Haemophilus influenzae Forms Biofilms on Airway Epithelia: Implications in Cystic Fibrosis

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Methods:

Bronchoalveolar lavage fluid (BALF) handling:

BALF samples were collected from cystic fibrosis CF patients as part of their standard care. Samples were immediately placed on ice before further processing. Quantitative cultures and cell counts with differential were performed by standardized protocols in the Clinical Pathology Laboratory at the University of Iowa. Briefly, sputum was diluted 1:1 in 2% N-acetyl cysteine in PBS, then diluted 1:5 in saline before plating on sheep blood, eosin and methylene blue, chocolate, and Burkholderia cepacia agar plates at 10^{-2} to 10^{-5} dilutions. Cell counts were performed by Wright's stain and manual counts. Remaining BALF was processed for transmission electron microscopy analysis.

BALF preparation for Scanning and Transmission Electron Microscopy:

BALF was centrifuged at 14,000 x g for 10 minutes at 4º C. For osmium-perfluorocarbon processing, pelleted samples were fixed in 1% osmium in perfluorocarbon for 2 hours at room temperature. Samples were then spun for 2 minutes at 14,000 x g and washed three times with 100% ethanol for 15 minutes. The pellets were then fixed and embedded in Epon as described below for electron microscopy sample preparation. For samples with adequate volume to additionally perform LR White (LRW) embedment, samples were fixed with 4% paraformaldehyde and 0.01% glutaraldehyde in PBS overnight at room temperature. The pellets then underwent successive 15-minute dehydrations with 25%, 50%, and 75% ethanol before a 30 minute wash with 95% ethanol. Pellets were next incubated with a 1:1 mixture of 95% ethanol:LR White medium grade acrylic (Ted Pella, Redding, CA) for one hour, then

incubated with 100% LRW for two hours, both at room temperature. The samples were then cured in a 50º C oven for 2-3 days.

Electron microscopy sample preparation:

Both SEM and TEM samples were fixed in 1% osmium tetroxide (EMS) dissolved in perfluorocarbon (Fluorinert FC-72; 3M, St. Paul, MN) for 2 hours at room temperature, then dehydrated by three washes with 100% ethanol for 15 minutes each. For SEM processing, samples were next washed in hexamethyldislazane (Ted Pella) twice for 15 minutes each, dried overnight and mounted on stubs before coating with argon, and imaged with a Hitachi S-4000 scanning electron microscope. For TEM processing, samples were next incubated with a 2:1 mixture of Epon (Ted Pella):ethanol for 60 minutes followed by a 1:2 mixture for 60 minutes, both at room temperature. The pellet was then incubated in 100% Epon for 60 minutes at room temperature before incubation at 65º C overnight. Samples were then sectioned (75 nm for TEM, 90 nm for immuno-TEM), uranyl acetate and lead citrate stained, and visualized on a Hitachi H-7000 or JEOL 1230 transmission electron microscope.

Batch biofilm:

Bacteria were stained with 1% crystal violet, rinsed in PBS, treated with ethanol, and absorbance at 570 nm measured using a VERSAmax™ microplate reader (Molecular Devices, Sunnyvale, CA) (n=6). Growth curves were performed on all NTHi isolates using an initial 1 ml of bacteria with an OD_{600} of 0.150 diluted in 24 ml of supplemented

BHI broth. Bacteria were then incubated in a shaker at 37º C and growth curves were plotted from hourly measurements of absorbance at 600 nm.

CCL20 ELISA:

Immulon 4, 96-well plates (MTX Lab Systems, Inc, Vienna, VA) were coated overnight at 4° C with 1 μg/ml anti-human MIP-3 alpha/CCL20 monoclonal antibody (MAB360, R&D Systems, Minneapolis, MN) diluted in BupH Carbonate-Bicarbonate Buffer Pack (Pierce Biotechnology, Inc, Rockford, IL). Wells were then incubated for 90 minutes at 37° C with CCL20 standard (Peprotech, Rocky Hill, NJ) or sample in dilution buffer. Dilution buffer consisted of PBS with 0.05% tween-80 and 1% bovine serum albumin. Wells were then incubated for 90 minutes at 37º C with 100 ng/ml of biotinylated affinity purified anti-human MIP-3 alpha/CCL20 polyclonal antibody (BAF360, R&D Systems) in dilution buffer. Wells were incubated with 0.1 ng/ml ImmunoPure Streptavidin, Alkaline Phosphatase Conjugated (Pierce) for 90 minutes at 37° C before development with p-nitrophenyl phosphate (pNPP) 20 mg tablets (Sigma N2765) in pNPP buffer per manufacturer's recommendations.

