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Bordetella bronchiseptica responses to physiological reactive nitrogen and oxygen stresses

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Abstract

Bordetella bronchiseptica can establish prolonged airway infection consistent with a highly developed ability to evade mammalian host immune responses. Upon initial interaction with the host upper respiratory tract mucosa, *B. bronchiseptica* are subjected to antimicrobial reactive nitrogen species (RNS) and reactive oxygen species (ROS), effector molecules of the innate immune system. However, the responses of *B. bronchiseptica* to redox species at physiologically relevant concentrations (nM-μM) have not been investigated. Using predicted physiological concentrations of nitric oxide (NO), superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) on low numbers of colony forming units (CFU) of *B. bronchiseptica*, all redox active species displayed dose-dependent antimicrobial activity. Susceptibility to individual redox active species was significantly increased upon introduction of a second species at sub-antimicrobial concentrations. An increased bacteriostatic activity of NO was observed relative to H_2O_2. The understanding of *Bordetella* responses to physiologically relevant levels of exogenous RNS and ROS will aid in defining the role of endogenous production of these molecules in host innate immunity against *Bordetella* and other respiratory pathogens.

Keywords

bacterial stress; nitric oxide; hydrogen peroxide; superoxide; reactive nitrogen species (RNS); reactive oxygen species (ROS)

INTRODUCTION

The *Bordetella* genus includes the closely related bacterial species *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. The human pathogens *B. pertussis* and *B. parapertussis*

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are etiological agents of whooping cough, a potentially lethal respiratory syndrome primarily affecting young children (Bjornstad & Harvill, 2005, Greenberg, et al., 2005). *B. bronchiseptica* infects a variety of mammals and can result in asymptomatic infection or symptomatic disease (Goodnow, 1980). *B. bronchiseptica* expresses most of the virulence factors identified in *B. pertussis*, with the notable exception of pertussis toxin. In both species, virulence factor expression is regulated by a two-component sensory system, *Bordetella* virulence gene activator-sensor (BvgAS) (Martinez de Tejada, et al., 1996, Cotter & Jones, 2003). Comparative analysis of the *B. pertussis* and *B. bronchiseptica* genomes underscores the conservation of their pathogenic machineries and supports the use of *B. bronchiseptica* as a model for *Bordetella* physiology and pathogenesis (Parkhill, et al., 2003).

Respiratory pathogens must overcome innate airway defenses including antimicrobial molecules secreted into the airway surface liquid (ASL) (Wilson, et al., 1996). Both reactive nitrogen species (RNS) and reactive oxygen species (ROS) can provide antimicrobial activity by inactivating bacterial cell enzymes through reaction with metal prosthetic groups, modification of specific amino acids, or by induction of DNA mutations (Imlay, 2003, Borisov, et al., 2004, Weber, et al., 2004). Bacterial responses to redox species are dependent on concentration, duration of exposure and the ability of the pathogen to detoxify specific RNS and ROS (Khelef, et al., 1996, Gardner, et al., 1998, Bryk, et al., 2000).

Nitric oxide (NO) is produced by NO synthases (NOS) in many cell types including respiratory epithelial cells (Donnelly & Barnes, 2002), where it may have antibacterial activity (Morris, et al., 1984, Schmidt & Walter, 1994, Fang, 1997, Nathan & Shiloh, 2000). Because NO can be oxidized to RNS such as nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3), and peroxynitrite ($ONOO^-$) (Ischiropoulos, et al., 1992), its antimicrobial effects may be due to alternate RNS species. Superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) are ROS produced by resident epithelial cells and/or migratory immune cells in the airway (Hampton, et al., 1996, Geiszt, et al., 2003, Forteza, et al., 2005, Pantano, et al., 2007). Bordetellae produce superoxide dismutase (SOD) and catalase to detoxify these compounds, and increased bacterial susceptibility to ROS has been observed in *B. pertussis* strains with mutations in SOD (Khelef, et al., 1996) or catalase (DeShazer, et al., 1994). In this study, we assessed antimicrobial effects of physiologically relevant concentrations of individual or combined RNS and ROS on low CFU of *B. bronchiseptica* to model the redox stress level in the airway during infection.

MATERIALS AND METHODS

Materials

Bordet Gengou (BG) agar plates, Bacto Agar, and Bacto Yeast Extract were purchased from Becton Dickinson (Sparks, Maryland). Stock H_2O_2 was from Acros Organics (Morris Plaines, New Jersey). Diethylenetriaminepentaacetic acid (DTPA), hypoxanthine (HX), SOD, and catalase were from Sigma-Aldrich (St. Louis, Missouri). Cytochrome c was from Calbiochem (San Diego, California). S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) was from Cayman Chemical (Ann Arbor, Michigan). Spermine NONOate (SPER/NO) was synthesized according to published procedures (Hrabie, et al., 1993). All other chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri), VWR (West Chester, Pennsylvania) or Fisher Scientific (Pittsburgh, Pennsylvania).

