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Haemophilus influenzae modulins in chronic obstructive pulmonary disease airway inflammation

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HAEMOPHILUS INFLUENZAE MODULINS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE AIRWAY INFLAMMATION

By

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DEDICATION

To my parents, my husband, and my brother

for their love, support, and encouragement.

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ABSTRACT

Nontypeable *H. influenzae* (NTHi) causes repeated respiratory infections characterized by a brisk inflammatory response that results in the expression and secretion of proinflammatory cytokines. We hypothesized that secreted and cell asssociated NTHi proteins mediate cellular interactions with respiratory epithelial cells, leading to the production of interleukin (IL)-8. We exposed human tracheal epithelial cells to *H. influenzae* strain Rd and compared the resulting profiles of IL-8 secretion. Putative *H. influenzae* Rd modulins were enriched from culture supernatant fluid. Proteome analysis of the enriched fractions revealed 27 candidate proteins. Further analysis of four proteins, ClpB, OmpP2, TonB, and RelA, suggested a potential role in the IL-8 response. A fifth protein, FtsH, showed no such response. Study of the other 22 proteins is required to understand their role in cytokine induction. These results suggest that factors other than lipooligosaccharide (LOS) contribute to IL-8 secretion.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

A. Nontypeable *Haemophilus influenzae* (NTHi) and Epidemiology

Chronic obstructive pulmonary disease (COPD) afflicts more than 15 million Americans, is responsible for more than 15 million physician office visits each year, and results in approximately 150 million days of disability per annum (2, 116). The total direct cost of medical care expenses because of this disease is approximately $15 billion per year (136). It is the fourth most common cause of death in the United States (121). Nontypeable *Haemophilus influenzae* (NTHi) is the most common cause of exacerbation among patients with COPD (96, 121, 128, 147). NTHi is also known to be associated with cystic fibrosis (CF), the most common autosomal recessive disease among Caucasians, affecting 1 in 2500 newborns (24). Isolates of NTHi account for 20-30% of all episodes of acute otitis media and perhaps a higher percentage of recurrent otitis media infections (47). Recent research has shown that this bacterium is also responsible for over 40% of the cases of chronic otitis media (110). Approximately one-third of the cases of acute or chronic sinusitis is caused by NTHi (39, 142). Apart from the above-mentioned diseases, NTHi is known to be associated with diseases like chronic bronchitis, pulmonary exacerbations, community-acquired pneumonia, meningitis, septic arthritis, and septicemia (86, 98).

NTHi causes pathogenesis in patientS with COPD by first colonizing the upper respiratory tract followed by contiguous spread in the adjacent areas. In most cases of

systemic disease due to NTHi, the patients have anatomic abnormalities or compromised immunities (86). The upper respiratory infection disrupts mucociliary activity, integrity of the mucosal lining, and neutrophil function, predisposing NTHi to cause several diseases (48, 84). For example, existing lung diseases such as CF, chronic bronchitis, and bronchiectasis impair the mucociliary escalator, thus allowing NTHi access to the lower respiratory tract, causing bronchitis and pneumonia (48, 84). Excessive exposure to cigarette smoke, air pollution, or viral infection results in goblet cell hyperplasia, mucus hypersecretion, and decreased respiratory epithelial cell ciliary function, which further increases the possibility of NTHi causing chronic respiratory tract infections (83, 95, 100, 131).

Apart from NTHi, other pathogens like *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Bordetella pertussis*, *Moraxella catarrhalis,* and *Streptococcus pneumoniae* are also known to be involved with the above-mentioned respiratory diseases (52, 120). All of these organisms produce several molecules that react with respiratory epithelial cells and cause the elevation of proinflammatory cytokines (68, 146, 148). These molecules can be bacterial cell associated or extracellular in nature and might include lipooligosaccharide (LOS), proteases, outer membrane proteins, adhesins, outer membrane vesicles, or secreted molecules (69, 146).

NTHi LOS produces the ciliotoxic effects of infection with the whole organism and is found to be 10-fold more potent than other virulence factors in stimulating the release of monocyte-derived inflammatory mediators such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6 (78, 56). NTHi LOS has been shown to stimulate cytokine and chemokine (e.g., interleukin (IL)-8 gene expression) in human

respiratory epithelial cells (43, 125, 138). Recent studies have shown that NTHi LOS accounts for approximately 50% of the proinflammatory cytokine stimulation from the human respiratory epithelial cells, suggesting that other bacterial molecules (e.g., modulins) play a role in the inflammatory response (23). The interaction of the modulins with the respiratory epithelial cells stimulates the secretion of proinflammatory cytokines and chemokines (17, 19, 29, 34, 57, 85). The chemokine IL-8, in particular, shows a high level of activity from respiratory epithelial cells when stimulated with NTHi modulins and is believed to be involved in the process of recruitment and activation of neutrophils and other leukocytes in the lungs of the patients with COPD (15, 75, 78, 133).

B. Nontypeable *Haemophilus influenzae* and the Proteins or Factors Involved in Pathogenesis

Haemophilus influenzae, belonging to the family Pasteurellaceae, is a Gram-negative, fastidious, nonspore-forming, coccobacillus-to-long-filament-shaped bacterium that was first described by Pfeiffer in 1892 (31, 107). It has derived its name *Haemophilus*, which means "blood-loving", from its dependence on heme-related molecules for growth under aerobic conditions (6). *H. influenzae* exists in two forms, encapsulated and nonencapsulated. Six antigenically and structurally distinct capsular polysaccharides, referred to as serotypes a-f, are expressed in the encapsulated strains (109). Nontypeable *H. influenzae* (NTHi) are unencapsulated strains that do not react with the antisera against the polysaccharide capsules (63).

NTHi is a common commensal organism in the human nasopharynx and occupies this niche as its natural habitat (63, 72, 88). The rate of nasopharyngeal colonization

increases from approximately 20% during the first year of life to over 50% by the age of 5-6 yrs, then remains high through adulthood (63, 72). Adults typically carry one strain, whereas children simultaneously harbor multiple strains (63, 88). β-lactam antibiotics, such as ampicillin and amoxicillin, are known to treat many disease incidences of NTHi; increasing resistance is becoming evident, however, and tends to be associated with the production of β-lactamase (12, 61, 108). Occasional isolates demonstrate penicillin resistance from an altered production of penicillin-binding protein and diminished affinity for β-lactam antibiotics. Resistance to other antibiotics, like trimethoprim-sulfamethoxazole, clarithromycin, and azithromycin, has been found (61,108).

An important requirement for NTHi in establishing infection is to first adhere to respiratory epithelium successfully, followed by persistence on the mucosal surface. Various studies have been performed that indicate many bacterial factors that aid in adherence and colonization on the mucosal lining. These include the outer membrane of NTHi that represents the interface between the organism and the human host, containing integral membrane proteins, surface associated proteins, and lipooligosaccharide (62). Lipopolysaccharide (LPS) is present as the major outer membrane component of many gram-bacilli and plays an important role in pathogenesis. In *H. influenzae* this LPS lacks an O-antigen and therefore is called lipooligosaccharide (LOS) (7,42). Studies performed *in vitro* have shown that the LOS from NTHi is responsible for the release of proinflammatory chemokines and cytokines from cultured human bronchial epithelial cells, middle ear epithelial cells, and monocytes (56,81,82,138). Moreover, LOS from NTHi also played a significant role in the stimulation of intercellular adhesion molecule-1 (ICAM-1) from the surface of human bronchial epithelial cells (81, 82). These surface

exposed ICAM-1 molecules, along with other cytokines, like IL-8, help in the recruitment of polymorphonuclear leukocytes (PMNs) to the site of infection caused by NTHi. Recently, another series of molecules, found in the soluble cytoplasmic fraction (SCF), were studied and found to be made up of lipopeptides (143). These SCF molecules were approximately $\leq$ 3-kDa and were associated with IL-8-inducing activity.

Apart from LOS, some of the other examples of bacterial cell surface factors that aid in adherence to the respiratory epithelium are molecules like pili, HMW proteins, Hia, and Hap (10, 11, 135). Pili are hairlike appendages protruding from the surface of the bacteria that help in adherence to the respiratory epithelium (135). The HMW adhesins consist of HMW1 (125-kDa) and HMW2 (120-kDa). They are part of the autotransporter family of proteins and are encoded by genes that encode for the adhesive molecules in different strains of NTHi (11, 129). Nearly one-fourth of the nontypeable strains of *H. influenzae* lack HMW proteins but still show the ability of adhering to the respiratory epithelium (130). For example, there are other proteins that play an important role in adhering to respiratory epithelial cell surface, like Hia (10). Hap is another such protein that has the ability to adhere to the bacterial surface (132). A recent study showed Hap protein's having no function in respiratory epithelial cells' cytokine stimulation (93, 128). Adhesive activities have been described in the work of Hartmann and Lingwood in NTHi; it was found that in response to heat shock, bacteria showed an increase in binding activity to sulfatoxygalactosylglycerol and sulfatoxygalactosylceramide (65). The increased adherence was due to two Hsp 70-related heat-shock proteins (65). Another protein from NTHi, known as OapA, has proved to be important for significant adherence and colonization in the nasopharynx of a rat model (144). The studies performed by

Kubeit and Ramphal showed that NTHi attaches to a major component of mucus, called mucin aiding, in the formation of colonies within the mucus layers, followed by the release of soluble factors that cause ciliostasis and sloughing of the ciliated cells in the upper respiratory tract (62, 87, 111).

After successful adherence to the epithelial surface, the next step for the bacteria is to persist inside the host successfully. One of the main ways of achieving this goal is by attacking the host immune system to derive nutrients such as heme and iron. Immunoglobin IgA, which is present on the mucosal surfaces of the host, inhibits the adherence and binding action of the bacteria by agglutination (18). NTHi secretes the endopeptidase IgA1 protease, which inactivates host IgA and facilitates bacterial colonization under such circumstances. This protease acts by cleaving the peptide bond present in the hinge region of the serum and secretory forms of IgA1 and helps in release of the antigen-binding domain, therefore further reducing the chances of any agglutination activity (97). In one of the recent studies, this gene showed the absence of any stimulation of proinflammatory cytokines from human respiratory epithelial cells (23, 74, 93).

Other proteins of NTHi playing important roles in the pathogenic process are the six major outer membrane proteins (OMP), P1 to P6. Of these six proteins, P2 is known to be the most abundant OMP (99). It forms a trimer and has porin activity, allowing molecules of up to 1400 Dalton to pass through the membrane (27, 62, 99). Omp P2 has been shown to act as a target for protective antibodies in an *H.influenzae* type b (Hib) experimental model (59). Reports showed that the Hib porin contributed to the signaling process in the inflammatory cascade during disease and that this porin mediated

proinflammatory cytokine induction by the TLR2/MyD88 pathway (45, 46). Omp P2 has been shown to be a facilitator for nicotinamide-based nucleotide transport (4).

Omp P6 is a highly conseved 16-kDa lipoprotein that has been associated with induction of proinflammatory cytokine and chemokines, mainly IL-10, TNF-α, and IL-8, from human macrophages (14). Omp P6 has also been found to activate NF-κB via TLR2-TAK1-dependent NIK-IKKα/β-IκBα and MKK3/6-p38 MAP kinase-signaling pathways (125). NF-κB, a transcription regulator, plays an essential contribution during the pathogenesis of diseases and has been shown to play an important role in the expression of many genes, including cytokines, chemokines, and other mediators, that are involved during the process of inflammation (67).

In addition to the above-mentioned major outer-membrane proteins, many minor outer-membrane proteins are also involved in the acquisition of heme (53, 145). Examples of such minor outer-membrane proteins involved in the binding and acquisition of heme are transferrin-binding proteins (Tbp1 and Tbp2) and several other heme-binding proteins, namely Hxu, Hgp, and Hit. The transferrin-binding proteins serve as surface receptors for human transferrin and help in the acquisition and transport of iron into the bacterial periplasm (54, 71, 119). This is a complex transport process that involves a second protein, called TonB (54, 71, 119). TonB is an energy-transducing transmembrane protein that assists in the transport of several vital metabolites after they bind to bacterial cell-surface receptors. Recent studies by Clemans et al. described *tbpA* and *tbpB* as having no such involvement with IL-8 induction from the human respiratory epithelial cells (74, 93, 128).

In vitro research has shown that heme, hemoglobin, heme complexed with hemopexin, or hemoglobin complexed with haptoglobin can also provide porphyrin and iron required by *H. influenzae* for aerobic growth of (28, 134). TonB fulfills this requirement of NTHi by actively transporting chemicals like heme, hemoglobin, hemoglobin:haptoglobin, heme-albumin, and heme:hemopexin and further transferring them into the periplasm (25, 76, 92).

Apart from the above-mentioned outer membrane proteins, there are several other proteins involved with *H. influenzae* that help in the acquisition of heme and thus further assist this bacterium in establishing pathogenesis inside the host environment. Proteins encoded by the *hxu* operon called HxuA, HxuB, and HxuC are associated with heme and hemopexin transport. HxuA, which is a 100-kDa protein, is required for utilization of heme bound to hemopexin (25). The 60-kDa HxuB is thought to be associated with facilitating the secretion of HxuA (25). HuxC, a 78-kDa protein is involved in the transport of the HxuA: heme complex into the bacterial cell (25). In an *in vitro* study, the phenotype of an *hxuC* mutant showed a lack of the ability to utilize heme at very low concentrations (25). *H. influenzae* can also bind to hemoglobin by the process that involves various proteins, including 120-kDa HgpA, 115-kDa HgpB, and 120-kDa HgpC. HgpA helps in binding and transport of hemoglobin:haptoglobin complexes and free hemoglobin; HgpB is associated with the binding and transport of hemoglobin; and HgpC is involved in binding and transport of hemoglobin:haptoglobin. Hgp proteins have shown no contribution in the stimulation of human respiratory epithelial cell cytokine activation (23, 74, 93).

C. *H. influenzae* and Inflammation

In order to establish itself inside a hostile host environment, *H. influenzae* has acquired various mechanisms to escape the innate as well as the adaptive immune responses elicited by its host. *H. influenzae* type b is more resistant to a host immune system than are other strains because it contains the polyribosyl ribitol phosphate polysaccharide capsule (139). The respiratory infection caused by NTHi has been associated with direct damage of the epithelial cells and has been found to be associated with elevated proinflammatory response.

In vitro experiments with NTHi have shown cytokines like IL-6, IL-8, and TNF-α (82) involved in mechanisms to up regulate inflammation. Recent studies demonstrated that the p38 mitogen-activated protein kinase (MAPK) and the Src-dependent Raf-1-Mek1/2-extracellular signal-regulated kinase mitogen-activated protein kinase (ERK MAPK) pathways are required for NTHI-induced IL-8 production (143). In chronic bronchitis and COPD, the airway bacteria are associated with an increase in concentrations of neutrophils, leukotriene B_4 (LTB_4), and TNF- α (30, 112). The rise in the levels of TNF-α during exacerbation leads to an increase in the activity of endothelial adhesion molecules that directly activates PMNs (1, 80, 122).

Studies have shown that during an NTHi infection in patients with COPD, inflammatory cells infiltrate the respiratory airways, limiting oxygen flow and manifesting increased breathlessness in the patients (33, 104, 113). During exacerbations, pulmonary inflammation becomes more prominent with the recruitment of eosinophils and an increase in the number of CD4+ lymphocytes (151). Release of elastase and different proteinases due to neutrophil degranulation causes respiratory

epithelial cell damage and reduced ciliary action (126). This further promotes the goblet cells to stimulate mucus secretion (102), leading to edema and protein exudation into the airway due to an increased amount of permeability of the bronchial mucosa (50). A decrease in the concentration of colonized bacteria shows reduction in the levels of inflammation. The cytokine found to be associated with the neutrophilic inflammatory changes during most severe episodes of the disease is IL-8. Thus, it is important to identify the modulins present in NTHi that might cause the release of these proinflammatory cytokines and chemokines from the human respiratory epithelial cells.

Further study on the NTHi proteins that play a role in respiratory epithelial cell inflammation will shed new light on the understanding of the intricate mechanisms involved between host-pathogen interaction in patients with COPD and other respiratory diseases.

D. Rationale and Hypothesis

Study from the literature supports our hypothesis that NTHi stimulate a variety of human cells to secrete proinflammatory cytokines and indicate that pathogenic *H. influenzae* are capable of recognizing several different cell-surface receptors. So far no studies have comprehensively analyzed all of the NTHi modulins that stimulate a proinflammatory response in host respiratory epithelial cells. Our hypothesis, therefore, is that the secreted and cell associated, non-LOS, NTHi modulins induce the production of proinflammatory cytokines from human respiratory epithelial cells. The goal of this study is to identify and characterize the secreted and cell-associated non-LOS modulins

from NTHi that stimulate the secretion of proinflammatory cytokines from respiratory epithelial cells.

CHAPTER 2

MATERIALS AND METHODS

A. Bacterial Strains and Growth Conditions

Table 1 describes the bacterial strains and plasmids used in this study. Mutant strains of *H. influenzae* were kindly provided by Tim Murphy (State University of New York, Buffalo). Strains of *H. influenzae* were cultured on chocolate agar plates (BBL, Becton Dickinson, Cockeysville, Md.) at 37^{o}C in 5% CO_2. *H. influenzae* were cultured in the chemically defined MIc liquid medium or the complex Brain Heart Infusion (BHI) (Difco, Becton Dickinson, Cockeysville, Md.) (9, 20). BHI is useful for growing fastidious bacterial strains like *H. influenzae*. Further, we shifted to work with BHI ultrafiltered with YM 30 membrane, as it showed fewer protein contaminants in the medium and thus gave a clearer background on the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. All *H. influenzae* growth media were supplemented with hemin (100 µg/ml) and β-NAD (nicotinamide adenine dinucleotide) (both from Sigma Chemical Co., St. Louis, Mo). *H. influenzae* mutants with antibiotic resistant markers were cultured in media containing 34 µg/ml chloramphenicol. *E. coli* strains containing the antibiotic resistance markers were grown in LB medium (Difco) containing 100µg/ml ampicillin. For long-term storage, the strains were stored at -80^{o}C in skim milk (*H. influenzae* strains) or 30% glycerol (*E.coli* strains).