Supplemental Figures:

Figure E1. Controls for NTHi specific immunolabeling using mAb 6E4. (A) No significant immunolabeling of patient #3 CF BALF with no primary antibody. (B) NTHi strain 2019 was prepared with 4% paraformaldehyde + 0.1% glutaraldehyde fixation and LR White embedment, followed by 6E4 immunolabeling and small immunogold with silver enhancement using the same protocol for BALF samples. NTHi 2019 immunolabeled with a similar pattern seen in CF patient #3 BALF.

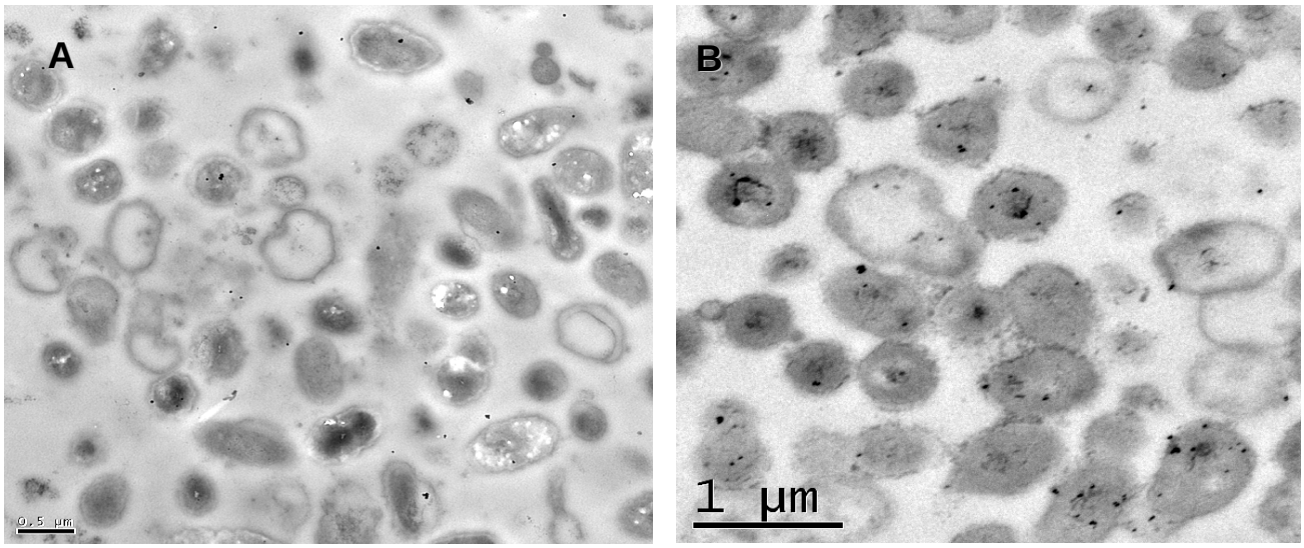

Figure E2. Pseudocolored SEMs of uninfected and infected cultures. Polarized airway epithelial cells are pseudocolored pink, NTHi dark green, biofilm/mucus light green, and Millicell membrane remains black and white. (A) Uninfected epithelia cultures show some mucus on the apical surface. (B) NTHi infected cultures display many bacteria and large NTHi biofilm structures. Scale bars indicate 10 μm.

