Expression of urease by *Haemophilus influenzae* during human respiratory tract infection and role in survival in an acid environment

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Abstract

**Background:** Nontypeable *Haemophilus influenzae* is a common cause of otitis media in children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD). Prior studies have shown that *H. influenzae* expresses abundant urease during growth in the middle ear of the chinchilla and in pooled human sputum, suggesting that expression of urease is important for colonization and infection in the hostile environments of the middle ear and in the airways in adults. Virtually nothing else is known about the urease of *H. influenzae*, which was characterized in the present study.

**Results:** Analysis by reverse transcriptase PCR revealed that the *ure* gene cluster is expressed as a single transcript. Knockout mutants of a urease structural gene (*ureC*) and of the entire *ure* operon demonstrated no detectable urease activity indicating that this operon is the only one encoding an active urease. The *ure* operon is present in all strains tested, including clinical isolates from otitis media and COPD. Urease activity decreased as nitrogen availability increased. To test the hypothesis that urease is expressed during human infection, purified recombinant urease C was used in ELISA with pre acquisition and post infection serum from adults with COPD who experienced infections caused by *H. influenzae*. A total of 28% of patients developed new antibodies following infection indicating that *H. influenzae* expresses urease during airway infection. Bacterial viability assays performed at varying pH indicate that urease mediates survival of *H. influenzae* in an acid environment.

**Conclusions:** The *H. influenzae* genome contains a single urease operon that mediates urease expression and that is present in all clinical isolates tested. Nitrogen availability is a determinant of urease expression. *H. influenzae* expresses urease during human respiratory tract infection and urease is a target of the human antibody response. Expression of urease enhances viability in an acid environment. Taken together, these observations suggest that urease is important for survival and replication of *H. influenzae* in the human respiratory tract.

**Background**

Nontypeable (non encapsulated) *Haemophilus influenzae* is an exclusively human pathogen whose primary ecological niche is the human respiratory tract. *H. influenzae* is a common and important human pathogen, causing otitis media in children and lower respiratory tract infection in adults with chronic obstructive pulmonary disease (COPD) [1-3]. The course of COPD, the fourth leading cause of death in the world, is characterized by intermittent worsening called exacerbations.

Approximately half of exacerbations are caused by bacterial infection, with *H. influenzae* being the most frequent bacterial cause [2]. In addition to causing exacerbations, *H. influenzae* also chronically colonizes the lower airways of adults with COPD. The normal human respiratory tract is sterile below the vocal cords, as determined by culture. However, in adults with COPD, the lower airways are colonized by bacteria, with *H. influenzae* as the most common pathogen in this setting [4-7].

The human respiratory tract is a hostile environment for bacteria. Nutrients and energy sources are limited. In the setting of COPD, airways are characterized by an oxidant/antioxidant imbalance and by an inflammatory...
milieu [8-12]. Thus to survive and cause infection in the human respiratory tract, *H. influenzae* must express proteins and other molecules to enable persistence in this unique environment.

In previous work, we characterized the proteome of *H. influenzae* that was grown in pooled human sputum obtained from adults with COPD in an effort to simulate the environment of the human airways in COPD [13]. In comparison to the same strain of *H. influenzae* grown in chemically defined media, 31 proteins were present in greater abundance in sputum grown-conditions at a ratio of > 1.5 compared to media-grown conditions. These included antioxidant proteins, stress response proteins, proteins that function in the uptake of divalent cations and proteins that function in the uptake of various molecules. Interestingly, the second most abundant protein with regard to the ratio of sputum-grown to media-grown analysis was urease C, the alpha subunit of urease, which was present in an abundance of 7-fold greater in sputum-grown conditions compared to media-grown conditions. This is an interesting finding in light of the observation by Mason et al [14] who monitored gene expression by *H. influenzae* in the middle ear of a chinchilla, the most widely used animal model of otitis media. The gene that encodes urease accessory protein, *ureH*, was induced 3.9-fold in bacterial cells in the middle ear compared to baseline. These two genes, *ureC* and *ureH* are part of the urease gene cluster and were among the most highly up regulated genes. These observations suggest that expression of urease is important for survival and growth of *H. influenzae* in the respiratory tract.

Ureases are nickel dependent enzymes that catalyze the hydrolysis of urea to form ammonia and carbon dioxide [15,16]. Urease is best studied as a virulence factor in *Helicobacter pylori* which colonizes the stomach and *Proteus mirabilis* which causes urinary tract infections [17-23]. Urease is also important for survival and pathogenesis of several bacterial species [24-27]. Urease functions to raise the pH of the environment, allowing survival in acidic media; urease also enables bacteria to use urea as a sole nitrogen source [28]. While these are the best known functions of urease, this protein also interacts with the human host and acts as virulence factor by several other mechanisms, including activation of macrophages [29], induction of inflammatory mediators [30-32], dysregulation of gastric epithelial tight junctions [33], apoptosis [34], activation of platelets, enhanced survival in macrophages [35,36] and others [37,38].

Virtually nothing is known about the urease of *H. influenzae*. In view of the high degree of up regulation of urease expression by *H. influenzae* in the respiratory tract and the importance of urease as a virulence factor in other bacteria, the goal of this study is to characterize the urease of *H. influenzae*. In particular we have constructed knockout mutants of *ureC* and the urease operon to assess urease activity by *H. influenzae*, characterized the urease transcript, determined the optimal pH for urease activity and demonstrated that the urease operon is present in clinical isolates from otitis media and COPD. Analysis of pre and post infection serum samples from adults with exacerbations of COPD caused by *H. influenzae* demonstrated directly that urease is expressed during human infection. Finally, we demonstrate that urease activity enhances survival of *H. influenzae* at a reduced pH.

### Results

**Identification of urease gene cluster**

The α subunit of urease, which was present in increased abundance in *H. influenzae* grown in pooled human sputum based on proteomic analysis, is a protein of 572 amino acids with a predicted molecular mass of 62 kilodaltons that is encoded by *ureC* [13]. The *ureC* gene is the third gene in the urease gene cluster, (Figure 1A); *ureA* and *ureB* encode the γ and β subunits respectively and *ureE*, *ureF*, *ureG* and *ureH* encode urease accessory proteins. These genes correspond to loci HI0535 through HI0541 in *H. influenzae* strain KW20 Rd (GenBank L42023.1) and to loci NTHI 0661 through NTHI 0667 in *H. influenzae* strain 86-028NP (GenBank CP000057).

