme-hemoglobin (data not shown). RhbpAV exhibited progressively reduced growth rates as heme or hemoglobin concentrations were lowered, and RhbpAC grew at the same rates as wild type, similar to results in Table 3. Conversely, no differences were observed between RXV and RdsbAV, suggesting that the residual levels of active HbpA in the ***dsbA*** mutant are 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 contribute to its decreased survival 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), isogenic HpbA+ parent (RdV), and the complemented strain (RhbpAC). Inocula were prepared from cultures grown anaerobically, a condition permissive for growth of the *hbpA* mutant (Table 2.3), and mice were inoculated by the IP route (Figure 2.3). Single strain infections indicated a decrease in recovery of bacterial CFU from mice inoculated with the RhbpAV compared to RdV (17-fold) or RhbpAC, (~60-fold); however, this trend did not achieve statistical significance (Figure 2.3A). To control for variation between animals, we repeated the experiment in the competition format. Each strain was co-inoculated with an equal number of cells of strain RdlacZ and competitive indices evaluated. The mutant exhibited a ~27-fold defect in competition relative to the 'vector only' and complemented strains, and these differences were statistically significant (Figure 2.3B). We conclude that survival of the *hbpA* mutant is attenuated in the bacteremia model, but to a lesser extent than is the *dsbA* mutant. Therefore, a decreased level of HbpA could contribute to the defect of *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 *H. influenzae* type b strain Eagan. The infant rat bacteremia model provides a

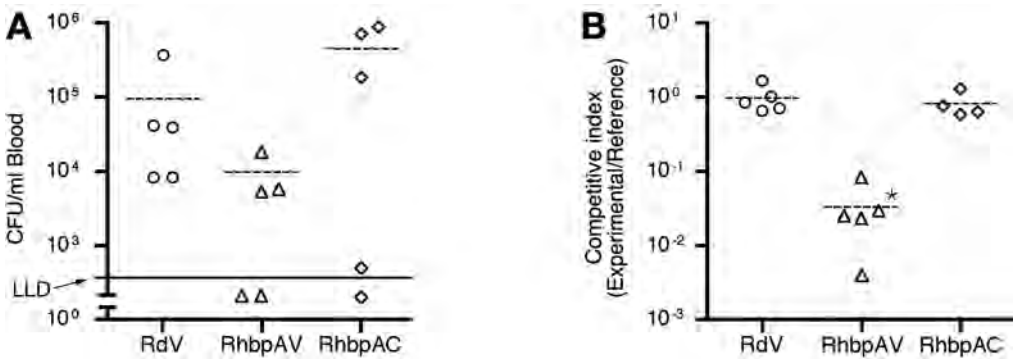

Figure. 2.3. Effect of *hbpA* mutation on survival of *H. influenzae* in the mouse model. Strains were inoculated IP into mice and bacteremia assessed after 24 hrs. Individual animals are represented by symbols and the dashed line represents the average. (A) Single strain infection. (B) Co-infections with the experimental strains indicated and reference strain (RdlacZ). Competitive indices are calculated as the ratio of $LacZ^-$ (experimental strain) to $LacZ^+$ (reference strain, RdlacZ). Asterisk indicates $p < 0.001$. Lower limit of detection (LLD) was 500 CFU/ml. Data points below the LLD indicate bacteremia was not detected.

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 at five days of age were inoculated IP with wild-type 'vector only' (HXV), *dsbA* mutant (HdsbAV), and the complemented strain (HdsbAC) and monitored for bloodstream infection at 12, 36, and 120 hours post-inoculation (Figure 2.4). At all times of sampling, recovery of *H. influenzae* CFU's from animals inoculated with the *dsbA* mutant was reduced by at least 100-fold compared with the parental or complemented strains, and this level of attenuation was statistically significant in all cases. Furthermore, by 120 hours post-inoculation only two of eleven animals inoculated with the *dsbA* mutant had detectable bacteremia, whereas most of the animals infected with wild type (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 magnitude of bacteremia in the infant rat model by a virulent clinical isolate of *H. influenzae* type b.

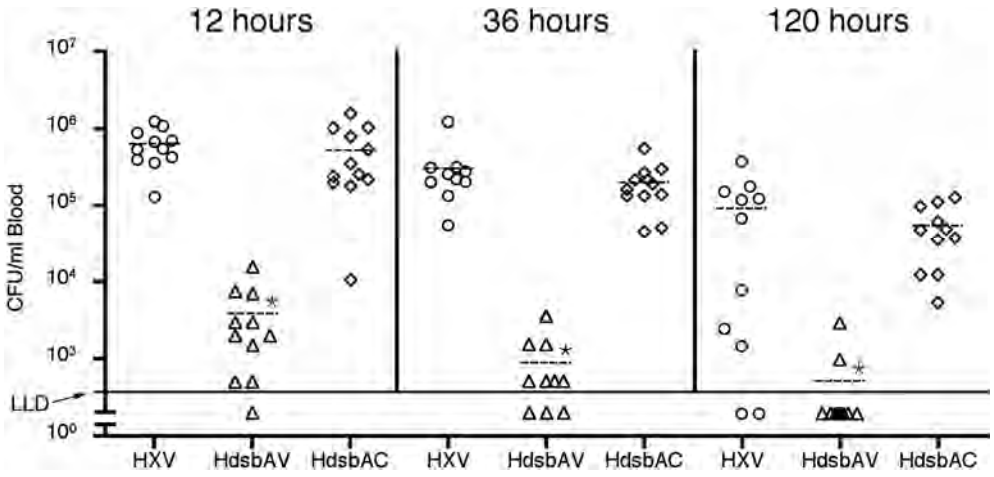

Figure. 2.4. Effect of *dsbA* mutation on virulence of *H. influenzae* type b in infant rats. Strains were inoculated IP into 5-day-old infant rats. Individual animals are represented by symbols and the dashed line represents the average. Asterisks indicate $p < 0.001$. The lower limit of detection (LLD) was 500 CFU/ml. Data points below the LLD indicate bacteremia was not detected.

DISCUSSION

We report a role for the *H. influenzae* disulfide oxidoreductase, DsbA, in bloodstream infection. Nonpolar *dsbA* deletion mutations in either the Rd or Hib Eagan strain backgrounds resulted in equal levels of attenuation in animal models, and 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 Rd and encapsulated type b strains of *H. influenzae*, 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 of LOS synthesis, evasion of complement deposition, and oxidative stress resistance. We detected no apparent role for *dsbA* in LOS synthesis or hydrogen peroxide resistance though 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 sufficient to account for the full defect of the *dsbA* mutant in pathogenesis. The results suggest that 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 amino acid sequence comparison 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 was in the presence of high concentrations (5 mM) of dithiothreitol, a condition that *H. influenzae* is unlikely to encounter *in vivo*. Reducing agents are present in plasma, such as glutathione which is found as a mixture of reduced and oxidized forms at a total concentration estimated to be ~5-30 mM (7). Growth in the presence of glutathione was tested at concentrations ranging from 0.005-1 mM, and growth of the *dsbA* mutant and parental strain was 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. Results of 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 (108, 189) and normal growth under anaerobic conditions (Table 2.3). 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 exhibits a defect in the murine bacteremia model, though not as pronounced as the defect of the *dsbA* mutant, and complementation restores its ability to promote bacteremia to that of 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 conditions of heme limitation, 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 on low heme. Nevertheless, both DsbA and HbpA participate in bloodstream infection. Survival *in vivo*, where diverse host factors efficiently sequester free heme, may exert a more stringent requirement for wild type levels of HbpA than the *in vitro* conditions tested here. Alternatively, it is possible that the *in vivo* growth defect of the *dsbA* mutant results from effects on a DsbA-dependent protein whose role in virulence remains to be identified. The partial attenuation of the *hbpA* mutant relative 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 2.2), are required for growth under zinc limiting conditions *in vitro* (166) and adhesion to mucosal epithelium during colonization

of the chinchilla nasopharynx (26), respectively. In addition, homologs of Pzp1 and P5 in several other bacterial species have been implicated in pathogenesis (5, 148, 240, 250, 292, 309). 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 zinc levels available to bacteria within the mammalian host are lower than those *in vitro*. A defect in levels or activity of Pzp1 in the *H. influenzae dsbA* mutant could contribute to its virulence defect and additional studies will be required to evaluate this hypothesis. In addition, a role for P5 during *H. influenzae* bacteremia has not been reported, and it will be of interest to investigate this possibility. Related outer-membrane proteins in other pathogens have been implicated in diverse aspects of pathogenesis, including complement resistance (240, 250, 292), 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 (Table 2.2) are not defined in *H. influenzae*, 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 pathogenesis by this bacterium, and may lead to novel approaches to treatment or prevention of invasive disease.

ACKNOWLEDGEMENTS

We thank Dr. Sanjay Ram for assistance and advice, and Dr. Jeffrey Gawronski for helpful comments. This work was supported in part by a grant from the American Heart Association and NIH NIAID 1RO1-AI49437 to B.J.A.

PREFACE TO CHAPTER III

This Chapter has been published

Charles V. Rosadini, Jeffrey D. Gawronski, Daniel Raimunda, José M. Argüello, and Brian J. Akerley. A novel zinc binding system, ZevAB, is critical for survival of nontypeable *Haemophilus influenzae* in a murine lung infection model. Infection and Immunity, Published Ahead of Print May 2011.

Jeffrey D. Gawronski generated the informatics-based list of DsbA substrates.

Charles V. Rosasdini and Daniel Raimunda performed the metal binding assays.

Charles V. Rosadini, José M. Argüello, and Brain J. Akerley designed the experiments.

Charles V. Rosadini performed all remaining experiments and wrote the manuscript.

CHAPTER III

A novel zinc binding system, ZevAB, is critical for survival of nontypeable *Haemophilus influenzae* in a murine lung infection model.

ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi) is a gram-negative bacterial pathogen that causes upper and lower respiratory infections. Factors required for pulmonary infection by NTHi are not well understood. Previously, using High-throughput Insertion Tracking by deep-Sequencing (HITS), putative lung colonization factors were identified. Also, previous research indicates that secreted disulfide-dependent factors are important for virulence of *H. influenzae*. In the present study, HITS data were compared with an informatics-based list of putative substrates of the periplasmic oxidoreductase, DsbA, to find and characterize secreted virulence factors. This analysis resulted in identification of the "zinc binding essential for virulence" (*zev*) locus consisting of *zevA* (HI1249) and *zevB* (HI1248). NTHi mutants of *zevA* and *zevB* grew normally in rich media, but were defective for colonization in a mouse lung model. Mutants also exhibited severe growth defects in media containing EDTA and were rescued by supplementation with zinc. Additionally, purified recombinant ZevA was found to bind to zinc with high affinity. Together, these data demonstrate that *zevAB* is a novel virulence factor important for zinc utilization of *H. influenzae* during conditions when zinc is limiting. Furthermore, evidence presented here suggests zinc limitation is likely an important mechanism for host defense against pathogens during lung infection.

INTRODUCTION

Nontypeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterial pathogen uniquely adapted to colonize the nasopharynx of healthy humans with a carriage frequency of approximately 20-80% (197). An opportunistic pathogen, NTHi resides asymptomatically in the upper airways of humans, but can disseminate into privileged anatomical locations causing infections such as otitis media, sinusitis, and pneumonia (201). NTHi is also one of the most prevalent microorganisms found in the lungs of patients with exacerbations of chronic obstructive pulmonary disease (202, 205, 251) and cystic fibrosis (86, 187, 245). An effective vaccine against NTHi strains has not yet been discovered, likely due to high variability of surface antigens between strains (251).

Little is known regarding factors required for survival of *H. influenzae* in the lung. To address this, we recently examined a transposon mutant library of *H. influenzae* Rd in a mouse lung infection model using a novel technique termed High-throughput Insertion Tracking by deep-Sequencing (HITS) (84). HITS analysis revealed a total of 136 genes required for survival in this site, including previously identified virulence determinants such as those involved in lipooligosaccharide synthesis (219) as well as several genes previously unrecognized to play a role in pathogenesis, some of which are implicated in diverse processes including DNA repair, membrane remodeling, and nutrient acquisition. However, many genes identified by HITS have unknown functions and are yet to be characterized.

Previously, the periplasmic disulfide-bond pathway was investigated to find virulence determinates of *H. influenzae* (241). Disulfide formation is mediated by the periplasmic oxidoreductase protein, DsbA, which directly catalyzes disulfide bond formation by transfer of a disulfide bond to free thiol groups of cysteine residues in secreted target proteins (79, 313). This system is important for providing structural stability and function to secreted virulence factors in a range of bacterial pathogens (113). DsbA was demonstrated to be required for pathogenesis of *H. influenzae* in a mouse model of bacteremia (241). Additionally, the heme-binding lipoprotein, HbpA, an important factor required for growth of *H. influenzae* on several heme sources (108, 189), was found to be a substrate of DsbA and was required for pathogenesis. However, the defect of an *hbpA* mutant was not as severe as that of a *dsbA* mutant suggesting that other, unidentified DsbA-dependent factors must be required for pathogenesis.

Since a DsbA-dependent protein was found to have a role in virulence of *H. influenzae,* we hypothesized that by examining genes required in the lung additional DsbA-dependent factors involved in infection could be found. We were particularly interested in investigating this set of proteins because their extracytoplasmic location and accessibility makes them attractive targets for development of therapeutics or vaccines for combatting NTHi infections. In this study, potential DsbA-substrates were identified based on predicted extracytoplasmic localization and putative disulfide bond formation. The whole-genome fitness data generated via the HITS procedure was used to identify candidate DsbA substrates required for survival of *H. influenzae* in the lung. This approach led us to characterize a locus important for zinc utilization, herein referred to as

"zinc binding essential for virulence" (*zev*). This locus contains an operon consisting of two genes: the potential DsbA-substrate *zevA* and a gene encoding a putative membrane protein called *zevB*. In contrast to the previously characterized ZnuABC zinc transport system which is required for optimal growth in rich culture medium in vitro (166), the *zevAB* system is specifically required for growth under severe zinc limitation. Results of this study suggest that the lung represents a niche that exposes infecting pathogens to severe zinc limitation and that the *zevAB* system is required for zinc homeostasis of *H. influenzae* during pathogenesis.

MATERIALS AND METHODS

Strains and culture conditions. *H. influenzae* RdAW (NZ_ACSM00000000), a capsule-deficient serotype d derivative (301), and pathogenic nontypable ***H. influenzae*** strain NT127 (NZ_ACSL01000014.1) were grown in brain heart infusion broth (BHI) supplemented with 10 µg/ml hemin and 10 µg/ml NAD (sBHI), or on sBHI agar plates at 35°C. To generate an anaerobic environment, strains were grown in anaerobic chambers with BBL GasPak Plus generators (Becton Dickenson and Company, Sparks, MD). Development of competence for transformation of ***H. influenzae*** was accomplished as previously described (15). For selection of Rd- and NTHi-derived strains, antibiotics were used at the following concentrations: 8 µg/ml tetracycline (Tc), 20 µg/ml kanamycin (Km), and 10 µg/ml gentamicin (Gm). For strain generation, plasmids and PCR products were constructed using standard molecular biology techniques (12). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, linearized and used to transform ***H. influenzae*** strains as previously described (301).

zevA* strain construction.** The NTHi *zevA* mutant strain NT1249G was constructed by replacement of the coding sequence of *zevA* (NT127 Locus_Tag HIAG_01363) with the Gm resistance cassette, *aacC1*. To do this, a 707-bp PCR product containing the 5' flanking region of ***ZevA-generated using primers 1249for and 1249B (Table 3.1), a 762-bp PCR product containing the coding region for the Gm resistance cassette generated with primers 5pGent1 and 3Gent2, and a 1,661-bp PCR product containing the 3' flanking

TABLE 3.1. Oligonucleotides used in this chapter

Primer	Sequence
1249for	CTTGTAGAATTTCACCAACAGAGGTGGGT
1249B	AGGCTTATGTCAATTCGAGAATTGAAAAAATCCTATTATTTTGAACGCTAGAATTTGA
5pGent1	CAATTCTCGAATTGACATAAGCCT
3Gent2	TTAGGTGGCGGTACTTGGGTCGAT
1249A	ACCCAAGTACCGCCACCTAAAAATGAAAAAATATAAAACTGGGCTCGT
1248rev	ACGAAAGTCGGAATGCAGAATGAATGGT
51249HA	AAAGCTCTTCAATGCTATAAAGATAATCATCTCTAAAATGCTCACGCT
31249R	TTTGCTCTTCTTTACTCACATTGAATTATTATTTTTTGCGCGA
1248BJBpArcA	TCTTTGTGATAGAATTTCGAGAATCTTCTACTCACATTGAATTATTACTTTTTGTGCGA
5parca	GATTCTCGAAATTCTATCACAAAGA
1248Ahib	ACCCAAGTACCGCCACCTAAAGTTAAAAATAAAGCCCATCAACATGA
1247revJB	AGGATAGATCGTAAAACGTGGGACAGCA
31248R	TTTGCTCTTCTTTAAAATAGGATTTTGCTTCCGCCAGTTGA
5ZnuA1	TGGCTAGAATAGATAATTACGAACAACGCT
3ZnuA1	AAGCATTGGTGCACCGTGCAGTCGATTTATGTTTCCTTACTTGTTGGTAGTAATTCT
IFTrcF	CGACTGCACGGTGCACCAATGCTT
3kan1.1	TTAGAAAAACTCATCGAGCATCAAATG
5ZnuA3	CATTTGATGCTCGATGAGTTTTTCTAAATCTTTCTAAAAACAATGACCGCATATAAAAGT
3ZnuA3	CTATTTTGGCGACTTTAACTGCATTG
5znuB1	CTAAGGTTTTAACATTTTTTCAGCTTCACGA
3ZnuB1	AAGCATTGGTGCACCGTGCAGTCGAAAATCGCCGTGTTTTCAACAATGGTTGG
5znuB3	CATTTGATGCTCGATGAGTTTTTCTAAGCTTGCCCAACCAGTGAAAACCAT
3znuB3	TTAGCAATAACCGTAAGACATTAAACGTTCATAGCGT
1249BL	AAAAAATCCTATTATTTTGAACGCTAGAATTTGA
LacFor2	TCTAGCGTTCAAAATAATAGGATTTTTTATGATTACGGATTCACTGGCCGTCGT
tetB	TGACGAAATAACTAAGCACTTGTCTCCTGTTTACT
1248AL	AGACAAGTGCTTAGTTATTTCGTCAAGTTAAAAATAAAGCCCATCAACTGGAT
31248gent	AGGCTTATGTCAATTCGAGAATTGTTATCAAAATAGGATTTTGCTTCCGCCAGTTGA

PXT10thyAF	AGGGCTTGAATCGCACCTCCA
3Revrfad1	AACAGGCTACGATAAACCATTCAAAACAGT
5'FNRcloning	CGGAATGTTGCATCAAGCACAC
3'FNR cloning	GGCTGCCACCAAATGGACATTAC
3FNRgent	AGGCTTATGTCAATTCGAGAATTGTTATTTAACTAATGTAAT TTTTGTTTTATTCGTACCA
5xylBgent	ACCCAAGTACCGCCACCTAATCAGAATTGGTTAATTGGTTG TAACACTGGCA
51249test	TCCCTAAATTAAATAGACACTTTAGGGCT
31249test	TCATCATTTCAATATCAAATTGGGTTCGT
5znuAtest	TGATAATAATGGAAACTGGCTTGGCA
3znuAtest	ACATAATATGCATAATGCTTTAATGGCGA
5znuBtest	CTTGCTCAACAAGCAAATGAAGTGGA
3znuBtest	TGATTTGCTCTAAAGCAAAAAGAATTTGA
51249JTOPO	GTGAAAACGTATTCATTATTACTCGCTCTA
31249JTOPO	GGACTGAAAATACAGGTTTTCGCCGCTGCTCTCACATTGAA TTATTACTTTTTGTGCGA
51249RT	AAACGTATTCATTATTACTCGGT
31249RT	TCCGCTTTAGTTCTAGCTTGT
51248RT	AAAAAATATAAAACTGGGCTCGT
31248RT	TGGTGCCTGCCTTGATGCT
RpoA5'	GTAGAAATTGATGGCGTATTG
RpoA3'	TCACCATCATAGGTAATGTCC

zevA generated with primers 1249A and 1248rev were joined by overlap extension PCR, via complementary ends, using primers 1249for and 1248rev. The resulting 3,086-bp fragment was introduced into competent cells of strain NTX, a derivative of NT127 in which the xylose locus was modified for efficient recombination with plasmid pXT10 and its derivatives (110). Gm resistant (Gm^R) transformants were selected on sBHI containing Gm creating strain NTzevAG. NTzevAG was transformed with linearized "empty vector" pXT10, and Tc^R transformants were isolated to create strain NTzevAV. Double crossovers into the *zevA* locus were verified using primers 51249test and 31249test, which bind outside the recombination junctions.

To complement the *zevA* deletion, *zevA* and upstream promoter elements were amplified by PCR with primers 51249HA and 31249R, which introduce SapI restriction sites at the termini of the fragment. The resulting 1,017-bp fragment was digested with SapI and cloned into SapI digested vector, pXT10, creating plasmid pX1249J. Plasmid pX1249J was linearized with ApaLI and introduced into competent cells of strain NT1249G. Tc^R recombinants were selected on sBHI containing Tc, resulting in strain NTzevAX.

***zevB* strain construction.** Deletion of *zevB* in NTHi was also performed by replacement of the coding region of *zevB* (HIAG_01364) with the Gm resistance gene. To increase efficiency of mutant selection, the aacC1 promoter was replaced with the *H. influenzae arcA* promoter. First, a 1,332-bp fragment containing the 5' flanking region of *zevB* was amplified by PCR using NT127 genomic DNA as a template with primers 1249for and

1248JBpArcA, a 762-bp fragment amplified from a pXT10 plasmid carrying the *arcA* promoter fused to the coding region of the Gm resistance gene from aacC1 using primers 5pArcA and 3Gent2, and a 1326 bp fragment containing the 3' flanking region of *zevB*, amplified from NT127 genomic DNA using primers 1248AHib and 1247RevJB were joined by overlap extension PCR using primers 1249for and 1247RevJB. The resulting 3,638-bp fragment was used to transform competent cells of strain NTX. Gm^R transformants were selected on sBHI-Gm plates resulting in strain NTzevBG. NTzevBG was then transformed with linearized vector pXT10, and Tc^r transformants were isolated to create strain NTzevBV. Double cross-overs into the *zevB* locus were verified using primers 51249test and 31249test, which bind outside the recombination junctions.

To complement the *zevB* mutant, the region consisting of *zevA, zevB* and upstream promoter elements were amplified by PCR with primers 51249HA and 31248R, which introduce SapI restriction sites at the termini of the fragment. The resulting 1,988-bp fragment was digested with SapI and cloned into SapI digested vector, pXT10, creating plasmid pX1248J. Plasmid pX1248J was linearized with ApaLI and introduced into competent cells of strain NT1248G. Tc^R recombinants were selected on sBHI containing Tc, resulting in strain NTzevBX.

For performing mixed infections with *zevA* and *zevB* mutant strains, a reference strain featuring a xylose inducible *lacZ* gene was generated by transforming NT127 with ApaLI digested plasmid pXELacZ2, which contains *lacZ* in place of *xylA***.** Tc^R recombinants were selected on sBHI containing Tc, resulting in strain NTlacZ.

***znuA* and *znuBC* strain construction.** To generate an NTHi *znuA* (HIAG_01677) mutant, a 971-bp fragment, containing the 5' flanking region of *znuA* was amplified from NT127 genomic DNA with primers 5ZnuA1 and 3ZnuA1. A 1,065-bp fragment containing the *trc* promoter fused to the Km resistance gene (*aphI*) was amplified from plasmid pENTtrcK (containing the mini-mariner transposon, *mmTrcK*, previously described (84)) with primers IFTrcF and 3kan1, and a 1,065 bp fragment, containing the 3' flanking region of *znuA*, amplified from NT127 genomic DNA with primers 5ZnuA3 and 3ZnuA3 were joined using overlap extension PCR by virtue for their complementary ends, with primers 5ZnuA1 and 3ZnuA3. The resulting 3,033-bp fragment was introduced into competent cells of strains NTV, NTzevAV and NTzevBV and Km resistant (Km^R) colonies were selected on sBHI Km, resulting in strains NTznuA, NTzevAznuA and NTzevBznuA, respectively. Double crossovers into the *znuA* locus were verified using primers 5znuAtest and 3znuAtest, which bind outside the recombination junctions.

To generate an NTHi *znuBC* (HIAG_00759 and HIAG_00758) mutant, a 1,592-bp fragment, containing the 5' flanking region of the *znuBC* operon was amplified from NT127 genomic DNA with primers 5ZnuB1 and 3ZnuB1, the 1,065-bp fragment described above, containing the trc promoter fused to the Km resistance gene, and a 1,027 bp fragment, containing the 3' flanking region of *znuBC*, amplified from NT127 genomic DNA with primers 5ZnuA3 and 3ZnuA3, were joined using overlap extension PCR by virtue for their complementary ends using primers 5ZnuB1 and 3ZnuB3. The resulting 3,700-bp fragment was introduced into competent cells of strains NTV,

NTzevAV and NTzevBV and Km^R colonies were selected on sBHI Km, resulting in strains NTznuBC, NTzevAznuBC and NTzevBznuBC, respectively. Double crossovers into the ***znuBC*** locus were verified using primers 5znuBCtest and 3znuBCtest, which bind outside the recombination junctions.

zevA* reporter strain construction.** To generate the reporter strain, R5, we replaced the coding region of ***zevAB (HI1249 and HI1248) in Rd, with a reporter construct containing the ***lacZ*** gene, designed to fuse the ***zevA*** promoter to the translational start of ***lacZ*** , and the ***tetAR*** Tc resistance operon as a selectable marker. To do this, a 683-bp fragment containing the 5' flanking region from ***zevA*** amplified by PCR from Rd genomic DNA with primers 1249for and 1249BL, a 6,869-bp fragment containing the ***lacZ*** gene and ***tetAR*** amplified from plasmid pXELacZ2 with primers LacFor2 and tetB, and a 689-bp fragment containing the 3' flanking region of ***zevB*** amplified from Rd genomic DNA with primers 1248AL and 1248rev, were joined using overlap extension PCR with primers 1249for and 1248rev. The resulting 8,188-bp fragment was used to transform Rd competent cells. Tc^R transformants were selected on media containing Tc resulting in strain R5.

To complement mutation of ***zevAB*** in R5, a 1,988-bp fragment containing the ***zevAB*** coding regions as well as upstream promoter elements was amplified by PCR from Rd genomic DNA with primers 51249HA and 31248R, which introduce SapI restriction sites at the termini. The resulting fragment was digested with SapI and cloned into SapI digested vector, pXT10, creating plasmid pX1248R. Next a 2,891-bp fragment containing

the *xylF* gene as well as the 5'flanking and coding regions of *zevAB* amplified from plasmid pX1248R with primers pXT10thyAF and 31248gent, the 762-bp Gm resistance cassette fragment described above, and a 1,794-bp fragment containing the *xylB* gene amplified from pXT10 with primers 5xylBgent and 3revrfad1, were joined using overlap extension PCR with primers pXT10thyAF and 3revrfad1. The resulting 5,447-bp fragment was used to transform competent cells of strain R5 resulting in R5X.

***fnr* mutant construction.** For mutation of *fnr* (HI1425**)**, a 4,230-bp fragment containing the *fnr* gene with a *magellan1* transposon insertion ~170-bp from the 5' end of the FNR translational start was amplified by PCR from previously described strain Rfnr (110) with primers 5FNRcloning and 3FNRcloning and used to transform competent R5 cells. Km^R transformants were selected on sBHI Km, and the mutation was verified by PCR resulting in strain R5fnr.