B. bronchiseptica cultures

Stocks of *B. bronchiseptica* strain RB50 (Cotter & Miller, 1994) were cultured on BG agar plates or in Stainer-Scholte medium (63 mM L-glutamic acid, 2 mM L-proline, 43 mM

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NaCl, 3.7 mM KH_2PO_4, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.14 mM $CaCl_2{\cdot}2H_2O$, 10g/L casamino acids and 39 mM Tris, pH 7.6) at 37°C with supplements (0.33 mM L-cysteine, 36 μM $FeSO_4{\cdot}7H_2O$, 32 μM nicotinic acid, 0.49 mM glutathione, and 2.3 mM ascorbic acid) added at the time of preparation of broth cultures (Stainer & Scholte, 1970). Airway surface liquid (ASL) mimic medium was prepared in phosphate buffered saline (PBS) with specific components derived from published chemical analysis of ASL (Robinson, et al., 1989, Joris, et al., 1993, Cowley, et al., 1997, Donaldson, et al., 2000, Kozlova, et al., 2005): 90.4 mM Na^+, 106.0 mM Cl^-, 10.0 mM Mg^{2+}, 1.0 mM Ca^{2+}, 0.1 mM NO_2^-, 0.1 mM NO_3^-, 20.0 mM K^+, 11.9 mM PO_4^-, 2.0 mM SO_4^{2-}, 4.0 g/L casamino acids and 550 nM ATP, pH 7.2.

In vitro generation of RNS and ROS

NO was produced by decomposition of the NO donor SPER/NO diluted in PBS (Keefer, et al., 1996). Peak absorption (250 nm) for SPER/NO stock in 10 mM NaOH was evaluated spectrophotometrically before each experiment to verify activity and effective concentration (ε of 8,000 $M^{-1}cm^{-1}$ (Maragos, et al., 1991)). Expired SPER/NO for control experiments was produced by incubation of stock solution at 37°C until complete loss of absorption at 250 nm. Steady state concentrations of NO (300–400 nM and 3–4 μM) in solution were measured directly for 6 μM and 62.5 μM SPER/NO respectively, using an ISO-NO Mark II isolated NO meter and SNAP as a NO standard (WPI, Sarasota, Florida). Due to the non-linear response of the ISO-NOPMC NO microchip sensor, NO concentrations at higher SPER/NO levels were estimated from a standard curve (12–15 μM steady state NO at 250 μM SPER/NO; 50–60 μM NO at 1,000 μM SPER/NO). The level of NO released from 1,000 μM SPER/NO likely represents a hyperphysiological concentration of NO.

H_2O_2 was diluted to the desired concentrations from a commercial stock solution. Because of its high reactivity (Riley, et al., 1991), $O_2^{\cdot-}$ gradients were established by continuous enzymatic production during oxidation of HX by xanthine oxidase (XO) in HX-buffer (500 μM HX, 50 μM DTPA in PBS, pH 7.2) containing 200 U/ml catalase and a concentration gradient of XO. The steady rate of synthesis for $O_2^{\cdot-}$ at 3.75×10^{-4} U/ml (0.1 μM/min) and 3.75×10^{-3} U/ml (1.0 μM/min) were determined by spectrophotometric monitoring of the reduction of cytochrome c at 550 nm (McCord & Fridovich, 1969). Higher steady state productions were estimated from standard curves. SOD or catalase (200 U/ml) were used to consume $O_2^{\cdot-}$, and H_2O_2, respectively. When both $O_2^{\cdot-}$ and H_2O_2 were required in bacterial challenge experiments, catalase was removed from the reaction mixture. HX-buffer was used as a control.

Exposure of *B. bronchiseptica* to RNS and ROS

Because growth phase can influence bacterial responses to redox stress (Gonzalez-Flecha & Demple, 1997, Michan, et al., 1999), all experiments were conducted with bacteria cultured at early to mid log phase from a standardized inoculum. Inocula were standardized by suspending 5 – 10 colonies from BG agar in PBS to achieve a working suspension with an OD_{600} of 0.3. A 50 μl aliquot of working suspension (~ 5.0×10^7 colony forming units (CFU)) was placed in 4.0 ml Stainer-Scholte medium and incubated at 37°C with shaking (250 rpm) for 19 – 20 h. Bacteria were pelleted, re-suspended in PBS to an OD_{600} of 0.3, and diluted 1,000-fold. Bacterial suspension (5 μl, or ~5,000 CFU) was added to redox challenge reactions in 96-well plates (100 μl reaction volume) or to control buffer for 5 h at 37°C with agitation (150 rpm). Sample aliquots were then plated in duplicate onto Luria-Bertani (LB) agar for analysis of bactericidal activity. Bacterial survival following challenge with RNS and/or ROS were determined by dividing the average number of CFU surviving the challenge by the number of CFU surviving incubation in buffer alone (100%).

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Analysis of colony morphology after 50% Lethal Dose (LD_{50}) challenge of *B. bronchiseptica* by NO or H_2O_2

B. bronchiseptica were exposed to 250 μM active or expired SPER/NO, 5 μM H_2O_2 or PBS. Aliquots were plated onto BG agar and incubated at 37°C for 48 h. Plates were imaged using a Chemi-Doc imaging system controlled by Quantity One Software (BioRad, Hercules, California). To quantify colony size after exposure of bacteria to RNS or ROS, individual colonies were excised with a sterile scalpel and immediately suspended into 1.0 ml PBS, vortexed and subjected to serial dilution. The determined number of CFU/colony represents the average of a total of 40 randomly selected colonies from at least two independent experiments for each treatment.

