Comprehensive Proteomic and Metabolomic Signatures of Nontypeable *Haemophilus influenzae*-Induced Acute Otitis Media Reveal Bacterial Aerobic Respiration in an Immunosuppressed Environment*

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A thorough understanding of the molecular details of the interactions between bacteria and host are critical to ultimately prevent disease. Recent technological advances allow simultaneous analysis of host and bacterial protein and metabolic profiles from a single small tissue sample to provide insight into pathogenesis. We used the chinchilla model of human otitis media to determine, for the first time, the most expansive delineation of global changes in protein and metabolite profiles during an experimentally induced disease. After 48 h of infection with nontypeable *Haemophilus influenzae*, middle ear tissue lysates were analyzed by high-resolution quantitative two-dimensional liquid chromatography-tandem mass spectrometry. Dynamic changes in 105 chinchilla proteins and 66 metabolites define the early proteomic and metabolomic signature of otitis media. Our studies indicate that establishment of disease coincides with actin morphogenesis, suppression of inflammatory mediators, and bacterial aerobic respiration. We validated the observed increase in the actin-remodeling complex, Arp2/3, and experimentally showed a role for Arp2/3 in nontypeable *Haemophilus influenzae* invasion. Direct inhibition of actin branch morphology altered bacterial invasion into host epithelial cells, and is supportive of our efforts to use the information gathered to modify outcomes of disease. The twenty-eight nontypeable *Haemophilus influenzae* proteins identified participate in carbohydrate and amino acid metabolism, redox homeostasis, and include cell wall-associated metabolic proteins. Quantitative characterization of the molecular signatures of infection will redefine our understanding of host response driven developmental changes during pathogenesis. These data represent the first comprehensive study of host protein and metabolite profiles in vivo in response to infection and show the feasibility of extensive characterization of host protein profiles during disease. Identification of novel protein targets and metabolic biomarkers will advance development of therapeutic and diagnostic options for treatment of disease.

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Our current understanding of the global changes that occur during infection is primarily based upon transcriptional profiles of either the host or the bacteria. However, a significant component of disease progression is dependent upon post-transcriptional processes. Recent advances in the application of two-dimensional tandem mass spectrometry have dramatically increased sensitivity to allow simultaneous analyses of multiple molecules from very small biological samples. In this study, we report the comprehensive proteomic and metabolomic profiling of middle ear tissues using samples that are smaller than those typically obtained from a human biopsy. Proteomic and metabolomic analyses of samples as small as biopsies will revolutionize the elucidation of the mechanisms...
of pathogenesis in experimental models of disease as well as in human samples. Otitis media (OM) is a significant disease of the pediatric population. The direct and indirect costs for the diagnosis and treatment of acute OM (AOM) in 2009 approached $3 billion and the number of all OM cases has estimated costs of treatment of up to $6 billion in the US annually (1–3). The scope of the problem is apparent with the observation that globally there are 709 million cases of AOM estimated per year of which 51% occur in children under the age of five (4). Moreover, children attending daycare outside the home, approximated at 4 million children in the US in 2011 (5), are fourfold more likely to have an incidence of AOM (6). As a result, OM is the most common reason for a doctor’s visit in school age children and is associated with significant morbidity. Long-term hearing loss is a direct consequence of untreated OM with effusion (OME) (4, 7, 8). Clinical management of OM has relied heavily on antibiotic therapies (8, 9), which has contributed to the emergence of antibiotic resistant strains of bacteria (10–13). Novel solutions are needed for this highly prevalent disease and require extensive description of the molecular mechanisms of disease to define novel therapeutic targets.

Nontypeable Haemophilus influenzae (NTHI) predominates in ~50% of cases of AOM and has a significant role in OME (14–16). To better understand and ultimately prevent disease, it is imperative that we define the bacteria-host interaction during active disease. A chinchilla model of human OM has long been used to investigate the role of NTHI in OM. This animal model of disease has been successfully used to develop candidate vaccines aimed to prevent NTHI-mediated OM (for example, (17–19)). In addition, mutational studies have identified NTHI gene products essential in bacterial pathogenesis (for example, (20–23)). A limited number of transcriptional studies have assessed NTHI gene expression during OM (for example, (24, 25)). However, global analyses of the chinchilla model of OM have been limited, primarily because of the small amount of host material that can be retrieved from an animal and, until now, the absence of a chinchilla genome sequence. We have thus developed and successfully implemented analyses that assess global changes in proteins and metabolites in the chinchilla model of AOM. Using quantitative approaches, we identified a group of 105 proteins and 66 metabolites as potential mediators of disease. These factors could lead to the development of rationally designed antimicrobial therapies and diagnostic tools. Moreover, these data represent the first global study of the host response during disease progression in vivo with techniques readily translatable to many diseases.

MATERIALS AND METHODS

Ethics Statement—Animal experiments were carried out in strict accordance with the accredited conditions in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (Welfare Assurance Number A3544–01) at The Research Institute at Nationwide Children's Hospital, AR13–00026. Suffering was minimized whenever possible.

Strain Description—NTHI strain 86–028NP was recovered from the nasopharynx of a child with chronic OM. Strain 86–028NP has been well characterized in vitro (26–28) and in chinchilla models of OM (20, 25, 29, 30). The genome sequence has been published (31).

Animal Infection—Sixteen, healthy adult chinchillas (Chinchilla lanigera, Rauscher’s Chinchilla Ranch, LaRue, OH), with no evidence of middle ear disease by pneumatic otoscopy, were used as the model organism for AOM in this study. Chinchillas were anesthetized with xylazine (2 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and ketamine (10 mg/kg) and middle ears challenged with NTHI cultured as follows. Strain 86–028NP was initially grown overnight at 37 °C, in a 5% CO₂ atmosphere on chocolate II agar plates (Fisher Scientific, Pittsburgh, PA). Cells were then transferred to an iron depleted Defined Iron Source (DIS) medium, a modification of a defined medium developed by Hasan et al. and Coleman et al. (32, 33) and previously used for the culture of strain 86–028NP (26, 34). The DIS medium was supplemented with 2 μg heme/mel and cells were grown statically at 37 °C in a 5% CO₂ atmosphere for 24 h. Bacteria were suspended in pyrogen-free sterile saline to an optical density (OD₅₆₅) of 0.37. Cultures were then serially diluted in pyrogen-free saline and ~300 bacteria were introduced via the transbular route, bilaterally, into the middle ears of a cohort of seven chinchillas. A second cohort of nine chinchillas was sham-treated with saline. After 48 h, the animals were euthanized, the middle ears dissected and the middle ear mucosae isolated. One ear from each NTHI-infected animal was processed for validation by microscopy or extraction of RNA or protein. Insufficient material was obtained from only one ear of the majority of sham-treated animals requiring pooling.
of the two ears from each animal for multomic analysis. Therefore, validation of the sham-treated animals was performed on independent ears from the same cohort. After washing with sterile saline, the mucosa for the omic analyses were placed in 50 mM ammonium bicarbonate, pH 8.0, homogenized and subjected to two passages through a high-pressure cell (20,000 psi; One Shot Model, Constant Systems Ltd., Kennesaw, GA). Lysates were frozen on dry ice and stored at −80 °C prior to analyses. For all subsequent proteomic and metabolomic analyses, we used tissues from six sham-treated animals and seven NTHI-infected animals.

Proteomic Analysis of Middle Ear Mucosae—

Sample Preparation and Protein Isolation—For the proteomics analysis, samples were thawed on ice, vortexed briefly, and 490 µl of each sample was mixed with 10 µl of 5% Rapigest SF Surfactant (Waters Corporation, Milford, MA). After solubilization, samples were further disrupted with three 5 s bursts of sonication using a probe sonicator. Samples were incubated on ice between bursts. Samples were then centrifuged at 21,000 × g for 5 min at 4 °C. Protein concentrations of the supernatants were determined by mini-Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA). Samples with concentrations ≥ 0.15 µg/µl were chosen for further sample processing and proteomic analysis. Six samples were from sham animals and seven samples were from infected animals. Twenty-five micrograms of protein from each sample was normalized to a protein concentration of ~0.15 µg/µl using 0.1% Rapigest SF Surfactant in 50 mM ammonium bicarbonate, pH 8.0. Samples were reduced with 10 mM DTT at 80 °C for 15 min then alkylated with 25 mM iodoacetamide at room temperature in the dark for 30 min. Trypsin was added at an enzyme to protein ratio (w/v) of 1:50 and samples were digested overnight at 37 °C. After digestion, all samples were acidified with 1% trifluoroacetic acid, 2% acetonitrile, heated to 60 °C for two hours to hydrolyze the Rapigest SF Surfactant, and then frozen before being lyophilized overnight. Before use, lyophilized samples were reconstituted to a final concentration of 1 µg/µl in 200 mM ammonium formate, pH 10, containing 25 fmol/µl of an alcohol dehydrogenase tryptic digest standard (MassPREP, Waters Corporation). A Quality Control (QC) Pool that contained 3 µl from each sample was generated to use for column conditioning and a technical reproducibility assessment.

