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Title: Nitrite Modulates Bacterial Antibiotic Susceptibility and Biofilm Formation in Association with Airway Epithelial Cells

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Abstract: *Pseudomonas aeruginosa* is the major pathogenic bacteria in cystic fibrosis and other forms of bronchiectasis. Growth in antibiotic resistant biofilms contributes to the virulence of this organism. Sodium nitrite has antimicrobial properties and has been tolerated as a nebulized compound at high concentrations in human subjects with pulmonary hypertension; however, its effects have not been evaluated on biotic biofilms or in combination with other clinically useful antibiotics. We grew *P. aeruginosa* on the apical surface of primary human airway epithelial cells to test the efficacy of sodium nitrite against biotic biofilms. Nitrite alone prevented 99% of biofilm growth. We then identified significant cooperative interactions between nitrite and polymyxins. For *P. aeruginosa* growing on primary CF airway cells, combining nitrite and colistimethate resulted in an additional log of bacterial inhibition compared to treating with either agent alone. Nitrite and colistimethate additively inhibited oxygen consumption by *P. aeruginosa*. Surprisingly, while the antimicrobial effects of nitrite in planktonic, aerated cultures are nitric oxide (NO) dependent, antimicrobial effects in other growth conditions are not. The inhibitory effect of nitrite on bacterial oxygen consumption and biofilm growth did not require NO as an intermediate as chemically scavenging NO did not block growth inhibition. These data suggest an NO-radical independent nitrosative or oxidative inhibition of respiration. The combination of nebulized sodium nitrite and colistimethate may provide a novel therapy for chronic *P. aeruginosa* airway infections, because sodium nitrite, unlike other antibiotic respiratory chain 'poisons', can be safely nebulized at high concentration in humans.



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Dr. Harry Ischiropoulos
Associate Editor
Free Radical Biology & Medicine Journal

Dear Dr. Ischiropoulos:

Attached please find our revised manuscript entitled "Nitrite modulates bacterial antibiotic susceptibility and biofilm formation in association with airway epithelial cells" by Anna Zemke, Sruti Shiva, Jane Burns, Samuel Moskowitz, Joseph Pilewski Mark Gladwin and Jennifer Bomberger (Manuscript No. FRBM-D-14-00520). We have addressed all of the comments by you and the reviewers, and we thank you for making important recommendations and suggestions. As a result of the revisions, the manuscript has greatly improved, and we hope it is now suitable for publication in the Free Radical Biology & Medicine Journal. Our responses to the comments are summarized below.

Reviewer #1:

1. The main issue I have is the title - I do not think that you have proved that nitrite is working through an NO independent mechanism. The evidence that you give for this is lack of inhibition of effect with CPTIO may be due to the inability of this substance to penetrate the biofilm in the experimental set-up. My understanding is that nitrate and nitrite will be toxic to *Pseudomonas* species if nitric oxide reductase enzymes are blocked. NO reductase rapidly reduced NO to N₂O. Could it be that the polymyxin interferes with NO reductase? This could be determined by measuring the formation of N₂O in these experiments, or, alternatively, looking at the effect of NO or an alternative NO donor when combined with the polymyxin antibiotic. At least the importance of *pseudomonas* NO reductase (which has been very well characterised - see <http://www.jbc.org/content/266/34/22785.short> by Braun - this paper shows that NO reductase inactivation is lethal to *pseudomonas* organisms exposed to nitrite) should be considered in the discussion.

At the reviewer's request, we have modified the title of the manuscript. We have also addressed a potential role for *P. aeruginosa* NO reductase in regulating the antimicrobial effect we observe in anaerobic conditions in the Discussion section (lines 413-418) and have referenced the Braun manuscript in the Introduction (lines 108-109).

Regarding the concern that CPTIO does not penetrate the biofilm: CPTIO is added one hour into the biofilm assay at which point microcolonies have begun to form but there is no EPS at this point. The concern is quite valid, particularly if experiments were to be done studying the effect of CPTIO and nitrite on mature biofilms. Because of these concerns, we have changed the title as suggested, and discussed this technical aspect in more detail (lines 424-426).

It is quite possible that the inner membrane damage induced by polymyxins also block denitrification pathway activity, however due to methodological constraints we did not test this hypothesis. As the reviewer points out, this may be an additional reason why *P. aeruginosa* is more sensitive to nitrite and polymyxins under anaerobic conditions (see the data in Table 1).

Reviewer #2:

1. Page 3, Line 4-6: This sentence is redundant since the issue elucidating the colistin effect was outlined one already in the prior paragraph.
Figure text figure 5. Second last line. Replace "of" by "or".

At the reviewer's request, we have corrected the described grammatical errors and removed the described text redundancy.

2. Please expand the issue of nitrite toxicity in the discussion section since the applied concentrations (300mM) are exorbitant high.

To address this important concern, we have performed additional experiments to measure the integrity of the airway epithelial barrier (via transepithelial electrical resistance measurements, TEER) and airway epithelial cytotoxicity (via lactate dehydrogenase release assays). Barrier function is a sensitive and early marker of epithelial dysfunction, so TEER measurements were done for both CFBE-wt and primary CF HBE cells. As reported in Figure 1F, epithelial barrier function was not compromised until 150 mM sodium nitrite for the CFBE-wt cell line and remained intact at 300 mM sodium nitrite in the primary CF HBE cells (noted in text). We retained the 150 mM concentration in Figure 1E to demonstrate that the antimicrobial effect of nitrite had plateaued by 50 mM, however because of concerns about cytotoxicity in later figures only concentrations at or below 50 mM were used with CFBE-wt cells. Lactate dehydrogenase (LDH) release assays were also performed as a secondary method to assess cytotoxicity and demonstrated no increase in LDH release with the sodium nitrite concentrations presented in the manuscript (data not shown). We have also added greater detail in the Results and Discussion sections to address concerns of cytotoxicity to the epithelium (see lines 382-390, 418-420).

Reviewer #3:

1. Results presented in Figure 1: Can the authors calculate a dose-response relationship from the current data, which could show the LC50s for nitrite on biofilm formation?