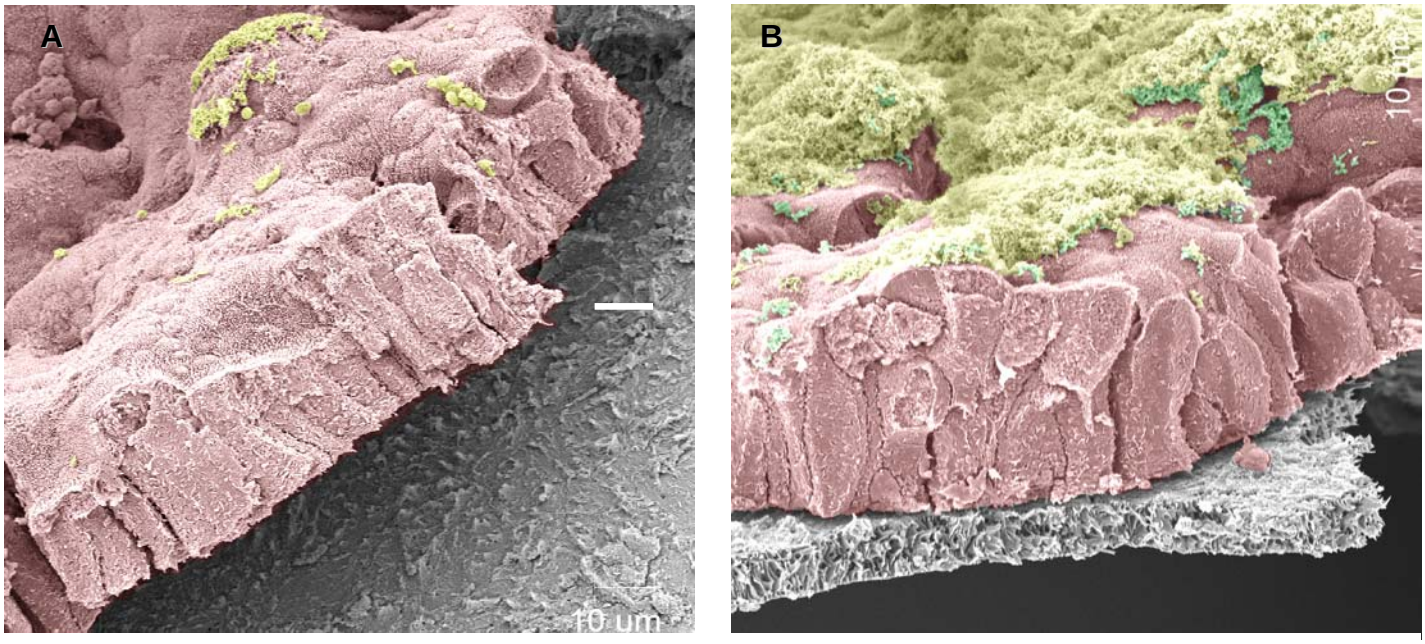

Figure E3. Variations in biofilm formation by CF NTHi isolates. Isolates (#3) and (#4) from figure 2 were co-cultured for 3 days on airway epithelia. Isolate #3 (top) formed robust biofilms while isolate #4 (bottom) formed biofilms poorly, corresponding to their relative biofilm density on plastic surfaces in figure 2. The 2500X images are magnified from the 500X images. Scale bars indicate 5 μm and 20 μm in the 2500 and 500 X images respectively.