Comparison of *B. bronchiseptica* growth

Following exposure to 250 μM SPER/NO, 5 μM H_2O_2 or PBS control, sample aliquots were plated on BG agar and incubated for 48 h. Ten to fifteen individual colonies (normal sized or microcolonies) were recovered from the agar plates and normalized in PBS to achieve an OD_{600} of 0.3. A 50 μl aliquot of bacterial suspension was used to seed 4.0 ml liquid cultures at 37°C. Growth in Stainer-Scholte medium was monitored spectrophotometrically at 600 nm at 3 h intervals. Growth in ASL mimic medium was assessed by plating samples from developing cultures on LB agar plates using a conventional serial dilution assay.

Statistical methods

Using Prism (GraphPad Software Inc., San Diego, California), one-way ANOVA with Bonferroni post-test was used to determine statistical significance within each experiment. Two-tailed unpaired student-t tests were used to analyze statistical significance between different experiments. For all comparisons, a value of $P < 0.05$ conferred statistically significant differences.

RESULTS

B. bronchiseptica is sensitive to prototypical redox species of the airway

To determine the responses of *B. bronchiseptica* to RNS and ROS, dose response analyses of antimicrobial activity were performed with NO, H_2O_2, and $O_2^{\cdot-}$ (Figure 1). The use of low CFU in log growth phase to mimic physiological infection allowed for the measurement of subtle yet significant changes in bacterial killing on a sub-logarithmic scale. *B. bronchiseptica* displayed high survival (95.1 ± 6.2%) after 5 h treatments with 6.0 μM SPER/NO (constant [NO] ~ 300–400 nM). However, as the concentration of SPER/NO increased, *B. bronchiseptica* survival significantly decreased (69.1 ± 10.5%, 58.8 ± 7.3% and 4.4 ± 1.0% of initial inoculum were recovered with 62.5, 250 and 1,000 μM SPER/NO, respectively). Expired SPER/NO was not bacteriocidal confirming that the observed antimicrobial effects were due to NO production and not accumulation of decomposition products or autoxidation of NO (e.g., spermine and/or nitrite/nitrate).

Incubation of *B. bronchiseptica* with H_2O_2 or $O_2^{\cdot-}$ also resulted in dose-dependent antimicrobial activity (Figure 1B, C). At the lowest H_2O_2 concentrations tested there were insignificant reductions in bacterial survival (83.3 ± 5.9%, and 85.4 ± 12.0% survival at 1.25 μM, and 2.5 μM, respectively). Significant reductions in *B. bronchiseptica* survival were observed after incubation with 5 or 10 μM H_2O_2 (63.4 ± 6.4% and 34.0 ± 3.4%, respectively). Despite an immediate reduction in *B. bronchiseptica* survival at the lowest $O_2^{\cdot-}$ concentration tested (68.7 ± 13.1% at 0.1 μM/min), the large variation between experiments prevented a significant change in survival except at 100 μM/min $O_2^{\cdot-}$ (32.6 ± 14.5% survival). Antimicrobial activity of NO, H_2O_2, and $O_2^{\cdot-}$ were observed against *B. bronchiseptica* within concentrations estimated to be relevant to mammalian ASL.

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Combinations of redox species increase the antimicrobial activity toward *B. bronchiseptica*

Because RNS and ROS are released continuously into ASL by airway epithelial cells, the total antimicrobial effect of redox species *in vivo* is likely the result of a synergistic effect. To determine whether NO augments the antimicrobial effect of H_2O_2 on *B. bronchiseptica*, challenges with SPER/NO were combined with a sub-antimicrobial concentration of H_2O_2 (2.5 μM). Survival of *B. bronchiseptica* after NO/H_2O_2 challenge was dose-dependent (Figure 2A), however, the decrease in bacterial survival was significant when compared to control, even at the lowest concentration of SPER/NO (72.7 ± 2.5%, 61.6 ± 9.1% and 69.6 ± 4.5% survival at 3.9, 15.6 and 62.5 μM, respectively). At 250 μM SPER/NO the combined antimicrobial effect of NO and H_2O_2 (29.4 ± 4.3% (Figure 2A)) was significantly increased over NO alone (58.8 ± 7.3% (Figure 1A)).

In complementary experiments SPER/NO was kept constant at a sub-antimicrobial concentration (15 μM) while the concentration of H_2O_2 was varied (Figure 2B). At the lowest supplemented concentration of H_2O_2 (1.25 μM), *B. bronchiseptica* survival was not significantly affected. All other concentrations of H_2O_2 reduced *B. bronchiseptica* survival when compared to controls (64.4 ± 10.7%, 49.3 ± 4.5% and 10.6 ± 3.8% survival at 2.5, 5.0 and 10.0 μM, respectively). Again, at the highest concentration of H_2O_2 tested (10.0 μM), the combined antimicrobial effect of NO and H_2O_2 (10.6 ± 3.8% (Figure 2B)) was significantly increased over H_2O_2 alone (34.0 ± 3.4 (Figure 1B)).

