Nontypeable Haemophilus influenzae Genetic Islands Associated with Chronic Pulmonary Infection

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Abstract

Background: Haemophilus influenzae (Hi) colonizes the human respiratory tract and is an important pathogen associated with chronic obstructive pulmonary disease (COPD). Bacterial factors that interact with the human host may be important in the pathogenesis of COPD. These factors, however, have not been well defined. The overall goal of this study was to identify bacterial genetic elements with increased prevalence among H. influenzae strains isolated from patients with COPD compared to those isolated from the pharynges of healthy individuals.

Methodology/Principal Findings: Four nontypeable H. influenzae (NTHi) strains, two isolated from the airways of patients with COPD and two from a healthy individual, were subjected to whole genome sequencing using 454 FLX Titanium technology. COPD strain-specific genetic islands greater than 500 bp in size were identified by in silico subtraction. Open reading frames residing within these islands include known Hi virulence genes such as lic2b, hgbA, iga, hmw1 and hmw2, as well as genes encoding urease and other enzymes involving metabolic pathways. The distributions of seven selected genetic islands were assessed among a panel of 421 NTHi strains of both disease and commensal origins using a Library-on-a-Slide high throughput dot blot DNA hybridization procedure. Four of the seven islands screened, containing genes that encode a methyltransferase, a dehydrogenase, a urease synthesis enzyme, and a set of unknown short ORFs, respectively, were more prevalent in COPD strains than in colonizing strains with prevalence ratios ranging from 1.21 to 2.85 (p<0.0002). Surprisingly, none of these sequences show increased prevalence among NTHi isolated from the airways of patients with cystic fibrosis.

Conclusions/Significance: Our data suggest that specific bacterial genes, many involved in metabolic functions, are associated with the ability of NTHi strains to survive in the lower airways of patients with COPD.

Introduction

The impaired lower airways in persons with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are especially susceptible to bacterial colonization and infection. Non-typeable H. influenzae (NTHi), which lack a polysaccharide capsule, are the most commonly isolated bacteria from lower respiratory tracts of adults with COPD and acquisition of new NTHi strains is often associated with exacerbation [1,2,3,4], likely because of its important role in stimulating immune responses in damaged respiratory cells and tissues leading to further damage [5,6]. COPD is the third leading cause of death in the United States [7], and mortality rates for COPD appear to be increasing worldwide [8].

Numerous studies have demonstrated the dynamic nature of H. influenzae asymptomatic colonization of the human pharynx, characterized by the carriage of multiple NTHi strains at any one time [9,10,11,12,13] and apparent rapid bacterial turnover [13,14,15,16]. Thus H. influenzae in the upper respiratory tract form a diverse pool of organisms from which organisms that infect the lower airway emerge. The lower respiratory tract, however, likely represents a different environmental niche than the upper airway and H. influenzae isolated from these sites show phenotypic differences. For example, in comparison to colonizing isolates from the upper airway, pulmonary isolates from the lower airway show enhanced expression of vacJ and ysd and increased serum resistance [17]. Thirty gene products, including anti-oxidant and stress-related proteins, as well as cofactor and nutrient uptake
systems were produced in greater abundance by *H. influenzae* grown in human sputum from COPD patients compared to broth-grown organisms, indicating a growth adaptation of *H. influenzae* in sputum [18]. We hypothesize that among the diverse pool of *H. influenzae* colonizing the human upper respiratory tract, strains expressing specific phenotypic characteristics exhibit a fitness advantage that allows them to persist in the lower airway and contribute to the inflammatory response that leads to COPD.

Genome comparison between pathogenic and nonpathogenic strains within a species is a powerful strategy for identifying candidate genes important for bacterial pathogenesis [19,20,21]. *H. influenzae* is well suited for such a comparison as gene content between strains varies considerably [22,23] and >50% of genes identified in *H. influenzae* are not found in all strains [21,24,25,26]. Associations of certain NTHi genes with otitis media have been well documented and include lic2B, which is involved in lipo polysaccharide biosynthesis, the *hmw* genes, which encode high-molecular-weight adhesins, and the *his* operon, which is responsible for histidine biosynthesis [27,28]. Further, HIG12 and HIG17, genetic islands first described in a type b strain [29], were significantly more prevalent in NTHi isolates from children with otitis media than in those from the throats of healthy children [30]. The objective in this study was to identify additional NTHi genes contributing to COPD pathogenesis. We used *in silico* whole NTHi genome subtraction to identify candidate COPD-associated gene regions, followed by population screening by DNA hybridization to identify candidate genes selectively enriched among isolates cultured from the airways of patients with COPD compared to strains from the airways of patients with cystic fibrosis (CF) or commensal strains isolated from the upper airways of healthy individuals. This analysis identified at least four genetic islands that are more associated with bronchial infections in COPD patients.

**Materials and Methods**

**Bacterial strains**

The bacterial strains used in this study included 421 NTHi, 25 typeable Hi, and 28 *Haemophilus haemolyticus*. All strains were initially identified as *H. influenzae* on the basis of colonial morphology during growth on chocolate agar with bacitracin, the requirement for X and V factors, porphyrin negativity, and lack of hemolysis of horse red blood cells [31,32,33]. These strains were further screened to confirm species designation based on the presence of *iga* and *lgtC* genes described previously [34,35]. In this study, we defined putative *H. influenzae* strains that were positive for *iga* and *lgtC* as *H. influenzae* and those negative as *H. haemolyticus*. The value of these markers to distinguish *H. influenzae* from *H. haemolyticus* has been documented by phylogenetic analyses [34,36,37] and the hybridization based method used here for species discrimination was validated previously [35]. Differentiating encapsulated from nontypeable *H. influenzae* was done by detecting the *bexA* and *bexB* genes of the capsule locus by PCR, based on our published method [38].