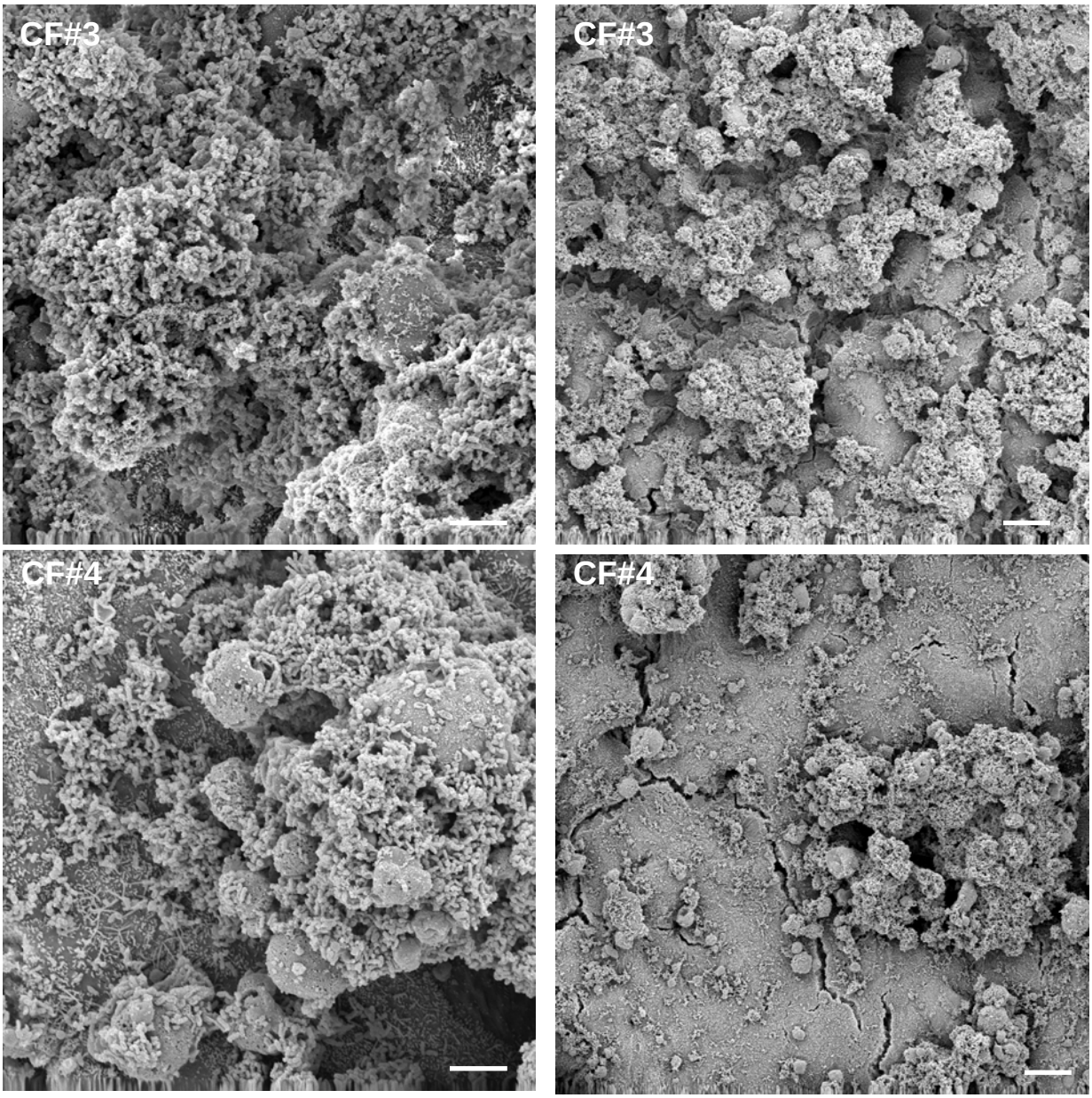

Figure E4. NTHi biofilms are adherent to airway epithelia. Representative example of adherent biofilms after 4 days of co-culture with vigorous daily washing shown at 10,000 and 2,500 X magnification. After infecting the cell cultures with NTHi, the epithelial surfaces were washed to remove non-adherent bacteria by vigorously pipetting 200 μl of PBS 5 times onto the apical surface. Apical washing was performed on a daily basis throughout the 4 days of co-culture. After 4 days of NTHi infection, bacteria were collected from the co-culture and manually counted and analyzed by SEM. Although NTHi biofilms are qualitatively smaller in appearance and had decreased recovered CFUs, large numbers of adherent bacteria in biofilm structures can be seen at day 4 that are qualitatively similar in appearance to day 2 biofilms without washing. Scale bars indicate 1 μm and 5 μm in the 10,000 and 2500 X images, respectively.

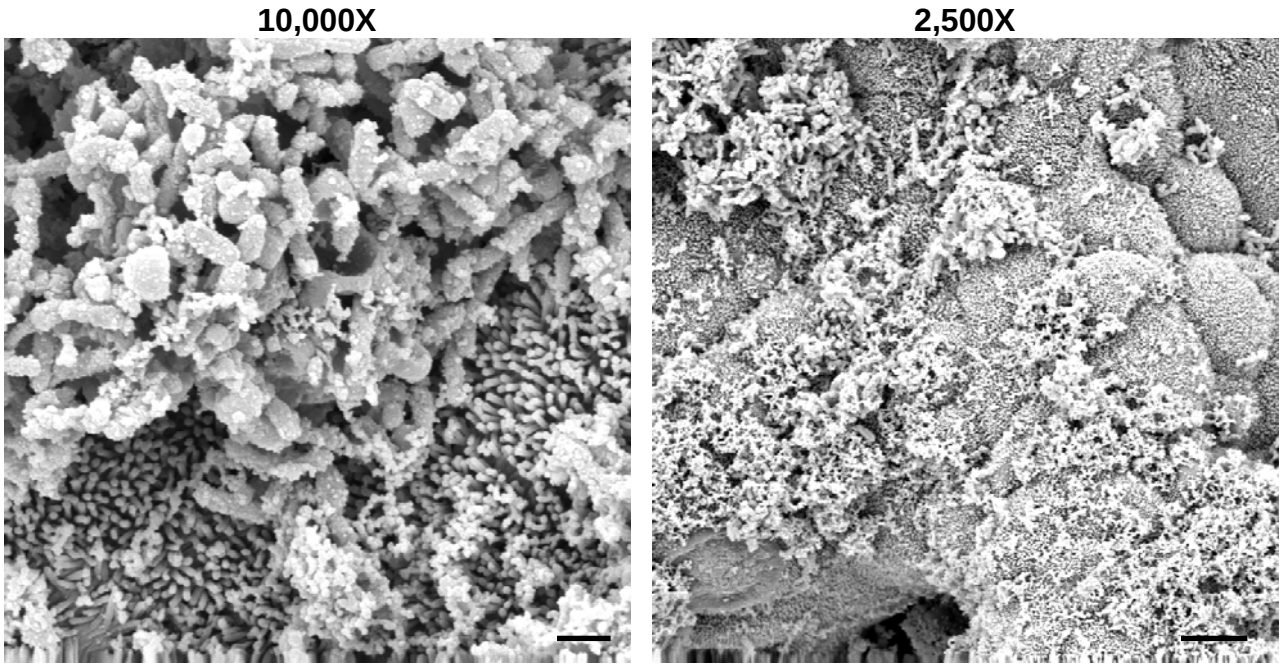

Figure E5. NTHi 2019wecA displays decreased biofilm formation. NTHi strain 2019 and 2019wecA labeled with GFP were co-cultured on Calu3 cells for 3 days. Representative 3-D rendered confocal microscopic images from one "stack" as shown from the side, top and at an angle. 600x images are shown for clarity of the images. Grid boxes indicate 10 μm.