**Characterization of mutants**

A *ureC* mutant was constructed in our prototype COPD exacerbation strain 11P6H by replacing the *ureC* gene with a non polar kanamycin resistance cassette by homologous recombination using overlap extension PCR (Figure 1B). The mutant construct was confirmed by PCR using oligonucleotide primers in and around the gene in the wild type strain and the kanamycin cassette in the mutant, and by sequencing through the region of homologous recombination. An immunoblot assay of whole cell lysates probed with rabbit antiserum raised to recombinant urease C reveals the presence of an ~62 kDa protein band in the wild type strain, corresponding to urease C, and no bands in the *ureC* mutant (Figure 2).

Complementation of the *ureC* mutation was accomplished by cloning a fragment corresponding to the promoter region of the urease operon upstream of *ureA* through *ureC* into plasmid pSPEC and transforming the plasmid into the *ureC* mutant [39]. The complemented mutant expresses urease C detected by specific antiserum (Figure 2, lane d).

A knockout of the entire urease gene cluster was constructed using a similar overlap extension PCR strategy (Figure 1C). The mutant construct was confirmed by PCR and sequencing through the region of homologous
recombination. An immunoblot assay of the whole bacterial cell lysate of the urease operon mutant probed with antiserum to urease C reveals an absence of a urease C band (Figure 2, lane c) that is present in wild type.

To further characterize the urease operon mutant, genomic DNA from wild type and urease operon mutant strains was purified, restricted with EcoRI and subjected to Southern blot assay. Probes that corresponded to the amino terminal region (ureA), the central region (ureC) and the carboxy terminal region (ureH) of the gene cluster and the kanamycin cassette revealed an absence of each of these 3 genes in the mutant and the presence of a kanamycin cassette as expected (Figure 3).

**Characterization of purified recombinant urease C**

Recombinant urease C was purified by elution from a metal affinity column and refolded by sequential dialysis in buffers that contained decreasing concentrations of arginine. Analysis of the purified protein by SDS PAGE

![Figure 1](https://example.com/figure1.png)  
**Figure 1**  
A. Diagram of urease gene cluster. Numbers above genes indicate length of genes in nucleotides and numbers below indicate nucleotides between gene coding sequences.  
B. Diagram of ureC knockout mutant.  
C. Diagram of urease operon knockout mutant.

![Figure 2](https://example.com/figure2.png)  
**Figure 2** Characterization of mutants and recombinant urease C protein. Left panel: Immunoblot assay probed with rabbit antiserum (1:50,000) raised to recombinant purified urease C and adsorbed with urease mutant 11P6H ureC-. Blots were probed with goat anti-rabbit IgG (1:1000) and color was developed with horseradish peroxide developer. Lanes contain whole cell lysates as follows: a) Wild type 11P6H; b) Urease C mutant 11P6HureC-; c) Urease operon mutant 11P6HureC-; d) Complemented urease C mutant 11P6HureC(pureC). Right panel. Coomassie blue stained polyacrylamide gel. Lane e) Purified recombinant urease C. Arrow denotes full size protein. The lower band is a fragment of the full size protein. Molecular mass standards are noted on the left of each panel in kilodaltons.

![Figure 3](https://example.com/figure3.png)  
**Figure 3** Southern blot assay. Purified genomic DNA of *H. influenzae* was restricted with EcoRI and hybridized with 200 bp probes corresponding to ureA, ureC, ureH and kanamycin cassette (kan) as noted at the bottom of each panel. Lanes a) wild type strain 11P6H; lanes b) urease operon mutant 11P6HureC-. Molecular size markers are noted on the left in kilobases.
showed a prominent band at the predicted size (Figure 2, lane e). Preparations of the purified protein also revealed a second band of varying intensity of a lower molecular mass. Immunoblot assay with antibody that recognizes the 6 histidine tag detected both bands, indicating that the smaller band resulted from proteolytic degradation of the full length protein (data not shown). Protease inhibitors were used during purification and storage; however the purified protein was prone to proteolytic degradation. The purified recombinant protein was used to raise antiserum in rabbits and to measure antibody by ELISA in human serum. Thus, this level of proteolytic degradation would not be expected to adversely affect these experiments.

**Characterization of urease activity**

Crude cell extracts of *H. influenzae* 11P6H were used to determine urease activity in wild type 11P6H and mutant strains. The *ureC* knockout mutant and the urease operon mutant both demonstrated no detectable urease activity compared to wild type and *ureC* complemented mutant when grown in laboratory media (Figure 4). We conclude that the *ureA-H* gene cluster accounts for all detectable urease activity of *H. influenzae* under the conditions of this assay. In addition, knocking out *ureC* alone, which encodes the major structural subunit of urease, completely abrogates urease activity.

The optimal pH of *H. influenzae* urease was determined by preparing whole cell extracts in phosphate buffers ranging in pH from 4 to 8. The optimal pH for urease was 7, with marked reduction in activity at lower pH (Figure 5).

To begin to assess factors that control urease expression in *H. influenzae*, the effect of nitrogen availability on urease production was measured by adding increasing concentrations of ammonium chloride to bacteria growing in broth culture. Urease production decreased as the concentration of added ammonium chloride increased (Figure 6).

**Analysis of urease transcript**

Reverse transcriptase PCR was performed to determine whether genes *ureA* through *ureH* of the urease gene cluster are transcribed as a single transcript or as
multiple transcripts. Reverse transcriptase PCR was performed using RNA isolated from *H. influenzae* 11P6H grown in broth using primers designed to correspond to transcripts that would span adjacent genes in the gene cluster (Figure 1). Figure 7 shows that the genes of the urease gene cluster are transcribed as a single transcript. Control assays confirmed that the purified RNA was free of contaminating DNA (Figure 7, lanes b).

**Presence of urease operon in clinical isolates**

To determine whether the urease operon is present in clinical isolates of *H. influenzae*, 20 clinical isolates, including 10 otitis media strains and 10 COPD strains were studied by PCR. Primers corresponding to genes located in the 5’ region (*ureA*), central region (*ureC*) and 3’ region (*ureH*) of the operon were designed. Amplicons of identical size were obtained from 20 of 20 clinical isolates with all 3 sets of primers (Figure 8). These results indicate that the urease operon is present in all strains tested and that no variation was observed in the lengths of these genes in diverse strains tested.

A BLASTn search with the sequence corresponding to the urease operon was performed to determine which strains of *H. influenzae* whose genomes are available in GenBank contained the urease operon. Five of 6 strains whose complete genome has been sequenced contain the urease operon. A high degree of sequence similarity in the urease operon is present among the 5 strains. In strain R2866, which is urease negative, the urease operon is replaced by a single gene with homology to the gonococcal *mtrF* gene [40]. Sequence analysis of the same region of 9 additional urease negative strains revealed sequence that is very similar to that of strain R2866 [40].