Previously collected strains were used in this study. Of the 421 NTHi, 96 were throat isolates collected from healthy individuals [32,39]; 101 were sputum isolates from patients with COPD collected at the University of Michigan Medical Center laboratories, or obtained from a prospective study at the Buffalo Veterans Administration Medical Center or obtained from Dr. David Hui (Chinese University of Hong Kong); 77 were isolates from patients with CF (71 sputum samples, 1 bronchioalveolar lavage sample, and 5 throat or nasopharyngeal samples) obtained from the University of Michigan Medical Center laboratories; and 147 isolates of various origins were collected from patients with clinical conditions other than COPD and CF at the University of Michigan. The 101 COPD isolates consisted of 37 isolates from patients without exacerbation and 64 isolates from patients with exacerbation.

Additional complete or partially sequenced *H. influenzae* were used as reference strains and included Rd (ATCC 51907), 86-028NP (from Lauren Bakaletz, Ohio State University), R2886 and R2846 (from Arnold Smith, University of Washington), and PitAA, PitBB, PitCC, PitDD, PitEE, PitFF, PitGG, PitHH, PitII and PitJJ from one of the authors (GDE), and a *H. haemolyticus* type strain, ATCC 33390.

The strains were collected from many colleagues over many years under approval by the Human Use Committees at each institution and stored at their institutions. Since the strains have no identifiers attached to them, the Univ of Michigan Human Use Committee approved their use on an EXEMPT status. “IRB EXEMPTION #4 (45 CFR 46.101(b)(4)); Research involving the collection or study of existing data, documents, records, pathological specimens, or diagnostic specimens, if these sources are publicly available or if the information is recorded by the investigator in such a manner that subjects cannot be identified, directly or through identifiers linked to the subjects.”

**H. influenzae genome sequencing**

Two COPD strains (6P18H1 and 7P49H1) and two throat strains (22-1.21, 22-4.21) were selected for initial comparative whole genomic analyses. Strains 6P18H1 and 7P49H1 were isolated from the expectorated sputum of two different adults with COPD followed in a prospective study performed at the Buffalo VA Medical Center. Based on molecular typing of strains collected from monthly cultures of sputum samples, each of these strains was initially acquired at the time of clinical evidence of an exacerbation of COPD. The acquisition of a new strain of NTHi simultaneous with the onset of symptoms of an exacerbation represents strong evidence that these strains caused exacerbations [40]. These strains were sequenced using 454 Lifesciences FLX pyrosequencing technology (454 Life Sciences) at the Center for Genomic Sciences, Allegheny-Singer Research Institute, Pittsburgh. Each genome was sequenced to a depth of 16x, or greater, and assembled into contigs using the Newbler de novo Assembler Software from 454 Life Sciences. The resulting numbers of contigs from the four genomes ranged from 18 to 53. The Microbial Genome Annotation Tools and Genome Annotation Pipeline from NCBI were used to predict and annotate the coding sequences (CDSs) (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html). The draft genomes have been deposited with GenBank and two genomes were used in an earlier analysis [25]. The accession numbers for these genome assemblies are AAZD00000000 (22.1–21), AAJ00000000 (22.4–21), ABVV00000000 (7P49H1), and ABWW00000000 (6P18H1).

**In silico genome subtraction**

To assemble separate contigs into a single genome sequence file, we used finished genome sequences of *H. influenzae* strains 86-028NP and Rd KW20 as references to order contigs based on sequence alignment. When contigs could not be mapped they were concatenated and added to the end of assembled genome. Both global and local sequence alignments among the four genome sequences were used to identify genomic differences between COPD strains and throat strains. Global whole genome alignments among the four genomes were constrained using Progressive Mauve algorithm under alignment parameters that are appropriate for aligning closely related genomes with moderate
to high amounts of genome rearrangement [41]. COPD strain-specific genomic islands greater than 500 bp were identified. These islands were further verified by local sequence alignments using BLASTn [42] by querying each island against all four genomes sequences.

Detection of genomic islands among the bacterial collections

The presence or absence of selected genomic islands identified by the in silico subtraction was determined by a high-throughput dot-blot hybridization on the Library-on-a-Slide (LOS) array platform developed previously in our laboratory [35,43,44].

To prepare genomic island-specific DNA probes, primers hybridizing the internal regions of each island were used to amplify DNA fragments using strain 6P18H1 as the template in a standard 30 cycles PCR reaction. The primer sequences and annealing temperatures are listed in Table 1. These PCR products were purified and fluorescein-labeled using the Fluorescein-High Prime kit from Roche Applied Science (Indianapolis, IN). In addition, a DNA concentration control probe, a mixture of seven H. influenzae MLST gene fragments (http://Haemophilus.mlst.net/) and the coding region of pepN, was prepared and labeled with digoxigenin (DIG) (DIG High Prime, Roche, Indianapolis).

Bacterial cells were harvested from chocolate agar plates (BBL) incubated overnight at 37°C with 5% CO2. Total genomic DNA was extracted using the GenElute Bacterial Genomic DNA kit from Sigma-Aldrich (St. Louis, MO). DNA from all strains was arrayed in duplicates onto a single nylon membrane coated slide (VividTM Gene Array Slides, Pall Life Sciences, Ann Arbor, MI) as described previously [35,44,45].

Hybridization and detection of gene probes and the analysis of the probing results have been described in detail elsewhere [35,43,44,45]. Briefly, each slide was first hybridized at 65°C in PerfectHyb Plus hybridization buffer (Sigma-Aldrich) with the digoxigenin-labeled control probe, serially washed with low- and high-stringency buffers, and analyzed. The slides were then stripped, washed, and rehybridized with a fluorescein-labeled genomic island specific probe. Spotfinder v.3.1.1 and MIDAS v.2.19 were used for spot signal extraction and normalization, respectively. The ratio of the log-transformed genomic island hybridization signal to the concentration-control signal was analyzed in the software “R”. A two-component Gaussian mixture model was fitted to classify the observed intensities into positive or negative spots [35].

