1 ***Moraxella catarrhalis*-produced Nitric Oxide Has Dual Roles in Pathogenicity**
2 **and Clearance of Infection in Bacterial-Host Cell Co-cultures**

3
4
5
6 Brian Mocca^{1a}, Dandan Yin^{1a}, Yamei Gao², and Wei Wang^{1*}
7

8
9
10 ¹Division of Bacterial, Parasitic and Allergenic Products, ²Division of Viral Products,
11 Office of Vaccines Research and Review, Center for Biologics Evaluation and Research,
12 Food and Drug Administration, Silver Spring, MD 20993-0002
13
14
15

16 Running Head: Nitric Oxide in pathogenicity and resolution
17
18
19

20 ^aBM and DY are co-first authors
21

22 ***Corresponding Author:**
23

24 Wei Wang, Ph.D.
25 FDA/CBER/OVRR/DBPAP
26 10903 New Hampshire Avenue
27 WO75-G638, HFM428
28 Silver Spring, MD 20993-0002
29
30 Telephone: 240-402-7380
31 E-mail: wei02.wang@fda.hhs.gov
32

ABSTRACT

In humans, the free radical nitric oxide ($\text{NO}\cdot$) is a concentration-dependent multifunctional signaling or toxic molecule that modulates various physiological and pathological processes, and innate immunity against bacterial infections. Because the expression of bacterial genes encoding nitrite reductase (AniA) and $\text{NO}\cdot$ reductase (NorB) is highly upregulated in biofilms in vitro, it is important to investigate whether bacterial $\text{NO}\cdot$ -metabolism might subvert host $\text{NO}\cdot$ signaling and play pathogenic roles during infection. The *Moraxella catarrhalis* AniA and NorB directly function in production and reduction of $\text{NO}\cdot$. Using *M. catarrhalis*-human bronchial epithelial cell (HBEC) co-cultures, we recently reported AniA/nitrite-dependent cytotoxic effects on HBECs, including altered protein profiles of HBECs and induced HBEC apoptosis, suggesting bacterial nitrite reduction likely dysregulates host cell gene expression. To further clarify whether nitrite reduction-derived $\text{NO}\cdot$ or nitrite-dependent stimulation of bacterial growth was responsible for adverse effects on HBECs, we monitored bacterial nitrite reduction, levels of $\text{NO}\cdot$ in co-cultures and resulted dynamic effects on HBEC proliferation and bacterial viability. This study demonstrated that *M. catarrhalis* nitrite reduction-derived $\text{NO}\cdot$ was responsible for observed adverse effects on HBECs at mid-to-late stages of infection. More importantly, our data showed that while nitrite promoted bacterial growth and biofilm formation at early hours of infection, nitrite reduction-derived $\text{NO}\cdot$ was toxic towards *M. catarrhalis* in maturing biofilms, suggesting nitrite reduction-derived $\text{NO}\cdot$ might be a possible dualistic mechanism by which *M. catarrhalis* promotes diseases and spontaneous resolutions.

INTRODUCTION

The free radical nitric oxide ($\text{NO}\cdot$) is a universal signaling molecule with concentration-dependent diverse functions in the mammalian system [reviewed in [1; 2]]. Mammalian cells utilize three types of $\text{NO}\cdot$ synthases (NOSs) to produce $\text{NO}\cdot$ by using *L*-arginine as substrate (Fig. 1A). Excess $\text{NO}\cdot$ can be scavenged via various means [1], for example, through hemoglobin and the common oxygen-involved conversions of $\text{NO}\cdot$ to nitrite/nitrate (Fig. 1A). $\text{NO}\cdot$ is an important modulator of the inflammatory response, and some microbial pathogens can be eliminated by inducible NOS-generated $\text{NO}\cdot$ (reviewed in [3; 4]). On the other hand, various truncated denitrification pathways (Fig. 1A) broadly exist in aerobic and anaerobic bacteria with $\text{NO}\cdot$ as an intermediate product. The bacterial nitrite reductases have been well studied with regard to their function in cellular respiration and bacterial growth under anaerobic or oxygen-limited conditions (reviewed in [5; 6]), and only recently for their possible roles in virulence [7-10].

Recent studies reported that some bacteria, including *M. catarrhalis*, express a nitrite/ $\text{NO}\cdot$ -sensing regulator, NsrR [11-16]. Upon sensing $\text{NO}\cdot$, likely through interactions between $\text{NO}\cdot$ and the iron-sulfur cluster of NsrR [17; 18], the repressor dissociates from the upstream DNA sequences of controlled genes to allow increased expressions. It was shown that bacterial NsrR-regulon [12-14; 19; 20] included genes encoding nitrite reductase NirK (also known as AniA, Fig. 1A), $\text{NO}\cdot$ reductase (NorB) and a flavohemoglobin protein HMP (a denitrosylase [21]). A bioinformatics analysis predicted that the *nsrR* gene and conserved NsrR binding sites upstream of $\text{NO}\cdot$ -metabolism genes exist widely in different microorganisms [22],

suggesting that NO $\cdot$ might be a common signaling molecule regulating the bacterial NO $\cdot$ -production and detoxification via NO $\cdot$ -sensing regulators.

Our main research interest is to understand how a respiratory tract disease-causing bacterium *M. catarrhalis* might utilize NO $\cdot$ to modulate host immune responses, to dysregulate host cell gene expression, and to control bacterial growth in bacterial-human airway epithelial cell co-cultures. This aerobic pathogen is one of the top three common bacterial causes of acute otitis media (AOM) and AOM with effusion (OME) in infants and young children (reviewed in [23]). It was reported that *M. catarrhalis* forms biofilms on mucosa of infected middle-ear [24]. Paradoxically, as disease progresses from OME to AOM with tympanic membrane perforation (AOMwiP), *M. catarrhalis* colonization declines significantly [25; 26]. Recent studies have shown that the expression of *aniA* and *norB* is highly upregulated in *M. catarrhalis* ATCC43617 biofilms formed on air-exposed tubing surfaces in a continuous-flow biofilm system [27] and in *Neisseria gonorrhoeae* biofilms in vitro [28]. The *M. catarrhalis* nitrite reductase is a homolog of a nitrite reductase (AniA) in *Neisseria* species [29] which was initially identified as a major anaerobically induced outer-membrane protein [30; 31]. Under aerobic growth conditions, the *M. catarrhalis* NorB is essential for this bacterium to detoxify nitrite reduction-derived NO $\cdot$ [14; 32]. Interestingly, the elements necessary for *M. catarrhalis* nitrite reduction and NO $\cdot$ production seemed to exist in vivo because studies have shown that *M. catarrhalis* likely expresses AniA in vivo [33], forms biofilms on the middle-ear mucosa of children experiencing OME [24]; and more importantly, pathophysiological levels of combined nitrate/nitrite in middle-ear effusions range from 0.5 to 1 mM [34]. Given that NO $\cdot$ is highly diffusible and has a short chemical half-life, we hypothesized that a portion of the *M. catarrhalis* nitrite reduction-

derived NO $\cdot$ might diffuse into the proximate environment and adversely affect adjacent host cells. To test this hypothesis, we recently optimized an in vitro bacteria-HBEC co-culturing model and revealed a nitrite- and AniA-dependent stimulation of host cells secretion of the tumor necrosis factor alpha (TNF- α) and interleukin 1 alpha (IL-1 α), alteration of HBEC expression of apoptotic protein profile and induction of HBEC apoptosis [10].

Here we further clarified whether nitrite reduction-derived NO $\cdot$ is responsible for the nitrite and AniA-dependent adverse effects on host cells, by measuring both nitrite reduction and levels of diffused NO $\cdot$ in co-cultures. This study focused on the dynamic correlation between bacterium-derived NO $\cdot$ and proliferation of host and growth of bacteria, more importantly, the bacterial biofilm physiology.

MATERIALS AND METHODS

Bacterial strains and *M. catarrhalis*-HBEC co-culture conditions. Table 1 lists the *M. catarrhalis* strains used in this study. Brain heart infusion (BHI, BD Bacto™) agar plates were supplemented with antibiotics kanamycin (15 µg/ml) or spectinomycin (15 µg/ml) as appropriate, and were used to grow *M. catarrhalis* strains at 37°C in a CO₂ (5% CO₂ and 95% air) incubator. The *M. catarrhalis* viability was monitored by determination of colony-forming units (CFU). It is important to note that, in co-cultures, CFU values represented *M. catarrhalis* attached to HBECs because unattached bacteria were removed by extensive washes, whereas in pure bacterial cultures (mono-cultures), CFU values reflected all *M. catarrhalis* in a culture owing to *M. catarrhalis* cell-aggregates did not persistently attach to the surface of tissue culture plates.

The culture conditions for HBECs and bacterial-HBEC co-cultures were as described [10]. *M. catarrhalis*-HBEC co-cultures initially comprised approximately $(9 \pm 1) \times 10^6$ CFU of *M. catarrhalis* and 2×10^5 HBECs so that the initial multiplicity of infection was ~50. Co-cultures were incubated at 37°C in a CO₂ incubator for a period of 24hr. The medium MEM* [10] was supplemented with nitrite (0 or 1 mM) or purified human hemoglobin (Hgb, 1mg/ml, MP Biomedicals, LLC, France) as appropriate.