**Transcription of the ureC during growth in pooled human sputum**

To assess expression of urease in conditions that simulate conditions in the human respiratory tract in COPD, transcription of *ureC* was measured in *H. influenzae* that was grown in the presence of pooled human sputum from adults with COPD in comparison to growth in the absence of human sputum using quantitative real time PCR. Results of *ureC* were normalized with *gyrA*, a gene that is constitutively expressed [14]. Transcription of *ureC* in media plus sputum was 3.32 ± 0.066 (mean ± standard deviation) fold greater than transcription of *ureC* in media alone (1.0 ± 0.223). We conclude that transcription of *ureC* is up regulated when *H. influenzae* grows in media with added human sputum compared to growth in laboratory media alone.

**Human antibody responses**

To determine whether urease was expressed by *H. influenzae* during infection of the human respiratory tract, 18 serum pairs from patients who experienced exacerbations due to *H. influenzae* were assayed for the development of antibody to purified recombinant urease following exacerbation.

The cutoff value for a significant percentage change between pre-exacerbation and post-exacerbation serum IgG levels was determined as previously described [41-44]. Eight control pairs of serum samples obtained 2 months apart (the same time interval for the experimental samples) from adults with COPD who were clinically stable and who had negative sputum cultures for *H. influenzae* were subjected to ELISA with the purified recombinant urease. The % change in OD$_{450}$ values between the paired
control samples was calculated. These paired control serum samples demonstrated a 3.36% ± 6.01 (mean ± SD) change when tested with urease. A change in OD of 9.37% represented the upper limit of the 99% confidence interval for the control samples. Therefore, any increase in value from pre to post exacerbation serum pairs of ≤ 9.37% was regarded as a significant change. A significant increase of serum IgG antibodies to urease was seen in 7 of 18 serum pairs (Figure 9). We conclude that H. influenzae expresses urease during infection of the human respiratory tract and is a target of human serum antibodies in adults with COPD.

**Susceptibility of H. influenzae to acid conditions**

The ability of wild type and urease mutant to survive exposure to acid was investigated in the presence and absence of urea. Incubation of H. influenzae at pH 4 in the absence of urea, resulted in ~35% survival of wild type and mutant strains. However, in the presence of either 50 mM or 100 mM urea, survival of the wild type strain increased whereas no change in survival was observed in the urease C mutant or the urease operon mutant (Figure 10). Survival of the complemented mutant closely paralleled that of wild type, supporting the conclusion that urease mediates survival in acid conditions.

**Discussion**

As an exclusively human pathogen, H. influenzae expresses molecules that mediate survival in the hostile conditions of the human respiratory tract. Previous studies in animal models and in conditions that simulate those in the human airways identified urease as a molecule that is expressed in high abundance by H. influenzae, providing evidence that urease plays a role in the pathogenesis of infection. Furthermore, urease activity may contribute to the pathogenesis of pulmonary infections due to Actinobacillus pleuropneumoniae in pigs [45]. These observations lead to the present study which is the first to characterize H. influenzae urease.

The H. influenzae urease gene cluster resembles that of other gram negative bacteria, possessing three contiguous structural genes (ureA, ureB and ureC) that encode the urease apoenzyme. Knocking out ureC alone by insertion of a nonpolar kanamycin cassette in its place resulted in complete loss of urease activity (Figure 4). Urease is a multi-subunit enzyme that requires an elaborate pathway for assembly in its active form. Associated with its three structural genes are 4 accessory genes which are necessary for synthesis of active enzyme. Based on available data from other organisms, ureEFG form a complex that keeps the apoenzyme in a conformation that will accept nickel. H. influenzae ureH, a structural homolog of ureD, is located downstream of the ureEFG, similar to the organization of the H. pylori urease gene cluster. H. influenzae does not have a ureR homolog, a regulatory gene that is present in some bacteria with urea-inducible urease [15]. Reverse transcriptase PCR demonstrated that the H. influenzae urease gene cluster is transcribed as a single transcript (Figure 7).

Urease activity in H. influenzae was dependent on nitrogen (ammonium chloride) availability as activity was maximal in the absence of added ammonium chloride and was markedly reduced as the concentration increased (Figure 6). This down regulation of urease expression by nitrogen sources is observed in other bacteria, including Brucella abortus and Klebsiella aerogenes and suggests that urease functions in assimilation of nitrogen from urea [23,25]. Because urea is translocated onto epithelial surfaces by secretory systems and in tissue exudates, urea is present in epithelial lining fluid of the human respiratory tract in concentrations approximately equal to that in plasma [46]. Thus, we speculate that the urease of H. influenzae facilitates nitrogen assimilation in the nutritionally limited environment of the human airways and the middle ear space.

Two indirect lines of evidence have suggested that H. influenzae expresses urease during human infection. Mason et al [14] showed that urease H is expressed during infection of the middle ear in chinchillas and Qu et al [13] showed that urease C was expressed in markedly increased abundance during growth in pooled human sputum. The present study advances those observations by showing directly that H. influenzae expresses urease
during airway infection in adults who experienced exacerbations of COPD. Paired pre and post infection serum samples were subjected to ELISA with purified recombinant urease C to characterize the antibody response to urease following infection. Because the pre infection serum samples were collected one month prior to acquisition of the infecting strain of *H. influenzae*, an increase in the level of antibody to urease indicates the development of new antibodies following infection. All serum samples had detectable levels of antibody to urease and 7 of 18 patients developed significantly increased levels following infection compared to their own pre infection levels (Figure 9). This frequency of antibody response following bacterial infection is typical as heterogeneity in immune responses to bacterial antigens among individuals is a hallmark of COPD [47,48]. Note also that recombinant purified urease C was used in the ELISA and this protein is only one of 3 proteins that comprise the urease complex; thus, a urease C-based ELISA may underestimate the frequency of new antibody responses to urease following infection. These results indicate that *H. influenzae* expresses urease during exacerbations of COPD and that urease is a target of human antibody responses.

An important result from the present study is the observation that urease functions to mediate survival of *H. influenzae* in an acid environment. Urease mediates survival in low pH as a virulence mechanism in other bacteria, notably *H. pylori* which must survive in the stomach. Other selected respiratory pathogens express urease but the role of urease in pathogenesis of respiratory tract infection is unclear [49,50]. Microenvironments in the human respiratory tract are likely low pH, consistent with the speculation that the high level of expression of urease in the respiratory tract mediates survival in acid microenvironments. Furthermore, *H. influenzae* is now known to invade and persist in respiratory epithelial cells and macrophages, suggesting that withstanding lower pH in intracellular compartments may be a virulence mechanism [51-53].