Statistical analyses

Prevalence ratios were calculated as the ratio of the proportion of clinical isolates possessing the tested genomic island to the proportion of isolates in the reference group, i.e., from throats of healthy individuals. Chi-square analysis or Fisher’s exact test was used to determine the significance of the differences in genomic island proportion between groups. Benjamini-Hochberg Step-Up FDR (false discovery rate)-controlling procedure was used to adjust for multiple comparisons [46]. An adjusted p value of ≤0.5 was considered significant. Statistical analyses were performed with SAS software (version 9.1).

Results

Identification of genetic islands (>500 bp) in genomic sequences of two COPD strains

As the first step in identifying H. influenzae candidate genes important in COPD pathogenesis, we conducted an in silico genome subtraction analysis between two COPD strains, 7P49H1 and 6P18H1 isolated from two different COPD patients, and two throat strains, 22.1–21 and 22.4–21 with very different multilocus sequence types isolated from one individual at two different time points [39]. With each genome sequenced to a depth of at least 16x, the sizes of the draft genome assemblies obtained ranged from 1.82Mb to 1.91Mb. These sizes are comparable to those of fully finished H. influenzae genomes, indicating high genome sequence coverage.

Sequence analysis of these four draft genomes showed both gene content and sequence variation comparable to those seen in an earlier analysis of 12 H. influenzae genomes [25]. Figure 1 displays a whole genome alignment of these four roughly assembled genomes

| Targeted genomic Island | Sequence (5’–3’) | Amplicon (bp) | Annealing temperature |
|-------------------------|-----------------|---------------|-----------------------|
| G1 F                    | GCACCTCAAGGGGCTAAG | 1046          | 55°C                  |
| R GAAGATAATACGGCGGAATTCAAT |                 |               |                       |
| G2 F                    | TCTAAATTCCATCGGAGTA | 570           | 48°C                  |
| R TTTTGGGGTATATATGTC     |                 |               |                       |
| G6 F                    | GTAAAGCCGTCGGTGATGCGAT | 1334         | 63°C                  |
| R CGCTGTTTGCCTGCGATCAAG |                 |               |                       |
| G8 F                    | ATGGGTATATATTCTGTC | 814           | 50°C                  |
| R AATCCCTTGTCCATCATCA   |                 |               |                       |
| G9 F                    | TAACCTCAAACATAATGGTCGCTCAAG | 444        | 52°C                  |
| R TATCCTCTATTTAACTTAC   |                 |               |                       |
| G10 F                   | AGGGCGTTTTATGTTTGGTAGA | 1603        | 58°C                  |
| R TTTTTGGGATATGTTTGGTTAG |                 |               |                       |
| G11 F                   | CAAAAGATTGCGTACCTA | 616           | 50°C                  |
| R GTTATATTTTCTTACACTTCC |                 |               |                       |

Note: Product sizes are based on 6P18H1 genome. F – forward primer; R – reverse primer.

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showing a common genomic backbone and genetic differences. We limited our bioinformatics analysis of the differences in the genomic contents to identify genetic islands greater than 500 bp in length so that a manageable number of genetic islands could be analyzed and to increase the probability of at least one or more coding regions within the islands. Using Progressive Mauve algorithm and BLASTn we identified 15 genetic islands (>500 bp) present in the genomes of the two NTHi strains isolated from patients with COPD and absent in two NTHi strains from healthy individuals. The sizes of these islands ranged from 628 bp to 5516 bp and totaled 33 kb in length (Table 2).

Each identified genetic island contained coding sequences, some representing known *H. influenzae* virulence factors while others are involved in metabolic functions whose role in pathogenesis is not clear. The glycosyl transferase gene lic2B, found in genetic island G5, was initially found to be important for NTHi pathogenesis based on epidemiological data [27, 47] and recently was shown to contribute to *H. influenzae* virulence by confirming serum resistance through a galactose addition to the LOS outer core [48].

Genetic island G3 contains a gene encoding an IgA protease which is different from the typical IgA protease encoding gene iga present in all *H. influenzae* and is similar to the more recently identified IgA protease encoding gene igaB [49, 50]. IgA1 protease activity was significantly higher in *H. influenzae* isolated from infected patients than in those isolated from throat swabs of asymptomatic carriers [51]. Recent prevalence studies showed that igaB is more common in strains recovered from adults with COPD than strains isolated from other clinical sources or from the throats of carriers [49, 50].

Sequences in genetic island G4 encode a hemoglobin and hemoglobin-haptoglobin binding-like protein (Hgp), important for iron acquisition and was shown to be associated with virulence in invasive infection of *H. influenzae* in animal model [52]. Genetic islands G12 to G15 all contained genes related to high-molecular-weight proteins (HMW). While labeled as four genetic islands because segments were found in four different contigs, they probably represent the two HMW loci known to be present in NTHi genomes. Islands G12 and G13 were located on contigs that mapped adjacent to each other as did islands G14 and G15. HMW mediate attachment to human epithelial cells [53] and hmaBC, the conserved elements in the operon, was shown to be more prevalent in NTHi isolates from the middle ears of children with otitis media than in isolates from the throats of healthy children [54].

Other genetic islands contained sequences encoding proteins involved in metabolic functions that included a SAM-dependent methyltransferase (G1), enzymes in urea metabolic pathway (G6), an aspartate-semialdehyde dehydrogenase (G8), another predicted glycosyltransferase that was different from Lic2B (G9), a likely ABC transporter (G10), and a probable TonB-dependent receptor (G11). Genetic island G2 contained multiple small open reading frames (ORFs) and its N terminus seemed to be part of a transcriptional regulator. Genetic island G7 likely originated from...