To examine the effects of exposure to combined ROS on *B. bronchiseptica* viability, bacterial survival was evaluated after challenge with continuous release of 0.1 μM/min of H_2O_2 and $O_2^{\cdot -}$, (Figure 2C). This exposure significantly reduced the level of *B. bronchiseptica* survival to 42.7 ± 8.9% as compared to control. Addition of SOD resulted in partial rescue of *B. bronchiseptica* survival (78.1 ± 22.3%) whereas addition of catalase had a less robust effect (52.7 ± 24.6%).

The antimicrobial activity of NO against *B. bronchiseptica* is in part bacteriostatic

In an effort to determine whether NO and H_2O_2 differed in their anti-microbial capacities, *B. bronchiseptica* colony morphology was evaluated after exposure to either SPER/NO or H_2O_2. In order to normalize the physiological pressure exerted by each reagent, the LD_{50} (250 μM SPER/NO and 5 μM H_2O_2) as determined from experiments presented above was used. *B. bronchiseptica* exposed to PBS in mock challenge experiments displayed a consistent colony morphology (Figure 3A). Challenge with SPER/NO resulted in the generation of microcolonies in approximately 60% of colonies after growth on solid medium (Figure 3B). In contrast, there was no evidence of reduced colony size after exposure to an LD_{50} of H_2O_2 (Figure 3C) or to expired SPER/NO (Figure 3D). These data were consistent with NO being a necessary component for the generation of microcolonies.

The number of CFU within each colony was determined for NO-induced microcolonies and compared to that of normal sized colonies from SPER/NO, H_2O_2, and control (PBS) treatments (Figure 3E). The average number of CFU in 48 h colonies from samples exposed to PBS was $6.57 \pm 0.56 \times 10^7$ CFU/colony. CFU from normal sized colonies obtained following exposure to 5.0 μM H_2O_2 or 250 μM SPER/NO were similar to that determined after exposure to PBS ($5.58 \pm 0.75 \times 10^7$ and $5.79 \pm 0.86 \times 10^7$ CFU/colony, respectively). The NO-induced microcolonies contained a significantly reduced number of CFU as compared to that determined for all other samples ($2.06 \pm 0.30 \times 10^7$ CFU/colony).

To determine if the reduced bacterial number in microcolonies was due to a permanent (e.g., DNA damage) or a transient (e.g., reversible metabolic inhibition) alteration, growth characteristics of bacteria recovered from each colony type were assessed in liquid culture

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with an optimal medium (Stainer-Scholte) and in an airway surface liquid-like medium (ASL mimic). In both cases, pre-exposure of *B. bronchiseptica* to redox stress did not adversely affect growth (Figure 4). Additionally, *B. bronchiseptica* sub-cultured from microcolonies resulted in normal sized colonies after subsequent plating onto solid medium (data not shown). These data are consistent with a transient bacteriostatic effect of NO in the development of *B. bronchiseptica* microcolonies.

DISCUSSION

For succesful colonization of the respiratory tract, pathogens must overcome innate immune defenses including mucociliary clearance and antimicrobial molecules that togther provide a non-specific, but robust, host defense (Ellerman & Bisgaard, 1997, Diamond, et al., 2000, Ganz, 2002, McCormack & Whitsett, 2002). *In vivo* analyses suggest RNS and ROS are important antimicrobial products in the airway. Loss of iNOS or the ROS-producing enzyme phagocyte oxidase in mice increases susceptibility to microbial infection (Nathan & Shiloh, 2000), including *B. pertussis* (Canthaboo, et al., 2002). The synergistic effects of these enzymes in immune defense are emphasized in double knockouts, where increased spontaneous infection by commensal microbes results in a high incidence of animal death (Shiloh, et al., 1999).

Both constitutive and inducible forms of NO synthases are expressed in the airway epithelium from a variety of *Bordetella* hosts (Asano, et al., 1994, Robbins, et al., 1994, Robbins, et al., 1994, Guo, et al., 1995, Rochelle, et al., 1998), allowing production of RNS near the cilia where bordetellae specifically bind *in vitro* and during colonization of the host airway (Goodnow, 1980, Soane, et al., 2000, Locht, et al., 2001, Edwards, et al., 2005). NO production has been measured in the proximity of a single endothelial cell *in vitro* at a concentration of 400–500 nM (Malinski & Taha, 1992). Because iNOS can predominate NOS species in respiratory epithelial cells, and the capacity of this isoform to produce NO is significantly higher than cNOS found in endothelial cells (Ricciardolo, 2003), we therefore considered 400 nM to be a lower concentration for NO in the ASL. Similarly, H_2O_2 has been measured in exhaled breath condensates at low micromolar levels (Jobsis, et al., 1998). Although sources for H_2O_2 are not as well defined as those for NO, airway epithelial cells can produce H_2O_2 for release into the ASL via nonphagocytic NADPH oxidases (Geiszt, et al., 2003, Lambeth, 2004, Moskwa, et al., 2007, Pantano, et al., 2007). The intimate interaction between bordetellae and ciliated epithelial cells of the host places this pathogen in an environmental niche enriched in redox species.