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Description of phenotype	Reference
H. influenzae		
Rd	Wild-type Rd strain	41
P2 mutant	Rd strain deficient in *P2,* Cm^r	26
13P24H1	Clinical isolate strain from COPD patient	124, 123
13P24H1 mutant	Clinical isolate strain deficient in *P2*, Cm^r	124, 123
E. coli		
JM109	Host strain used for cloning experiments	Promega
Plasmids		
pGEMT-Easy	TA cloning vector, Amp^r	Promega
clpB: pGEMT-Easy	*clpB* gene from strain Rd in TA cloning vector	This study
relA: pGEMT-Easy	*relA* gene from strain Rd in TA cloning vector	This study
tonB: pGEMT-Easy	*tonB* gene from strain Rd in TA cloning vector	This study

Cm^r – Chloramphenicol resistant cassette
Amp^r – Ampicillin resistant cassette

B. Cell Culture

The human respiratory epithelial cell line 9HTEo- was obtained from Dieter C. Gruenert (University of California, San Francisco) (55). These cells were cultured in 75 - cm^2 tissue culture flasks (Techno Plastic Products, Trasadingen, Switzerland) in 20 ml of Eagle's minimum essential medium (Gibco, Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin solution, and 1% L-glutamine (all from Gibco). After the formation of confluent monolayers, the adhered

cells were released from the plastic surface of the flask with 0.05% trypsin, 0.53 mM EDTA (Gibco) for 10 minutes at 37°C and 5% CO_2. Viable cells were enumerated in a hemocytometer through the use of trypan blue dye (Gibco) exclusion. Cells were resuspended in cell-culture freezing medium (Gibco) and cryopreserved for extended periods under liquid nitrogen.

C. Ultracentrifugation of the Supernatant Fluid Obtained from *H. influenzae* Strain Rd

H. influenzae strain Rd was grown in sBHI (supplemented brain heart infusion) broth at 37°C in a shaking water bath set at 100 rpm. The overnight culture of bacteria containing the whole bacterial cells with the broth they had been growing in is referred to as the culture fluid (CF). All the experiments beyond this point were carried out at 4°C to retain the protein activity. Bacterial CF was filtered by use of 0.22-µm nitrocellulose filters, and the resulting filtrate devoid of bacterial whole cells is referred to as the supernatant. Further, the two fractions of greater than and less than 100-kDa were generated by the process of ultrafiltration of the supernatant fraction with YM100 membranes (Millipore Corporation, Bedford, Mass.). These fractions were ultracentrifuged at 100,000 xg for 2 hrs to recover potential membrane-containing fractions (37). The pellets obtained after separation from the supernatant fluid were washed with phosphate-buffered saline (Gibco). Each of the fractions obtained were assayed with Bio-Rad protein assay to determine the protein concentration (Bio-Rad Laboratories, Hercules, Calif.).

D. Enrichment of Modulin from *H. influenzae* Strain Rd

The culture fluid (CF) was obtained after growing *H. influenzae* strain Rd to the stationary phase. All further steps were carried out at 4°C to prevent the loss of any protein activity. The culture fluid was centrifuged (Sorvall superspeed RC2-B, Ivan Sorvall Inc., Norwalk, Conn.) inside 250-ml GSA centrifuge bottles (Nalgene, Rochester, N.Y.) at 3020 xg for 20 minutes. The pellet obtained was discarded, and the supernatant was carefully decanted into a fresh, sterile bottle. This supernatant fluid was filtered with a 0.22-µm nitrocellulose filter (Millipore) to eliminate the whole bacterial cells. Protease inhibitor cocktail (Cat.# P-8465, Sigma) was added to the filtered supernatant fluid at a ratio of 500µl of protease inhibitor to 500 ml of the supernatant fluid. An ultrafiltration cell (Amicon Corporation, Denvers, Mass.) equipped with a YM100 membrane (Millipore) was used to concentrate 2.5 liters of culture fluid to 4-5 ml and generate >100-kDa and <100-kDa fractions. The highly concentrated >100-kDa fraction was washed 3-4 times with 20 mM Tris pH8.5, 50 mM NaCl buffer and then dialysed three times with 20 mM Tris, pH8.5, 50 mM NaCl buffer. Protein concentration was quantified with the Bio-Rad protein reagent (Bio-Rad).

E. Ion Exchange Chromatography

Ion exchange chromatography was performed with the use of 5-ml, HiTrap Q sepharose high-performance anion exchange columns (Amersham Biosciences, Piscataway, N.J.) at 4°C. The concentrated and dialysed, >100-kDa protein fraction (approximately 90 µg) generated in section D, was loaded on the column and washed with buffer containing 20 mM Tris, pH 8.5, 50 mM NaCl. Fractions were batch-eluted

with 20 mM Tris, pH 8.5 buffer containing increasing concentration of NaCl (0.1 M, 0.2 M, 0.3 M, 0.5 M, and 1 M). Each of the batches were eluted three times with 50 ml of buffer, pooled together, and further concentrated to a volume of 3-4 ml with a YM10 ultrafiltration membrane. Care was taken to avoid overlap between one fraction and another; the column was washed until it showed zero absorbance, measuring at wavelength 280 nm. This was repeated before the column washes were shifted from one concentration of NaCl to another. Each fraction was further concentrated to 250 µl with a Centricon C-10 concentrator (Millipore).

F. Stimulation of 9HTEo- Cells with *H. influenzae* Strain Rd Modulins and Other Strains

Six-well tissue culture plates (Techno Plastic Products, Switzerland) or 24-well tissue culture plates (Techno Plastic Products) were seeded in the concentration of 2 X 10^5 cells per mm^2 in 3 ml or 1.5 ml of fresh supplemented Eagle's minimal essential medium (Gibco), respectively. On the second day, the medium was replaced with the respective volume of serum-free medium (SAGM from Clonetics, Cambrex Corporation, East Rutherford, N.J.) and allowed to grow overnight. On the third day of growth, the cells were approximately 95% confluent. The growth medium was replaced with 3 ml or 1.5 ml of fresh serum-free medium for the 6-well or 24-well tissue culture plates, respectively.

A final protein concentration of 20 µg/ml from each of the samples was used to stimulate individual wells containing the 9HTEo- cells. For the experiments involving the study of the role of a particular gene, the culture of *E. coli* cells in LB medium was

incubated with agitation at 37°C overnight. Bacterial cell concentration was determined spectrophotometrically at 610 nm. 6×10^7 bacterial cells were added to each of the wells in the 24-well plate already containing 6×10^5 9HTEo- cells per well. This corresponds to a ratio of 100:1 bacterial cells to human respiratory epithelial cells.

All experiments included unstimulated (negative) control wells, positive control well(s) containing IL-1β (20 ng/ml: BD Pharmingen, San Diego, Calif.), and experimental wells containing various levels of stimuli.

Cell culture supernatant fluid was harvested after 16 hours of incubation in 5% CO_2 at 37°C and stored at -80°C. The schematic diagram of the above experiment is represented in Fig. 1.

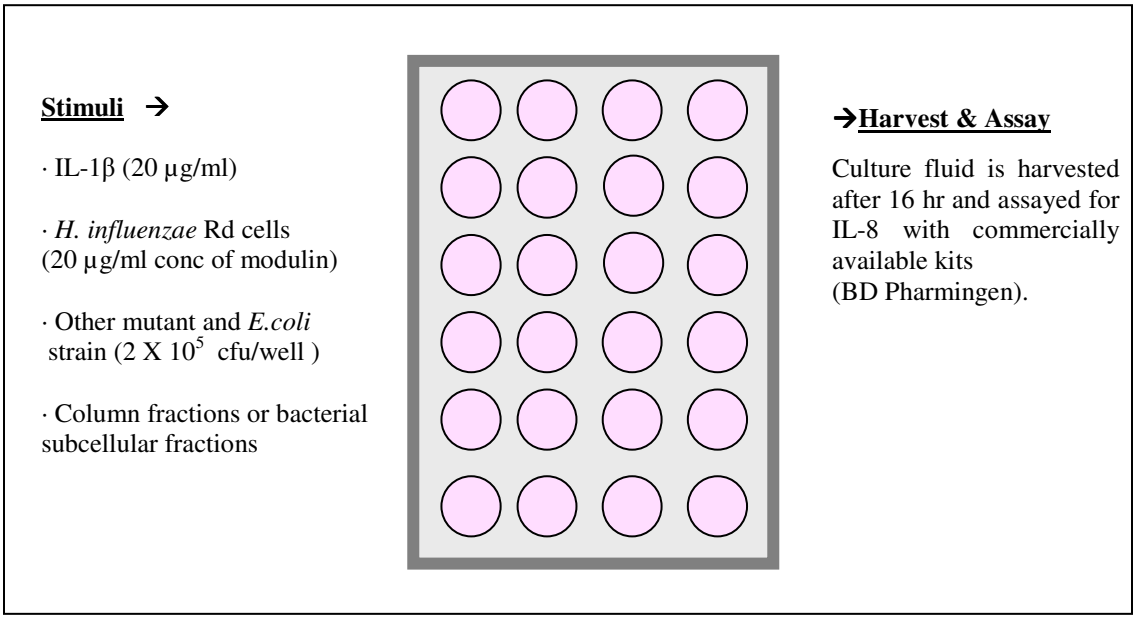

Fig. 1. In vitro assay system monolayers of 9HTEo- cells in serum-free medium.

G. Enzyme-Linked Immunosorbent Assay (ELISA) of IL-8

The amount of IL-8 secreted into the cell culture medium following 16 h of incubation was determined by an enzyme-linked immunosorbent assay (ELISA). ELISAs were performed with BD Pharmingen IL-8 sets according to the manufacturer's instructions. For assay diluent preparation, the fetal bovine serum used was from Gibco. ELISA assays were performed in Immulon 2HB assay plates (Labsystems, Milford, Mass.). The wells were developed with the substrate tetramethylbenzidine (TMB) and hydrogen peroxide (BD Pharmingen).

The cell culture supernatant fluid was diluted in the assay diluent in the ratio of 1:10, and the IL-1β was diluted in the ratio 1:50.

H. Statistical Analysis

Summary data from various groups were expressed as mean $\pm$ S.E.M. Experimental treatments were compared with controls by the Students's *t* test for two-sample. Statistical analysis was performed with the GraphPad version 3.0 for Windows [GraphPad Software, San Diego, Calif. (http://www.graphpad.com)].

I. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed under denaturing and reducing condition on Novex 4-12% tris-glycine polyacrylamide pre-cast gels (Invitrogen). The samples were mixed with 2X tris-glycine SDS sample buffer (Invitrogen) and boiled for 10 minutes. They were loaded inside the wells of the SDS-PAGE gel and electrophoresed at 125 V in an

XCell sure-lock electrophoresis cell (Novex, Invitrogen Corporation). After electrophoresis, the gels were stained with Sypro ruby protein gel stain (Cambrex) and scanned with the help of an electronic U.V. transilluminator (Ultra Lum, Inc., Paramount, Calif.). The bands that corresponded to the stimulatory fractions were chosen for MALDI-TOF (matrix-assisted laser desorption/ionization-time-of-flight) mass spectral analysis.

J. MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time-of-Flight) Mass Spectral Analysis

The specific bands in the SDS-PAGE gels relating to high IL-8 activity were submitted to the Michigan Proteome Consortium, University of Michigan, for mass spectrometry (MS) and tandem mass spectrometry (MS/MS) by MALDI-TOF technique. It is a device for prediction and confirmation regarding identification of given peptides to a mass accuracy up to 20 ppm. The digest of the selected protein bands was performed with trypsin. The tools used for interpretation were MS-Fit (for MS data) and MS-Tag (for MS/MS data). The protein sequence and mass spectral data obtained were used to search and identify the *H. influenzae* putative modulin genes. The databases used to search and identify the putative modulin genes were Protein Prospector (http://prospector.ucsf.edu), Mascot (http://www.matrix-science.com), the National Center for Biotechnology Information (NCBI; http://www.ncbi.nih.gov) and The Institute for Genomic Research (TIGR; http://www.tigr.org). The parameters used for the database searches were hydrogen for peptide N terminus, free acid for peptide C terminus, and carbamidomethylation for cysteine modification, and the maximum

percentage of unmatched ions were kept at 50. The putative hit proteins finally considered were the ones that corresponded to the correct band sizes from where they were excised and had pI > 8.5.

K. Polymerase Chain Reaction (PCR) and Plasmid Construction

PCR was performed with PCR master mix (Promega, Madison, Wis.). We obtained the *H. influenzae* Rd genomic DNA protocol from the manufacturer's directions provided in the Wizard genomic kit (Promega). The amplification of the various PCR products from the *H. influenzae* Rd genomic DNA were performed through the use of oligonucleotide primers listed in Table 2. Fifty nanograms of chromosomal DNA and 20 pg of each primer (forward and reverse) were used in a total reaction mixture of 50 µl. The 50 µl reaction mixture also included the master mix (Promega) containing Mg^{+2}(3 mM), buffer, taq polymerase (50 units/ml) and dNTPs (400 µM each dATP, dGTP, dCTP, and dTTP). The cycling conditions consisted of 2 min of initial denaturation at 95°C, cycle melt at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 4 min, and final extension at 72°C for 5 min. Under these conditions, a total of 30 cycles were repeated inside a thermocycler. PCR products were mixed with 6X blue/orange loading dye (cat# G1881, Promega) and run on a 0.8% agarose gel with 1 kb DNA ladder (Promega) to confirm the amplified sequences. Purification of the PCR products for nucleotide sequence analysis after agarose gel electrophoresis, the standard manufacturer's protocol of the Wizard SV gel and PCR clean-up system, was used (Promega).

Purified PCR products were ligated into the TA cloning vector pGEM-T Easy (Promega) by using the protocol provided in the standard manufacturer's directions in the pGEM-TEasy vector system-I kit (Promega). An additional step was taken to include a 0.5-kb extra sequence on either side of the primers designed for the *H. influenzae* genes (*clpB, relA, tonB*, *and ftsH*) in order to include the promoter regions. This was done to make sure that the genes were expressed. These plasmids were transformed into *E. coli* JM109 (Promega).

Recombinant plasmids were isolated according to the protocol provided in the Wizard plus SV minipreps DNA purification system (Promega). The DNA inserts were confirmed after *Eco*RI (Promega) restriction analysis and 0.8% agarose gel electrophoresis.

Table 2. Sequences of the primers used in this study

Name of gene	**Primer sequences**
relA (forward) *relA* (reverse)	5'- AGA TTT ATT TTG CGG CAT GG -3' 5'- GGC GAG ATA AAA TTG CGG TA -3'
clpB (forward) *clpB* (reverse)	5'- CGC ACT GAA ATC CGA AAA AT -3' 5'- CTC GCA CAC TAC GCG ATT TA -3'
tonB (forward) *tonB* (reverse)	5'-TTG CTA CCA TTT ATC TTC CAT ATC A -3' 5'-CAA AAA TGT GGC AAT TGT TTC T -3'
ftsH (forward) *ftsH* (reverse)	5'-GTC TTG GCA AGC AAA GGA AG -3' 5'-TTT CCG AAG AAA GGC TTT GA -3'

The different genes of H. *influenzae* studied in the experiments were amplified with the commercially synthesized primers (Integrated DNA Technologies, Coralville, Iowa).

CHAPTER 3

RESULTS

A. IL-8 Stimulation from 9HTEo- Epithelial Cells

Haemophilus influenzae strain Rd was chosen as a model system for our experiments because its genome has been completely sequenced and annotated, and it showed a similar profile of cytokine stimulation from human respiratory epithelial cells as did selected NTHi clinical isolates (23, 41). *H. influenzae* strain Rd used to be a capsular type d, but with the accumulation of mutations, it converted to a noncapsular form known as nontypeable (nonencapsulated) (41).

Previous experiments by Clemans et al. showed that the modulin activity was located in the secretory fraction of bacterial cells cultured in Levinthal broth, sBHI, and MIc media (23, 74). Heat lability and proteinase-K sensitivity experiments suggested that the modulin was a protein (23, 74). Ultrafiltration of the secreted fraction revealed that the stimulation of IL-8 from the human respiratory epithelial cells was higher in the >100-kDa fraction as compared to the <100-kDa fraction (Fig. 2). When compared to the unstimulated controls, bacterial cell culture fluid showed a 73.75-fold increase, the cell-free supernatant fraction showed a 28.2-fold increase, bacterial <100-kDa fraction showed a 25.4-fold increase, and the >100-kDa fraction showed a 66-fold increase in IL-8 secretion. The co-incubation of sterile sBHI broth with epithelial cells (negative control) showed a 4.2-fold increase in IL-8 secretion over the unstimulated control. These results suggested that the factors playing key roles in stimulation of IL-8 activity might be located in the >100-kDa fraction. The 100-kDa fraction showed approximately

a 2.5-fold greater IL-8 secretion value over the <100-kDa size fraction (Fig. 2). Perria and Clemans showed that the >100-kDa fraction lost activity upon heat and protease treatment, thus proving that some protein(s) might be playing a role in this kind of high stimulatory activity (74). The positive control (monolayers of 9HTEo- stimulated with 20 ng per ml IL-1β) showed an IL-8 activity measuring 13,620.4 pg/ml.

The differences in the IL-8 secretions between <100-kDa and >100-kDa obtained after stimualtion of human respiratory epithelial cells were statistically significant ($p \leq 0.002$).

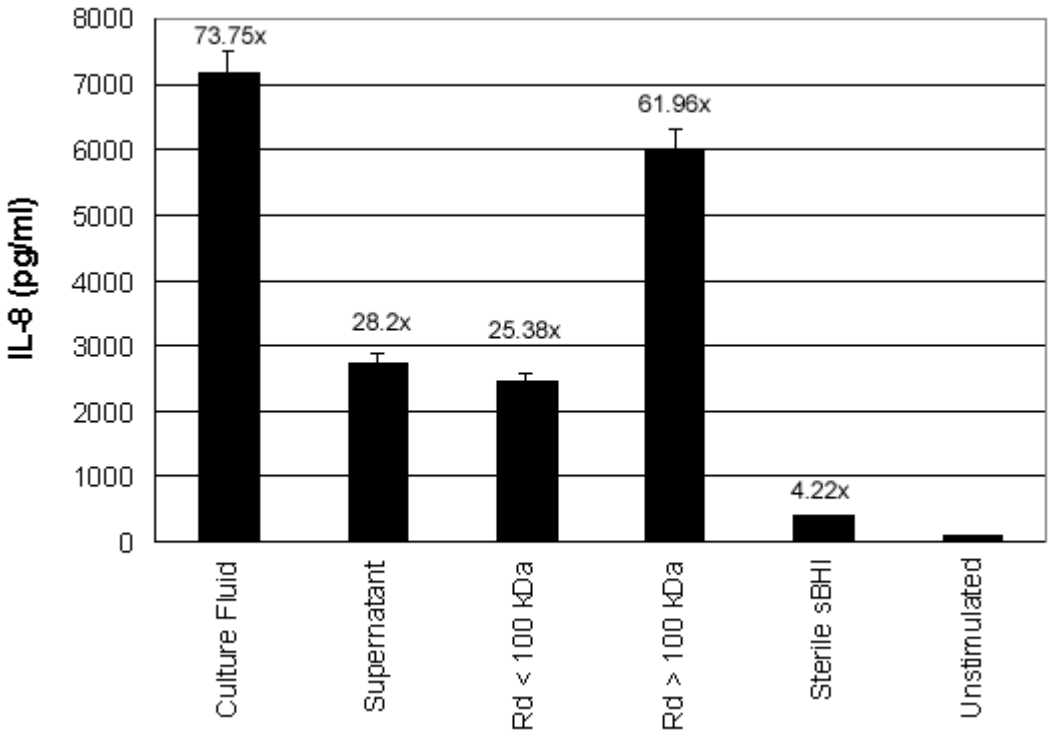

Fig. 2. Comparison of IL-8 response after treating the 9HTE0- cell line with different fractions obtained after ultrafiltration of culture fluid from *Haemophilus influenzae* strain Rd. Cultured monolayers of human respiratory epithelial 9HTE0- transfer cell line were coincubated with 20 μg/ml of protein from cell culture fluid, supernatant fluid (0.22 μm filtered), <100-kDa fraction, >100-kDa fraction, sterile sBHI (bacterial growth medium), and tissue culture medium alone (unstimulated). Twenty nanograms of IL-1β per ml (IL-8 secretion was measured as 13,620.4 pg/ml) was used as a positive control. Supernatant fluid was harvested after 16 h of coincubation with the monolayered cells. Secreted cytokine IL-8 was measured with the commercially available ELISA kits. The data shown are from one experiment of three similarly performed experiments. The numerical value represented above each bar in the ELISA graph is the fold increase value of that particular fraction over the unstimulated control. The differences in the IL-8 expression between the two fractions, <100-kDa, and >100-kDa, were statistically significant ($p < 0.002$).