The potency of antibiotics (and a particular organisms' susceptibility to any particular antibiotic) is typically expressed as minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC). There are no widely established methodologies for determining an MBC for biofilms in general or biotic biofilms specifically. Even less established are methodologies for determining values such as LC50 in biofilm models, consequently we are not reporting the data as an LC50.

2. In Figure 1 very high nitrite concentrations were used as final concentrations. Is this toxic for any kind of cell - also including alveolar epithelium? What are dose-response curves for nitrite and alveolar epithelium particularly in comparison to bacteria.

To address this important concern, we have performed additional experiments to measure the integrity of the airway epithelial barrier (via transepithelial electrical resistance measurements) and airway epithelial cytotoxicity (via lactate dehydrogenase release assays). Please see the response to Reviewer 2, comment 2 above.

3. In Figure 1 very high millimolar concentrations were used to assess the toxicity on *Pseudomonas aeruginosa*. Using e.g. nitrite inhalation will such high concentrations be reached in the lungs? The review recalls from the authors previous studies that - at least in plasma - nitrite inhalation causes an increase in the micromolar range only. Please comment.

The concentration of inhaled nitrite is slightly over 1 M and roughly 1 ml is inhaled. The airway surface liquid in a healthy lung is 7 ml, so low millimolar concentrations are probably achievable in the airway surface liquid. Because ASL cannot be sampled noninvasively, these are estimates. While Figure 1 goes up to 150 mM, the main effect is between 5-50mM. Low mM nitrite concentrations in this range in the airway surface liquid are likely achievable.

4. In Figure 2 (effects for the combination of colistimethate and nitrite). Can the authors use e.g. 2-way ANOVA or any kind linear regression testing to assess the statistical effect of both treatments alone as well as their combination?

At the reviewer's request, we have reanalyzed the results of Figure 2 and Figure 3, using a 2-way ANOVA (using GraphPad Prism software package). The data presented in both figures is significant, as assessed by 2-way ANOVA (Figure 2 p value of <0.0001 and Figure 3 p value of 0.0056, respectively).

We thank you and the reviewers for the time and effort in reviewing our manuscript, and recommending the necessary additions and changes that resulted in improvement of the manuscript. We sincerely hope that our paper is now suitable for publication in the FRBM Journal.

Sincerely,

A handwritten signature in purple ink that reads "Jennifer Bomberger". The signature is cursive and stylized.

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*Highlights (for review)

- Nitrite prevents *P. aeruginosa* biofilms on human CF airway epithelial cells.
- Nitrite and polymyxins additively inhibit bacterial respiration.
- Nitrite and colistimethate additively inhibit biofilm growth on airway cells.

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3 Nitrite Modulates Bacterial Antibiotic Susceptibility and Biofilm Formation in Association with Airway
4 Epithelial Cells

5 Running title: Colistin and nitrite prevent *P. aeruginosa* biofilms

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27 Conflict of interest: Dr. Gladwin is listed as a co-inventor on an NIH government patent for the use of
28 nitrite salts in cardiovascular diseases. Dr. Gladwin consults with Mast-Aires Pharmaceuticals on the
29 development of a phase II proof of concept trial using inhaled nitrite for pulmonary arterial hypertension.

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40

Abstract

Pseudomonas aeruginosa is the major pathogenic bacteria in cystic fibrosis and other forms of bronchiectasis. Growth in antibiotic resistant biofilms contributes to the virulence of this organism. Sodium nitrite has antimicrobial properties and has been tolerated as a nebulized compound at high concentrations in human subjects with pulmonary hypertension; however, its effects have not been evaluated on biotic biofilms or in combination with other clinically useful antibiotics. We grew *P. aeruginosa* on the apical surface of primary human airway epithelial cells to test the efficacy of sodium nitrite against biotic biofilms. Nitrite alone prevented 99% of biofilm growth. We then identified significant cooperative interactions between nitrite and polymyxins. For *P. aeruginosa* growing on primary CF airway cells, combining nitrite and colistimethate resulted in an additional log of bacterial inhibition compared to treating with either agent alone. Nitrite and colistimethate additively inhibited oxygen consumption by *P. aeruginosa*. Surprisingly, while the antimicrobial effects of nitrite in planktonic, aerated cultures are nitric oxide (NO) dependent, antimicrobial effects in other growth conditions are not. The inhibitory effect of nitrite on bacterial oxygen consumption and biofilm growth did not require NO as an intermediate as chemically scavenging NO did not block growth inhibition. These data suggest an NO-radical independent nitrosative or oxidative inhibition of respiration. The combination of nebulized sodium nitrite and colistimethate may provide a novel therapy for chronic *P. aeruginosa* airway infections, because sodium nitrite, unlike other antibiotic respiratory chain 'poisons', can be safely nebulized at high concentration in humans.

Keywords

Pseudomonas aeruginosa, biofilm, sodium nitrite, colistimethate, colistin, polymyxin

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INTRODUCTION

68 In cystic fibrosis (CF), chronic airway infection results in bronchiectasis and cycles of airway
69 inflammation that ultimately lead to early death from respiratory failure. *Pseudomonas aeruginosa* is the
70 most common pathogen in teenagers and adults with CF. Once chronic airway infection is established, *P.*
71 *aeruginosa* becomes very difficult to eradicate because of multiple resistance mechanisms including
72 intrinsic antibiotic tolerance, selection of antibiotic-resistant subpopulations by decades of antibiotic
73 exposure, and bacterial growth in biofilms (1-3).

74 The high metabolic activity of *P. aeruginosa* and neutrophils in and around mucus plaques
75 depletes oxygen at the airway surface, such that biofilm growth is largely anaerobic, with bacteria
76 subsisting through denitrification (4). *Ex vivo* evidence of denitrification in the airway was recently
77 established by detecting nitrous oxide, a unique product of bacterial denitrification, in sputum samples
78 from patients with CF (5). Anaerobic growth confers resistance to many antibiotics that clinicians
79 commonly use against *P. aeruginosa*, in part through alterations in efflux pump expression (1, 6). Further
80 complicating the search for new antimicrobial approaches to *P. aeruginosa* is the difference in behavior of
81 biofilms grown in the presence of airway cells compared to those grown on abiotic surfaces (glass or
82 plastic). The former, known as “biotic” biofilms, can be >100-fold more resistant to antibiotics than
83 predicted by conventional susceptibility testing. This makes extrapolation of data derived from
84 planktonic experiments difficult to translate to performance against biotic biofilms and limits our
85 understanding of the *in vivo* activity of existing drugs (2).