Label-Free Quantitative Analysis of Proteins from Middle Ear Mucosae—Quantitative two-dimensional liquid chromatography tandem mass spectrometry (LC/LC-MS/MS) was performed once on 3 µg of protein digest per sample, and the pool was analyzed four times with 3 µg injections evenly across the run queue using the acquisition method described below. The method used two-dimensional liquid chromatography in a high-low pH reversed phase/reversed phase configuration on a nanoACQUITY UltraPerformance liquid chromatography system coupled to a Synapt G2 High Definition Mass Spectrometer (both Waters Corporation) with nano-electrospray ionization as similarly described previously (35–37). Peptides were first trapped at 2 µl/min at 97/3 v/v 20 mM ammonium formate in water, pH 10 acetonitrile on a 5 µm XBridge BEH130 C18 300 µm × 50 mm column (Waters Corporation). A series of step-elutions of acetonitrile at 2 µl/minute was used to elute peptides from the first-dimension column. Five steps of 10.8%, 14.0%, 16.7%, 20.4%, and 50.0% acetonitrile were used for the unbiased analyses; these percentages were optimized to deliver an approximately equal load to the second-dimension column for each fraction. For second-dimension separation, the eluent from the first dimension was first diluted 10-fold online with 99.8/0.2 v/v 50 mM ammonium bicarbonate/formic acid and trapped on a 5 µm Symmetry C18 180 µm × 20 mm trapping column (Waters Corporation). The second-dimension separations were performed on a 1.7 µm Acquity BEH130 C18 75 µm × 150 mm column (Waters Corporation) using a linear gradient of 7 to 35% acetonitrile with 0.1% formic acid over 37 min, at a flow rate of 0.5 µl/minute and a column temperature of 35 °C. Data collection on the Synapt G2 mass spectrometer was performed in ion-mobility assisted data-independent acquisition (IMS-DIA, also called HDMSE3) mode, using 0.6 s alternating cycle time between low (6V) and high (27–50V) collision energy (CE) in the transfer region after the ion mobility cell, with data collected in “resolution” mode, Rs ~ 20,000 at 785.8 m/z. Scans performed at low CE measured peptide accurate mass and intensity (abundance), whereas scans at elevated CE allowed for qualitative identification of the resulting peptide fragments via database searching. The pool was also injected once by DDA (data-dependent acquisition) and once using a method to optimize collision energies at the end of the run queue for supplemental qualitative-only peptide identifications (the pool was injected a total of six times). The total analysis cycle time for each sample analysis was ~6 h. After data collection, raw data were imported into Rosetta Elucidator v3.3 (Rosetta Bio-software, Inc, Seattle, WA), and all LC/LC-MS runs were aligned based on the accurate mass and retention time of detected ions (“features”) using the PeakTeller algorithm. The relative peptide abundance was calculated based on area-under-the-curve (AUC) of aligned features across all runs. The MS/MS data was searched against a custom database containing 35,615 forward entries, including chinchilla sequences (GenBank Accession AGCD00000000, http://www.ncbi.nlm.nih.gov/nuccore/AGCD0000000000), H. influenzae strain 86–028NP sequences (NCBI Accession number NC_007146), as well as six exogenous proteins that were common contaminants or surrogate standards (ADH1_YEAST, ENO1_YEAST, ALBU_BOVIN, PYGM_RABIT, CASA1_BOVIN, CASA2_BOVIN). The database was appended with a reversed-sequence “decoy” database for false discovery rate (FDR) determination. ProteinLyx Global Server (PLGS) v2.5.2 (Waters Corporation) was used to produce fragment ion spectra from the IMS-DIA runs and to perform the database searches in PLGS, whereas Mascot v2.4 (Matrix Science, Inc., Boston, MA) was used to search DDA data. A 10 ppm precursor and 0.04 Da product ion tolerance was used, with tryptic specificity allowing two missed cleavages. Included in the database searches were variable modifications on Met (oxidation) and Asn/Gln (deamidation), and fixed modification on Cys (carbamidomethyl). All search results from both search engines were imported into Elucidator without FDR cutoff initially, followed by individual peptide scoring using PeptideProphet (38) within the Elucidator software, and final annotation at a 1.2% peptide false discovery rate for the aggregate of DIA and DDA peptide searches, or a minimum PeptideTeller score of 0.94. This analysis yielded identifications for 2773 peptides and 746 proteins across all samples; the nature of label-free alignment allows for these peptides to be quantified in all samples. For quantitative processing, the data was first curated to contain only high quality peptides with appropriate chromatographic peak shape, peptide quantities across all five LC/LC fractions were summed, and the data set was intensity scaled to the robust mean across all samples analyzed; the final quantitative data set was based on 2398 peptides and contains 384 proteins with at least two unique peptides. Relative protein abundances for statistical comparison were calculated as the sum of the individual peptide intensities, using peptides assigned under the principles of Occam’s Razor. Summaries for the proteomics data are contained in supplemental Table S1 (sample identifier information), supplemental Table S2 (peptide level qualitative and quantitative data), and supplemental Table S3 (protein-level quantitative data). The %CV for the technical replicates analyzed throughout the study showed excellent analytical reproducibility, with a mean of 15.0% and median of 11.3%. Biological replication measures are also reported in supplemental Table S3. Finally, the ADH1_YEAST spike-in reference protein was used to make a quantitative estimate of the molar abundance of individual proteins in the sample, based on the top three best ionizing peptides.
to each protein using the method of Silva et al. (39). This was used primarily for simple assessments of the major components of the previously-unknown proteome of the ear mucosa, and the summary of this calculation is found in supplemental Table S4. Raw proteomics data generated are available via the Chorus Project consortium and can be downloaded at https://chorusproject.org/download/bulk?experiment=1525&lab=.

Broad Spectrum Targeted Metabolomic Analyses of Middle Ear Mucosa—

Sample Preparation—Ear mucosa lysates were prepared using the AbsoluteIDQ p180 kit (Biocrates, Innsbruck, Austria) following the manufacturer protocol. Ten microliters of a solution containing stable-isotope labeled internal standards and 15 μl of each middle ear mucosa extract was added to a 96-well extraction plate and the plate dried under a gentle stream of nitrogen. An additional 15 μl of each middle ear mucosa extract was then added to the wells and the drying process repeated. The samples were derivatized with phenyl isothiocyanate then eluted with unbuffered 5 mM ammonium acetate in methanol. Samples were diluted 4:1 v/v (5X dilution) with 40% methanol in water for ultra performance liquid chromatography (UPLC) analysis or 19:1 v/v (20X dilution) with running solvent (a proprietary mixture provided by Biocrates) for flow injection analysis. A QC pool based on equal volumes of all 24 samples was created. The pooled sample was prepared exactly as the experimental samples and injected once before, once during, and once after the experimental samples to measure the performance of the assay during sample analysis.

Quantitative Analysis of Metabolites from Middle Ear Mucosa—

Separation of amino acids and biogenic amines was performed using an ACQUITY UPLC System (Waters Corporation) using an ACQUITY 2.1 mm × 50 mm 1.7 μm BEH C18 column fitted with an ACQUITY BEH C18 1.7 μm VanGuard guard column, and quantified by calibration curve using a linear regression with 1/x fit. Acylcarnitines, sphingolipids, and glycerophospholipids, were analyzed by flow injection analysis tandem mass spectrometry (FIA-MS/MS), quantified by ratio to a stable-isotope labeled internal standard in the same analyte class. Thus, FIA analytes are reported as semiquantitative values except where a stable-isotope labeled internal standard of the exact analyte was used. Samples for both UPLC and flow injection analysis were introduced directly into a Xevo TQ-S mass spectrometer (Waters Corporation) using positive electrospray ionization operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time using tune files and acquisition methods provided in the AbsoluteIDQ p180 kit from Biocrates. The UPLC data were imported into TargetLynx (Waters Corporation) for peak integration, calibration, and concentration calculations. The UPLC data from TargetLynx and flow injection data were analyzed using Biocrates’ MetIDQ software. The data for the p180 Kit are reported in supplemental Table S5 (sample IDs indicated in S1), along with a color-coded key denoting samples that were below the limit of detection (<LOD), below the lowest calibration standard (<LLOQ), or quantified based on a ratio to a class-based internal standard (semiquantitative). Each analyte was normalized for the total protein in the sample, concentrations shown in supplemental Table S5.

Oxylipin Assay of Middle Ear Mucosa—

Preparation of Oxylipin Standards—Stable isotope labeled (SIL) oxylipin standard solutions were purchased from Cayman Chemical (Ann Arbor, MI). All standards were combined to generate a stock SIL mixture in methanol, which was further diluted to a final concentration of 1 μM with 1:1 methanol/acetoniitrile (IS Solution). Calibration standards and QC’s were prepared in 50 mg/ml bovine serum albumin (BSA) in 50 mM ammonium bicarbonate, pH 8.0. The concentrations of the calibration standards were 10, 25, 50, 100, 250, 500, 1000, 5000, 10,000, and 50,000 pg/ml. Calibration standards were analyzed in duplicate and bracketed the study samples. QC samples were prepared at three concentrations 400, 4000, and 40,000 pg/ml. QC samples were analyzed in triplicate. An antioxidant solution of 10 mg butylated hydroxytoluene (BHT)/ml anhydrous ethanol and a solution of 80:20:2 water/methanol/formic acid were also prepared. Equilibration buffer was prepared by combining water, methanol, and formic acid in a 9:0:5 ratio.