Ion exchange chromatography was used to enrich the protein modulins from the >100-kDa fraction. The protein(s) obtained in this experiment were from the *H. influenzae* strain Rd grown in MIc liquid medium (9). Fig. 3 shows the results of the stimulation of 9HTEo- monolayers with different fractions from ion exchange chromatography. The batch elutions of 0.1 M NaCl, 0.3 M NaCl, and 0.5 M NaCl showed activity of about 6.6-fold, 8.5-fold, and 8.6-fold increases, respectively, over the unstimulated control (Fig. 3). This indicated that the putative proteins that might be responsible for high level of cytokine IL-8 stimulation must be located in these fractions. The starting material, which consisted of very highly concentrated >100-kDa fraction, showed the highest activity of about 10.2-fold over the unstimulated control, whereas the flow through wash 1 and wash 2 showed 8-fold, 4.3-fold, and 3.2-fold increases, respectively, over the unstimulated control. The reason behind the high stimulation by flow through and wash fractions could be the overloading of the ion exchange column. The positive control stimulated with IL-1β showed IL-8 induction of about 16,391.2 pg per ml. The comparisons of the results of the unstimulated fraction with different fractions were all statistically significant ($p \leq 0.005$). The comparisons between the wash fractions were statistically significant [starting material–flow through ($p < 0.003$); 0.1 M NaCl wash–0.5 M NaCl wash ($p < 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.003$)].

The chemically defined MIc medium showed some problems with precipitation of media components and subsequent clogging of the ion exchange column during the course of performing the experiments. The SDS-PAGE gels with the proteins recovered from the MIc-cultured *H. influenzae* strain Rd cells are shown in Figures 5 and 6.

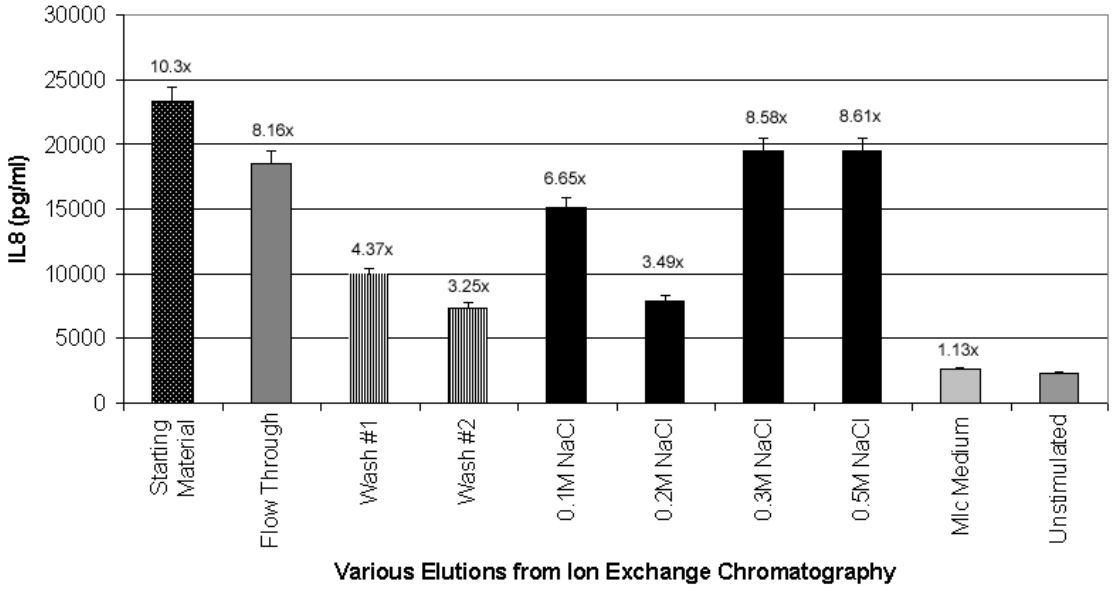

Fig. 3. Secretion of IL-8 from 9HTEo- cells after stimulation with various fractions obtained after performing ion exchange chromatography from *Haemophilus influenzae* strain Rd cultured in MIc liquid medium. Cultured human respiratory epithelial cells 9HTEo- were coincubated with starting material (highly concentrated >100 kDa fraction), flow through, three buffer washes (each 50 ml), and different fractions obtained after the washing of the column with increasing NaCl concentrations (0.1 M, 0.2 M, 0.3 M, and 0.5 M) during ion exchange chromatography. Twenty nanograms of IL-1β per ml (16,391.2 pg/ml was the IL-8 secretion detected from this positive control), sterile MIc liquid medium (bacterial growth medium), and only SAGM (tissue culture medium) were used as controls for the experiment. The supernatant cell culture fluid was harvested after 16 h, followed by ELISA to determine IL-8 activity with the use of commercially available kits. The data shown are from one experiment of several similarly performed experiments. The numerical value represented above each bar in the ELISA graph is the fold increase value of that particular fraction over the unstimulated control. The comparisons between the following fractions were statistically significant [0.1 M NaCl wash–0.5 M NaCl wash ($p < 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.003$)].

Ion exchange chromatography of samples from sBHI cultured bacteria (Fig. 4) showed a similar elution profile to that of samples from MIc-cultured bacteria (Fig. 3). SDS-PAGE gels from the sBHI-based samples are shown in Figures 5 and 6. NaCl washes of 0.2 M, 0.3 M, and 0.5 M showed very high IL-8 activity of about 50-fold, 41-fold, and 54.6-fold increases, respectively, over the unstimulated control. Compared to the above three batch elutions, 0.1 M and 1 M NaCl washes showed lower fold values of 4.6-fold and 16.23-fold increases, respectively, over the unstimulated control (Fig. 4). The starting material showed a high IL-8 activity of about a 35-fold increase over the unstimulated control, followed by flow through that showed a 20.8-fold increase. Washes 1 and 2, along with control containing only sterile bacterial growth medium, sBHI, showed very low or undetectable cytokine activity. The IL-1β positive control showed about 16,391.2 pg per ml of IL-8. The comparisons of the unstimulated fraction with the different elution fractions obtained after NaCl wahes were all statistically significant ($p \leq 0.005$). The comparisons between the wash fractions were statistically significant [starting material–flow through ($p < 0.003$); 0.2 M NaCl wash–0.3 M NaCl wash ($p \leq 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.004$); 0.2 M NaCl wash–0.5 M NaCl wash ($p < 0.004$)].

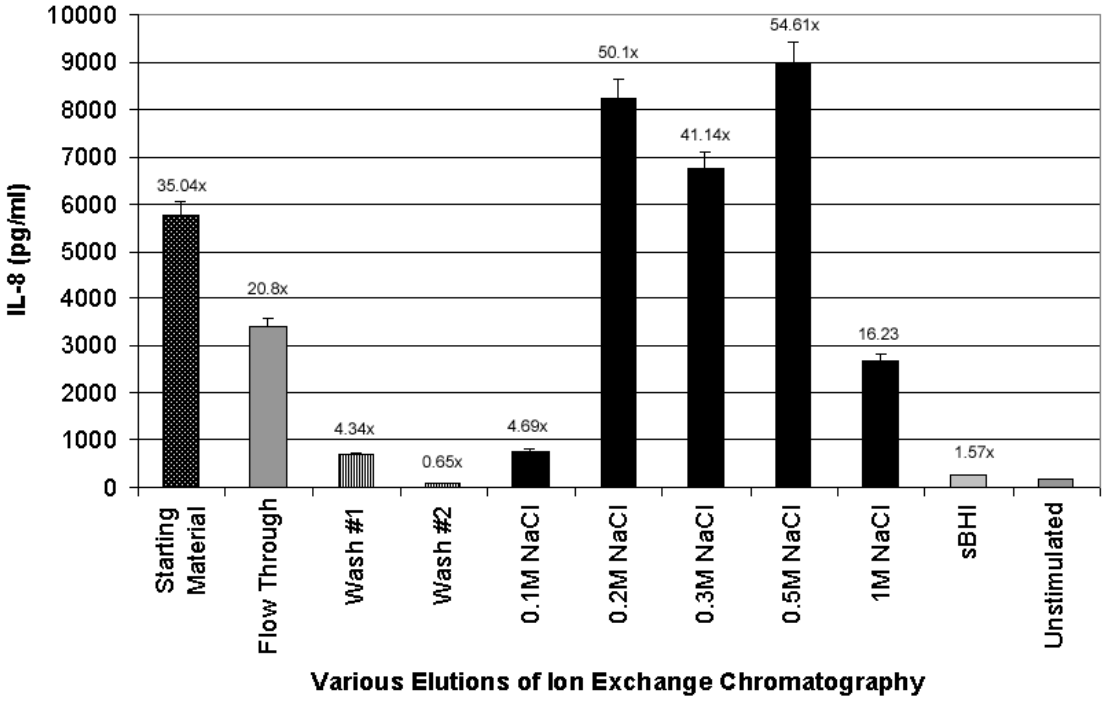

Fig. 4. Secretion of IL-8 from 9HTEo- cells after stimulation with various fractions obtained after performing ion exchange chromatography from *Haemophilus influenzae* strain Rd cultured in sBHI medium. Each of the fractions shown in the figure are same as in Figure 3 except that one more wash with 1 M NaCl was performed that was not performed with MIc medium. Also, sterile sBHI was used instead of sterile MIc as one of the negative controls. The data shown are representative from one experiment of several similarly performed experiments. The numerical value represented above each bar in the ELISA graph is the fold increase value of that particular fraction over the unstimulated control. The comparisons between the following fractions were statistically significant [starting material–flow through ($p < 0.003$); 0.2 M NaCl wash–0.3 M NaCl wash ($p < 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.004$); 0.2 M NaCl wash–0.5 M NaCl wash ($p < 0.004$)].

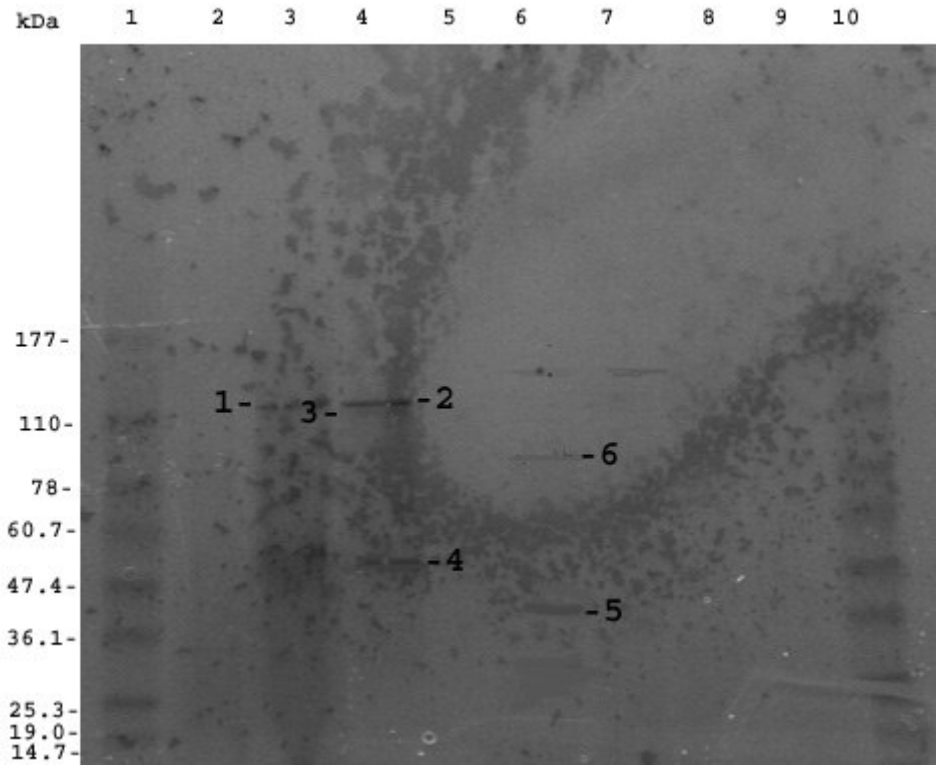

Fig. 5. Coomassie Blue stained 8-12% polyacrylamide gel with the numbered bands selected for MALDI-TOF analysis. Lanes 1 and 10, molecular weight standards; lanes 2 through 5, 0.1, 0.2, 0.3, and 0.5 M NaCl washes, respectively, obtained after ion exchange chromatography from >100-kDa fraction from strain Rd. Lanes 6 through 9 also follow the same pattern of NaCl washes (i.e., washes with 0.1, 0.2, 0.3, and 0.5 M NaCl). The concentrated protein in lanes 2 through 5 was obtained from bacteria grown in MIc liquid medium, and lanes 6 through 9 had the protein obtained from bacteria grown in sBHI medium. The hits from the searches showed bands 2 and 3 as containing gene *pon*A; band 4 and 5 had hypothetical proteins HI0523 and HI1505. Band 4 also showed the presence of *moa*A. Band 3 showed the presence of *rel*A. This gel representative corresponds to the data shown in Figures 3 and 4.

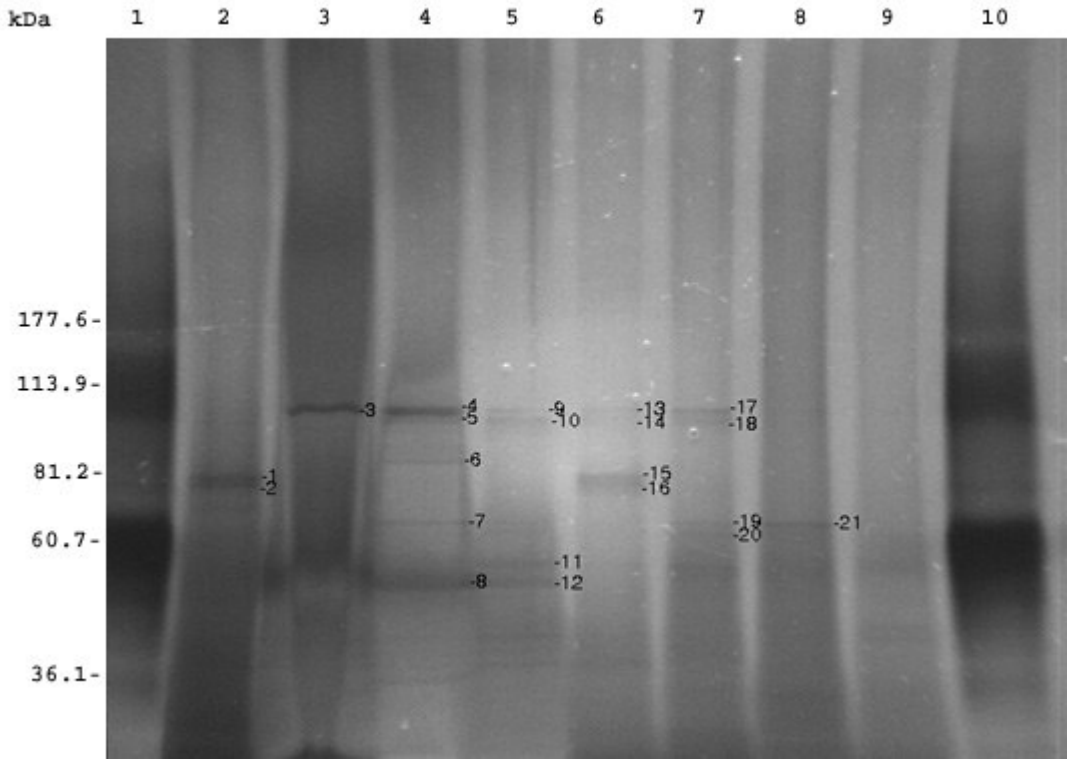

Fig. 6. Silver stained 8-12% polyacrylamide gel with the numbered bands selected for MALDI-TOF analysis. Lanes 1 and 10, molecular weight standards; lanes 2 through 5, 0.1, 0.2, 0.3, and 0.5 M NaCl washes, respectively, obtained after ion exchange chromatography from >100-kDa fraction from strain Rd. Lanes 6 through 9 also follow the same pattern of washes. The concentrated protein in lanes 2 through 5 was obtained from bacteria grown in MIc liquid medium, and lanes 6 through 9 had the protein obtained from bacteria grown in sBHI medium. *ftsH* was found in the search result from bands 2, 16, 19, and 21; *ptsI* from band 2; *tonB* from band 3; *mglA* from band 6; *metE* from bands 2, 3, 10, and 14; *IgA* protease and HI1369 from band 3; *ponA* from band 5; *oapA* from band 7; *cysS* from band 17; *moaA* from band 18; and *clpB* from bands 5 and 17. This gel representative corresponds to the data shown in Figures 3 and 4.

The graph showing the IL-8 activity of the various fractions obtained with ultrafiltered sBHI are shown in Fig. 7. Fractions eluted from the ion exchange column with 0.1 M, 0.2 M, 0.3 M, 0.5 M, and 1 M NaCl washes showed 10.6-fold, 2.6-fold, 21.7-fold, 15.8-fold, and 21.8-fold increases, respectively, when compared to the unstimulated control. These results suggested that the proteins actively participating in IL-8 secretion from human respiratory epithelial cells were most likely located in the 0.3 M, 0.5 M, and 1 M NaCl washes' fractions. Both the unstimulated cells and cells stimulated with growth medium showed very low IL-8 secretion from the 9HTEo- cells. The positive control, stimulated with IL-1β, showed a very high IL-8 value of about 20, 552 picogram per ml. The comparisons of the IL-8 activity were found to be statistically significant when each of the fractions (0.1 M, 0.2 M, 0.3 M, 0.5 M, and 1 M NaCl) were compared to the unstimulated control ($p < 0.05$). The comparisons between the wash fractions from ion exchange chromatography were statistically significant [starting material–flow through ($p \leq 0.01$); 0.1 M NaCl wash–0.3 M NaCl wash ($p \leq 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.003$); 1 M NaCl wash–0.5 M NaCl wash ($p < 0.03$)].