86 Sodium nitrite has long been known to have antimicrobial properties as a food preservative.
87 Nitrite may also contribute to host defense against *Helicobacter pylori* and *Clostridium difficile* (7, 8). The
88 antimicrobial action of the nitrite moiety is due in part through generation of NO, inactivation of Fe-S
89 containing proteins, and inhibition of bacterial respiration (9-11). Within the lung, inhaled nitrite salts
90 are converted to NO through reductive reactions with heme- and molybdenum-containing enzymes such

91 as hemoglobin and xanthine oxidoreductase, and potentially through bacterial metabolism. NO has a
92 half-life of milliseconds *in vivo* (reviewed in (12, 13)). There are ongoing attempts to adapt inhaled NO as
93 an antimicrobial agent, but the short half-life makes delivery cumbersome (14, 15). Nitrite has a half-life
94 of 50-60 minutes *in vivo* when delivered intravenously, allowing intermittent dosing (16). An ongoing
95 Phase 2b clinical trial is currently evaluating the safety and efficacy of nebulized sodium nitrite in
96 pulmonary hypertension; inhaled nitrite has thus far been well tolerated at concentrations near 1 molar
97 (ClinicalTrials.gov locator NCT01431313, M. Gladwin personal communication). The availability of
98 pharmaceutical-grade sodium nitrite for inhalation improves the feasibility of using nitrite as an
99 antimicrobial agent in CF airway infection.

100 Previous work has shown that sodium nitrite has pH-dependent antimicrobial activity against *P.*
101 *aeruginosa*, with activity at pH 6.5 being best studied (hereafter “nitrite” refers to sodium nitrite in
102 solution at pH 6.5)(17). At micromolar concentrations, nitrite is a potential substrate for anaerobic
103 energy generation through denitrification, however at millimolar concentrations it inhibits anaerobic
104 growth (11, 18). Beyond direct bactericidal effects, nitrosative stress also affects virulence of *P.*
105 *aeruginosa* by inactivating pyocyanin and increasing production of alginate (19, 20). Additionally, nitrite
106 has antimicrobial activity against a wide variety of other pathogens found in CF, including *Burkholderia*
107 *cepacia* complex and *Staphylococcus aureus* (21, 22). When nitric oxide reductase activity is lost, nitrite
108 becomes growth inhibitory in *P. stutzeri* (23). Whether nitrite itself or the NO produced from nitrite can
109 prevent biotic biofilm growth is unknown. The interactions between nitrite and commonly used
110 antibiotics are also poorly understood. The goals of this study were to determine the effect of sodium
111 nitrite on *P. aeruginosa* biotic biofilms and screen nitrite for interactions with other commonly used
112 antibiotics in the CF population. We focused primarily on colistin because previous work has shown that
113 it targets the inner core of highly structured abiotic biofilms where the oxygen tension is lowest,
114 suggesting activity against anaerobically growing *P. aeruginosa* (24, 25). The effect of polymyxins on *P.*
115 *aeruginosa* clinical isolates grown under anaerobic conditions has not been extensively studied but
116 available data suggest decreased susceptibility when compared to aerobic growth (6).

117 Polymyxins are polycationic lipopeptide antibiotics that interact with negatively charged
118 lipopolysaccharide at the outer membrane of Gram-negative bacteria. Polymyxins initially increase outer
119 membrane permeability and, after diffusing across the periplasmic space, disrupt the inner (cytoplasmic)
120 membrane. Bacterial death ensues within minutes (26). Colistimethate is a prodrug of colistin
121 (polymyxin E) that can be administered by inhalation for the treatment of chronic airway infections in
122 both CF and non-CF bronchiectasis (27). In 2005, 9% of CF patients in the United States routinely inhaled
123 an aerosol form of intravenous colistimethate for the treatment of chronic airway infection, and as of
124 2012, approximately 11% of adult CF patients were doing so (28, 29). A dry powder colistimethate
125 inhaler approved to treat CF airway infection in Europe is designed to make delivery more convenient
126 (30).

127 The primary objective of this study was to investigate the role of nitrite in preventing biotic
128 biofilm growth and the potential interactions between nitrite and colistimethate. We tested the
129 hypotheses (1) that the inhibition of biofilm growth by nitrite is NO-independent and (2) that the
130 cooperative interaction between nitrite and colistimethate is due to NO independent respiratory
131 inhibition.

132 MATERIALS AND METHODS

133
134 **Bacterial strains:** The following bacterial strains were studied: *Pseudomonas aeruginosa* strains PA14
135 and PA01 (gift of George O'Toole, Geisel School of Medicine at Dartmouth)(31), ten "late" *P. aeruginosa*
136 clinical isolates from the University of Washington collection previously published in (32), 8 previously
137 published *Achromobacter sp.* isolates and ten *Burkholderia sp.* isolates from the Cystic Fibrosis
138 Foundation *Burkholderia cepacia* Research Laboratory and Repository at the University of Michigan (33)
139 and colistin resistant *P. aeruginosa* isolates (described in (34)).

140 **Reagents:** Colistin sulfate, sodium nitrite, hydrogen peroxide, potassium cyanide, sodium azide,
141 paraquat, Luria broth (LB), Luria broth agar, polymyxin B sulfate and polymyxin b nonapeptide were all
142 obtained from Sigma (St. Louis MO), Other reagents obtained as follows: colistimethate (XGen

143 pharmaceuticals, Lot A72649 and A99296, Big Flats NY), Carboxy-PTIO (Cayman Chemicals, Ann Arbor
144 MI).