During the initial phase of a respiratory tract infection the bacterial load can be extremely low; *B. bronchiseptica* airway infection can be established in healthy animals with doses below 20 CFU (Harvill, et al., 1999). The experiments outlined in this report, where low bacterial numbers (~5,000 CFU) were used to assess survival against physiological concentrations (nM-μM) of redox active species, represent at least a 20-fold reduction in the bacterial density as compared to studies in which high concentrations of RNS and/or ROS were used to induce logarithmic reductions in bacterial viability (Brunelli, et al., 1995, Pacelli, et al., 1995, Hurst & Lymar, 1997). However, the low bacterial load during the initial stage of an infection suggests the need for only a slight bias towards the host in the host-pathogen interplay for an effective innate immune function. Moreover, adverse effects of RNS and ROS on the airway mucosa prevent the release of high levels of redox active species into ASL as an innate immune mechanism. In fact, prolonged incubations (>24 h) of hamster tracheal organ cultures with *B. pertussis* induced sufficient NO production to damage the airway epithelium and favor bacterial colonization (Flak & Goldman, 1996, Flak & Goldman, 1999). Similarly, high concentrations of H_2O_2 in prolonged exposure experiments (>25 μM for 24 h) reduced the viability of cultured human airway epithelial

cells (Spencer, et al., 1995). The synchronized production of NO and H_2O_2 to lower effective redox concentrations can limit damage to host cells and, as illustrated in Figure 2, simultaneously potentiate the antibacterial activity of the redox active species. In summary, the low CFU and physiologically relevant concentrations of redox-active species used herein may be more representative of the bacteria-to-reactant ratios found in the respiratory tract and thus allow for a more relevant evaluation of the actual contribution of RNS/ROS in airway innate immunity.

The mechanisms of antimicrobial activity induced by a particular redox species is dependent upon a number of factors including concentration, chemical stability, ability to cross hydrophobic membranes, reactivity, and spatio-temporal relationship with other redox species. Figure 5 illustrates some of the complexity of RNS and ROS reactions derived from enzymes known to be expressed in airway epithelial cells (Asano, et al., 1994,Rochelle, et al., 1998,Forteza, et al., 2005,Moskwa, et al., 2007). For example, in the presence of CO_2 the reaction between NO and $O_2{}^{\cdot -}$ (Espey, et al., 2002) can give rise to the free radicals, $NO_2{}^{\cdot}$ and $CO_3{}^{\cdot -}$ (Squadrito & Pryor, 2002), which themselves can have antimicrobial activity. Furthermore, although NO and H_2O_2 do not directly react with each other, $NO_2{}^{-}$, the end product of aqueous NO autoxidation can be oxidized to NO_2 by peroxidases as well as free heme and metals (Kono, et al., 1994,van der Vliet, et al., 1997,Sampson, et al., 1998,Wu, et al., 1999,Thomas, et al., 2002). At present, we cannot identify specific targets for bacteriocidal or bacteriostatic activity of individual redox-active species produced by airway epithelial cells. However, differences between NO and H_2O_2 in inducing microcolony formation indicate that NO is both bacteriocidal and bacteriostatic and thus that NO might have several targets within the bacterial cell.

In this study, the responses of low CFU of *B. bronchiseptica* to redox species at physiologically relevant doses have been established, and the data show that minor alterations in redox stress level can result in significant antibacterial effects. Our model for the analysis of RNS and ROS is, to our knowledge, the first such model established using a pathogen highly adapted to colonization of the healthy conducting airway. Further, the established antibacterial effects of low concentrations of redox active species together with the complexity of reactions involving redox active species in ASL encourages analyses to identify specific effector molecules and their targets within the bacterial cells during *in vitro* exposure or following controlled production by the airway epithelium *in vivo*.

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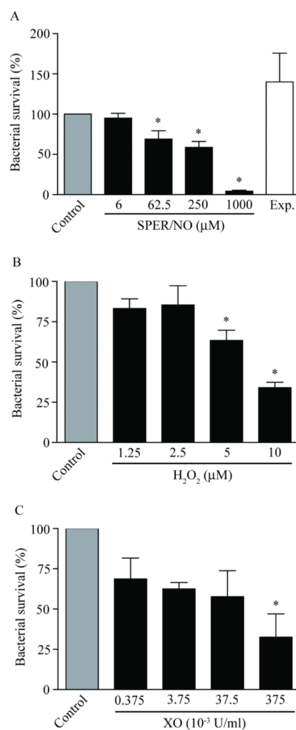

Figure 1. Dose-dependent antimicrobial activity of prototypical airway redox species

Approximately 5,000 CFU *B. bronchiseptica* were exposed to A) NO (generated from SPER/NO at the listed micromolar concentrations); B) H_2O_2 at final μM concentrations from diluted stock; or C) $O_2^{\cdot -}$ (reported as xanthine oxidase concentration in U/ml). All the redox species tested displayed dose-dependent antimicrobial activity against *B. bronchiseptica*. Bacterial survival was determined after a 5 h exposure period (± SEM) as compared to buffer controls and are representative of at least three independent experiments. "Exp" in A indicates experiment conducted with expired SPER/NO (1000 μM). "*" indicates statistical significance relative to matched controls ($P < 0.05$).