**Conclusions**

The present study demonstrates that 1) The *ureA-ureH* gene cluster of *H. influenzae* is exclusively responsible for urease production because knock out mutants show no urease activity. 2) Genes of the urease gene cluster are transcribed as a single transcript. 3) Urease expression is regulated in response to nitrogen availability. 4) The optimal
pH for urease activity is 7.0. 5) The urease operon is present in all strains of *H. influenzae* tested including otitis media and COPD isolates. 6) Transcription of the ure operon is up regulated when *H. influenzae* grows in human sputum, consistent with the earlier observation established by proteomics analysis [13]. 7) Urease is expressed in the human airways during infection in adults with COPD and is the target of human antibody responses. And 8) Urease mediates survival of *H. influenzae* in an acid environment. In view of the high level of expression of urease in the respiratory tract, future work will focus on elucidating the role of urease as a virulence factor for *H. influenzae* infection of the human respiratory tract.

**Methods**

**Bacterial strains and growth conditions**

*H. influenzae* 11P6H was isolated from the sputum of an adult with COPD who was experiencing an exacerbation as part of a prospective study at the Buffalo VA Medical Center [54]. The following strains were also isolated from the sputum of adults with COPD as part of the same study: 14P14H1, 24P17H1, 27P5H1, 33P18H1, 43P2H1, 55P3H1, 66P33H1, 74P16H1, 91P18H1. Each strain was isolated from a different subject. *H. influenzae* strains 1749, 1826, 6699, 6700, 4R, 17R, 26R, 47R, P86 and P113 were isolated from middle ear fluid obtained by tympanocentesis from children with otitis media in either Buffalo NY or Rochester NY. All strains were identified as *H. influenzae* by growth requirement for hemin and nicotinamide adenine dinucleotide (NAD), absence of porphyrin production and absence of hemolysis. Each isolate was also subjected to immunoblot assay with monoclonal antibody 7F3 that recognizes outer membrane P6 to exclude the possibility of non hemolytic *H. haemolyticus* [55].

*H. influenzae* was grown on chocolate agar at 37°C in 5% CO₂ or in brain heart infusion broth supplemented with hemin and NAD each at 10 µg/ml with shaking at 37°C. In selected experiments, *H. influenzae* was grown in chemically defined media (Table 1).

Chemically competent *E. coli* strains Top10 and BL21 (DE3) were obtained from Invitrogen (Carlsbad, CA) and were grown at 37°C on Luria-Bertani (LB) plates or in LB broth supplemented with antibiotics as noted in individual experiments. Plasmid pSPEC1 was kindly provided by Lauren Bakaletz and Robert Munson [39].

**Construction of mutants**

A mutant lacking ureC, the gene that encodes the alpha subunit of urease, was constructed using overlap

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**Table 1 Composition of chemically defined media (CDM)**

| Reagent                  | Concentration |
|--------------------------|--------------|
| NaCl                     | 0.1 M        |
| K₂SO₄                    | 5.75 mM      |
| Na₂EDTA                  | 4 mM         |
| NH₄Cl                    | 4 mM         |
| K₂HPO₄                   | 2 mM         |
| KH₂PO₄                   | 2 mM         |
| Thiamine HCl             | 6 µM         |
| Thiamine pyrophosphate   | 1 µM         |
| Pantothentic acid         | 8 µM         |
| d-Biotin                 | 12 µM        |
| Glucose                  | 0.5%         |
| Hypoxanthine             | 0.375 mM     |
| Uracil                   | 0.45 mM      |
| L-aspartic acid          | 3.75 mM      |
| L-glutamic acid HCl      | 7.5 mM       |
| L-arginine               | 0.875 mM     |
| Glycine HCl              | 0.225 mM     |
| L-serine                 | 0.475 mM     |
| L-leucine                | 0.7 mM       |
| L-isoleucine             | 0.225 mM     |
| L-valine                 | 0.525 mM     |
| L-tyrosine               | 0.4 mM       |
| L-cysteine HCl           | 0.35 mM      |
| L-cystine                | 0.15 mM      |
| L-proline                | 0.45 mM      |
| L-tryptophan             | 0.4 mM       |
| L-threonine              | 0.425 mM     |
| L-phenylalanine          | 0.15 mM      |
| L-asparagine             | 0.2 mM       |
| L-glutamine              | 0.35 mM      |
| L-histidine HCl          | 0.125 mM     |
| L-methionine             | 0.11 mM      |
| L-alanine                | 1.125 mM     |
| L-lysine                 | 0.35 mM      |
| Glutathione reduced      | 0.15 mM      |
| HEPES                    | 42 mM        |
| NaHCO₃                   | 0.125 mM     |
| Na acetate trihydrate    | 6.25 mM      |
| Choline chloride salt    | 0.05 mM      |
| Myo-inositol             | 1 µM         |
| MgCl₂                    | 2.5 mM       |
| CaCl₂                    | 0.6 mM       |
| Fe(NO₃)₃                 | 0.1 mM       |
| Nicotinamide adenine dinucleotide | 0.02 mM |
| Protoporphyrin IX        | 0.02 mM      |
| Histidine                | 6 µM         |
| Triethanolamine          | 0.01%        |
extension PCR. The transforming DNA to accomplish this was composed of 3 fragments: 1) a ~1 kb fragment of DNA corresponding to sequence upstream of ureC, 2) the nonpolar kanamycin resistance cassette AphA-3 [56], 3) a ~1 kb fragment of DNA corresponding to sequence downstream of ureC. Primers for each of the fragments were designed with 10 bp overlaps with complementary overlapping regions with the adjacent fragment (Table 2). The 3 fragments were amplified using the high fidelity DNA polymerase Pfu (Stratagene, Cedar Creek, TX) and were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA). Amplicons were mixed in the absence of additional primers in a PCR with Pfu and were subjected to a PCR program consisting of 10 cycles with a denaturing step at 94°C for 30 sec, an annealing step at 50°C for 1 min and an elongation step at 72°C for 5 min. The fusion product was subsequently amplified by Pfu with primers 539frag1 5' and 539frag3 3' (Table 2). This amplicon consisted of 1020 bp sequence upstream of ureC and 1029 bp sequence downstream of ureC flanking the kanamycin cassette.

The fragment was transformed into H. influenzae strain 11P6H which was made competent by the method of Herriott et al [57] using the transformation protocol of Poje and Redfield [58] as previously described [59]. Transformants were selected on chocolate agar containing 15 μg/ml of robustimycin. A mutant was obtained (11P6HureC-) and allelic exchange was verified by PCR analysis and sequencing as detailed in Results.