Determination of the expression of *M. catarrhalis* *aniA* and *norB* genes. *M. catarrhalis* O35E total RNA was isolated from 6-hr cultures in MEM* with agitation (planktonic culture, PT) or 6-, 10- and 24-hr cultures without agitation (biofilm-like cell-aggregates

formation, BF) using a Mini RNeasy kit (Qiagen, Valencia, CA). RNA samples were used to detect expression of *aniA* and *norB* by *M. catarrhalis* cell-aggregates and by planktonically grown cells using a quantitative real-time RT-PCR analysis (qRT-PCR) and the ORF1234 was used as an endogenous control as described previously [27]. A previous DNA microarray analysis determined that the expression of ORF 1234 was unchanged in planktonic and biofilm growth conditions [27].

Monitoring the *M. catarrhalis* biofilm formation in co-cultures using Scanning Electron Microscopy (SEM) analysis. A poly-L-lysine (PLL)-coated glass coverslip (Neuvitro, Germany) was placed in a well of a tissue-culture plate before seeding HBECs. HBECs were seeded and infected with *M. catarrhalis* as described [10]. At each post-infection time-point, medium was removed from co-cultures and samples were washed three times with phosphate-buffered (0.1 M) saline (PBS) , pH 7.4, to remove unattached *M. catarrhalis*. The samples were fixed with 2.5% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in PBS at room temperature overnight, followed by two washes with phosphate-buffered saline at room temperature. The PLL-coated coverslips were removed from tissue culture plates and processed as followings: (1) post-fixed in 1% osmium tetroxide solution (Electron Microscopy Sciences) at room temperature for 1 hr, followed by three washes in distilled water; (2) dehydrated in a graded series of ethanol (50%, 70%, 95%, 100%); (3) dried using a Critical Point Dryer auto program (Leica EM CPD 300); and (4) mounted to Hitachi S-450 aluminum stubs (Electron Microscopy Sciences) and sputter coated with gold using a DESK IV® sputter system (Denton Vacuum, NJ) prior to viewing and imaging using a JSM-6390LV Scanning Electron Microscope (JEOL, Tokyo, Japan).

Detection of *M. catarrhalis* nitrite reduction. Nitrite reduction was measured by detection of remaining nitrite levels at different time-points using a Griess reaction-based [14; 32] assay. Briefly, at each time-point a 100- μ l aliquot of culture medium was sampled and analyzed using the Nitrate/Nitrite Colorimetric Assay kit (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer's protocol.

Determination of diffused NO $\cdot$ from *M. catarrhalis* nitrite reduction in co-cultures. In a proof-of-concept study to demonstrate that NO $\cdot$ was indeed produced by the *M. catarrhalis* nitrite reduction in co-cultures, the levels of diffused NO $\cdot$ in co-cultures was assessed directly using a TBR 4100 Free Radical Analyzer (World Precision Instruments, Inc. WPI, Sarasota, FL) and L-shaped NO $\cdot$ microsenors (ISO-NOPF-200-L10, WPI) following the manufactures protocol. Briefly, NO $\cdot$ microsenors were polarized overnight in 0.1M CuCl₂ solution and calibrated using NO-releasing chemical S-nitroso-N-acetyl-D, L-penicillamine (SNAP, an NO $\cdot$ -releasing chemical, Sigma) to compose a liner calibration curve of signal outputs (in pA) against concentrations of SNAP (in nM). The NO $\cdot$ microsensor was then immersed in a co-culture proximally close to bottom of tissue culture plate. The manufacturer's software (LabScribe, v3) was programmed to record signal outputs every minute for up to 24 hr and to display NO $\cdot$ concentration (in nM). The difference of NO $\cdot$ concentrations in co-cultures of HBECs with O35E wild-type and O35E *aniA* mutant in MEM* supplemented with 1mM of nitrite was depicted as the levels of diffused NO $\cdot$ from *M. catarrhalis* AniA-mediated nitrite reduction. Alternatively, the difference of NO $\cdot$ concentrations in co-cultures of HBECs-O35E wild-type in MEM* with or without 1mM of nitrite was detected.

Monitoring HBEC proliferation in bacteria-HBEC co-cultures. To evaluate dynamic effects of bacterial nitrite reduction-derived NO $\cdot$ on the host cell proliferation, the confluence of HBEC monolayer in each co-culture was monitored using an IncuCyte (ESSEN BioScience, MI) live-cell imaging system (10 $\times$ objective) at 1-hr intervals over a period of 24 hr. At each time-point post-infection, the IncuCyte took micrographic images from four fields-of-view and determined a mean confluence which was used to plot a HBEC growth curve by the manufacturer's software. At the end of 24-hr co-culturing, the co-cultures were stained and the morphology of a HBEC monolayer was examined using an Olympus IX51 microscope (Objective 20 $\times$) system as described [10].

Transcriptomic study of host-cell production of antibacterial cytokines and chemokines. To exam effects of bacterium-derived NO $\cdot$ on host HBEC expression of antibacterial cytokines and chemokines, expression profiles of HBEC cultured alone, or co-cultured with O35E wild-type or *aniA* mutant strains in medium containing 1 mM nitrite were determined. First, RNA samples were isolated from HBECs in culture or co-cultures by using QIAshredder spin column (Qiagen) and RNeasy Mini kit (Qiagen). RNA samples were further digested using the MessageClean kit (GenHunter, Nashville, TN) to remove DNA contamination. The absence of bacterial RNA contamination was confirmed by a real-time RT-PCR analysis using oligonucleotide primer pairs for *M. catarrhalis* ORF1234 and *aniA* as described [27].

Then, the above HBEC RNA samples were used as templates to synthesize cDNA samples using the SABiosciences RT² First Strand kit (Qiagen). Each cDNA sample was mixed with the SABiosciences RT² qPCR Master Mixture to prepare a PCR cocktail which was then loaded onto a RT²Profiler™ PCR Array (Human Inflammatory Cytokines and Receptors 96-well PCR Array, SABiosciences, a Qiagen Company). Quantitative real-time PCR analysis ($\Delta\Delta C_T$) was then performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The expression of a glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to normalize the array. The fold-change in expression of antibacterial cytokines and chemokines in HBECs infected with O35E cells relative to in uninfected HBECs were analyzed using the manufacturer's Relative Quantification (RQ) Study Software (version 1.2.1).

In addition, the levels of chemokines CCL3 and CXCL2 produced by HBECs in co-cultures were measured using a Human CCL3 ELISA kit (R&D *SYSTEMS*®) and a Human CXCL2 ELISA kit (KA2189, version 13, Abnova, Inc).

RESULTS

***M. catarrhalis* express higher levels of *aniA* and *norB* in cell-aggregates and formed biofilms on the surface of HBECs.** A previous study reported that the expression of *M. catarrhalis aniA* and *norB* was highly upregulated in ATCC 43617 biofilms formed on a surface of air-exposed tubing in a continuous flow biofilm system [27]. Because we and other groups have not been able to construct genetic mutants in the ATCC 43617 strain, we used *M. catarrhalis* O35E strains for this study and verified the expression of O35E *aniA* and *norB* under tissue culture condition. When cultured without agitation, *M. catarrhalis* O35E cells formed cell-aggregates (microbial communities). Similar to the *M. catarrhalis* ATCC 43617 biofilms [27], the expression of O35E *aniA* and *norB* genes was highly upregulated in the 6-, 10- and 24-hr *M. catarrhalis* O35E cell-aggregates (Fig. 1B, grey and shaded bars for *aniA* and *norB*), whereas the expression of ORF1020 was increased slightly yet significantly at early time-point but insignificantly at later time-points (*t*-tests, paired two-sample) under both growth conditions (Fig. 1B, grey and shaded bars for ORF1020) by using the ORF1234 as an endogenous control for the real-time RT-PCR analysis.

It was noted that the O35E cell-aggregates did not attach to a surface of plastic tissue culture plate in pure bacterial cultures (mono-cultures), as reported previously that O35E was a biofilm-negative strain based on a crystal violet staining assay in vitro [35]. However, we previously noted that the vast majority of *M. catarrhalis* O35E wild-type strain tenaciously attached to the surface of HBECs in MEM*: approximately 10^7 CFU attached to HBEC vs. approximately 10^5 CFU in medium suspension [10]. To exam whether HBEC-attached *M.*

catarrhalis cell-aggregates are biofilms in co-cultures, we performed SEM analysis which is a standard method of visualizing fine structures of bacterial biofilms. As described in the Materials and Methods section, HBEC-*M. catarrhalis* co-culturing samples were washed extensively to remove unattached bacteria. Therefore, cell-aggregates in the SEM micrographic images (Fig. 1C) were *M. catarrhalis* O35E cells that attached to the surface of HBECs in co-cultures. SEM images revealed some typical features of biofilms in *M. catarrhalis* cell-aggregates at a late time-point post infection, such as water-channels (Fig. 1C, 24hr, pointed with open arrows). Importantly, SEM images showed that *M. catarrhalis* cell-aggregates possibly attached to or entangled with HBEC surface microspike-like protrusions with various lengths (Fig. 1C, pointed with white arrows), revealing possible important host factors that support formation of biofilm by this bacterium. Based on the basic definition of biofilms as surface attached microbial communities that present differential gene expression profiles [36; 37], data from this study indicated that the HBEC-surface attached *M. catarrhalis* O35E cell-aggregates were biofilms.