Initially we used MIc, as we thought it was a cleaner system because it would contained fewer protein contaminants. MIc medium, however, had severe precipitation problems that hindered the ion exchange chromatography. The complex medium sBHI contained several protein contaminants that interfered with SDS-PAGE analysis. Finally, use of ultrafiltered sBHI in this experiment gave a much cleaner background in the SDS-PAGE gels because of the absence of protein contaminants present in the medium (Figures 8, 9 and 10). The protein bands were more prominent and visible against this

lighter background, unlike as compared to the proteins bands that were not that easily visible (Figures 5 and 6) when plain sBHI was used as the bacterial growth medium.

Compared to the IL-8 induction pattern obtained from the various fractions when MIc (Fig. 3) and sBHI (Fig. 4) were used, very similar trends in the cytokine stimulation were found from the different fractions of ion exchange chromatography in this experiment with UF sBHI (Fig. 7).

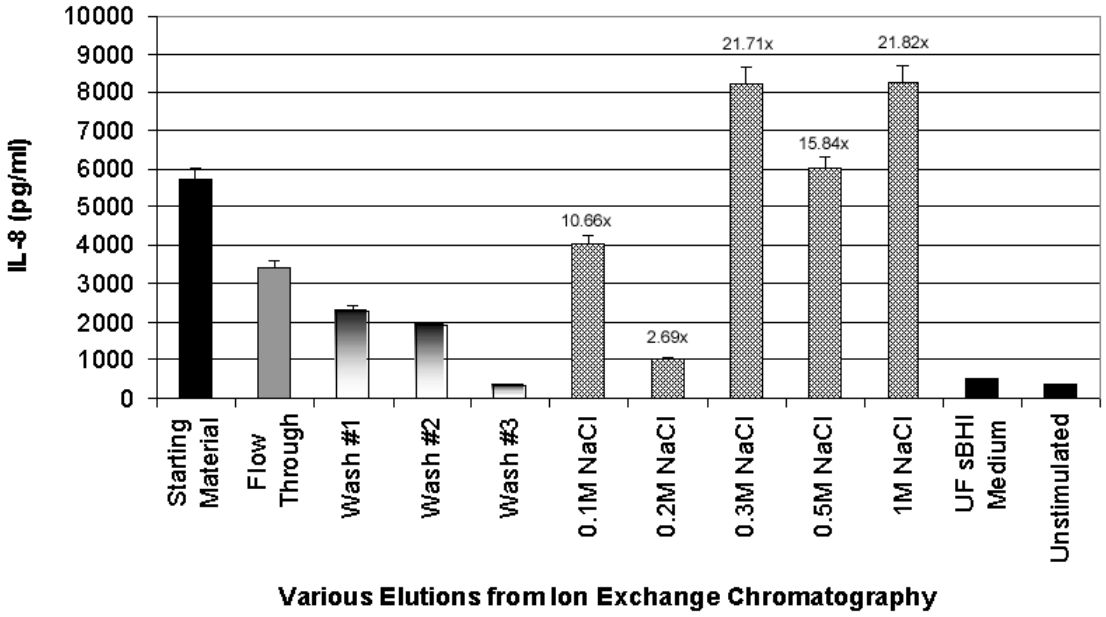

Fig. 7. Secretion of IL-8 from 9HTEo- cells after stimulation with various fractions obtained after performing ion exchange chromatography from *Haemophilus influenzae* strain Rd cultured in ultrafiltered sBHI. Each of the fractions shown in the figure is the same as in Figure 3 except that one more wash with 1M NaCl was performed that was not done with MIc medium. Also, sterile UF sBHI was used instead of sterile MIc or sBHI medium as one of the negative controls. From the positive control stimulated with 20 ng/ml IL-1β, we detected 20,552 pg/ml IL-8 induction. The data shown are representative from one experiment of several similarly performed experiments. The numerical value represented above each bar in the ELISA graph is the fold increase value of that particular fraction over the unstimulated control. The comparisons between the following fractions were statistically significant [starting material–flow through ($p < 0.01$); 0.1 M NaCl wash–0.3 M NaCl wash ($p < 0.001$); 0.3 M NaCl wash–0.5 M NaCl wash ($p < 0.003$); 1 M NaCl wash–0.5 M NaCl wash ($p < 0.03$)].

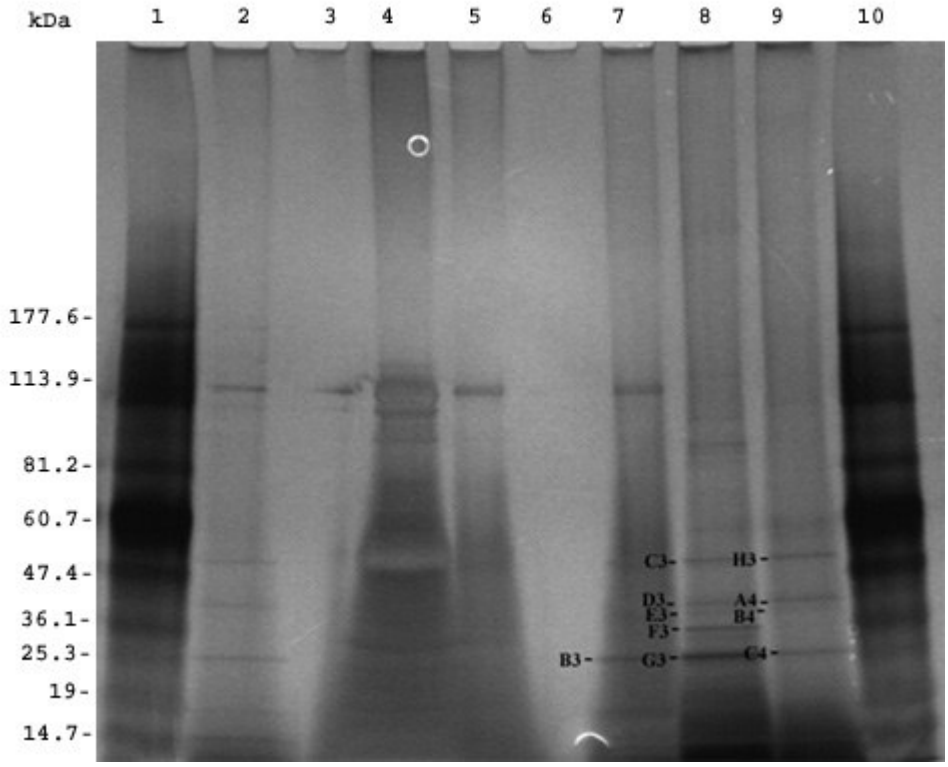

Fig. 8. Silver stained 8-12% polyacrylamide gel with the numbered bands selected for MALDI-TOF. The *H. influenzae* strain Rd was cultured in UF sBHI broth. Lanes 1 and 10, molecular weight standards; lane 2 is the starting material; lanes 4, 5, 7, 8, and 9 correspond to 0.1 M, 0.2 M, 0.3 M, 0.5 M, and 1 M NaCl washes respectively, of 100-kDa strain Rd. *kdsA* was the hit from the searches from bands B3 and C4. This representative gel corresponds to data from Fig. 7.

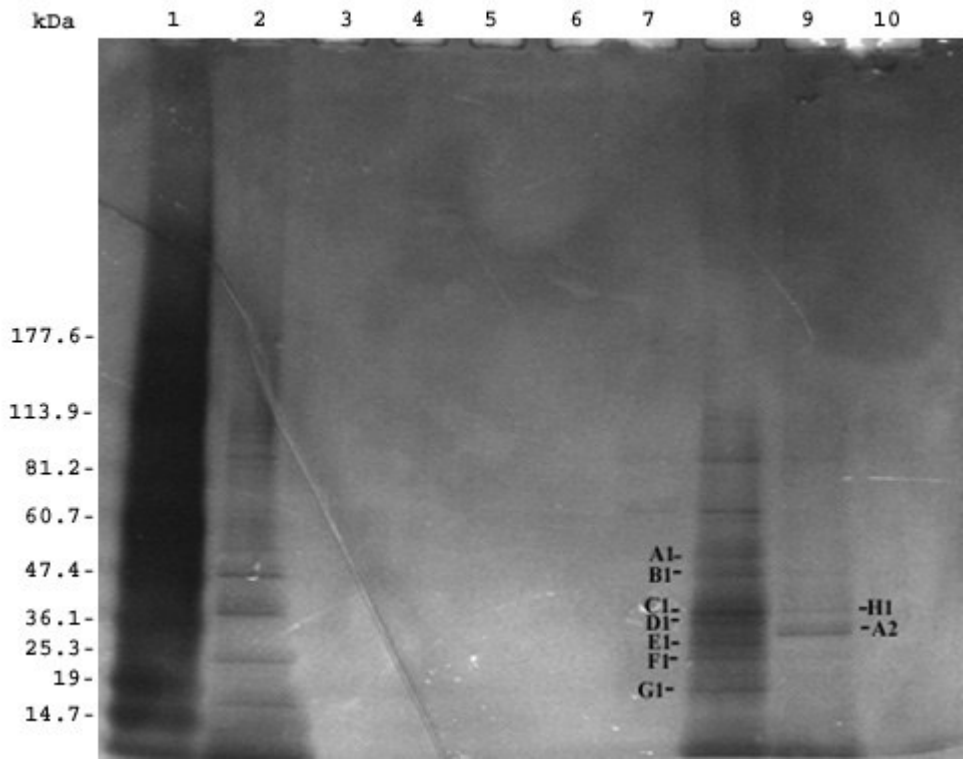

Fig. 9. Silver stained 8-12% polyacrylamide gel with the numbered bands selected for MALDI-TOF. The *H. influenzae* strain Rd was cultured in UF sBHI broth. Lane 1 corresponds to the molecular weight standards. Lanes 2 through 9 correspond to the different fractions obtained from ion exchange chromatography: lane 2 for starting material (>100-kDa); lane 3 for flow through; lanes 4 and 5 for the washes; lanes 6 through 10 for 0.1, 0.2, 0.3, 0.5 M, and 1M NaCl washes. Search results from band A1 are *hgbA* and *huxC*; from C1 is *ompP2*; from E1 are *radC* and *trpX*; from band F1 are *kdsA* and *trpX*; and from band H1 is rRNA methylase. This representative gel is obtained from one of the several ion-exchange chromatography runs using UF sBHI.

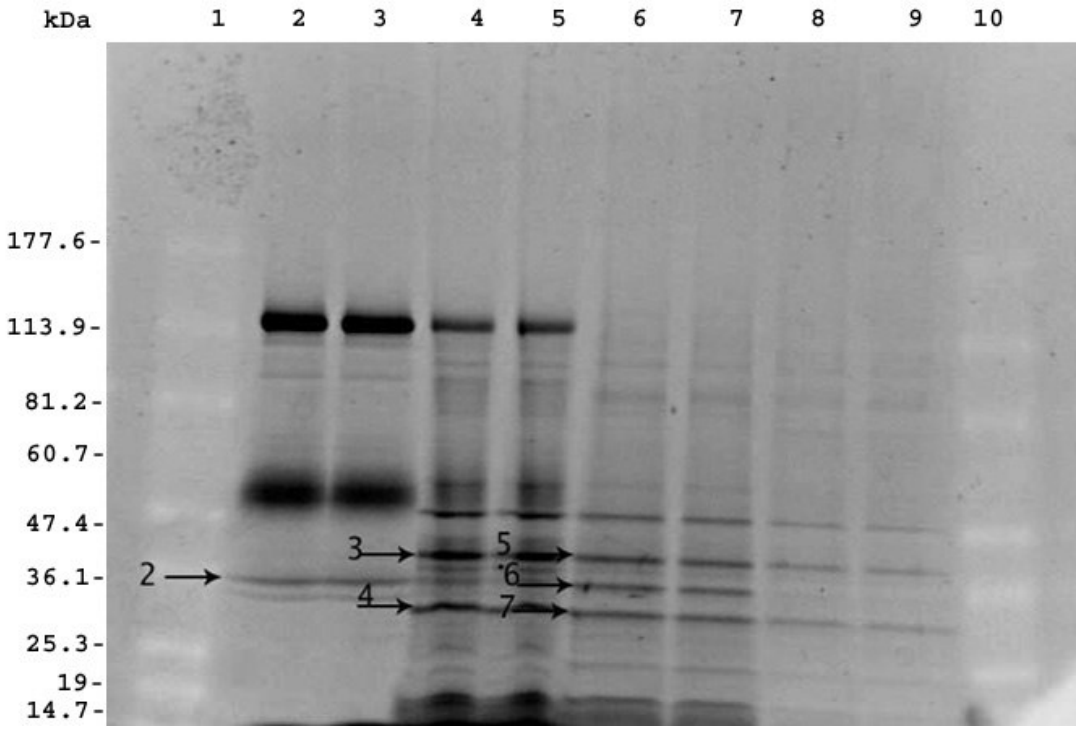

Fig. 10. Numbers showing the bands chosen for MALDI-TOF analysis after SDS-PAGE and Sypro ruby staining. Lanes 1 and 10 correspond to the molecular weight standards. The *H. influenzae* strain Rd was cultured in UF sBHI broth. Lanes 2 through 9 correspond to the different fractions obtained from ion-exchange chromatography: lanes 2 and 3 for 0.2 M; lanes 4 and 5 for 0.3 M; lanes 6 and 7 for 0.5M; and lanes 8 and 9 for 1 M NaCl. The search result from band 2 is *acpD*; from bands 3 and 5, they are *ompP2*, and from band 6, they are *ppiB* and *rps3*. This representative gel was obtained from one of the several ion-exchange chromatography runs with UF sBHI.

B. MALDI-TOF Mass Spectral Analysis

Various batches of protein bands corresponding to the active or high IL-8-stimulating fraction were selected from the gels (Figures 5, 6, 8, 9 and 12) for MALDI-TOF mass spectral analysis. With the results of the peptide fingerprints and tandem MS data provided by Michigan Proteome Consortium (University of Michigan), further searches (MS-Fit, Mascot) were performed to identify the putative modulin proteins in the various samples (Table 3). Several peak lists were generated, and the greater number of matched peptides showing higher mowse scores and better coverage of proteins were considered. The peptide with a reasonably high score was further analyzed for pI and the molecular weight. Proteins with a wide range of pIs and molecular weights were obtained, but the ones within the range of pI < 8.5 (as molecular activity from *H. influenzae* strain Rd was negatively charged at pH 8.5) and corresponding molecular weights were finally selected. In addition, information such as the function of each protein was also obtained from the database searches. The significant hits of the proteins obtained on analysis revealed several important proteins that were categorized into four different functionally distinct groups (i.e., stress proteins, nutrient transport proteins, biosynthetic proteins. and hypothetical proteins) (Table 3).

Table 3. List of protein obtained from the database searches after performing MALDI-TOF

Name	Accession#	Roles	Gel figure	Cellular location
Stress proteins				
ClpB	P44403	Degradation of proteins, peptides, & glycopeptides	Fig. 6	Cytoplasm
FtsH	P71377	Cell division	Fig. 6	Membrane
RelA	P44644	Regulatory functions	Fig. 5	Cytoplasm
Nutrient transport proteins				
HxuC	33330978	Transport & binding proteins, heme/hemopexin	Fig. 9	Membrane
HgbA	AF221059	Transport & binding proteins, hemoglobin/haptoglobin	Fig. 9	Membrane
MglA	P44884	Transport & binding proteins: carbohydrates, org. alcohols, & amino acids	Fig. 6	Periplasm
OppA	P71370	Transport & binding proteins: peptides, amines & amino acids	Fig. 6	Periplasm
PtsI	P43922	Transport & binding proteins: carbohydrates, org. alcohols, & amino acids	Fig. 6	Cytoplasm
TonB	P42872	Transport & binding proteins: cations	Fig. 6	Membrane
AcpD	P43013	Fatty acid and phospholipid metabolism	Fig. 10	Cytoplasm
Biosynthetic proteins				
MetE	P45331	Amino acid biosynthesis: aspartate family	Fig. 6	Cytoplasm

(Table continued)

Table 3. (continued)

MoaA	P45311	Biosynthesis of cofactors, prosthetic groups & carriers: molydopterin	Figs. 5 and 6	Cytoplasm
PonA	P31776	Cell envelope: biosynthesis of murein sacculus & peptidoglycan	Figs. 5 and 6	Cytoplasm
IgA protease	P45386	Cleaves peptide bonds	Fig. 6	Cytoplasm
OmpA	16273088M	Outer membrane protein P5		Membrane
OmpP2	23429714	Outer membrane protein P2	Fig. 9 and Fig. 10	Membrane
KdsA	16273457M	Cell envelope: biosynthesis & degradation of surface polysaccharide	Fig. 9	Cell wall
TrpX	16272042M	Protein synthesis: tRNA & rRNA modification	Fig. 9	Cytoplasm
PpiB	P44499	Protein fate: protein folding and stabilization	Fig. 10	Cytoplasm
Rps3	P44372	Protein synthesis: ribosomal proteins: synthesis and modification	Fig. 10	Cytoplasm
rRNA methylase	16272372M	Protein synthesis: tRNA & rRNA modification	Fig. 9	Cytoplasm
RadC	P44952	DNA replication, recombination, and repair	Fig. 9	Cytoplasm
HemY	P44772	Protoheme IX synthesis	Fig. 12	Membrane
CysS	P43816	Protein synthesis: tRNA aminoacylation	Fig. 6	Cytoplasm
Hypothetical proteins				
HI0523	P44011	Hypothetical protein: conserved	Fig. 5	Unknown

(Table continued)

Table 3. (continued)

HI1369	P45182	Hypothetical protein: conserved	Fig. 6	Unknown
HI1505	P44227	Hypothetical protein: conserved	Fig. 5	Unknown

C. Stimulation of 9HTEo- Cells with the Fractions Obtained from Ultracentrifugation of *H. influenzae* Strain Rd Supernatant Fluid

Outer membrane vesicles (OMV) of *Haemophilus influenzae* type b have been shown to play an important role in induction of meningeal inflammation (101). This experiment was performed to assess the role of various fractions obtained from the supernatant fluid (which might contain OMV) in stimulating an IL-8 response. Both the pellets obtained after ultracentrifugation of the >100-kDa fraction and the culture fluid fraction showed high IL-8 secretion (Fig. 11). The pellets obtained from >100-kDa and 0.22 µm filtered culture fluid showed 15.2-fold and 14.6-fold increases, respectively, whereas the supernatant fractions from the >100-kDa fraction and 0.22 µm culture fluid showed 13.4-fold and 8.6-fold increases, respectively, when compared to the unstimulated control. These results suggested that the pellet fraction, because of the presence of some specific proteins that might be absent in the supernatant fraction, shown higher IL-8 activity. The gels obtained after running all these fractions on SDS-PAGE are shown in Fig. 12.