145 **Bacterial Growth Methods:** 5ml bacterial cultures were grown on a roller drum at 100 rpm for 16-18
146 hours prior to the start of the experiment in LB. Unless otherwise indicated, bacteria were “returned to
147 log phase” by diluting overnight cultures 1:100 in fresh LB and placing cultures on a roller drum at 37°C
148 for 2 hours. During experiments combining colistimethate, colistin sulfate, azide, cyanide and CPTIO,
149 200µl of “log phase” cultures were grown for additional 5.5 hours in a 96 well plate at 37°C on a nutating
150 rocker.

151 **Biotic Biofilm Imaging:** To image epithelial-bacterial co-cultures, live-cell imaging was used as
152 described in (31). Briefly, we used a FCS2 closed system, live-cell chamber from Biopetechs (Butler, PA).
153 The immortalized human bronchial cell line (CFBE41o-) derived from a $\Delta F508/\Delta F508$ patient with
154 stable expression of wild-type CFTR was used (gift from Bruce Stanton, referred to as CFBE-wt). Biofilms
155 of GFP expressing *P. aeruginosa* strain PAO1 were grown on CFBE-wt cells seeded onto glass coverslips in
156 a flow chamber. Bacteria were inoculated at approximately 25 MOI and allowed to attach to airway
157 epithelial cells for two hours without media flowing. Biofilms were then grown for four hours with
158 minimum essential media (MEM) flowing at 20 ml/hr. After a total of six hours, z-stack images were
159 taken of 6-10 random fields from each chamber using a Nikon Ti-inverted microscope. Biofilm biomass
160 was measured with COMSTAT image analysis software(35). Nitrite treated chambers were exposed to
161 15mM sodium nitrite in pH 6.5 minimal essential medium for 4 hours following bacterial attachment,
162 while control chambers were exposed to pH 6.5 MEM alone. The experiment was done in triplicate.
163 More detail for this protocol can be found in the descriptions by Moreau-Marquis (31, 36).

164 **Liquid Culture Experiments:** For time-kill assays to measure bactericidal activity of polymyxins with
165 other compounds, overnight cultures were diluted in pH 6.5 LB, returned to log phase growth at 37°C,
166 and then treated with nitrite and/or polymyxins and grown for an additional 5.5 hours. The number of
167 live bacteria was then determined by plating in a colony forming unit assay (CFU assay). For nitrite
168 pretreatment assays: bacteria were grown as above in 15mM nitrite and after 5.5 hours the cultures were

169 pelleted at 6000xg for 5 min and re-suspended in media containing colistimethate. The cultures were
170 then incubated for 60 minutes and bacteria were counted in a CFU assay.

171 **Co-culture experiments:** Conducted as previously described (31) with the following modifications:.

172 Primary human bronchial epithelial cells (HBE) were cultured from explanted lungs of patients with CF,
173 under an Institutional Review Board approved protocol at the University of Pittsburgh (PRO11070367
174 and IRB970946). Cells were enzymatically dissociated, expanded in growth media, and seeded onto
175 Transwell inserts at air-liquid interface. Cultures were used when well polarized and differentiated (37).
176 For other experiments, the cell line CFBE-wt stably expressing wild-type CFTR were used as described
177 (31). CFBE-wt cells were seeded onto Transwell filters, grown at air-liquid interface, and used 7-10 days
178 after seeding. Both cell lines were used in *P. aeruginosa* – epithelial co-culture experiments as follows
179 (described in detail in (31)). Biofilm prevention assays were done as follows: overnight cultures of *PAO1*
180 were rinsed once and added to the apical surface of the epithelial cells in 500 μ l of minimal essential
181 media with glutamate at an MOI of 25. After the 60 minute bacterial attachment period, developing
182 biofilms were treated with 300 mM sodium nitrite, 20 μ g/ml colistimethate, both agents or 300mM
183 sodium chloride as a tonicity control for the subsequent 5 hours. At the end of this period, biofilms were
184 removed with 0.1% Triton-X 100 and live bacteria were plated in a colony forming unit assay.

185 **Agar Dilution MIC under aerobic and anaerobic conditions:** Agar dilution minimum inhibitory
186 concentration (MIC) experiments were used to screen multiple clinical isolates for nitrite/polymyxin
187 cooperativity. The MIC protocol by Wiegand et al. was used with the following modifications (38).
188 Checkerboards included two-fold dilutions of colistin sulfate from 0.125 μ g/ml to 16 μ g/ml. Nitrite
189 concentrations from 0.5mM to 32mM were used. The pH of molten agar was adjusted to pH 6.5 with HCl.
190 The agar was cooled to 50°C and increasing concentrations of colistin sulfate and/or nitrite were added.
191 Plates were poured and used immediately. For aerobic experiments, plates were read 18 -20 hours
192 later. For highly colistin resistant isolates, plates were grown for 48-72 hours because control growth of
193 these organisms is very slow. To determine anaerobic MICs for *Pseudomonas aeruginosa* clinical isolates,

194 MHB plates were made as described above with the addition of 1% potassium nitrate (Sigma). An
195 anaerobic environment was created using GasPak EZ Anaerobe Container System and BBL Dry Anaerobic
196 Indicator Strips (BD Franklin Lakes, NJ) were used for every experiment to assure an anaerobic
197 environment. Plates were incubated for 2 days for anaerobic MIC determinations.

198 **Oxygen Consumption measurements:** Bacteria were diluted in LB and treated with nitrite and/or
199 colistimethate for 30 min at 37°C. Oxygen consumption was measured with a Clark-type Instech Oxygen
200 Electrode, model #125-05 with a YSE Biological Oxygen Monitor, model 5300A and calculated from the
201 slope. Measurements were done in triplicate on different days and data are displayed as % control
202 oxygen consumption with all points from each day normalized to the control oxygen consumption from
203 that day. Representative tracings show the raw data. The tracing have been overlayed for easier visual
204 comparison.

205 **Cytotoxicity Assays:** Lactate dehydrogenase release assays were done using the CytoTox 96 Non-
206 Radioactive Cytotoxicity Assay kit (Promega Madison, WI), following the manufacturer's instructions.