To complement the *fnr* mutation, a 2,201-bp fragment containing *xylF*, the *fnr* promoter and *fnr* (amplified by PCR from strain RfnrC with primers pxt10thyAF and 3FNRgent), the 762-bp Gm resistance cassette fragment described above, and a 1,794-bp fragment containing the *xylB* gene (amplified from pXT10 with primers 5xylBgent and 3revrfad1) were joined using overlap extension PCR with primers pXT10thyAF and 3revrfad1. The resulting 4,733-bp fragment was used to transform competent cells of strain R5fnr and the mutation was verified by PCR resulting in strain R5fnrX.

His-tagged ZevA construction. To generate the ZevA-His fusion, a 618-bp fragment containing the *zevA* gene was amplified from NT127 genomic DNA template with

primers 51249JTOPO and 31249JTOPO. Primer 31249TOPO adds a tobacco etch virus (TEV) protease cleavage site to the 3' end of the *zevA* gene (275). The 618-bp fragment was then TA cloned into the pBAD-TOPO-TA cloning vector (Invitrogen, Carlsbad, CA), which adds a C-terminal 6X his tag (immediately after the TEV cleavage site), and transformed into Oneshot® TOP10 *E. coli* (Invitrogen, Carlsbad, CA) creating strain ECZevAHIS, harboring plasmid pZevAHIS. Plasmid pZevAHIS was verified by sequencing.

Genetic footprinting. *H. influenzae* Rd genomic DNA from input and output libraries was used as a template for genetic footprinting analysis as previously described (3, 255). PCR reactions were performed using the transposon-specific primer, marout, and gene-specific primer 3129rev that binds 661-bp downstream of the *zevB* gene and visualized on a 0.9% agarose gel stained with ethidium bromide. Migration distances and gene positions were determined as described previously (84, 246).

Murine lung infection model. Standing overnight cultures were used to inoculate 25 ml of sBHI in a 50 ml flask to a final optical density at 600 nm (OD_{600}) of 0.01. The resulting cultures were incubated shaking at 250 rpm and 35°C until mid-log phase. Experimental strains were either mixed (mixed infections) with the NTlacZ reference strain at a 1:1 ratio or not mixed (single strain infections), washed, and diluted in HBSS to obtain a final concentration of 5×10^8 bacteria per ml. Forty microliters (2×10^7 CFU total) were inoculated into the nares of female 6.5-week-old C57BL/6 mice (Charles River Laboratories, Boston, MA) anesthetized with ketamine (65 mg/kg) and xylazine

(6.5 mg/kg) by i.p. injection. At 40 h of infection, lungs were harvested and homogenized using a Fisher Tissuemiser. Dilutions of the lung homogenate were plated on sBHI agar plates with or without 1 mM D-xylose and X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; Sigma-Aldrich, St. Louis, MO) for CFU enumeration. For statistical analysis of mixed infections, the ratio of each experimental strain to NTlacZ was calculated, multiplied by 100, $\log_{10}$ transformed, and analyzed using one-way ANOVA with Tukey's multiple-comparison test (Prism 5.03; GraphPad Software, La Jolla, CA). For single strain infections, numbers of CFU/lung were calculated and analyzed by t-test. All animal procedures were conducted in accordance with NIH guidelines and with prior approval by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

EDTA sensitivity and growth curve analysis. Strains were inoculated from standing overnight cultures at an OD_{600} of 0.01 into 25 ml of sBHI in a 50 ml flask and incubated shaking at 250 rpm and 35°C until mid-log phase. These cultures were used to inoculate sBHI media containing EDTA (at the final concentrations specified) at an OD_{600} of 0.02 and 100 µl was dispensed into the wells of a 96-well microtiter plate (Corning) prefilled with 100 ul sBHI broth containing EDTA (final concentrations specified in results). When necessary, media was supplemented with $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuSO_4$, $ZnSO_4$, or Na_2MoO_4 to final concentrations of 30 or 60 µM. As a control, strains were also grown in sBHI media without additions. For double mutant analysis, strains were grown in media containing various concentrations of EDTA (final concentration of 0.0025 mM, 0.005 mM, or 0.01 mM), or media containing various

concentrations of $ZnSO_4$ (final concentration of 0.001 mM, 0.003 mM, or 0.05 mM). Microtiter plates were incubated at 35°C for 10 h in a $Versa_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA) set to read the absorbance at 600 nm every 10 min. Effects of EDTA concentration and metal supplementation on strains were assessed by relative growth yields and generation times (Prism 5.03; GraphPad Software, La Jolla, CA).

Purification of ZevA-His. To purify the ZevA-His fusion protein, 1 liter cultures of strain ECZevAHis were inoculated from 10 ml overnight cultures and grown in Luria-Bertani broth containing 100 µg/ml ampicillin with shaking at 200 rpm and 37°C. When cultures achieved an OD_{600} between 0.6 and 0.8, L-arabinose was added to a final concentration of 0.02%, and cultures were incubated for an additional 3 hrs. After incubation, cells were pelleted by centrifugation at 5,000 x g for 10 min, resuspended in 25 mM Tris-ClH (pH 8.0), 500 mM NaCl and lysed using a French pressure cell. Lysates were cleared by ultracentrifugation at 30,000 x g and protein was purified by affinity chromatography using a Ni^{2+}-nitrilotriacetic acid (Ni-NTA) column (Qiagen, Valencia, CA). Protein was eluted using buffer containing 150 mM imidazole and concentrated by centrifugation in an Amicon Ultra, 10 kDa cutoff filter (Millipore, Billerica, MA). To remove the His-tag, ZevA-His fusion protein was incubated with purified 6X His-tagged TEV protease (1:1 molar ratio) for 1 hr at 22°C in buffer containing 50 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione. After incubation, TEV-His was removed by affinity purification with Ni-NTA resin (Figure 3.1). Protein concentration was determined using the Bradford reagent (Sigma-

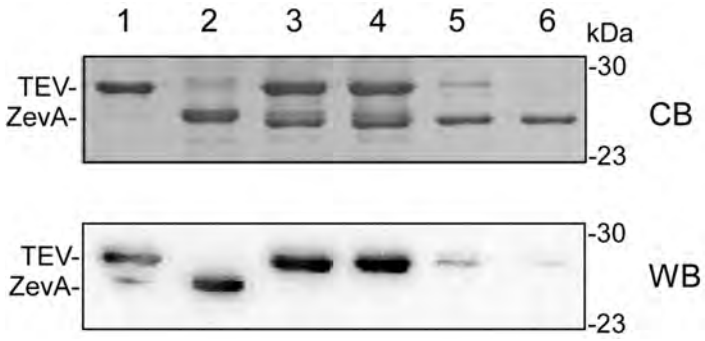

Figure 3.1. Removal of the tag from recombinant ZevA-His fusion. Equivalents of each sample were loaded in lanes of Coomassie Brilliant Blue stained gel (top panel) and corresponding lanes of western blot (bottom panel). Lane 1 represents TEV-His fusion alone, Lane 2 represents ZevA-His fusion alone, Lane 3 and 4 represent the cutting reaction after a 1 hr incubation, and Lane 5 and 6 represent ZevA recovered after incubation with Ni-NTA resin to remove TEV-His. Lane 6 represents purified untagged-ZevA protein used in Zn^{2+} binding experiments. Purified untagged-ZevA was determined by densitometry to be at least 99% pure of ZevA-His (Lane 2 versus Lane 6) and 98% pure of TEV-His (Lane 4 versus Lane 6).

Aldrich, St. Louis, MO). Purity was assessed by Coomassie Brilliant Blue staining of overloaded SDS-PAGE and by immunostaining western blots with rabbit anti-His polyclonal primary antibody (GenScript, Piscataway,NJ, USA) and, goat anti-rabbit IgG secondary antibody (horseradish peroxidase conjugate; GenScript, Piscataway,NJ, USA) (Figure 3.1). For Zn^{2+} binding assays, reaction buffer was exchanged with 50 mM Hepes-NaOH (pH 7.5), 200 mM NaCl, and 10% glycerol by centrifugation in an Amicon Ultra, 10 Kd cutoff filters (Millipore, Billerica, MA).

Zn^{2+} binding stoichiometry determination. Stoichiometry assays were performed as described previously (68). ZevA protein (0.8 µM) was incubated for 1 min at room temperature in presence or absence of 50 µM $ZnSO_4$. Buffer (50 mM Hepes-NaOH pH 7.5, 200 mM NaCl, and 10% glycerol) containing 50 µM $ZnSO_4$ was incubated in parallel as a control. After incubation, excess Zn^{2+} was removed by size exclusion with Sephadex G-25 columns. Eluted protein was acid digested with concentrated nitric acid overnight at room temperature. Following digestion, samples were treated with H_2O_2 to a final concentration of 1.5%. The concentration of Zn^{2+} in each sample was measured using atomic absorption spectroscopy (AAS) (AAnalyst 300; PekinElmer, Waltham, MA). Average background Zn^{2+} levels detected for control samples were <2% of levels determined for ZevA+Zn^{2+} samples.

Determination of ZevA affinity for Zn^{2+}. Binding affinity of ZevA for Zn^{2+} was determined using the Zn binding chromophore, mag-fura-2 (Invitrogen, Carlsbad, CA) as

previously described (68, 163, 279). Briefly, 10 µM ZevA protein and 20 µM mag-fura-2 were mixed and titrated with 1 mM Zn^{2+}. Free mag-fura-2 was determined by monitoring OD_{366}, using $_{366}$ = 29,900 $M^{-1}cm^{-1}$ (285). Free Zn^{2+} concentrations were calculated using the equation $K_I = [I \cdot Zn^{2+}]/[I_{free}][Zn^{2+}]$, where I is the concentration of mag-fura-2 and K_I is the association constant of magfura-2 for Zn^{2+} (K_I = 5 x 10^7 M^{-1}) (163, 285). The Zn^{2+}-protein association constant (K_a) and the apparent stoichiometry (n) of ZevA, were calculated by fitting the data to $v = nK_a[Metal]_f/(1 + K_a[Metal]_f)$, where v is the ratio of moles of metal bound to total protein and n is the number of binding sites (100).

qRT-PCR. RNA was prepared from liquid cultures of Rd using the RNeasy mini kit and on column DNase I kit (Qiagen, Valencia, CA). The RNA samples (5 µg) were used as templates for cDNA synthesis with Random Primer 9 (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 over time using the DNA Engine Opticon II system (MJ Research, Waltham, MA). For transcript analysis of *zevA* and *zevB*, qRT-PCR performed with primers 51249RT and 31249RT or 51248RT and 31248RT, respectively. Primers RpoA5' and RpoA3' 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 (302). Results of mock reverse transcription reactions, containing RNA and all reagents except reverse transcriptase,

confirmed, that results obtained were not due to contaminating genomic DNAs (data not shown).

Reporter expression analysis. Strains were plated at a density of 2 x 10^5 CFU per plate on sBHI agar with or without 100 mM $ZnSO_4$, addition and incubated at 35°C overnight in either an open air incubator or an anaerobic chamber. Cells were swabbed from plates and diluted into Z-buffer (183) to a concentration of 1 x 10^9 bacteria per ml (OD_{600} of 0.333). Alternatively, cells were cultured in sBHI broth containing 0.5 mM EDTA shaking at 250 rpm and 35°C until mid-log phase before being diluted into Z-Buffer. The β-galactosidase assay was then performed as described (183). The optical density at 420 nm (OD_{420}) was recorded using a $Versa_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA), and converted to Miller units with the equation: Miller Units = (1000 x OD_{420}) / (T x V x OD_{600}) where T is time in minutes and V is volume of the reaction.

RESULTS

Identification of secreted factors involved in *H. influenzae* pathogenesis. An informatics-based approach was utilized to compile a database of the exported proteins in *H. influenzae* that are likely to require DsbA for oxidative folding. First, web-based prediction servers, signalP (19) and LipoP (135) were used to identify polypeptides with predicted cleavage sites for signal peptidase I (soluble exported proteins) or signal peptidase II (lipoproteins), respectively. Next, the list was refined to include only mature proteins (excluding leader peptides) with two or more cysteine residues, as these are expected to be DsbA substrates (206). The β-barrel Outer Membrane Protein predictor (BOMP) (22) and membrane topology prediction servers TMHMM (258) and phobius (142) were used to predict the locations of cysteines in the loops of membrane proteins thereby excluding proteins with cysteines that are predicted to be cytoplasmic and inaccessible to DsbA. The final database includes secreted proteins involved in a variety of functional processes, which are cataloged based on Clusters of Orthologous Groups (COG) categories in Table 3.2.

Next, to identify the putative substrates of DsbA that are required for pathogenesis of *H. influenzae,* we cross-referenced the list of genes in Table 3.2 with the set of genes previously implicated in survival of *H. influenzae* Rd in the lung as identified by the HITS technique (84). In that study, tranposon mutant libraries were acquired before and after selection for survival in the mouse lung model, and transposon/chromosomal junctions were amplified from genomic DNA, captured,

TABLE 3.2. Potential *H. influenzae* DsbA-substrates identified using informatics techniques

Category	Gene ID	Name	Predicted function / description
Translation, ribosomal structure			
	HI0526	- *	Hypothetical protein HI0526
Cell wall/membrane/envelope biogenesis			
	HI0197	*mepA*	Penicillin-insensitive murein endopeptidase
	HI0275	-	Hypothetical protein HI0275
	HI0364	-	Penicillin-binding protein 7
	HI0366	-	Fimbrial biogenesis and twitching motility protein
	HI0383	*tolA*	Cell envelope integrity inner membrane protein
	HI0389	-	Outer membrane protein
	HI0706	*nlpD*	Lipoprotein
	HI0730	-	Organic solvent tolerance protein
	HI0990	*iga1*	Immunoglobin A1 protease
	HI1138	*murG*	N-acetylglucosaminyl transferase
	HI1164	*ompA*	Outer membrane protein P5
	HI1330	*dacB*	D-alanyl-D-alanine carboxypeptidase/endopeptidase
Posttranslational modification, chaperones			
	HI0271	*djlA*	DNA-J like membrane chaperone protein
	HI0428	*dsbB*	Disulfide bond formation protein B
	HI0846	*dsbA*	Periplasmic oxidoreductase
	HI0885	*dipZ*	Thiol:disulfide interchange protein precursor
	HI0934	*nrfF*	Cytochrome C-type biogenesis protein
	HI0935	*dsbE*	Thiol-disulfide interchange protein
	HI1213	*dsbC*	Thiol-disulfide interchange protein
	HI1453	*purL*	Phosphoribosylformylglycinamidine synthase
	HI1455	*msrA*	Bifunctional methionine sulfoxide reductase
	HI1541	*sppA*	Protease IV
Signal transduction mechanisms			
	HI0267	*narQ*	Nitrate/nitrite sensor protein NarQ
	HI1378	*phoR*	Phosphate regulon sensor protein PhoR
Intracellular trafficking and secretion			
	HI0015	*lepB*	Signal peptidase I
	HI0264	*hxuA*	Heme-hemopexin utilization protein A
Energy production and conversion			

	HI0345	*napG*	Quinol dehydrogenase periplasmic component
	HI0347	*napB*	Periplasmic nitrate reductase
	HI0348	*napC*	Cytochrome C-type protein
	HI0643	*bisC*	Biotin sulfoxide reductase
	HI0644	*yecK*	Cytochrome C-like protein
	HI1047	*dmsA*	Anaerobic dimethyl sulfoxide reductase chain A
	HI1067	*nrfC*	Nitrite reductase Fe-S protein
	HI1586	-	Isoleucyl-tRNA synthetas
	HI1684	-	Electron transport complex protein RnfB
Amino acid transport and metabolism			
	HI0213	*oppA*	Oligopeptide transporter periplasmic-binding protein
	HI0745	*ansB*	L-asparaginase II
	HI0853	*hbpA*	Heme-binding lipoprotein
	HI1048	-	Hypothetical protein HI1048
	HI1179	*artI*	Arginine ABC transporter periplasmic-binding protein
	HI1638	*sapA*	Anti-peptide resistance ABC transporter periplasmic protein
Nucleotide transport and metabolism			
	HI0583	*cpdB*	Bifunctional 2',3'-cyclic nucleotide 2'-phosphodiesterase/3'-nucleotidase periplasmic precursor protein
Coenzyme transport and metabolism			
	HI0118	-	Hypothetical protein HI0118
	HI1019	*tbpA*	Thiamin ABC transporter periplasmic-binding protein
	HI0113	*hemR*	Hemin receptor
	HI0119	*znuA*	High-affinity zinc transporter periplasmic component
	HI0131	*afuA*	Ferric ABC transporter protein
	HI0712	-	Hemoglobin-binding protein
	HI0994	*tbp1*	Transferrin-binding protein 1 precursor
	HI0995	*tbp2*	Transferrin-binding protein 2 precursor
	HI1050	*merP*	Mercuric ion scavenger protein
	HI1068	*nrfB*	Cytochrome c-type protein NrfB
	HI1069	*nrfA*	Cytochrome c nitrite reductase
	HI1217	-	Transferrin-binding protein
	HI1427	-	Hypothetical protein HI1427
2° metabolites biosynthesis and transport			
	HI0733	-	Truncated suppressor of ftsI protein
Function unknown			

	HI0120	-	Hypothetical protein HI0120
	HI0178	-	Hypothetical protein HI0178
	HI0246	-	Hypothetical protein HI0246
	HI0310	-	Hypothetical protein HI0310
	HI0449	-	Hypothetical protein HI0449
	HI0696	-	Hypothetical protein HI0696
	HI0930	-	Hypothetical protein HI0930
	HI0941	-	Hypothetical protein HI0941
	HI0966	-	Hypothetical protein HI0966
	HI0973	-	Hypothetical protein HI0973
	HI0983	-	Hypothetical protein HI0983
	HI1236	-	Hypothetical protein HI1236
	HI1262	*sanA*	SanA
	HI1413	-	Hypothetical protein HI1413
	HI1452	-	Hypothetical protein HI1452
General function prediction only			
	HI0036	-	ABC transporter ATP-binding protein
	HI0331	*oapB*	Opacity associated protein OapB
	HI0436	*comD*	Competence protein D
	HI0495	*aphAm*	Acid phosphatase/phosphotransferase
	HI0933	-	Hypothetical protein HI0933
	HI1005	-	Hypothetical protein HI1005
	HI1064	-	Hypothetical protein HI1064
	HI1249	*zevA*	Hypothetical protein HI1249
	HI1655	*yraM*	Antigen

* Gene name unavailable

sequenced and aligned with the *H. influenzae* genome sequence. Results of the analysis were reported as a survival index (s.i.), which denotes the number of mutations detected in a given gene in the lung-selected output library divided by mutations detected in the in vitro grown input library. Genes required during lung infection were identified on the basis of two parameters: having s.i. of 0.3 or lower and sustaining insertions in >40% of the potential TA dinucleotide insertion sites, specific to the *himar1*-derived transposon used in the study (84). Comparison of our list of potential DsbA substrates to results of HITS revealed three genes that fit these criteria: ***znuA*** (s.i. 0.101), encoding the periplasmic component of a high affinity zinc uptake transporter (166, 221), ***nlpD*** (s.i. 0.000), encoding a lipoprotein suggested to be involved in daughter cell separation (267, 272), and HI1249 (s.i. 0.106), encoding a putative protein herein referred to as ZevA. The role of ***znuA*** in virulence has been studied by several groups (5, 32, 55, 83, 161, 244, 309) and ***nlpD*** has been implicated in virulence of *Yersinia pestis* and *Yersinia enterocolitica* (53, 267). However, ***zevA*** has not been previously investigated and its role in virulence was unknown. Additionally, the putative gene 2-bp downstream of the 3' end of the ***zevA*** coding sequence, HI1248, herein referred to as ***zevB,*** was also found to be required for lung survival (s.i. 0.103).

Results of HITS were verified using genetic footprinting on our mutant libraries. In this technique, transposon insertions in a given region are physically mapped using PCR with a chromosomal-specific primer paired with a transposon-specific primer (3, 255). Sizes of amplified products correspond to the distance between the chromosome-specific primer and the transposon insertion within that gene. In agreement with HITS,

footprinting of the library recovered from lung infection reveals a reduction in transposon insertions within the *zevAB* locus compared with footprints of the input library (Figure 3.2). These data indicate that *zevA and zevB* are essential for lung colonization by *H. influenzae* Rd.

***zevAB* is required for efficient NTHi infection of the mouse lung.** Mutants of *zevA* and *zevB* were generated in an NTHI clinical isolate, NT127 (110) to evaluate roles of these genes during in vitro growth and lung infection. Mutants were constructed by replacement of coding regions with a drug resistance marker to generate non-polar deletions and complementation was conducted using an exchange vector for delivery and expression at the xylose locus (301). The complete strain set consisted of the parent strain carrying the "empty vector" (NTV), *zevA* mutant carrying the empty vector (NTzevAV), *zevB* mutant carrying the empty vector (NTzevBV), and complemented *zevA* and *zevB* mutants (NTzevAX and NTzevBX, respectively) (Table 3.3). When grown aerobically in sBHI media, all strains had equivalent growth yields and generation times (NTV: 45.4 ± 1.7 min, NTzevAV: 44.1 ±1.3 NTzevBV: 41.6±0.6, NTzevAC: 43.0 ± 0.5, and NTzevBC: 44.2±1.9). Ratios of CFU to optical density at 600 nm were indistinguishable between these strains.

Next, these strains were evaluated for their ability to infect the lungs of C57Bl/6 mice. This experiment was performed using mixed infections in which a competition was performed between each strain and strain NTlacZ, which expresses *E. coli lacZ* at the *xyl* locus. At 40 hrs after inoculation, average competitive indices (c.i.) were ~3 fold lower

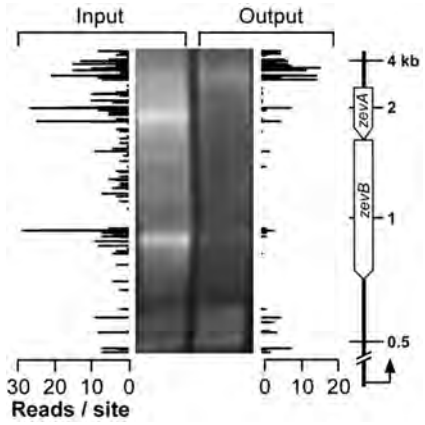

Figure 3.2. Analysis of transposon insertions within the *zevAB* locus. In vitro selected (Input) and in vivo selected (Output) libraries were compared using HITS and genetic footprinting data. Bars represent the number of sequencing reads at each individual insertion site within the *zevAB* region. MW standards are displayed to the right and apply to both HITS plots and footprints. Positions of the *zevA* and *zevB* genes are indicated. Genetic footprinting was performed by PCR with transposon-specific primer marout and chromosomal-specific primer 31248rev (represented by the arrow) which binds 661-bp downstream of *zevB.*

TABLE 3.3. Strains and plasmids used in this chapter

Strains or plasmids	Genotypes, description, and/or relevant features	Reference or source
Strains		
NT127	Nontypeable *H. influenzae* clinical isolate	(110)
NTV	NT127 *xylA*$\Delta_{4\text{-}804}$::*tetAR*; *tetAR* sequence from pXT10 replaces *xylA*	(110)
NTzevAV	NT127 Δ*zevA::aacCI, xylA*$\Delta_{4\text{-}804}$::*tetAR*; *zevA* deletion mutant with *tetAR* tet^R cassette replacing *xylA*	This study
NTzevAX	NT127 Δ*zevA::aacCI; xylA*$\Delta_{4\text{-}804}$::*zevA*; *zevA* deletion mutant complemented with zevA expressed via the zevA promoter in place of *xylA*	This study
NTzevBV	NT127 Δ*zevB::aacCI; xylA*$\Delta_{4\text{-}804}$::*tetAR*; *zevA* mutant replacing *xylA*	This study
NTzevBX	NT127 Δ*zevB::aacCI; xylA*$\Delta_{4\text{-}804}$::*zevAB*; *zevB* deletion mutant complemented with zevAB expressed via the *zevA* promoter in place of *xylA*	This study
NTlacZ	NT127 *xylA*$\Delta_{4\text{-}804}$::*lacZ*; *lacZ* coding sequence expressed via the *xylA* promoter replacing *xylA*	This study
NTznuA	NT127 Δ*znuA::aphI, xylA*$\Delta_{4\text{-}804}$::*tetAR*; *znuA* deletion mutant with tetAR tet^R cassette replacing *xylA*	This study
NTzevAznuA	NTzevAV Δ*znuA::aphI; zevA* and *znuA* double mutant	This study
NTzevBznuA	NTzevBV Δ*znuA::aphI; zevB* and *znuA* double mutant	This study
NTZnuB	NT127 Δ*znuBC::aphI, xylA*$\Delta_{4\text{-}804}$::*tetAR*; *znuBC* deletion mutant with *tetAR* tet^R cassette replacing *xylA*	This study
NTzevAznuBC	NTzevAV Δ*znuBC::aphI; zevA* and *znuBC* double mutant	This study
NTzevBznuBC	NTzevBV Δ*znuBC::aphI; zevB* and *znuBC* double mutant	This study
Rd AW	Wild type; *H. influenzae* capsule-deficient type d	(301)
R5	Rd Δ*zevAB*::*lacZ*; *zevAB* deletion mutant with *lacZ* expressed via the *zevA* promoter (*zevA* Reporter strain)	This study
R5X	R5 *xylA*$\Delta_{4\text{-}804}$::*zevAB;* Strain R5 complemented with *zevAB* expressed via the *zevA* promoter replacing *xylA*	This study
R5fnr	R5 *fnr'*::*nptII*; *fnr* mutant of strain R5 with Km^R transposon insertion in *fnr*	This study
R5fnrX	R5fnr *xylA*$\Delta_{4\text{-}804}$::*fnr*; *fnr* mutant of strain R5 complemented with *fnr* expressed via the *fnr* promoter in place of *xylA*	This study
RdlacZ	Rd *xylA*$\Delta_{4\text{-}804}$::*lacZ*; *lacZ* coding sequence expressed via the *trc* promoter replacing *xylA*	(302)
Plasmids		
pXT10	Delivery vector for chromosomal expression at the xylose locus of *H. influenzae* containing *xylF*, *xylB*, *xylA*Δ4-804, and the *tetAR* tetracycline resistance cassette	(301)
P1249J	pXT10 carrying *zevA* from NT127 expressed from the *zevA* promoter	This study
p1248J1	pXT10 carrying *zevAB* from NT127 expressed from the *zevA* promoter	This study
pX1248R	pXT10 carrying *zevAB* from Rd expressed from the *zevA* promoter	This study
pXELacZ2	pXT10 carrying *lacZ* expressed from the *xylA* promoter	This study

for the *zevA* mutant (NTzevAV; c.i. 0.364) and ~22 fold lower for the *zevB* mutant, (NTzevBV; c.i. 0.064), when compared to the parent strain (NTV; c.i. 1.22) (Figure 3.3). Complementation restored the ability of the mutants to survive in the lung, verifying that defects are specific to mutation of *zevA* and *zevB*. The complemented strains, NTzevAX and NTzevBX, exhibited competitive indices that were slightly higher than the parent strain likely due to increased expression of the complementing genes due to the presence of both the *zevA* and *xylA* promoters in the constructs. To examine the possibility that mixed infection influences relative survival of the mutants, the *zevA* mutant was evaluated in the lung model in single strain infections. Consistent with results obtained with mixed infections, average CFU recovery at 40 hrs post infection was significantly reduced ($p=0.027$) by 3.5 fold for the *zevA* mutant compared to the parent strain (data not shown). In agreement with HITS analysis in Rd, these data indicate that the *zevAB* genes are required for colonization and survival of pathogenic NTHi in the mouse lung but, are not required for normal growth in rich media.