Nitrite enhances *M. catarrhalis* biofilm development at an early stage of infection but exerts nitrite reduction-dependent toxicity to HBECs at mid-to-late stages of co-culturing. Compared to 6-hr biofilms in MEM* (Fig. 1C, 6hr), biofilms of O35E wild-type (Fig. 2, top panel, column of HBEC-O35E wt) and *aniA* mutant (Fig. 2, top panel, column of HBEC-O35E *aniA*) strains were visually better developed in MEM* supplemented with 1mM of nitrite. Again, *M. catarrhalis* biofilms were entangled with microspikes-like protrusions and attached to the surface of HBECs but not to a plastic tissue culture plate, suggesting that host cell surface protrusions might be crucial elements for *M. catarrhalis* biofilm formation in co-cultures.

At 24 hr post infection in MEM* supplemented with 1mM nitrite, HBEC monolayer was severely damaged in co-cultures with *M. catarrhalis* O35E wild-type, and O35E wild-type biofilms (Fig. 2, bottom panel, HBEC-O35E wt, pointed with open arrow) mainly attached to apoptotic bodies or apoptotic HBECs (Fig. 2, bottom panel, HBEC-O35E wt, pointed with white arrows), whereas O35E *aniA* mutant biofilms still attached to seemingly healthy HBECs (Fig. 2, bottom panel, HBEC-O35E *aniA*).

The *M. catarrhalis* biofilm-induced nitrite reduction and NO $\cdot$ production in bacteria-HBEC co-cultures. First, we monitored nitrite reduction in cultures of HBEC alone or in bacteria-HBEC co-cultures in MEM* supplemented with 1mM of nitrite. Nitrite reduction was insignificant in all co-cultures at early time points (Fig. 3A, all bars at 0 and 4 hr). A significant nitrite reduction occurred only in co-cultures with the O35E wild-type strain at 5–8 hr post-infection (Fig. 3A, black bars at 5–8 hr), which functionally corroborated the biofilm-upregulated expression of *aniA* (Fig. 1B). These results demonstrated that nitrite was reduced predominantly by the *M. catarrhalis* AniA at mid-to-late stages of infection in vitro.

Furthermore, in a proof-of-concept study to demonstrate that NO $\cdot$ was indeed produced from the *M. catarrhalis* nitrite reduction in co-cultures, we used a NO $\cdot$ -specific microsensor to detect diffused NO $\cdot$ in medium suspensions of co-cultures. The total diffused NO $\cdot$ in co-cultures of HBEC-O35E wild-type or HBEC-O35E *aniA* mutant may consist of diffused NO $\cdot$ that was produced by bacterial nitrite reduction and by HBEC NOS (Fig. 3B). In the presence of nitrite, the difference of NO $\cdot$ levels in co-cultures of HBEC-wt and HBEC-O35E *aniA* mutant represented a net increase of diffused NO $\cdot$ from bacterial nitrite reduction (Fig. 3C), which

indicated that high levels of NO $\cdot$ were produced by *M. catarrhalis* nitrite reduction at mid-to-late stages of infection in vitro.

***M. catarrhalis* nitrite reduction-derived NO $\cdot$ inhibits HBEC proliferation in mid-to-late stages of infection in vitro.** To study the dynamic effects of *M. catarrhalis* nitrite reduction-derived NO $\cdot$ on HBECs, we monitored HBEC proliferation using an IncuCyte live-cell imaging system (10 $\times$ objective). HBECs seemed to grow well in co-cultures with different O35E strains in MEM* (Fig. 4A, top panel) or with AniA-negative strains in MEM* with 1 mM nitrite (Fig. 4A, bottom panel for *aniA* and *aniA*(pWW115)). In MEM* supplemented with 1 mM nitrite, HBECs grew well initially (about 0–6 hr post-infection) with O35E wild-type strain (Fig. 4A, bottom panel for wt). As level of nitrite reduction-derived NO $\cdot$ increased (Fig. 3), however, host-cell proliferation was arrested and then declined during the mid-to-late stages of infection in vitro (Fig. 4A, bottom panel for wt; arrow indicates when host-cell proliferation peaked). HBEC proliferation was arrested sooner when co-cultured with the O35E *norB* mutant or a genetically complemented O35E *aniA*(pWW161) strain (Fig. 4A, bottom panel for *norB* or *aniA*(pWW161)) compared with HBECs co-cultured with O35E wild type. The increased toxicity towards HBECs might be due to higher levels of NO $\cdot$ because the O35E *norB* mutant is unable to detoxify NO $\cdot$ [32] and the complemented O35E *aniA*(pWW161) strain [10] expresses a high level of AniA from a plasmid-borne multi-copy *aniA* gene. Interestingly, similar to its parental wild-type strain, the O35E *nsrR* mutant (which generates a high flux of NO $\cdot$ from nitrite owing to increased expression of AniA and NorB [14]) also inhibited HBEC proliferation (Fig. 4A, bottom panel for *nsrR*).

Furthermore, the addition of a natural NO $\cdot$ scavenging agent (such as purified human Hgb, 1 mg/ml) was able to prevent above observed cytotoxicity. *M. catarrhalis* nitrite reduction damaged the integrity of HBECs monolayer resulting Swiss Cheese-like disrupted host cell monolayer at 24hr PI (Fig. 4B, MEM* + nitrite). Based on observations from SEM (Fig. 2, bottom panel, HBEC-O35E wt) and previous live-cell confocal microscopic image studies [10], the dark and round-up HBECs mostly on the edge of monolayers (for example, Fig. 4B, MEM* + nitrite, arrows) were apoptotic bodies or apoptotic HBECs covered with O35E wild-type biofilms as seen above (Fig. 2, bottom panel, HBEC-O35E wt, 24hr). The addition of NO $\cdot$ -scavenger Hgb not only protected the integrity of HBEC monolayer (Fig. 4B, MEM* + nitrite + Hgb), but also seemed to promote bacterial growth because much bigger cell-aggregates were seen (for example, Fig. 4B, MEM* + nitrite +Hgb, arrows). This data supported that *M. catarrhalis* nitrite reduction-derived NO $\cdot$ was responsible for nitrite-dependent cytotoxicity to host cells. Importantly, this study revealed a dynamic correlation between increased level of nitrite reduction-derived NO $\cdot$ (Fig. 3C) and the infection stages at which HBEC proliferation peaked (Fig. 4A, bottom panel, arrows). The stages at which HBEC growth peaked might indicate a transition of bacterial-host cell interaction from commensalism to pathogenicity in vitro.

Nitrite and *M. catarrhalis* nitrite reduction-derived NO $\cdot$ have growth phase-dependent effects on bacterial viability in co-cultures. To address an important critique and question of whether nitrite-enhanced bacterial growth or nitrite reduction-derived NO $\cdot$ is responsible for the observed cytotoxicity towards HBEC, we monitored bacterial viability at the beginning of co-culture and at post-infection time-points that encompass the stage when HBEC

proliferation peaked. Initially, similar amounts of O35E wild-type or O35E *aniA* mutant strains were attached to HBECs in MEM* with or without 1 mM nitrite (Fig. 5A, black and gray bars at 0 hr). At 6 hr post-infection, the CFU of both O35E wild-type and *aniA* mutant strains were significantly higher in co-cultures with nitrite (Fig. 5A, black and gray “+” bars at 6 hr) than in co-cultures without nitrite (Fig. 5A, black and gray “-” bars at 6 hr). This result indicated that nitrite promoted the growth of *M. catarrhalis* in an AniA-independent manner during an early stage of bacterial infection in vitro, which validated the data of a nitrite-dependent and AniA-independent promotion of *M. catarrhalis* biofilm development during early hours of infection (Fig. 2, top panel). Unexpectedly yet importantly, there was a nitrite reduction-dependent antibacterial activity during the mid-to-late stages of infection in vitro, as evidenced by a nitrite-dependent significant decrease in the viability of O35E wild-type strain (Fig. 5A, black + bars at 8, 10 and 24hr) whereas the viability of O35E *aniA* mutant was not reduced by nitrite (Fig. 5A, gray + bars at 8, 10 and 24hr). Although at late stages of co-cultures, the O35E *aniA* mutant viability was much higher than that of O35E wild-type strain in MEM* supplemented with nitrite (Fig. 5A, gray and black + bars, at 8-24hr), the O35E *aniA* mutant did not exert apparent adverse effects on HBECs but O35E wild-type strain did (Figs 2 and 4A, bottom panel), demonstrating that increased levels of nitrite reduction-derived NO $\cdot$ was responsible for nitrite-derived toxicity to HBECs.