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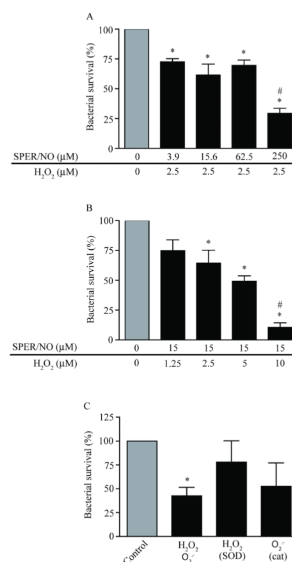

Figure 2. Combinations of NO and H_2O_2 augment their antimicrobial activity

A) Approximately 5,000 CFU of *B. bronchiseptica* were exposed to a sub-antimicrobial concentration of H_2O_2 (2.5 μM) in combination with a concentration gradient of SPER/NO. B) *B. bronchiseptica* were exposed to a sub-antimicrobial concentration of SPER/NO (15 μM) in combination with a concentration gradient of H_2O_2. The addition of a sub-antimicrobial concentration of either NO or H_2O_2 augmented the antimicrobial activity of the other redox agent. C) *B. bronchiseptica* were subjected to incubation in reaction buffer containing both H_2O_2 and $O_2^{\cdot-}$. SOD and catalase were included as experimental controls to verify the augmentative effect of H_2O_2 and $O_2^{\cdot-}$ in combination. In each case significant antimicrobial activity was measured at concentrations of redox species that were not effective individually. Bacterial survival was determined after a 5 h exposure period (± SEM) as compared to buffer controls and are representative of at least three independent experiments. "*" indicates statistical significance relative to control ($P < 0.05$). "#" indicates a statistically significant difference as compared to either 250 μM SPER/NO alone (Figure 2A) or to 10 μM H_2O_2 alone (Figure 2B) ($P < 0.05$).

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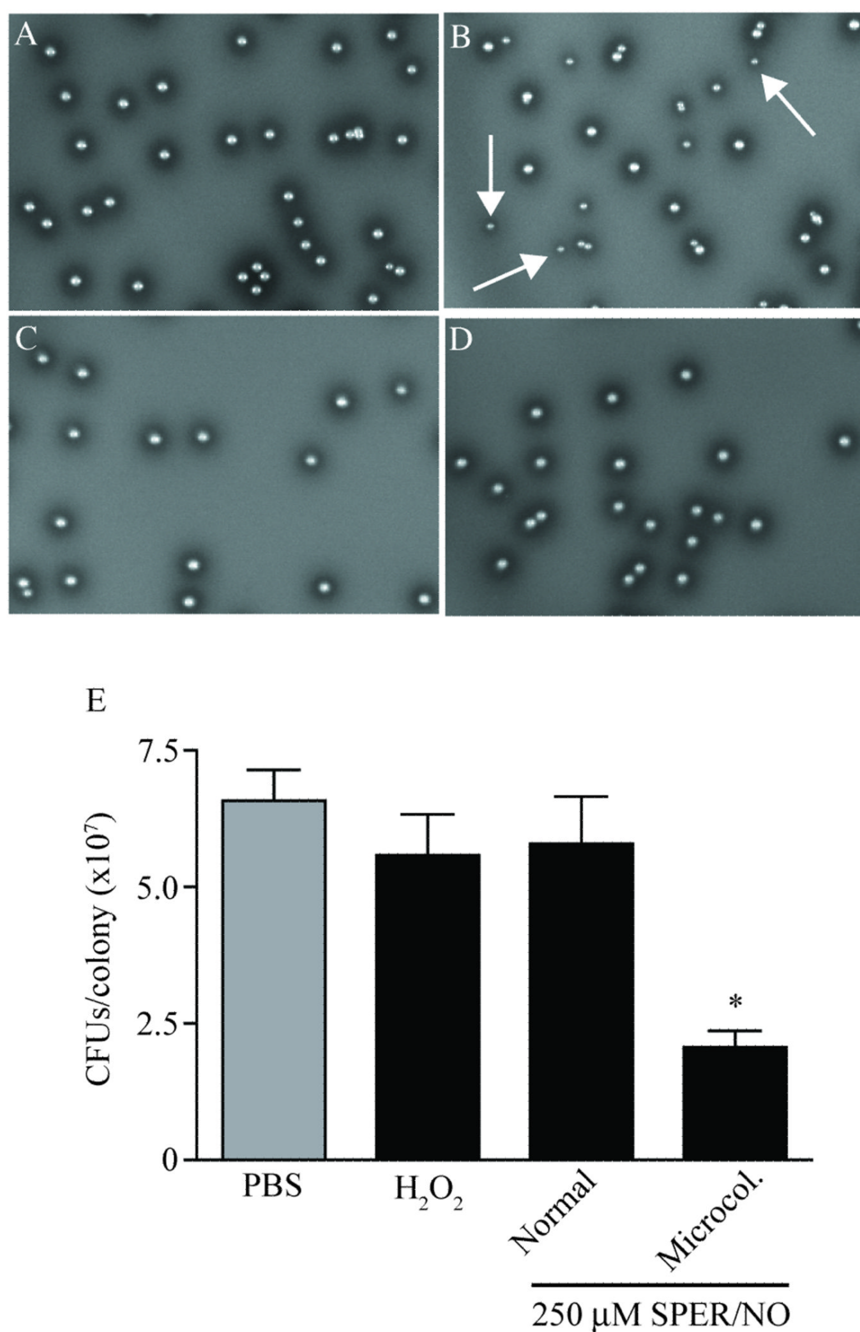