207 **Transepithelial Electrical Resistance Measurements:** Transepithelial electrical resistance (TEER) was
208 measured hourly for five hours on air-liquid interface differentiated CFBE-wt airway epithelial cells and
209 primary human airway epithelial cells (HBE) treated with sodium nitrite or sodium chloride using a
210 Ag/AgCl electrode (EVOM meter).

211 **Statistics:** All data are plotted as mean +/- standard deviation. Data were log transformed and tested by
212 one-way ANOVA for an effect (or two-way ANOVA as applicable). If an effect was present, a Tukey test
213 was used for individual comparisons using PRISM software (La Jolla, California).

215 RESULTS

216
217 **Nitrite prevents *P. aeruginosa* biofilm formation on airway epithelial cells.**

218 To test the ability of nitrite to prevent biofilm formation on airway epithelial cells, immortalized human
219 airway cells were grown as a confluent monolayer on glass coverslips. The *P. aeruginosa* strain PAO1
220 stably expressing green fluorescent protein (PAO1-GFP) was added to the surface of these cells in a flow
221 chamber and biofilm development was imaged as described (31). Under control conditions, robust
222 biofilm growth (seen in green in Figures 1A and 1C) is seen. In the presence of 15mM sodium nitrite,
223 biofilm growth was nearly absent (Figure 1B and 1D). Quantification of biofilm biomass revealed a 90%
224 reduction in biomass (numbers on panels 1C and 1D). *P. aeruginosa* biofilms grown in this model have
225 been previously characterized for expression of biofilm marker genes (*tolA*), repression of planktonic
226 marker genes (*fliC*), exopolysaccharide production and increased antibiotic resistance (31). To define the
227 dose-response relationship, a static co-culture system was used where *P. aeruginosa* biofilms were grown
228 on airway epithelial cells at an air-liquid interface for five hours (31). Dose-dependent prevention of
229 biofilm growth was observed in the range of 1.5 and 50 mM nitrite, with an effect ceiling at 50 mM nitrite
230 (Figure 1E). Transepithelial electrical resistance (TEER), a measure of epithelial barrier integrity,
231 decreased in CFBE-wt cells at nitrite doses exceeding 50 mM (Figure 1F), but there was no consistent
232 LDH release at doses up to 150 mM (data not shown). Further CFBE-wt experiments were conducted
233 with nitrite concentrations below 50mM. Of note, comparable decreases in transepithelial electrical
234 resistance were seen with sodium chloride at the same concentrations, so the decrease in TEER may be
235 due to hypertonicity rather than nitrite per se.

236
237 **Colistin sulfate demonstrates cooperative antimicrobial activity with nitrite against biotic**
238 **biofilms.** Colistimethate is in wide clinical use as an inhaled antibiotic, and previous work has shown
239 antimicrobial activity against abiotic biofilms (24, 25). The magnitude of activity of polymyxins against
240 biotic biofilms (i.e. biofilms grown in association with airway epithelial cells) is not known, so we used
241 the static co-culture system to test the ability of colistin to disrupt established biofilms. Figure 2A shows
242 that 2 µg/ml colistin sulfate disrupted one-half log of mature biofilm, while 20 µg/ml colistin sulfate
243 disrupted 3 logs (or 99.9%) of established biofilms. The airway concentration of active polymyxin

244 achieved through colistimethate nebulization are poorly characterized, however estimated
245 concentrations of colistin are near 10-20 $\mu\text{g}/\text{ml}$ (39, 40). In comparison to reported data using the
246 identical model system, the biotic biofilm antimicrobial activity of colistin sulfate was 2 logs better than
247 that reported for imipenem, 1 log better than ciprofloxacin and comparable to tobramycin at doses that
248 are achieved in the airway (31).

249 We next examined if nitrite could improve the antimicrobial activity of polymyxins. To determine
250 the interaction between polymyxins and nitrite, modified time-kill assays were used. Robust growth was
251 seen in the presence of up to 30 mM nitrite. No bacterial killing was seen with 70 $\mu\text{g}/\text{ml}$ colistimethate
252 (CMS) or 1.5 $\mu\text{g}/\text{ml}$ colistin sulfate (COL) in well aerated cultures (doses that are subinhibitory in *P.*
253 *aeruginosa* PA14 under these conditions). However, when nitrite was combined with subinhibitory
254 concentrations of colistin sulfate (Figure 2B) or colistimethate (Figure 2C) up to 4 additional logs of
255 bacteria were killed. Representative images of cultures grown with 15mM nitrite, 20 $\mu\text{g}/\text{ml}$
256 colistimethate or both agents are shown in Figure 2D, demonstrating large differences in turbidity.
257 Similarly, increased bacterial killing was seen with polymyxin B and 12mM nitrite at sub-inhibitory
258 concentrations of both compounds (Figure 4B). These results suggest that nitrite and polymyxins may
259 have cooperative antimicrobial activity.

260 Because there is great diversity in the antibiotic susceptibility of bacterial clinical isolates, we used
261 agar dilution checkerboard testing to assess for antimicrobial synergy in a collection of *P. aeruginosa*
262 clinical isolates from cystic fibrosis patients. Synergy is defined as a Fractional Inhibitory Concentration
263 (FIC) of ≤ 0.5 . FIC is calculated by dividing the MIC of each drug when used in combination with the MIC
264 of each drug when used alone. Anaerobic MICs were determined adding 1% KNO_3 to the growth media
265 to support denitrification and growing the plates for 48 hours prior to interpretation to account for
266 slower growth under anaerobic conditions. Three isolates grew too slowly anaerobically to give
267 interpretable MICs by this method. Eight of nine isolates were more susceptible to colistin under
268 anaerobic conditions. In addition, isolates were 2-4 fold more susceptible to nitrite under anaerobic
269 conditions, in agreement with previously published data (17). Only the lab strain PA14 grown under

270 anaerobic conditions met the FIC cutoff of 0.5 that defines synergy. The MIC was very consistently
271 lowered by 2-fold when nitrite and colistin were combined (corresponds to the FICs of >0.5 and <1). The
272 FIC cutoff of 0.5 was set in part to account for the 1 dilution variation that is accepted for MIC assays. In
273 these experiments, we consistently saw a 1-dilution decrease (and never an increase) in colistin sulfate
274 MIC when subinhibitory nitrite was present. Despite not meeting the 0.5 FIC threshold for synergy, the
275 consistent decrease in MIC suggested that nitrite and colistimethate might have cooperative activity
276 against biofilms.