Furthermore, the addition of Hgb blocked nitrite reduction-dependent toxicity towards biofilm-grown O35E wild-type strain at 24 hr post-infection (Fig. 5B, black + + bar). Overall, these data indicated that the observed nitrite-derived cytotoxicity towards HBECs and *M. catarrhalis* in biofilms was associated with the *M. catarrhalis* nitrite reduction-derived NO $\cdot$.

***M. catarrhalis*-produced NO $\cdot$ stimulates production of pro-inflammatory cytokines and antibacterial chemokines in HBECs.** Initially, the nitrite reduction-dependent toxicity towards biofilm-grown *M. catarrhalis* wild-type strain were unexpected because previous studies showed that a higher level of nitrite (5 mM) has no apparent adverse effects on the aerobic growth of O35E wild-type strain due to an innate functional NorB to protect *M. catarrhalis* from nitrite-derived cytotoxicity [14; 32]. An intuitive hypothesis was that *M. catarrhalis*-generated NO $\cdot$ might induce host-cell expression of antibacterial cytokines and chemokines, as a previous report showed a suite of human chemokines (e.g. CCL1, CCL20, CXCL1, CXCL2, CXCL3) with potent bactericidal activities against gram negative bacteria (including *M. catarrhalis*) in vitro [38]. Therefore, we performed transcriptomic studies in co-cultures in MEM* containing 1 mM nitrite to exam the expression of human cytokines and antibacterial chemokines by HBECs that were cultured alone (Fig. 6A, open bars), co-cultured with O35E wild-type (Fig. 6A, black bars) or *aniA* mutant strains (Fig. 6A, grey bars) for 24 hr. The upregulated expression of the host-cell chemokines CCL4 and CXCL14 seemed to be AniA-independent but seemed to be further enhanced by *M. catarrhalis* nitrite reduction-derived NO $\cdot$ (Fig. 6A, black and grey bars for CCL4 and CXCL14). Interestingly, the expression of an array of cytokines and antibacterial chemokines was significantly upregulated in co-cultures with O35E wild-type strain when NO $\cdot$ was produced (Fig. 6A, black bars of IL-1 α , IL-1 β , IL8, IL17, TNF- α , CCL1, CCL3, CCL20, CXCL1, CXCL2 and CXCL3). This data confirmed a previous proteomic study that increased secretion of TNF- α , IL-1 α , and IL8 by HBECs is associated with *M. catarrhalis* nitrite reduction [10]. In addition, NO $\cdot$ -stimulated secretion of CCL3 (Fig. 6B, black column) and CXCL2 (Fig. 6C, black column) was confirmed by ELISA-base study results.

382
383 However, it was noted that the concentration of CXCL2 secreted by host cells in co-
384 cultures was at most 60 pg/ml (Fig. 6C, black bars), which was significantly lower than the
385 concentrations (at $\mu\text{g per ml}$ levels) used in bactericidal assay [38]. We also tested if any
386 possible toxic molecules might be released from dead cells by using medium containing 50% or
387 100% of spent media from HBEC-O35E wild-type co-cultures to culture HBECs or HBEC-
388 O35E *aniA* cells. No apparent toxic effects towards either host or bacteria were observed (data
389 not shown). Together, these data suggested that *M. catarrhalis* nitrite reduction-derived $\text{NO}\cdot$,
390 instead of $\text{NO}\cdot$ -induced antibacterial chemokines, might be the major bactericidal factors in co-
391 cultures.

392
393 **Nitrite reduction-derived $\text{NO}\cdot$ is toxic to *M. catarrhalis* biofilm-grown cells in co-**
394 **cultures.** To verify that upregulated production of $\text{NO}\cdot$ in *M. catarrhalis* biofilms might be toxic
395 to *M. catarrhalis* in biofilms, we examined *M. catarrhalis* nitrite reduction and bacterial viability
396 in pure bacterial cultures. Similar to in co-cultures, after a short period of incubation without
397 agitation (cell-aggregates formation), nitrite was reduced in O35E wild-type mono-cultures (Fig.
398 7A, O35E wt), whereas nitrite was not reduced in mono-cultures of the *aniA* mutant strain (Fig.
399 7A, O35E *aniA*).

400
401 In aerobic cultures (with agitation), similar amount of O35E wild-type strain ($\sim 10^7$ CFU)
402 were recovered after a 24-hr incubation in MEM* with or without 1 mM nitrite, corroborating
403 previous reports that the aerobic growth of O35E wild type is not affected by nitrite [14; 32].
404 However, this was not the case when O35E cells were cultured without agitation. Similar

amounts of O35E wild-type and *aniA* mutant strains were used for inoculation (Fig. 7B, black and gray bars at 0 hr). Unlike what was observed in co-cultures, there was no nitrite-dependent stimulation of bacterial growth at the 6-hr time-point (Fig. 7B, black and gray “+” bars at 6 hr). Importantly, similar to in co-cultures, nitrite reduction–dependent cytotoxicity was observed in the mono-culture of O35E wild-type strain at the 8- and 10-hr time-points (Fig. 7B, black “+” bars at 8 and 10 hr). These results corroborated that nitrite reduction-derived NO $\cdot$ was detrimental to *M. catarrhalis* grown in biofilms in co-cultures. It remains to be fully elucidated, however, why *M. catarrhalis* NorB was unable to protect against NO $\cdot$ toxicity in biofilms even though the expression of *norB* was also upregulated in biofilms.

Interestingly, we noted that the viability of *M. catarrhalis* biofilms formed on HBEC monolayers in co-cultures (Fig. 7C, co bars) was two orders of magnitude greater than the viability in mono-cultures (Fig. 7C, mono bars) in MEM* after 24hr incubation, indicating that nutrients in biofilms (especially in mono-cultures) might be depleted. This beneficial commensalism was independent of AniA because the viability of the O35E *aniA* mutant (Fig. 7C, gray bars) was virtually the same as that of wild type (Fig. 7C, black bars) under identical growth conditions.

DISCUSSION

NO $\cdot$ is a reactive and highly diffusible radical with concentration-dependent diverse functions in modulating physiological and pathophysiological processes in mammalian cells (reviewed in [1; 2; 39]). Upon microbial infection, higher levels of NO $\cdot$ can be produced by a mammalian inducible NOS to kill pathogens (reviewed in [40]). Recent studies indicated that bacterial pathogens might utilize NO $\cdot$ -sensing regulators, including NsrR [11; 13-15; 17] and FNR [41] or through a two-component system [12; 42], to upregulate the expression of NO $\cdot$ -detoxification proteins such as HMP and NorB. The reports that *aniA* and *norB* are highly upregulated in *M. catarrhalis* biofilms [27] and in gonococcal biofilms [28] raised our interest in exploring possible roles for bacterium-generated NO $\cdot$ and biofilms in pathogenesis. Using a bacteria-HBEC co-culture model, our recent [10] and the present studies demonstrate that *M. catarrhalis* nitrite reduction-derived NO $\cdot$ stimulates host-cell production of TNF- α and IL-1 α , which are two potent activators of NF- κ B and endogenous pyrogens [43; 44], suggesting that *M. catarrhalis*-generated NO $\cdot$ likely interferes with host-cell NO $\cdot$ signaling. Other recent studies have indicated that NO $\cdot$ detoxification by bacterial enzymes might also interfere with host NO $\cdot$ signaling, as illustrated by the *Neisseria meningitidis* NorB-mediated modification of host-cell cytokine profiles [45] and the bacterial NO $\cdot$ -detoxification protein affects host-cell S-nitrosothiol formation [46]. NO $\cdot$ -induced alteration of cytokines and chemokines profile (Fig.6A) might play important roles in host innate immunity, such as in neutrophil recruitment and in clearance of bacterial infection in animals [47; 48]. Overall, these studies suggest that NO $\cdot$ signaling is an integral aspect of cellular functions across the kingdoms of life and modulates bacteria-host cell interactions.

Previous results have shown that direct contact between bacteria and host cells is required for maximum NO $\cdot$ cytotoxicity [10], which might be due to a possible NO $\cdot$ concentration gradient generated by *M. catarrhalis* biofilms: as NO $\cdot$ diffuses, the NO $\cdot$ concentration and concentration-dependent NO $\cdot$ toxicity decrease as well. The majority NO $\cdot$ -generated by AniA-mediated nitrite reduction is further reduced by NorB [32], or consumed by various biochemical or cellular processes (as reviewed in [1]), and only a portion of bacterium-generated NO $\cdot$ might be diffused into medium. Similarly, a portion of host cell generated NO $\cdot$ might also diffuse into co-culturing medium. Because co-cultures were incubated without agitation, the detected levels of NO $\cdot$ (diffused into medium, Fig. 3B) might be lower than levels of NO $\cdot$ inside or on surface of NO $\cdot$ -producing cells. In the presence of nitrite, the difference of NO $\cdot$ levels between HBEC-wt co-culture and HBEC-*aniA* mutant co-culture denoted a net increase of diffused NO $\cdot$ from bacterial nitrite reduction (Fig. 3C). The diffused NO $\cdot$ from *M. catarrhalis* nitrite reduction (Fig. 3C) at different stages of co-cultures might affect different basic cellular processes of human host cells, based on concentration-dependent NO $\cdot$ functions (reviewed in [1]).