![Figure 1. Whole genome comparison of assembled/concatenated contigs of four genomes.](image-url)
Prevalence of selected genetic islands among *proteins* and other small hypothetical proteins. A transposon as it contains genes that encode transposase-like proteins and other small hypothetical proteins.

**Prevalence of selected genetic islands among *H. influenzae* from different sources**

To evaluate the disease-related importance of the genetic islands identified through *in silico* subtraction, we examined by DNA hybridization their distributions among a panel of both disease-associated and commensal *H. influenzae* strains that included a random sample of 96 throat isolates from healthy individuals representing commensal NTHi, 101 disease isolates from the airways of COPD patients and 77 isolates from the airways of patients with cystic fibrosis (CF). In addition, the screening assay tested 147 NTHi strains from patients with and without exacerbation.

Distribution of genetic islands between COPD isolates from patients with and without exacerbation

Other investigators, Fernaays et al, have hypothesized that differences among strains of *H. influenzae* contributed to different clinical presentations in COPD-associated infection [49]. Our COPD collection consisted of 64 strains isolated from patients with exacerbation and 37 strains isolated from patients without exacerbation. We performed a stratified comparison to test whether selected genetic islands might be associated with exacerbation (Table 4). The prevalences of six of seven genetic islands were similar (PRs close to 1) in the two groups. While PR comparisons, many of these differences between commensal strains and disease strains were statistically significant. Four of the 7 genetic islands, G2, G6, G8 and G10, were found to be significantly more prevalent among COPD strains compared to commensal strains. In contrast to COPD strains, only one genetic island (G1, containing a SAM-dependent methyltransferase) showed a significant difference in prevalence in CF strains compared to throat strains and it was significantly less, rather than more, frequent in CF strains than in throat strains. Compared to CF strains, NTHi isolates that originated from other clinical conditions were more similar to COPD strains in the distribution of these 7 genetic islands. The four genetic islands more prevalent in COPD strains were significantly or marginally significantly more prevalent in other clinical strains. However, the absolute prevalence of these genetics islands in those strains was lower than in COPD strains.

Although only a small number of typeable Hi and *H. haemolyticus* strains were included in the screening, the distribution of the genetic islands in these two groups showed an interesting contrast to NTHi. In general, island G1 was more and islands G2, G8 and G9 were less frequently found in typeable Hi and *H. haemolyticus*, a non-pathogenic organism closely related to *H. influenzae*, that also colonizes the human pharynx were included in the comparisons.

Our population screening focused on probing with genetic islands containing sequences that were previously not defined as virulence factors or only minimally studied. Thus, we excluded genetic islands G3 (IgA), G4 (Hgbs), G5 (Lic2B), and G12 to G15 (HMW) in this analysis. In addition, island G7 was also excluded because it contained mostly transposon elements, leaving seven genetic islands eligible for prevalence analysis.

The prevalence of each selected genetic island was tabulated for the comparisons. Many of these differences between commensal strains and disease strains were statistically significant. Four of the 7 genetic islands, G2, G6, G8 and G10, were found to be significantly more prevalent among COPD strains compared to commensal strains. In contrast to COPD strains, only one genetic island (G1, containing a SAM-dependent methyltransferase) showed a significant difference in prevalence in CF strains compared to throat strains and it was significantly less, rather than more, frequent in CF strains than in throat strains. Compared to CF strains, NTHi isolates that originated from other clinical conditions were more similar to COPD strains in the distribution of these 7 genetic islands. The four genetic islands more prevalent in COPD strains were significantly or marginally significantly more prevalent in other clinical strains. However, the absolute prevalence of these genetics islands in those strains was lower than in COPD strains.

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**Table 2. CODP strain specific genetic islands identified by *in silico* subtraction.**

| Island | Size (bp) | Location Contig (starting bp position into contig) | No. of ORFs | Blast Match/Perdition |
|--------|-----------|-------------------------------------------------|-------------|-----------------------|
| G1     | 1215      | 21 (22976)                                       | 1           | SAM-dependent methyltransferase |
| G2     | 714       | 23 (267)                                         | multiple    | Potential transcriptional regulator and other small ORFs |
| G3     | 3813      | 23 (21413)                                       | 1           | IgA-specific serine endopeptidase |
| G4     | 2818      | 27 (6519)                                        | 2           | Carbamoyl kinase and hemoglobin and hemoglobin-haptoglobin binding like protein |
| G5     | 1918      | 48 (34610)                                       | 2           | Glycosyltransferase Lic2B and undefined protein |
| G6     | 5516      | 48 (39066)                                       | 7           | Urease operon (UreA, B, C, E, F, G, H) |
| G7     | 2705      | 42 (1193)                                        | 6           | Transposase-like proteins and other small hypothetical proteins |
| G8     | 1617      | 43 (277467)                                      | 1           | Aspartate-semialdehyde dehydrogenase |
| G9     | 628       | 43 (289778)                                      | 1           | Hypothetical protein (predicted glycosyltransferase) |
| G10    | 2778      | 6 (65373)                                        | 1           | ABC transporter ATP-binding |
| G11    | 697       | 6 (68252)                                        | 1           | Probable TonB-dependent receptor |
| G12    | 829       | 7 (2916)                                         | 1           | High-molecular-weight proteins (hmw locus 1) |
| G13    | 2857      | 17 (1290)                                        | 1           | High-molecular-weight proteins (hmw locus 1) |
| G14    | 3781      | 20 (1)                                           | 2           | High-molecular-weight proteins (hmw locus 2) |
| G15    | 962       | 44 (256)                                         | 1           | High-molecular-weight proteins (hmw locus 2) |

