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A proteomic characterization of NTHi lysates

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ABSTRACT

Background: Non-typeable *Haemophilus influenzae* (NTHi) is a ubiquitous bacterial pathogen which accounts for a majority of human upper respiratory tract infections. Laboratory lysate preparations from this bacterium are commonly utilized to investigate the promulgation of inflammatory responses in respiratory and middle ear epithelium both *in vivo* and *in vitro*. We undertook an unbiased proteomics based analysis of NTHi lysate preps to: (a) identify abundant bacterial proteins present in these lysates that could play a role in NTHi biological effects and (b) determine the protein content variability in different lysate prep batches from the same NTHI strain.
Study design: Proteomic analysis of laboratory NTHi lysate preparations from clinical strain 12.
Methods: NTHi lysates were denatured, gel-fractionated, digested by trypsin and the generated peptides were identified using a liquid chromatography tandem mass spectrometry (LC–MS/MS). Western blot analyses for the important proinflammatory enhancer, outer membrane protein 6 (OMP6), was performed to validate the MS findings. Luciferase assays for NF-kB activation were used to measure the pro-inflammatory biologic effects from each NTHi lysate preparation.
Results: The MS identified 793 unique NTHi proteins. Most common and abundant proteins found have been described to either contribute to biofilm formation, elude the innate immune system, or activate epithelial pro-inflammatory pathways such as Toll Like Receptor 2 (TLR-2) signaling and NF-kB transcription factor. Strong positive signal for OMP6 was found in each of the NTHi lysate preparations. Significant NF-kB promoter response activation as expected with NTHi stimulation over control was also noted for each NTHi lysate preparation.
Conclusions: Proteomics was a successful technique to broadly define the protein content of NTHi lysates. This is the first report of the proteome of NTHi lysates widely used in laboratories to study the biological effect of NTHi. Despite the variability of the protein composition from different preps, all the batches of NTHi lysates induced similar NFκB activation.
Level of evidence: NA

1. Introduction

Non-typeable Haemophilus influenzae (NTHi) is a gram negative bacterium lacking capsular polysaccharides. A ubiquitous human respiratory pathogen, it has become the most common infectious pathogen in upper airway diseases such as acute otitis media (AOM) [1,2] and acute sinusitis. Although less systemically virulent and invasive than encapsulated *H. influenzae* strains, NTHi strains contribute to a majority of respiratory tract infections [3] primarily due to its ability to adhere to respiratory mucosa [4], it's high pediatric nasopharynx carrier rates [5] and through its ability to form biofilms over respiratory mucosal surfaces [6]. NTHi clinical strain 12 in is one of the most studied strains, particularly in terms of its ability to adhere to respiratory surfaces [4], and is a common pathogen in acute otitis media [7]. Importantly, nearly complete genomic data is now available for this strain of NTHi [8,9].

Bacterial lysis due to innate immunity defense molecules such as lactoferrin or defensins, due to the bactericidal action of

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antibiotics, or due to autolysis, can result in the release of a plethora of proteins and bacterial products which have been shown to further potentiate a proinflammatory response including an activation of MAP kinase signaling [10] and NF-kappaB [11,12] in epithelial cells. Laboratory preparations of NTHi bacterial lysates, most often from clinical strain 12, are commonly used to investigate the substantiation of inflammatory responses in respiratory and middle ear epithelium by us and other groups [11,13–15], yet the actual protein content of lysates from this clinical strain has not been profiled. A comprehensive analysis of the lysates would help understand variation in protein presence from lysate to lysate that could account for subsequent experimental effect variability.

In order to better understand the global composition of NTHi lysates from clinical strain 12 we undertook an unbiased proteomics based analysis of 3 separate lysate preps from this bacterial strain. Our goal was to potentially elucidate and identify novel bacterial mediators of inflammatory regulation along with defining a list of abundant bacterial proteins in this clinical strain.

2. Materials and methods

Preparation of NTHi lysates. NTHi clinical strain 12 was generously provided by Dr. Xin-Xing Gu (NIDCD, Bethesda, MD). Bacteria were grown on chocolate agar at 37 °C in 5% CO_2 overnight and inoculated in brain heart infusion (BHI) broth supplemented with 10 mg of nicotinamide adenine dinucleotide per mL. After overnight incubation, bacteria were subcultured into 500 mL of fresh brain heart infusion (BHI) and upon reaching log phase growth NTHi were washed and suspended in phosphate-buffered saline (PBS) followed by sonication for lysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and peptide preparation for mass spectrometry (MS) analysis. NTHi lysates (50 μg) were dissolved in Laemili buffer containing 0.1 mM DTT and were run in a one-dimensional SDS gel electrophoresis gel at 200 V for 50 min. The gel was fixed with methanol and stained with Coomassie for protein visualization (Fig. 1). Each gel lane was sliced into 30 segments, and each slice was digested with trypsin as follows. Briefly, the gel cuts were placed in 100 μL of water and then subjected to two washes with a 1:1 by volume solution of water and acetonitrile. The gel pieces were then dehydrated with acetonitrile and rehydrated using 100 mM ammonium bicarbonate, followed by a 1:1 by volume wash of 100 mM ammonium bicarbonate and acetonitrile. The gels were then dehydrated with acetonitrile, resuspended in digestion buffer containing 12.5 ng/μL of MS grade Trypsin Gold (Promega Corp., Madison, WI), and incubated overnight at 37 °C. Extraction of peptides from the gel was then conducted *via* two washes with 25 mM of ammonium bicarbonate, followed by two washes with a 1:1 by volume solution of 5% formic acid and acetonitrile. The extracted peptides were then completely dried in a SpeedVac (ThermoScientific, Waltham, MA).