Experimental Sample Preparation for Oxylipin Analysis—Middle ear mucosa lysates were thawed, mixed, and prepared using an ISOLUTE SLE+ Supported Liquid Extraction 96-well plate (Biotage, Uppsala, Sweden). For each blank, calibration standard, QC, and study sample, 100 μl Equilibration Buffer was added to a well of a 96-well plate. Ninety-five microliters of 80:20:2 water:methanol:formic acid were added to each well to be used for double blanks with 5 μl BHT solution. Three hundred microliter aliquots of the blank, calibration standards, and QC samples were added to the appropriate wells. Ear mucosa study samples were added in 300 μl aliquots when possible. When less than 300 μl was available for experimental samples, the volumes added to the extraction plate were noted. One sample (ID13677) for which less than 300 μl was available was made up to 300 μl with 50 mM ammonium bicarbonate, pH 8.0. The plate was then capped and mixed for 10 min at room temperature. After mixing, all samples were transferred to the SLE+ plate, loaded and incubated for 5 min at room temperature. The samples were then eluted with 900 μl 75:25 hexane/ethyl acetate. The eluate was dried under a gentle stream of nitrogen. Using 900 μl 75:25 hexane/ethyl acetate, the SLE+ plate was then eluted a second time into the collection plate containing the dried samples. The collected samples were again dried under a gentle stream of nitrogen then reconstituted in 50 μl 1:1 acetonitrile/methanol. Five microliters were injected for LC/MS/MS analysis.

Quantitative Assessment of Oxylipins from Middle Ear Mucosa—LC separation was performed using a Waters Acuity UPLC with a Waters Acuity 2.1 mm × 100 mm 1.7 μm BEH C18 column. Mobile phase A was 0.1% acetic acid in water and mobile phase B was 90:10 acetonitrile/isoproply alcohol. Flow rate was 0.6 ml/min, and the gradient used was 25 to 95% B in 10 min at 40 °C. Samples were introduced directly into a Xevo TQ-S mass spectrometer (Waters Corporation) using negative ion electrospray ionization operating in the Multiple Reaction Monitoring (MRM) mode. MRM transitions (compound-specific precursor to product ion transitions) for each analyte and internal standard were collected over the appropriate retention time. The MRM data were imported into TargetLynx (Waters Corporation) for peak integration, calibration, and concentration calculations (supplemental Table S6). Analytes for which analytical standards were not included were quantified against the standard curve of an analyte from the same or similar compound class as shown in supplemental Table S7. The SRM targets and retention times have been made publicly available as a Skyline document at the following link: https://discovery.genome.duke.edu/express/resources/3728/Oxylipin_Panel_June2015.sky.zip.

Data were acquired for oxylipins from 14 compound classes: alcohols, diols, epoxides, epoxyxins, hydroxyperoxides, isoprostanes, ketones, leukotrienes, lipoxins, prostanoids, protectins, resolvins, thromboxanes, and triols. Isoprostanes, leukotrienes, lipoxins, resolvins, and thromboxanes were not detected in the assay in study samples, though they were contained in the targeted method. Data for the triol compounds were not reportable because of chromatographic interferences. supplemental Table S6 summarizes the data observed by compound class and individual analyte (sample IDs are indicated in supplemental Table S1), and supplemental Table S7 gives the list of compounds along with internal standards and refer-
nance standard compounds used for quantification of each compound. Concentrations were not reported in supplemental Table S6 for compounds where more than 50% of the samples were below the level of quantitation. Each analyte was normalized for the total protein concentration based on the Bradford assay.

Characterization of Protein Changes during AOM—Both qualitative and quantitative information about the abundance of proteins in the middle ear mucosae was obtained. All proteins with two or more peptides to match are indicated in supplemental Table S4. To make a first approximation of the abundance of each protein identified, the method of Silva et al. was used with a single-point protein calibrant as described in the methods (39). The top 25 proteins are ranked in order of abundance across the samples in this study (supplemental Fig. S2). These proteins make up ~60% of the measured proteome by mass, showing the dynamic range in concentration of the mucosal proteome. Albumin, hemoglobin, serotransferrin, and other serum proteins predominated.

To identify proteins that are differentially expressed between sham and NTHI-infected cohorts, we used protein intensity calculated as the sum of all peptide intensities (supplemental Table S3). This method provides a more precise measure of protein relative abundance, because all peptides available are used (40). Intensities of each protein identified were averaged within each treatment group, and fold-changes calculated between groups. Significance was calculated using a multiple $t$ test after log2 scaling; and data with a q value of 1% (Benjamini-Hochberg FDR-correction method, $p < 0.05$) was considered significant. Proteins passing quantitative thresholds are displayed in Table I.

Chinchilla proteins that exhibited changes in expression because of NTHI infection were categorized based on Gene Ontology (GO, (41)). For each chinchilla protein to be categorized, its human ortholog was identified. Two input files were generated that contained the human genes that were orthologous to the chinchilla genes whose expression was either up-regulated or down-regulated after NTHI infection. Each file was then analyzed using the Biological Networks Gene Ontology tool, BINGO, an application that calculates the GO terms overrepresented in a data set and integrates with Cytoscape for visualization of the data (42, 43). A $p$ value was calculated for each node using a hypergeometric test with a Benjamini and Hochberg FDR correction. A node with a $p$ value <0.05 was determined to be overrepresented within the data set.

Proteomic Validation—Middle ear mucosa from ears of NTHI-infected and sham-treated animals were isolated concurrently with the mucosae used for proteomic and metabolic analyses. These mucosae were used to validate the proteomic data as follows.

Quantitative Reverse Transcriptase PCR—Mucosa were placed directly into 1 ml TRIzol reagent (Life Technologies Grand Island, NY) and frozen at −80 °C prior to RNA purification. After thawing, samples were extracted with chloroform and RNA purified with an RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA concentration was determined using a NanoDrop spectrophotometer (NanoDrop Products, Wilmington, DE), and each sample was analyzed for integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Only samples that passed these quality controls were used in subsequent analyses. Quantitative reverse transcription-PCR (qRT-PCR) was performed as in Harrison et al. (20). All threshold cycle (CT) values were normalized to the endogenous control gene, which encodes β-glucuronidase. Relative quantitation was calculated from the median CT value using ΔΔCT. Statistical significance could not be determined because of poor quality RNA, leaving RNA from only one sham tissue available for analysis.

Visualization of the Chinchilla Middle Ear Epithelium and Mucin Production—Middle ears from a sham-treated and an infected chinchilla were fixed in 4% paraformaldehyde in Dulbecco’s phosphate-buffered saline (DPBS), decalified overnight, paraffin embedded, and sectioned. Sections were deparaffinized in xylene followed by hydration to water through graded alcohols. Sections were then placed into 0.05% periodic acid for 5 mins, rinsed in distilled water and subsequently placed in room temperature Schiff’s reagent for 15 min. Slides were washed in warm running water for 3 mins then counterstained in Shandon hematoxylin for 1 min. Following a tap water wash, slides were dehydrated in graded alcohols (twice in 95% ethanol, twice in 100% ethanol) and cleared in xylene. Slides were mounted with PermaSlip (Alban Scientific Inc, St. Louis, MO) and examined by light microscopy using an Axio Lab.A1 light microscope and an AxioCam ERC 5s camera (Carl Zeiss Inc., Thornwood, NY).

Arp2/3 Expression in NTHI Infected Mucosae—Western blot Analysis—Protein concentrations of middle ear mucosal lysates from NTHI infected and sham-treated animals were quantified using Bradford's reagent. Mucosal lysates were mixed with Laemmli sample buffer, boiled for 10 min, and —100 ng of each sample resolved on a 12% SDS-polyacrylamide gel. Proteins were transferred then into a nitrocellulose membrane by electrophoresis and the membrane probed with an anti-human Arp2/3 complex antibody (EMD Millipore, Billerica, MA) and horse radish peroxidase (HRP) conjugated to goat anti-mouse IgG (Life Technologies) and detected using a chemiluminescent substrate ECL (GE Healthcare, Pittsburgh, PA).

Arp2/3 Expression in NTHI Infected Mucosae – Immunofluorescence Microscopy—Arp2/3 was visualized in situ as follows. Paraffin embedded sections of middle ears from sham-treated and NTHI-infected chinchillas were deparaffinized in xylene then rehydrated stepwise in graded alcohols (three times in 100% ethanol, once in 95% ethanol), then placed in water. Slides were then placed in Antigen Retrieval AR-10 Solution (Biogenex, Fremont, CA) and rapidly boiled for 15 min by microwaving. After cooling, slides were washed twice with water, then twice with DPBS. Unless noted otherwise, slides were washed twice with DPBS between all steps. Slides were blocked for 10 min with 0.01% sodium borohydride blocked for a further 10 min with CAS-Block Histochemical Reagent (Life Technologies), then directly incubated for 30 min in Image-IT FX Signal Enhancer (Life Technologies). Slides were then incubated overnight with anti-human Arp2/3 complex antibody diluted 1:5000 in CAS-Block Histochemical Reagent. As controls, slides were also incubated overnight at 4 °C with either CAS-Block or with normal mouse IgG2a (Santa Cruz Biotechnology, Dallas, TX) diluted 1:5000 in CAS-Block. All slides were then incubated for one hour with Protein A conjugated to Alexa Fluor 488 (Life Technologies) diluted 1:100 in CAS-Block. Slides were stained with wheat germ agglutinin (WGA)-Alexa Fluor 594 (LifeTechnologies), followed by Hoescht 33342 (LifeTechnologies) and examined by fluorescent microscopy using an Axiovert 200 m Inverted epifluorescence microscope equipped with an Axiocam MRM CCD camera (Carl Zeiss Inc.).