A mutant in which the entire urease gene cluster was knocked out and replaced with a kanamycin cassette was constructed (11P6Hure-) with the same strategy using the primers noted in Table 2. The mutant was verified by PCR analysis, Southern blot assay and sequencing.

**Complementation of ureC mutant**

Complementation was accomplished by using the plasmid pSPEC1 [39]. A fragment containing the ureC gene and 740 bp upstream to include the promoter of the urease operon and 300 bp downstream was amplified from genomic DNA of strain 11P6H and ligated into pSPEC1 at BamHI and EcoRI restriction sites (Table 2). After confirming the insert sequence of the resulting plasmid (p UreCspe), H. influenzae 11P6H was electroporated with p UreCspe that had been methylated with CpG methylase (New England Biolabs) in a 0.1-cm cuvette (200 Ω, 2.5 kV, 25 μF). Cells were plated on chocolate agar containing 200 μg/ml of spectinomycin, incubated overnight and the complemented mutant 11P6H ureC (p UreCspe) was obtained. This complemented mutant was grown in the presence of spectinomycin for all experiments.

**Southern blot assay**

Southern blot assays were performed with genomic DNA restricted with EcoRI with the Hoefer TransVac vacuum blotting unit following the manufacturer’s instructions (Hoefer, SanFrancisco, CA). Probes were biotinylated with the NEBlot Phototope kit (New England BioLabs) and blots were developed with the Phototope-Star Detection Kit (New England BioLabs) using the manufacturer’s instructions.

**Measurement of urease activity**

Urease activity was determined by measuring the amount of ammonia released from urea [25,60]. To prepare whole bacterial cell extracts, overnight cultures (5 ml) were centrifuged at 2500 × g for 10 min at 4°C and the pellet was suspended in 5 ml of phosphate buffered saline (PBS) pH 7.5. Cells were disrupted by sonication with three 10 second bursts (Branson Sonifier 450, output control 5). One ml of the resulting suspension was centrifuged at 16,000 × g for 2 min to remove unbroken cells and 10 μl of the sonic extract were added to 200 μl of PBS containing 50 mM urea and incubated at 37°C for 30 min. To perform the urease assay, 125 μl of sonic extract were mixed with 250 μl of lysine hydrochlorite, 250 μl phenol nitroprusside and 1 ml of water and the assay was incubated for 30 min at 37°C. A volume of 200 μl was removed and placed into wells of a 96 well plate and the OD595 was measured in an ELISA plate reader. Urease activity was determined by the use of a standard curve using NH₄Cl (0.156 mM to 2.5 mM) performed simultaneously with each assay. Urease activity was expressed in μmoles of urea hydrolyzed per minute.

**Expression and purification of recombinant protein encoded by ureC**

The ureC gene was amplified by PCR from genomic DNA of H. influenzae strain 11P6H using oligonucleotide primers noted in Table 2 and cloned into pET101 D-TOPO (Invitrogen, Carlsbad, CA), which places a 6 histidine tag on the carboxy terminus of the recombinant protein, using manufacturer’s instructions. Chemically competent E. coli TOP10 cells were transformed with the recombinant plasmid and transformants were selected by plating on LB plates containing 50 μg/ml of carbenicillin. The plasmid (p539) from a transformant was confirmed to have the ureC gene by PCR and by sequence determination. Plasmid p539 was purified using the Qiagen plasmid mini purification system and transformed into chemically competent E. coli BL21 (DE3) for expression. To express recombinant protein, 2.5 ml of overnight culture was used to inoculate 50 ml of LB broth containing 300 μg/ml of carbenicillin. When the culture reached an OD₆₀₀ of ~0.6, expression
### Table 2 Oligonucleotide primer sequences

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| 539 frag1 5’   | ureC upstream         | Forward   | 5’-GACCTTTACCCACAGCTAAT-3’ |
| 539 frag1 3’   | ureC upstream         | Reverse   | 5’-TAGTTAGTCATTGAAATTGTA ATGCCC-3’ |
| 539 frag2 5’   | Kanamycin cassette    | Forward   | 5’-CAATTCTAGTACTAATCTAGGA GGAATA-3’ |
| 539 frag2 3’   | Kanamycin cassette    | Reverse   | 5’-TGACCCAATGCTATTCCCTCC AGGTA-3’ |
| 539 frag3 5’   | ureC downstream       | Forward   | 5’-GGGAATAATGCTATTGGTCAGC GATA-3’ |
| 539 frag3 3’   | ureC downstream       | Reverse   | 5’-ATGGAACACCGAGTTTG-3’ |

#### Cloning of ureC into pSPEC1 to complement ureC mutation

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| 539promoter F1 | ureA upstream         | Forward   | 5’-GAGAGGATCCGTTAATACTCGT ACTTTCG-3’ |
| 539C R1        | ureC downstream       | Reverse   | 5’-ATATTTTCTTTATTCCTCCA GGTAC-3’ |

#### Construction of urease operon mutant

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| 539 Op frag1 F1| ureA upstream         | Forward   | 5’-TACACTTCTTGCACG-3’ |
| 539 Op frag1 R1| ureA upstream         | Reverse   | 5’-TAGTTAGTCATTTCATCTCCTTAAT-3’ |
| 539 Op frag2 F1| Kanamycin cassette    | Forward   | 5’-GAATTAAATGCTACTTAG GGAATA-3’ |
| 539 Op frag2 R1| Kanamycin cassette    | Reverse   | 5’-ACCAATTTCCATTATCCCTCCA GGTAC-3’ |

#### Construction of urease operon mutant

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| 539 Op frag3 F1| ureA downstream       | Forward   | 5’-GGGAATAATGGAATAATGGTAG GCTAT-3’ |
| 539 Op frag3 R1| ureA downstream       | Reverse   | 5’-CAGATTTTGCTCCTGACAGCA-3’ |

#### Subjecting multiple strains to PCR to assess presence of urease operon

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| UreaseA F1     | ureA                  | Forward   | 5’-ATGCACTTAAACTTCCAGAG-3’ |
| UreaseA R1     | ureA                  | Reverse   | 5’-TTATCTGATTGGAATTATGC-3’ |
| 539 F1         | ureC                  | Forward   | 5’-CAACATGGCATTAACAATTTCAAG-3’ |
| 539 R1         | ureC                  | Reverse   | 5’-TTAGAATAGGAAATATCGCTG-3’ |

#### Cloning of ureC gene to express recombinant protein

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| 539 F1         | ureC                  | Forward   | 5’-CAACATGGCATTAACAATTTCAAG-3’ |
| 539 R1         | ureC                  | Reverse   | 5’-TTAGAATAGGAAATATCGCTG-3’ |