Figure 3. Differential antimicrobial effects on *B. bronchiseptica* for NO and H_2O_2 at LD_{50}

B. bronchiseptica were cultured on BG agar for 48 h following exposure of ~5,000 CFU to A) PBS, B) 250 µM SPER/NO, C) 5 µM H_2O_2, or D) 250 µM expired SPER/NO. The production of microcolonies was observed only after exposure to SPER/NO (B, white arrows). E) Microcolonies contained a significantly reduced number of CFU as compared to normal size colonies when grown on BG agar plates. Values are presented ± SEM. Results represent data from a total of 40 colonies collected from at least two independent experiments. "*" indicates statistical significance relative to all other samples ($P < 0.05$).

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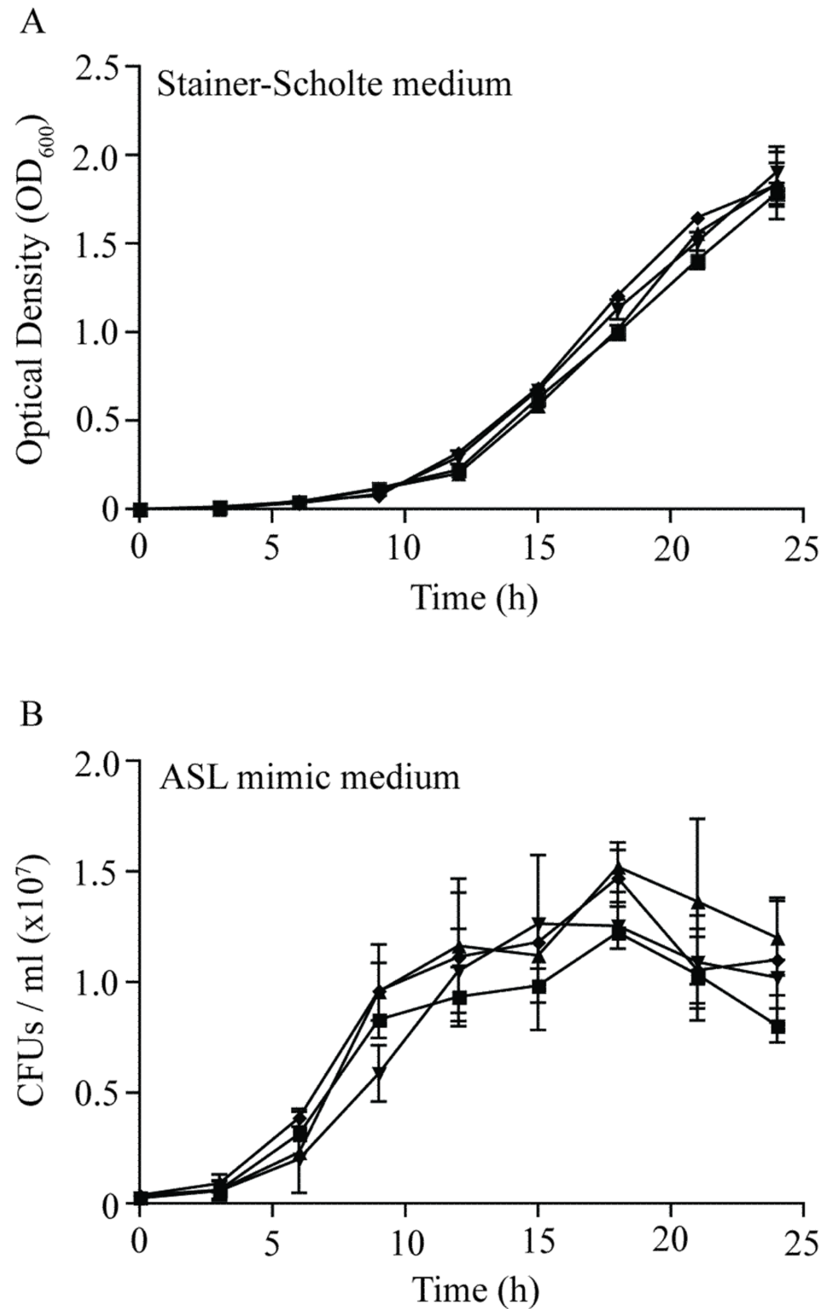

Figure 4. *B. bronchiseptica* growth in optimal and sub-optimal medium following exposure to redox stress

B. bronchiseptica cultured on BG agar following exposure to redox stress were resuspended in PBS to an OD_{600} of 0.3 and transferred to liquid medium for growth analysis in A) fully supplemented Stainer-Scholte medium or B) minimal medium representative of the ASL. ν - PBS; σ - microcolonies (250 μM SPER/NO); τ - normal sized colonies (250 μM SPER/NO); υ - H_2O_2. Growth in Stainer-Scholte medium was monitored spectrophotometrically (OD_{600}) while growth in ASL mimic medium was monitored by determination of CFU/ml at 3 h intervals. Pre-exposure to redox agents did not adversely affect growth. These data are

consistent with a transient bacteriostatic effect of NO to produce microcolonies that is not evident in the H_2O_2 challenge or control experiments.