Bioinformatic analysis of *zevA* and *zevB*. Blast (4) reveals that *zevA* and *zevB* of *H. influenzae* are conserved in other bacterial species within the *Pastuerellaceae* and *Enterobacteriaceae* including several human pathogens such as *Klebsiella pnemoniae*, *Yersinia pestis, Proteus mirablilis,* and *Salmonella typhimurium* (Table 3.4). In the Conserved Domains Database (CDD) (170) ZevA and ZevB are annotated by structural domain classification as the periplasmic substrate-binding protein (PBP) and permease subunit of an ATP-binding cassette (ABC) transporter, respectively. ZevB is also annotated as member of the NiCoT superfamily of potential nickel/cobalt importers and

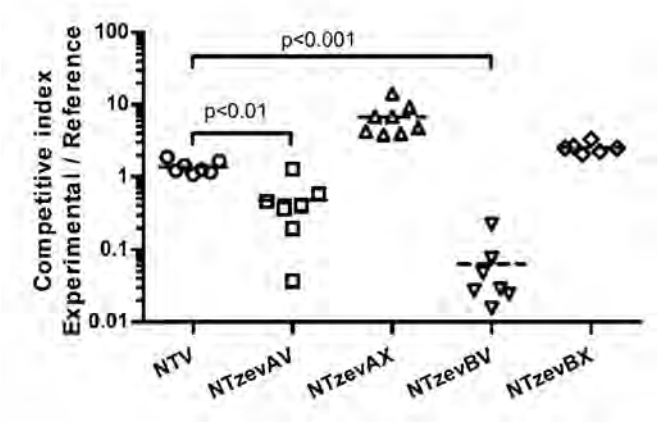

Figure 3.3. Effect of *zevA* and *zevB* mutations on survival of NTHi in the mouse lung infection model. Mice were coinfected with reference strain NTlacZ and either the parent strain carrying the "empty vector" (NTV), ***zevA*** mutant (NTzevAV), complemented ***zevA*** mutant (NTzevAX), ***zevB*** mutant (NTzevBV), or complemented ***zevB*** mutant (NTzevBX). Bacteria were recovered from lung homogenates after 40 hours. Competitive indices (c.i.) are calculated as the ratio of recovered CFU of the $LacZ^{-}$ experimental strain to recovered CFU of the $LacZ^{+}$ reference strain. The symbols indicate data for individual animals, and the dashed lines indicate the averages. The lower limit of detection (LLD) was c.i. = 0.001. Relevant statistical comparisons were indicated by brackets and p-values were determined using ANOVA with Tukey's multiple comparison test. For NTV n=8, NTzevAV n=7, NTzevAX, n=8, NTzevBV n=7, and NTzevBX n=6.

TABLE 3.4. *H. influenzae* Rd ZevA and ZevB homologs in other pathogens

Organism	Blast Results							
	ZevA (HI1249, Length 206)				ZevB (HI1248, Length 322)			
	Locus ID	Identity (%)	Length	Expect	Locus ID	Identity (%)	Length	Expect
H.influenzae NT127	HIAG_01363	97	206	2e-117	HIAG_01364	96	322	5e-165
K. pnemoniae 342	Kpk_1251	42	199	7e-42	Kpk_1252	36	326	2e-40
Y. pestis KIM 10	y1329	43	224	3e-41	y1330	38	340	7e-52
P. mirabilis HI4320	PMI1519	37	221	2e-32	PMI1518	38	333	8e-47
S. typhimurium LT2	STM2552	37	212	1e-33	STM2551	38	328	1e-50

exporters (4). This superfamily is comprised of a diverse group of eight transmembrane segmented secondary transporters, with a characteristic HX_4DH sequence in their second transmembrane span (66). ZevB does not show these characteristics. Moreover, modeling of ZevB using the consensus prediction of membrane protein topology server (TOPCONS) (21) indicates that it contains only the typical six transmembrane spans present in each of the two transmembrane subunits observed in ABC ATPases (54). This evidence suggests that ZevA and ZevB are partnering components of an ABC ATPase transport system probably involved in substrate influx.

ZevA and ZevB are important for growth of *H. influenzae* during zinc limitation. Metal restricted growth conditions can be generated by titration of the media with increasing concentrations of EDTA (5, 32, 223). To determine if ***zevAB*** is involved in metal acquisition in ***H. influenzae***, strains were grown in media containing 0.25, 0.5, or 0.75 mM EDTA. Mutants of ***zevA*** and ***zevB*** were able to acquire essential nutrients for optimal growth in rich media as evidenced by the lack of apparent growth defects in vitro (Figure 3.4A). However, media containing 0.5 mM EDTA inhibited growth of the ***zevA*** and ***zevB*** mutants, in which growth yields at 10 hrs were reduced by 81% and 87% respectively, compared to the parent or complemented strains that grew normally (Figure 3.4B). Supplementation with 0.25 mM EDTA had no effect on growth of any strains whereas 0.75 mM significantly reduced growth of the parent and complemented strains in addition to the mutants (data not shown).

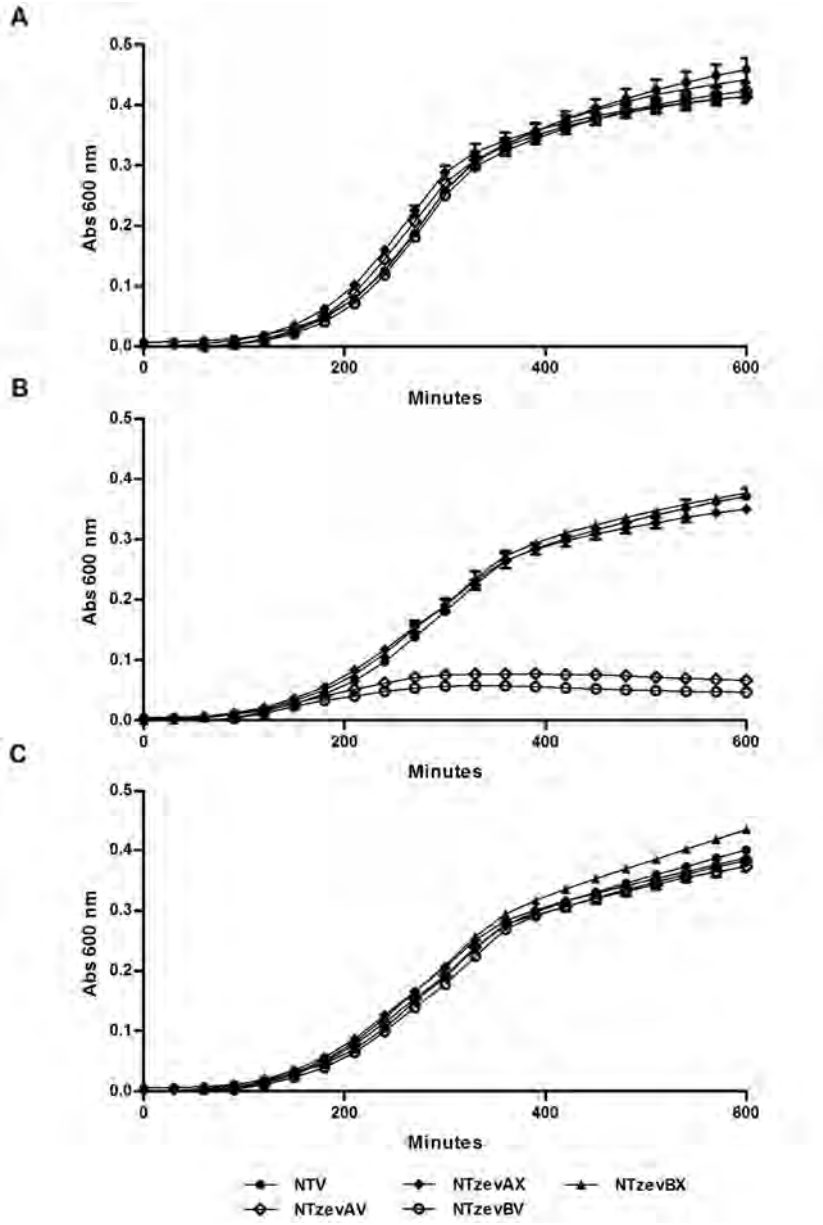

Figure 3.4. Growth phenotypes of *zevA* and *zevB* mutants in liquid media. Parent strain (NTV), *zevA* mutant (NTzevAV), complemented *zevA* mutant (NTzevAX), *zevB* mutant (NTzevBV), complemented *zevB* mutant (NTzevBX), grown in (A) sBHI, (B) sBHI containing 0.5 mM EDTA, or (C) sBHI containing 0.5 mM EDTA supplemented with 60 µM $ZnS0_4$. Symbols represent average absorbance (Abs) readings of three cultures. Error bars indicate the SD.

In preliminary studies, specific metals were added to media chelated with EDTA in attempt to promote growth of the mutants. At concentrations of 30 or 60 µM, the addition of $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_3$, $CoCl_2$, $NiCl_2$, $CuSO_4$, or Na_2MoO_4 at either concentration had no significant effect on growth of the ***zevB*** mutant in the presence of 0.5 mM EDTA (data not shown). In contrast, addition of 30 µM $ZnSO_4$, partially restored growth of the ***zevB*** mutant and addition of 60 µM $ZnSO_4$ completely restored growth to the level of the parent strain. Figure 3.4C shows that in the presence of 0.5 mM EDTA, addition of 60 µM zinc restores both the ***zevA*** and ***zevB*** mutants to levels of the parent and complemented strains. These data suggest that ***zevAB*** is important for growth of ***H. influenzae*** during conditions when free zinc is limiting.

ZevA protein binds zinc with high affinity. Metal binding assays were performed to determine whether ZevA binds Zn^{2+} and therefore can participate in metal uptake. Recombinant ZevA was cloned, expressed in *Escherichia coli*, affinity purified, and the metal binding tag was removed. After incubation in the presence of Zn^{2+}, the metal bound to ZevA was determined by atomic absorption spectroscopy (AAS). Results indicate that ZevA binds 1.9±0.3 Zn^{2+} ions per mol of ZevA. The affinity of ZevA for Zn^{2+} was determined in metal competition assays including ZevA and mag-fura-2. The fluorescent metal chelator mag-fura-2 binds Zn^{2+} with a K_a of 5 x 10^7 M^{-1} and this is associated with a shift from 366 nm to 325 nm in the chelator's spectrum maximum. Thus, the decrease in OD_{366} was used to calculate the concentration of the various species in the assay. Figure 3.5 shows the results of these assays. Cooperativity in binding to the two Zn^{2+} binding sites was not observed. Fitting the data to a Langmuir equation yielded an

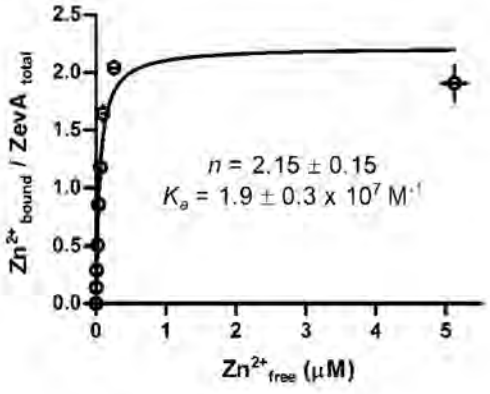

Figure 3.5. Zn^{2+} binding to purified recombinant ZevA. The association constant (K_a) and the number of metal binding sites (n) of ZevA, were calculated by fitting the data to $v = nK_a[\text{Metal}]_f/(1 + K_a[\text{Metal}]_f)$, where v is the ratio of moles of metal bound to total protein and n is the number of binding sites. Symbols represent the average of three independent measurements and error bars indicate S.E.

association constant of ZevA for Zn^{2+} of 1.9 ± 0.3 x 10^7 M^{-1}. This affinity is similar to those observed in other known Zn^{2+} binding proteins (60, 90, 163).

Relative contributions of *zevAB* and *znuABC* in zinc utilization. Aside from the ZevAB system presented here, only one other system has been implicated in zinc utilization of *H. influenzae*, the high affinity zinc transporter ZnuABC. An *H. influenzae znuA* (originally called *pzp1*) mutant was shown to exhibit growth defects in normal media that could be rescued by addition of zinc, and recombinant ZnuA bound up to 5 molecules of zinc per protein (166). The *znuBC* genes have also been demonstrated to participate in zinc utilization in other organisms (32, 221) including *Pasteurella multocida* (83), a close relative of *H. influenzae,* and are thought to encode the ATPase (*znuC*) and membrane permease (*znuB*) components of the transporter (220, 221). To evaluate conditions in which ZevAB and ZnuABC are needed, their relative contributions to growth of *H. influenzae* were examined through single and double mutant analysis. First, a *znuA* mutant was generated by replacement of its coding region with an antibiotic resistance marker and compared to growth of the *zevA* and *zevB* mutants. The *znuA* mutant (NTznuA) exhibited an ~16% increase in generation time in sBHI broth relative to the parent strain (NTV), *zevA* mutant (NTzevAV) and *zevB* mutant (NTzevBV) (Figure 3.6A). Addition of 60 µM zinc restored growth of the *znuA* mutant in rich media to wild-type levels (data not shown), similar to results previously reported for *znuA* mutants of other *H. influenzae* strains (166). When grown in 0.1 mM EDTA, the *znuA* mutant exhibited both a 21-fold reduction in growth yield at 10-hrs and a 2.3-fold increase in generation time compared with growth of this strain in sBHI media, whereas

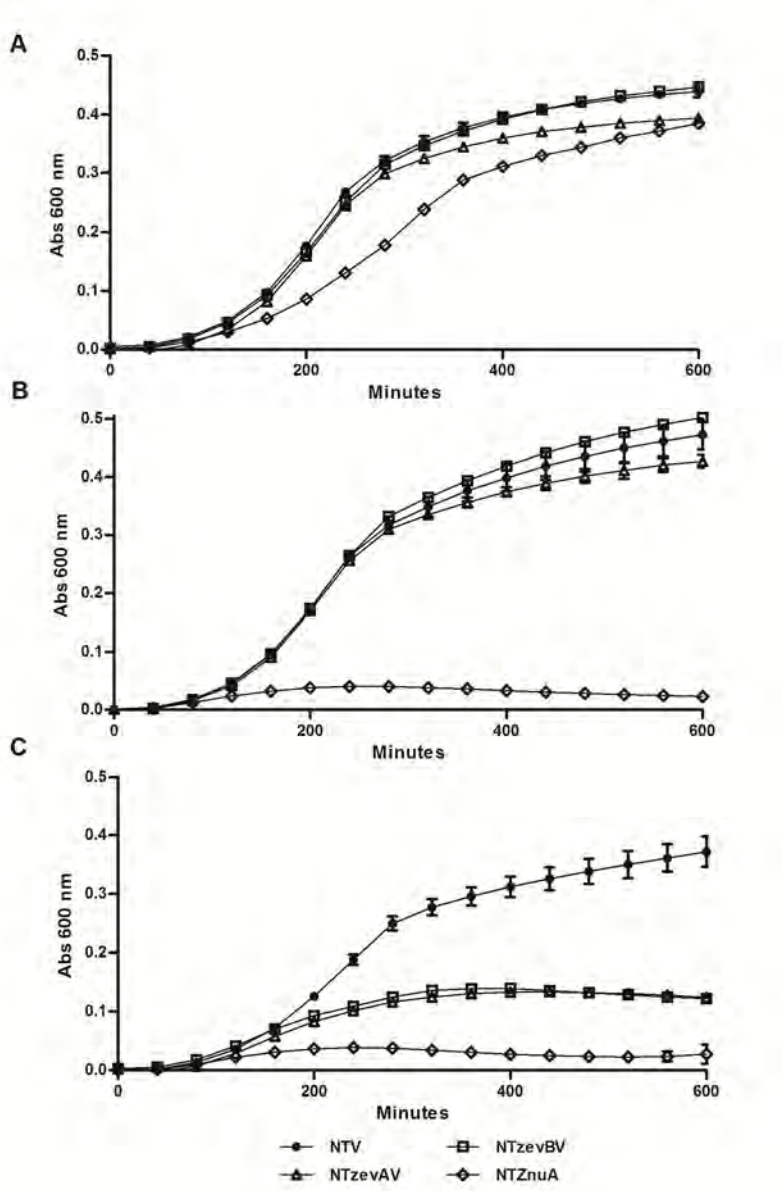

Figure 3.6. Growth phenotypes of *zevA*, *zevB*, and *znuA* mutnats in liquid media. Parent strain (NTV), *zevA* mutant (NTzevAV), *zevB* mutant (NTzevBV), and *znuA* mutant (NTznuA), grown in (A) sBHI, (B) sBHI containing 0.1 mM EDTA, or (C) sBHI containing 0.5 mM EDTA. Symbols represent average absorbance (Abs) readings of three cultures. Error bars indicate the SD.

the parent strain, *zevA* mutant, and *zevB* mutant grew normally in this condition (Figure 3.6B). In the presence of 0.5 mM EDTA, growth yield of the *znuA* mutant was also reduced by 21-fold compared to the parent (Figure 3.6C). Additionally, growth yields of the *zevA* and *zevB* mutants were reduced by 67% compared with the parent strain, similar to data reported for these strains in Figure 3.4B (Figure 3.6C). A *znuBC* deletion, which was also constructed by gene replacement, exhibited defects similar to the *znuA* mutant for growth in rich media or media containing 0.5 mM EDTA (data not shown) providing evidence that ZnuBC functions together with ZnuA in NTHi as components of the zinc transport system described previously in other organisms (32, 83, 221) .

Next, to examine the role of *zevAB* in zinc uptake in the absence of the *znuABC* pathway, double mutants were generated by deletion of *znuA* or *znuBC* in either the *zevA* or *zevB* mutant backgrounds. Growth phenotypes of double mutants were equal in severity to *znuA* or *znuBC* mutants in sBHI media, sBHI with various concentrations of EDTA (0.0025 mM, 0.005 mM, or 0.01 mM), or sBHI supplemented with various concentrations of zinc (0.001 mM, 0.003 mM, or 0.05 mM). The lack of an additive effect of *zevA* or *zevB* mutation in combination with *znuA* or *znuBC* mutations indicates that ZevAB does not substitute for ZnuABC for zinc uptake, at these zinc concentrations. Taken together, these data suggest that ZevAB functions in a specialized pathway needed only when the concentration of free zinc is low in contrast to ZnuABC, which is essential for growth over a wide range of free zinc concentrations.

FNR participates in the regulation of ZevAB operon. In many species that have been examined, transcriptional regulation of bacterial genes involved in zinc uptake is

mediated by the zinc responsive metalloregulatory protein, Zur (109). When zinc is abundant in the cell, dimerized Zur binds to a specific palindromic sequence in promoters to repress transcription of zinc uptake genes such as *znuABC* of *E. coli* (221) and *zinT* of *S. typhimurium* (223). However, previous studies, as well as our own Blast searches, indicate that the *Pasturellaceae* do not contain a *zur* homolog (83). In these organisms, zinc uptake is likely regulated by other mechanisms. For example, in *Pasteurella multocida*, *znuABC* is regulated by the iron response regulator Fur (83).

To investigate the potential regulation of *zevAB*, the 330 bp region directly upstream of the *zevA* translational start site from the start codon of HI1250 (negative strand relative to the *zevA* coding sequence) to the start codon of *zevA* (positive strand) was analyzed for potential transcriptional regulator binding sites. A palindromic sequence between 235 and 248 bp upstream of the HI1250 start that was conserved in both Rd and NT127 strains was identified (Figure 3.7A). This sequence matches the known consensus binding site for the oxygen responsive transcriptional regulator, FNR (65, 177). FNR directly senses a range of low oxygen conditions and binds to DNA to either repress genes required for aerobic growth or activate those needed for growth in low oxygen (110, 156).

To determine the expression profile of *zevA* and *zevB,* we first examined transcription of these genes in the Rd strain. Cultures were grown in liquid media and transcript levels were determined by quantitative PCR, revealing that *zevA* and *zevB* are expressed similarly relative to transcript levels of the reference gene *rpoA* which encodes

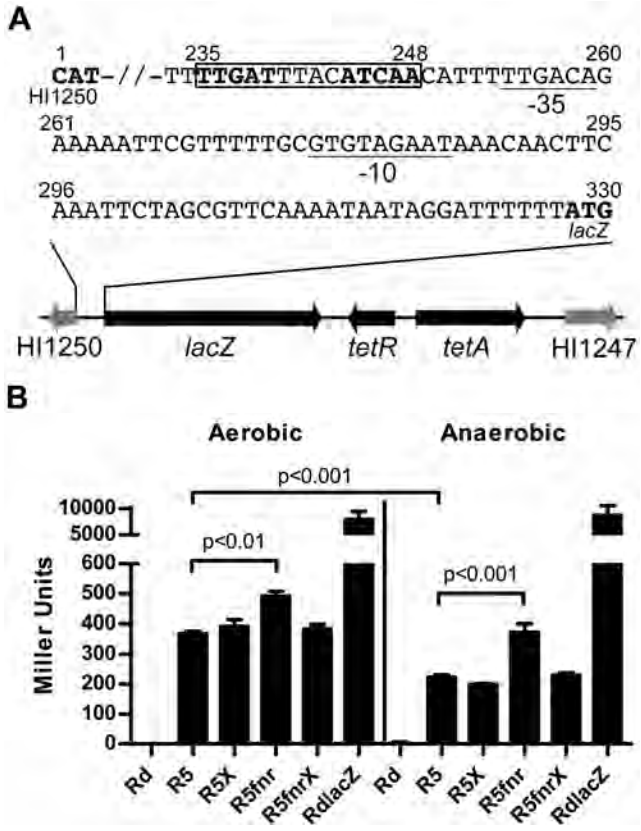

Figure 3.7. FNR-mediated regulation of the *zevA* promoter. (A) Schematic of *zevA* promoter-*lacZ* fusion construct inserted into the Rd genome at the *zevAB* locus. The 330 bp intergenic region, from the start codon of HI1250 to the start codon of *lacZ* is displayed above. A potential FNR binding site is indicated by a box and bold letters indicate the palindromic sequence. The predicted -10 and -35 promoter elements are underlined. (B) β-galactosidase activity. Parent strain (Rd), reporter strain (R5), complemented reporter strain (R5X), reporter strain carrying an *fnr* mutation (R5fnr), complemented *fnr* mutant (R5fnrX), and positive control strain (RdlacZ), were plated at a density of 5 x 10^8 CFU, grown in aerobic and anerobic environments and assayed for production of β-galactosidase. Values are displayed in Miller Units. Relevant statistical comparisons are indicated by brackets and p-values were determined using ANOVA with Tukey's multiple comparison test. Bars represent the average measurements of three technical replicates. Error bars indicate the SD.

RNA polymerase. Together with their genomic organization, these data suggest that *zevA* and *zevB* may be expressed via the same promoter.

Next, to determine if the *zevAB* genes are regulated in response to oxygen, reporter strain R5 was constructed as a *zevA* promoter *lacZ* fusion with deletion of *zevAB* in the Rd background (Figure 3.7A). A complemented reporter strain, R5X, was also generated in which the *zevAB* genes are expressed via their native promoter at the *xyl* locus in trans to the *zevA* promoter-*lacZ* fusion. When grown aerobically (sBHI agar plate), β-gal activities were similar between the reporter strain R5 and complemented reporter strain R5X (Figure 3.7B). When bacteria were grown anaerobically, β-gal activity was reduced for both strains by ~50% compared with aerobic levels. A strain carrying an *fnr* disruption mutation in the R5 background, R5fnr, exhibited ~80% greater β-gal activity compared to the parent-reporter strain or complemented-reporter strain in the anaerobic condition, effectively restoring activity to levels observed for the parent strain in the aerobic condition. The *fnr* mutant also exhibited an ~25% increase in β-gal activity compared with the parent strain in the aerobic condition, likely due to oxygen availability decreasing as culture density increases on the plate. Complementation restored levels of fusion expression in the *fnr* mutant to that of wild-type. Additionally, RdlacZ, a strain expressing *lacZ* via a constitutive promoter produced equivalent β-galactosidase (β-gal) levels in both the aerobic and anaerobic environments, suggesting that β-gal activity itself is not affected by the growth condition (Figure 3.7B).

To examine whether free zinc concentrations affect *zevA* reporter expression, the complemented reporter strain, R5X, was grown in liquid media in the presence or absence of 0.5 mM EDTA in the aerobic condition. In this assay, β-gal levels of R5X were indistinguishable between conditions (data not shown). When R5X was grown in either an aerobic or anaerobic condition in media supplemented with 100 mM zinc (a concentration non-toxic to *H. influenzae*), β-gal levels were similar to those observed for this strain in normal sBHI media (data not shown). Taken together, these data indicate that expression from the *zevA* promoter is unaffected by the presence or absence of *zevAB* or changes in free zinc concentrations and is repressed by FNR under oxygen limiting conditions. Furthermore, a higher level of *zevA* promoter activity in the aerobic environment is consistent with the requirement for *zevAB* for lung infection, in which *H. influenzae* is likely to encounter high oxygen concentrations.

DISCUSSION

Secreted bacterial proteins mediate critical aspects of pathogenesis and often require disulfide bond formation in the periplasm to stabilize their mature structures. Due to their extracytoplasmic location and accessibility, identification of these factors may aid in design of novel therapeutics or vaccines for combatting NTHi infections. Here, we generated a list of potential substrates of the periplasmic disulfide oxidoreductase protein, DsbA (Table 3.2), and integrated our findings with results of a previous study in which HITS technology was used to identify genes required for lung infection (84). Using this strategy, we identified three genes that were potentially important for lung infection including two, *znuA* and *nlpD,* that have been previously implicated in virulence of several organisms (5, 32, 53, 55, 83, 161, 244, 267, 309), and a third gene, *zevA,* whose function had not been determined. Therefore, *zevA* and its neighboring gene, *zevB,* were investigated for their roles in pathogenesis of a clinical NTHi isolate. NTHi mutants of *zevA* and *zevB* were not defective for growth in vitro on rich medium compared with parent or complemented strains but exhibited significant survival defects in a mouse lung infection model (Figure 3.3). These data confirmed that *zevA* and *zevB* are indispensable for virulence of *H. influenzae* strains in the murine lung. Interestingly, several potential DsbA-dependent proteins that were not required in the lung model as determined by HITS are important for *H. influenzae* pathogenesis in other sites of infection. For example, the hemopexin utilization protein, HxuA, has been shown to be required for wild-type bacteremic levels in an infant rat model of *H. influenzae* type-B infection (191) and the outer membrane protein P5 (OmpA) is required for virulence of NTHi in a

chinchilla model of otitis media (256). This suggests that examination of the requirement for DsbA-substrates in other *H. influenzae* infection models is likely to reveal additional mechanisms involved in pathogenesis.