Inhibition of Arp2/3—Normal Human Bronchial Epithelial Cell (NHBE) Infection Model—NHBE cells (Lonza, Walkersville, MD) were seeded into 24-well plates containing sterile coverslips and grown in Bronchial Epithelial Cell Growth Medium (BEGM; Lonza) at 37 °C in a 5% CO2 atmosphere to near confluency. The GFP-expressing strain of NTHI strain 86–028NP (pGM1.1) (30) was grown overnight at 37 °C, in a 5% CO2 atmosphere on chocolate II agar plates. The next morning the colonies were resuspended in brain heart infusion broth supplemented with 2 μg/ml heme and 2 μg/ml β-NAD (sBHI) and grown at 37 °C with shaking at 180 rpm until the cells were in mid-log phase (~5 generations). Prior to infection, the BEGM was removed from the NHBE cells and replaced with BEGM that contained either 200 μM CK-636 (Sigma-Aldrich, St. Louis, MO), an inhibitor of Arp2/3 function, or 0.2% DMSO, the vehicle for CK-636. The toxicity of these concentrations of CK-636 and DMSO under the conditions used in these experiments was determined for NHBE cells using a CellTiter 96
### Table I

Chinchilla proteins that exhibited significant changes in amount due to NTHI-induced AOM (* Amino acid identity to human orthologue)

| Protein accession no. | Function                                             | Fold change of infected/sham | Identity (%)* |
|-----------------------|------------------------------------------------------|------------------------------|---------------|
| XP_005375096.1        | neutrophil cytosol factor 2 isoform X2               | 13.2                         | 88.7          |
| XP_005379227.1        | neutrophil cytosol factor 4                          | 6.2                          | 89.1          |
| XP_005414659.1        | protein S100-A8                                      | 6.2                          | 73.6          |
| XP_005409872.1        | protein-arginine deiminase type-4                    | 4.3                          | 78.5          |
| XP_005396685.1        | uridine phosphorylase 1 isoform X4                   | 4.1                          | 78.8          |
| XP_005415265.1        | lysozyme C-like                                      | 4.0                          | 84.8          |
| XP_005393368.1        | grancalcin isoform X1                               | 3.7                          | 68.6          |
| XP_005378904.1        | tyrosine-protein phosphatase non-receptor type 6 isoform X1 | 3.6                          | 94.3          |
| XP_005414660.1        | protein S100-A9                                      | 3.5                          | 65.1          |
| XP_005383459.1        | 15 kDa protein A-like                               | 3.4                          | 31.7          |
| XP_005415392.1        | aldehyde dehydrogenase 1                            | 3.3                          | 91.2          |
| XP_005382437.1        | neutrophil gelatinase-associated lipocalin-like      | 3.2                          | 62.0          |
| XP_005413882.1        | peptidoglycan recognition protein 1                  | 3.2                          | 71.7          |
| XP_005377232.1        | polyserase-2-like                                    | 3.1                          | 68.3          |
| XP_005389257.1        | arginase-1 isoform X1                               | 3.0                          | 31.0          |
| XP_005406075.1        | neutrophil elastase                                  | 3.0                          | 72.3          |
| XP_005399838.1        | glia maturation factor gamma isoform X1              | 2.9                          | 95.7          |
| XP_005373598.1        | high mobility group protein B2                      | 2.8                          | 98.1          |
| XP_005412491.1        | CD177 antigen isoform X1                            | 2.8                          | 52.7          |
| XP_005414274.1        | glucose-6-phosphate 1-dehydrogenase isoform X1      | 2.7                          | 72.8          |
| XP_005375305.1        | chitotriosidase-1 isoform X1                        | 2.6                          | 77.5          |
| XP_005380964.1        | dextrin                                              | 2.6                          | 100.0         |
| XP_005401229.1        | uncharacterized protein                              | 2.6                          | 94.7          |
| XP_005409156.1        | plasmin-2                                            | 2.6                          | 97.8          |
| XP_005413229.1        | uncharacterized protein                              | 2.6                          | 68.2          |
| XP_005385769.1        | UTP-glucose-1-phosphate uridylyltransferase isoform X4 | 2.5                          | 99.4          |
| XP_005397993.1        | actin-related protein 2/3 complex subunit 1B isoform X3 | 2.5                          | 98.1          |
| XP_005412491.1        | CD177 antigen isoform X1                            | 2.5                          | 99.2          |
| XP_005414274.1        | glucose-6-phosphate 1-dehydrogenase isoform X1      | 2.5                          | 92.2          |
| XP_005387330.1        | myeloperoxidase                                     | 2.5                          | 89.4          |
| XP_005394087.1        | sorcin isoform X2                                    | 2.5                          | 93.8          |
| XP_005402983.1        | galectin-9-like                                      | 2.5                          | 95.4          |
| XP_005405241.1        | coronin-1A                                          | 2.3                          | 95.4          |
| XP_005403838.1        | osteoclast-stimulating factor 1                      | 2.3                          | 97.1          |
| XP_005385769.1        | protein S100-A11 isoform X2                         | 2.2                          | 85.2          |
| XP_005374289.1        | olfactomedin-4                                       | 2.1                          | 82.2          |
| XP_005378835.1        | glyceraldehyde-3-phosphate dehydrogenase            | 2.1                          | 95.8          |
| XP_005381261.1        | thrombospondin-1                                    | 2.1                          | 96.3          |
| XP_005388181.1        | F-actin-capping protein subunit alpha-2             | 2.1                          | 97.8          |
| XP_005404076.1        | 6-phosphogluconate dehydrogenase, decarboxylating    | 2.1                          | 94.2          |
| XP_005409388.1        | lamin-B1 isoform X1                                 | 2.1                          | 97.9          |
| XP_005389933.1        | metalloproteinase inhibitor 1 isoform X2            | 2.0                          | 75.6          |
| XP_005379277.1        | galectin-1 isoform X4                               | 2.0                          | 85.2          |
| XP_005377495.1        | heterogeneous nuclear ribonucleoproteins A2/B1 isoform X5 | 2.0                          | 100.0         |
| XP_005388642.1        | annexin A5 isoform X1                               | 2.0                          | 92.8          |
| XP_005393859.1        | tropomysin alpha-4 chain isoform X8                 | 2.0                          | 99.9          |
| XP_005405252.1        | fructose-bisphosphate aldolase A isoform X4         | 2.0                          | 98.4          |
| XP_005373090.1        | serpin B4                                           | 2.0                          | 62.3          |
| XP_005384889.1        | adenosylhomocysteinase                              | 2.0                          | 96.1          |
| XP_005389440.1        | N(G),N(G)-dimethylarginine dimethylaminohydroxyl    | 2.0                          | 96.5          |
| XP_005394874.1        | chloride intracellular channel protein 4            | 2.0                          | 98.8          |
| XP_005406050.1        | ATP synthase subunit delta, mitochondrial isoform X2 | 2.0                          | 89.9          |
| XP_005406282.1        | hydroxyacyl-coenzyme A dehydrogenase, mitochondrial isoform X2 | 2.0                          | 89.8          |
| XP_005373784.1        | dihydroypropimidine-related protein 2 isoform X1    | 2.0                          | 98.5          |
| XP_005389984.1        | adenosine kinase isoform X1                         | 2.0                          | 93.5          |
| XP_005407967.1        | glutamate dehydrogenase 1, mitochondrial-like        | 2.0                          | 97.6          |
| XP_005412123.1        | purine nucleoside phosphorylase isoform X2          | 2.0                          | 85.4          |
| XP_005402983.1        | galectin-9-like                                     | 2.0                          | 53.2          |
| XP_005415261.1        | neuroblast differentiation-associated protein        | 2.0                          | 69.0          |
| XP_005374506.1        | lumican                                             | 2.0                          | 85.2          |
| XP_005377593.1        | aldose reductase                                    | 2.0                          | 84.5          |
Protein accession no. | Function | Fold change of infected/sham | Identity (%) *
--- | --- | --- | ---
XP_005381747.1 | protein disulfide-isomerase A3 | -2.5 | 87.9
XP_005382153.1 | stress-70 protein, mitochondrial | -2.5 | 98.1
XP_005386908.1 | aldo-keto reductase family 1 member C1 homolog | -2.5 | 81.1
XP_005388949.1 | glutathione S-transferase Mu 1-like isoform X2 | -2.5 | 84.4
XP_005398907.1 | thioredoxin domain-containing protein 5 | -2.5 | 90.7
XP_005405560.1 | non-muscle caldesmon isoform X7 | -2.5 | 89.8
XP_005406092.1 | basigin isoform X1 | -2.5 | 67.3
XP_005409541.1 | tropomyosin alpha-4 chain | -2.5 | 99.0
XP_005374508.1 | decorin isoform X2 | -2.6 | 91.7
XP_005380133.1 | hemoglobin subunit beta | -2.6 | 81.6
XP_005386118.1 | beta-hexosaminidase subunit beta | -2.6 | 79.2
XP_005406364.1 | lambda-crystallin homolog | -2.6 | 84.7
XP_005414854.1 | neuroblast differentiation-associated protein | -2.6 | 59.8
XP_005375324.1 | prolargin | -2.7 | 91.4
XP_005406777.1 | tubulin alpha-1A chain-like | -2.7 | 100.0
XP_005388953.1 | glutathione S-transferase Mu 5-like isoform X1 | -2.8 | 89.3
XP_005393042.1 | ceruloplasmin isoform X1 | -2.9 | 83.6
XP_005379942.1 | serpin H1 isoform X2 | -23.0 | 94.7
XP_005405733.1 | tartrate-resistant acid phosphatase type 5-like isoform X2 | -23.0 | 90.8
XP_005411945.1 | methyltransferase-like protein 7A | -23.0 | 84.3
XP_005414575.1 | selenium-binding protein 1 isoform X2 | -23.0 | 86.4
XP_005388267.1 | annexin A3 isoform X3; D | -23.1 | 90.1
XP_005388944.1 | glutathione S-transferase Mu 2 isoform X2 | -23.1 | 80.3
XP_005408960.1 | mimecan | -23.1 | 86.2
XP_005374405.1 | cysteine and glycine-rich protein 2 | -23.2 | 99.0
XP_005375980.1 | collagen alpha-1(VI) chain | -23.2 | 90.2
XP_005392386.1 | prostacyclin synthase | -23.2 | 82.9
XP_005399681.1 | beta-enolase isoform X3 | -23.2 | 99.0
XP_005391729.1 | hemoglobin subunit alpha-like | -23.3 | 84.5
XP_005392450.1 | matrix metalloproteinase-9 | -23.3 | 79.2
XP_005411706.1 | prelamin-A/C isoform X3 | -23.3 | 97.0
XP_005387798.1 | arachidonate 5-lipoxygenase-activating protein | -23.4 | 92.6
XP_005394586.1 | band 3 anion transport protein isoform X2 | -23.5 | 80.9
XP_005403866.1 | retinal dehydrogenase 1-like | -23.5 | 87.3
XP_005373031.1 | aldehyde oxidase-like | -23.8 | 78.2
XP_005382035.1 | carbonic anhydrase 2 | -4.0 | 83.5
XP_005379664.1 | tetraicopeptide repeat protein 36 | -4.2 | 85.5
XP_005380714.1 | dihydropyrimidinase-related protein 3 isoform X2 | -4.6 | 97.4
XP_005375783.1 | carbonyl reductase [NADPH] 1-like | -4.7 | 87.3
XP_005392435.1 | beta-actin-like protein 2 isoform X2 | -4.7 | 86.2
XP_005391730.1 | hemoglobin subunit alpha-like | -5.5 | 62.0
XP_005383679.1 | 40S ribosomal protein SA-like | -6.5 | 76.1