Note: Size and location are based on 6P18H1 genome. G12 and G13 are on two adjacent contigs representing two split parts of one *hmw* locus in the genome. G14 and G15 are on two adjacent contigs as well representing two split parts of the second *hmw* locus in the genome.
Table 3. Prevalence of seven genetic islands in *H. influenzae* isolates of different origins.

| Island | No. (%) with island | No. (%) with island | P value | PR (95% CI) |
|--------|---------------------|---------------------|---------|-------------|
| G1     | 226 (23.6)          | 170 (17.0)          | 0.0091  | 1.36 (1.17, 1.58) |
| G2     | 145 (15.0)          | 53 (5.2)            | <0.0001 | 2.77 (2.41, 3.18) |
| G3     | 60 (6.1)            | 27 (2.7)            | 0.0020  | 2.30 (1.66, 3.18) |
| G4     | 142 (14.5)          | 61 (6.0)            | 0.0041  | 2.34 (1.66, 3.28) |
| G5     | 75 (7.6)            | 29 (2.9)            | 0.0003  | 2.61 (1.70, 3.96) |
| G6     | 121 (12.3)          | 98 (9.7)            | 0.0007  | 1.25 (1.05, 1.49) |
| G7     | 38 (3.9)            | 17 (1.7)            | 0.0031  | 2.26 (1.49, 3.43) |
| G8     | 35 (3.6)            | 24 (2.4)            | 0.0031  | 1.45 (1.02, 2.06) |
| G9     | 30 (3.1)            | 11 (1.1)            | 0.0041  | 2.67 (1.59, 4.52) |
| G10    | 121 (12.3)          | 61 (6.0)            | 0.0041  | 2.00 (1.34, 3.00) |
| G11    | 2 (0.2)             | 8 (0.8)             | 0.0020  | 2.30 (1.66, 3.28) |

*PR = prevalence ratio.*

Note: P values were adjusted by Benjamini-Hochberg Step-Up FDR procedure to account multiple comparisons.

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Discussion

While bacteria often utilize complex regulatory systems to alter gene expression in response to environmental changes, *H. influenzae*, with a small yet highly variable genome and a niche limited to the human respiratory tract, also appear to rely on natural selection and clonal expansion of selected strains to survive in varying environments [55]. This study exploited the process of natural selection that occurs as NTHi evolve in the human host, and uses epidemiologic analyses to identify specific *H. influenzae* genes that have been disproportionally preserved, and thus more critical to survival and disease initiation, among isolates cultured from patients with COPD compared to commensal NTHi isolated from throats of healthy individuals. Previously, we used a similar strategy that successfully identified NTHi genes associated with otitis media in which subtractive genomic hybridization was used to generate candidate sequences [27,47]. In this study, we took advantage of next generation DNA sequencing technology to generate whole genome sequences used in *in silico* genome subtraction to generate candidate COPD-specific sequences. Among 15 genetic islands found initially, seven—those that didn’t possess previously known Hi virulence factors—were subjected to population prevalence analyses utilizing a panel of 421 NTHi to identify genes significantly more prevalent among COPD strains than commensal strains. Four of these seven genetic islands (G2, G6, G8, and G9) were found to be significantly associated with NTHi isolated from the airways of COPD patients.

The genetic island G2 had the strongest association with COPD strains compared to throat strains with a prevalence ratio of 2.85. The potential function, however, of the G2 was difficult to infer from its sequence. The 714 bp fragments contained multiple ORFs no greater than 51 amino acids in size. This sequence could contain short sequences involved in gene regulation or encode small peptides. G2 was found to be highly similar to a DNA fragment 183UM identified in an early study that attempted to identify genes associated with exacerbations of COPD [49]. In that study, an alternative hypothesis was put forward that the 183UM region was found to be associated with exacerbation in our univariate analysis. As additional genes are for island G11 is 4.04, its absolute prevalence in both groups was very low and not statistically different.
exacerbation strains similar to the analysis performed in the strains compared to throat strains while it was similarly distributed strains, i.e. it was significantly less prevalent (PR = 0.36) in CF COPD strains. Island G1 showed a negative association with CF islands among CF strains differed from the distribution among islands among COPD and commensal strains. G1 encodes a type of S-adenosyl-L-methionine (AdoMet)-dependent methyltransferase (MTases), enzymes that usually transfer methyl groups to compounds on substrates such as nucleic acids, proteins, and many small molecules [62] and alter the targeting and timing of gene expression and activity of certain enzymes [63]. The genetic island frequency data indicated that NTHi factors important in bronchial infection in CF patients were different than in COPD patients.

Bronchial infections in patients with COPD and CF share important clinical and pathogenic features, including long term chronicity with episodic exacerbations characterized by increased dyspnea, increased sputum volume and increased sputum purulence; progressive cycles of chronic lung inflammation and infection; and abnormal structural remodeling of the lower airways as a result of chronic inflammation and infection. The primary pathogenesis of these two conditions, however, is different. CF is an inherited disorder characterized by mutation of the cystic fibrosis transmembrane regulator (CFTR), an adenosine triphosphate-dependent chloride channel that leads to exocrine gland dysfunction. Resulting dehydrated respiratory secretions decrease normal mucociliary clearance of bacteria and facilitate chronic infection and inflammation that lead to progressive suppurative obstructive lung disease. COPD is an acquired disorder caused by airway damage, primarily from smoking, with resultant impaired bacterial clearance that leads to inflammation, chronic infection and end-airway obstruction.