#### Performing RT PCR

| Primer         | Gene                  | Direction | Sequence¹ |
|----------------|-----------------------|-----------|-----------|
| UreA-UreB F1   | ureA, ureB            | Forward   | 5’-ATGAGTGTTACCGGAAGTG-3’ |
| UreA-UreB R1   | ureA, ureB            | Reverse   | 5’-ACGTTTTAACCGATCCGGA-3’ |
| UreB-UreC F1   | ureB, ureC            | Forward   | 5’-TGAAACCAATATGGCATCCT-3’ |
| UreB-UreC R1   | ureB, ureC            | Reverse   | 5’-TGGATGCGCGCTCATTGCAGC-3’ |
| UreC-UreE F1   | ureC, ureE            | Forward   | 5’-GGATTGGCGGACATATTGG-3’ |
| UreC-UreE R1   | ureC, ureE            | Reverse   | 5’-GAATGACTGTGAAATATGCC-3’ |
| UreE-UreF F1   | ureE, ureF            | Forward   | 5’-GCTGAAACCAAGATGATG-3’ |
| UreE-UreF R1   | ureE, ureF            | Reverse   | 5’-TGAATTGCGGAGCATATTGG-3’ |
| UreF-UreG F1   | ureF, ureG            | Forward   | 5’-TGCCCTGATGCAATGGAC-3’ |
| UreF-UreG R1   | ureF, ureG            | Reverse   | 5’-AGCAACAAAAGAATGAGCC-3’ |
| UreG-UreH F1   | ureG, ureH            | Forward   | 5’-AGTTTGCACCGCACAGGC-3’ |
| UreG-UreH R1   | ureG, ureH            | Reverse   | 5’-GTTCGTCATTCAACACC-3’ |

¹Underlined sequence denotes restriction enzyme site or cloning site
was induced by the addition of IPTG to a concentration of 4 mM. Cells were harvested by centrifugation after 4 hours and recombinant protein was purified with Talon Metal Affinity resin (Clontech, Mountain View, CA) using manufacturer’s instructions.

The purified recombinant protein was refolded by dialysis in buffer with sequentially decreasing concentrations of L-arginine. The buffer contained 0.15 M NaCl, 20 mM tris pH 9, with decreasing concentrations (1 M, 0.5 M, 5 mM) of L-arginine. Protease Arrest™ (EMD Chemicals, Gibbstown NJ) was added to purified protein.

Development of antiserum to urease C
Purified recombinant urease C was sent to Covance (Denver, PA) for antibody production in New Zealand white rabbits using a 59 day protocol. All applicable regulations for animal treatment were followed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (http://oacu.od.nih.gov/regs/guide/guide.pdf). Specific pathogen free rabbits received 250 μg of purified protein with complete Freund’s adjuvant subcutaneously on day 0, followed by 125 μg of protein with incomplete Freund’s adjuvant subcutaneously on days 21 and 49. Blood was obtained on day 59.

The rabbit antiserum was adsorbed with 11P6H ureC (urease C mutant) to remove background rabbit antibodies to H. influenzae. To accomplish this, bacteria were grown to log phase in broth, centrifuged to pellet bacteria, washed in PBS and suspended in 1 ml of a 1:1000 dilution of rabbit antiserum. After incubation for 30 min at 4°C, bacteria were removed by centrifugation. This process was repeated 3 more times. After the last adsorption, the serum was filter sterilized.

Reverse transcriptase-PCR
Bacteria were grown in chemically defined media (Table 1) and RNA was isolated using a QIAGEN RNeasy kit and a Qiashredder column (QIAGEN, Valencia, CA) following the manufacturer’s instructions, with an additional incubation with RNase-free DNaseI (Promega) for 30 min at 37°C. Reverse transcriptase PCR (RT-PCR) was performed using a QIAGEN OneStep RT-PCR kit and RNaseOut inhibitor (Invitrogen, Carlsbad, CA). Primers were designed to amplify fragments that would be predicted to correspond to transcripts that span adjacent genes in the urease gene cluster (Table 2). To exclude the possibility of contaminating DNA, parallel reactions with Taq DNA polymerase (HotMaster Mix; Eppendorf, Hamburg, Germany) were performed. Following amplification, samples were electrophoresed in 1% agarose gels and stained with ethidium bromide.

COPD Study Clinic
The COPD study clinic at the Buffalo Veterans Affairs Medical Center is an ongoing prospective study that was started in 1994 [54]. The study was approved by the Health Sciences Institutional Review Board of the University at Buffalo and the Human Studies Subcommittee of the Western New York Veterans Affairs Healthcare System. All study participants provided written informed consent. To be included in this study, patients must have chronic bronchitis as defined by the American Thoracic Society [61] and must be willing to attend the study clinic monthly. Patients with asthma, malignancies, or other immunocompromising illnesses were excluded. Patients were seen monthly and at times when an exacerbation was suspected. At each visit clinical criteria were used to determine whether patients were experiencing an exacerbation or whether they were clinically stable as previously described [54]. Additionally at each visit, serum and expectorated sputum samples were collected. Bacteria present in the sputum were identified using standard techniques. Serum and bacteria obtained from sputum cultures were stored at -80°C.

An exacerbation of COPD caused by H. influenzae was defined by the onset of clinical symptoms of an exacerbation simultaneous with the acquisition of a new strain of H. influenzae that had not previously been isolated from that patient based on molecular typing [54]. Serum samples collected one month prior to acquisition of the strain and one month following the exacerbation were used to analyze human serum antibody responses to the purified recombinant urease C.

Pooled human sputum
Expectorated sputum samples were collected from subjects in the COPD Study Clinic and were processed for culture as previously described [54,62]. Briefly, sputum samples were homogenized by incubation at 37°C for 15 minutes with an equal volume of 0.1% dithiothreitol. After an aliquot was removed for quantitative culture, sputum samples were centrifuged at 27,000 × g for 30 minutes at 4°C and supernatants were stored at -80°C until used. Samples from patients who were receiving antibiotics and samples that grew potential pulmonary bacterial pathogens in culture were excluded. Supernatants from approximately 100 sputum samples from 30 individuals were pooled for the purpose of growing bacteria in pooled sputum supernatants [13]. To render the sputum supernatants sterile, the pooled samples were placed in Petri dishes and exposed to UV light in a cell culture hood for approximately 10 minutes. An aliquot was plated on chocolate agar and no growth was detected after overnight incubation.
Quantitative real time PCR

*H. influenzae* was grown in the presence pooled human sputum from adults with COPD to simulate conditions in the human respiratory tract. To assess transcription of *ureC*, strain 11P6H was grown overnight in chemically defined media (CDM) at 37°C with shaking to which pooled human sputum supernatant of 20% of the volume of the culture was added [13]. A second culture was grown simultaneously in CDM to which PBS containing 0.1% dithiothreitol was added to 20% of the total volume as a control for the sputum supernatant. Cells were harvested by centrifugation at 10,000 × g for 10 minutes at 4°C. Cells were washed by suspending in cold PBS and centrifuging again using the same conditions. Bacterial RNA was isolated as described above (Reverse Transcriptase-PCR).