Non-Radioactive Cell Proliferation Assay kit (Promega Corporation, Madison WI). No significant toxicity was shown (data not shown). Strain 86–028NP(pGM1.1) was then added to the NHBE cells at a multiplicity of infection of 50 bacteria to one NHBE cell and incubated at 37 °C in a 5% CO₂ atmosphere for one hour. The NHBE cells were then washed twice with DPBS before being placed back in BEGM that contained either 200 μM CK-636 or 0.2% DMSO. Cells were incubated at 37 °C in a 5% CO₂ atmosphere for 24 or 48 h. At each time point, cells were stained with WGA-Alexa Fluor 594, fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) in DPBS, made permeable with 0.1% Triton X-100 then stained with phalloidin- Alexa Fluor 350. The NHBE cells were examined by fluorescence microscopy using an Axiovert 200M inverted epifluorescence microscope equipped with an Axiscam MRM CCD camera. Experiments were repeated in triplicate and representative examples are shown.

RESULTS

Descriptive Analysis of the Experimental AOM Proteome—We determined the proteomic profiles by LC/LC-MS/MS of 3 μg middle ear tissue infected for 2 days with NTHI for comparison with sham treated middle ear tissues. The mucosae in the middle ear bullae is typically a few cell layers thick that is closely juxtaposed with the underlying bone (Fig. 1, sham). The presence of NTHI results in a rapid expansion in the number of cells and the thickness of the mucosal tissue in the middle ear (Fig. 1, infected). There is 5-log increase in bacterial numbers over the first 48 h of infection. On the surface of the epithelial cell a biofilm begins to develop that is composed of bacteria and early infiltrating immune cells. The majority of these infiltrating immune cells transverse the mucosae to become an integral component of the biofilm that is closely associated with the epithelial surface. Also, given the architecture of the middle ear, the biofilm is not evenly distributed along the entirety of the epithelium at this early time point. To obviate bias because of the differential distribution of the biofilm, we removed the entire middle ear mucosae...
Infection of chinchilla middle ears with NTHI influences host-bacterial responses. Images are representative of chinchilla middle ears inoculated with sterile saline (Sham; A, C) or NTHI (Infected; B, D). A, B, Histopathology was assessed 2 days after inoculation by examination of hematoxylin and eosin stained thin sections to observe the mucosa and the biofilm. C, D, The production of mucin was assessed by glycan staining using the Periodic acid-Schiff (PAS) stain. Inset of panel D depicts positive PAS labeling of chinchilla middle ear epithelium. Mucin is stained red and readily identified at the interface of the mucosa and the lumen. Scale bar = 50 μm.

including the associated biofilm. Although the analyses of heterogeneous cell populations limit the ability to assign specific proteins to individual cell types, the use of the entire middle ear tissue provides a unique snapshot of the complex changes and microenvironmental cues that both the host and the bacteria respond to early during infection. The release of the chinchilla genome sequence provided the platform for improved accuracy in identification of the proteomic profiles the bacteria respond to early during infection. The release of microenvironmental cues that both the host and middle ear tissue provides a unique snapshot of the complex specificity of proteins to individual cell types, the use of the entire middle ear tissue provides a unique snapshot of the complex changes and microenvironmental cues that both the host and the bacteria respond to early during infection. The release of the chinchilla genome sequence provided the platform for improved accuracy in identification of the proteomic profiles.

Gene Ontology Analyses—The levels of 105 proteins changed significantly (44 proteins increased, 61 proteins decreased) because of infection with NTHI (Table I). BLAST analysis showed strong identity between the chinchilla proteins and their human orthologs. Chinchilla proteins that exhibited changes in production after NTHI infection could thus be categorized with Gene Ontology (GO) tools designed for the analyses of human data. The Biological Networks Gene Ontology tool (BINGO; (42)) identified proteins overrepresented within three ontologies: biological process, molecular function, or cellular component (41) and visualized with Cytoscape (43). These analyses identified nodes significantly overrepresented within each data set (supplemental Figs. S5–S10, summarized in Fig. 2). Within biological processes, overrepresented nodes that contain up-regulated proteins are primarily related to host immune functions, whereas nodes that represent down-regulated proteins encompass small molecule metabolic and catabolic processes, response to chemical stimuli and the cellular response to calcium (supplemental Tables S8 and S9). For molecular functions, up-regulated proteins are in nodes that represent carbohydrate, polysaccharide, bacterial cell surface, calcium, actin, and actin-filament binding. Conversely, proteins down-regulated are in nodes of which 40% are related to aspects of oxidoreductase activity and 29% are involved in molecular binding (supplemental Tables S10 and S11). For cellular components, seven nodes for up-regulated proteins contained proteins with roles in actin filament morphogenesis and actin-related functions. Finally, for the cellular component proteins whose levels decreased because of NTHI infection, approximately one-third

should tightly group, because they are technical replicates, and drift in these analyses over the study should be minimal compared with the biological variance (viewed as spread in the principal components). The technical reproducibility during the proteomics study is clear because the QC pools (red) are tightly grouped in the PCA plots. The three highest-weighted principal components for the QC pools and the six sham and seven NTHI-infected samples are depicted in supplemental Fig. S3. Two sham samples (1L&R and 2L&R) were removed because of the presence of NTHI peptides (supplemental Fig. S3).

NTHI Infection of Chinchilla Middle Ears Induces Transcriptional Changes That Mirror Changes in Protein Amount—qRT-PCR was used to confirm that the changes in the amount of select proteins because of AOM caused by NTHI were because of changes in transcription. RNA was purified from middle ear tissue of both sham-treated and NTHI infected chinchillas collected concurrently with the tissues used in the proteomic and metabolomic studies. Although these tissues were limited in number, sufficient RNA was isolated to test expression of seven genes. In all cases, changes in gene expression because of NTHI infection showed a similar trend to changes in the amount of protein detected (supplemental Fig. S4).

Voltage Control Using Principal Components Analysis—To screen for potential outliers and observe the general technical reproducibility of the analysis versus the biological variability, a principal component analysis (PCA) was performed using z-scored protein intensities from supplemental Table S3. The expectation for a high-quality analysis is that the QC Pools
of the associated nodes have roles in actin-myosin based functions (supplemental Tables S12 and S13).

**Actin Filament Regulation in AOM**—Actin cytoskeleton plasticity is critical for many cellular functions (49–54). During NTHI-induced AOM in the chinchilla, 14 of the 105 proteins identified participate in actin morphogenesis. Within this subset of proteins, seven exhibited increased levels, while seven exhibited decreased levels (Table II). At this stage of the infection, actin rearrangements could be involved in epithelial cell proliferation, immune cell migration, or phagocytosis of NTHI.