Information on *M. catarrhalis* biofilm formation and dispersal is limited. The *M. catarrhalis* O35E strain was determined as a biofilm-negative strain using a crystal violet staining assay [35] because O35E cells do not persistently attach to surface of plastic tissue culture plates. On the other hand, several surface-exposed *M. catarrhalis* outer-membrane proteins, including Hag, UspA1, and type IV pili, have been shown to facilitate *M. catarrhalis* biofilm formation and adherence to human epithelial cells in vitro [49-51]. A recent study showed that the majority (over 99.99%) of *M. catarrhalis* grown attached to HBEC in co-

cultures [10]. This study indicated that the nonmotile bacterium *M. catarrhalis* seems to lack a mechanism for dislodging itself from biofilms, as evidenced by the robust biofilm development during the 6- to 24-hr co-culture period (Fig. 1C, 6 and 24 hr) while bacterial viability decreased from $(2.74 \pm 0.99) \times 10^7$ CFU at 6 hr to $(1.55 \pm 0.57) \times 10^7$ CFU at 24 hr (Fig. 5A, black – bar at 6 and 24hr). Furthermore, SEM data revealed that microspike-like protrusions on the surface of HBECs might be important elements for *M. catarrhalis* biofilm formation, and that nitrite reduction-derived $\text{NO}\cdot$ seemed to affect the morphology of bacterial biofilms. While chemically-generated $\text{NO}\cdot$ function in biofilm dispersal has been reported in other bacteria ([52; 53] and reviewed in [54]), our study suggested that *M. catarrhalis* might generate cytotoxic levels of $\text{NO}\cdot$ from nitrite reduction to disperse a biofilm.

M. catarrhalis is considered as one of the top three most important bacterial causes of AOM and OME, followed after *Streptococcus pneumonia* and *Haemophilus influenzae* (reviewed in [23] and [55]). The proinflammatory cytokines, IL-1 α and TNF- α , are important hallmarks of *M. catarrhalis* disease [56; 57] and are proven endogenous pyrogens in mammals [43]. Our observations from the present and a previous study [10] show that *M. catarrhalis* nitrite reduction-generated $\text{NO}\cdot$ induces host-cell secretion of IL-1 α and TNF- α , suggesting that *M. catarrhalis*-generated $\text{NO}\cdot$ might play pathogenic roles in *M. catarrhalis*-associated otitis media diseases. Paradoxically, clinical studies showed that *M. catarrhalis* is rarely isolated from patients experiencing AOMwiP [26]. Thus, *M. catarrhalis* is largely discounted as a cause of AOMwiP according to Koch's postulate number two, which requires "the microorganism must be isolated from a diseased organism and grown in pure culture" to establish a causative relationship between a microorganism and a disease. As mentioned in the introduction section,

the essential elements necessary for *M. catarrhalis* biofilm-induced NO $\cdot$ production—such as bacterial biofilms, expression of AniA, and the presence of 0.5–1 mM nitrite—most likely exist in OME [24; 33; 34; 58]. In our studies, *M. catarrhalis* nitrite reduction-derived NO $\cdot$ induces the adjacent HBECs undergoing apoptosis and damage the HBEC monolayer (Fig. 4, and [10]), suggesting that *M. catarrhalis* nitrite reduction-derived NO $\cdot$ may contribute to tympanic membrane perforation in AOMwiP. More importantly, the novel finding of NO $\cdot$ cytotoxicity towards biofilm-grown *M. catarrhalis* (Fig. 5A, black + bars at 8, 10 and 24hr) provides a plausible explanation for the *M. catarrhalis* disease paradox: *M. catarrhalis* loads largely disappear as disease progresses from OME to AOMwiP [25; 26]. It remains to be investigated why the innate NorB failed to protect against AniA-generated NO $\cdot$ in biofilms in vitro and what critical molecular targets of NO $\cdot$ in biofilm-grown *M. catarrhalis*. The beneficial commensalism for *M. catarrhalis* growth (Fig. 7C) and *M. catarrhalis* O35E wild-type biofilms attached to apoptotic HBECs (Fig. 2, HBEC-O35E wt, 24hr) raised interesting questions as if apoptotic bodies of host cells might provide nutrients to support growth of few survived *M. catarrhalis* (Fig. 5A, black + bar at 24hr). It remains to be further elucidated if *M. catarrhalis* utilized its AniA and NorB as a pair of “spear-and-shield” (in terms of production and detoxification of NO $\cdot$) during infections, nevertheless, this study might provide interesting insights into a possible dualistic mechanism of nitrite reduction-derived NO $\cdot$ during *M. catarrhalis* infection, i.e., it might promote pathogenesis yet facilitate the spontaneous resolution of infection.

ACKNOWLEDGEMENTS

This study was supported by the FDA/CBER Operation Fund to W. Wang. A Research Participation Program administered by the Oak Ridge Institute for Science and Education (ORISE, through an interagency agreement between the U.S. Department of Energy and the U.S. FDA) provided support to ORISE fellows. The authors thank Dieter C. Gruenert for providing HBECs, Eric J. Hansen for providing *M. catarrhalis* O35E strains, Joaquin Jaramillo Sabogal and Irene Yang (Summer Interns, administrated by ORISE) for technical support, the FDA Nanotechnology Core Facility and Dr. Jiwen Zheng for SEM instrument use and technical assistance.

523 Table 1. *M. catarrhalis* O35E Strains

524

Strain	Description or Genotype	Reference
O35E	Wild-type strain	[59]
O35E <i>aniA</i>	<i>aniA</i> ::Kan ^{r†}	[14]
O35E <i>nsrR</i>	<i>nsrR</i> ::Kan ^r	[14]
O35E <i>norB</i>	<i>norB</i> ::Kan ^r	[32]
O35E <i>aniA</i> (pWW115)	O35E <i>aniA</i> containing pWW115 vector	[10]
O35E <i>nsrR</i> (pWW150)	O35E <i>aniA</i> containing a cloned wild-type <i>aniA</i> gene from ATCC 43617	[10]

525

526

527

† Kan^r, kanamycin resistance gene.

References

- [1] Thomas,D.D., Ridnour,L.A., Isenberg,J.S., Flores-Santana,W., Switzer,C.H., Donzelli,S., Hussain,P., Vecoli,C., Paolocci,N., Ambs,S., Colton,C.A., Harris,C.C., Roberts,D.D., Wink,D.A. The chemical biology of nitric oxide: Implications in cellular signaling, *Free Radic. Biol. Med.* 45 (2008) 18-31.
- [2] Kim,Y.M., Bombeck,C.A., Billiar,T.R. Nitric oxide as a bifunctional regulator of apoptosis, *Circ. Res.* 84 (1999) 253-256.
- [3] Bogdan,C., Rollinghoff,M., Diefenbach,A. The role of nitric oxide in innate immunity, *Immunol. Rev.* 173 (2000) 17-26.
- [4] Fang,F.C. Antimicrobial reactive oxygen and nitrogen species: concepts and controversies, *Nat. Rev. Microbiol.* 2 (2004) 820-832.
- [5] Zumft,W.G. Cell biology and molecular basis of denitrification, *Microbiol. Mol. Biol. Rev.* 61 (1997) 533-616.
- [6] Zumft,W.G. Nitric oxide reductases of prokaryotes with emphasis on the respiratory, heme-copper oxidase type, *J. Inorg. Biochem.* 99 (2005) 194-215.
- [7] Rock,J.D., Mahnane,M.R., Anjum,M.F., Shaw,J.G., Read,R.C., Moir,J.W. The pathogen *Neisseria meningitidis* requires oxygen, but supplements growth by denitrification. Nitrite, nitric oxide and oxygen control respiratory flux at genetic and metabolic levels, *Mol. Microbiol.* 58 (2005) 800-809.