2.1. Mass spectometry (MS) and protein identification

Dried peptides were resuspended in 10 μL of 0.1% trifluoroacetic acid (TFA). Each sample (6 μL) was injected *via* an autosampler and loaded onto a C18 trap column (5 μm, 300 μm i.d. X 5 mm, LC Packings) for 10 min at a flow rate of 10 L/min, 100% A. The sample was subsequently separated by a C18 reverse-phase column (3.5 μm, 75 μm X 15 cm, LC Packings) at a flow rate of 250 nL/min using an Eksigent Nano-HPLC System (Dublin, CA). The mobile phases consisted of water with 0.1% formic acid (A) and 90% acetonitrile with 0.1% formic acid (B). A 65-min linear gradient from 5 to 60% B was used. Eluted peptides were introduced into the mass spectrometer *via* a 10-μm silica tip (New Objective Inc., Ringoes, NJ) adapted to a nano-electrospray source (Thermo Fisher Scientific). The spray voltage was set at 1.2 kV and the heated capillary at 200 °C. The linear trap quadrupole (LTQ) mass spectrometer (ThermoFisher Scientific) was operated in data-dependent mode with dynamic exclusion in which one cycle of experiments consisted of a full-MS (300–2000 m/z) survey scan and five subsequent MS/MS scans of the most intense peaks. Proteins with more than 2 peptide hits in at least one of the batches were considered unique and positively identified. The reasoning for this cut-off is that 2 peptides per protein is the standard minimum number of peptide 'hits' needed to confidently state the protein has been identified by MS, and is the cutoff previously used in protemics reports from our group [16–18].

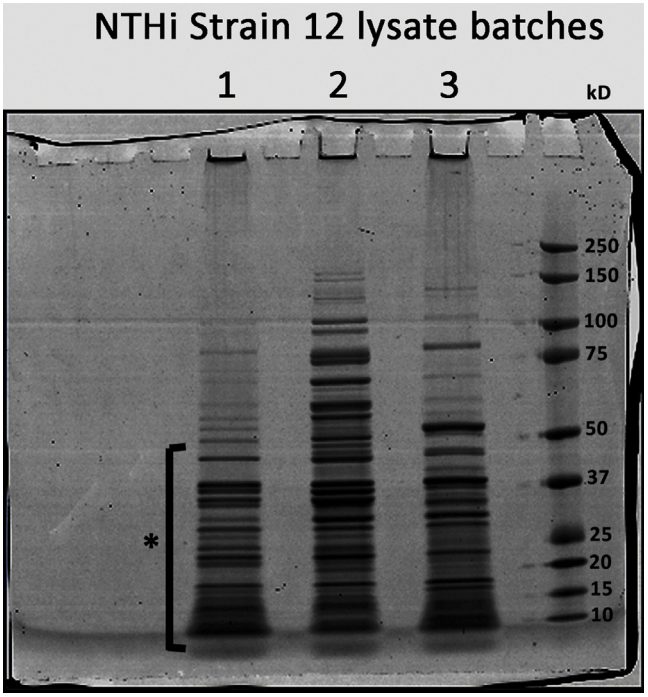

Fig. 1. *SDS–PAGE of NTHi lysate batches stained by Coomassie blue.* SDS–PAGE was performed with three lysate preparations, the gel was fixed with a mixture of acetone and methanel and then stained with Coomassie to visualize the proteins. Each individual sample gel lane was cut into 30 gel segment bins where protein bands were noted with the staining. The cuts were performed for each lysate sample prior to in gel digestion and LC-MS/MS analysis for bacterial peptide identification as described in the Methods section. Notably, a majority of the common protein bands were identified for proteins 50 kD and under in size (as denoted by the asterisk). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.2. Cell lines

The mouse middle ear epithelial cell line mMEEC was graciously provided by Dr. Jizhen Lin (University of Minnesota, Minneapolis, MN). These cells are immortalized by a temperature sensitive simian virus 40 (SV40), allowing for a proliferative phenotype at 33 °C and for differentiation at 37 °C [19]. mMEEC were maintained and passaged in full growth media (FGM) as previously described [20]. Prior to experimentation, cells were transferred to a 37 °C, 5% CO_2 humidified atmosphere to inactivate the SV-40 virus.

2.3. Transient transfection and luciferase assays

The pIgκBLuc reporter construct containing three immunoglobulin G-κ chain NF-κB binding sites upstream of the luciferase gene has been previously described [21] and was generously