The microbial components of chronic infection in patients with COPD and cystic fibrosis vary with the stage of the infection. Among patients with cystic fibrosis, Staphylococcus aureus and H. influenzae predominate in young children whereas older children and adults exhibit chronic infection with Pseudomonas aeruginosa, Stenotrophomonas maltophilia and Burkholderia cepacia [64]. Irrespective of the stage of COPD, H. influenzae, Streptococcus pneumoniae, and Moraxella catarrhalis are the most commonly isolated pathogens from the lower respiratory tract [40]. The explanation for the differences in the flora of these two seemingly similar disease processes remains unclear, but may rest with differences in the local milieu-nutritional, chemical, or physical-in the airways. Thus, different environmental conditions, either host or microbial community driven, in the airways of patients with COPD or cystic fibrosis may explain the differences in prevalence of specific H. influenzae genes as seen in this study, i.e. possession of certain genes and expression of their gene products may predispose different strains of H. influenzae to successful chronic infection in patients with cystic fibrosis or COPD.

### Table 4. Prevalence of seven genetic islands in COPD NTHi isolates from patients with and without exacerbations.

| Island | Isolates from COPD patients without exacerbations (n = 37) | Isolates from COPD patients with exacerbations (n = 64) | PR* | P value |
|--------|----------------------------------------------------------|----------------------------------------------------------|-----|---------|
| G1     | 10(27.0)                                                 | 15(25.8)                                                 | 0.87| 0.6871  |
| G2     | 16(43.2)                                                 | 26(40.6)                                                 | 0.94| 0.797   |
| G6     | 37(100)                                                  | 61(95.3)                                                 | 0.97| 0.3714  |
| G8     | 28(75.7)                                                 | 49(76.6)                                                 | 1.01| 0.9196  |
| G9     | 33(89.2)                                                 | 53(82.8)                                                 | 0.95| 0.5417  |
| G10    | 18(48.6)                                                 | 35(54.7)                                                 | 1.12| 0.5582  |
| G11    | 1(2.7)                                                   | 7(10.9)                                                  | 4.04| 0.2713  |

*PR = prevalence ratio.

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screened we plan to perform multivariate analyses to identify sets of combined genes that differentiate exacerbation and non-exacerbation strains similar to the analysis performed in the Fernaays study [49].

Island G6 contains the entire urease operon. Urease is a nickel metalloenzyme that catalyzed urea into carbon dioxide and ammonia, generates nitrogen for bacterial growth, and allows bacteria to survive in acid environments [56]. In other bacteria, increased urease expression occurs in nitrogen-limited environments, in acid environments, and by urea induction. Thus, urease production may be beneficial for NTHi survival and infection of the chronically inflamed airways of COPD patients while simultaneously damaging the respiratory epithelium, either by increasing the local pH or by direct ammonia toxicity. Genetic island G8, predicted to encode aspartate-semialdehyde dehydrogenase (Asd), was also strongly associated with COPD strains. Asd forms an early branch point in the metabolic pathway forming lysine, methionine, leucine, and isoleucine from aspartate and generates diaminopimelic acid (DAP), an essential component of the Gram-negative bacterial peptidoglycan [57]. To maintain membrane integrity, asd auxotrophs require diaminopimelic acid (DAP) [58], which may be more abundant in the pharynx than in airways of COPD patients.

Island G9 encodes a predicted glycosyltransferase that is iron and heme inducible [59]. The neighboring genes HI1384 and HI1385 encode ferritin subunits which form a macromolecular structure that stores and detoxifies Fe when cellular levels become elevated [60]. The functional relationship of the glycosyltransferase to the ferritin is unknown but human ferritin is glycosylated [61], and thus, the bacterial ortholog may also require glycosylation for function and may in turn be important in pathogenesis.

The distribution of genetic islands among NTHi strains isolated from patients with clinical conditions other than COPD and CF mirrored that seen in COPD isolates but prevalences of these islands were comparably lower. Three of the four COPD-associated islands were also significantly more prevalent in those strains than in throat strains. Given that this collection was a mixture of strains from different sources and likely contained some lower airway NTHi pathogens with different growth requirements from those required for survival in the airways of COPD patients, we would expect lower PRs for these genetic islands when compared to that in the well-defined COPD collection.

One surprising finding was that the distribution of the genetic islands among CF strains differed from the distribution among COPD strains. Island G1 showed a negative association with CF strains, i.e. it was significantly less prevalent (PR = 0.36) in CF strains compared to throat strains while it was similarly distributed among COPD patients with and without exacerbations.
Another observation was the relatively or extremely lower frequencies of those COPD-associated genetic islands (G2, G6, G11, and G19) in *H. haemolyticus*. Since *H. haemolyticus* is generally considered to be non-pathogenic and does not cause disease or live in normally sterile sites [27,36], such findings could be taken as an additional evidence to support the hypothesis that COPD-associated genetic islands might be important in NTHI pathogenesis.

In summary, this study used a molecular epidemiologic approach that combined *in silico* subtraction and population prevalence analysis to identify NTHI genes associated with lower airway infections. The results showed that several genetic sequences were associated with infections in COPD. Future studies will be directed to screen additional sequences and analyze the joint effects of these sequences epidemiologically. In addition, functional studies will be needed to elucidate the mechanisms by which these identified genes contribute to the pathogenesis.

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**Author Contributions**

Conceived and designed the experiments: LZ CFX JRG. Performed the experiments: JX MP AB AA JE. Analyzed the data: LZ GDE AA JE. Contributed reagents/materials/analysis tools: DC TFWM. Wrote the paper: LZ.