**Role of Actin Morphogenesis in NTHI Invasion into Epithelial Cells**—To investigate the biological consequences of actin morphogenesis in epithelial cells, we evaluated the contribution of the actin-related protein complex, Arp2/3, in NTHI invasion. Arp2/3 is comprised of two actin-like proteins (Arp2, Arp3) and five accessory proteins (ArpC1–5). This complex has a critical role in the nucleation of branched actin structures to generate force at the lamellipodia, within phagocytic cups, for endocytic vesicle morphogenesis and autophagy, among others (55–57). Two of the subunits of the Arp2/3 complex (ArpC1, ArpC4) showed increased levels in middle ears infected with NTHI, as compared with sham-infected middle ears. The increase in Arp2/3 levels observed in infected tissues was validated by immunoblot analysis of mucosal lysates (Fig. 3). Moreover, bright punctate foci of Arp2/3 (green) were readily apparent at the leading edges of Ly6C positive neutrophils (red) within the NTHI biofilm in the infected middle ear (Fig. 4). The foci were observed only in the presence of antibody directed against Arp 2/3 (Figs. 3, 4). In addition, Arp2/3 was readily observed at the membrane surface of epithelial cells in the infected sample and rarely in the sham sample (Fig. 3). This increase in Arp2/3 activity in the epithelium is consistent with the increased proliferation observed during infection (Fig. 1). A role for Arp2/3 in epithelial cell actin remodeling in response to NTHI infection was evaluated through the inhibition of Arp2/3 function in cultured epithelial cells using the small molecule CK-636 (58). Consist-

![Graphical summary of overrepresented proteins categorized by GO function.](image)

Genes that encoded proteins who increased (green) or decreased (red) in amount because of NTHI-induced AOM were categorized by Gene Ontology. For each ontological group; the number of nodes generated are represented by the larger circles, whereas the number of nodes that are the overrepresented are shown as the smaller circles (*). The size of each circle is scaled to the number of nodes contained within. The top 10 significantly overrepresented nodes within each ontological category are listed on the figure, whereas all nodes are listed in supplemental Tables S8–S13.
ent with previous reports (22), in the absence of CK-636 viable internalized NTHI were not observed in NHBE cells following 24 and 48 h of infection. When Arp2/3 function was inhibited, we observed gross morphological changes in actin structure and an increase in the number of extracellular bacteria indicating that bacterial uptake was reduced (Fig. 5). These data suggest that Arp2/3 participates in NTHI internalization with subsequent intracellular trafficking to the endolysosomal pathway (59).

**Action of Actin-Associated Proteins Identified during AOM**

**Regulation of Actin Ultrastructure**—We observed increased levels of proteins (e.g. glia maturation factor G (GMFG), coronin-1A, coflin, L-plastin) (Table II; supplemental Fig. S11) that have predicted roles in the production of new actin filaments, in concert with the destabilization of existing actin filaments, primarily through interactions with Arp2/3 (60–66). Consistent with the plasticity of actin polymerization at this time point, we also observed decreases in levels of proteins (e.g. caldesmon, CapZA) that have predicted roles in the stabilization of actin filaments (67–71). Actin capping proteins are thus critical to direct local polymerization to facilitate lamellipodia formation and motility and cell velocity (72). Taken together, we suggest that the changes in these protein profiles observed during AOM will result in the net destabilization of actin polymers to facilitate actin filament remodeling and branch formation.

**Analysis of the Experimental AOM Metabolome**—Classical approaches to elucidate the molecular mechanisms of disease focus on changes in transcriptional profiles. It is clear that certain biological metabolites can act as markers of pathological conditions and as novel therapeutic targets. However, little is known in regard to the role of these diverse molecules during bacterial infection, particularly in the context of animal models or human samples. We evaluate multiple classes of bioactive metabolites, using two independent analyses from the same samples used for proteomic analysis, to provide a more comprehensive assessment of the molecular changes that occur during AOM.

**Metabolite Profile Indicates that the Middle Ear is Immuno-suppressed on Day 2**—The first five classes of metabolites targeted were: acylcarnitines, biogenic amines, amino acids, glycerophospholipids, and sphingolipids. Of the 186 analytes assayed, 75 were measured at quantifiable levels. Of these, 66 showed statistically significant changes because of NTHI infection. Acylcarnitines stimulate proinflammatory responses, in part, through IL-8 (73). However, acylcarnitines were not detected. We observed a significant reduction in the levels of other regulatory metabolites in the infected tissues as compared with the sham treated tissues. Biogenic amines (e.g. histamine, dopamine, and serotonin) regulate a diverse array of processes. Of the 21 biogenic amines analyzed, 10 were detected at quantifiable levels, and seven showed statistically significant changes. Six of these were significantly decreased at day 2, indicating that host metabolism is modulated by NTHI (Fig. 6). Taurine is a potent antioxidant and is produced at higher levels in response to oxidative stress and the only molecule significantly increased (74). We interpret the presence of the detected metabolites to indicate that the previously nonoxidative environment of the middle ear is transitioning to an oxidative environment. Of 17 amino acids quantified, three significantly increased during infection (Lys, Gln, Orn; Fig. 6).

The lipid species measured by flow injection using the Biocrates p180 kit must be treated with some caution as they may contain interference from isobaric analytes, thus, may be less specific than those lipids measured using higher resolution (LC-MS/MS) approaches. Nevertheless, because many lipid species in the same class are moving in concert, using this kit produced lipid measurements that characterized significant trends in the lipid species present in the infected samples. Sphingolipids are important signaling molecules involved in cellular growth, differentiation, inflammation, and immunity, including activation of cyclooxygenase (75, 76). The

| Protein accession no. | Function | Peptide match | Fold change of infected/sham | p value |
|-----------------------|----------|---------------|-----------------------------|---------|
| XP_005398838.1        | F-actin-capping protein subunit alpha 2     | 2                           | 2.9                           | 2.5132E-05 |
| XP_005413091.1        | actin-related protein 2/3 complex subunit 4 | 7                           | 2.8                           | 7.17E-07   |
| XP_005380964.1        | destrin                                             | 2                           | 2.6                           | 2.41E-05   |
| XP_005409156.1        | plastin – 2                                        | 18                          | 2.8                           | 3.81E-06   |
| XP_005397993.1        | actin-related protein 2/3 complex subunit 1B isoform X3 | 5                           | 2.5                           | 3.51E-05   |
| XP_005405451.1        | coronin-1A                                         | 10                          | 2.3                           | 7.17E-05   |
| XP_005388181.1        | tropomyosin alpha-4 chain isoform X8              | 2                           | 2.1                           | 1.88E-03   |
| XP_005393859.1        | tropomyosin alpha-4 chain isoform X8              | 5                           | –2.1                         | 1.46E-04   |
| XP_005415261.1        | neuroblast differentiation-associated protein     | 4                           | –2.4                         | 7.89E-07   |
| XP_005409541.1        | tropomyosin alpha-4 chain isoform X8              | 2                           | –2.5                         | 4.25E-04   |
| XP_005405560.1        | non-muscle caldesmon isoform X7                  | 4                           | –2.5                         | 2.75E-06   |
| XP_005414854.1        | neuroblast differentiation-associated protein     | 10                          | –2.6                         | 7.89E-07   |
| XP_005380714.1        | dihydropyrimidinase-related protein 3 isoform X2 | 4                           | –4.6                         | 4.00E-05   |
| XP_005392735.1        | beta-actin-like protein 2 isoform X2             | 2                           | –4.7                         | 2.03E-04   |
sphingomyelin molecules were decreased in all the infected samples (Fig 6D, 6E). These molecules are known proinflammatory mediators during other infections. The levels of detected sphingolipids are below that of the sham treated tissues, therefore, we hypothesize that NTHI initially suppresses metabolites that stimulate immune responses. A number of phosphatidylcholine derivatives were statistically different between the sham and infected cohorts. The magnitude of some phosphatidylcholine derivatives increased, whereas others decreased in response to NTHI infection (Fig. 7). In other systems, these molecules play important roles in mucous production, actin polymerization, and host signaling (77–80).

Suppression of Immunostimulatory Oxylipins—We quantified 22 of a possible 128 oxylipins, half of which were significantly changed because of NTHI infection. Interestingly, although some of the actin and actin associated proteins as well as neutrophil cytosolic factor 2 identified in the infected proteome are associated with neutrophils; we did not detect the neutrophil-derived metabolite 5-HETE (81). In addition, although variable, we observed a reduction of 13(S)-hydroperoxyoctadeca-9Z,11E-dienoic acid (13-HpODE) in the infected samples. We were unable to detect the chemokine monocyte chemoattractant protein-1 known to be positively regulated by 13-HpODe (82) (Fig. 8). These analyses suggest that 2 days following infection, immune cells, although clearly
present in the middle ear (Figs. 3, 4), lack detectable metabolic activity typically associated with bacterial clearance. A decrease in the infected tissue was also observed for 15-hydroxyeicosatetraenoic acid (15-HETE) (Fig. 8), which other studies have shown participates in vascular remodeling and increases both iNOS and mucin production (83–85). 15-HETE is also a precursor in lipoxin production and suppresses epithelial differentiation (86–89). Therefore, decreased levels of 15-HETE in our samples may explain the increased epithelial expansion associated with infection and may contribute to the immunosuppressed status (Fig. 8). In the context of experimental OM, mucin production requires the lipoxygenase pathway including 15-HETE (90, 91). Consistent with this observation, mucin production was not observed in infected middle ears (Fig. 1). Moreover, other studies have shown reduced mucin expression early during infection that increases as the disease progresses (J.E.K., unpublished results). We observed statistically significant decreases in the levels of other products of autoxidation of arachidonic acids (8-, 9-, and 11-HETE; Fig. 8) in the infected samples. Although not significant, we also observed a decrease in 5- and 12-HETE (supplemental Table S6). There was also a significant decrease in the proinflammatory oxylipin 9-HODE (92) in the infected samples (Fig. 8).

Simultaneous Detection of NTHI Proteins—Characterization of bacterial and host proteins from the same sample illuminates the dynamic interplay between host and pathogen during disease progression. Approximately 1% of the total peptides identified were derived from NTHI proteins. We identified proteins involved in carbohydrate metabolism, amino acid biosynthesis, redox homeostasis, protein synthesis, as well as cell wall-associated proteins involved in transport and membrane integrity. These proteins provide insight into the metabolic state of NTHI during the early stages of infection.