Quantitative real time PCR was performed using the BioRad MyiQ Real-Time PCR Detection System. Oligonucleotide primers pairs (Table 2) were designed with Primer 3 software. Each reaction mixture contained 5 ng purified RNA, 100 nM of each primer, 12.5 μl 2 × Sybr Green Supermix (BioRad), 0.125 μl reverse transcriptase and 6.375 μl water. Controls lacking reverse transcriptase or RNA template contained the appropriate volume of water in place of enzyme or template. Each purified RNA sample was tested for DNA contamination prior to proceeding with the real time PCR assay. Results with *gyrA*, a constitutively expressed gene, were measured in both growth conditions and used to normalize the results with *ureC* in the corresponding growth condition. These normalized results were used to calculate the fold change expression of *ureC* during growth in CDM plus sputum compared to CDM alone. BioRad iQ5 software was used to analyze data.

Enzyme-linked immunosorbent assay (ELISA)

Eighteen pre and post exacerbation serum pairs from adults with COPD followed in the COPD Study Clinic were subjected to ELISA to detect the development of new IgG antibodies in serum to urease C [48]. The change in antibody level from pre-exacerbation to post-exacerbation samples was calculated using the following formula: % change = \[((\text{post OD} - \text{pre OD}) / \text{pre OD}) \times 100\]. Paired pre-exacerbation and post-exacerbation samples were always tested in the same assay. The cutoff value for a significant percentage change between pre-exacerbation and post-exacerbation serum IgG levels was determined by studying 8 control pairs of serum samples obtained 2 months apart (the same time interval for the experimental samples) from patients who were clinically stable and who had negative sputum cultures for *H. influenzae* as described previously [42,43,48,63].

Susceptibility of *H. influenzae* to acid

*H. influenzae* wild type and mutant strains were grown in broth to log phase, harvested by centrifugation and suspended to a concentration of ~10^7 colony forming units/ml in PBS adjusted to varying pH. Cells were incubated in the presence or absence of urea (50 mM or 100 mM) and dilutions of bacteria were plated at time 0 and at 30 min. Bacteria were counted after overnight incubation on chocolate agar.

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Authors’ contributions

TFM was responsible for the conception and design of the study, analysis and interpretation of data, and drafting the manuscript. ALB made substantial contribution to the design of the study, acquired the data by performing the experiments and contributed important intellectual content to revisions of the manuscript. Both authors read and approved the final manuscript.

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References

1. Murphy TF, Faden H, Bakalez LQ, Kyd JM, Forsgren A, Campos J, Virji M, Petlon SJ. Nontypeable *Haemophilus influenzae* is a pathogen in children. *Pediatr Infect Dis J* 2009, 28(1):43-48.
2. Sethi S, Murphy TF. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2008, 179(22):235S-236S.
3. Murphy TF. Respiratory infections caused by non-typeable Haemophilus influenzae. *Curr Opin Infect Dis* 2003, 16(2):120-134.
4. Zalacain R, Sobradillo V, Amilibia J, Baron J, Achtotelu V, Pipio JL, Llorente JL. Predisposing factors to bacterial colonization in chronic obstructive pulmonary disease. *Eur Respir J* 1999, 13:43-48.
5. Soler N, Torres A, Ewig S, Gonzalez J, Celis R, El-Ebiary M, Hernandez C, Rodriguez-Rosin R. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. *Am J Respir Crit Care Med* 1995, 152:1498-1505.
6. Sethi S, Maloney J, George L, Wrona C, Benerson CS. Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2005, 172(9):991-998.
7. Monsio E, Ruiz J, Rosell A, Manerola J, Fiz J, Moreira J, Ausina V. 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* 1995, 152:1316-1320.
8. Droth EM, Skwarshi KM, Sauleda L, Soler N, Roca J, Agusti A, MacNee W. Oxidative stress and airway inflammation in severe exacerbations of COPD. *Thorax* 2005, 60(4):293-300.
9. Gemmisen WB, Asin J, Zanen P, van den Bosch JM, Haas FJ. Markers of inflammation and oxidative stress in exacerbated chronic obstructive pulmonary disease patients. *Respir Med* 2005, 99(1):84-90.
10. Dekhuijzen PN, Aben KK, Dekker J, Aarts LP, Welders PL, van Hervenader CL, Baal A. Increased exhalation of hydrogen peroxide in patients with stable and unstable chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 1996, 154(3):Pt 1:813-816.
11. Barnes PJ. The cytokine network in chronic obstructive pulmonary disease. *Am J Respir Cell Mol Biol* 2009, 41(6):631-638.
The role of Helicobacter pylori infection: the role of bacterial virulence factors. Dig Dis Sci 2010, 55(4):1042-1050.

12. Maroncle N, Rich C, Forestier C: Molecular biology of microbial ureases. Microbiol Rev 1995, 59(3):451-480.

13. Mason KM, Munson RS Jr: Urease. Trends Microbiol 2000, 10(11):e774-780.

14. Mobley HL, Island MD, Hausinger RP: Acid survival of Helicobacter pylori: Physiology and Genetics. Edited by: Mobley HLT, Mendez GL, Hazell SL. Washington DC: ASM Press; 2001, 2001/02/04 edn.

15. Molnar B, Galamb O, Sipos F, Leiszter K, Tulassay Z: Molecule pathogenesis of Helicobacter pylori infection: how does urease activity trigger cytoplasmic pH homeostasis? Trends Microbiol 2002, 10(2):70-74.

16. Molnar B, Galamb O, Sipos F, Leiszter K, Tulassay Z: Characterization of the urease operon of Brucella abortus and assessment of its role in virulence of the bacterium. Infect Immun 2007, 75(2):774-780.

17. Moralez N, Rich C, Foster RN: The role of Klebsiella pneumoniae urease in intestinal colonization and resistance to gastrointestinal stress. Res Microbiol 2006, 157(2):184-193.

18. Olivera-Severo D, Wassermann GE, Carlini CR: Urease of Helicobacter pylori and its implications for the mechanism of urease-dependent acid tolerance at pH 1. J Bacteriol 2002, 184(11):3053-3060.