**References**

1. Murphy TF (2003) Respiratory infections caused by non-typeable *Haemophilus influenzae*. Curr Opin Infect Dis 16: 129–134.
2. Murphy TF, Sethi S (2002) Chronic obstructive pulmonary disease: role of bacteria and guide to antibacterial selection in the older patient. Drugs Aging 19: 761–773.
3. Sethi S, Murphy TF (2001) Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clin Microbiol Rev. 14: 356–363.
4. Wilson R (2001) Bacteria, antibiotics and COPD. Eur Respir J 17: 995–1007.
5. Murphy TF, Brauer AL, Sethi S (2001) Persistent colonization by *Haemophilus influenzae* in chronic obstructive pulmonary disease. Am J Respir Crit Care Med 167: 966–272.
6. Patel IS, Seemungal TAR, Wills M, Lloyd-Owen SJ, Donaldson GC, et al. (2002) Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. Thorax 57: 759–764.
7. Minuth AM, Xu, J, Kochanek KD (2006) Deaths: Preliminary Data for 2005. Natl Vital Stat Rep 55: 1–52.
8. Mannino DM (2002) COPD: epidemiology, prevalence, morbidity and mortality, and disease heterogeneity. Chest 121(S Suppl): 121S–128S.
9. Granten TJ, Montgomery J, Georgs G, Granten H, Siwi H, et al. (1989) Multiple colonization of the upper respiratory tract of Papua New Guinea children with *Haemophilus influenzae* and *Streptococcus pneumoniae*. Southeast Asian J Trop Med Public Health 20: 301–309.
10. Miller LV, Regelnig AK, Grassel WE, Dankert-Rolee JE, Dankert J, et al. (1995) Multiple *Haemophilus influenzae* strains and strain variants coexist in the respiratory tract of patients with cystic fibrosis. J Infect Dis 172: 1388–1392.
11. Smith-Vaughan RC, Leach AJ, Shelby-James TM, Kemp K, Kemp DJ, et al. (1996) carriage of multiple ribotypes of non-encapsulated *Haemophilus influenzae* in aboriginal infants with otitis media. Epidemidol Infection 116: 177–183.
12. Trotter S, Stenberg K, Svanborg-Edén C (1989) Turnover of non-typeable *Haemophilus influenzae* in the nasopharynges of healthy children. J Clin Microbiol 27: 2175–2179.
13. Dhooe J, Vanhemontghe M, Clays G, Verschraegen G, Van Cauwenberge P (2000) Turnover of *Haemophilus influenzae* isolates in otitis-prone children. Int J Pediatr Otorhinolaryngol. 54: 7–12.
14. Faden H, Duffy L, Williams A, Krystofik DA, Wolf J (1995) Epidemiology of *Haemophilus influenzae* bexB in chronic obstructive pulmonary disease. Curr Opin Infect Dis 27: 2175–2179.
15. Nakamura S, Shchepetov M, Dalia AB, Clark SE, Murphy TF, et al. (2011) Molecular basis of increased serum resistance among pulmonary isolates of non-typeable *Haemophilus influenzae*. PLoS Pathog 7(1): e1001247.
16. Qiu J, Lesse AJ, Brauer AL, Can J, Gill SR, et al. (2010) Proteomic expression profiling of *Haemophilus influenzae* grown in pooled human sputum from adults with chronic obstructive pulmonary disease reveal antioxidant and stress responses. BMC Microbiol 10: 162.
17. Schoenhol GR (2002) Functional and comparative genomics of pathogenic bacteria. Curr Opin Microbiol 5: 29–36.
18. Whittam TS, Bumbaugh AC (2002) Inferences from whole genome sequences of bacterial pathogens. Curr Opin Genet Develop 12: 719–725.
19. Gilsdorf JR, Marts CF, Foxman B (2004) *Haemophilus influenzae*: genetic variability and natural selection to identify virulence factors. Infect Immun 72: 2457–2461.
20. Boucher Y, Neslo CL, Doolittle WF (2001) Microbial genomes: dealing with diversity. Curr Opin Microbiol 4: 285–289.
21. Munson RS Jr, Harrison A, Gillaspay A, Ray WC, Carson M, et al. (2004) Partial analysis of the genomes of two nontypeable *Haemophilus influenzae* otitis media isolates. Infect Immun 72: 3002–3010.
22. Erwin AL, Smith AL (2007) Nontypeable *Haemophilus influenzae*: understanding virulence and commensal behavior. Trends Microbiol 15: 355–362.
23. Hogg JS, Ho FZ, Juon B, Bouy R, Hayes J, et al. (2007) Characterization and modeling of the *Haemophilus influenzae* core and supragenomes based on the complete genomic sequences of Rd and 12 clinical nontypeable strains. Genome Biol 8(6): R103.
24. Gilsdorf JR, Ahmed A, Juon B, Earl J, Hall BJ, Hogg J, et al. (2011) Comparative supragenomic analyses among the pathogens *Streptococcus aureus*, *Streptococcus pneumoniae*, and *Haemophilus influenzae* using a modification of the finite supragenome model. BMC Genomics: 12:167.
25. Xie J, Juliao PC, Gilsdorf JR, Glish D, Patel M, et al. (2006) Identification of new genetic regions in nontypeable *Haemophilus influenzae* otitis media strains than in throat strains. J Clin Microbiol 44: 4316–4323.
26. Juliao PC, Marts CF, Xie J, Gilsdorf JR (2007) Histidine auxotrophy in commensal and disease-causing nontypeable *Haemophilus influenzae*. J Bacteriol 189: 4994–5001.
27. Bergman NH, Akerley BJ (2003) Position-based scanning for comparative genomes and identification of genetic islands in *Haemophilus influenzae* type b. Infect Immun 71: 1091–1108.
28. Sandstedt SA, Marts CF, Patel M, Hirasaawa H, Zhang L, et al. (2010) Prevalence of *Haemophilus influenzae* type b genetic islands among clinical and communal *H. influenzae* and *Haemophilus haemolyticus*. J Clin Microbiol 48: 2565–2568.