Informatics-based analysis of ZevAB suggested potential roles in metal transport and this possibility was explored. Mutants of *zevA* and *zevB* were highly defective for growth in the presence of 0.5 mM EDTA (Figure 3.4B), suggesting that these genes are important during metal restricted growth. Supplementation with 60 µM zinc, but not other metals, was able to rescue growth of the *zevA* and *zevB* mutants in the presence of 0.5 mM EDTA to levels of the parent and complemented strains (Figure 3.4C). The concentration of zinc in sBHI media was determined to be 40 µM using AAS. Therefore, upon chelation with EDTA, addition of 60 µM likely restores free zinc concentrations to the normal range found in sBHI media. Together, these data indicate that *zevAB* is a novel zinc utilization pathway important for growth of *H. influenzae* when free zinc concentrations are limiting. Consistent with this observation, we also demonstrated that ZevA protein binds to two zinc ions with high affinity (Figure 3.5), similar to periplasmic binding proteins of other high affinity zinc transporters (60, 90).

H. influenzae encodes at least two zinc utilization systems, the ZnuABC system and the ZevAB system described here. Mutants of individual genes in both pathways were compared for growth in rich or metal restricted media and growth defects of the *znuA* mutant were consistently more severe than defects exhibited by the parent strain, *zevA*, or *zevB* mutants in rich media or media containing EDTA (Figure 3.6A, B, and C).

These data are consistent with the role of ZnuABC as a primary zinc transport system of *H. influenzae*, required over a wide range of conditions, whereas contributions from ZevAB are important for supporting growth only when zinc concentrations are limiting. Because double mutant analysis revealed that strains lacking both Znu and Zev pathways are not completely inhibited for growth in rich media, it is likely that an additional pathway is involved in zinc acquisition of this organism. One possibility is the PitA-dependent inorganic phosphate utilization pathway which was proposed to be a low affinity zinc transporter of *E. coli* (18). Whole-genome mutant fitness analysis via HITS suggested that PitA is required for optimal growth in vitro as well as for lung infection (84). Together, these observations suggest that *H. influenzae* contains several zinc utilization pathways whose functions are specialized to maintain zinc homeostasis in diverse environments.

Consistent with our observation that these proteins participate in zinc utilization, bioinformatic analysis of ZevA and ZevB suggested that they constitute PBP and membrane permease components of an ABC-type transporter, respectively. However, the *zevAB* locus does not encode a protein containing the Walker-A/B nucleotide binding sequences known to be required by ABC-transporters for activity (54). HITS analysis reveals seven genes required for lung infection that are predicted to encode ABC-type ATPases (84). Possibly ZevAB utilizes one of these ATPases for function, however further investigation will be needed to address this question.

In many bacterial species, genes important for zinc acquisition and homeostasis are repressed in response to elevated intracellular zinc by the transcriptional regulator Zur

(109, 223). Because *H. influenzae* does not contain a homolog of this regulator and expression of *zevAB* was not influenced by exogenous zinc levels, we sought to determine if another regulator was involved. Expression analysis using *zevA* promoter-*lacZ* fusion reporter strains revealed that *zevAB* is repressed in low oxygen conditions by the oxygen responsive transcriptional regulator, FNR (Figure 3.7B). Of note, FNR has been shown to positively regulate transcription of metal transporters such as *nikABCDE* nickel transport genes and *feoABC* iron uptake genes of *E. coli* (144, 305) and *feoABC, sitABCD,* and *fhuA* iron uptake genes of *Shigella flexneri* (28, 307). To our knowledge, FNR mediated regulation of genes involved in zinc acquisition or homeostasis has not been previously demonstrated.

Potential explanations for repression of *zevAB* by FNR are that maximum expression of *zevAB* may be detrimental to survival of *H. influenzae* in the anaerobic environment due to excessive zinc uptake or *zevAB* may be dispensable during anaerobic growth and repressed to conserve resources. However, an *fnr* mutant and a wild-type strain were equally sensitive to a range of toxic zinc concentrations during anaerobic growth, and the defect in growth of a *zevB* mutant in zinc limiting medium was not significantly different between aerobic and anaerobic conditions (Rosadini, C.V. unpublished results). Ultimately, regulation of *zevAB* by FNR may serve as an important strategy for increasing this system's expression exclusively in sites of infection where the bacterium encounters zinc limitation, as is likely to occur at airway mucosal surfaces, and is consistent with other reports indicating that oxygen is an important signal for modulation of virulence factor expression in *H. influenzae* (300, 302, 303).

Bacteria are thought to contain high affinity zinc transporters as a strategy to overcome zinc limitation during infection in which the acute phase response has been shown to result in reduced plasma zinc concentrations (82, 164) with upregulation and release of the zinc binding complex calprotectin in the blood and lungs (111, 134, 234). In accord with this hypothesis, we demonstrated here that *H. influenzae* requires a specialized high affinity zinc utilization pathway for virulence. The result that *zevAB* is needed exclusively during growth under low zinc conditions and is required for lung infection, suggests that bacteria growing in the lung experience zinc limitation that may represent an immune defense for controlling pulmonary infection.

ACKNOWLEDGMENTS

This work was supported by NIH grants 2R56AI049437-07 (B.J.A.) and 1R21AI082484-01 (J.M.A.). We thank Mr Don Pellegrino for his valuable assistance with AAS determinations.

Preface to Chapter IV

The data presented in this Chapter is unpublished

Charles V. Rosadini, Sanjay Ram, and Brian J. Akerley. The role of outer membrane protein P5 in serum resitance of *Haemophilus influenzae.*

Sanjay Ram performed the C3, C4, and antibody binding assays and generated the C4BP depleted serum.

Charles V. Rosadini performed all other experiments.

Charles V. Rosadini, Sanjay Ram, and Brian J. Akerley designed the experiments.

CHAPTER IV

The role of outer membrane protein P5 in serum resitance of *Haemophilus influenzae*

ABSTRACT

Defense against serum complement is an important mechanism for survival of *Haemophilus influenzae* during infection. Previously, we demonstrated that the periplasmic disulfide oxidoreductase, DsbA, was important for serum resistance of *H. influenzae*, however the DsbA-dependent factors required defense against serum bactericidal activity are not known. In the present study, we determine that the outer membrane protein P5 is important for resistance of pathogenic nontypeable *H. influenzae* (NTHi) to normal human serum. Mutants of P5 were found to bind increased levels of complement components C3 and C4, as well as increased levels of IgM. Together these data suggest that P5 decreases serum bactericidal activity by inhibiting binding of IgM to the surface of *H. influenzae*. Additionally, an Rd P5 mutant complemented with an NTHi P5 allele was more serum resistant than wild-type Rd, suggesting that variation in P5 between *H. influenzae* isolates is a contributor to strain specific serum resistance.

INTRODUCTION

The complement system is an important first line of defense for mammalian hosts against invading pathogens. This system consists of over 30 proteins which can mediate lysis of pathogens, serve as opsonins, and stimulate the inflammatory response (286). Complement resistance is an important feature for *H. influenzae* survival during infection. This is supported by the fact that patients deficient in complement component 3 (C3) are more susceptible to *H. influenzae* type-B (Hib) infection (299). Additionally, when infected with Hib, C3-depleted infant rats develop elevated levels of bacteremia and increased mortality rates compared with control animals (46). The importance of complement in clearance of *H. influenzae* was also extended to capsule deficient strains, where C3-depleted infant rats were found to be more susceptible to infection than controls rats (43).

Hib has been shown to encounter complement during bloodstream infection (46, 214) and is highly resistant to serum complement by virtue of its outer surface polysaccharide capsule (43, 212, 265). Conversely, nontypeable strains (NTHi), which are typically less resistant to complement than encapsulated strains, are thought to be exposed to complement at mucosal surfaces during infections of the inner ear (208, 209) and respiratory tract (9, 94, 278). However, several examples of invasive NTHi strains have been recently documented (31, 34, 47, 110, 211, 215) and complement resistance is likely important for their survival in the bloodstream. NTHi strains have been found to utilize their outer surface lipooligosaccharide (LOS) (117, 303), and sialic acid (75, 123)

for protection against complement. Several outer membrane proteins have also been implicated in this process and are thought to participate in recruitment of complement regulatory factors (102, 106), dissociate complement components, and prevent binding of protective antibody and subsequent complement mediated killing (105).

Previously, DsbA was shown to contribute to serum resistance of *H. influenzae* (241), however, the DsbA-dependent factors important for defense against serum bactericidal activity have not been identified. To determine what they might be, a list of potential DsbA substrates (Rosadini *et al*. 2011, submitted to Infection and Immunity) was examined for proteins that have been implicated in serum resistance of other organisms. The outer membrane protein P5 was identified because it is ~50% identical to *E. coli* outer membrane protein A (OmpA) (199), a factor previously shown to be important for serum resistance (292). *E. coli* OmpA has also been implicated in binding of complement regulatory factor, C4b-binding protein (C4BP) (227) allowing *E. coli* to survival more efficiently in serum (304). NTHi P5, a β-barrel protein with eight transmembrane spans and four outer surface loops (288), was shown to be required for virulence in a chinchilla ear infection model (256) and has also been implicated in adhesion of *H. influenzae* to various mucosal surface structures (26, 115, 132, 186, 236). However, its role in serum resistance has not been previously examined.

Here we demonstrate that P5 is a critical factor involved in defense against serum complement. Results suggest that P5 is important for inhibiting binding of natural serum antibody to surface structures of *H. influenzae*. Additionally, heterogeneity within P5

alleles may be a determining factor in varying levels of serum resistance between different *H. influenzae* strains.

MATERIALS AND METHODS

Strains and culture conditions. *H. influenzae* RdAW (NZ_ACSM00000000), a capsule-deficient serotype d derivative (301), and nontypable ***H. influenzae*** clinical isolate NT127 (NZ_ACSL01000014.1) (110) were grown in brain heart infusion broth (BHI) supplemented with 10 µg/ml hemin and 10 µg/ml NAD (sBHI), or on sBHI agar plates at 35°C. Development of competence for transformation of ***H. influenzae*** was accomplished as previously described (15). For selection of Rd- and NTHi-derived strains, antibiotics were used at the following concentrations: 8 µg/ml tetracycline (Tc), 20 µg/ml kanamycin (Km), and 10 µg/ml gentamicin (Gm). For strain generation, plasmids and PCR products were constructed using standard molecular biology techniques (12). For complementation of mutants, DNA fragments were amplified by PCR and cloned between adjacent SapI restriction sites of the chromosomal delivery vector pXT10, linearized and used to transform ***H. influenzae*** strains as previously described (301).

P5 mutant strain construction. Rd P5 mutant strain, RP5G, was constructed by replacement of the coding sequence of P5 with the gentamicin resistance gene from the *aacC1* Gm resistance cassette via PCR "stitching". First, three overlapping fragments were generated representing the 1,008 bp region immediately 5' of the P5 translational start codon (primers 5omp1 5'-TGCTACTCTCACTTAATTCAAGCGCAT-3' and 3omp1 5'-TGCTGCTGCGTAACAtTTTGATGTCCTCTATTTAGTGATCGAATAGT-3'), the 537-bp coding region of the gentamicin resistance gene (primers 5gent2 5'-ATGTTACGCAGCAGCAACGATGTT-3' and 3gent2 5'-

TTAGGTGGCGGTACTTGGGTCGAT-3'), and a 1,477-bp region immediately 3' of the P5 translational termination codon (primers 5omp2 5'-AAGTACCGCCACCTAaTTTTAGTATTTGTTTAACGAAAGATTAAATACAGCA-3' and 3omp2 5'-TTAGATAAACTAACTCGTTATCCAGATGCGA-3'). Subsequently, these fragments were assembled using overlap extension PCR with primers 5omp1 and 3omp2. The resulting 2,990-bp exchange fragment was transformed into competent cells of strain RdAW or strain NTV (a version or NT127 carrying a modified xylose locus for efficient recombination with plasmid pXT10 and its derivatives (110)) and selected on media containing Gm to create strains RP5G and NTP5V, respectively.

To complement the P5 mutation in RP5G, a 1,503-bp fragment containing the coding region of Rd P5 and 441-bp of sequence immediately upstream of the P5 translational start site, was generated using primers 5pOmpAHA (5'-aaagctcttcaATGAAAAAAACTGCAATCGCATTAGTAGT-3') and 3OmpAS (5'-tttgctcttctttaTTTAGTACCGTTTACCGCGATTTCTACA-3') which introduce SapI sites in the termini of the fragment. The resulting 1,458-bp fragment was digested with SapI and ligated between SapI restriction sites of the chromosomal delivery vector pXT10, which does not replicate in *H. influenzae* (301). Ligated products were used to transform RP5G and Tc resistant colonies were isolated creating strain RP58. Strain RP58 contains a co-integrate structure in which the entire pXT10 vector is integrated via single crossover into *xylF*. Next, a 1,373-bp fragment including 932-bp of 5' *xylA*-flanking sequence fused to the 441-bp putative promoter region of P5 generated from strain RP58 (primers pXT10thyAF 5'-AGGGCTTGAATCGCACCTCCA-3' and 3P51 5'-

TTTGATGTCCTCTATTTAGTGATCGAATAGT-3'), a 1,063-bp fragment containing the P5 coding sequence amplified from Rd (primers p5switch 5'-actattcgatcactaaatagaggacatcaaaATGAAAAAAACTGCAATCGCATTAGTAGT-3' and 3ompkan1 5'-CATCAGAGATTTTGAGACACGGGCCTCTTATTTAGTACCGTTTACCGCGATTTCTACA-3'), and a 2,716-bp PCR product containing the Km resistance (Km^R) gene and homology to *xylB* amplified from a kanamycin-marked derivative of pXT10 (primers 5pkan1 5'-GAGGCCCGTGTCTCAAAATCTCTGATG-3' and 3revRfaD1 5'-AACAGGCTACGATAAACCATTCAAAACAGT-3') were joined by PCR stitching (primers pXT10thyAFand 3revRfaD1). The resulting 5,072-bp fragment was introduced into strain RP5G and transformants were selected on Km generating strain RP5X.

To complement the mutation in NTP5V and generate an RdP5 mutant expressing NT127 P5, the 1,373-bp P5 promoter fragment, a 1,063-bp fragment containing the P5 coding sequence from NT127 amplified with primers p5stitch and 3ompAkan, and the 2,716-bp KmR-*xylB* fragment were joined by PCR stitching (primers pXT10thyAFand 3revRfaD1). The resulting 5,072-bp fragment was introduced into strains NTP5V and RP5G and transformants were selected on Km generating strains NTP5X and $RP5X_{NT}$, respectively.

Serum bactericidal assay. The sensitivity of P5 mutants to serum was determined as previously described (233). Briefly, strains were inoculated in triplicate 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. At log phase, 2,000 CFU from each culture were diluted in Hank's Balanced Salt Solution (HBSS) and incubated at 37°C for 30 min with or without 1, 2, or 3% (final concentration) pooled normal human serum (NHS) or rat serum (RS) (Innovative research, Novi, Michigan) in a 150-µl reaction mixture. The reaction was also performed with or without 10 mM Mg^{2+} EGTA (final concentration). 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. Results of the serum bactericidal assay are reported as percent survival, which is calculated by dividing the CFU recovered from serum treated samples by the CFU recovered from the untreated sample. Statistical analyses were performed using one-way ANOVA with Tukey's multiple-comparison test (Prism 5.03; GraphPad Software, La Jolla, CA).

Polymyxin B assay. For evaluating sensitivity to polymyxin B, strains were inoculated in triplicate from overnight cultures into 25 ml of sBHI in a 50-ml Erlenmeyer flask at an OD_{600} of 0.01 and incubated at 35°C with shaking at 250 RPM. When cultures reached log-phase, they were diluted in sBHI to 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 polymyxin B to final concentrations of 100, 200, 300, 400, 500, and 600 nM, or with sBHI alone in control wells. The plate was then incubated at 35°C for 16 hours in a $Versa_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA) set to read absorbance at 600 nm every 10 min. Sensitivity was scored as a relative growth yield as assessed by OD_{600} at the end of the incubation period.

Complement binding. Western blotting for assessment of binding of iC3b was performed as previously described (75, 231). Briefly, cultures of strains were grown as described above for serum bactericidal assays, washed and suspended in HBSS containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$ (final reaction mixture volume, 0.5 ml). NHS was added to a final concentration of 10% and incubated for 30 min at 37°C. Bacteria were lysed in 1x SDS-PAGE sample buffer and lysates were separated on 4-12% SDS-PAGE gels and analyzed by immunoblotting using primary antibodies to human iC3b (Sigma-Aldrich, St. Louis, MO) and alkaline phosphatase-conjugated secondary anti-human antibodies as described previously (231). Band densities were calculated by densitometry using ImageJ (National Institutes of Health, Bethesda, MD).

Flow cytometry. Measurement of complement component C4, IgG, or IgM binding was performed as described previously (232). Briefly, strains were cultured as described above for serum bactericidal assays. Log-phase bacteria were washed and suspended in HBSS containing 0.15mM $CaCl_2$ and 1mM $MgCl_2$at a concentration of 10^8 CFU/ml. They were then incubated with 5% NHS for 30 min at 37°C. Detection was performed using anti-C416, IgM (Abcam), and IgG (Sigma) FITC-conjugated polyclonal antibodies (Sigma).

RESULTS

P5 is important for serum resistance of *H. influenzae*. P5 mutants were constructed by replacement of coding regions with a drug resistance marker to generate non-polar deletions in Rd and NT127, a clinical NTHI strain isolated from the cerebrospinal fluid of a patient with meningitis (110) that was observed to be more serum resistant than Rd (data not shown). Complementation was conducted using PCR-based exchange vectors described in Materials and Methods for delivery and expression at the xylose locus. The complete strain set consisted of parent strain (Rd), Rd P5 mutant (RP5G), complemented Rd P5 mutant (RP5X), NT127parent strain carrying the "empty vector" (NTV), NT127 P5 mutant carrying the empty vector (NTP5V), and complemented NT127 P5 mutant (NTP5X) (Table 4.1). First, strains were evaluated for in vitro growth in rich media. The Rd P5 mutant, RP5G, exhibited similar generation times (~ 32 min) and growth yields (0.6 OD_{600}) to the parent strain, Rd, or complemented strain, RP5X. However, the NT127 P5 mutant NTP5V exhibited an ~46% increase in generation time (~62 min) and a ~20% decrease in growth yield (0.4 OD_{600}) compared with parent strain, NTV (~40 min, 0.5 OD_{600}) or complemented strain, NTP5X (~40 min, 0.5 OD_{600}). This data suggests that P5 is important for optimal growth of NTHi strains, but is not required for growth of Rd.

To determine if P5 is important for serum resistance of *H. influenzae*, strains were assayed for survival in the presence of normal human serum (NHS). To control for variable growth rates between strains, experiments were performed in growth independent conditions described in Materials and Methods. When incubated in 1%

Table 4.1. Strains used in this chapter

Strains	Genotypes, description, and/or relevant features	Reference or source
NT127	Nontypeable *H. influenzae* clinical isolate	(110)
NTV	NT127 *xylA*$\Delta_{4\text{-}804}$::*tetAR*; *tetAR* sequence from pXT10 replaces *xylA*	(110)
NTP5V	NT127 ΔP5*::aacCI, xylA*$\Delta_{4\text{-}804}$::*tetAR*; P5 deletion mutant with *tetAR* tet^R cassette replacing *xylA*	This study
NTP5X	NT127 ΔP5*::aacCI; xylA*$\Delta_{4\text{-}804}$::P5; P5 deletion mutant complemented with P5 expressed via the P5 promoter in place of *xylA*	This study
Rd	Rd AW, wild type; *H. influenzae* capsule-deficient type d	(300)
RP5G	Rd ΔP5*::aacCI*; P5 deletion mutant	This study
RP5X	Rd ΔP5*::aacCI; xylA*$\Delta_{4\text{-}804}$::P5; P5 deletion mutant complemented with Rd P5 expressed via the Rd P5 promoter in place of *xylA*	This study
$RP5X_{NT}$	Rd ΔP5*::aacCI; xylA*$\Delta_{4\text{-}804}$::P5; P5 deletion mutant complemented with NT127 P5 expressed via the Rd P5 promoter in place of *xylA*	This study

NHS, survival of the Rd P5 mutant, RP5V, was reduced to levels below the lower limit of detection (LLD) of our assay of 1.5%, whereas survival of the parent strain, RDV, and complemented strain, RP5X was unaffected (Figure. 4.1A). For comparison, viability of a strain carrying a disruption mutation in the coding region of *galU*, which encodes UDP-glucose pyrophosphorylase an essential enzyme for synthesis of the LOS outer core and complement resistance (229), was significantly reduced in this assay (survival 3.45%). Heat inactivation abrogated the effect of serum on the P5 and *galU* mutants, consistent with the essential role for serum complement in this assay (data not shown).

When NTHi strains were evaluated for resistance to 1% NHS, survival of the NT127 P5 mutant strain, NTP5V, was reduced by ~70%, compared with the parent strain, NTV, or complemented P5 mutant, NTP5X, which were unaffected (Figure 4.1B). Additionally, treatment with 2% NHS reduced survival of NTP5V by ~99% compared with survival of the parent and complemented strains which were both reduced by ~40%. Moreover, treatment with 3% NHS reduced the survival of the P5 mutant to less than the LLD of 0.25%, which was 584-fold lower than the parent strain and 272-fold lower than the complemented strain at this concentration. Heat treatment of NHS eliminated killing of NTHi strains at all concentrations (data not shown). Together, these results indicate that P5 is required for resistance of both Rd and an NTHi clinical isolate to the bactericidal activity of NHS. Furthermore, the effect of NHS on the P5 mutants is heat labile suggesting serum complement is important in killing of these strains.

Membrane integrity of a P5 mutant. Mutation of P5 may result in disruption of the

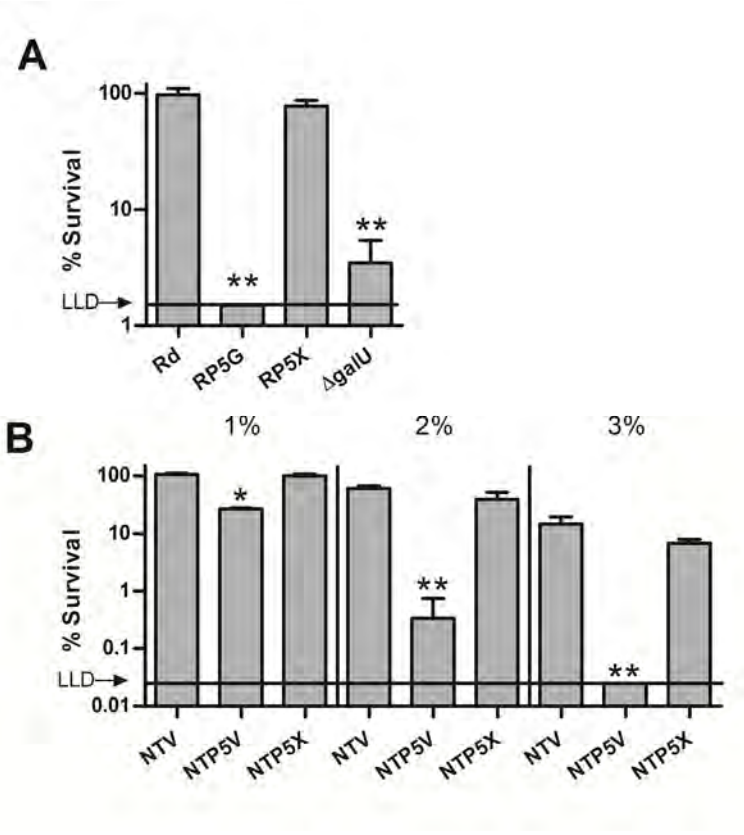

Figure 4.1. Effect of P5 mutation of resistance of *H. influenzae* to human serum. Strains were treated with NHS for 30 min at 37°C and plated for survivors. (A) Parent stain (Rd), Rd P5 mutant (RP5G), complemented Rd P5 mutant (RP5X), and *galU* mutant (ΔgalU), were treated with 1% NHS. The lower limit of detection (LLD) was 5%. (B) NTHi parent strain (NTV), P5 mutant (NTP5V), and complemented P5 mutant (NTP5X), treated with 1, 2, or 3% NHS. The LLD was 0.05%. Bars represent the mean % Survival (CFU treated /CFU untreated) of 3 replicates and error bars indicate the S.E. Statistical comparisons between parent strains and mutants are indicated by asterisks (single: $p<0.01$, double: $p<0.001$) and were determined using ANOVA with Tukey's multiple comparison test.

outer membrane resulting in increased serum sensitivity compared to the parent strain. This mechanism has been proposed for increased serum sensitivity of mutants of the peptidoglycan-associated outer membrane protein of *H. influenzae,* P6 (204). To examine whether mutation of P5 alters membrane structure, the parent strain, RDV, mutant strain, RP5V, and complemented P5 mutant, RP5X, were treated with 100, 200, 300, 400, 500, or 600 nM of the positively charged polypeptide antibiotic, polymyxin B, and assayed for growth in liquid media. The minimum concentration of polymyxin B needed for complete growth inhibition of all strains was 400 nM and no differences in survival were observed between the wild-type, mutant, and complemented strains (data not shown). These data suggest that mutation of P5 does not result in significant membrane alterations compared with parent or complemented strains.

C3 binding to P5 mutants. To determine if P5 mutants are more susceptible to killing by complement, strains were evaluated for binding of complement component C3, a central component of the pathway. Binding of C3 was assessed by incubating strains in 5 % NHS and performing Western blots with anti-iC3b mAb, which detects iC3b, a cleavage product of C3b. Differences in binding were calculated using densitometry. First, when analyzed for iC3b binding, non-opsonized bacteria produced minimal signal on Western blots (Figure 4.2; lane 6). Next, the P5 mutant strain, RP5V, was evaluated for C3 binding and was found to bind ~45% more iC3b than the parent strain, RdV, or complemented P5 mutant, RP5X. This was assessed by densitometric analysis of the ~67 kDa band which is likely to represent the iC3b-LOS complex (303)(Grey arrow; Figure 4.2; Lanes 1, 2 and 3). Binding of iC3b to the mutant was also increased on structures

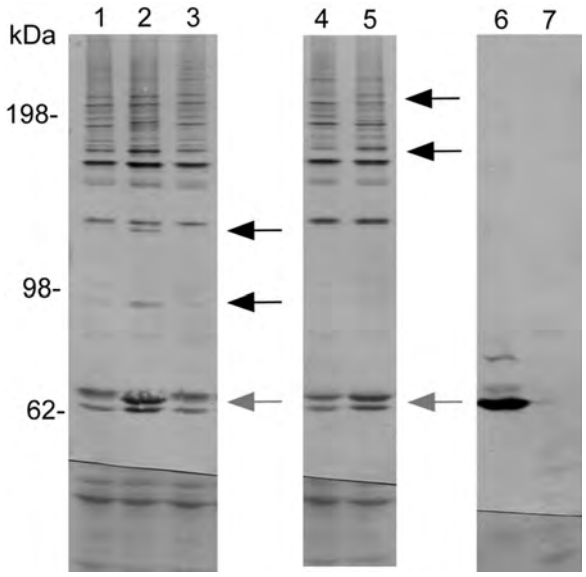

Figure 4.2. Effect of P5 mutation on binding of complement component C3. Parent strain (Rd) (lane 1), Rd P5 mutant (RP5G) (lane 2), and complemented P5 mutant (RP5CX) (lane 3) or NTHi parent strain (NTV) (lane 4) and NTHi P5 mutant (NTP5V) (lane 5) were incubated with 2% NHS for 30 min at 37°C. C3 binding was analyzed by Western blot with primary anti-human iC3b antibody and secondary anti-human alkaline phosphatase conjugated antibody. Lane 6 is purified iC3b and lane 7 is non-opsonized Rd. The bottom portion of the gel was stained with Coomassie blue to serve as a loading control. Black arrows indicate structures with increased C3 binding on P5 mutants compared to parent strains. Grey arrows indicate LOS + iC3b complex.

that were not detected with the parent or complemented strain (Black arrows; Figure 4.2; Lanes 1, 2, and 3). These data suggest that mutation of P5 "unmasks" additional binding sites for linkage of iC3b to the surface of Rd, but also increases overall C3 binding to normal surface exposed structures.