277 To determine whether nitrite would confer polymyxin susceptibility to bacteria with high innate
278 polymyxin resistance, eight highly colistin resistant clinical isolates were also tested under aerobic
279 conditions in the presence of nitrite. All isolates had a colistin MIC >512 µg/ml in both the presence and
280 absence of 3 mM (subinhibitory) sodium nitrite. *Achromobacter spp.* and *Burkholderia spp.* are pathogens
281 that are difficult to treat within the CF population because of high innate antibiotic resistance.
282 *Burkholderia spp.* have high innate resistance to polymyxins. Accordingly, the MIC to colistin was >512
283 µg/ml with or without nitrite, although growth of these organisms was inhibited by 18 mM nitrite alone.
284 Amongst *Achromobacter spp.*, increased susceptibility to colistin in the presence of nitrite was seen in 4 of
285 8 clinical isolates (Supplementary Table 1). Taken together, among isolates of *P. aeruginosa* that do not
286 have high innate polymyxin resistance, nitrite increases the susceptibility to polymyxins for a majority of
287 isolates, and for some of the *Achromobacter* isolates.

288 Biofilms grow more robustly on CF airway cells than on those expressing wild-type CFTR (31).
289 We have shown that nitrite causes an increase in susceptibility to colistin in planktonic culture and on
290 nutrient agar. To determine whether nitrite augmented the antimicrobial activity of polymyxins for
291 biotic biofilms on primary CF HBE cells, the polymyxin pro-drug colistimethate was combined with
292 nitrite in a biofilm prevention assay (Figure 3). In this experiment, bacteria were allowed to attach to the
293 surface of primary CF HBE cells for one hour. The developing biofilms were then treated with nitrite,
294 colistimethate or both agents. When nitrite was not used, sodium chloride was added to control for
295 tonicity. Nitrite alone prevented 1-2 logs of bacterial biofilm growth. Of note, primary CF HBE cells

296 tolerate higher concentrations of nitrite than CFBE-wt cells. Transepithelial electrical resistance did not
297 drop with up to 300 mM nitrite (control TEER 678 mΩ•cm² vs 300 mM nitrite 597 mΩ•cm²).
298 Colistimethate (20 μg/ml) prevented 3 logs of growth and the combination of the agents prevented 4 logs
299 of growth. The cooperative effect of this combination in biofilm prevention led us to undertake further
300 mechanistic studies of nitrite and colistimethate.

301
302 **Nitrite and polymyxins additively inhibit bacterial respiration.** Nitrite has many effects on bacterial
303 physiology. In the case of *P. aeruginosa*, concentrations of nitrite in the 1-20 mM range inhibit aerobic
304 respiration (as measured by NADH oxidase activity), oxygen uptake, and both aerobic and anaerobic
305 growth (11, 18, 41, 42). Polymyxins initially bind to the outer membrane and increase its permeability;
306 subsequently they disrupt the cytoplasmic membrane (43). Polymyxins appear to inhibit bacterial
307 respiration, presumably a consequence of damage to the cytoplasmic membrane (26). To explore the
308 mechanism of action of combined nitrite and colistin, we tested the hypotheses that (1) polymyxins
309 increase outer membrane permeability and thus promote increased intracellular concentrations of nitrite
310 and (2) polymyxins and nitrite cooperatively inhibit bacterial respiration to exert antimicrobial activity.

311 If increased bacterial killing by nitrite and colistin were a consequence of increased intracellular
312 nitrite concentration, then nitrite and colistin would need to be present simultaneously for increased
313 killing to occur. To test this prediction, bacteria were treated with 15 mM nitrite, rinsed and treated with
314 increasing concentrations of colistimethate in aerobic culture. Pretreatment with nitrite increases the
315 susceptibility to colistimethate (Figure 4A). The timing of these effects excludes increased intracellular
316 concentration of nitrite due to polymyxins because extracellular nitrite has been removed from the
317 system prior to exposure to colistimethate. Polymyxin B nonapeptide, which lacks a lipid tail, increases
318 outer membrane permeability without causing bacterial death or cytoplasmic membrane damage (44).
319 Increased susceptibility to polymyxin b nonapeptide was not seen with nitrite (Figure 4B) in contrast to
320 the increased bacterial killing seen with the parent compound polymyxin b and nitrite in a time-kill assay.
321 The lack of killing in combination with polymyxin b nonapeptide and the increased susceptibility to

322 polymyxins with nitrite pretreatment both exclude increased intracellular accessibility to nitrite as a
323 cause of this effect.

324 Both nitrite and polymyxins inhibit bacterial respiration, although in the case of polymyxins this
325 may be a secondary effect to massive cytoplasmic membrane damage. We next tested if other agents that
326 inhibit cellular respiration sensitize *P. aeruginosa* to polymyxins. In time-kill assays, the combinations of
327 colistin sulfate and colistimethate with potassium cyanide or sodium azide all showed at least 4 logs of
328 additional killing than any of the agents alone (Figure 5A). To show more quantitatively that nitrite and
329 polymyxins were inhibiting respiration, oxygen consumption was measured. For these experiments low
330 concentrations of nitrite and colistimethate were chosen that did not affect overall bacterial viability in
331 combination so that the oxygen consumption measurements would not be confounded by bacterial death.
332 Nitrite, as previously described, inhibits oxygen consumption with a linear dose-response at
333 concentrations below the MIC as measured using a Clark-type electrode (representative tracings shown
334 in Figure 5B). 5mM nitrite and 30 μ g/ml colistimethate both cause a 20% reduction in oxygen uptake
335 under the conditions tested. In combination the agents caused a statistically significant, 50% decrease in
336 oxygen uptake (Figure 5C). Next, to determine if the effect was NO dependent, the scavenger CPTIO was
337 added in modified time-kill assays. As shown in Figure 5D, CPTIO did not block the increased bacterial
338 killing by nitrite with colistimethate. Similar results were obtained using oxyhemoglobin as the
339 scavenger (data not shown). If respiratory blockade were part of the mechanism, then respiratory
340 blockade in this situation should also be independent of NO. As shown in Figure 5E, treatment of cultures
341 with CPTIO and hemoglobin did not protect *P. aeruginosa* against the inhibitory effects of nitrite on
342 oxygen uptake.