29. Campos JM (1999) *Haemophilus*. In: Murray PR, Barson E, Fallon MA, Tenover FC, Yokken RH, editors. Manual of clinical microbiology, 7th ed. ASM Press, Washington, D.C. 604–613.
30. Farjo RS, Foxman B, Patel M, Zhang L, Pettigrew MM, et al. (2004) Diversity and sharing of *Haemophilus influenzae* strains colonizing healthy children attending day-care centers. Pediatr Infect Dis J 23: 41–46.
31. Kilian M (2005) Genus III. *Haemophilus*. Winslow, Broadhurst, Buchanan, Kravetswie, Rogers and Smith 1917, 561AL. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM, editors. Bergey’s manual of systematic bacteriology, 2nd ed. Springer-Verlag, New York, N.Y. 883–904.
32. McCrea KW, Xie J, Laerou N, Patel M, Mukundan D, et al. (2008) Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. J Clin Microbiol 46: 406–416.
33. Sandstedt SA, Zhang L, Patel M, McCrea KW, Qin Z, et al. (2008) Comparison of laboratory-based and phylogenetic methods to distinguish between *Haemophilus influenzae* and *H. haemolyticus*. J Microbiol Methods 75: 369–371.
34. Davis GS, Sandstedt SA, Patel M, McCrea KW, Gilsdorf JR (2011) Use of *bexB* to detect the capsule locus in *Haemophilus influenzae*. J Clin Microbiol 49: 2594–2601.
35. St Sauver J, Marts CF, Foxman B, Sonesel P, Madera R, et al. (2000) Risk factors for otitis media and carriage of multiple strains of *Haemophilus influenzae* and *Streptococcus pneumoniae*. Emerg Infect Dis 6: 629–630.
36. Sethi S, Evans N, Grant BJ, Murphy TF (2002) New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. N Engl J Med 347: 463–471.
43. Zhang L, Foxman B, Gilsdorf JR, Marrs CF (2005) Bacterial genomic DNA isolation using sonication for microarray analysis. Biotechniques 39: 640–644.
44. Zhang L, Srinivasan U, Marrs CF, Ghosh D, Gilsdorf JR, et al. (2004) Library on a slide for bacterial comparative genomics. BMC Microbiol 4: 12.
45. Kong Y, Cave MD, Zhang L, Foxman B, Marrs CF, et al. (2006) Population-based study of deletions in five different genomic regions of Mycobacterium tuberculosis and possible clinical relevance of the deletions. J Clin Microbiol 44: 3940–3946.
46. Benjamin Y, Hochberg Y (1995) Controlling the false discovery rate: A practical and powerful approach to multiple testing. Journal of the Royal Statistical Society, Series B 57: 289–300.
47. Pettigrew MM, Foxman B, Marrs CF, Gilsdorf JR (2002) Identification of the lipooligosaccharide biosynthesis gene lic2B as a putative virulence factor in strains of nontypeable Haemophilus influenzae that cause otitis media. Infect Immun 70: 3551–3556.
48. Wong SM, St Michael F, Cox A, Ram S, Akerley BJ (2011) ArcA-regulated glycosyltransferase Lic2B promotes complement evasion and pathogenesis of nontypeable Haemophilus influenzae. Infect Immun 79: 1971–1983.
49. Fernaays MM, Lesse AJ, Sethi S, Cai X, Murphy TF (2006) Differential genome contents of nontypeable Haemophilus influenzae strains from adults with chronic obstructive pulmonary disease. Infect Immun 74: 3366–3374.
50. Fernaays MM, Lesse AJ, Cai X, Murphy TF (2006) Characterization of igA1, a second immunoglobulin A1 protease gene in nontypeable Haemophilus influenzae. Infect Immun 74: 3065–3072.
51. Vitovski S, Dunkin KT, Howard AJ, Sayers J (2002) Non-attachment to human epithelial cells. Proc Natl Acad Sci U S A 90: 2075–2079.
52. Ecevit IZ, McCrea KW, Pettigrew MM, Sen A, Marrs CF, et al. (2004) Prevalence of the licRC, licA1A, lic2A, licC, and rha genes in Haemophilus influenzae isolates. J Clin Microbiol 42: 3065–3072.
53. Weiser JN (2000) The generation of diversity by Haemophilus influenzae. Trends Microbiol 8: 433–435.
54. Sachs G, Kraut JA, Wren Y, Feng J, Scott DR (2006) Urea transport in bacteria: acid acclimation by gastric Helicobacter spp. J Membr Biol 212: 71–82.
55. Schliefer KH, Kandler O (1972) Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol Rev 36: 407–447.
56. Harb OS, Abu Kwaik Y (1998) Identification of the aspartate-beta-semialdehyde dehydrogenase gene of Legionella pneumophila and characterization of a null mutant. Infect Immun 66: 1898–903.
57. Whitby PW, VanWagoner TM, Scale TW, Morton DJ, Stull TL (2006) Transcriptional profile of Haemophilus influenzae: effects of iron and heme. J Bacteriol 188: 5640–5645.
58. Whitby PW, Scale TW, VanWagoner TM, Morton DJ, Stull TL (2009) The iron/heme regulated genes of Haemophilus influenzae: comparative transcriptional profiling as a tool to define the species core modulon. BMC Genomics 10: 6.
59. Abdul-Tehrani H, Hudson AJ, Chang YS, Timms AR, Hawkins C, et al. (1999) Ferritin mutants of Escherichia coli are iron deficient and growth impaired, and fur mutants are iron deficient. J Bacteriol 181: 1415–1428.
60. Jeltsch A (2002) Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases. ChemBioChem 3: 274–293.
61. Schubert HL, Blumenthal RM, Chen X (2003) Many paths to methyltransfer: a chronicle of convergence. Trends Biochem Sci 28: 329–335.