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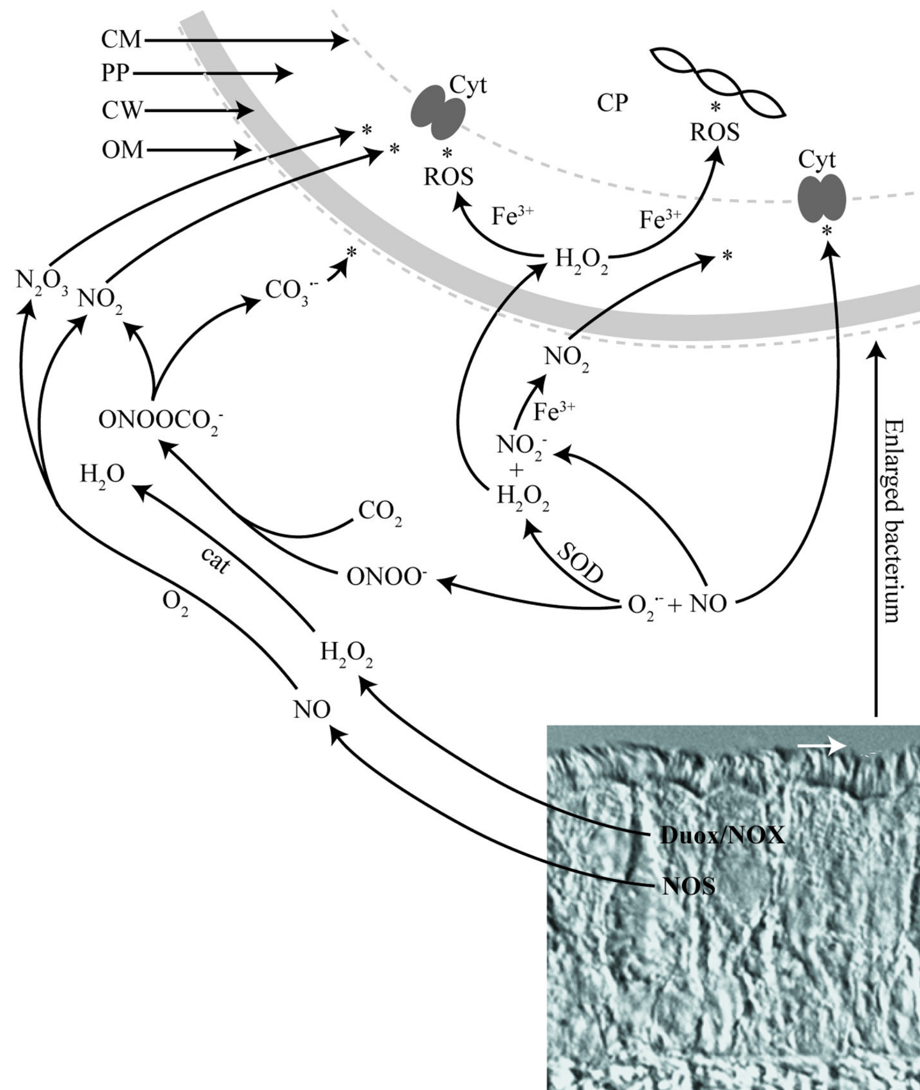

Figure 5. Role of RNS and ROS as antimicrobial agents at the airway mucosa

DIC of tracheal section (Rabbit) showing pseudostratified airway epithelium with ciliated lining oriented toward the tracheal lumen. The white arrow points to the area where bacteria (in white) first interact with airway host cells on the ciliated surface. Theoretical reactions based on release of NO and H_2O_2 and potential targets (asterisks) within an invading bacterium (enlarged) are illustrated. The site of activity for individual redox species depends in part on their ability to cross biological membranes. NO can arise from NOS while H_2O_2/ $O_2^{\cdot-}$ can be produced from NOX or Duox in airway epithelial cells. Release of RNS and ROS into the ASL can support generation of numerous redox species with potential antibacterial activity. In addition to the antibacterial potential of redox species, ASL components including surfactant and mucins also contribute to the antibacterial defense of the airway (not shown). The combined effects of all antibacterial components (not shown) at the airway mucosa constitute the full chemical barrier of the airway innate immune system. *In vitro* analysis shows that NO and H_2O_2 dependent antimicrobial activity can contribute significantly to the chemical barrier against *B. bronchiseptica*. Abbreviations: CP,

cytoplasm; CM. cytoplasmic membrane; PP, periplasm; CW, cell wall; OM, outer membrane; Cyt, cytochrome.

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