Because outer surface structures vary in composition between *H. influenzae* isolates, C3 binding sites exposed by mutation of P5 in the Rd strain may not be present in an NTHi isolate. Therefore, NTV and NTP5V were also examined for binding of iC3b. Results of densitometric analysis of the LOS-iC3b band indicated that the NTP5V bound ~40% more iC3b than the parent strain (Grey arrow; Figure 4.2; Lanes 4 and 5). However, iC3b binding sites were common to both the parent and mutant strains (Black arrows; Figure 4.2; Lanes 4 and 5). This suggests that increased sensitivity of NTHi P5 mutants to NHS is not dependent on "unmasking" of new binding sites as a result of deletion of P5 as observed with Rd, but is due to an overall increase in C3 deposition on normal surface structures of *H. influenzae*. Together, these data demonstrate that increased sensitivity of the P5 mutant to serum correlates with increased binding of complement components.

Complement activation by P5 mutants. Previous reports indicate C3 deposition on the *H. influenzae* cell surface is largely mediated by classical complement pathway activation (298). To determine if the classical/MBL- pathways are required for killing of P5 mutants, Rd strains were treated with NHS in buffer with or without 10 mM Mg^{2+} EGTA. Incubation with Mg^{2+} EGTA alone had no effect on viability of the strains, indicating that

EGTA does not interfere with their survival at this concentration (Figure 4.3). When incubated in 1% or 2% NHS, survival of the P5 mutant was decreased significantly compared with parent and complemented strains (Figure 4.3). Addition of Mg^{2+} EGTA eliminated the lethal effects of both concentrations of serum on all strains (Figure 4.3). Taken together, these data suggest that the classical/MBL pathway is required for killing of P5 mutants.

C4 binding to P5 mutants. C4 is an essential component of the classical C3 convertase and increases in C4 binding or activation can lead to greater bactericidal activity (117). To determine if mutation of P5 affects C4 binding to *H. influenzae,* strains were incubated in ~5 % NHS and evaluated for total C4 binding using flow cytometry. Binding of C4 to the P5 mutant was ~10-fold greater than to the parent strain or complemented strain as determined by fluorescence intensity (Figure 4.4). These data suggest that P5 plays a role in inhibition of the initial steps of the complement cascade. Furthermore, increased C4 binding to the P5 mutant is consistent with increased C3 binding observed in Figure 4.2A.

Complement regulatory component C4BP. NTHi strains have been previously shown to bind the complement regulatory factor C4b-binding protein (C4BP) (103). Binding of C4BP to C4b prevents formation and promotes dissociation of the classical C3 convertase C4b2a (85). C4BP also participates in degradation of C4b by functioning as a co-factor for the serine proteinase factor I (80, 81). Because P5 mutants bound elevated levels of C4 compared to parent strains and, a putative P5homolog of *E.coli,* OmpA, binds C4BP

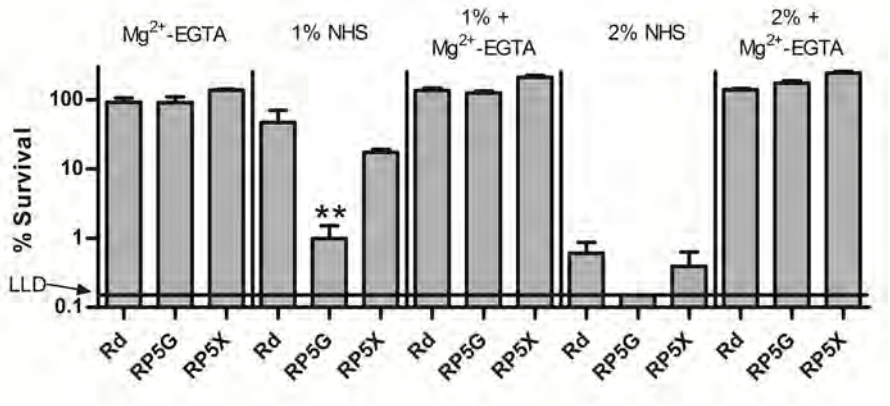

Figure 4.3. Effect of P5 mutation of resistance of *H. influenzae* to human serum in the presence or absence of Mg^{2+}-EGTA. Parent stain (Rd), Rd P5 mutant (RP5G), and complemented Rd P5 mutant (RP5X) were treated with or without NHS in the presence of absence of 10 mM Mg^{2+}-EGTA for 30 min at 37°C and plated for survivors. The LLD was 0.15%. Bars represent the mean % Survival (CFU treated /CFU untreated) of 3 replicates and error bars indicate the S.E. Statistical comparison between parent strain and mutant is indicated by a double asterisk ($p<0.001$) and was determined using ANOVA with Tukey's multiple comparison test.

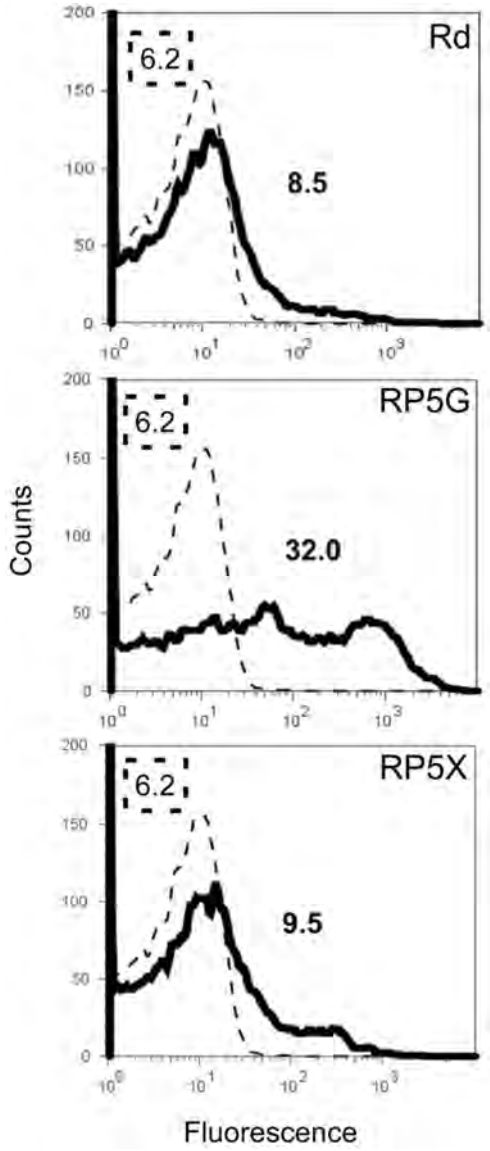

Figure 4.4. Effect of P5 mutation on binding of complement component C4. Parent stain (Rd), P5 mutant (RP5G), and complemented P5 mutant (RP5X) were incubated with 5% NHS for 30 min at 37°C and assayed for binding of complement component C4 using flow cytometry. Log_{10} fluorescence is represented on the *x*-axis. Dashed line represents the background fluorescence and the solid line represents fluorescence of strains indicated. Values on each graph represent the mean fluorescence for strains indicated (bold number) and background (number within dashed box).

(227), the possibility that P5 may participate in interaction with C4BP was examined. To test this hypothesis, Rd strains were incubated in C4BP-depleted NHS (immuno-depleted to <2% of normal C4BP levels) or C4BP-depleted serum, supplemented with purified C4BP. The parent strain, RdV, exhibited equivalent survival of ~35% in both the presence and absence of C4BP (data not shown). Similar to data observed in Figure 4.1A, the P5 mutant, RP5V, exhibited ~230-fold lower survival compared to the parent strain in both C4BP-replete and C4BP-depleted serum (data not shown). Together, these data suggest that P5 does not play a role in binding of regulatory factor C4BP. Additionally, C4BP binding contributes minimally to protection of Rd strains from bactericidal activity of NHS.

Immunoglobulin binding to P5 mutants. Antibody binding to the surface of a pathogen is an important step in complement activation via the classical pathway. To determine if P5 effects antibody binding to the surface of *H. influenzae*, Rd strains were incubated with NHS and antibody levels were examined using flow cytometry. Levels of IgG binding were found to be similar between the parent, P5 mutant, and complemented strain (Figure 4.5A). However, IgM binding to the P5 mutant was increased by ~100% over the parent and ~120% over the complemented strain (Figure 4.5B). Similar results were obtained for these strains in separate experiments (data not shown). Increased IgM binding is consistent with the observation that P5 mutants are more sensitive to classical complement pathway activation.

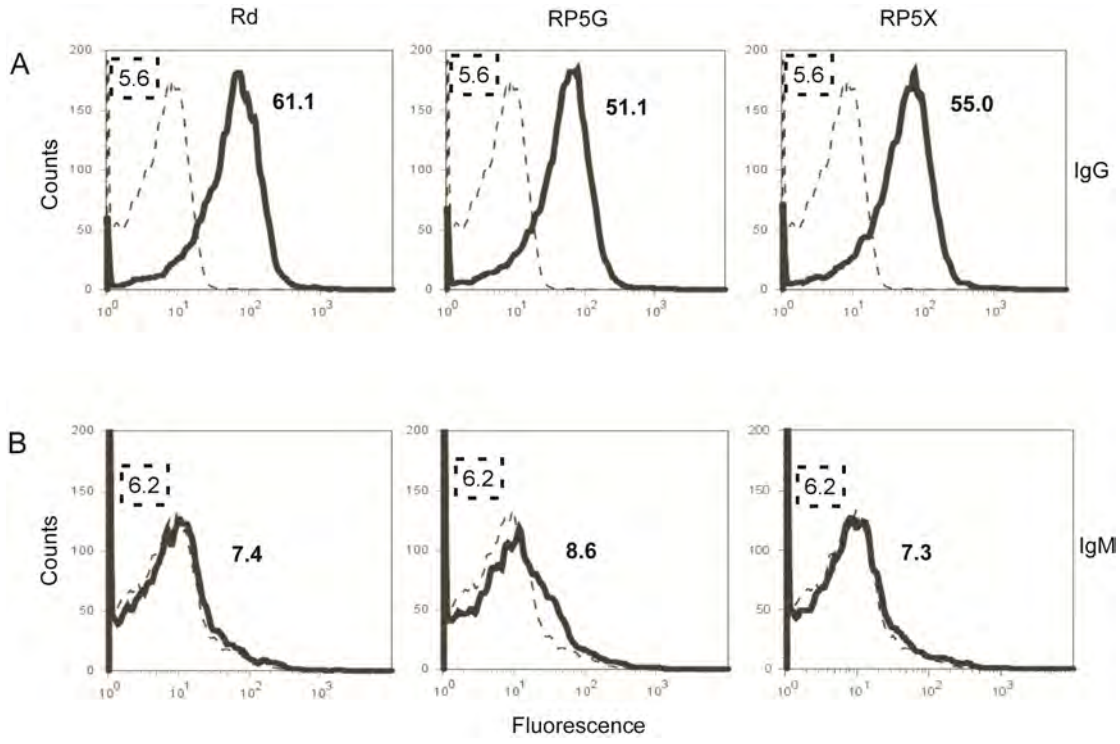

Figure 4.5. Effect of P5 mutation on binding of serum antibodies. Parent stain (Rd), P5 mutant (RP5G), and complemented P5 mutant (RP5X), were incubated with 5% NHS for 30 min at 37°C and assayed for binding of (A) IgG or (B) IgM using flow cytometry. Log_{10} fluorescence is represented on the *x*-axis. Dashed line represents the background fluorescence and the solid line represents fluorescence of strains indicated. Values on each graph represent the mean fluorescence for strains indicated (bold number) and background (number within dashed box).

Sensitivity of a P5 mutant to rat serum. Because humans are repeatedly colonized by *H. influenzae*, NHS may contain specific antibodies to this organism. To determine if the effect of mutation of P5 on serum resistance was exclusive to human sera, Rd strains were exposed to 1 or 2% pooled rat sera (RS) which is unlikely to contain specific *H. influenzae* antibody. In 1% RS, survival of the P5 mutant, RP5V, was ~74 to ~75-fold lower than the parent strain, RdV or complemented strain, RP5X, respectively (Figure 4.6A). Next, NTHi strains were tested for survival in 1, 2, or 3% RS. Survival of the P5 mutant strain, NTP5V, was below the LLD for the assay at all concentrations, whereas the parent strain NTV and complemented strain, NTP5X survived to ~100%, ~30%, and ~15% in 1, 2, and 3 % RS, respectively (Figure 4.6B). These data indicate that P5 is required for resistance of *H. influenzae* to RS and suggest that specific *H. influenzae* antibody is not required for complement mediated killing of P5 mutants.

Heterogeneity of P5 alleles effects serum resistance. Surface exposed residues on P5 have been previously shown to be variable between NTHi clinical isolates, likely due to antigenic pressure (62, 288). In accordance with this, alignments of Rd and NT127 P5 alleles show variability in predicted outer surface loops, whereas other portions of the proteins were largely identical in sequence (Figure 4.7A). To determine if variability in P5 alleles contributes to differences in serum resistance between Rd and NT127, Rd P5 mutants were complemented with either the P5 allele from Rd, (strain RP5X) or the P5 allele from NT127 (strain $RP5X_{NT}$). To exclude possible differences in gene expression, complementing alleles were expressed via the Rd P5 promoter. When assayed for survival in 1% NHS, complementation of the P5 mutant with Rd P5 (strain RP5X)

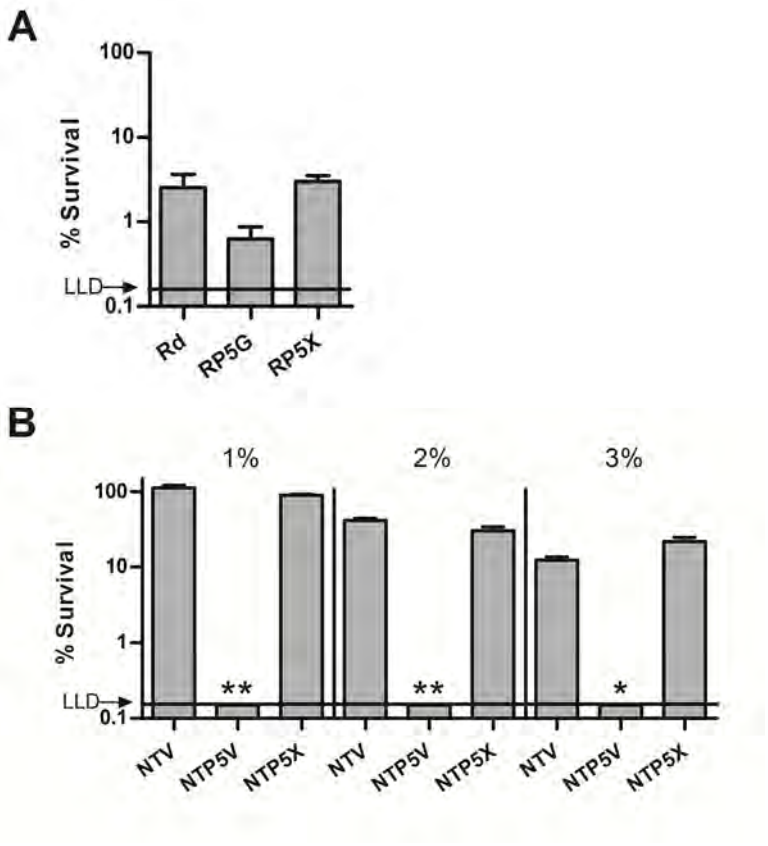

Figure 4.6. Effect of P5 mutation of resistance of *H. influenzae* to rat serum. Strains were treated with RS for 30 min at 37°C and plated for survivors. (A) Parent stain (Rd), Rd P5 mutant (RP5G), and complemented Rd P5 mutant (RP5X), were treated with 1% RS. The lower limit of detection (LLD) was 0.15%. (B) NTHi parent strain (NTV), P5 mutant (NTP5V), and complemented P5 mutant (NTP5X), treated with 1, 2, or 3% RS. The LLD was 0.15%. Bars represent the mean % Survival (CFU treated /CFU untreated) of 3 replicates and error bars indicate the S.E. Statistical comparisons between parent strains and mutants are indicated by asterisks (single: $p<0.01$, double: $p<0.001$) and were determined using ANOVA with Tukey's multiple comparison test.

restored the survival defect of the Rd P5 mutant to near parent levels (Fig. 4.7B). However, survival of the Rd P5 mutant complemented with NT127 P5, $RP5X_{NT}$, was significantly increased by 3-fold over RP5X (Figure 4.7B). When strains were treated with 2% NHS, survival of $RP5X_{NT}$ was increased by ~13-fold over Rd or RP5X (Figure 4.7B). In 3% NHS, survival of stains Rd, RP5G, and RP5X was below the LLD, whereas strain $RP5X_{NT}$ exhibited a percent survival of 0.06. Together, these data demonstrate that P5 is not only necessary for resistance to NHS, but also a determinant of strain specific serum resistance.

A

```
 Identities = 283/353 (81%), Positives = 312/353 (89%), Gaps = 3/353 (0%)
                                                          Loop 1
Rd       1   MKKTAIALVVAGLAAASVAQAAPQENTFYAGVKAGQASFHDGLRALAREYKVGYHRNSFT  60
NT127    1   MKKTAIALVVASLAAASVAQAAPQENTFYAGVKAGQGSFHDGVRAMGKQFSAGHYRNTFT  60
Consensus    MKKTAIALVVA LAAASVAQAAPQENTFYAGVKAGQ SFHDG+RA+ +++  G++RN+FT
                                                  Loop 2
Rd      61   YGVFGGYQILNQNNLGLAVELGYDDFGRAKGREKGKTVVKHTNHGTHLSLKGSYEVLEGL  120
NT127   61   YGVFGGYQILNQDNFGLAAELGYDDFGRAKLKTTGKLTGKHTNHGTHLSLKGSYEVLDGL  120
Consensus    YGVFGGYQILNQ+N GLA ELGYDDFGRAK +  GK   KHTNHGTHLSLKGSYEVL+GL
                                          Loop 3
Rd     121   DVYGKAGVALVRSDYKLYNENSSTLKKLGEHHRARASGLFAVGAEYAVLPELAVRLEYQW  180
NT127  121   DVYGRLGVALIRSDYKYYDQ--GVRQRAKSEHSSRVSPVFAAGLEYAVLPELAVRVEYQW  178
Consensus    DVYG+ GVAL+RSDYK Y++     ++    H +R S +FA G EYAVLPELAVR+EYQW
                                  Loop 4
Rd     181   LTRVGKYRPQDKPNTALNYNPWIGSINAGISYRFGQGAAPVVAAPEVVSKTFSLNSDVTF  240
NT127  179   VNQIGKLRSHDN-NRVYDYNPSVGSINAGISYRFGQGEAPVVAAPEMVSKTFSLNSDVTF  237
Consensus    + ++GK R  D  N   +YNP +GSINAGISYRFGQG APVVAAPE+VSKTFSLNSDVTF

Rd     241   AFGKANLKPQAQATLDSIYGEMSQVKSAKVAVAGYTDRIGSDAFNVKLSQERADSVANYF  300
NT127  238   AFGKANLKPQAQATLDSVYGEISQVKSAKVAVAGYTDRIGSDAFNVKLSQERADSVANYF  297
Consensus    AFGKANLKPQAQATLDS+YGE+SQVKSAKVAVAGYTDRIGSDAFNVKLSQERADSVANYF

Rd     301   VAKGVAADAISATGYGKANPVTGATCDQVKGRKALIACFAPDRRVEIAVNGTK  353
NT127  298   VAKGVAADAISATGYGEANPVTGATCDQVKGRKALIACLAPDRRVEIAVNGTK  350
Consensus    VAKGVAADAISATGYG+ANPVTGATCDQVKGRKALIAC APDRRVEIAVNGTK
```

B

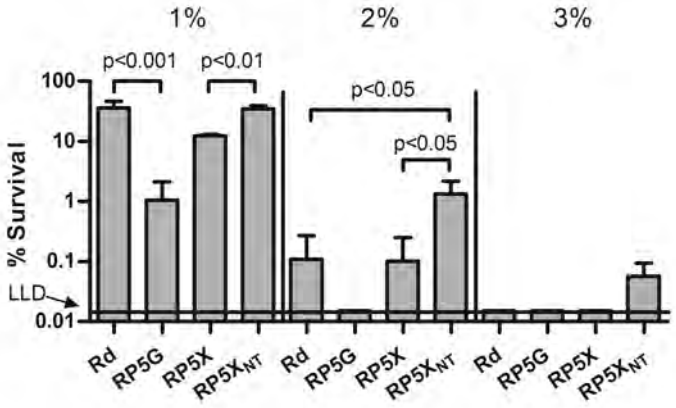

Figure 4.7. Effect of P5 allele heterogeneity on serum resistance. (A) Comparison of Rd and NT127 P5 amino-acid sequences. Alignments were performed using BLAST. Putative surface exposed regions were described previously (283) and are indicated by boxes. (B) Parent stain (Rd), Rd P5 mutant (RP5G), Rd P5 mutant complemented with Rd P5 (RP5X), and Rd P5 mutant complemented with NT127 P5 (RP5X$_{NT}$) were treated

with 1, 2, or 3% NHS for 30 min at 37°C and plated for survivors. The LLD was 0.015%. Bars represent the mean % Survival (CFU treated /CFU untreated) of 3 replicates and error bars indicate the S.E. Relevant statistical comparisons are indicated on the graph and were determined using ANOVA with Tukey's multiple comparison test.

DISCUSSION

NTHi strains utilize several outer surface structures including LOS and sialic acid for defense against serum complement. Outer surface proteins are also thought to participate in serum resistance; however their role in this process is not well understood. In this study, we demonstrated that P5 is required for serum resistance of both a lab passaged strain, Rd, and a clinical NTHi isolate, strain NT127. The effects of NHS on our strains were abolished by addition of EGTA and heat treatment, consistent with the essential role for complement in the assay. These data also suggested antibody agglutination is not a significant mechanism responsible for killing in our assays. Additionally, polymyxin B assays suggest that P5 mutants do not exhibit membrane disruptions which have been shown to result in increased sensitivity to serum (204). Together, these data implicate P5 in resistance of *H. influenzae* to complement.

The mechanism by which P5 participates in serum resistance was investigated. An increase in C3-binding was observed for Rd and NTHi P5 mutants versus wild-type strains. Results from this analysis suggested that increased sensitivity of P5 mutants to complement is not a result of unmasking of additional C3 binding sites on the surface, but due to an overall increase in C3 deposition on normal surface structures of *H. influenzae*. Mg-EGTA eliminated the effect of NHS on all of the strains in our assays, suggesting that the majority of the complement activation in the assay is mediated through the classical or MBL pathway. In accordance with this hypothesis, P5 mutants exhibited increased binding of C4 compared with parent or complemented strains. Together, these

results suggested that P5 mediates serum resistance through inhibition of the initial stages of complement activation.

Increased binding of C4 to P5 mutants suggested that P5 may participate in binding of the classical pathway inhibitor, C4BP. This possibility was also suggested by previous reports showing that the putitive P5 homolog, *E. coli* OmpA, binds C4BP (227) and that this mechanism is important for survival of *E. coli* in NHS (304). However, data presented here does not support a role for C4BP in P5 mediated serum resistance of *H. influenzae*.

Due to the apparent role of classical pathway activation in killing of P5 mutants, we examined immunoglobulin binding to our strains. P5 mutants were found to bind increased levels of IgM compared to parent and complemented strains. These results were consistent with previous reports suggesting that increases in IgM, a potent activator of complement, results in serum sensitivity of *H. influenzae*. For example, serum sensitivity of mutants of *vacJ* and *yrb*, which encode factors important for control of phospholipid turnover in the outer leaflet of NTHi, correlated with increased IgM binding (207). Furthermore, deletion of *dsrA* of *Haemophilus dureyi*, which encodes an outer membrane protein required for infection of humans (25), resulted in IgM dependent serum sensitivity (1, 67). Therefore, it is likely that increased IgM binding is responsible for serum sensitivity of P5 mutants.

Because, colonization of the human respiratory tract by NTHi has been shown to stimulate humoral immunity against other NTHi strains, human serum may contain

specific antibody against *H. influenzae* surface epitopes (149). To determine whether serum sensitivity was due to specific or natural antibody, P5 mutants were assayed for survival in rat serum, which does not likely contain specific anti-*H. influenzae* antibody. P5 mutants were found to be more sensitive to rat serum than parent or complemented strains, suggesting that the bactericidal effect of serum on P5 mutants is not dependent on pre-exposure of the serum donor to *H. influenzae*. We cannot rule out the potential for cross-reactive responses to resident bacterial flora, however, rats were housed in SPF conditions and therefore are not expected to have encountered a wide range of pathogens to elicit strong cross-reactive antibody responses. Hence, complement activation on P5 mutants is likely mediated by natural IgM present in serum, consistent with previous studies which found that natural antibody in NHS that is bactericidal for *H. influenzae* is predominantly IgM (70, 87, 207).

Multiple reports have concluded that strain specific serum resistance is mediated by variation in the outer surface structures of *H. influenzae* including heterogeneity in oligosaccharide components (70, 75, 117, 123, 295, 303), phospholipid trafficking (207), and membrane protein heterogeneity (105). We observed that predicted surface exposed regions of P5 were also heterogeneous between our strains. Modifications to P5 may be influenced by selective pressure imposed by host antibody and complement (62). Therefore, we postulated that heterogeneity in P5 alleles may contribute to varied levels of serum resistance between strains. Accordingly, a P5 allele from the more serum resistant NTHi strain, NT127, was found to enhance resistance of the low serum resistant strain, Rd, to NHS. These results suggest that variation in P5 contributes to strain specific

serum resistance. Greater resistance of $RP5X_{NT}$ to NHS compared with RP5X may involve inhibition of IgM-binding, however, when examined by flow cytometry, IgM binding was indistinguishable between these strains (Rosadini C.V. unpublished data). Perhaps this assay was not sensitive enough to detect minute changes in IgM binding between strains. One other possibility is that expression of P5 may participate in inhibition of C4b deposition on *H. influenzae*, similar to the mechanism reported for participation of LOS biosynthesis gene, *lgtC* (117). Further analysis will have to be performed to address these possibilities.

A P5 mutant has been shown to be impaired for colonization of the chinchilla nasopharynx and middle ear (256). Subsequently, a role for P5 in adherence to the mucosal epithelium via CEACAM1 receptors or mucin was suggested to be responsible for in vivo defects (26, 115, 132, 186, 236). The role of complement in clearance of NTHi P5 mutants from the chinchilla middle ear was not addressed. However, other reports have shown that complement is important for clearance of NTHi from this site (75). Possibly, P5 mediates several functions in vivo including complement resistance and adherence allowing for efficient colonization. Taken together, our understanding of the role of P5 in colonization and virulence of NTHi is not complete, and future analysis will need to be performed to examine requirements for P5 at various sites of infection.

In summary, the complement pathway is an important immune defense for mammalian hosts against invading pathogens. To defend themselves, bacteria utilize diverse strategies to subvert complement activation. The data presented here suggests that

the outer membrane protein P5 inhibits binding of natural IgM present in NHS to the surface of *H. influenzae*. By doing so, P5 inhibits activation of the classical complement pathway and subsequent killing of the cell. Furthermore, variation in P5 is a contributor to strain specific serum resistance. Thus, P5 is a critical factor for *H. influenzae* resistance to serum complement.