The comparisons of the IL-8 activity from different fractions after comparison with the unstimulated control obtained from human respiratory epithelial cells after stimualtion with the different fractions obtained after the ultracentrifugation experiment

were statistically significant and within $p < 0.003$. The following comparisons were also shown to be statistically significant [>100-kDa fraction's pellet–0.22-µm filtered culture fluid pellet with ($p < 0.003$); >100-kDa fraction's supernatant– supernatant from 0.22-µm filtered culture fluid ($p < 0.001$)].

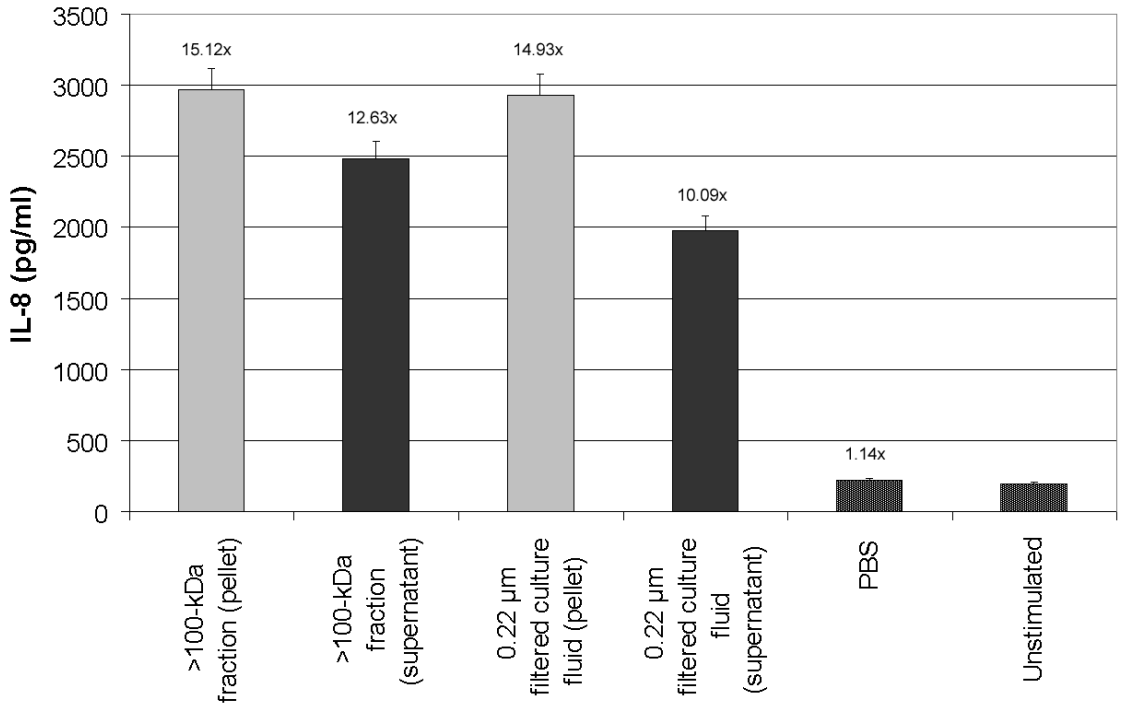

Fig. 11. Induction of IL-8 from 9HTEo- cells upon stimulation with *Haemophilus influenzae* strain Rd proteins after ultracentrifugation at 100,000xg. Human respiratory epithelial cells were co-incubated with 20 µg/ml of proteins each from the >100-kDa fraction's pellet, the >100-kDa fraction's supernatant, pellets from 0.22-µm filtered culture fluid, and supernatant from 0.22 µm filtered culture fluid, PBS, and tissue culture medium (unstimulated). Twenty nanograms of IL-1β per ml was recorded as positive control (IL-8 secretion = 6652.7 pg/ml). IL-8 values given in picogram per milliliter units were tested with commercially available ELISA kits. IL-8 secretion was measured after 16 h incubation of the 24-well plates containing all the stimuli. The data shown are from one experiment of the three similarly performed experiments. The numerical value represented above each bar in the ELISA graph is the fold increase value of that particular fraction over that of the unstimulated control. The comparisons of the IL-8 activity between different fractions were statistically significant [>100-kDa fraction's pellet– 0.22-µm filtered culture fluid pellet with ($p < 0.003$); >100-kDa fraction's supernatant– supernatant from 0.22-µm filtered culture fluid ($p < 0.001$)].

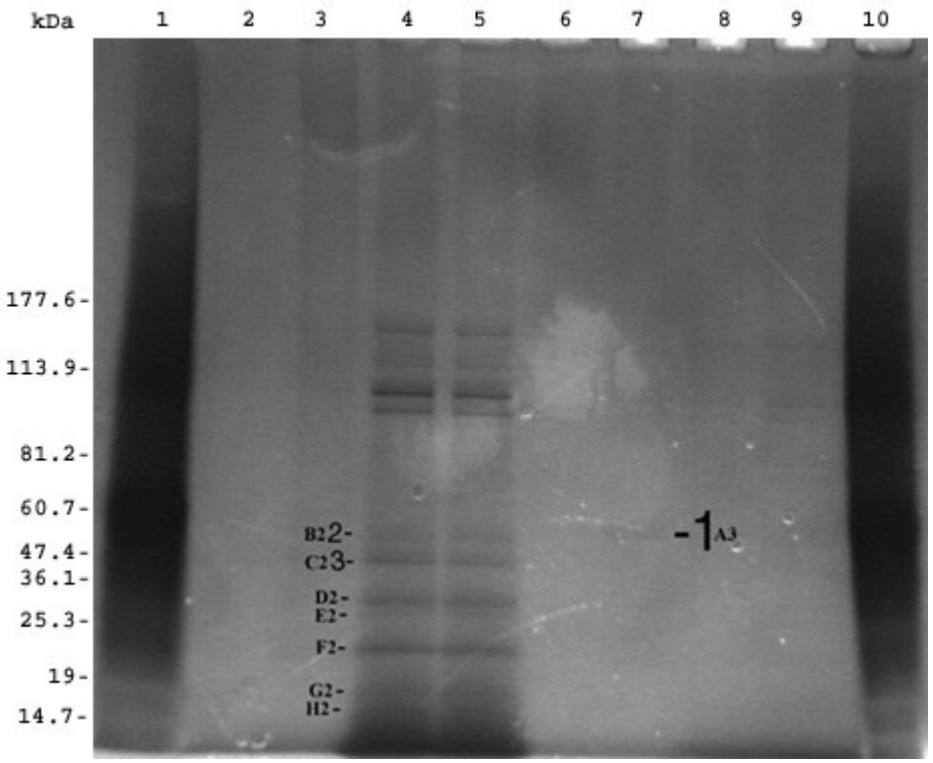

Fig. 12. Silver stained 8-12% polyacrylamide gel with the numbered bands selected for MALDI-TOF mass spectral analysis from the experiment with prepared NTHi membranes. Lanes 1 and 10 are molecular weight standards; lanes 2 and 3 are 0.22 μm filtered fraction; lanes 4 and 5 are pellets obtained from 0.22 μm filtered fraction; lanes 6 and 7 are >100-kDa fraction supernatant; lanes 8 and 9 are >100-kDa fraction pellet. *hemY* was found as the search result from band C23 after mass spectral analysis. This representative gel corresponds to Fig. 11.

D. Analysis of the Role of Selected Proteins

Outer membrane protein P2

Omp P2, found from the 0.5-M NaCl wash fraction during ion exchage chromatography, is the most abundant and highly variable major outer membrane protein of NTHi. Various studies have been performed that deal with the structural and transport characteristics of this protein (27). We chose to study this protein in terms of its contribution to IL-8 activity from human respiratory epithelial cells, as no such study has been previously performed dealing with this aspect of Omp P2. To analyze the expression of IL-8 activity by Omp P2, four different strains were studied. The first two strains were the wild type (13P24HI) and the mutant type (13P24HI-P2 mutant) of a clinical isolate strain obtained from a patient with COPD (123, 124). The other two strains were the Omp P2 mutant and the wild type from *H. influenzae* strain Rd (123, 124). We used mutants of Omp P2 in *H. influenzae* strains, whereas for other proteins (ClpB, RelA, TonB and FtsH), we chose recombinant *E. coli* to express the *H. influenzae* genes. This decision was based on the availability of Omp P2 mutants from Dr. Tim Murphy's laboratory (123, 124). Comparison of the IL-8 stimulation profile for all strains is shown in Fig. 13.

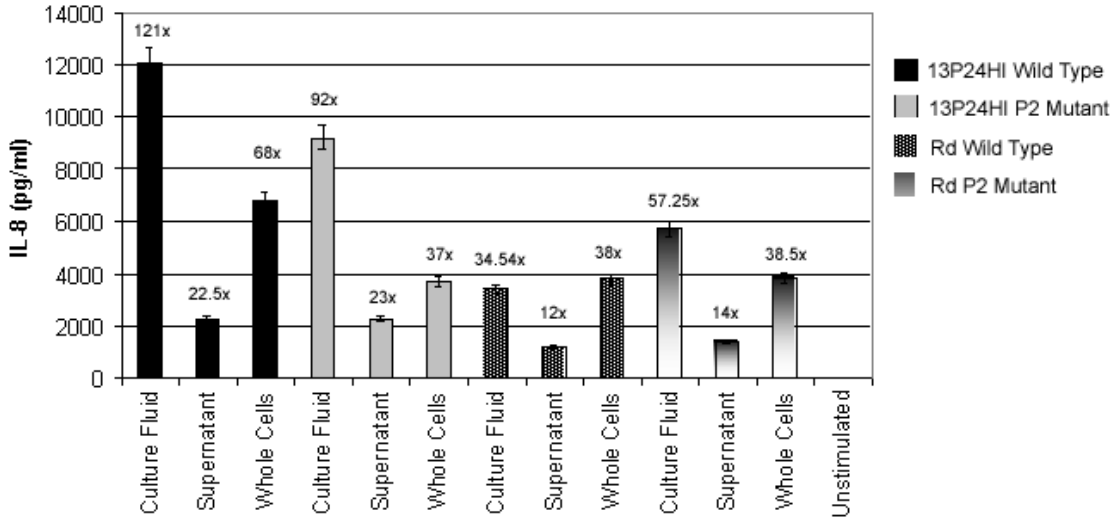

Fig. 13. Role of P2 in IL-8 secretion in *Haemophilus influenzae* from a clinical isolate strain 13P24H1 and strain Rd. 6 x 10^5 human respiratory epithelial cells 9HTE0- were coincubated with 6 x 10^7 bacterial cells, and the wells were stimulated with culture fluid (CF), cell-free supernatant fluid, and whole cells (WC) obtained from the various bacterial strains. Tissue culture medium was used alone (unstimulated), and 20 ng of IL-1β per ml was used as the positive control (IL-8 secretion = 11,505 pg/ml). The chemokine IL-8 activity was assayed with commercially available ELISA kits. The results were replicated three times, and this is one of the representative data from one of those three. The difference while comparing IL-8 expression between the following fractions was also found to be statistically significant [WC from strain 13P24HI–WC from strain 13P24HI P2 mutant ($p < 0.04$); CF from strain Rd–CF from strain Rd P2 mutant ($p < 0.05$); CF from strain 13P24HI–CF from strain 13P24HI P2 mutant ($p < 0.03$)].

The result from this experiment showed that culture fluid (CF) from the wild-type 13P24HI clinical isolate strain obtained from COPD patients induced a 121-fold increase in IL-8 secretion, compared to a 92-fold increase by the same fraction in the *P2* mutant strain 13P24HI over the unstimulated controls in 9HTE0- cells after 16 h incubation ($p < 0.003$). The whole cells from the wild-type 13P24HI clinical isolate strain induced a 68-fold increase in IL-8 secretion, compared to a 37-fold increase by the 13P24HI *P2* mutant strain over unstimulated controls ($p < 0.001$). On the other hand, culture fluid (CF) from the wild-type strain Rd induced a 34.5-fold increase in IL-8 secretion, compared to a 57.25-fold increase by *P2* mutant strain Rd when compared over unstimulated controls in 9HTE0- cells ($p < 0.01$). The difference while comparing IL-8 expression between the following fractions was also found to be statistically significant [WC from strain 13P24HI – WC from strain 13P24HI *P2* mutant ($p < 0.04$); CF from strain Rd – CF from strain Rd *P2* mutant ($p < 0.05$); CF from strain 13P24HI – CF from strain 13P24HI *P2* mutant ($p < 0.03$)].

The supernatant fractions from all the strains showed very little difference in the induction of IL-8 activity, and their differential values were not statistically significant. The negative control (unstimulated monolayers of 9HTEo- cells) showed no detectable IL-8 activity. The responses of IL-8 activity from all three fractions (culture fluid, supernatant, and whole cells) in both the mutant and the wild-type strains after each of them were compared with the unstimulated control were statistically significant ($p < 0.01$). These results suggest that Omp P2 plays a positive role in elevation of the cytokine IL-8 in the clinical strain 13P24HI isolated from a COPD patient. In contrast, the *ompP2* mutant from strain Rd showed higher activity in the secretion of cytokine.

ClpB

ClpB was selected for study because it is a stress protein (heat shock) that is found in almost all organisms studied to date (22). Various studies have shown that ClpB, a molecular chaperone, is a highly conserved heat shock protein that is essential for thermotolerance in bacteria and eukaryotes (22). In an *in vitro* study of ClpB in *E. coli*, close resemblance was found with another group of chaperones, like DnaK, DnaJ, and GrpE. These proteins, along with ClpB, worked together in suppressing and reversing aggregation of proteins during heat shock (152). Recently, work by Yuan et al. showed that the *clpB* gene from *Porphyromonas gingivalis* is involved with the capacity to invade human epithelial and endothelial cells and thus could play some key role in periodontal disease (73).

To assess the role of *H. influenzae clpB* gene in the stimulation of epithelial cell cytokine production, sets of *E. coli* strain JM109 were tested with and without the *clpB* gene inserts (Table 1). The data for the IL-8 secretion obtained after ELISA is presented in Fig. 14. Compared to the unstimulated fraction, the epithelial cells stimulated with *E. coli* cells transformed with the plasmid having the *clpB* gene insert showed approximately a 24-fold increase, whereas epithelial cells stimulated with *E. coli* transformed with pGEM-T Easy plasmid with no inserts showed 11.46-fold more production of IL-8 over the untreated control. The 9HTEo- cells treated with *H. influenzae* strain Rd whole cells demonstrated a 35-fold stimulation of IL-8 over the unstimulated control. The results obtained after comparison of the unstimulated control fraction with that of recombinant *E. coli* containing *H. influenzae* strain Rd *clpB* gene were statistically significant ($p < 0.05$). The comparison between IL-8 expression

responses from the recombinant *E. coli* cells with the *clpB* gene and *E. coli* cells without the *clpB* gene was statistically significant ($p < 0.03$).

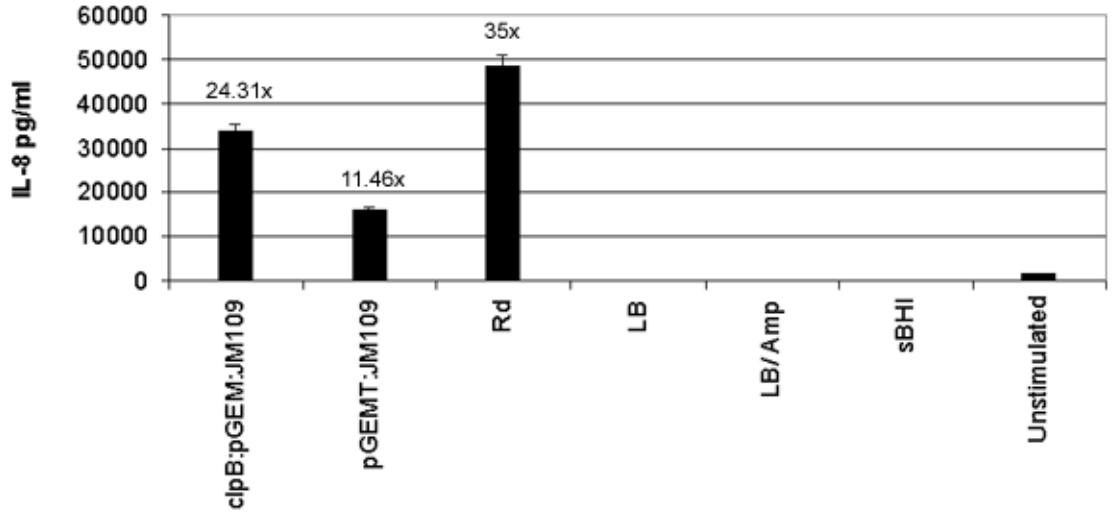

Fig. 14. Experiment showing the role of clpB gene in IL-8 induction. Cultured human respiratory epithelial cells (9HTEo-) were coincubated with 6 X 107 CFU of bacterial cells (*E. coli/H.influenzae* strain Rd). The number of epithelial cells used was 6 X 105 CFU/well. This corresponds to a 100:1 bacterial to epithelial cell ratio; bacterial growth medium (LB and sBHI); bacterial growth medium supplemented with antibiotic (ampicillin), and tissue culture medium only (unstimulated). Twenty nanograms per milliliter of IL-1β per milliliter was used as positive control (IL-8 production = 22,004.05 pg/ml). After coincubation time of 16 h, supernatant fluid was harvested to test the IL-8 activity with a commercially available IL-8 kit. This experiment is a representative from one of the three similarly performed experiments. The difference found with comparison between IL-8 expression levels from the recombinant *E. coli* cells with the *relA* gene and *E. coli* without the *relA* gene was statistically significant ($p < 0.003$).

RelA

RelA is a protein that is expressed under the condition of starvation for amino acids or other compounds (21, 115, 117). Activation of the *relA* gene further leads to the expression of other virulence genes (121). Studies have shown the *relA* gene contributing toward pathogenesis in bacteria like *Vibrio cholera Pseudomonas aeruginosa* and *Legionella pneumophila* (38, 58, 60, 147, 148).

To assess the contribution of RelA in the stimulation of cytokine secretion from human respiratory epithelial cells, the *relA* gene from *H. influenzae* strain Rd was amplified and cloned into plasmid pGEM-T Easy and transformed into *E. coli* strain JM109. The data showing the secretion pattern for IL-8 are presented in Fig. 15.