344 **Anti-biofilm effects of nitrite are NO independent.**

345 Previous work indicates that scavenging NO prevents the anti-bacterial effects of nitrite on *P.*
346 *aeruginosa* in planktonic culture(17). First we determined the nitrite concentrations required for growth
347 inhibition in this system by exposing aerated, planktonic cultures of *P. aeruginosa* to increasing

348 concentrations of nitrite (Figure 6A). Similar to the previous work, introduction of NO scavengers
349 (hemoglobin and CPTIO) protected *P. aeruginosa* against nitrite induced growth inhibition (Figure 6B).
350 Because the metabolic demands of biotic biofilm lifestyle differ from those of a planktonic lifestyle, we
351 next examined whether biofilm prevention by nitrite was also dependent on NO. In contrast to the effects
352 of nitrite under aerobic conditions, the addition of 1 mM CPTIO (far in excess of the 500 nM NO generated
353 by nitrite in culture as reported in (17)) did not protect biotic biofilms from the activity of nitrite (Figure
354 6C). In conclusion, nitrite prevents *P. aeruginosa* biofilm formation on the apical surface of airway
355 epithelial cells. Unlike the antibacterial activity in aerated planktonic culture, scavenging NO does not
356 block the antibacterial activity of nitrite under biofilm conditions.

357 358 **DISCUSSION**

359 We have demonstrated that sodium nitrite prevents biotic biofilm formation by *P. aeruginosa* grown
360 on the surface of CF primary human airway cells. While it was previously known that nitrite could inhibit
361 growth of *P. aeruginosa* in planktonic conditions, in abiotic biofilms and in expectorated sputum, this is
362 the first demonstration of antimicrobial activity against biotic biofilms. In concordance with previously
363 published work, we found that anaerobic conditions increased the susceptibility of *P. aeruginosa* to
364 nitrite. The oxygen tension in our epithelial co-culture systems is very low as the perfusate for the flow
365 chamber is not actively oxygenated and the static co-cultures are submerged under several millimeters of
366 media despite having $>10^6$ metabolically active bacteria/ml growing on the surface of the airway cells.
367 While the available evidence suggests that mucus plugs within the CF airway provide an anaerobic
368 environment for bacterial growth, regions of aerobic growth cannot be excluded, especially during
369 periods of biofilm dispersal. It is noteworthy that nitrite effectively prevented bacterial growth under
370 both aerobic and anaerobic conditions.

371 The millimolar concentrations of nitrite we used in this study are much higher than typical
372 concentrations of currently used antibiotics, which may cause concern regarding cytotoxicity. Yoon *et al*
373 clearly demonstrated that nitrite concentrations up to 300 mM do not cause epithelial cytotoxicity as

374 measured by lactate dehydrogenase release, effect on short circuit current, permeability to dextran, or IL-
375 8 secretion (17). In our studies, CFBE-wt cells tolerated up to 50mM nitrite without a loss of barrier
376 function and well-differentiated primary CF HBE cells did not show loss of barrier integrity even with
377 exposure to 300 mM sodium nitrite. Further, inhalation of approximately 1 molar sodium nitrite has
378 been well tolerated by human subjects in phase I and II studies (M. Gladwin, personal communication).
379 These data support the pursuit of clinical trials using nitrite as a nebulized antibiotic against *P.*
380 *aeruginosa* in CF.

381 While our study investigated the interactions between colistimethate and nitrite, inhaled
382 colistimethate is used less frequently than inhaled tobramycin and inhaled aztreonam in the CF
383 population. Previous reports demonstrated that NO protects bacteria against a wide range of antibiotics
384 (45). However, these reports do not address the interaction between NO and antimicrobial peptides
385 such as the polymyxins, which have a unique mechanism of action. The possibility that nitrite may have
386 adverse interactions with other antibiotics is a valid concern, warranting further investigation.

387 Despite widespread clinical use, the activity of colistimethate against biotic biofilms has not been
388 clearly described. We have shown that colistin sulfate has activity against biotic biofilms that is at least
389 comparable to that of ciprofloxacin. The activity of polymyxins under anaerobic conditions also has not
390 been extensively studied. In the report by Hill et al, anaerobic growth was supported by Muller Hinton
391 broth in the absence of exogenous nitrate, which makes the results difficult to interpret. Polymyxin
392 susceptibility is also pH dependent. This study used pH 6.5, the reported pH of the CF airway surface
393 liquid, thus the colistin sulfate MICs for these isolates may be slightly different in this study than when
394 MIC testing is performed at pH 7.4. Additionally, ionic strength can affect polymyxin activity, and
395 hypertonicity can cause additional effects on the epithelial cells used in our co-culture experiments (43).
396 To account for this possibility, sodium chloride was used as a tonicity control in the static co-culture
397 experiments.

398 The strengths of combining polymyxins with nitrite are the activity under anaerobic conditions
399 and the activity against biotic biofilms, which are limitations of our current antibiotic approaches.

400 Additionally, the proposed mechanism of interaction is unique. Existing antibiotics do not target
401 bacterial respiration, although the combination of fosfomycin and tobramycin has recently been shown to
402 suppress denitrification (46). While the current study focused on the interaction of nitrite and
403 polymyxins in aerobic metabolism, a similar effect is possible regarding anaerobic respiration where
404 polymyxin induced inner membrane damage may indirectly disrupt the function of the denitrification
405 enzymes allowing accumulation of toxic intermediates. Because of the rapidity of bacterial killing by
406 polymyxins we did not directly test the hypothesis that polymyxins inhibit denitrification. However, the
407 proposed mechanism that nitrite and polymyxins are both inhibiting aerobic respiration is concordant
408 with the observation that flow cell biofilms are killed by the combination of colistin and the respiratory
409 chain uncoupler CCCP (24). The advantage of our approach is that, unlike CCCP and other respiratory
410 chain poisons, nitrite can be safely nebulized.