- 548 [8] Akhtar,S., Khan,A., Sohaskey,C.D., Jagannath,C., Sarkar,D. Nitrite reductase NirBD is
549 induced and plays an important role during in vitro dormancy of Mycobacterium
550 tuberculosis, J. Bacteriol. 195 (2013) 4592-4599.
- 551 [9] Baek,S.H., Rajashekara,G., Splitter,G.A., Shapleigh,J.P. Denitrification genes regulate
552 *Brucella* virulence in mice, J. Bacteriol. 186 (2004) 6025-6031.
- 553 [10] Mocca,B., Wang,W. Bacterium-generated nitric oxide hijacks host tumor necrosis factor
554 alpha signaling and modulates the host cell cycle in vitro, J. Bacteriol. 194 (2012) 4059-
555 4068.
- 556 [11] Bodenmiller,D.M., Spiro,S. The yjeB (nsrR) gene of *Escherichia coli* encodes a nitric
557 oxide-sensitive transcriptional regulator, J. Bacteriol. 188 (2006) 874-881.
- 558 [12] Overton,T.W., Whitehead,R., Li,Y., Snyder,L.A., Saunders,N.J., Smith,H., Cole,J.A.
559 Coordinated regulation of the *Neisseria gonorrhoeae*-truncated denitrification pathway
560 by the nitric oxide-sensitive repressor, NsrR, and nitrite-insensitive NarQ-NarP, J. Biol.
561 Chem. 281 (2006) 33115-33126.
- 562 [13] Rock,J.D., Thomson,M.J., Read,R.C., Moir,J.W. Regulation of denitrification genes in
563 *Neisseria meningitidis* by nitric oxide and the repressor NsrR, J Bacteriol. 189 (2007)
564 1138-1144.
- 565 [14] Wang,W., Richardson,A.R., Martens-Habbena,W., Stahl,D.A., Fang,F.C., Hansen,E.J.
566 Identification of a repressor of a truncated denitrification pathway in *Moraxella*
567 *catarrhalis*, J. Bacteriol. 190 (2008) 7762-7772.

- 568 [15] Gilberthorpe,N.J., Lee,M.E., Stevanin,T.M., Read,R.C., Poole,R.K. NsrR: a key
569 regulator circumventing *Salmonella enterica* serovar Typhimurium oxidative and
570 nitrosative stress in vitro and in IFN-gamma-stimulated J774.2 macrophages,
571 Microbiology 153 (2007) 1756-1771.
- 572 [16] Karlinsey,J.E., Bang,I.S., Becker,L.A., Frawley,E.R., Porwollik,S., Robbins,H.F.,
573 Thomas,V.C., Urbano,R., McClelland,M., Fang,F.C. The NsrR regulon in nitrosative
574 stress resistance of *Salmonella enterica* serovar Typhimurium, Mol. Microbiol. 85 (2012)
575 1179-1193.
- 576 [17] Tucker,N.P., Hicks,M.G., Clarke,T.A., Crack,J.C., Chandra,G., Le Brun,N.E., Dixon,R.,
577 Hutchings,M.I. The transcriptional repressor protein NsrR senses nitric oxide directly via
578 a [2Fe-2S] cluster, PLoS. ONE. 3 (2008) e3623.
- 579 [18] Yukl,E.T., Elbaz,M.A., Nakano,M.M., Moenne-Loccoz,P. Transcription Factor NsrR
580 from *Bacillus subtilis* Senses Nitric Oxide with a 4Fe-4S Cluster, Biochemistry(2008).
- 581 [19] Isabella,V.M., Lapek Jr,J.D., Kennedy,E.M., Clark,V.L. Functional analysis of NsrR, a
582 nitric oxide-sensing Rrf2 repressor in *Neisseria gonorrhoeae*, Mol. Microbiol.(2008).
- 583 [20] Heurlier,K., Thomson,M.J., Aziz,N., Moir,J.W. The nitric oxide (NO)-sensing repressor
584 NsrR of *Neisseria meningitidis* has a compact regulon of genes involved in NO synthesis
585 and detoxification, J. Bacteriol. 190 (2008) 2488-2495.
- 586 [21] Hausladen,A., Gow,A., Stamler,J.S. Flavohemoglobin denitrosylase catalyzes the
587 reaction of a nitroxyl equivalent with molecular oxygen, Proc. Natl. Acad. Sci. U. S. A 98
588 (2001) 10108-10112.

- 589 [22] Rodionov,D.A., Dubchak,I.L., Arkin,A.P., Alm,E.J., Gelfand,M.S. Dissimilatory
590 metabolism of nitrogen oxides in bacteria: comparative reconstruction of transcriptional
591 networks, PLoS. Comput. Biol. 1 (2005) e55.
- 592 [23] Murphy,T.F., Parameswaran,G.I. *Moraxella catarrhalis*, a human respiratory tract
593 pathogen, Clin. Infect. Dis. 49 (2009) 124-131.
- 594 [24] Hall-Stoodley,L., Hu,F.Z., Gieseke,A., Nistico,L., Nguyen,D., Hayes,J., Forbes,M.,
595 Greenberg,D.P., Dice,B., Burrows,A., Wackym,P.A., Stoodley,P., Post,J.C.,
596 Ehrlich,G.D., Kerschner,J.E. Direct detection of bacterial biofilms on the middle-ear
597 mucosa of children with chronic otitis media, JAMA 296 (2006) 202-211.
- 598 [25] Binks,M.J., Cheng,A.C., Smith-Vaughan,H., Sloots,T., Nissen,M., Whiley,D.,
599 McDonnell,J., Leach,A.J. Viral-bacterial co-infection in Australian Indigenous children
600 with acute otitis media, BMC. Infect. Dis. 11 (2011) 161.
- 601 [26] Mackenzie,G.A., Carapetis,J.R., Leach,A.J., Morris,P.S. Pneumococcal vaccination and
602 otitis media in Australian Aboriginal infants: comparison of two birth cohorts before and
603 after introduction of vaccination, BMC. Pediatr. 9 (2009) 14.
- 604 [27] Wang,W., Reitzer,L., Rasko,D.A., Pearson,M.M., Blick,R.J., Laurence,C., Hansen,E.J.
605 Metabolic Analysis of *Moraxella catarrhalis* and the Effect of Selected In Vitro Growth
606 Conditions on Global Gene Expression, Infect. Immun. 75 (2007) 4959-4971.
- 607 [28] Falsetta,M.L., Bair,T.B., Ku,S.C., Vanden Hoven,R.N., Steichen,C.T., McEwan,A.G.,
608 Jennings,M.P., Apicella,M.A. Transcriptional profiling identifies the metabolic
609 phenotype of gonococcal biofilms, Infect. Immun. 77 (2009) 3522-3532.

- 610 [29] Barth,K.R., Isabella,V.M., Clark,V.L. Biochemical and genomic analysis of the
611 denitrification pathway within the genus *Neisseria*, Microbiology 155 (2009) 4093-4103.
- 612 [30] Mellies,J., Jose,J., Meyer,T.F. The *Neisseria gonorrhoeae* gene aniA encodes an
613 inducible nitrite reductase, Mol. Gen. Genet. 256 (1997) 525-532.
- 614 [31] Hoehn,G.T., Clark,V.L. The major anaerobically induced outer membrane protein of
615 *Neisseria gonorrhoeae*, Pan 1, is a lipoprotein, Infect. Immun. 60 (1992) 4704-4708.
- 616 [32] Wang,W., Kinkel,T., Martens-Habben, W., Stahl,D.A., Fang,F.C., Hansen,E.J. The
617 *Moraxella catarrhalis* Nitric Oxide Reductase Is Essential for Nitric Oxide Detoxification,
618 J. Bacteriol. 193 (2011) 2804-2813.
- 619 [33] Ruckdeschel,E.A., Kirkham,C., Lesse,A.J., Hu,Z., Murphy,T.F. Mining the *Moraxella*
620 *catarrhalis* genome: identification of potential vaccine antigens expressed during human
621 infection, Infect. Immun. 76 (2008) 1599-1607.
- 622 [34] John,E.O., Russell,P.T., Nam,B.H., Jinn,T.H., Jung,T.T. Concentration of nitric oxide
623 metabolites in middle ear effusion, Int. J. Pediatr. Otorhinolaryngol. 60 (2001) 55-58.
- 624 [35] Pearson,M.M., Laurence,C.A., Guinn,S.E., Hansen,E.J. Biofilm formation by *Moraxella*
625 *catarrhalis* in vitro: roles of the UspA1 adhesin and the Hag hemagglutinin, Infect.
626 Immun. 74 (2006) 1588-1596.
- 627 [36] O'Toole,G., Kaplan,H.B., Kolter,R. Biofilm formation as microbial development, Annu.
628 Rev. Microbiol. 54 (2000) 49-79.

629 [37] Donlan,R.M., Costerton,J.W. Biofilms: survival mechanisms of clinically relevant
630 microorganisms, Clin. Microbiol Rev. 15 (2002) 167-193.

631 [38] Yang,D., Chen,Q., Hoover,D.M., Staley,P., Tucker,K.D., Lubkowski,J., Oppenheim,J.J.
632 Many chemokines including CCL20/MIP-3alpha display antimicrobial activity, J.
633 Leukoc. Biol. 74 (2003) 448-455.

634 [39] Blaise,G.A., Gauvin,D., Gangal,M., Authier,S. Nitric oxide, cell signaling and cell death,
635 Toxicology 208 (2005) 177-192.

636 [40] Blantz,R.C., Munger,K. Role of nitric oxide in inflammatory conditions, Nephron 90
637 (2002) 373-378.

638 [41] Cruz-Ramos,H., Crack,J., Wu,G., Hughes,M.N., Scott,C., Thomson,A.J., Green,J.,
639 Poole,R.K. NO sensing by FNR: regulation of the Escherichia coli NO-detoxifying
640 flavohaemoglobin, Hmp, EMBO J. 21 (2002) 3235-3244.