The *E. coli* strain carrying the *H. influenzae relA* gene showed 24.31-fold greater IL-8 secretion when compared to the unstimulated control (Fig. 15). The *E. coli* strain lacking the *H. influenzae relA* gene showed only 11.46-fold greater IL-8 secretion over the unstimulated control. The difference found with comparison between IL-8 expression levels from the recombinant *E. coli* cells with the *relA* gene and those of *E. coli* without the *relA* gene was statistically significant ($p < 0.003$). The results obtained after each of the fractions (*E. coli* with and without *H. influenzae relA* gene) was compared with the unstimulated control were statistically significant ($p < 0.003$).

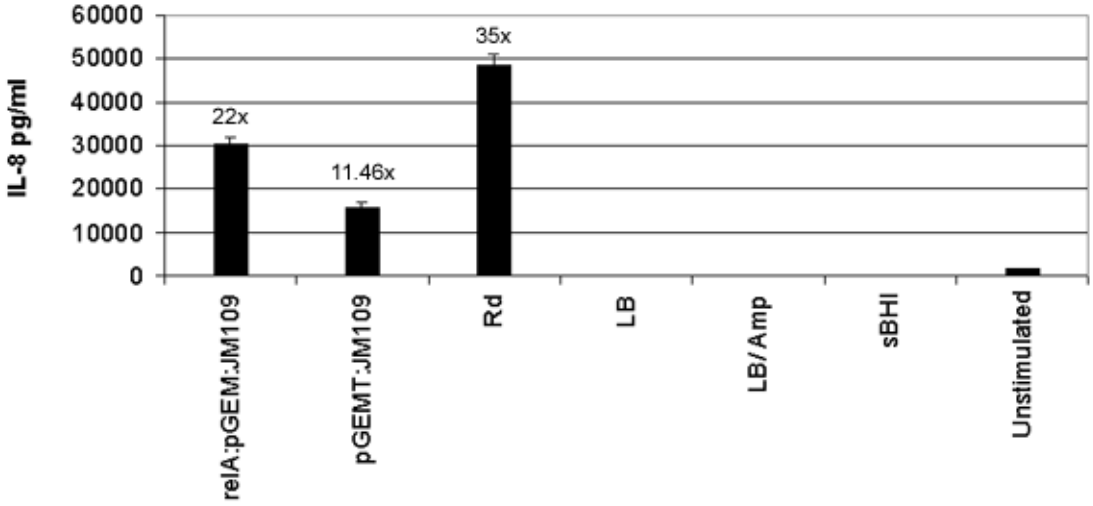

Fig. 15. Experiment showing the role of *relA* gene in IL-8 induction. The same standard was followed as was mentioned in Fig. 14. The data shown are from one experiment of three similar performed experiments. The difference found with comparison between IL-8 expression levels from the recombinant *E. coli* cells with *relA* gene and *E. coli* without *relA* gene was statistically significant ($p < 0.003$).

TonB

TonB plays a key role in *H. influenzae* heme transport. Inactivation of the *tonB* gene restricts heme utilization and aerobic growth of *H. influenzae* (77). To assess the contribution of TonB in the stimulation of cytokine secretion from human respiratory epithelial cells, the *tonB* gene from *H. influenzae* strain Rd was amplified and cloned into plasmid and transformed into *E. coli* strain JM109 pGEM-T Easy (Fig. 16).

Secretion of IL-8 from 9HTEo- cells after coincubation of 16 hrs followed by ELISA showed that this cytokine was 20-fold more stimulated from the *E. coli* strain JM109 carrying the *relA* gene and 11.46-fold more stimulated in the same strain deficient of *tonB* gene when compared to the unstimulated control. The difference in the values obtained between IL-8 induction from the recombinant *E. coli* cells with *tonB* gene and *E. coli* cells without *tonB* gene was statistically significant ($p < 0.005$). The results obtained after each of the fractions (*E. coli* with and without *H. influenzae tonB* gene) were compared with the unstimulated control were statistically significant ($p < 0.003$).

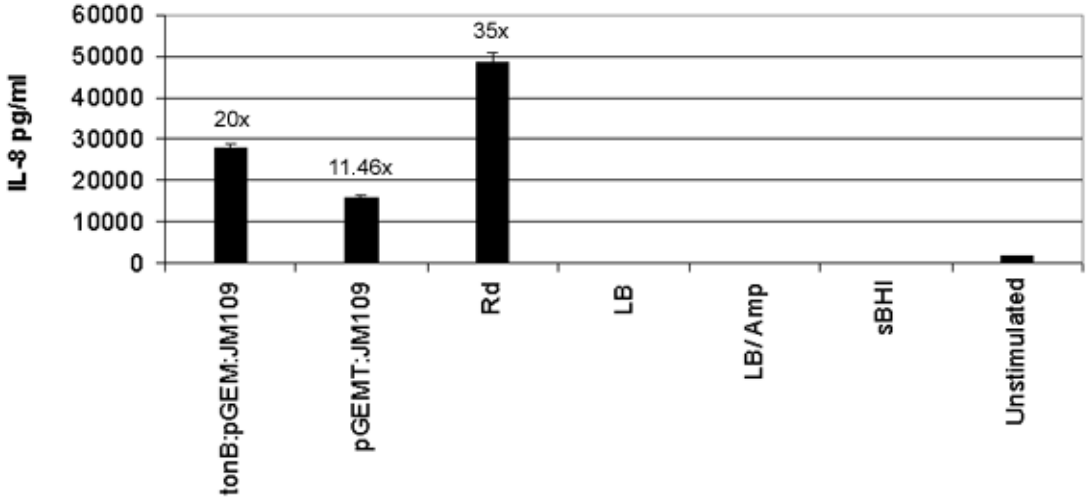

Fig. 16. Experiment showing the role of *tonB* gene in IL-8 induction. The same standard was followed as was mentioned in Fig. 14. The data shown are from one experiment of three similar performed experiments. The difference in the values obtained between IL-8 induction from the recombinant *E. coli* cells with *tonB* gene and *E. coli* cells without *tonB* gene was statistically significant ($p < 0.005$).

FtsH

FtsH is an ATP-dependent protease that degrades other integral membrane proteins (44, 51). Bacterial cells respond to the accumulation of proteins in both membranes and cytosol; FtsH degrades these accumulated membrane proteins (3). The absence of the *ftsH* gene has shown many defects, namely, reduced viability under starvation conditions (91). These defects include slower growth rates, sensitivity to stress conditions like salt and acids, and enhanced expression in the murine skin lesion model of pathogenecity (91). In wild-type *E. coli* cells, FtsH has been found to exist as a large, complex holo-enzyme (136).

To analyze the contribution of the *ftsH* gene from *H. influenzae* to cytokine secretion, sets of *E. coli* strains (Table 1) were studied with and without this gene from *H. influenzae* strain Rd (Fig. 17). No difference was seen between the *E. coli* strains containing the *H. influenzae* strain Rd *ftsH* gene and those lacking it. All the results obtained after each of the fractions was compared with the unstimulated control were statistically significant ($p < 0.05$). Also, the comparison between IL-8 secretion from the recombinant *E. coli* cells with the *ftsH* gene and *E. coli* cells lacking the *ftsH* gene were found to be statistically not significant.

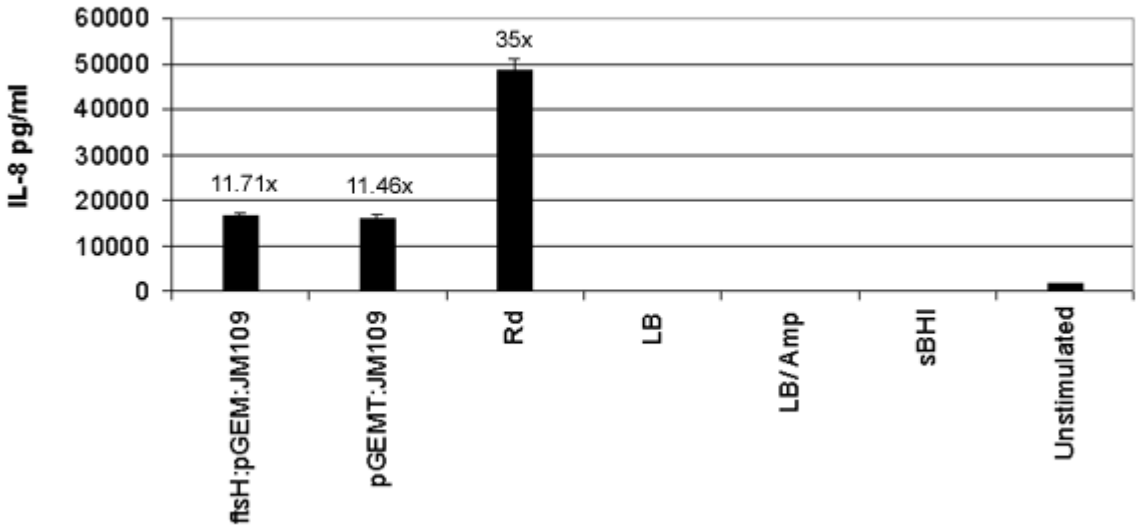

Fig. 17. Experiment showing the role of *ftsH* gene in IL-8 induction. The same standard was followed as was mentioned in Fig. 14. The comparison between IL-8 secretion from the recombinant E. coli cells with the *ftsH* gene and *E. coli* cells lacking the *ftsH* gene were found to be statistically not significant. The data shown are from one experiment of three similar performed experiments.

CHAPTER 4

DISCUSSION

H. influenzae is a major respiratory pathogen associated with chronic obstructive pulmonary disease. Better knowledge of how this bacteria causes pathogenesis will provide new insights into the development of new medicines and adjunct therapies to successfully treat the respiratory diseases caused by it. In this study, we have identified several proteins that were associated with *H. influenzae* and initiated a systematic study of those putative modulins on respiratory epithelial cytokine stimulation.

Studies by Clemans et al. have shown that NTHi LOS is responsible for approximately 50% of the proinflammatory cytokine stimulation from human respiratory epithelial cells and also that bacterial adherence did not play any specific role in cytokine production (23). Further studies suggested that in *H. inflluenzae* strain Rd modulin activity was greater than 100-kDa in size and had a pI < 8.5 (74, 112). MALDI-TOF mass spectrometry was used to identify 27 putative *H. influenzae* modulins ranging in size from 25.3-kDa to 110-kDa. The putative modulins were represented by proteins from all parts of the bacterial cell, including those from membrane fractions, cytoplasm, and periplasm (Table 3). Seventeen proteins were obtained from the searches in which MIc liquid medium was used for culturing *H. influenzae.* Six and 16 proteins were recovered from a growth medium containing sBHI and UF sBHI. These data further refine the initial studies with the >100-kDa fraction and suggest that proteins other than secreted proteins participate toward induction of IL-8 secretion from respiratory epithelial

cells. Another reason for the presence of cytoplasmic proteins in the secretory fraction could be that as cells grow old and lyse, the cytoplamic proteins might be released in the supernatant fluid (Table 3). A recent study has shown that a soluble cytoplasmic fraction (SCF) from NTHi stimulated higher cytokine IL-8 induction from human epithelial cells than did LOS and other envelope proteins from NTHi (143). Therefore, the high activity from the supernantant fraction in the ultracentrifugation experiment could be related to the fact that it consisted of cytoplasmic proteins (Fig. 11).

Furthermore, the cell walls of *H. influenzae* release outer-membrane vesicles (50-250 nm in diameter and spherical, bilayered and membranous structure) from their cell surfaces during growth (16, 79, 149). These membrane vesicles, while blebbing from the outer membrane, carry with them small parts of Gram-negative cell wall consisting of LPS, periplasmic constituents, phospholipids, cytoplasmic components, and membrane proteins (149). This could be one of the reasons that the pellet fraction obtained after ultracentifugation at 100,000 xg showed such high IL-8 value (Fig. 11); it could have contained membrane-associated proteins like Omp P2, Omp P5, and Omp P6, all of which have shown high IL-8 activity from macrophages (14, 45).

Outer-membrane proteins play an important role in the establishment of bacteria in the host environment (90). Omp P2 is the most abundant outer-membrane protein in *H. influenzae* and varies in molecular weight from 36-kDa to 42-kDa (99). In addition, Omp P2 is associated with binding of bacterial cells to human nasopharyngeal mucin (93). Our studies showed that the *omp P2* mutant from strain 13P24HI (isolated from COPD patient) showed lower IL-8 stimulation when compared to the wild-type strain of 13P24HI. This result suggests that Omp P2 plays a role in IL-8 stimulation.

In contrast, however, the *omp P2* mutant from strain Rd showed higher IL-8 activity when compared to wild-type strain Rd (Fig. 13). One possibility behind the higher IL-8 activity from the strain Rd mutant compared to the wild type is that in absence of Omp P2, other proteins, like Omp P5 and Omp P6, become dominant and are overexpressed. Both Omp P5 and Omp P6 have been shown to stimulate epithelial cytokine secretion (14, 45). Studies have also found that IL-8 produced by Omp P6 is comparable to the IL-8 induced by total Omps and LOS. This induction was many-fold higher than the IL-8 stimulated by Omp P2 (14).

Another reason behind the differences in the pattern of IL-8 activity from one strain to another could be the fact that the amino acid sequences of Omp P2 vary considerably from one strain to another because of accumulation of point mutations in specific loops of the protein structure (5). In *H. influenzae,* the variations due to mutations were clustered in the surface-exposed amino acid chains or loops (13). During the occurrence of chronic bronchitis, these amino acid changes were found for the coding region for loops 5 and 6 of the protein, which resulted in antigenic drift (35, 127). Apart from this, variation in the clonal population has also been seen during the time of an existing infection (35). Depending upon the strain, it might either upregulate or down-regulate the IL-8 stimulation mechanism from the respiratory epithelial cells. Studies in the past have shown that under *in vitro* conditions, *omp P2* mutants are living but attenuated when dealing with the animal models, therefore suggesting an important role for bacterial physiology and infection during *in vivo* conditions (140). Recent study by Berenson et al. showed Omp P2 purified from NTHi 1479 only marginally induced the IL-8 activity in human macrophages (14).

Even though *H. influenzae* shows dissimilarities in the IL-8 induction pattern in the culture fluid and whole cells fraction in both the clinical isolate strain 13P24HI and strain Rd, the supernatant fractions continued to generate almost the same level of IL-8 activity from human respiratory epithelial cells. This could be because Omp P2 is not secreted but is a membrane-associated protein (99). Therefore, this might be the factor for which the graph does not demonstrate much difference in the IL-8 activity in the secreted fraction devoid of whole cells in both types of strains, including their mutants. Further fractionation experiments can be performed in order to understand the distribution of Omp P2 in the cell.

In future, *in vitro* studies can be performed with other cell lines to study the IL-8 pattern generated upon stimulation with Omp P2 protein. Purified Omp P2 from different NTHi strains can also be used to study the stimulation pattern of proinflammatory cytokines from various strains. Further *in vivo* studies can be performed to detect cytokine response by raising anti-Omp P2 antibodies. These experiments will shed new light on how Omp P2 plays a role in inflammation and cytokine regulation in the animal-model system.

Like P2, another protein obtained after mass spectral analysis, called ClpB, was also studied to determine its activity toward stimulation of IL-8 from human respiratory epithelial cells. But unlike the *Omp P2* gene, which was inactivated in *H. influenzae* itself, the *clpB* gene from *H. influenzae* was cloned in *E. coli* strain JM109. Many studies have been performed in which *H. influenzae* genes were overexpressed in *E. coli* cells (89). Several studies have been performed to determine the activity of this ATP-dependent molecular chaperone in rescuing proteins that were damaged by heat-shock.

No work has been performed yet to understand whether ClpB plays any role in epithelial cytokine production (103, 105, 106, 118, 141). Apart from stress tolerance, in many Gram-negative bacteria, proteins secreted from a type III secretion system (a factor involved in pathogenesis) have been shown to require small cytosolic chaperones to maintain the level of secreted substrates in a secretion-competent state (32). Clp-mediated proteolysis has been shown to be involved in the virulence of several bacterial pathogens by helping survival inside the host environment or turning the activity of virulence-causing factors (137). For example, studies have found ClpB responsible for regulation of virulence in pathogens like *Yersinia enterocolitica*, in which it affects bacterial invasiveness and motility (8). In the bacterium *Salmonella enterica* serovar Typhimurium, the inactivation of the *clpP* gene (belonging to the same family as the *clpB* gene) showed inhibition of growth and survival inside macrophages (70, 150).

In our study, we transformed *E. coli* with the plasmid containing the *clpB* gene amplified from *H. influenzae* strain Rd to study the role of this gene in cytokine IL-8 production. One advantage in choosing *E. coli* was that this bacterium possesses two small heat-shock proteins (IbpA and IbpB) that aid in the production and stability of recombinant proteins (94). Our studies suggested that the ClpB from *H. influenzae* strain Rd played a positive role in IL-8 stimulation. The presence of this gene almost doubled the amount of cytokine IL-8 production when 9HTEo- monolayers were stimulated with the recombinant bacterial strain.

The interpretation of these results is based on the premise that the *H. influenzae* *clpB* gene is expressed in *E. coli*. Care was taken to clone the entire gene with its promoter, but confirmation of the expression of this gene in *E. coli* has yet to be done.

The study of RelA also brought some interesting information in regard to cytokine IL-8 induction that was previously unknown to us. RelA stimulates the production of the nucleotide guanosine tetraphosphate (ppGpp) during the condition of amino acid starvation or other forms of nutrient limitation that cause arrest in the growth (21). RelA plays an important role during this type of stringent response by associating itself with ribosome to further produce ppGpp (21). Studies have also shown that ppGpp plays a vital role in controlling different virulence features in several bacteria (49).

This study demonstrated that the *E. coli* strain carrying the *H. influenzae* strain Rd *relA* gene showed a little over two times more cytokine production compared to the strain that carries no *relA* gene. This suggests that RelA plays a positive role in cytokine stimulation from human respiratory epithelial cells monolayers. As with ClpB, the reasoning behind this result supporting the contribution of RelA in IL-8 induction is based upon the premise that the *relA* gene is expressed in *E. coli*, as care was taken to include the promoter sequence. Further experiments are needed in order to verify the expression of the *H. influenzae relA* gene in *E. coli* cells. Also, because were using a high-copy-number plasmid, the *relA* gene might be overexpressed when compared to normal *in vivo* conditions. Therefore, future *in vivo* studies are required in order to understand the specific role of this gene in cytokine stimulation in COPD patients.