DISCUSSION

Rationale for a Multiomics Approach to the Study of Disease—The drive to understand disease has become increasingly dependent on multifaceted approaches. Identification of proteins and metabolites from the same sample provides a cross platform validation of the analysis of the host-pathogen interactions that influence disease progression. Using the chinchilla model, we simultaneously defined the protein and metabolite signatures of middle ears infected with NTHI compared with sham treatment. We observed striking differences in the architecture of the middle ear mucosal tissue as well as early infiltration of immune cells in the chinchillas that developed experimental OM as compared with sham treatment (Fig. 1). We showed that this multifaceted approach is possible for the identification of both therapeutic targets and biomarkers of a human disease. In addition to the small sample size, we show the feasibility of using outbred animals in the identification of significantly changed molecules. Therefore,
this approach is amenable to other experimental models of disease as well as with human samples.

Actin Morphogenesis in Pathogenesis and Immune Signaling—Approximately 13% of chinchilla proteins that exhibited significant changes in amount because of AOM were involved in actin remodeling. Based upon the known roles for these proteins in other systems, the changes observed will likely lead to depolymerization, debranching, and a reduction in bundling of actin filaments, mechanisms that increase the pool of monomeric actin for subsequent actin cytoskeleton morphogenesis. As well as contributing to these structural functions, actin related proteins also participate in regulation of host cell signaling in response to bacteria. GMFG negatively regulates lipopolysaccharide (LPS) induced activation of the NF-κB, MAPK and IRF3 signaling pathways in macrophages through the control of endosomal sorting of TLR4 (93). GMFG can also stimulate neutrophil migration and polarity, whereas coronin-1A and L-plastin control neutrophil phagocytosis and CD18 activation, respectively (94–99). Coronin-1A is localized to phagocytic vacuoles of neutrophils where the actin cytoskeleton mediates both phagocytic killing and binds p40phox to activate NADPH oxidase driven production of superoxide. This latter process is also plastin mediated (96, 100, 101). Plastins further participate in cell invasion by the intracellular pathogens Salmonella and Shigella (100). Similarly, AHNAK complexes with actin, S100A10 and annexin A2 to mediate architectural remodeling of the cell membrane and cortical cytoskeleton to promote invasion of Salmonella into epithelial cells (100, 102–105). We hypothesize that the increased levels of these actin-associated proteins suggest actin remodeling enhances recruitment of immune cells and invasion of epithelial cells. Concurrent with this hypothesis, we observed a significant decrease in the lysosomal enzyme hexosaminidase B that plays a role in degradation of internalized Staphylococcus (106). Taken together, the regulatory systems described generate precise control of actin filament and network formation and participate in multiple aspects of pathogenesis (107–112).

Biological Consequences of Actin-Associated Proteins during Pathogenesis—The importance of actin morphogenesis during NTHI infection of the middle ear was shown by our proof-of-concept study in which the function of Arp2/3 was inhibited. We showed that Arp2/3 plays important roles in NTHI invasion into NHBE cells. We have previously shown that the NTHI responses to nutritional immunity promote intracellular invasion and survival within host cells (113). We therefore suggest that therapeutic modulation of actin remodeling to prevent invasion will increase the vulnerability of NTHI to the host immune responses and antimicrobial therapies.

Role of Amino Acids during AOM—Three amino acids were increased in amount because of AOM. Of these, a role for lysine during bacterial pathogenesis is unknown. Glutamine attenuates local inflammation through the NF-κB pathway (114), but has no known role in AOM (114). Ornithine is the
precursor for polyamine production, regulatory molecules that provide protection against acid stress in other systems. Our observed accumulation of ornithine, in the absence of polyamines, further suggests that the microenvironment within the middle ear on day 2 of AOM is transitioning into a stressful environment. Valine suppresses competence in *H. influenzae* (115) (Fig. 6), a mechanism used to obtain DNA as a nutritional source during periods of starvation (116, 117). Thus, we hypothesize that the observed decrease in valine leads to increased competence and greater uptake of DNA for genetic plasticity and/or a nutritional source for NTHI.

Potential Roles for Lipid Species Early during AOM—Diacyl phosphatidylcholine (PC aa) derivatives increase the viscosity of mucous and are precursors for prostaglandin synthesis (77). Reduction in the content of PC aa in the gastrointestinal mucosae leads to increased invasion of pathogens (118). PC aa derivatives in the plasma membrane inhibit actin polymerization, inhibit phagosomal actin assembly, stimulate *Mycobacterium* survival in phagosomes, and increase nitric oxide production (78, 118, 119). These PC aa functions are also complementary with the proposed increase in actin rearrangements suggested by the proteomics analysis (Fig. 7). Acyl-alkyl phosphatidylcholines (PC ae) are platelet activating factor (PAF) mimetics that can be ligands or potent agonists of the PAF receptor (79, 80). PAF stimulates inflammatory responses and mucin production in respiratory epithelial cells.

**Fig. 7.** Changes in the levels of diacyl phosphatidylcholine and acyl-alkyl phosphatidylcholine derivatives of glycerophospholipids because of AOM. Diacyl phosphatidylcholine (A, B, C) and acyl-alkyl phosphatidylcholine (D, E, F) derivatives of glycerophospholipids were identified in the sham (gray) and infected (red) middle ear tissue samples. The derivatives were stratified according to the magnitude detected in the samples for ease of visualization. Statistical significance was determined using a two-tailed unpaired *t* test (*, *p* < 0.05; **, *p* < 0.009; ***, *p* < 0.0008). Data were derived from six sham infected mucosae and seven NTHI infected mucosae.
Future studies will delineate the role of these molecules during early AOM.

NTHI depends upon host phosphatidylcholine (PC) as the source of choline for phosphorylcholine (ChoP) modification of glycans. ChoP modification is important for NTHI colonization and persistence in AOM because of roles in biofilm formation, adherence to cells through the PAF receptor, survival of complement-mediated lysis, as well as resistance to antimicrobial peptides (121–127). The significant reduction of PC derivatives could be because of decreases in biosynthesis or use as a preferential substrate for glycan modification by NTHI. Further investigations are needed to distinguish between these two possibilities.

Pathways and Functions Identified by both Proteomics and Metabolomics—Concordance between proteomic and metabolomic data derived simultaneously from a single tissue sample strengthens the validity of the approach used to identify signatures of disease. For example, prostaglandin I2 synthase is the last enzyme in the cyclooxygenase pathway for the metabolism of arachidonic acid. Although this was the only protein in the cyclooxygenase pathway significantly decreased because of infection, three oxylipin metabolites of the pathway (12S-HHTe, 11-HETe, 13-HDoHe) were also significantly reduced, providing additional support that this pathway is not yet activated on day 2 post infection (Fig. 8). We also observed multiple examples of proteins and metabolites involved in inflammation that were all significantly increased during infection (e.g. S100A8/9, annexin A1, lactotransferrin, neutrophil cytoplasmic factor 2, taurine and phosphatidylcholine). Previous studies showed up-regulation of S100A8/9 during OM and the antibacterial effects on NTHI (128). Because we observed down-regulation of glutathione S-transferase, known to inhibit S100A8/9 inflammatory activity by glutathionylation (129), we predict that unmodified S100A8/9 primarily mediates neutrophil influx into the middle ear with attendant increased antibacterial activity. Similarly, we observed increases in metabolites that are known biomarkers for inflammation in other diseases and our data suggest that they may be potential biomarkers for OM (e.g. phosphatidylcholine derivatives, taurine and peptidyl arginine deiminase (PAD)). PAD deiminates cathelicidin and impairs the antimicrobial activity against NTHI (130). We observed increases in both the chinchilla ortholog of human antimicrobial peptide-cathelicidin (15 kDa protein A-like) and PAD during AOM (Table I). Taken together, we propose that deimination reduces cathelicidin activity and so benefits NTHI survival at the early stages of infection.

Inflammation includes the production of inflammatory markers and oxidant species by the host. Although we observed inflammatory markers in the middle ear, multiple proteins involved in the generation of, or protection from, oxidative stress are significantly decreased during AOM. For example, the selenium importing proteins selenium-binding protein 1 and anion exchange protein 1, are reduced in NTHI-infected tissue. Thus, our data suggest that the function of selenium containing proteins, the majority of which protect against oxidative stress (131, 132), will be impaired (131,132). Crystalin, glutathione S-transferases, and purine nucleoside...
phosphorylase are reduced during AOM. We predict that a decrease in purine nucleoside phosphorylase results in a reduced NAD pool and NADPH oxidase activity. Significant decreases were also observed in oxylipins that are produced through nonenzymatic modification in oxidative environments (9-HETE, 10-HDoHE, 16-HDOE). Taken together, bioactive metabolites and protein profiles suggest that the middle ear remains immunologically quiescent. However, some precursors of key mediators for inflammation are beginning to increase, suggesting a transition in the immune responses during AOM.