641 [42] Kinkel,T.L., Roux,C.M., Dunman,P.M., Fang,F.C. The Staphylococcus aureus SrrAB
642 two-component system promotes resistance to nitrosative stress and hypoxia, MBio. 4
643 (2013) e00696-13.

644 [43] Dinarello,C.A., Cannon,J.G., Wolff,S.M., Bernheim,H.A., Beutler,B., Cerami,A.,
645 Figari,I.S., Palladino,M.A., Jr., O'Connor,J.V. Tumor necrosis factor (cachectin) is an
646 endogenous pyrogen and induces production of interleukin 1, J. Exp. Med. 163 (1986)
647 1433-1450.

- 648 [44] Dinarello,C.A., Cannon,J.G., Mier,J.W., Bernheim,H.A., LoPreste,G., Lynn,D.L.,
649 Love,R.N., Webb,A.C., Auron,P.E., Reuben,R.C., . Multiple biological activities of
650 human recombinant interleukin 1, J. Clin. Invest 77 (1986) 1734-1739.
- 651 [45] Stevanin,T.M., Laver,J.R., Poole,R.K., Moir,J.W., Read,R.C. Metabolism of nitric oxide
652 by *Neisseria meningitidis* modifies release of NO-regulated cytokines and chemokines by
653 human macrophages, Microbes Infect. 9 (2007) 981-987.
- 654 [46] Laver,J.R., Stevanin,T.M., Messenger,S.L., Lunn,A.D., Lee,M.E., Moir,J.W., Poole,R.K.,
655 Read,R.C. Bacterial nitric oxide detoxification prevents host cell S-nitrosothiol
656 formation: a novel mechanism of bacterial pathogenesis, FASEB J. 24 (2010) 286-295.
- 657 [47] Miller,L.S., Pietras,E.M., Uricchio,L.H., Hirano,K., Rao,S., Lin,H., O'Connell,R.M.,
658 Iwakura,Y., Cheung,A.L., Cheng,G., Modlin,R.L. Inflammasome-mediated production
659 of IL-1beta is required for neutrophil recruitment against *Staphylococcus aureus* in vivo,
660 J. Immunol. 179 (2007) 6933-6942.
- 661 [48] Lindell,D.M., Standiford,T.J., Mancuso,P., Leshen,Z.J., Huffnagle,G.B. Macrophage
662 inflammatory protein 1alpha/CCL3 is required for clearance of an acute *Klebsiella*
663 *pneumoniae* pulmonary infection, Infect. Immun. 69 (2001) 6364-6369.
- 664 [49] Luke,N.R., Jurgisek,J.A., Bakaletz,L.O., Campagnari,A.A. Contribution of *Moraxella*
665 *catarrhalis* type IV pili to nasopharyngeal colonization and biofilm formation, Infect.
666 Immun. 75 (2007) 5559-5564.

- 667 [50] Lafontaine,E.R., Cope,L.D., Aebi,C., Latimer,J.L., McCracken,G.H., Jr., Hansen,E.J.
668 The UspA1 protein and a second type of UspA2 protein mediate adherence of *Moraxella*
669 *catarrhalis* to human epithelial cells in vitro, J. Bacteriol. 182 (2000) 1364-1373.
- 670 [51] Holm,M.M., Vanlerberg,S.L., Sledjeski,D.D., Lafontaine,E.R. The Hag protein of
671 *Moraxella catarrhalis* strain O35E is associated with adherence to human lung and
672 middle ear cells, Infect. Immun. 71 (2003) 4977-4984.
- 673 [52] Barraud,N., Hassett,D.J., Hwang,S.H., Rice,S.A., Kjelleberg,S., Webb,J.S. Involvement
674 of nitric oxide in biofilm dispersal of *Pseudomonas aeruginosa*, J. Bacteriol. 188 (2006)
675 7344-7353.
- 676 [53] Marvasi,M., Chen,C., Carrazana,M., Durie,I.A., Teplitski,M. Systematic analysis of the
677 ability of Nitric Oxide donors to dislodge biofilms formed by *Salmonella enterica* and
678 *Escherichia coli* O157:H7, AMB. Express 4 (2014) 42.
- 679 [54] Arora,D.P., Hossain,S., Xu,Y., Boon,E.M. Nitric Oxide Regulation of Bacterial
680 Biofilms, Biochemistry 54 (2015) 3717-3728.
- 681 [55] Ruohola,A., Meurman,O., Nikkari,S., Skottman,T., Salmi,A., Waris,M., Osterback,R.,
682 Eerola,E., Allander,T., Niesters,H., Heikkinen,T., Ruuskanen,O. Microbiology of acute
683 otitis media in children with tympanostomy tubes: prevalences of bacteria and viruses,
684 Clin Infect. Dis 43 (2006) 1417-1422.
- 685 [56] Sethi,S., Muscarella,K., Evans,N., Klingman,K.L., Grant,B.J., Murphy,T.F. Airway
686 inflammation and etiology of acute exacerbations of chronic bronchitis, Chest 118 (2000)
687 1557-1565.

- [57] Parameswaran,G.I., Wrona,C.T., Murphy,T.F., Sethi,S. *Moraxella catarrhalis* acquisition, airway inflammation and protease-antiprotease balance in chronic obstructive pulmonary disease, BMC. Infect. Dis. 9 (2009) 178.
- [58] Murphy,T.F., Bakaletz,L.O., Smeesters,P.R. Microbial interactions in the respiratory tract, Pediatr. Infect. Dis. J. 28 (2009) S121-S126.
- [59] Helminen,M.E., Maciver,I., Latimer,J.L., Cope,L.D., McCracken,G.H., Jr., Hansen,E.J. A major outer membrane protein of *Moraxella catarrhalis* is a target for antibodies that enhance pulmonary clearance of the pathogen in an animal model, Infect. Immun. 61 (1993) 2003-2010.

FIGURE LEGENDS

Figure 1. Mechanisms of NO $\cdot$ turnover and *M. catarrhalis* biofilm formation in bacteria-HBEC co-cultures. (A) Selected mechanisms of NO $\cdot$ turnover. Mammalian production of NO $\cdot$ from *L*-arginine by three types of NOS is depicted in grey. Depicted in black are the oxygen-involved conversion of NO $\cdot$ to nitrite (NO $_2^-$), or into nitrate (NO $_3^-$) by Hgb or bacterial HMP and a truncated bacterial denitrification pathway, including the reduction of NO $_3^-$ to NO $_2^-$ by the nitrate reductase complex NarGHJI, the reduction of NO $_2^-$ to NO $\cdot$ by the nitrite reductase NirK or AniA, and the reduction of NO $\cdot$ to nitrous oxide (N $_2$ O) by the NO $\cdot$ reductase NorB. Bacterial NsrR and its repression of the expression of *aniA*, *norB* and *hmp* are also shown. (B) Detection of differential expression of *M. catarrhalis* O35E *aniA* and *norB* by real-time RT-PCR. The fold-change of expression of *aniA* and *norB* (*aniA* and *norB* bars) in cultures with agitation (planktonic growth, PT, open bars) or without agitation (biofilm-like cell-aggregates, BF) was shown. The ORF1234 was used as an endogenous control, and the expression of ORF1020 was also assessed. The data represent the mean $\pm$ standard error from three biological replicates. The expression of *aniA* and *norB* in 6, 10 or 24 hr BF was significantly increased (*t*-tests, paired two-sample) relative to in 6 hr PT, whereas the expression of ORF1020 was increased significantly in 6 hr BF but was not significant in 10 or 24 hr BF (*t*-tests, paired two-sample). (C) SEM analysis to monitor *M. catarrhalis* biofilm formation in HBEC-bacteria co-cultures. The SEM study was carried out twice. Possible water channels in matured biofilms were pointed with open arrows, and HBEC-surface microspike-like protrusions were pointed with white arrows. At each time-point, three fields of view were examined, and images were taken with different magnifications ($\times 1000$, $\times 2000$, $\times 5000$ and $\times 10000$) for each

field of view. Shown are a representative set of images ($\times 5000$) of *M. catarrhalis* O35E wild-type biofilms in MEM* at 6- and 24-hr post-infection.

Figure 2. Nitrite enhanced *M. catarrhalis* biofilm formation at early stage of infection and exerts nitrite reduction-dependent adverse effects on HBECs at mid-to-late stages of co-culturing. The SEM study was carried out twice. At each time-point, three fields of view were examined, and images were taken with different magnifications ($\times 1000$, $\times 2000$ and $\times 5000$) for each field of view. Shown is a representative set of images of the O35E wild-type biofilms (HBEC-O35E wt) or *aniA* mutant biofilms (HBEC-O35E *aniA*) formed on surfaces of HBECs in MEM* supplemented with 1 mM nitrite at 6 and 26hr post-infection in co-cultures. Top panel: nitrite-dependent and AniA-independent enhancement of *M. catarrhalis* biofilm formation at early stage of infection. SEM images showed visually better developed biofilms (compared with biofilm showed in Fig. 1C, 6hr) of *M. catarrhalis* O35E wild-type (top panel, HBEC-O35E wt) and O35E *aniA* mutant (top panel, HBEC-O35E *aniA*) that were formed on surfaces of HBECs at 6hr post-infection. Bottom panel: Nitrite- and AniA-dependent cytotoxicity to HBECs. SEM image showed, at 24hr post-infection, O35E wild-type biofilms on surfaces of apoptotic bodies of HBEC (bottom, HBEC-O35E wt) and O35E *aniA* mutant biofilms (bottom, HBEC-O35E *aniA*) on surfaces of seemingly healthy HBECs.