NTHi has a strict requirement for heme when cultured under aerobic conditions (40). Studies have shown the lack of the ability to utilize heme after inactivation of the *tonB* gene in NTHi (77). A functional *H. influenzae tonB* gene is required for this bacterium to grow with transferrin as the sole source of iron (76). TonB is considered a potent virulence factor in *H. influenzae* because of its role in the uptake of iron, heme,

hemoglobin, transferrin, and various other vital metabolites (25, 76, 77, 149). Beyond these roles of the TonB in the utilization of heme and other metabolites, our study suggested that TonB also played a role in the induction of cytokine IL-8 from human respiratory epithelial cells. This addresses the fact that TonB has some contribution in the overall ability of *H. influenzae* to stimulate proinflammatory responses. This evaluation is based upon the fact that the *tonB* gene from *H. influenzae* is expressed inside the *E. coli* cells. Further analysis is required in order to confirm the expression.

Further study can be performed to understand the role of this protein in induction of other cytokines that are involved during the pathogenesis caused by NTHi. Stimulation of human respiratory epithelial cells with purified TonB can also be done to quantitate the level of IL-8 induction by this protein. *In vivo* analysis can be perfomed with anti-TonB antibodies in rabbit or rat models to study the inflammation during the course of an infection.

Unlike ClpB, RelA, and TonB, FtsH showed no similar contribution to induction of IL-8 from human repiratory epithelial cells. Though several studies have shown the expression of *H. influenzae* genes in *E. coli* cells (36) and care was taken to include the promoter sequences from this gene, experiments need to be performed to verify its expression. Further experiments must be performed with FtsH in human monocytes and macrophages to analyze any IL-8 induction from them. This protein moreover can be purified with methods that retain its antigenic reactivity to further study the proinflammatory cytokine induction.

This overall study dealt with the important aspect of identifying and understanding the roles of selected protein molecules that play a role in stimulating an

elevated inflammatory response from human respiratory epithelial cells. Further experiments must be performed to understand the other components that might be present in the secreted fraction that could be responsible for such high IL-8 activity after stimulation of epithelial cells with that fraction. The other proteins found from proteome analysis (Table 3) can also be studied to understand their contributions to induction of various cytokines and other mediators. These potential proteins can also be studied to find out if they play any role in activating various signaling pathways that could play critical roles in regulating the gene expression of various cytokines and chemokines. Further, *in vivo* analysis will help us to understand how these proteins affect the host and could prove to be very important in our understanding of severe respiratory diseases like COPD.

CHAPTER 5

CONCLUSION

This study throws light on the fact that there are many proteins that are or could be involved in the process of stimulation of IL-8 from human respiratory epithelial cells. Roles of some specific proteins that show contributions to elevation of IL-8 activity were recognized in this study. Our findings support the fact that many factors are involved in the complicated cellular interaction between the host and NTHi. Identifying the role(s) played by the proteins associated with NTHi in inflammatory response will futher help studies involved with the development of new drugs and treatments for the patients suffering with severe respiratory diseases caused by NTHi.

REFERENCES

1. Aaron, S., J. B. Angel, M. Lunau, K. Wright, C. Fex, N. Saux, and R. E. Dales. 2001. Granulocyte inflammatory markers and airway infection during acute exacerbation of chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **163:**349-55.
2. Adams, P. F., G. E. Hendershot, and M. A. Marano. 1999. Current estimates from the National Health Interview Survey. Vital Health Stat. **10:**1-212.
3. Akiyama, Y., and I. Koreaki. 2000. Roles of multimerization and membrane association in the proteolytic functions of FtsH (HflB). EMBO J. **19:**3888-3895.
4. Andersen, C., E. Maier, G. Kemmer, J. Blass, A. K. Hilpert, R. Benz, and J. Reidl. 2003. Porin Omp P2 of *Haemophilus influenzae* shows specificity for nicotinamide-derived nucleotide substrates. J. Biol. Chem. **278:**24269-24276.
5. Annette, G. R., D. Dahan, L.V. M. Möller, J. W. Coulton, P. Eijk, P. V. Ulsen, J. Dankert, and L. V. Alphen. 1999. Variation in the composition and pore function of major outer membrane pore protein P2 of *Haemophilus influenzae* from cystic fibrosis patients antimicrobial agents and chemotherapy. Antimicrob. Agents Chemother. **43:**226-232.
6. Aubrey, R., & C. Tang. 2003. The pathogenesis of disease due to type b *Haemophilus influenzae.* Methods Mol. Med. **71**:29-50.
7. Aubrey, R., & C. Tang. 2003. The pathogenesis of disease due to type b *Haemophilus influenzae.* In M. A. Herbert, D. W. Hood, & R. E. Moxon (Eds.),

Haemophilus influenzae protocols (p. 35-36). Totowa, New Jersey: Humana Press.

8. Badger, J. L., B. M. Young, A. J. Darwin, and V. L. Miller. 2000. *Yersinia enterocolitica* ClpB affects levels of invasin and motility. J. Bacteriol. **182:**5563-71.

9. Barcak, G. J., M. S. Chandler, R. J. Redfield, and Tomb J. F. 1991. Genetic systems in *Haemophilus influenzae*. Methods Enzymol. **204:**321-42

10. Barenkamp, S.J. and J.W. St. Geme, III. 1996. Identification of a second family of high-molecular-weight adhesion proteins expressed by nontypeable *Haemophilus influenzae*. Mol. Microbiol. **19:**1215-1223.

11. Barenkamp, S. L., and E. Leininger. 1992. Cloning, expression, and DNA sequence analysis of genes encoding nontypeable *Haemophilus influenzae* high-molelular-weight proteins related to filamentous hemagglutinin of *Bordatella pertussis*. Infect. Immun. **60:**1302-1313.

12. Barry, A. L., M. A. Phaller, P. C. Fuchs, and R. R. Parker. 1994. *In vitro* activities of 12 orally administered agents against four species of bacterial respiratory pathogens from US medical centers in 1992 and 1993. Antimicrob. Agents. Chemother. **38:**2419-2425.

13. Bell, J., S. Grass, D. Jeanteur, and R. S. Munson, Jr. 1994. Diversity of the P2 protein among nontypeable *Haemophilus influenzae* isolates. Infect. Immun. **62:** 2639-2643.

14. Berenson, C. S., T. F. Murphy, C. T. Wrona, and S. Sethi. 2005. Outer membrane protein P6 of nontypeable *Haemophilus influenzae* is a potent and

selective inducer of human macrophage proinflammatory cytokines. Infect. Immun. **73:**2728-2735.

15. Bernstein, J. M., R. Hard, Z. D. Cui, N. So, J. Fisher, and P. L. Ogra. 1990. Human adenoidal organ culture: a model to study nontypable *Haemophilus influenzae* (NTHI) and other bacterial interactions with nasopharyngeal mucosa--implications in otitis media. Otolaryngol. Head Neck Surg. **103:**784-791.

16. Beveridge, T. J., and J. L. Kadurugamuwa. 1996. Periplasm, periplasmic spaces, and their relation to bacterial wall structure: novel secretion of selected periplasmic proteins from *Pseudomonas aeruginosa*. Microb. Drug Resist. **2:**1-8.

17. Bonfield, J. K., and R. Staden. 1996. Experiment files and their application during large-scale sequencing projects. DNA Seq. **6:**109-17.

18. Brandtzaeg, P. 1992. Humoral immune response patterns of human mucosae: induction and relation to bacterial tract infections. J. Infect. Dis. **165:**S167-S176.

19. Broug-Holub, E., G. B. Toews, J. F. van Iwaarden, R. M. Stricter, S. L. Kunkel, R. Paine, III, and T. J. Standiford. 1997. Alveolar macrophages are required for protective pulmonary defenses in murine *Klebsiella pneumonia*: elimination of alveolar macrophages increases neutrophil recruitment but decreases bacterial clearance and survival. Infect. Immun. **65:**1139-46.

20. Poje, G., and R. J. Redfield. 2003. General methods for culturing *Haemophilus influenzae.* Methods Mol. Med. **71**:51-6.

21. Cashel, M. 1996. The stringent response. In *Escherichia coli* and salmonella: cellular and molecular biology (p 1458-1496). Washington DC, ASM Press.

22. Clarke, A. K., and M. J. Eriksson. 2000. The truncated form of the bacterial heat shock protein ClpB/HSP100 contributes to development of thermotolerance in the cyanobacterium *Synechococcus* sp. strain PCC 7942. J. Bacteriol. **182:**7092-7096.

23. Clemans, D. L., R. J. Bauer, J. A. Hanson, M. V. Hobbs, J. W. St. Geme, III, C. F. Marrs, and J. R. Gilsdorf. 2000. Induction of proinflammatory cytokine from human respiratory epithelial cells after stimulation by nontypeable *Haemophilus influenzae*. Infect. Immun. **68:**4430-4440.

24. Collins, F. S. 1992. Cystic fibrosis: molecular biology and therapeutic implications. Science **256:**774-9.

25. Cope, L. D., R. Yogev, U. Muller-Eberhard, and E. J. Hansen. 1995. A gene cluster involved in the utilization of both free heme and heme:hemopexin by *Haemophilus influenzae* type b. J. Bacteriol. **177:**2644-2653.

26. Cope, L. D., S. E. Thomas, J. L. Latimer, C. A. Slaughter, U. Muller-Eberhard, and E. J. Hansen. 1994. The 100-kDa haem: haemopexin-binding protein of *Haemophilus influenzae*: structure and localization. Mol. Microbiol. **13:**863-73.

27. Coulton, J. W., A. C. Chin, and V. Vachon. 1992. Recombinant porin of *Haemophilus influenzae* type b. J. Infect. Dis. **165:**S188-S191.

28. Coulton, J. W., and J. C. S. Pang. 1983. Transport of hemin by *Haemophilus influenzae* type b. Curr. Microbiol. **9:**93-98.

29. Cromwell, O., Q. Hamid, C. J. Corrigan, J. Barkans, Q. Meng, P. D. Collins, and A. B. Kay. 1992. Expression and generation of interleukin-8, IL-6 and granulocyte-macrophage colony-stimulating factor by bronchial epithelial cells and enhancement by IL-Iβ and tumor necrosis factor-α. Immunology **77:**330-7.

30. Crooks, S. W., D. L. Bayley, S. L. Hill, and R. A. Stockley. 2000. Bronchial inflammation in acute bacterial exacerbations of chronic bronchitis: the role of leukotriene B4. Eur. Respir. J. **15:**274-280.

31. Davis, B. D., R. Dulbecco, H. N. Eisen, H. S. Ginsberg, and W. B. Wood, Jr. 1973. The *Haemophilus-Bordetella group* . Microbiology **9:**792-800.

32. Delahay, R. M., R. K. Shaw, S. J. Elliott, J. B. Kaper, S. Knutton, and G. Frankel. 2002. Functional analysis of the enteropathogenic *Escherichia coli* type III secretion system chaperone CesT identifies domains that mediate substrate interactions. Mol. Microbiol. **43:**1365-2958.

33. Di Stefano, A., A. Capelli, M. Lusuardi, P. Balbo, C. Vecchio, P. Maestrelli, C. E. Mapp, L. M. Fabbri, C. F. Donner, and M. Saetta. 1998. Severity of airflow limitation is associated with severity of airway inflammation in smokers. Am. J. Respir. Crit. Care. Med. **158**:1277-85.

34. DiMango, E., H. J. Zar, R. Bryan, and A. Prince. 1995. Diverse *Pseudomonas aeruginosa* gene products stimulate respiratory epithelial cells to produce interleukin-8. J. Clin. Invest. **96:**2204-10.44.

35. Duim, B., L. V. Alphen, P. Eijk, H. M. Jansen, and J. Dankert. 1994. Antigenic drift of non-encapsulated *Haemophilus influenzae* major outer membrane protein P2 in patients with chronic bronchitis is caused by point mutations. Mol. Microbiol. **11:**1181-1189.

36. El-Adhami, W., J. M. Kyd, D. A. Bastin, and A. W. Cripps. 1999. Characterization of the gene encoding a 26-kilodalton protein (OMP26) from

nontypeable *Haemophilus influenzae* and immune responses to the recombinant protein. Infect. Immun. **67:**1935-1942.

37. Elkins, C. 1995. Identification and purification of a conserved heme-regulated hemoglobin-binding outer membrane protein from *Haemophilus ducreyi*. Infect. Immun. **63:**1241-1245.

38. Erickson, D. L., J. L. Lines, E. C. Pesci, V. Venturi, and D. G. Storey. 2004. *Pseudomonas aeruginosa relA* Contributes to Virulence in *Drosophila melanogaster.* Infect. Immun. **72:**5638-45.

39. Evans, F. O., Jr., J. B. Sydnor, W. E. C. Moore, G. R. Moore, J. L. Manwaring, A. H. Brill, R. T. Jackson, S. Hanna, J. S. Skaar, L. V. Holdeman, S. Fitz-Hugh, M. A. Sande, and J. M. Gwaltney. 1975. Sinusitis of the maxillary antrum. N. Engl. J. Med. **293:** 735-739.

40. Evans, N. M., D. D. Smith, and A. J. Wicken. 1974. Haemin and nicotinamide adenine dinucleotide requirements of *Haemophilus influenzae* and *Haemophilus parainfluenzae.* J. Med. Microbiol. **7:**359-365.

41. Fleischmann, R. D., M. D. Adams, O.White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenny, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. I. Liu, A. Glodek, J. M. Kelley, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritch, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C.

Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496-512.

42. Flesher, A. R., and R. A. Insel. 1978. Characterization of lipopolysaccharide of *Haemophilus influenzae*. J. Infect. Dis. **138:**719-30.

43. Frick, A.G., T. D. Joseph, L. Pang, A. M. Rabe, J. W. St. Geme, III, and D. C. Look. 2000. *Haemophilus influenzae* stimulates ICAM-1 expression on respiratory epithelial cells. J. Immunol. **164:**4185-4196.

44. Gaillot, O. 2004. ATP-dependant proteolysis and bacterial pathogenesis. Ann. Biol. Clin. **62:**7-14.

45. Galdiero, M., E. Finamore, F. Rossano, M. Gambuzza, M. R. Catania, G. Teti, A. Midiri, and G. Mancuso. 2004. *Haemophilus influenzae* porin induces toll-like receptor 2-mediated cytokine production in human monocytes and mouse macrophages. Infect. Immun. **72:**1204-1209.

46. Galdiero, M., M. D'Amico, F. Gorga, C. Di Filippo, M. D'Isanto, M. Vitiello, A. Longanella, and A. Tortora. 2001. *Haemophilus influenzae* porin contributes to signaling of the inflammatory cascade in rat brain. Infect. Immun. **69:**221-227.

47. Giebink, G. S. 1989. The microbiology of otitis media. Pediatr. In. Dis. J. **8:**S18-S20.

48. Gilligan, P. H. 1991. Microbiology of airway disease in patients with cystic fibrosis. Clin. Microbiol. Rev. **4:**35-51.

49. Godfrey, H. P., J. V. Bugrysheva, and F. C. Cabello. 2000. The role of the stringent response in pathogenesis of bacterial infections. Trends Microbiol. **10:**349-351.

50. Gompertz, S., C. O'Brien, D. L. Bayley, S. L. Hill and R. A. Stockley. 2001. Changes in bronchial inflammation during acute exacerbations of chronic bronchitis. Eur. Respir. J. **17:**1112-9.

51. Gottesman, S. 1999. Regulation by proteolysis: developmental switches. Curr. Opin. Microbiol. **2:**142-147.

52. Govan, J. R., and J. W. Nelson. 1992. Microbiology of lung infection in cystic fibrosis. Br. Med. Bull. **48:**912-30.

53. 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.

54. Gray-Owen, S. D., S. Loonsmore, and A. B. Schryvers. 1995. Identification and characterization of genes encoding the human transferrin-binding proteins from *Haemophilus influenzae.* Infect. Immun. **63:**1201-1210.

55. Gruenert, D. C., C. B. Basbaum, M. J. Welsh, M. Li, W. E. Finkbeiner, and J. A. Nadel. 1998. Characterization of human tracheal epithelial cells transformed by an origin-defective simian virus 40. Proc. Natl. Acad. Sci. USA. **85:**5951-5955.

56. Gu, X. X., C. M. Tsai, M. A. Apicella, and D. Lim. 1995. Quantitation and biological properties of released and cell-bound forms of lipooligosaccharides from nontypeable *Haemophilus influenzae*. Infect. Immun. **63:**4115-4120.

57. Hakansson, A., I. Carlstedt, J. Davies, A. K. Mossberg, H. Sabharwal, and C. Svanborg. 1996. Aspects on the interaction of *Streptococcus pneumoniae* and *Haemophilus influenzae* with human respiratory tract mucosa. Am. J. Respir. Crit. Care Med. **154:** S187-S191.

58. Hammer, B. K., E. S. Tateda, and M. S. Swanson. 2002. A two-component regulator induces the transmission phenotype of stationary-phase *Legionella pneumophila.* Mol. Microbiol. **44:**107-18.

59. Hansen, E. J., F. R. Gonzales, N. R. Chamberlain, M. V. Norgard, E. E. Miller, L. D. Cope, S. E. Pelzel, B. Gaddy, and A. Clausell. 1988. Cloning of the gene encoding the major outer membrane protein of *Haemophilus influenzae* type b. Infect. Immun. **56:**2709-2716.

60. Haralalka, S., S. Nandi, and R. K. Bhadra. 2003. Mutation in the relA gene of *Vibrio cholerae* affects in vitro and in vivo expression of virulence factors. J. Bacteriol. **185:**4672-4682.

61. Hardy, G. G., S. M. Tudor, and J. W. St. Geme, III. 2003. The pathogenesis of disease due to nontypeable *Haemophilus influenzae* (p. 2). In M. A. Herbert, D. W. Hood, and R. E. Moxon (Eds.), *Haemophilus influenzae protocols*. Totowa, NJ: Humana Press.

62. Hardy, G. G., S. M. Tudor, and J. W. St. Geme, III. 2003. The pathogenesis of disease due to nontypeable *Haemophilus influenzae* (p. 3). In M. A. Herbert, D. W. Hood, & R. E. Moxon (Eds.), *Haemophilus influenzae protocols.* Totowa, NJ: Humana Press.

63. Hardy, G. G., S. M. Tudor, and J. W. St. Geme, III. 2003. The pathogenesis of disease due to nontypeable *Haemophilus influenzae* (p. 3). In M. A. Herbert, D. W. Hood, & R. E. Moxon (Eds.), *Haemophilus influenzae protocols.* Totowa, NJ: Humana Press.

64. Hardy, G. G., S. M. Tudor, and St. Geme, J. W., III. 2003. The pathogenesis of disease due to nontypeable *Haemophilus influenzae* (p. 5). In M. A. Herbert, D. W. Hood, & R. E. Moxon (Eds.), *Haemophilus influenzae protocols.* Totowa, New Jersey: Humana Press.

65. Hardy, G. G., S. M. Tudor, and J. W. St. Geme, III. 2003. The pathogenesis of disease due to nontypeable *Haemophilus influenzae* (p. 6). In M. A. Herbert, D. W. Hood, & R. E. Moxon (Eds.), *Haemophilus influenzae protocols.* Totowa, NJ: Humana Press.