CHAPTER V

Conclusions and future directions

Various aspects of *H. influenzae* pathogenesis including proteins and nutrients required for infection of specific anatomical locations, such as the bloodstream and lung, are not well understood. The goal of this thesis was to identify and characterize some of these virulence proteins and by doing so, develop an understanding of specific nutrients that this organism requires to survive during disease. In many Gram-negative organisms, proteins important for virulence are often secreted and require periplasmic disulfide bond formation for function and stability. Consistent with this, data described in this thesis demonstrates that mutation of *dsbA*, which encodes the major disulfide oxidoreductase in the periplasm, results in reduced colonization and virulence of *H. influenzae*. DsbA was not required for in vitro growth, but was indispensable for blood stream infection in both Rd and Hib in the mouse and infant rat infection models, respectively. Additionally, preliminary data indicates that DsbA is required for *H. influenzae* colonization of the mouse nasopharynx (Rosadini, C.V. unpublished results) and lung (84). Using in vitro assays, *dsbA* mutants were shown to be defective for resistance of *H. influenzae* to serum complement. Together these data suggested that some of the targets of the disulfide pathway must be necessary for this organism's pathogenesis and at least one of them is involved in serum resistance.

The set of disulfide-dependent proteins of *H. influenzae* was not previously known. Therefore, to address the hypothesis that DsbA-dependent factors are important

for virulence, informatics-based approaches were utilized to produce a database of potential DsbA-substrates. Using literature based analyses and data from a genome-wide screen to identify genes required during murine lung infection, the list of substrates was examined for factors that were potentially important for pathogenesis of ***H. influenzae***. Results of these analyses led to identification of a factor required for bloodstream infection, HbpA, as well as novel virulence mechanisms including zinc utilization via ZevAB and serum resistance via P5 (Figure 5.1). HbpA was shown to contain a DsbA-dependent disulfide and preliminary data suggests that ZevA also contains a disulfide bond (Rosadini C.V. unpublished results). Additionally, sequence comparisons revealed that cysteine pairs within ZevA and HbpA, as well as other virulence factors including ZnuA and P5, are highly conserved in their homologs in the ***Pasteurellaceae*** and ***Enterobacteriaceae*** (4). Moreover, many of the 81 potential DsbA-dependent proteins of ***H. influenzae*** described in this thesis are conserved in other species, including members of the above families (4). Taken together, results of this thesis demonstrate that evaluating DsbA substrates for virulence properties is a viable way to determine pathogenic mechanisms of ***H. influenzae*** and suggest that searching for these substrates in other Gram-negative organisms is likely to be a productive area for future research.

Several potential DsbA-dependent proteins were not required in the lung model, but are important for ***H. influenzae*** pathogenesis in other sites of infection. For example, the hemopexin utilization protein, HxuA, has been shown to be required for wild-type bacteremic levels in an infant rat model of ***H. influenzae*** type-B infection (191) and the outer membrane protein P5 (OmpA) is required for virulence of NTHi in a chinchilla

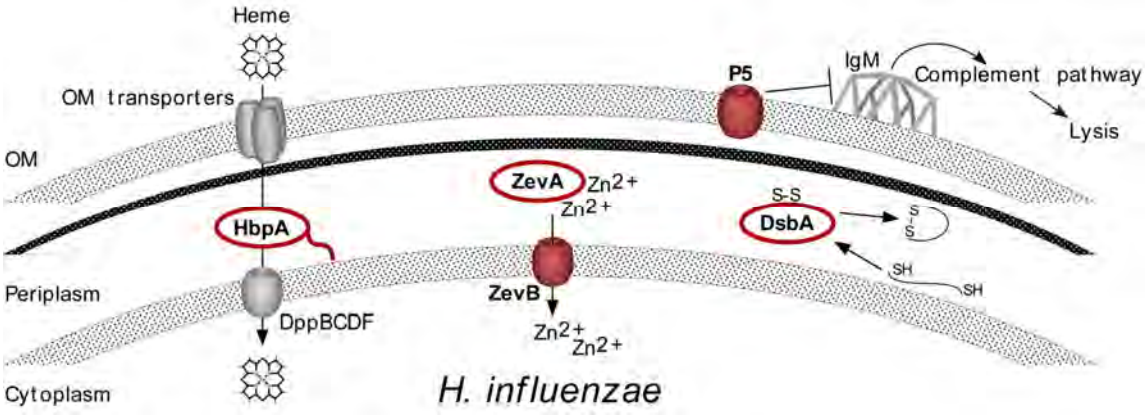

Figure 5.1. Summary of *H. influenzae* virulence factors presented in this thesis. Proteins highlighted in red were examined for their participation in various pathogenesis related phenotypes of *H. influenzae*. Items in grey were not characterized by this work. The disulfide bond oxidoreductase DsbA was found to be required for bloodstream infection by Hib and Rd. HbpA, although characterized previously by other groups as important for heme binding and utilization of *H. influenzae*, was first found to be important for bloodstream infection by work described in this thesis. The ZevAB system was characterized as a zinc binding system necessary for zinc utilization during conditions when zinc is limiting and is required for survival in the mammalian lung. Lastly, the outer membrane protein P5 was found to prevent complement mediated killing likely through inhibition of natural IgM binding. OM is outer membrane and IgM is displayed in the "staple" confirmation.

model of otitis media (256). Therefore, we can conclude that DsbA-dependent virulence factors are differentially required at diverse sites of infection. Examination of the requirement for DsbA-substrates in sites such as the bloodstream, nasopharynx, or middle ear may reveal additional mechanisms involved in pathogenesis. Understanding of processes needed for *H. influenzae* to survive within specific host environments might aid in developing strategies for preventing its transition from asymptomatic colonization to invasive infection.

Heme utilization

Although there are many known systems in *H. influenzae* for binding heme at the outer surface, little is understood about how heme is transported to the cytoplasm. Previously, several reports had demonstrated that HbpA was important for heme binding and utilization by *H. influenzae* and concluded that HbpA is a periplasmic binding protein for a heme specific ABC transporter (108) composed of the putative dipeptide transport system DppBCDF (192). However, the role of HbpA during infection was not known. The result that HbpA is required for *H. influenzae* bloodstream infection was first demonstrated by the work discussed in Chapter II. Consistent with our results, recent studies indicated that HbpA was required for Hib infection of 30 day old rats (190). However, unpublished data from our lab as well as other reports indicated that HbpA is not required for Rd or NTHi colonization of the mouse and chinchilla nasopharynx, respectively (190) (Rosadini C.V. unpublished results). Moreover, HbpA was not required by NTHi for infection of the chinchilla ear or by Hib for infection of the

bloodsteam in 5-day old rats (190). The fact that HbpA is not always required during infection suggests that it is not the only pathway available for ***H. influenzae*** to transport heme sources across the inner membrane. Interestingly, the periplasmic antimicrobial peptide resistance protein, SapA, has been recently shown to bind heme and is required for optimal growth of ***H. influenzae*** in vitro (171, 174). The Sap system has been shown to be upregulated in the chinchilla middle ear and is required for ***H. influenzae*** colonization of the chinchilla middle ear and nasopharynx (172). Potentially, SapA represents a second pathway for heme transport across the inner membrane (174). Therefore, the result, presented in this thesis, that HbpA is required for bloodstream infection, combined with results from others, have provided necessary insight into mechanisms for ***H. influenzae*** to import heme, a key molecule which is required for its success in vivo.

Zinc utilization

One of the highlights in this thesis is the discovery of the ZevAB zinc utilization pathway. Our results suggest that ZevAB functions as a high-affinity zinc importer that likely transports zinc across the inner membrane. This pathway was found to be essential for NTHi survival in the mouse lung infection model; however, it is not necessary for in vitro growth on rich media. ZevAB is required for zinc utilization exclusively during conditions when zinc is limiting. This is in contrast to the ***H. influenzae*** ZnuABC zinc transporter which was shown to be required over a wide range of zinc concentrations in this work and by others (166). Therefore, these results suggest that ZnuABC is the

primary zinc uptake pathway of *H. influenzae* and ZevAB is an alternate transporter system needed to sustain growth during severe zinc limitation.

ZnuABC is required for virulence of several Gram-negative pathogens at different sites of infection, including the gut, tissues, and blood, suggesting that zinc is not readily available to pathogens in vivo (5, 32, 55, 83, 161, 244, 309). However, the requirement for ZevAB for *H. influenzae* survival in the lung suggests that this infection site also represents a condition where pathogens encounter severe zinc limitation. Potentially these results could reflect host immune processes which are thought to promote sequestration of zinc to inhibit growth of invading pathogens during disease (145). For instance, initiation of the acute phase response during infection has been shown to result in up-regulation of the host intracellular metal binding protein metallothionein in hepatocytes and increased expression of cellular zinc import systems, such as the hepatic ZIP14 transporter, resulting in reduced plasma zinc concentrations (82, 164). Possibility, the metallothionein-null (MT -/-) mouse model (180) might be employed to address the role of this pathway in zinc limitation during *H. influenzae* infection. Previous studies have indicated that even at extremely high plasma zinc concentrations, MT -/- mice fail to store zinc in their livers (44). This suggests that during activation of the inflammatory response due to challenge with pathogens such as *H. influenzae*, MT -/- mice will be unable to reduce the level of zinc available. Potentially, mutants of *znuABC* or *zevAB* described in this thesis will exhibit enhanced survival during infection of the MT -/- mouse.

In addition to increased zinc storage in the liver, the acute phase response also stimulates up-regulation and release of the antimicrobial zinc binding complex calprotectin in the blood and lungs (36, 111, 134, 234, 257, 262). Several reports indicate that calprotectin is antimicrobial when applied to microbes in vitro (257, 262). Perhaps the effect of calprotectin on ***zevAB*** mutants could be examined to explore the role of these genes in ***H. influenzae*** defense against host immune functions. In vivo studies could also be performed using calprotectin-null mice which are not able to produce this highly expressed antimicrobial complex (118).

The role of zinc limitation in growth inhibition of ***H. influenzae*** during lung infection might also be explored by supplementation of mice with zinc during challenge with wild-type NTHi compared with ***zevAB*** mutants. Previous studies indicate that alteration of dietary zinc intake can affect the levels of zinc in the lungs of mammals (14). Possibly, zinc supplementation will overcome the requirement for ***zevAB*** in vivo. This result would suggest that attenuation of ***zevAB*** mutants during infection is zinc specific. Interestingly, zinc is an essential element for proper immune function in mammals and severe zinc deficiency has been liked to failure of the host to defend against bacterial infection and sepsis (14). However, restoration of host tissue and serum zinc levels through dietary zinc supplementation or administration of zinc directly into the serum or tissues before or during infection results in increased immune response and improved host survival (14, 269). Therefore, zinc supplementation in humans might help boost immunity against organisms like ***H. influenzae*** which contain mechanisms to overcome the hosts attempt at halting their growth through zinc limitation. Overall, an

important area for future investigation would be to examine the role of host zinc sequestration mechanisms in defense against *H. influenzae* during infection.

The discovery of ZevAB and its role is zinc uptake has potential implications for other pathogens. For example, with the hypothesis that zinc limitation likely occurs during infection, Desrosiers and co-workers recently examined pathways important for *Y. pestis* to acquire zinc in vivo (59). Similar to reports on other organisms, they determined that ZnuABC is required for in vitro growth on low zinc. However, they found that this pathway is not required for *Y. pestis* pathogenesis in mouse bubonic and pneumonic plague models (59). These researchers concluded that a yet undiscovered high-affinity zinc uptake pathway is likely to support growth and survival of *Y. pestis* during infection (59). Blast searches performed in Chapter III indicate that *Y. pestis* contains putative *zevA* and *zevB* homologs. Possibly, the ZevAB system could be the critical transporter required for *Y. pestis* pathogenesis.

In addition to discovery of the role of ZevAB in infection, the work presented here describes biochemical characteristics of the transporter. Specifically, the putative periplasmic binding protein ZevA was shown to bind two Zn^{2+} ions with high-affinity. However, the residues important for substrate specificity have not been determined. Studies analyzing the functionally related "cluster 9" family of Zn^{2+} and Mn^{2+} transporters illustrate the critical amino acids involved in zinc binding. For example, the primary zinc binding site of *E. coli* consists of three His residues and a Glu that bind Zn^{2+} in a nearly tetrahedral geometry (162, 310). Additionally, periplasmic binding proteins

PsaA of *Streptococcus pneumoniae* and TroA of *Treponema pallidum* also contain at least two His residues in their primary metal binding sites (310). Although ZevA is not classified as a cluster 9 family periplasmic binding protein, analysis of its amino acid sequence reveals that this protein contains four His residues, three of which are highly conserved in ZevA homologs in other species. Potentially, these residues could participate in binding of Zn^{2+}; however, further analysis will be needed to determine their role in this process.

The predicted permease ZevB also contains interesting features that could participate in function of the transporter. Analysis of ZevB secondary structure revealed that this protein has six potential transmembrane spans with three large cytoplasmic loops. The second cytoplasmic loop in ZevB, as well as ZevB homologs of other bacterial pathogens including members of the *Pastuerellaceae* and *Enterobacteriaceae,* contains a highly conserved sequence with the consensus C(S/G)CGHXH (where X is Q, K, R or A). Due to its high conservation, this sequence is likely important for function of the transporter. Although a motif similar to this has not been previously described, pairs of Cys and His residues are common among zinc binding domains (153). Possibly the residues in this motif are important for mediating substrate specificity of the transporter. Alternatively, this motif may serve as part of a sensory domain for regulation of the transporter. Overall, investigation of the second cytoplasmic loop of ZevB may reveal novel mechanisms for transport by this system.

Serum resistance

The outer membrane protein P5 was found to be important for serum resistance of *H. influenzae*. Results suggest that P5 inhibits binding of natural IgM to the surface of *H. influenzae,* thus preventing activation of the classical complement pathway and subsequent killing of the cell. In the P5 mutant, the absence of this outer membrane protein may reveal epitopes for binding of IgM or alternatively generate a more favorable binding site for IgM to conform to the staple conformation, its most potent complement activating state (88). However, further analysis will be needed to fully address the role of P5 in inhibition of IgM binding to *H.influenzae*. For example, depletion of IgM from normal human serum has been reported for addressing the role of IgM in killing of bacterial mutants (1). Additionally, purified IgM from NHS has also been used in conjunction with a complement source such as baby rabbit serum to reconstitute IgM specific bactericidal activity against bacterial mutants (87, 207). Potentially these strategies could be employed to examine the role of IgM in P5 mediated serum sensitivity. Alternatively, P5 might participate in preventing binding of C4 and/or C3 to the surface of *H. influenzae*. This could be addressed by identification of the surface targets of C4 and C3 using immunoblotting and mass spectrometry techniques on P5 mutants versus WT as has been described previously (117). Identification of the C4 and C3 binding sites might indicate a novel role for P5 in blocking complement component binding to specific surface structures on *H. influenzae*. Overall, the results presented here suggest that P5 is a critical factor for *H. influenzae* to avoid detection by host antibody or complement and prevent complement mediated clearance.

When examining the possibility that P5 plays a role in strain specific serum resistance, an Rd strain expressing a P5 allele from the more serum resistant NTHi strain, NT127, was found to be more resistant to serum than Rd expressing its own P5 allele. Although both alleles were expressed via the Rd promoter to control for differences in expression between strains, we acknowledge that we did not address possible membrane protein concentration differences between these strains which could account for differences in serum resistance between them. Potentially, future analysis using Coomassie Brilliant Blue stained gels or Western blots using an anti-P5 antibody might be used to address this possibility. In either case, the majority of the amino acid sequence heterogeneity between NT127 P5 and Rd P5 is contained within their predicted outer surface loops. Therefore, differences in serum resistance conferred by each allele might be attributed to variability within these loops. Heterogeneity between outer loops of P5 is likely due to adaptations in response to immune pressure encountered during infection and is thought to be a critical feature for preventing host recognition (62). However, the loops of P5 are also important for mediating specific processes. For example, outer surface loops 3 and 4 were shown to be important for binding to CEACAM1 (26). Taken together, variation of outer surface loops of P5 may reflect both functional adaptations for adherence as well as defensive adaptations for avoiding host recognition. Perhaps evolution of outer surface loops requires a delicate balance between functional and defensive roles; however, future research will be necessary to investigate this possibility.

Concluding remarks

The work presented in this thesis identifies four secreted virulence factors of *H. influenzae* including those involved in disulfide formation, heme and zinc acquisition, as well as resistance to complement (Figure 5.1). Potentially, due to their extracytoplasmic location, these factors may be useful targets for development of vaccines or therapeutics against NTHi strains. Additionally, this work provides insight into nutrient requirements for *H. influenzae* to survive within the bloodstream and lung. This knowledge could prove valuable in development of strategies for preventing dissemination of *H. influenzae* from the nasopharynx to other sites within the body. Lastly, the results of this thesis indicate that future research into the secreted factors within other Gram-negative pathogens is likely to result in discovery of additional mechanisms allowing for bacteria to cause disease.

References

1. **Abdullah, M., I. Nepluev, G. Afonina, S. Ram, P. Rice, W. Cade, and C. Elkins.** 2005. Killing of *dsrA* mutants of *Haemophilus ducreyi* by normal human serum occurs via the classical complement pathway and is initiated by immunoglobulin M binding. Infect Immun **73:**3431-3439.
2. **Ahrén, I. L., H. Janson, A. Forsgren, and K. Riesbeck.** 2001. Protein D expression promotes the adherence and internalization of non-typeable *Haemophilus influenzae* into human monocytic cells* 1. Microbial pathogenesis **31:**151-158.
3. **Akerley, B. J., and D. J. Lampe.** 2002. Analysis of gene function in bacterial pathogens by GAMBIT. Methods Enzymol **358:**100-108.
4. **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.
5. **Ammendola, S., P. Pasquali, C. Pistoia, P. Petrucci, P. Petrarca, G. Rotilio, and A. Battistoni.** 2007. High-affinity Zn2+ uptake system ZnuABC is required for bacterial zinc homeostasis in intracellular environments and contributes to the virulence of *Salmonella enterica*. Infect Immun **75:**5867-5876.
6. **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.
7. **Anderson, M. E., and A. Meister.** 1980. Dynamic state of glutathione in blood plasma. Journal of Biological Chemistry **255:**9530.
8. **Anderson, P., R. B. Johnston, Jr., and D. H. Smith.** 1972. Human serum activities against *Hemophilus influenzae*, type b. The Journal of clinical investigation **51:**31-38.
9. **Andersson, M., L. Michel, J. B. Llull, and U. Pipkorn.** 1994. Complement activation on the nasal mucosal surface--a feature of the immediate allergic reaction in the nose. Allergy **49:**242-245.
10. **Armstrong, D. S., K. Grimwood, J. B. Carlin, R. Carzino, J. P. Gutierrez, J. Hull, A. Olinsky, E. M. Phelan, C. F. Robertson, and P. D. Phelan.** 1997. Lower airway inflammation in infants and young children with cystic fibrosis. American journal of respiratory and critical care medicine **156:**1197-1204.
11. **Arredondo, S. A., T. F. Chen, A. F. Riggs, H. F. Gilbert, and G. Georgiou.** 2009. Role of dimerization in the catalytic properties of the *Escherichia coli* disulfide isomerase DsbC. Journal of Biological Chemistry **284:**23972.
12. **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.

13. **Bader, M., W. Muse, D. P. Ballou, C. Gassner, and J. C. Bardwell.** 1999. Oxidative protein folding is driven by the electron transport system. Cell **98:**217-227.
14. **Bao, S., M. J. Liu, B. Lee, B. Besecker, J. P. Lai, D. C. Guttridge, and D. L. Knoell.** 2010. Zinc modulates the innate immune response in vivo to polymicrobial sepsis through regulation of NF-kappaB. American Journal of Physiology-Lung Cellular and Molecular Physiology **298:**L744.
15. **Barcak, G. J., M. S. Chandler, R. J. Redfield, and J. F. Tomb.** 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol **204:**321-342.
16. **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 U S A **90:**1038-1042.
17. **Bardwell, J. C., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation in vivo. Cell **67:**581-589.
18. **Beard, S. J., R. Hashim, G. Wu, M. R. Binet, M. N. Hughes, and R. K. Poole.** 2000. Evidence for the transport of zinc(II) ions via the pit inorganic phosphate transport system in *Escherichia coli*. FEMS Microbiol Lett **184:**231-235.
19. **Bendtsen, J. D., H. Nielsen, G. von Heijne, and S. Brunak.** 2004. Improved prediction of signal peptides: SignalP 3.0. J Mol Biol **340:**783-795.
20. **Berkmen, M., D. Boyd, and J. Beckwith.** 2005. The nonconsecutive disulfide bond of *Escherichia coli* phytase (AppA) renders it dependent on the protein-disulfide isomerase, DsbC. Journal of Biological Chemistry **280:**11387.
21. **Bernsel, A., H. Viklund, A. Hennerdal, and A. Elofsson.** 2009. TOPCONS: consensus prediction of membrane protein topology. Nucleic Acids Res **37:**W465-468.
22. **Berven, F. S., K. Flikka, H. B. Jensen, and I. Eidhammer.** 2004. BOMP: a program to predict integral beta-barrel outer membrane proteins encoded within genomes of Gram-negative bacteria. Nucleic Acids Res **32:**W394-399.
23. **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. Journal of Biological Chemistry **274:**7784.
24. **Bishai, W. R., N. S. Howard, J. A. Winkelstein, and H. O. Smith.** 1994. Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. Infection and immunity **62:**4855-4860.
25. **Bong, C. T., R. E. Throm, K. R. Fortney, B. P. Katz, A. F. Hood, C. Elkins, and S. M. Spinola.** 2001. DsrA-deficient mutant of *Haemophilus ducreyi* is impaired in its ability to infect human volunteers. Infect Immun **69:**1488-1491.
26. **Bookwalter, J. E., J. A. Jurcisek, S. D. Gray-Owen, S. Fernandez, G. McGillivary, and L. O. Bakaletz.** 2008. A carcinoembryonic antigen-related cell adhesion molecule 1 homologue plays a pivotal role in nontypeable *Haemophilus influenzae* colonization of the chinchilla nasopharynx via the outer membrane protein P5-homologous adhesin. Infect Immun **76:**48-55.
27. **Bouchet, V., D. W. Hood, J. Li, J. R. Brisson, G. A. Randle, A. Martin, Z. Li, R. Goldstein, E. K. Schweda, S. I. Pelton, J. C. Richards, and E. R. Moxon.**

2003. Host-derived sialic acid is incorporated into *Haemophilus influenzae* lipopolysaccharide and is a major virulence factor in experimental otitis media. Proceedings of the National Academy of Sciences of the United States of America **100:**8898-8903.

28. **Boulette, M. L., and S. M. Payne.** 2007. Anaerobic regulation of *Shigella flexneri* virulence: ArcA regulates Fur and iron acquisition genes. Journal of bacteriology **189:**6957-6967.
29. **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. Journal of bacteriology **189:**4860.
30. **Burall, L. S., J. M. Harro, X. Li, C. Lockatell, S. D. Himpsl, J. R. Hebel, D. E. Johnson, and H. L. T. Mobley.** 2004. *Proteus mirabilis* genes that contribute to pathogenesis of urinary tract infection: identification of 25 signature-tagged mutants attenuated at least 100-fold. Infection and immunity **72:**2922.
31. **Campos, J., M. Hernando, F. Roman, M. Perez-Vazquez, B. Aracil, J. Oteo, E. Lazaro, and F. de Abajo.** 2004. Analysis of invasive *Haemophilus influenzae* infections after extensive vaccination against H. influenzae type b. J Clin Microbiol **42:**524-529.
32. **Campoy, S., M. Jara, N. Busquets, A. M. Perez De Rozas, I. Badiola, and J. Barbe.** 2002. Role of the high-affinity zinc uptake *znuABC* system in *Salmonella enterica* serovar typhimurium virulence. Infect Immun **70:**4721-4725.
33. **CDC.** 2002. From the Centers for Disease Control and Prevention. Progress toward elimination of *Haemophilus influenzae* type b invasive disease among infants and children-- United States, 1998-2000. JAMA **287:**2206-2207.
34. **Cerquetti, M., M. L. Ciofi degli Atti, G. Renna, A. E. Tozzi, M. L. Garlaschi, and P. Mastrantonio.** 2000. Characterization of non-type B *Haemophilus influenzae* strains isolated from patients with invasive disease. The HI Study Group. J Clin Microbiol **38:**4649-4652.
35. **Chen, J., J. Song, S. Zhang, Y. Wang, D. Cui, and C. Wang.** 1999. Chaperone activity of DsbC. Journal of Biological Chemistry **274:**19601.
36. **Clohessy, P., and B. Golden.** 1995. Calprotectin Mediated Zinc Chelation as a Biostatic Mechanism in Host Defence. Scandinavian journal of immunology **42:**551-556.
37. **Coleman, H. N., D. A. Daines, J. Jarisch, and A. L. Smith.** 2003. Chemically defined media for growth of *Haemophilus influenzae* strains. Journal of clinical microbiology **41:**4408-4410.
38. **Cope, L. D., Z. Hrkal, and E. J. Hansen.** 2000. Detection of phase variation in expression of proteins involved in hemoglobin and hemoglobin-haptoglobin binding by nontypeable *Haemophilus influenzae*. Infection and immunity **68:**4092.
39. **Cope, L. D., R. P. Love, S. E. Guinn, A. Gilep, S. Usanov, R. W. Estabrook, Z. Hrkal, and E. J. Hansen.** 2001. Involvement of HxuC outer membrane

protein in utilization of hemoglobin by *Haemophilus influenzae*. Infection and immunity **69:**2353.

40. **Cope, L. D., S. E. Thomas, Z. Hrkal, and E. J. Hansen.** 1998. Binding of heme-hemopexin complexes by soluble HxuA protein allows utilization of this complexed heme by *Haemophilus influenzae*. Infection and immunity **66:**4511.
41. **Cope, L. D., S. E. Thomas, J. L. Latimer, C. A. Slaughter, U. Müller Eberhard, and E. J. Hansen.** 1994. The 100 kDa haem: haemopexin binding protein of *Haemophilus influenzae*: structure and localization. Molecular microbiology **13:**863-873.
42. **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. Journal of bacteriology **177:**2644.
43. **Corrall, C. J., J. A. Winkelstein, and E. R. Moxon.** 1982. Participation of complement in host defense against encapsulated *Haemophilus influenzae* types a, c, and d. Infect Immun **35:**759-763.
44. **Coyle, P., J. Philcox, and A. Rofe.** 1995. Hepatic zinc in metallothionein-null mice following zinc challenge: in vivo and in vitro studies. Biochemical Journal **309:**25.
45. **Crack, J., J. Green, and A. J. Thomson.** 2004. Mechanism of oxygen sensing by the bacterial transcription factor fumarate-nitrate reduction (FNR). The Journal of biological chemistry **279:**9278-9286.
46. **Crosson, F. J., Jr., J. A. Winkelstein, and E. R. Moxon.** 1976. Participation of complement in the nonimmune host defense against experimental *Haemophilus influenzae* type b septicemia and meningitis. Infect Immun **14:**882-887.
47. **Cuthill, S. L., M. M. Farley, and L. G. Donowitz.** 1999. Nontypable *Haemophilus influenzae* meningitis. Pediatr Infect Dis J **18:**660-662.
48. **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. Infection and immunity **65:**2700.
49. **Dailey, F. E., and H. C. Berg.** 1993. Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. Proceedings of the National Academy of Sciences of the United States of America **90:**1043.
50. **Daines, D. A., L. A. Cohn, H. N. Coleman, K. S. Kim, and A. L. Smith.** 2003. *Haemophilus influenzae* Rd KW20 has virulence properties. Journal of medical microbiology **52:**277.
51. **Dakin, C. J., A. H. Numa, H. Wang, J. R. Morton, C. C. Vertzyas, and R. L. Henry.** 2002. Inflammation, infection, and pulmonary function in infants and young children with cystic fibrosis. American journal of respiratory and critical care medicine **165:**904-910.
52. **Darby, N. J., and T. E. Creighton.** 1995. Catalytic mechanism of DsbA and its comparison with that of protein disulfide isomerase. Biochemistry **34:**3576-3587.