**Bacterial Proteins Indicate Aerobic Respiration of NTHI on Day 2 of AOM**—Half of the NTHI proteins identified in this study participate in carbohydrate and amino acid metabolism during aerobic respiration (Table III). The presence of proteins involved in oxidative phosphorylation associated with both glycolysis and TCA cycle generation of NADH and ATP suggest that NTHI uses glucose for aerobic respiration during AOM. Adenylate kinase generates pools of ADP for oxidative phosphorylation and coincides with identification of both phosphopyruvate hydratase and transaldolase B, enzymes that generate pyruvate through glycolysis and glyceraldehyde-3-phosphate dehydrogenase, respectively. Glyceraldehyde-3-phosphate can then be converted to D-glycerate 1,3-bisphosphate by glyceraldehyde-3-phosphate dehydrogenase (also observed during AOM) as part of the glycolytic activity of NTHI. Uridylic kinase (133) catalyzes the conversion of ATP and UMP to ADP and UDP (133), molecules important for storage of glucose during glycogenesis. Haemophilus strains contain intact pathways necessary for glycolysis and generation of acetyl-CoA, a key substrate for the TCA cycle. However, KEGG analysis (http://www.genome.jp/kegg/) shows that, similar to *H. influenzae* Rd and type b strains, NTHI strain 86–028NP lacks citrate synthase, aconitase, isocitrate dehydrogenase, and succinate dehydrogenase preventing entry into the TCA cycle via acetyl-CoA (supplemental Fig. S12). Asparaginyl-tRNA synthetase uses L-glutamine (both increased during infection (Fig. 6)) as a

| Protein accession no. | NTHI no. | Gene | Function |
|-----------------------|----------|------|----------|
| AAX87406              | NTHI0468 | adk  | ATP-AMP transphosphorylase |
| AAX87533              | NTHI0609 | atpD  | ATP synthase F0F1 subunit beta |
| AAX88688              | NTHI1935 | lpdA  | dihydrolipoamide dehydrogenase |
| AAX87004              | NTHI0001 | gapA  | glyceraldehyde-3-phosphate dehydrogenase |
| AAX88208              | NTHI1381 | mdh   | malate dehydrogenase |
| AAX87971              | NTHI1103 | eno   | enolase |
| AAX88128              | NTHI1293 | talB  | transaldolase B |
| AAX88075              | NTHI1225 | pyrH  | uridylicate kinase |
| AAX87733              | NTHI0831 | tnaA  | tryptophanase |
| AAX88414              | NTHI1615 | asnS  | asparaginyl-tRNA synthetase |
| YP_001621483.1        | NTHI2055 |       | beta-lactamase TEM |
| AAX87492              | NTHI0567 | mrcA  | penicillin-binding protein 1A |
| AAX87905              | NTHI1028 | clpB  | ClpB |
| AAX87199              | NTHI0225 | ompP2 | outer membrane protein P2 |
| AAX87824              | NTHI0937 | rpsJ  | 30S ribosomal protein S10 |
| AAX87848              | NTHI0963 | rpsK  | 30S ribosomal protein S11 |
| AAX87829              | NTHI0942 | rpsS  | 30S ribosomal protein S19 |
| AAX87660              | NTHI0746 | rpsG  | 30S ribosomal protein S7 |
| AAX87565              | NTHI0642 | rplA  | 50S ribosomal protein L1 |
| AAX87566              | NTHI0643 | rplK  | 50S ribosomal protein L11 |
| AAX87836              | NTHI0951 | rplN  | 50S ribosomal protein L14 |
| AAX87838              | NTHI0953 | rplE  | 50S ribosomal protein L5 |
| AAX87841              | NTHI0956 | rplF  | 50S ribosomal protein L6 |
| AAX87661              | NTHI0747 | fusA  | elongation factor G |
| AAX87662              | NTHI0748 | tufB2 | elongation factor Tu |
| AAX87555              | NTHI0632 | rbsB  | D-ribose transporter subunit RbsB |
| Unknown               | NTHI0696 |       | hypothetical protein |
substrate for the synthesis of aminoacylated tRNA. A pool of L-glutamate can be catabolized via L-glutamate dehydrogenase to generate α-ketoglutarate (supplemental Fig. S12), an alternative entry point into the TCA cycle. The cycle therefore drives exclusively through the succinate-fumarate-malate branch, which requires malate dehydrogenase (also detected) (Table III). Thus, generation of pyruvate via glycolysis and α-ketoglutarate as an entry point into the TCA cycle are critical metabolic processes active in NTHI during AOM. We detected dihydrolipoamide dehydrogenase, a key metabolic enzyme in aerobic respiration and essential functional subunit of both pyruvate dehydrogenase (catalyzes the conversion of pyruvate to acetyl-CoA), and α-ketoglutarate dehydrogenase (catabolism of α-ketoglutarate in the TCA cycle) (134). Because acetyl-CoA is not used in the TCA cycle in Haemophilus, the increased pool of acetyl-CoA may be used for synthesis of fatty acids. Herbert and colleagues previously suggested that an enhanced lipid bilayer because of fatty acid synthesis is necessary for in vivo resistance against reactive oxygen intermediates (134). Our proteomic data support their hypothesis that the noncyclic, branched TCA pathway in NTHI likely evolved as a consequence of NTHI adaptive physiology and overall fitness to the host microenvironment (134).

Our data provide the first evidence that NTHI use aerobic respiration during AOM. However, shifts in NTHI metabolic activity, such as amino acid metabolism, suggest changes in nutritional requirements and availability in the host environment. We observed tryptophanase, the catalyst of tryptophan metabolism, which produces indole, pyruvate, and ammonia. Indole is an important intra- and interspecies signaling molecule that regulates bacterial behaviors such as biofilm formation and antibiotic resistance (135, 136). In E. coli, indole increases production of multidrug exporter proteins leading to antibiotic resistance, yet decreases biofilm formation through repression of motility and cell adherence (137-140). Further, endogenous oxidative stress leads to increased tryptophanase, increased indole and delayed biofilm formation (141). Evidence of host-derived oxidative stress in the middle ear (Table III), concurrent with NTHI-derived tryptophanase, suggests indole production. Indole can inhibit N-acylated homoserine lactone-mediated quorum signaling associated with virulence and biofilm formation in Vibrio species (140). Decreases in indole concentration through changes in bacterial metabolic activity or release of factors that interfere with tryptophanase production, coincides with increased bacterial persistence and increased biofilm formation (142, 143).

Although NTHI biofilm formation is critical for manifestation of disease in the middle ear, mechanisms of NTHI adaptation to this host niche, including the transition from acute disease to biofilm formation and persistence associated with chronic disease, is not well characterized. Soluble mediators, released by bacteria, modulate the composition of NTHI lipooligosaccharides that effects biofilm maturation and persistence in vivo (144). Swords and colleagues showed that RbsB participates as a quorum regulated protein required for uptake of and response to the signaling molecule Al-2 (145). The consequence of a tryptophanase driven increase in indole on biofilm inhibition may be diminished through increased transport of Al-2.

A number of stress response proteins are also detected during NTHI infection, which includes the ClpB heat shock protein (Table III) (up-regulated in NTHI biofilms) (146), and detection of β-lactamase (Table III) (antibiotic resistance). Penicillin-binding protein 1A mediates bacterial membrane stability and has been shown to promote resistance to antimicrobial peptides in Streptococcal species (147). Our observation suggests that penicillin-binding protein 1A may provide resistance against the host cathelicidin (15kDa protein A-like) observed during AOM. Finally, the observation of P2 was not surprising because this is the most abundant outer membrane protein of NTHI (148). Despite extensive antigenic heterogeneity among strains, identification of conserved epitopes in P2 could provide a useful therapeutic target for vaccination (149).

Impact of Findings and Future Investigations in OM—We show one of the proteins identified in our analyses has a role in the invasion of NTHI, revealing a new mechanistic insight into OM. Our data thus show the feasibility of our approach to identify host and bacterial processes that can be targeted for therapeutic interventions for AOM (Fig. 9). In addition, specific proteomic and metabolic signatures may hallmark stages of infection that could be implemented into metabolic biomarkers for diagnosis.
It will be important to provide mechanistic insight into disease progression in the middle ear considering proteome and metabolome signatures of disease. Future investigations will examine immunomodulatory responses contributing to early suppression of inflammatory mediators and subsequent transition to inflammatory responses and the effect of these changing environments on bacterial responses. In this context, we are particularly interested in examining the modification of antimicrobial peptides during disease and the consequences on bacterial persistence and disease severity.

We also characterized specific metabolic indicators that indicate a shift to an oxidative stress environment that coincides with production of mediators that affect host immune cell influx. It will be important to investigate the consequence of these stressors on bacterial gene expression and protein production, modulation of bacterial lipid composition, and changes in bacterial metabolic state. Although AOM coincides with the presence of enzymes and products of aerobic metabolism, we provide evidence for a role in amino acid metabolism, particularly increased tryptophan metabolism and presumed production of indole. Future studies will examine the consequences of indole production on biofilm formation, antibiotic resistance, and role in establishment of polymicrobial infections in the middle ear.

CONCLUDING REMARKS

Comprehensive molecular profiling data must be integrated and evaluated based upon current understanding of the state of the field in all inflammatory processes, yet provide novel insights as advanced technologies provide a more in depth description of these processes. The use of high throughput, unbiased proteomics, as well as targeted quantitative metabolomics has now been successfully applied to an animal model of human infection (Fig. 9). Moreover, the approach revealed proteins from both the host and the bacteria, dramatically increasing the association of the proteins with the metabolic and environmental status of the middle ear early during AOM. In addition, we would expect that the NTHI-host interactions revealed here would likely have applicability to other sites of NTHI infection and potentially other bacterial-mediated infections. We provide experimental evidence for the identification of an actin-related protein previously not described in NTHI mediated disease, and the utility of targeting this protein as a potential adjunct therapy. Current investigations will focus on the best combinatorial approach for the use of this therapy in the chinchilla model of otitis media to quell infection with the ultimate outlook of implementing novel therapeutics to treat human disease. The amount of material used in the analyses was much less than normally generated by a standard tissue biopsy (150). Application of these approaches to other inflammatory conditions could potentially lead to an expansive understanding of many other diseases and so expedite the development of both vaccine and therapeutic targets as well as the identification of potential biomarkers of disease.

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