Figure 3. Monitoring of *M. catarrhalis* biofilm-induced nitrite reduction and diffused $\text{NO}\cdot$ from bacterial nitrite reduction in co-cultures. (A) Upregulated nitrite reduction in *M. catarrhalis* biofilms in MEM* supplemented with 1 mM nitrite. A Griess assay was performed to measure nitrite levels in cultures of HBECs alone (open bars), co-cultures with

M. catarrhalis O35E wild-type strain (black bars), or with O35E *aniA* mutant strain (grey bars) at various time-points post-infection. The data represent the mean $\pm$ standard error from two biological replicates (each experiment comprised three technical replicates). The differences of nitrite levels (open bars vs. black bars) were not significant at 0 and 4 hr post-infection time-points, but were significant (*t*-tests, paired two-sample) at 5 to 8 hr time-points. (B) Direct detection of diffused NO $\cdot$ in co-cultures. Diffused NO $\cdot$ in medium suspension of HBEC-O35E wild-type (solid line) or HBEC-O35E *aniA* mutant co-cultures in MEM* with 1 mM of nitrite was detected using a TBR 4100 Free Radical Analyzer (World Precision Instruments, Inc. WPI, Sarasota, FL) and L-shaped NO $\cdot$ microsensors (ISO-NOPF-200-L10, WPI). (C) The difference of NO $\cdot$ detected in co-cultures of HBECs with O35E wild-type and O35E *aniA* mutant in MEM* supplemented with 1mM of nitrite was depicted as diffused NO $\cdot$ generated by *M. catarrhalis* nitrite reduction. A representative set of data (B and C) from four biological replicates was shown.

Figure 4. Monitoring dynamic effects of *M. catarrhalis* nitrite reduction-derived NO $\cdot$ on HBEC proliferation in vitro. (A) Dynamic effects of *M. catarrhalis* nitrite reduction-produced NO $\cdot$ on HBEC proliferation. Growth curves for HBECs that were co-cultured with *M. catarrhalis* O35E wild-type, O35E *aniA* mutant, O35E *norB* mutant, O35E *nsrR* mutant, O35E *aniA*(pWW115) mutant, or complemented O35E *aniA*(pWW161) strain in MEM* (top panel) or MEM* with 1 mM nitrite (bottom panel). Arrows (bottom panel) indicate when HBEC proliferation peaked. *x* axis: time (0–24 hr); and *y* axis: relative confluences of host-cell monolayer (22–90%). Shown is a representative set of data from three biological replicates. (B) Confirmation of *M. catarrhalis* nitrite reduction-NO $\cdot$ is responsible for cytotoxicity to HBECs.

Micrographs of HBEC monolayer in co-cultures of HBEC–*M. catarrhalis* O35E wild-type strain in MEM* supplemented with 1mM of nitrite (MEM* + nitrite, arrows pointed to apoptotic HBECs) or in MEM* supplemented with both 1mM of nitrite and 1mg/ml of Hgb (MEM* + nitrite + Hgb, arrows pointed at bigger *M. catarrhalis* cell-aggregates). Shown is a representative set of data from three biological replicates.

Figure 5. Growth phase-dependent effects of nitrite and nitrite reduction on *M. catarrhalis* viability in biofilms formed on surfaces of HBECs. (A) *M. catarrhalis* O35E wild-type (black bars) or *aniA* mutant (grey bars) biofilm viability was determined at different post-infection time-points in co-culture with HBECs in MEM* with (bars +) or without (bars –) initial supplementation with 1 mM nitrite. The data represent the mean $\pm$ standard error from two (biologically repeated experiments, each experiment comprising three technical replicates). (B) Confirmation of *M. catarrhalis* nitrite reduction-NO $\cdot$ is responsible for cytotoxicity to biofilm-grown *M. catarrhalis*. The viabilities of *M. catarrhalis* O35E wild-type (black bars) and *aniA* mutant (grey bars) biofilms in co-cultures with HBECs at 24 hr post-infection were detected. Media used were MEM* with or without the supplementation of 1mM of nitrite (Nitrite – or +) and/or 1 mg/ml of human hemoglobin (Hgb – or +). The data represent the mean $\pm$ standard error from three biological replicates (each experiment comprised two technical replicates).

Figure 6. *M. catarrhalis*–generated NO $\cdot$ stimulates host-cell expression of pro-inflammatory cytokines and antibacterial chemokines. (A) Identification of NO $\cdot$ -induced cytokines and chemokines expressed by HBECs at 24hr post-infection. A RT²Profiler™ PCR

792 Array (Human Inflammatory Cytokines and Receptors 96-well PCR Array, manufactured by
793 SABiosciences, a Qiagen company) was used to determine HBEC cytokine expression profiles
794 by following the manufacturer's protocol. Each well of a 96-well PCR array was preloaded with
795 a gene-specific oligonucleotide primer-pair (triplicates per gene). RNA samples were isolated
796 from HBECs that were cultured alone, co-cultured with O35E wild-type or with *aniA* mutant
797 strains in the presence of nitrite (1mM). The HBEC RNA preparations were then used to
798 determine cytokine expression profiles by using a 96-well PCR array and quantitative real-time
799 RT-PCR (qRT-PCR, $\Delta\Delta C_T$) analysis. The results of total nine arrays from three experimental
800 repeats were analyzed using a Relative Quantification (RQ) Study Software (Applied
801 Biosystems) to determine fold-change of cytokine expression by HBECs in a co-culture relative
802 to in culture of HBEC alone. The RQ Study Software used the expression of GAPDH as an
803 endogenous control to normalize arrays, and used gene expression by HBECs in cultures of
804 HBEC only (open bar) as calibrator to determine gene expression fold-change in co-cultures of
805 HBEC-O35E wild-type (black bars) or HBEC-O35E *aniA* (grey bar). Genes whose expression
806 was increased at least twofold (black bar vs. open bar) were shown, along with gene expression
807 in co-cultures of HBEC-*aniA* mutant (grey bars). The difference between gene expression
808 levels (black bar vs. open bar) is significant (*t*-tests, paired two-sample) for all genes, except
809 GAPDH. The expression of CCL4 and CXCL14 was significantly increased (*t*-tests, paired
810 two-sample) and the expression of IL-1 α and IL-1 β was significantly decreased (*t*-tests, paired
811 two-sample) by HBEC in co-cultures of HBEC-O35E *aniA* (grey bars) relative to in cultures of
812 HBEC only (open bars). (B, C) ELISA-based confirmation of NO $\cdot$ -induced production of the
813 chemokine CCL3 (B) or CXCL2 (C) by HBECs that were cultured alone (open bars) or co-
814 cultured with O35E wild-type (black bars) or *aniA* mutant (grey bars). The results in (B) and (C)

represent the mean $\pm$ standard error from three biological replicates, and *t*-tests (paired two-sample).

Figure 7. The upregulated *M. catarrhalis* nitrite reduction is cytotoxic for cell-aggregates in mono-cultures. (A) Reduction of nitrite by *M. catarrhalis* O35E wild-type (solid line) or *aniA* mutant (dotted line) strains in pure bacterial cultures (mono-cultures) incubated without agitation. Nitrite levels were determined hourly for a period of 8 hr. The data represent the mean $\pm$ standard error from three biological replicates. The difference of nitrite levels between solid line and dotted line was significant (*t*-tests, paired two-sample) at all-time points but except 1 and 2 hr time points. (B) Viability of *M. catarrhalis* cell-aggregates in mono-cultures incubated without agitation. Viability of the O35E wild-type (black bars) or *aniA* mutant (grey bars) strain was determined at different time-points in mono-cultures in MEM* with (bars +) or without (bars -) supplementation of 1 mM nitrite. The data represent the mean $\pm$ standard error from three biological replicates and *t*-tests (paired two-sample). (C) AniA-independent beneficial commensalism. The MEM* was used in this study. The viability of O35E wild-type (wt bars) or *aniA* mutant (*aniA* bars) biofilms formed on an HBEC monolayer in co-cultures (co- bars) or in pure bacterial cultures (mono- bars) was determined by measuring CFU. The data represent the mean $\pm$ standard error from at least three biological replicates, and *t*-tests (paired two-sample).

Figure 1

HBEC-O35E wt

HBEC-O35E *aniA*

Figure 2

A **B** **C** **Figure 3**

Figure 4

Figure 5

Figure 6

Figure 7