53. **Darwin, A. J., and V. L. Miller.** 1999. Identification of *Yersinia enterocolitica* genes affecting survival in an animal host using signature-tagged transposon mutagenesis. Mol Microbiol **32:**51-62.
54. **Davidson, A. L., E. Dassa, C. Orelle, and J. Chen.** 2008. Structure, function, and evolution of bacterial ATP-binding cassette systems. Microbiol Mol Biol Rev **72:**317-364, table of contents.
55. **Davis, L. M., T. Kakuda, and V. J. DiRita.** 2009. A *Campylobacter jejuni znuA* orthologue is essential for growth in low-zinc environments and chick colonization. J Bacteriol **191:**1631-1640.
56. **Dawid, S., S. J. Barenkamp, and J. W. St Geme.** 1999. Variation in expression of the *Haemophilus influenzae* HMW adhesins: a prokaryotic system reminiscent of eukaryotes. Proceedings of the National Academy of Sciences of the United States of America **96:**1077.
57. **De Souza-Hart, J. A., W. Blackstock, V. Di Modugno, I. B. Holland, and M. Kok.** 2003. Two-component systems in *Haemophilus influenzae*: a regulatory role for ArcA in serum resistance. Infection and immunity **71:**163-172.
58. **Depuydt, M., S. E. Leonard, D. Vertommen, K. Denoncin, P. Morsomme, K. Wahni, J. Messens, K. S. Carroll, and J. F. Collet.** 2009. A periplasmic reducing system protects single cysteine residues from oxidation. Science **326:**1109.
59. **Desrosiers, D. C., S. W. Bearden, I. Mier, Jr., J. Abney, J. T. Paulley, J. D. Fetherston, J. C. Salazar, J. D. Radolf, and R. D. Perry.** 2010. Znu is the predominant zinc importer in *Yersinia pestis* during in vitro growth but is not essential for virulence. Infect Immun **78:**5163-5177.
60. **Desrosiers, D. C., Y. C. Sun, A. A. Zaidi, C. H. Eggers, D. L. Cox, and J. D. Radolf.** 2007. The general transition metal (Tro) and Zn2+ (Znu) transporters in *Treponema pallidum*: analysis of metal specificities and expression profiles. Mol Microbiol **65:**137-152.
61. **Dibden, D. P., and J. Green.** 2005. In vivo cycling of the *Escherichia coli* transcription factor FNR between active and inactive states. Microbiology **151:**4063-4070.
62. **Duim, B., L. D. Bowler, P. P. Eijk, H. M. Jansen, J. Dankert, and L. van Alphen.** 1997. Molecular variation in the major outer membrane protein P5 gene of nonencapsulated *Haemophilus influenzae* during chronic infections. Infect Immun **65:**1351-1356.
63. **Duim, B., L. Vogel, W. Puijk, H. M. Jansen, R. H. Meloen, J. Dankert, and L. van Alphen.** 1996. Fine mapping of outer membrane protein P2 antigenic sites which vary during persistent infection by *Haemophilus influenzae*. Infection and immunity **64:**4673-4679.
64. **Dutton, R. J., D. Boyd, M. Berkmen, and J. Beckwith.** 2008. Bacterial species exhibit diversity in their mechanisms and capacity for protein disulfide bond formation. Proceedings of the National Academy of Sciences **105:**11933.
65. **Eiglmeier, K., N. Honore, S. Iuchi, E. C. Lin, and S. T. Cole.** 1989. Molecular genetic analysis of FNR-dependent promoters. Mol Microbiol **3:**869-878.

66. **Eitinger, T., J. Suhr, L. Moore, and J. A. Smith.** 2005. Secondary transporters for nickel and cobalt ions: theme and variations. Biometals **18:**399-405.
67. **Elkins, C., K. J. Morrow, Jr., and B. Olsen.** 2000. Serum resistance in Haemophilus ducreyi requires outer membrane protein DsrA. Infect Immun **68:**1608-1619.
68. **Eren, E., D. C. Kennedy, M. J. Maroney, and J. M. Arguello.** 2006. A novel regulatory metal binding domain is present in the C terminus of Arabidopsis Zn2+-ATPase HMA2. J Biol Chem **281:**33881-33891.
69. **Erwin, A. L., P. J. Bonthuis, J. L. Geelhood, K. L. Nelson, K. W. McCrea, J. R. Gilsdorf, and A. L. Smith.** 2006. Heterogeneity in tandem octanucleotides within *Haemophilus influenzae* lipopolysaccharide biosynthetic gene *losA* affects serum resistance. Infection and immunity **74:**3408-3414.
70. **Erwin, A. L., Y. A. Brewah, D. A. Couchenour, P. R. Barren, S. J. Burke, G. H. Choi, R. Lathigra, M. S. Hanson, and J. N. Weiser.** 2000. Role of lipopolysaccharide phase variation in susceptibility of *Haemophilus influenzae* to bactericidal immunoglobulin M antibodies in rabbit sera. Infect Immun **68:**2804-2807.
71. **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*. Infection and immunity **73:**5853-5863.
72. **Evans, N. M., D. D. Smith, and A. J. Wicken.** 1974. Haemin and nicotinamide adenine dinucleotide requirements of *Haemophilus influenzae* and *Haemophilus parainfluenzae*. Journal of medical microbiology **7:**359-365.
73. **Fan, X., H. Goldfine, E. Lysenko, and J. N. Weiser.** 2001. The transfer of choline from the host to the bacterial cell surface requires *glpQ* in *Haemophilus influenzae*. Molecular microbiology **41:**1029-1036.
74. **Farley, M. M., D. S. Stephens, S. L. Kaplan, and E. O. Mason.** 1990. Pilus-and Non-Pilus-Mediated Interactions of *Haemophilus influenzae* Type b with Human Erythrocytes and Human Nasopharyngeal Mucosa. Journal of Infectious Diseases **161:**274.
75. **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.
76. **Fink, D. L., and B. A. Green.** 2002. The *Haemophilus influenzae* Hap autotransporter binds to fibronectin, laminin, and collagen IV. Infection and immunity **70:**4902.
77. **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, and J. M. Merrick.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496.

78. **Fox, K. L., J. Li, E. K. Schweda, V. Vitiazeva, K. Makepeace, M. P. Jennings, E. R. Moxon, and D. W. Hood.** 2008. Duplicate copies of lic1 direct the addition of multiple phosphocholine residues in the lipopolysaccharide of *Haemophilus influenzae*. Infect Immun **76:**588-600.
79. **Frech, C., M. Wunderlich, R. Glockshuber, and F. X. Schmid.** 1996. Preferential binding of an unfolded protein to DsbA. EMBO J **15:**392-398.
80. **Fujita, T., I. Gigli, and V. Nussenzweig.** 1978. Human C4-binding protein. II. Role in proteolysis of C4b by C3b-inactivator. J Exp Med **148:**1044-1051.
81. **Fujita, T., and V. Nussenzweig.** 1979. The role of C4-binding protein and beta 1H in proteolysis of C4b and C3b. J Exp Med **150:**267-276.
82. **Gabay, C., and I. Kushner.** 1999. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med **340:**448-454.
83. **Garrido, M. E., M. Bosch, R. Medina, M. Llagostera, A. M. Perez de Rozas, I. Badiola, and J. Barbe.** 2003. The high-affinity zinc-uptake system *znuACB* is under control of the iron-uptake regulator (*fur*) gene in the animal pathogen *Pasteurella multocida*. FEMS Microbiol Lett **221:**31-37.
84. **Gawronski, J. D., S. M. Wong, G. Giannoukos, D. V. Ward, and B. J. Akerley.** 2009. Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for *Haemophilus* genes required in the lung. Proc Natl Acad Sci U S A **106:**16422-16427.
85. **Gigli, I., T. Fujita, and V. Nussenzweig.** 1979. Modulation of the classical pathway C3 convertase by plasma proteins C4 binding protein and C3b inactivator. Proc Natl Acad Sci U S A **76:**6596-6600.
86. **Gilligan, P. H.** 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin Microbiol Rev **4:**35-51.
87. **Gnehm, H. E., S. I. Pelton, S. Gulati, and P. A. Rice.** 1985. Characterization of antigens from nontypable *Haemophilus influenzae* recognized by human bactericidal antibodies. Role of *Haemophilus* outer membrane proteins. J Clin Invest **75:**1645-1658.
88. **Goldsby, R. A.** 2003. Immunology, 5th ed. W.H. Freeman, New York.
89. **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 microbiology letters **198:**125-128.
90. **Graham, A. I., S. Hunt, S. L. Stokes, N. Bramall, J. Bunch, A. G. Cox, C. W. McLeod, and R. K. Poole.** 2009. Severe zinc depletion of *Escherichia coli*: roles for high affinity zinc binding by ZinT, zinc transport and zinc-independent proteins. J Biol Chem **284:**18377-18389.
91. **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.
92. **Grauschopf, U., A. Fritz, and R. Glockshuber.** 2003. Mechanism of the electron transfer catalyst DsbB from *Escherichia coli*. The EMBO journal **22:**3503-3513.

93. **Grauschopf, U., J. R. Winther, P. Korber, T. Zander, P. Dallinger, and J. C. Bardwell.** 1995. Why is DsbA such an oxidizing disulfide catalyst? Cell **83:**947-955.
94. **Greiff, L., M. Andersson, J. S. Erjefalt, C. G. Persson, and P. Wollmer.** 2003. Airway microvascular extravasation and luminal entry of plasma. Clin Physiol Funct Imaging **23:**301-306.
95. **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. Infection and immunity **73:**7022.
96. **Griffin, R., A. D. Cox, K. Makepeace, J. C. Richards, E. R. Moxon, and D. W. Hood.** 2005. Elucidation of the monoclonal antibody 5G8-reactive, virulence-associated lipopolysaccharide epitope of *Haemophilus influenzae* and its role in bacterial resistance to complement-mediated killing. Infection and immunity **73:**2213-2221.
97. **Groeneveld, K., L. van Alphen, C. Voorter, P. P. Eijk, H. M. Jansen, and H. C. Zanen.** 1989. Antigenic drift of *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. Infection and immunity **57:**3038-3044.
98. **Guest, J.** 1992. Oxygen-regulated gene expression in *Escherichia coli*. The 1992 Marjory Stephenson Prize Lecture. Microbiology **138:**2253-2263.
99. **Guilhot, C., G. Jander, N. L. Martin, and J. Beckwith.** 1995. Evidence that the pathway of disulfide bond formation in *Escherichia coli* involves interactions between the cysteines of DsbB and DsbA. Proceedings of the National Academy of Sciences of the United States of America **92:**9895-9899.
100. **Guo, J., and D. P. Giedroc.** 1997. Zinc site redesign in T4 gene 32 protein: structure and stability of cobalt(II) complexes formed by wild-type and metal ligand substitution mutants. Biochemistry **36:**730-742.
101. **Ha, U. H., Y. Wang, and S. Jin.** 2003. DsbA of *Pseudomonas aeruginosa* is essential for multiple virulence factors. Infect Immun **71:**1590-1595.
102. **Hallstrom, T., A. M. Blom, P. F. Zipfel, and K. Riesbeck.** 2009. Nontypeable *Haemophilus influenzae* protein E binds vitronectin and is important for serum resistance. J Immunol **183:**2593-2601.
103. **Hallstrom, T., H. Jarva, K. Riesbeck, and A. M. Blom.** 2007. Interaction with C4b-binding protein contributes to nontypeable *Haemophilus influenzae* serum resistance. J Immunol **178:**6359-6366.
104. **Hallstrom, T., F. Resman, M. Ristovski, and K. Riesbeck.** 2010. Binding of complement regulators to invasive nontypeable *Haemophilus influenzae* isolates is not increased compared to nasopharyngeal isolates, but serum resistance is linked to disease severity. J Clin Microbiol **48:**921-927.
105. **Hallstrom, T., and K. Riesbeck.** 2010. *Haemophilus influenzae* and the complement system. Trends Microbiol **18:**258-265.
106. **Hallstrom, T., E. Trajkovska, A. Forsgren, and K. Riesbeck.** 2006. *Haemophilus influenzae* surface fibrils contribute to serum resistance by interacting with vitronectin. J Immunol **177:**430-436.

107. **Hallstrom, T., P. F. Zipfel, A. M. Blom, N. Lauer, A. Forsgren, and K. Riesbeck.** 2008. *Haemophilus influenzae* interacts with the human complement inhibitor factor H. J Immunol **181:**537-545.
108. **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.
109. **Hantke, K.** 2005. Bacterial zinc uptake and regulators. Curr Opin Microbiol **8:**196-202.
110. **Harrington, J. C., S. M. Wong, C. V. Rosadini, O. Garifulin, V. Boyartchuk, and B. J. Akerley.** 2009. Resistance of *Haemophilus influenzae* to reactive nitrogen donors and gamma interferon-stimulated macrophages requires the formate-dependent nitrite reductase regulator-activated *ytfE* gene. Infect Immun **77:**1945-1958.
111. **Henke, M. O., A. Renner, B. K. Rubin, J. I. Gyves, E. Lorenz, and J. S. Koo.** 2006. Up-regulation of S100A8 and S100A9 protein in bronchial epithelial cells by lipopolysaccharide. Exp Lung Res **32:**331-347.
112. **Heras, B., M. A. Edeling, H. J. Schirra, S. Raina, and J. L. Martin.** 2004. Crystal structures of the DsbG disulfide isomerase reveal an unstable disulfide. Proceedings of the National Academy of Sciences of the United States of America **101:**8876.
113. **Heras, B., S. R. Shouldice, M. Totsika, M. J. Scanlon, M. A. Schembri, and J. L. Martin.** 2009. DSB proteins and bacterial pathogenicity. Nat Rev Microbiol **7:**215-225.
114. **Herbert, M., S. Hayes, M. Deadman, C. Tang, D. Hood, and E. Moxon.** 2002. Signature tagged mutagenesis of *Haemophilus influenzae* identifies genes required for in vivo survival. Microbial pathogenesis **33:**211-223.
115. **Hill, D. J., M. A. Toleman, D. J. Evans, S. Villullas, L. Van Alphen, and M. Virji.** 2001. The variable P5 proteins of typeable and non-typeable *Haemophilus influenzae* target human CEACAM1. Mol Microbiol **39:**850-862.
116. **Hiniker, A., and J. C. Bardwell.** 2004. In vivo substrate specificity of periplasmic disulfide oxidoreductases. J Biol Chem **279:**12967-12973.
117. **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.
118. **Hobbs, J. A. R., R. May, K. Tanousis, E. McNeill, M. Mathies, C. Gebhardt, R. Henderson, M. J. Robinson, and N. Hogg.** 2003. Myeloid cell function in MRP-14 (S100A9) null mice. Molecular and cellular biology **23:**2564.
119. **Hong, W., K. Mason, J. Jurcisek, L. Novotny, L. O. Bakaletz, and W. E. Swords.** 2007. Phosphorylcholine decreases early inflammation and promotes the establishment of stable biofilm communities of nontypeable *Haemophilus influenzae* strain 86-028NP in a chinchilla model of otitis media. Infection and immunity **75:**958-965.
120. **Hood, D. W., A. D. Cox, M. Gilbert, K. Makepeace, S. Walsh, M. E. Deadman, A. Cody, A. Martin, M. Mansson, E. K. Schweda, J. R. Brisson, J.**

C. Richards, E. R. Moxon, and W. W. Wakarchuk. 2001. Identification of a lipopolysaccharide alpha-2,3-sialyltransferase from *Haemophilus influenzae*. Molecular microbiology **39:**341-350.

121. **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.
122. **Hood, D. W., M. E. Deadman, M. P. Jennings, M. Bisercic, R. D. Fleischmann, J. C. Venter, and E. R. Moxon.** 1996. DNA repeats identify novel virulence genes in *Haemophilus influenzae*. Proceedings of the National Academy of Sciences of the United States of America **93:**11121.
123. **Hood, D. W., K. Makepeace, M. E. Deadman, R. F. Rest, P. Thibault, A. Martin, J. C. Richards, and E. R. Moxon.** 1999. Sialic acid in the lipopolysaccharide of *Haemophilus influenzae*: strain distribution, influence on serum resistance and structural characterization. Mol Microbiol **33:**679-692.
124. **Huber-Wunderlich, M., and R. Glockshuber.** 1998. A single dipeptide sequence modulates the redox properties of a whole enzyme family. Fold Des **3:**161-171.
125. **Humphries, H. E., and N. J. High.** 2002. The role of *licA* phase variation in the pathogenesis of invasive disease by *Haemophilus influenzae* type b. FEMS Immunology & Medical Microbiology **34:**221-230.
126. **Inaba, K., and K. Ito.** 2008. Structure and mechanisms of the DsbB-DsbA disulfide bond generation machine. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research **1783:**520-529.
127. **Inaba, K., S. Murakami, M. Suzuki, A. Nakagawa, E. Yamashita, K. Okada, and K. Ito.** 2006. Crystal structure of the DsbB-DsbA complex reveals a mechanism of disulfide bond generation. Cell **127:**789-801.
128. **Inaba, K., Y. H. Takahashi, and K. Ito.** 2005. Reactivities of quinone-free DsbB from *Escherichia coli*. The Journal of biological chemistry **280:**33035-33044.
129. **Ito, T., H. Shibata, M. Nakazawa, M. Myokai, K. Ikegaya, K. Tsuchiya, and T. Kamimaki.** 2011. Meningitis and septicemia caused by nontypeable *Haemophilus influenzae* in a previously healthy 2-year-old girl. J Infect Chemother.
130. **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.
131. **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 U S A **91:**11552-11556.
132. **Jiang, Z., N. Nagata, E. Molina, L. O. Bakaletz, H. Hawkins, and J. A. Patel.** 1999. Fimbria-mediated enhanced attachment of nontypeable *Haemophilus*

influenzae to respiratory syncytial virus-infected respiratory epithelial cells. Infect Immun **67:**187-192.

133. **Jin, H., Z. Ren, P. W. Whitby, D. J. Morton, and T. L. Stull.** 1999. Characterization of *hgpA*, a gene encoding a haemoglobin/haemoglobin-haptoglobin-binding protein of *Haemophilus influenzae*. Microbiology **145:**905.
134. **Johne, B., M. K. Fagerhol, T. Lyberg, H. Prydz, P. Brandtzaeg, C. F. Naess-Andresen, and I. Dale.** 1997. Functional and clinical aspects of the myelomonocyte protein calprotectin. Mol Pathol **50:**113-123.
135. **Juncker, A. S., H. Willenbrock, G. Von Heijne, S. Brunak, H. Nielsen, and A. Krogh.** 2003. Prediction of lipoprotein signal peptides in Gram-negative bacteria. Protein Sci **12:**1652-1662.
136. **Jurcisek, J., L. Greiner, H. Watanabe, A. Zaleski, M. A. Apicella, and L. O. Bakaletz.** 2005. Role of sialic acid and complex carbohydrate biosynthesis in biofilm formation by nontypeable *Haemophilus influenzae* in the chinchilla middle ear. Infection and immunity **73:**3210-3218.
137. **Jurcisek, J. A., J. E. Bookwalter, B. D. Baker, S. Fernandez, L. A. Novotny, R. S. Munson Jr, and L. O. Bakaletz.** 2007. The PilA protein of non typeable *Haemophilus influenzae* plays a role in biofilm formation, adherence to epithelial cells and colonization of the mammalian upper respiratory tract. Molecular microbiology **65:**1288-1299.
138. **Kadokura, H., and J. Beckwith.** 2002. Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA. The EMBO journal **21:**2354-2363.
139. **Kadokura, H., and J. Beckwith.** 2010. Mechanisms of oxidative protein folding in the bacterial cell envelope. Antioxid Redox Signal **13:**1231-1246.
140. **Kadokura, H., F. Katzen, and J. Beckwith.** 2003. Protein disulfide bond formation in prokaryotes. Annu Rev Biochem **72:**111-135.
141. **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.
142. **Kall, L., A. Krogh, and E. L. Sonnhammer.** 2007. Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. Nucleic Acids Res **35:**W429-432.
143. **Kamitani, S., Y. Akiyama, and K. Ito.** 1992. Identification and characterization of an *Escherichia coli* gene required for the formation of correctly folded alkaline phosphatase, a periplasmic enzyme. The EMBO journal **11:**57-62.
144. **Kammler, M., C. Schon, and K. Hantke.** 1993. Characterization of the ferrous iron uptake system of *Escherichia coli*. Journal of bacteriology **175:**6212-6219.
145. **Kehl-Fie, T. E., and E. P. Skaar.** 2010. Nutritional immunity beyond iron: a role for manganese and zinc. Curr Opin Chem Biol **14:**218-224.
146. **Ketterer, M. R., J. Q. Shao, D. B. Hornick, B. Buscher, V. K. Bandi, and M. A. Apicella.** 1999. Infection of primary human bronchial epithelial cells by *Haemophilus influenzae*: macropinocytosis as a mechanism of airway epithelial cell entry. Infection and immunity **67:**4161.

147. **Kilian, M., J. Mestecky, and R. E. Schrohenloher.** 1979. Pathogenic species of the genus *Haemophilus* and *Streptococcus pneumoniae* produce immunoglobulin A1 protease. Infection and immunity **26:**143.
148. **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.
149. **King, P. T., J. Ngui, D. Gunawardena, P. W. Holmes, M. W. Farmer, and S. R. Holdsworth.** 2008. Systemic humoral immunity to non-typeable *Haemophilus influenzae*. Clin Exp Immunol **153:**376-384.
150. **Kishigami, S., and K. Ito.** 1996. Roles of cysteine residues of DsbB in its activity to reoxidize DsbA, the protein disulphide bond catalyst of *Escherichia coli*. Genes to Cells **1:**201-208.
151. **Kishigami, S., E. Kanaya, M. Kikuchi, and K. Ito.** 1995. DsbA-DsbB interaction through their active site cysteines. Evidence from an odd cysteine mutant of DsbA. The Journal of biological chemistry **270:**17072-17074.
152. **Klein, J. O.** 1997. Role of nontypeable *Haemophilus influenzae* in pediatric respiratory tract infections. Pediatr Infect Dis J **16:**S5-8.
153. **Klug, A., and J. Schwabe.** 1995. Protein motifs 5. Zinc fingers. The FASEB journal **9:**597.
154. **Kobayashi, T., S. Kishigami, M. Sone, H. Inokuchi, T. Mogi, and K. Ito.** 1997. Respiratory chain is required to maintain oxidized states of the DsbA-DsbB disulfide bond formation system in aerobically growing *Escherichia coli* cells. Proceedings of the National Academy of Sciences of the United States of America **94:**11857-11862.
155. **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.
156. **Lazazzera, B. A., H. Beinert, N. Khoroshilova, M. C. Kennedy, and P. J. Kiley.** 1996. DNA binding and dimerization of the Fe-S-containing FNR protein from *Escherichia coli* are regulated by oxygen. J Biol Chem **271:**2762-2768.
157. **Lee, B. C.** 1995. Quelling the red menace: haem capture by bacteria. Molecular microbiology **18:**383-390.
158. **Leichert, L. I., and U. Jakob.** 2004. Protein thiol modifications visualized in vivo. PLoS Biol **2:**e333.
159. **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.
160. **Levinson, G., and G. A. Gutman.** 1987. Slipped-strand mispairing: a major mechanism for DNA sequence evolution. Molecular biology and evolution **4:**203.
161. **Lewis, D. A., J. Klesney-Tait, S. R. Lumbley, C. K. Ward, J. L. Latimer, C. A. Ison, and E. J. Hansen.** 1999. Identification of the *znuA*-encoded periplasmic zinc transport protein of *Haemophilus ducreyi*. Infect Immun **67:**5060-5068.

162. **Li, H., and G. Jogl.** 2007. Crystal structure of the zinc-binding transport protein ZnuA from *Escherichia coli* reveals an unexpected variation in metal coordination. Journal of molecular biology **368:**1358-1366.
163. **Liu, J., A. J. Stemmler, J. Fatima, and B. Mitra.** 2005. Metal-binding characteristics of the amino-terminal domain of ZntA: binding of lead is different compared to cadmium and zinc. Biochemistry **44:**5159-5167.
164. **Liuzzi, J. P., L. A. Lichten, S. Rivera, R. K. Blanchard, T. B. Aydemir, M. D. Knutson, T. Ganz, and R. J. Cousins.** 2005. Interleukin-6 regulates the zinc transporter Zip14 in liver and contributes to the hypozincemia of the acute-phase response. Proc Natl Acad Sci U S A **102:**6843-6848.
165. **Loeb, M., E. Connor, and D. Penney.** 1988. A comparison of the adherence of fimbriated and nonfimbriated *Haemophilus influenzae* type b to human adenoids in organ culture. Infection and immunity **56:**484.
166. **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.
167. **Magnet, S., S. Bellais, L. Dubost, M. Fourgeaud, J. L. Mainardi, S. Petit-Frère, A. Marie, D. Mengin-Lecreulx, M. Arthur, and L. Gutmann.** 2007. Identification of the L, D-transpeptidases responsible for attachment of the Braun lipoprotein to *Escherichia coli* peptidoglycan. Journal of bacteriology **189:**3927.
168. **Mannino, D. M., and V. A. Kiriz.** 2006. Changing the burden of COPD mortality. Int J Chron Obstruct Pulmon Dis **1:**219-233.
169. **Marc, M. M., P. Korosec, M. Kosnik, I. Kern, M. Flezar, S. Suskovic, and J. Sorli.** 2004. Complement factors c3a, c4a, and c5a in chronic obstructive pulmonary disease and asthma. Am J Respir Cell Mol Biol **31:**216-219.
170. **Marchler-Bauer, A., S. Lu, J. B. Anderson, F. Chitsaz, M. K. Derbyshire, C. DeWeese-Scott, J. H. Fong, L. Y. Geer, R. C. Geer, N. R. Gonzales, M. Gwadz, D. I. Hurwitz, J. D. Jackson, Z. Ke, C. J. Lanczycki, F. Lu, G. H. Marchler, M. Mullokandov, M. V. Omelchenko, C. L. Robertson, J. S. Song, N. Thanki, R. A. Yamashita, D. Zhang, N. Zhang, C. Zheng, and S. H. Bryant.** 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res **39:**D225-229.
171. **Mason, K. M., M. E. Bruggeman, R. S. Munson, and L. O. Bakaletz.** 2006. The non typeable *Haemophilus influenzae* Sap transporter provides a mechanism of antimicrobial peptide resistance and SapD dependent potassium acquisition. Molecular microbiology **62:**1357-1372.
172. **Mason, K. M., R. S. Munson Jr, and L. O. Bakaletz.** 2005. A mutation in the sap operon attenuates survival of nontypeable *Haemophilus influenzae* in a chinchilla model of otitis media. Infection and immunity **73:**599.
173. **Mason, K. M., R. S. Munson Jr, and L. O. Bakaletz.** 2003. Nontypeable *Haemophilus influenzae* gene expression induced in vivo in a chinchilla model of otitis media. Infection and immunity **71:**3454.
174. **Mason, K. M., F. K. Raffel, W. C. Ray, and L. O. Bakaletz.** 2011. Heme Utilization by Nontypeable *Haemophilus influenzae* is Essential and Dependent on Sap Transporter Function. Journal of bacteriology**:**JB. 01313-01310v01311.