19. Coker C, Poole CA, Li X, Mobley HL: Pathogenicity of Proteus mirabilis urinary tract infection. Microbes Infect 2000, 2(12):1497-1505.

20. Dattelbaum JD, Lockard CV, Johnson DE, Mobley HL: Urel, the transcriptional activator of the Proteus mirabilis urease gene cluster, is required for urease activity and virulence in experimental urinary tract infections. Infect Immun 2003, 71(2):1026-1030.

21. Island MD, Mobley HL: Proteus mirabilis urease: operon fusion and linker insertion analysis of ure gene organization, regulation, and function. J Bacteriol 1995, 177(19):5653-5660.

22. Burns RA, Chen YY: Bacterial ureases in infectious diseases. Microbes Infect 2000, 2(5):533-542.

23. Sangari FJ, Seoane A, Rodriguez MC, Aguero J, Garcia Lobo JM: Characterization of the urease operon of Brucella abortus and assessment of its role in virulence of the bacterium. Infect Immun 2007, 75(2):774-780.

24. Harris PR, Ernst PR, Kawabata S, Kiyono H, Graham MF, Smith PD: Recombinant Helicobacter pylori urease activates primary murine macrophages. J Infect Dis 1998, 178(5):1516-1520.

25. Zhang JY, Liu T, Guo H, Liu XF, Zhuang Y, Yu S, Chen L, Wu C, Zhao Z, Tang B, Luo P, Mao XH, Guo G, Shi Y, Zou QM: Expression of a Th17 cell response by Helicobacter pylori urease subunits B. Immunobiology 2010.

26. Tanahashi T, Kita M, Kodama T, Tamaoka Y, Sawai N, Ohno T, Mitsufuji S, Wai YP, Kishima K, Imanishi J: Cytochrome expression and production by purified Helicobacter pylori urease in human gastric epithelial cells. Infect Immun 2000, 68(2):654-671.

27. Harris PR, Mobley HL, Perez-Perez GI, Blaser MJ, Smith PD: Helicobacter pylori urease is a potent stimulus of mononuclear phagocyte activation and inflammatory cytokine production. Gastroenterology 1996, 111(2):412-418.

28. Wroblewski LE, Shen L, Ogden S, Romero-Gallo J, Lapereere LA, Israel DA, Turner JR, Peek RM Jr: Helicobacter pylori dysregulation of gastric epithelial tight junctions by urease-mediated myosin II activation. Gastroenterology 2009, 136(1):236-246.

29. Harris PR, Ernst PB, Kawabata S, Kiyono H, Graham MF, Smith PD: Helicobacter pylori urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. J Immunol 2000, 165(4):1918-1924.

30. Schwartz JT, Allen LA: Role of urease in megasomal formation and Helicobacter pylori survival in macrophages. J Leukoc Biol 2006, 79(6):1214-1225.

31. Maroncle N, Rich C, Forestier C: Highly significant role of Helicobacter pylori urease in phagocytosis and production of oxygen metabolites by human granulocytes. J Infect Dis 1998, 173(3):803-808.

32. Kuwahara H, Miyamoto Y, Akane T, Kubota T, Sawa T, Okamoto S, Maeda H: Helicobacter pylori urease suppresses bacteraidal activity of peroxynitrite via carbon dioxide production. Infect Immun 2000, 68(8):4378-4383.

33. Fan X, Gunasena H, Cheng Z, Espoo R, Crowe SE, Ernst PB, Reyes VE: Helicobacter pylori urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. J Immunol 2000, 165(4):1918-1924.

34. Schwartz JT, Allen LA: Role of urease in megasomal formation and Helicobacter pylori survival in macrophages. J Leukoc Biol 2006, 79(6):1214-1225.

35. Maroncle N, Rich C, Forestier C: Highly significant role of Helicobacter pylori urease in phagocytosis and production of oxygen metabolites by human granulocytes. J Infect Dis 1998, 173(3):803-808.

36. Kuwahara H, Miyamoto Y, Akane T, Kubota T, Sawa T, Okamoto S, Maeda H: Helicobacter pylori urease suppresses bacteraidal activity of peroxynitrite via carbon dioxide production. Infect Immun 2000, 68(8):4378-4383.

37. Harris PR, Ernst PB, Kawabata S, Kiyono H, Graham MF, Smith PD: Helicobacter pylori urease binds to class II MHC on gastric epithelial cells and induces their apoptosis. J Immunol 2000, 165(4):1918-1924.

38. Schwartz JT, Allen LA: Role of urease in megasomal formation and Helicobacter pylori survival in macrophages. J Leukoc Biol 2006, 79(6):1214-1225.
54. Sethi S, Evans N, Grant BJB, Murphy TF: New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med 2002, 347:465-471.

55. Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ: Haemophilus haemolyticus: a human respiratory tract commensal to be distinguished from Haemophilus influenzae. J Infect Dis 2007, 195(1):81-89.

56. Menard R, Sansonetti PJ, Parsot C: Nonpolar mutagenesis of the ipa genes defines IpaB, IpaC, and IpaD as effectors of Shigella flexneri entry into epithelial cells. J Bacteriol 1993, 175(1):590-596.

57. Hemiot RT, Meyer EP, Vogt M, Modan M: Defined medium for growth of Haemophilus influenzae. J Bacteriol 1970, 101:513-516.

58. Poje G, Redfield RJ: Transformation of Haemophilus influenzae. In Haemophilus influenzae protocols. Edited by: Herbert M, Wood D, Moxon E. Totowa, NJ: Humana Press; 2003:57-70.

59. Murphy TF, Kirkham C, Lesse AJ: Construction of a mutant and characterization of the role of the vaccine antigen P6 in outer membrane integrity of nontypeable Haemophilus influenzae. Infect Immun 2006, 74(9):5169-5176.

60. Senior BW, Bradford NC, Simpson DS: The ureases of Proteus strains in relation to virulence for the urinary tract. J Med Microbiol 1980, 13(4):507-512.

61. American Thoracic Society: Standards for the diagnosis and care of patients with chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1995, 152(5 Pt 2):S77-S121.

62. Sethi S, Muscarella K, Evans N, Klingman KL, Grant BJB, Murphy TF: Airway inflammation and etiology of acute exacerbations of chronic bronchitis. Chest 2000, 118:1557-1565.

63. Murphy TF, Kirkham C, Liu DF, Sethi S: Human immune response to outer membrane protein CD of Moraxella catarrhalis in adults with chronic obstructive pulmonary disease. Infect Immun 2003, 71(3):1298-1304.

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