66. Hartmann, E., and C. Lingwood. 1997. Brief heat shock treatment induces a long-lasting alteration in the glycolipid receptor binding specificity and growth rate of *Haemophilus influenzae*. Infect. Immun. **65:**1729-1733.

67. Hatada, E. N., D. Krappmann, and C. Scheidereit. 2000. NF-kappaB and the innate immune response. Curr. Opin. Immunol. **12:**52-8.

68. Heiss, L. N., S. A. Moser, E. R. Unanue, and W. E. Goldman. 1993. Interleukin-1 is linked to the respiratory epithelial cytopathology of pertussis. Infect. Immun. **61:**3123-3128.

69. Henderson, B., S. Poole, and M. Wilson. 1996. Bacterial modulins: a novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol. Rev. **60:**316-341.

70. Hensel, M., J. E. Shea, C. Gleeson, M. D. Jones, E. Dalton, and D. W. Holden. 1995. Simultaneous identification of bacterial virulence genes by negative selection. Science **269:**400-403.

71. Herrington D. A., and P. F. Sparling. 1985. *Haemophilus influenzae* can use human transferrin as a sole source for required iron. Infect. Immun. **48:**248-51.

72. Howard, A. J., K. T. Dunkin, and G. W. Millar. 1988. Nasopharyngeal carriage and antibiotic resistance of *Haemophilus influenzae* in healthy children. Epidemiol. Infect. **100:**193-203.

73. Identification and characterization of *ClpB* and *HtrA* mutants of *Porphyromonas gingivalis* (L. Yuan, and A. Progulske-Fox, Abstr., Am. Society of Microbiol. 104th General Meeting, abstr. B-183, 2004).

74. Induction of Interleukin (IL)-8 from human tracheal epithelial cells after stimulation of secreted nontypeable *H. influenzae* (C. Perria and D. Clemans, Abstr., Am. Society of Microbiol. 102nd General Meeting, abstr. B-292, 2002).

75. Janson, H., B. Carl, A. Cervin, A. Forsgren, A. B. Magnusdottir, S. Lindberg, and T. Runer. 1999. Effects on the ciliated epithelium of protein D-producing and nonproducing nontypeable *Haemophilus influenzae* in nasopharyngeal tissue cultures. J. Infect. Dis. **180:**737-746.

76. Jarosik, G. P., I. Maciver, and E. Hansen. 1995. Utilization of transferin-bound iron by *Haemophilus influenzae* requires an intact *tonB* gene. Infect. Immun. **63:**710-713.

77. Jarosik, G. P., J. D. Sanders, L. D. Cope, U. Muller-Eberhard, and E. J. Hansen. 1994. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type b. Infect. Immun. **62:**2470-2477.

78. Johnson, A. P., and T. J. Inzana. 1986. Loss of ciliary activity in organ cultures of rat trachea treated with lipo-oligosaccharide from *Haemophilus influenzae*. J. Med. Microbiol. **22:**265-268.

79. Kadurugamuwa, J. L., and T. J. Beveridge. 1997. Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. J. Antimicrob. Chemother. **40:**615-621.

80. Keatings, V. M., P. D. Collins, D. M. Scott, and P. J. Barnes. 1996. Differences in interleukin-8 and tumor necrosis factor-alpha in induced sputum from patients with chronic obstructive pulmonary disease or asthma. Am. J. Respir. Crit. Care Med. **153:**530-4.

81. Khair, O. A., J. L. Devalia, M. M. Abdelaziz, R. J. Sapsford, and R. J. Davies. 1995. Effect of erythromycin on *Haemophilus influenzae* endotoxin-induced release of IL-6, IL-8 and sICAM-1 by cultured human bronchial epithelial cells. Eur. Respir. J. **8:**1451-7.

82. Khair, O. A., J. L. Devalia, M. M. Abdelaziz, R. J. Sapsford, H. Tarraf, and R. J. Davies. 1994. Effect of *Haemophilus influenzae* endotoxin on the synthesis of IL-6, IL-8, TNF-α and expression of ICAM-1 in cultured human bronchial epithelial cells. Eur. Respir. J. **7:**2109-2116.

83. Khair, O. A., R. J. Davies, and J. L. Devalia. 1996. Bacterial-induced release of inflammatory mediators by bronchial epithelial cells. Eur. Respir. J. **9:**1913-1922.

84. Kilbourn, J. P., H. Haas, J. F. Morris, and S. Samson. 1983. *Haemophilus influenzae* biotypes and chronic bronchitis. Am. Rev. Respir. Dis. **128:**1093-1094.

85. Konstan, M. W., and M. Berger. 1997. Current understanding of the inflammatory process in cystic fibrosis: onset and etiology. Pediatr. Pulmonol. **24:**137-42.

86. Krasan, G. P., D. Cutter, S. L. Block, and J. W. St. Geme, III. 1999. Adhesin expression in the matched nasopharyngeal and middle ear isolates of nontypeable *Haemophilus influenzae* from children with acute otitis media. Infect. Immun. **67:**449-454.

87. Kubiet, M., and R. Ramphal. 1995. Adhesion of nontypeable *Haemophilus influenzae* from blood and sputum to human tracheobronchial mucins and lactoferrin. Infect. Immun. **63:**899-902.

88. Kuklinska, D., and M. Kilian. 1984. Relative proportions *of Haemophilus* species in the throat of healthy children and adults. Eur. J. Clin. Microbiol. **3:**249-252.

89. Kulkarni, A. S., N. Khalap, and V. P. Joshi. 2004. *Haemophilus influenzae* UvrA: overexpression, purification, and in cell complementation. Protein Expr. Purif. **37:**462-7.

90. Lin, J., S. Huang, and Q. Zhang. 2002. Outer membrane proteins: key players for bacterial adaptation in host niches. Microbes. Infect. **4:**325-31.

91. Lithgow, J. K., E. Ingham, and S. J. Foster. 2004. Role of the *hprT–ftsH* locus in *Staphylococcus aureus.* Microbiology **150:**373-81.

92. Maciver, I., J. L. Latimer, H. H. Liem, U. Muller-Eberhard, Z. Hrkal, and E. J. Hansen. 1996. Identification of an outer membrane protein involved in the utilization of hemoglobin-haptoglobin complexes by nontypeable *Haemophilus influenzae*. Infect. Immun. **64:**3703-3712.

93. Marrs, C. F., G. P. Krasan, K. W. McCrea, D. L. Clemans, and J. R. Gilsdorf. 2001. *Haemophilus influenzae*-human specific bacteria. Frontiers in Bioscience **6:** e41-60.

94. Mee-Jung, H., S. J. Park, T. J. Park, and S. Y. Lee. 2004. Roles and applications of small heat shock proteins in the production of recombinant proteins in *Escherichia coli*. Biotechnol. Bioeng. **88:**426-36.

95. Mills, P. R., R. J. Davies, and J. L. Devalia. 1999. Airway epithelial cells, cytokines, and pollutants. Am. J. Respir. Crit. Care Med. **160:** S38-S43.

96. Monso, E., J. Ruiz , A. Rosell, J. Manterola, J. Fiz, J. Morera, and V. Ausina. 1995. Bacterial infection in chronic obstructive pulmonary disease. A study of stable and exacerbated outpatients using the protected specimen brush. Am. J. Respir. Crit. Care Med. **152:**1316-1320.

97. Mulks, M. H., S. J. Kornfeld, and A. G. Plaut. 1980. Specific proteolysis of human IgA by *Streptococcus pneumoniae* and *Haemophilus influenzae*. J. Infect. Dis. **141:** 450-456.

98. Murphy, T. F., and S. Sethi. 1992. Bacterial infection in chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. **146:**1067-1083.

99. Murphy, T. F., K. C. Dudas, J. M. Mylotte, and M. A. Apicella. 1983. A subtyping system for nontypeable *Haemophilus influenzae* based on outer-membrane proteins. J. Infect. Dis. **147:**838-846.

100. Murphy, T. F., and S. Sethi. 1992. Bacterial infection in chronic obstructive pulmonary disease. Am. Rev. Respir. Dis. **146:**1067-1083.

101. Mustafa, M. M., O. Ramilo, G. A. Syrogiannopoulos, K. D. Olsen, G. H. McCracken, Jr., and E. J. Hansen. 1989. Induction of meningeal inflammation by outer membrane vesicles of *Haemophilus influenzae* type b. J. Infect. Dis. **159:**917-22.

102. Nadel, J. A. 2000. Role of neutrophil elastase in hypersecretion during COPD exacerbations, and proposed therapies. Chest **17:**386-9S.

103. Neuwald, A. F., L. Aravind, J. L. Spouge, and E. V. Koonin. 1999. Aaa+: a class of chaperone-like atpases associated with the assembly, operation, and disassembly of protein complexes. Genome Res. **9:**27-43.

104. O'Shaughnessy, T. C., T. W. Ansari, N. C. Barnes, and P. K. Jeffery. 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. Am. J. Respir. Crit. Care Med. **155:**852-7.

105. Ogura, T., and A. J. Wilkinson. 2001. AAA+ superfamily ATPases: common structure-diverse function. Genes Cells **6:**575-597.

106. Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist. 1994. Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372**:475–478.

107. Pfeiffer, R. 1892. Vorlaufige meitteilungen uber die erreger der *influenza.* Dtsch. Med. Wochenschr. **18:**28-34.

108. Pichichero, M. E., and C. L. Pichichero. 1995. Persistent acute otitis media I. Causative pathogens. Pediatr. Infect. Dis. J. **14:**178-183.

109. Pittman, M. 1931. Variation and type specificity in the bacterial species *Haemophilus influenzae.* J. Exp. Med. **53:**471-493.

110. Rayner, M. G., Y. Zhang, M. C. Gorry, Y. Chen, J. C. Post, and G. D. Ehrlich. 1998. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. JAMA **279:**296-299.

111. Read, R., R. Wilson, A. Rutman, V. Lund, H. Todd, A. Brian, P. Jeffery, and P. Cole. 1991. Interaction of nontypeable *Haemophilus influenzae* with human respiratory mucosa *in vitro*. J. Infect. Dis. **163:**549-558.

112. Roles of various modulins from Haemophilus influenzae Rd in Interleukin-8 production (G. Kundu, J. W. Perry, M. J. Metropoulos, A. Morton, and D. Clemans, Abstr., Am. Society of Microbiol 104th General Meeting, abstr. B-199, 2004).

113. Saetta, M., A. Di Stefano, G. Turato, F. M. Facchini, L. Corbino, C. E. Mapp, P. Maestrelli, A. Ciaccia, and L. M. Fabbri. 1998. CD8+ T-lymphocytes in the peripheral airways of smokers with chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **157:**822-6.

114. Saikawa, N., A. Yoshinori, and K. Ito. 2004. FtsH exists as an exceptionally large complex containing HflKC in the plasma membrane of *Escherichia coli.* J. Struct. Biol. **146:**123-9.

115. Sands, M. K., and R. B. Roberts. 1952. The effects of a tryptophan-histidine deficiency in a mutant of E. coli. J. Bacteriol. **63:**505-511.

116. Schappert, S. M. 1997. Ambulatory care visits to physician offices, hospital outpatient departments, and emergency departments: United States. Vital Health Stat. **13:**1–39.

117. Collins, J. G. 1997. Prevalence of selected chronic conditions: United States, 1990–1992. Vital Health Stat **10**:1-89.

118. Schirmer, E. C., J. R. Glover, M. A. Singer, and S. Lindquist. 1996. Hsp100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem. Sci. **21**:289-296.

119. Schryvers, A. B. 1989. Identification of the transferrin- and lactoferrin-binding proteins in *Haemophilus influenzae*. J. Med. Microbiol. **29:**121-30.

120. Segal, N., N. Givon-Lavi, E. Leibovitz, P. Yagupsky, A. Leiberman, and R. Dagan. 2005. Acute otitis media caused by *Streptococcus pyogenes* in children. Clin. Infect. Dis. **41:**35-41.

121. Sethi, S., and T. F. Murphy. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin. Microbiol. **14:**336-363.

122. Sethi, S., B. S. Muscarella, N. Evans, K. L. Klingman, B. J. Grant, and T.F. Murphy. 2000. Airway inflammation and etiology of acute exacerbations of chronic bronchitis. Chest **118:**1557-65.

123. Sethi, S., C. Wrona, B. J. B. Grant, and T. F. Murphy. 2003. Strain-specific immune response to *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. **169:**448-53.

124. Sethi, S., N. Evans, R. N. Brydon, J. B. Grant, and T. F. Murphy. 2002. New strains of bacteria and exacerbation of chronic obstructive pulmonary disease. N. Engl. J. Med. **347:**465-471.

125. Shuto, T., H. Xu, B. Wang, J. Han, H. Kai, X. X. Gu, T. F. Murphy, D. J. Lim, and J. D. Li. 2001. Activation of NF-kappa B by nontypeable *Hemophilus influenzae* is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK alpha /beta-I kappa B alpha and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. Proc. Natl. Acad. Sci. **98:**8774-8779.

126. Smallman, L. A., S. L. Hill, and R. A. Stockley. 1984. Reduction of ciliary beat frequency in vitro by sputum from patients with bronchiectasis: a serine proteinase effect. Thorax **39:**663-7.

127. Smith-Vaughan, H. C., K. S. Sriprakash, J. D. Mathews, and D. J. Kemp. 1997. Nonencapsulated *Haemophilus influenzae* in aboriginal infants with otitis media: Prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. Infect. Immun. **65:**1468-1474.

128. Soler, N., A. Torres, S. Ewig, J. Gonzalez, R. Celis, M. El-Ebiary, C. Hernandez, and R. Rodriguez-Roisin. 1998. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. Am. J. Respir. Cri. Care Med. **157:**1498-1505.

129. St. Geme, J. W., III., S. Falkow, and S. J. Barenkamp. 1993. High-molecular-weight proteins of nontypable *Haemophilus influenzae* mediate attachment to human epithelial cells. Proc. Natl. Acad. Sci. **90:**2875-2879

130. St. Geme, J. W., III., V. V. Kumar, D. Cutter, and S. J. Barenkamp. 1998. Prevalence and distribution of the hmw and hia genes and the HMW and Hia adhesins among genetically diverse strains of nontypeable *Haemophilus influenzae*. Infect. Immun. **66:**364-368.

131. St. Geme, J. W., III. 1993. Nontypeable *Haemophilus influenzae* disease: epidemiology, pathogenesis, and prospects for prevention. Infect. Agents Dis. **2:**1-16.

132. St. Geme, J. W., III, M. L. de la Morena, and S. Falkow. 1994. *Haemophilus influenzae* IgA protease-like protein promotes intimate interaction with human epithelial cells. Mol. Microbiol. **14:**217-233.

133. Stricter, R. M., J. A. Belperio, and M. P. Keane. 2002. Cytokines in innate host defense in the lung. J. Clin. Invest. **109:**699-705.

134. Stull, T. L. 1987. Protein sources of heme for *Haemophilus influenzae*. Infect. Immun. **55:**148-153.

135. Stull, T. L., P. M. Mendelman, J. E. Haas, M. A. Schoenborn, K. D. Mack, and A. L. Smith. 1984. Characterization of *Haemophilus influenzae* type b pili. Infect. Immun. **46:**787-796.

136. Sullivan, S. D., S. D. Ramsey, and T. A. Lee. 2000. The economic burden of COPD. Chest **117:**5S-9S.

137. Swanson, M. S., and B. K. Hammer. 2000. *Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages. Annu. Rev. Microbiol. **54:**567-613.

138. Tong, H. H., C. M. James, J. Van Deusen, D. B. Welling, and T. F. DeMaria. 2001. Expression of cytokine and chemokine genes by human middle ear epithelial cells induced by formalin-killed *Haemophilus influenzae* or its lipooligosaccharide htrB and rfaD mutants. Infect. Immun. **69:**3678-3684.

139. Turk, D. C., and J. R. May. 1967. *Haemophilus influenzae*, its clinical importance. London: The English Universities Press, Ltd.

140. Vachon, V., D. J. Lyew, and J. W. Coulton. 1985. Transmembrane permeability channels across the outer membrane of *Haemophilus influenzae* type b. Bacteriol. **162:**918-24.

141. Vale, R. D. AAA proteins: lords of the ring. 2000. J. Cell Biol. **150**:F13–F19.

142. Wald, E. R., G. J. Milmoe, A. D. Bowen, J. Ledesma-Medina, N. Salamon, and C. D. Bluestone. 1981. Acute maxillary sinusitis in children. N. Eng. J. Med. **304:**749-754.

143. Wang, B., P. P. Cleary, H. Xu, and J. Li. 2003. Up-regulation of interleukin-8 by novel small cytoplasmic molecules of nontypeable *Haemophilus influenzae* via p38 and extracellular signal-regulated kinase pathways. Infect. Immun. **71:**5523-5530.

144. 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. Mol. Microbiol. **17:**555-564.

145. White, D. C., and S. Granick. 1963. Hemin biosynthesis in *Haemophilus*. J. Bacteriol. **85:**842-850.

146. Wilson, M., R. Seymour, and B. Henderson. 1998. Bacterial perturbation of cytokine networks. Infect. Immun. **66:**2401-2409.

147. Wilson, R. 1998. The role of infection in COPD. Chest **113:**242S-248S.

148. Wilson, R., R. B. Dowling, and A. D. Jackson. 1996. The effects of bacterial products on airway cells and their function. Am. J. Respir. Crit. Care Med. **154:** S197-S201.

149. Wispelwey, B., E. J. Hansen, and W. M. Scheld. 1989. *Haemophilus influenzae* outer membrane vesicle-induced blood-brain barrier permeability during experimental meningitis. Infect. Immun. **57:**2559-62.

150. Yamamoto, T., H. Sashinami, A. Takaya, T. Tomoyasu, H. Matsui, Y. Kikuchi, T. Hanawa, S. Kamiya, and A. Nakane. 2001. Disruption of the genes for ClpXP protease in *Salmonella enterica* serovar Typhimurium results in persistent infection in mice, and development of persistence requires endogenous gamma interferon and tumor necrosis factor alpha. Infect. Immun. **69:**3164-3174.

151. Zhu, J., Y. S. Qiu, S. Majumdar, E. Gamble, D. Matin, G. Turato, L. M. Fabbri, N. Barnes, M. Saetta, and Peter K. Jeffery 2001. Exacerbations of bronchitis: bronchial eosinophilia and gene expression for interleukin-4, interleukin-5, and eosinophil chemoattractants. Am. J. Respir. Crit. Care Med. **164:**109-16.

152. Zolkiewski, M. 1999. ClpB cooperates with DnaK, DnaJ, and GrpE in suppressing protein aggregation. J. Biol. Chem. **40:**28083-6.