175. **Masoud, H., E. R. Moxon, A. Martin, D. Krajcarski, and J. C. Richards.** 1997. Structure of the variable and conserved lipopolysaccharide oligosaccharide epitopes expressed by *Haemophilus influenzae* serotype b strain Eagan. Biochemistry **36:**2091-2103.

176. **McCarthy, A. A., P. W. Haebel, A. Törrönen, V. Rybin, E. N. Baker, and P. Metcalf.** 2000. Crystal structure of the protein disulfide bond isomerase, DsbC, from *Escherichia coli*. Nat Struct Mol Biol **7:**196-199.

177. **Melville, S. B., and R. P. Gunsalus.** 1996. Isolation of an oxygen-sensitive FNR protein of *Escherichia coli*: interaction at activator and repressor sites of FNR-controlled genes. Proc Natl Acad Sci U S A **93:**1226-1231.

178. **Messens, J., J. F. Collet, K. Van Belle, E. Brosens, R. Loris, and L. Wyns.** 2007. The oxidase DsbA folds a protein with a nonconsecutive disulfide. Journal of Biological Chemistry **282:**31302.

179. **Metheringham, R., L. Griffiths, H. Crooke, S. Forsythe, and J. Cole.** 1995. An essential role for DsbA in cytochromec synthesis and formate-dependent nitrite reduction by*Escherichia coli* K-12. Archives of microbiology **164:**301-307.

180. **Michalska, A. E., and K. Choo.** 1993. Targeting and germ-line transmission of a null mutation at the metallothionein I and II loci in mouse. Proceedings of the National Academy of Sciences of the United States of America **90:**8088.

181. **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.

182. **Miki, T., N. Okada, Y. Kim, A. Abe, and H. Danbara.** 2008. DsbA directs efficient expression of outer membrane secretin EscC of the enteropathogenic *Escherichia coli* type III secretion apparatus. Microbial pathogenesis **44:**151-158.

183. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

184. **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. Proceedings of the National Academy of Sciences of the United States of America **90:**7084.

185. **Missiakas, D., F. Schwager, and S. Raina.** 1995. Identification and characterization of a new disulfide isomerase-like protein (DsbD) in *Escherichia coli*. The EMBO journal **14:**3415.

186. **Miyamoto, N., and L. O. Bakaletz.** 1996. Selective adherence of non-typeable *Haemophilus influenzae* (NTHi) to mucus or epithelial cells in the chinchilla eustachian tube and middle ear. Microb Pathog **21:**343-356.

187. **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.

188. **Morton, D. J., L. O. Bakaletz, J. A. Jurcisek, T. M. VanWagoner, T. W. Seale, P. W. Whitby, and T. L. Stull.** 2004. Reduced severity of middle ear

infection caused by nontypeable *Haemophilus influenzae* lacking the hemoglobin/hemoglobin-haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. Microbial pathogenesis **36:**25-33.

189. **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.
190. **Morton, D. J., T. W. Seale, L. O. Bakaletz, J. A. Jurcisek, A. Smith, T. M. VanWagoner, P. W. Whitby, and T. L. Stull.** 2009. The heme-binding protein (HbpA) of *Haemophilus influenzae* as a virulence determinant. International journal of medical microbiology : IJMM **299:**479-488.
191. **Morton, D. J., T. W. Seale, L. L. Madore, T. M. VanWagoner, P. W. Whitby, and T. L. Stull.** 2007. The haem-haemopexin utilization gene cluster (*hxuCBA*) as a virulence factor of *Haemophilus influenzae*. Microbiology **153:**215-224.
192. **Morton, D. J., T. W. Seale, T. M. VanWagoner, P. W. Whitby, and T. L. Stull.** 2009. The *dppBCDF* gene cluster of *Haemophilus influenzae*: Role in heme utilization. BMC research notes **2:**166.
193. **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.
194. **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 and infection **9:**932-939.
195. **Moxon, E. R.** 2009. Bacterial variation, virulence and vaccines. Microbiology **155:**997.
196. **Moxon, E. R.** 1992. Molecular basis of invasive *Haemophilus influenzae* type b disease. Journal of Infectious Diseases **165:**S77.
197. **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.
198. **Munson Jr, R., and K. Sasaki.** 1993. Protein D, a putative immunoglobulin D-binding protein produced by *Haemophilus influenzae*, is glycerophosphodiester phosphodiesterase. Journal of bacteriology **175:**4569.
199. **Munson, R. S., Jr., S. Grass, and R. West.** 1993. Molecular cloning and sequence of the gene for outer membrane protein P5 of *Haemophilus influenzae*. Infect Immun **61:**4017-4020.
200. **Murphy, G. L., T. D. Connell, D. S. Barritt, M. Koomey, and J. G. Cannon.** 1989. Phase variation of gonococcal protein II: regulation of gene expression by slipped-strand mispairing of a repetitive DNA sequence. Cell **56:**539-547.
201. **Murphy, T. F.** 2003. Respiratory infections caused by non-typeable *Haemophilus influenzae*. Curr Opin Infect Dis **16:**129-134.

202. **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.
203. **Murphy, T. F., H. Faden, L. O. Bakaletz, J. M. Kyd, A. Forsgren, J. Campos, M. Virji, and S. I. Pelton.** 2009. Nontypeable *Haemophilus influenzae* as a pathogen in children. The Pediatric infectious disease journal **28:**43-48.
204. **Murphy, T. F., C. Kirkham, and A. J. Lesse.** 2006. Construction of a mutant and characterization of the role of the vaccine antigen P6 in outer membrane integrity of nontypeable *Haemophilus influenzae*. Infect Immun **74:**5169-5176.
205. **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.
206. **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.
207. **Nakamura, S., M. Shchepetov, A. B. Dalia, S. E. Clark, T. F. Murphy, S. Sethi, J. R. Gilsdorf, A. L. Smith, and J. N. Weiser.** 2011. Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*. PLoS Pathog **7:**e1001247.
208. **Narkio-Makela, M., J. Hellwage, O. Tahkokallio, and S. Meri.** 2001. Complement-regulator factor H and related proteins in otitis media with effusion. Clin Immunol **100:**118-126.
209. **Narkio-Makela, M., A. M. Teppo, and S. Meri.** 2000. Complement C3 cleavage and cytokines interleukin-1beta and tumor necrosis factor-alpha in otitis media with effusion. Laryngoscope **110:**1745-1749.
210. **Nickitenko, A. V., S. Trakhanov, and F. A. Quiocho.** 1995. 2. ANG. Resolution Structure of DppA, a Periplasmic Dipeptide Transport/Chemosensory Receptor. Biochemistry **34:**16585-16595.
211. **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.
212. **Noel, G. J., A. Brittingham, A. A. Granato, and D. M. Mosser.** 1996. Effect of amplification of the Cap b locus on complement-mediated bacteriolysis and opsonization of type b *Haemophilus influenzae*. Infect Immun **64:**4769-4775.
213. **Noel, G. J., S. K. Hoiseth, and P. J. Edelson.** 1992. Type b capsule inhibits ingestion of *Haemophilus influenzae* by murine macrophages: studies with isogenic encapsulated and unencapsulated strains. The Journal of infectious diseases **166:**178-182.
214. **Noel, G. J., S. Katz, and P. J. Edelson.** 1988. Complement-mediated early clearance of *Haemophilus influenzae* type b from blood is independent of serum lytic activity. J Infect Dis **157:**85-90.
215. **O'Neill, J. M., J. W. St Geme, 3rd, 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.

216. **Okamoto, K., T. Baba, H. Yamanaka, N. Akashi, and Y. Fujii.** 1995. Disulfide bond formation and secretion of *Escherichia coli* heat-stable enterotoxin II. Journal of bacteriology **177:**4579.
217. **Ostberg, K. L., M. W. Russell, and T. F. Murphy.** 2009. Mucosal immunization of mice with recombinant OMP P2 induces antibodies that bind to surface epitopes of multiple strains of nontypeable *Haemophilus influenzae*. Mucosal Immunol **2:**63-73.
218. **Panek, H., and M. R. O'Brian.** 2002. A whole genome view of prokaryotic haem biosynthesis. Microbiology **148:**2273-2282.
219. **Pang, B., D. Winn, R. Johnson, W. Hong, S. West-Barnette, N. Kock, and W. E. Swords.** 2008. Lipooligosaccharides containing phosphorylcholine delay pulmonary clearance of nontypeable *Haemophilus influenzae*. Infect Immun **76:**2037-2043.
220. **Patzer, S. I., and K. Hantke.** 2000. The zinc-responsive regulator Zur and its control of the *znu* gene cluster encoding the ZnuABC zinc uptake system in *Escherichia coli*. J Biol Chem **275:**24321-24332.
221. **Patzer, S. I., and K. Hantke.** 1998. The ZnuABC high-affinity zinc uptake system and its regulator Zur in *Escherichia coli*. Mol Microbiol **28:**1199-1210.
222. **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 U S A **89:**6210-6214.
223. **Petrarca, P., S. Ammendola, P. Pasquali, and A. Battistoni.** 2010. The Zur-regulated ZinT protein is an auxiliary component of the high-affinity ZnuABC zinc transporter that facilitates metal recruitment during severe zinc shortage. J Bacteriol **192:**1553-1564.
224. **Phillips, N. J., M. A. Apicella, J. M. Griffiss, and B. W. Gibson.** 1993. Structural studies of the lipooligosaccharides from *Haemophilus influenzae* type b strain A2. Biochemistry **32:**2003-2012.
225. **Pittman, M.** 1931. Variation and Type Specificity in the Bacterial Species *Hemophilus Influenzae*. The Journal of experimental medicine **53:**471-492.
226. **Poolman, J., L. Bakaletz, A. Cripps, P. Denoel, A. Forsgren, and J. Kyd.** 2000. Developing a nontypeable *Haemophilus influenzae* (NTHi) vaccine. Vaccine **19:**S108-S115.
227. **Prasadarao, N. V., A. M. Blom, B. O. Villoutreix, and L. C. Linsangan.** 2002. A novel interaction of outer membrane protein A with C4b binding protein mediates serum resistance of *Escherichia coli* K1. J Immunol **169:**6352-6360.
228. **Prasadarao, N. V., E. Lysenko, C. A. Wass, K. S. Kim, and J. N. Weiser.** 1999. Opacity-associated protein A contributes to the binding of *Haemophilus influenzae* to Chang epithelial cells. Infection and immunity **67:**4153.
229. **Priebe, G. P., C. R. Dean, T. Zaidi, G. J. Meluleni, F. T. Coleman, Y. S. Coutinho, M. J. Noto, T. A. Urban, G. B. Pier, and J. B. Goldberg.** 2004. The *galU* Gene of *Pseudomonas aeruginosa* is required for corneal infection and

efficient systemic spread following pneumonia but not for infection confined to the lung. Infect Immun **72:**4224-4232.

230. **Prinz, W. A., F. Åslund, A. Holmgren, and J. Beckwith.** 1997. The role of the thioredoxin and glutaredoxin pathways in reducing protein disulfide bonds in the *Escherichia coli*cytoplasm. Journal of Biological Chemistry **272:**15661.
231. **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.
232. **Ram, S., M. Cullinane, A. M. Blom, S. Gulati, D. P. McQuillen, B. G. Monks, C. O'Connell, R. Boden, C. Elkins, and M. K. Pangburn.** 2001. Binding of C4b-Binding Protein to Porin. The Journal of experimental medicine **193:**281.
233. **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.
234. **Raquil, M. A., N. Anceriz, P. Rouleau, and P. A. Tessier.** 2008. Blockade of antimicrobial proteins S100A8 and S100A9 inhibits phagocyte migration to the alveoli in streptococcal pneumonia. J Immunol **180:**3366-3374.
235. **Read, R. C., R. Wilson, A. Rutman, V. Lund, H. C. Todd, A. P. R. Brain, P. K. Jeffery, and P. J. Cole.** 1991. Interaction of nontypable *Haemophilus influenzae* with human respiratory mucosa in vitro. Journal of Infectious Diseases **163:**549.
236. **Reddy, M. S., J. M. Bernstein, T. F. Murphy, and H. S. Faden.** 1996. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. Infect Immun **64:**1477-1479.
237. **Reidl, J., and J. J. Mekalanos.** 1996. Lipoprotein e (P4) is essential for hemin uptake by *Haemophilus influenzae*. The Journal of experimental medicine **183:**621.
238. **Ren, Z., H. Jin, D. J. Morton, and T. L. Stull.** 1998. *hgpB*, a gene encoding a second *Haemophilus influenzae* hemoglobin-and hemoglobin-haptoglobin-binding protein. Infection and immunity **66:**4733.
239. **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. Journal of bacteriology **179:**6602.
240. **Ristow, P., P. Bourhy, F. W. da Cruz 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.
241. **Rosadini, C. V., S. M. Wong, and B. J. Akerley.** 2008. The periplasmic disulfide oxidoreductase DsbA contributes to *Haemophilus influenzae* pathogenesis. Infect Immun **76:**1498-1508.
242. **Rosenfeld, M., R. L. Gibson, S. McNamara, J. Emerson, J. L. Burns, R. Castile, P. Hiatt, K. McCoy, C. B. Wilson, A. Inglis, A. Smith, T. R. Martin,**

and B. W. Ramsey. 2001. Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. Pediatr Pulmonol **32:**356-366.

243. **Rubin, L. G., and J. St Geme 3rd.** 1993. Role of lipooligosaccharide in virulence of the Brazilian purpuric fever clone of *Haemophilus influenzae* biogroup aegyptius for infant rats. Infection and immunity **61:**650.
244. **Sabri, M., S. Houle, and C. M. Dozois.** 2009. Roles of the extraintestinal pathogenic *Escherichia coli* ZnuACB and ZupT zinc transporters during urinary tract infection. Infect Immun **77:**1155-1164.
245. **Saiman, L.** 2004. Microbiology of early CF lung disease. Paediatr Respir Rev **5 Suppl A:**S367-369.
246. **Schaffer, H. E., and R. R. Sederoff.** 1981. Improved estimation of DNA fragment lengths from Agarose gels. Anal Biochem **115:**113-122.
247. **Schlör, S., M. Herbert, M. Rodenburg, J. Blass, and J. Reidl.** 2000. Characterization of ferrochelatase (*hemH*) mutations in *Haemophilus influenzae*. Infection and immunity **68:**3007.
248. **Schweda, E. K., J. C. Richards, D. W. Hood, and E. R. Moxon.** 2007. Expression and structural diversity of the lipopolysaccharide of *Haemophilus influenzae*: implication in virulence. International journal of medical microbiology : IJMM **297:**297-306.
249. **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. Infection and immunity **74:**6213.
250. **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.
251. **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.
252. **Sevier, C. S., and C. A. Kaiser.** 2002. Formation and transfer of disulphide bonds in living cells. Nat Rev Mol Cell Biol **3:**836-847.
253. **Shao, F., M. W. Bader, U. Jakob, and J. C. A. Bardwell.** 2000. DsbG, a protein disulfide isomerase with chaperone activity. Journal of Biological Chemistry **275:**13349.
254. **Shevchik, V. E., G. Condemine, and J. Robert-Baudouy.** 1994. Characterization of DsbC, a periplasmic protein of Erwinia chrysanthemi and *Escherichia coli* with disulfide isomerase activity. The EMBO journal **13:**2007.
255. **Singh, I. R., R. A. Crowley, and P. O. Brown.** 1997. High-resolution functional mapping of a cloned gene by genetic footprinting. Proc Natl Acad Sci U S A **94:**1304-1309.
256. **Sirakova, T., P. E. Kolattukudy, D. Murwin, J. Billy, E. Leake, D. Lim, T. DeMaria, and L. Bakaletz.** 1994. Role of fimbriae expressed by nontypeable

Haemophilus influenzae in pathogenesis of and protection against otitis media and relatedness of the fimbrin subunit to outer membrane protein A. Infect Immun **62:**2002-2020.

257. **Sohnle, P. G., M. J. Hunter, B. Hahn, and W. J. Chazin.** 2000. Zinc-Reversible Antimicrobial Activity of Recombinant Calprotectin (Migration Inhibitory Factor—Related Proteins 8 and 14). Journal of Infectious Diseases **182:**1272.
258. **Sonnhammer, E. L., G. von Heijne, and A. Krogh.** 1998. A hidden Markov model for predicting transmembrane helices in protein sequences. Proc Int Conf Intell Syst Mol Biol **6:**175-182.
259. **St Geme 3rd, J.** 1994. The HMW1 adhesin of nontypeable *Haemophilus influenzae* recognizes sialylated glycoprotein receptors on cultured human epithelial cells. Infection and immunity **62:**3881.
260. **St Geme 3rd, J., M. De La Morena, and S. Falkow.** 1994. A *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. Molecular microbiology **14:**217.
261. **St Geme, J. W., 3rd.** 2002. Molecular and cellular determinants of non-typeable *Haemophilus influenzae* adherence and invasion. Cell Microbiol **4:**191-200.
262. **Steinbakk, M., C. Naess-Andresen, M. Fagerhol, E. Lingaas, I. Dale, and P. Brandtzaeg.** 1990. Antimicrobial actions of calcium binding leucocyte L1 protein, calprotectin. The Lancet **336:**763-765.
263. **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.
264. **Sutton, A., R. Schneerson, S. Kendall-Morris, and J. B. Robbins.** 1982. Differential complement resistance mediates virulence of *Haemophilus influenzae* type b. Infection and immunity **35:**95-104.
265. **Swift, A. J., E. R. Moxon, A. Zwahlen, and J. A. Winkelstein.** 1991. Complement-mediated serum activities against genetically defined capsular transformants of *Haemophilus influenzae*. Microb Pathog **10:**261-269.
266. **Swords, W. E., B. A. Buscher, K. Ver Steeg Ii, A. Preston, W. A. Nichols, J. N. Weiser, B. W. Gibson, and M. A. Apicella.** 2000. Non typeable *Haemophilus influenzae* adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Molecular microbiology **37:**13-27.
267. **Tidhar, A., Y. Flashner, S. Cohen, Y. Levi, A. Zauberman, D. Gur, M. Aftalion, E. Elhanany, A. Zvi, A. Shafferman, and E. Mamroud.** 2009. The NlpD lipoprotein is a novel *Yersinia pestis* virulence factor essential for the development of plague. PLoS One **4:**e7023.
268. **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.
269. **Tocco-Bradley, R., and M. J. Kluger.** 1984. Zinc concentration and survival in rats infected with *Salmonella typhimurium*. Infection and immunity **45:**332.

270. **Tomb, J. F.** 1992. A periplasmic protein disulfide oxidoreductase is required for transformation of *Haemophilus influenzae* Rd. Proc Natl Acad Sci U S A **89:**10252-10256.
271. **Turk, D. C.** 1984. The pathogenicity of *Haemophilus influenzae*. Journal of medical microbiology **18:**1-16.
272. **Uehara, T., K. R. Parzych, T. Dinh, and T. G. Bernhardt.** 2010. Daughter cell separation is controlled by cytokinetic ring-activated cell wall hydrolysis. EMBO J **29:**1412-1422.
273. **Van Alphen, L., P. Eijk, L. Geelen-van Den Broek, and J. Dankert.** 1991. Immunochemical characterization of variable epitopes of outer membrane protein P2 of nontypeable *Haemophilus influenzae*. Infection and immunity **59:**247.
274. **van Alphen, L., J. Poole, and M. Overbeeke.** 1986. The Anton blood group antigen is the erythrocyte receptor for *Haemophilus influenzae*. FEMS microbiology letters **37:**69-71.
275. **van den Berg, S., P. A. Lofdahl, T. Hard, and H. Berglund.** 2006. Improved solubility of TEV protease by directed evolution. J Biotechnol **121:**291-298.
276. **van Ham, S. M., L. van Alphen, F. R. Mooi, and J. P. M. van Putten.** 1993. Phase variation of *H. influenzae* fimbriae: transcriptional control of two divergent genes through a variable combined promoter region. Cell **73:**1187-1196.
277. **van Schilfgaarde, M., L. van Alphen, P. Eijk, V. Everts, and J. Dankert.** 1995. Paracytosis of *Haemophilus influenzae* through cell layers of NCI-H292 lung epithelial cells. Infection and immunity **63:**4729.
278. **Van Zele, T., F. Coppieters, P. Gevaert, G. Holtappels, P. Van Cauwenberge, and C. Bachert.** 2009. Local complement activation in nasal polyposis. Laryngoscope **119:**1753-1758.
279. **VanZile, M. L., N. J. Cosper, R. A. Scott, and D. P. Giedroc.** 2000. The zinc metalloregulatory protein *Synechococcus* PCC7942 SmtB binds a single zinc ion per monomer with high affinity in a tetrahedral coordination geometry. Biochemistry **39:**11818-11829.
280. **Vertommen, D., M. Depuydt, J. Pan, P. Leverrier, L. Knoops, J. P. Szikora, J. Messens, J. C. A. Bardwell, and J. F. Collet.** 2008. The disulphide isomerase DsbC cooperates with the oxidase DsbA in a DsbD independent manner. Molecular microbiology **67:**336-349.
281. **Vimr, E., and C. Lichtensteiger.** 2002. To sialylate, or not to sialylate: that is the question. Trends in microbiology **10:**254-257.
282. **Vitovski, S., K. T. Dunkin, A. J. Howard, and J. R. Sayers.** 2002. Nontypeable *Haemophilus influenzae* in carriage and disease. JAMA: The Journal of the American Medical Association **287:**1699.
283. **VITOVSKI, S., R. C. READ, and J. O. N. R. SAYERS.** 1999. Invasive isolates of *Neisseria meningitidis* possess enhanced immunoglobulin A1 protease activity compared to colonizing strains. The FASEB journal **13:**331.
284. **Vogel, L., B. Duim, F. Geluk, P. Eijk, H. Jansen, J. Dankert, and L. vanAlphen.** 1996. Immune selection for antigenic drift of major outer membrane

protein P2 of *Haemophilus influenzae* during persistence in subcutaneous tissue cages in rabbits. Infection and immunity **64:**980.

285. **Walkup, G. K.** 1997. Fluorescent Chemosensors for Divalent Zinc Based on Zinc Finger Domains. Enhanced Oxidative Stability, Metal Binding Affinity, and Structural and Functional Characterization. J. Am. Chem. Soc **119:**3443-3450.
286. **Walport, M. J.** 2001. Complement. First of two parts. N Engl J Med **344:**1058-1066.
287. **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 U S A **92:**4927-4931.
288. **Webb, D. C., and A. Cripps.** 1998. Secondary structure and molecular analysis of interstrain variability in the P5 outer-membrane protein of non-typable *Haemophilus influenzae* isolated from diverse anatomical sites. Journal of medical microbiology **47:**1059.
289. **Weiser, J., D. Maskell, P. Butler, A. Lindberg, and E. Moxon.** 1990. Characterization of repetitive sequences controlling phase variation of *Haemophilus influenzae* lipopolysaccharide. Journal of bacteriology **172:**3304.
290. **Weiser, J. N., S. T. H. Chong, D. Greenberg, and W. Fong.** 1995. Identification and characterization of a cell envelope protein of *Haemophilus influenzae* contributing to phase variation in colony opacity and nasopharyngeal colonization. Molecular microbiology **17:**555-564.
291. **Weiser, J. N., J. B. Goldberg, N. Pan, L. Wilson, and M. Virji.** 1998. The phosphorylcholine epitope undergoes phase variation on a 43-kilodalton protein in *Pseudomonas aeruginosa* and on pili of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Infect Immun **66:**4263-4267.
292. **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.
293. **Weiser, J. N., A. Lindberg, E. Manning, E. Hansen, and E. Moxon.** 1989. Identification of a chromosomal locus for expression of lipopolysaccharide epitopes in *Haemophilus influenzae*. Infection and immunity **57:**3045.
294. **Weiser, J. N., J. M. Love, and E. R. Moxon.** 1989. The molecular mechanism of phase variation of *H. influenzae* lipopolysaccharide. Cell **59:**657-665.
295. **Weiser, J. N., N. Pan, K. L. McGowan, D. Musher, A. Martin, and J. Richards.** 1998. Phosphorylcholine on the lipopolysaccharide of *Haemophilus influenzae* contributes to persistence in the respiratory tract and sensitivity to serum killing mediated by C-reactive protein. J Exp Med **187:**631-640.
296. **Whitby, P. W., T. W. Seale, D. J. Morton, T. M. VanWagoner, and T. L. Stull.** 2010. Characterization of the *Haemophilus influenzae tehB* gene and its role in virulence. Microbiology **156:**1188.
297. **White, D. C., and S. Granick.** 1963. Hemin Biosynthesis in *Haemophilus*. Journal of bacteriology **85:**842-850.

298. **Williams, B. J., G. Morlin, N. Valentine, and A. L. Smith.** 2001. Serum resistance in an invasive, nontypeable *Haemophilus influenzae* strain. Infect Immun **69:**695-705.
299. **Winkelstein, J. A., and E. R. Moxon.** 1992. The role of complement in the host's defense against *Haemophilus influenzae*. J Infect Dis **165 Suppl 1:**S62-65.
300. **Wong, S. M., and B. J. Akerley.** 2005. Environmental and genetic regulation of the phosphorylcholine epitope of *Haemophilus influenzae* lipooligosaccharide. Mol Microbiol **55:**724-738.
301. **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.
302. **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.
303. **Wong, S. M., F. St Michael, A. Cox, S. Ram, and B. J. Akerley.** 2011. ArcA-regulated glycosyltransferase Lic2B promotes complement evasion and pathogenesis of nontypeable *Haemophilus influenzae*. Infect Immun.
304. **Wooster, D. G., R. Maruvada, A. M. Blom, and N. V. Prasadarao.** 2006. Logarithmic phase *Escherichia coli* K1 efficiently avoids serum killing by promoting C4bp-mediated C3b and C4b degradation. Immunology **117:**482-493.
305. **Wu, L. F., M. A. Mandrand-Berthelot, R. Waugh, C. J. Edmonds, S. E. Holt, and D. H. Boxer.** 1989. Nickel deficiency gives rise to the defective hydrogenase phenotype of *hydC* and *fnr* mutants in *Escherichia coli*. Mol Microbiol **3:**1709-1718.
306. **Wunderlich, M., and R. Glockshuber.** 1993. Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. Protein science : a publication of the Protein Society **2:**717-726.
307. **Wyckoff, E. E., M. L. Boulette, and S. M. Payne.** 2009. Genetics and environmental regulation of *Shigella* iron transport systems. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine **22:**43-51.
308. **Yamanaka, H., M. Kameyama, T. Baba, Y. Fujii, and K. Okamoto.** 1994. Maturation pathway of *Escherichia coli* heat-stable enterotoxin I: requirement of DsbA for disulfide bond formation. Journal of bacteriology **176:**2906.
309. **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.
310. **Yatsunyk, L. A., J. A. Easton, L. R. Kim, S. A. Sugarbaker, B. Bennett, R. M. Breece, Vorontsov, II, D. L. Tierney, M. W. Crowder, and A. C. Rosenzweig.** 2008. Structure and metal binding properties of ZnuA, a periplasmic zinc transporter from *Escherichia coli*. J Biol Inorg Chem **13:**271-288.
311. **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.

312. **Zapun, A., J. C. Bardwell, and T. E. Creighton.** 1993. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation in vivo. Biochemistry **32:**5083-5092.
313. **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.
314. **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.
315. **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.