411 While the antimicrobial effects of nitrite in planktonic, aerated culture are NO-dependent,
412 antimicrobial effects under biofilm growth conditions are likely not NO-dependent. In these experiments,
413 the scavenger CPTIO was added while the biofilms were microcolonies with minimal matrix production,
414 so inability of the scavenger to penetrate the colony is unlikely to be solely responsible for these results.
415 There are several potential explanations for the difference in NO-radical dependence of growth inhibition
416 by nitrite. First, *P. aeruginosa* has multiple mechanisms for detoxifying NO including expression of
417 flavohemoglobins and the anaerobic expression catalase (47, 48). The relative efficiency of these
418 detoxification systems, and thus organismal exposure to NO vs nitrite, may vary with growth conditions.
419 Second, the targets of nitrosative stress are not clearly understood in *P. aeruginosa*, especially when the
420 metabolic versatility of the bacterium is considered. Nitrite and NO can cause S-nitrosation of cysteine
421 and the inactivation of Fe-S containing proteins. Targets of S-nitrosation have been identified in *Borrelia*
422 *burgdorferi*, *Mycobacterium tuberculosis*, *Escherichia coli* and *Helicobacter pylori*, but to date not in *P.*
423 *aeruginosa* (49-51). Multiple enzymes within central metabolism are potential targets for inactivation by
424 nitrosative stress including NADH dehydrogenase, the terminal cytochrome c oxidases, and aconitase. In
425 eukaryotes, mitochondrial complex I is a target for cysteine S-nitrosation, which confers protection from

426 ischemia/perfusion injury (52, 53). In contrast, work in *Salmonella enterica* suggested the site of
427 respiratory inhibition by the NO donor spermine NONOate to be nitrosylation of *heme d* in the terminal
428 cytochrome oxidases while bacterial NADH dehydrogenase activity remained uninhibited. The sources
429 of nitrosative stress vary between these reports, and more importantly the role of NADH dehydrogenase
430 in the organism vary (*S. enterica* is viable in the absence of both NADH dehydrogenases). Growth
431 conditions may dictate the bacterial targets of nitrosative stress (and thus the chemical targets for nitrite
432 or NO). The study by Ren *et al* is informative. *E. coli* growth in minimal media requires synthesis of
433 branched chain amino acids catalyzed by dihydroxyacid dehydratase (IlvD). NO inhibits IlvD through
434 formation of dinitrosyl iron complexes, thus inhibiting growth and rendering the bacteria dependent on
435 supplementation of branched chain amino acids. Growth inhibition by NO in this situation is avoided by
436 supplementing the growth media with the needed amino acids (54). One could imagine that had the
437 experiment performed in *E. coli* been done in rich media, a different target of nitrosative stress might
438 have been found (albeit with a higher stress required)(55). In the current study, the bacterial substrate
439 usage in aerated LB is likely very different from that of a low-oxygen tension biofilm grown in apposition
440 to an epithelial cell, which may then in turn dictate which metabolic pathways are susceptible to
441 inhibition. Teasing out the targets of NO vs nitrite under biofilm conditions as compared to planktonic
442 growth will be a complex task.

443 In conclusion, nitrite shows promise as an antimicrobial agent targeting biotic biofilms in the
444 lungs of patients with *P. aeruginosa* infection. Combining nitrite with colistimethate may increase the
445 effectiveness of the latter. Even in isolation, inhaled nitrite may be a useful addition to our antimicrobial
446 armamentarium because of its broad range of activity, including activity against anaerobically growing *P.*
447 *aeruginosa*, anti-biofilm activity, and activity against other pathogens such as *Achromobacter sp.* and
448 *Burkholderia sp.* for which few effective antimicrobials are available. This study provides further
449 motivation to pursue human trials of nebulized nitrite as an antimicrobial in patients with airway
450 colonization by resistant organisms.

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612 **Figure 1: Nitrite prevents *P. aeruginosa* biofilm formation on airway epithelial cells.** To test if
613 nitrite prevented biotic biofilm formation, *Pseudomonas aeruginosa* biofilms were grown on CFBE-wt
614 cells seeded onto coverslips and imaged by live cell microscopy after 6 hours of growth. DAPI (blue)
615 staining shows the epithelial cell nuclei. (A) Untreated biofilms showed abundant growth of (GFP)-*P.*
616 *aeruginosa* in characteristic clusters. (B) Biofilms treated with 15mM showed very few bacteria present.
617 Volumetric projections of control (C) vs 15 mM (D) treated biofilms demonstrate the decreased bacterial
618 biomass with 15 mM nitrite exposure. COMSTAT biomass quantification showed an 80% reduction in
619 biomass (numbers on panels). (E) A static co-culture model was used to establish a dose-response
620 relationship for nitrite in biofilm prevention. Increasing concentrations of sodium nitrite were added to
621 epithelial – *P. aeruginosa* co-cultures following an attachment period. Bacterial colony forming units
622 (CFU) were counted 5 hours later. All concentrations caused a statistically significant decrease in bacteria
623 (one-way ANOVA followed by Tukey test). (F) Transepithelial electrical resistance measurements were
624 used to assess epithelial integrity in the presence of nitrite. Resistance was not significantly different
625 from control at 15mM and 50 mM nitrite (one-way ANOVA followed by Tukey test, $p > 0.05$).

627 **Figure 2: Nitrite sensitizes *P. aeruginosa* to polymyxins.** (A) The static co-culture model was used to
628 determine the efficacy of colistin sulfate at disrupting established *P. aeruginosa* biotic biofilms. *P.*
629 *aeruginosa* was added to the apical surface of CFBE-wt cells. After 6 hours of maturation, the biofilms
630 were treated with colistin sulfate for 90 minutes and the number of bacteria was enumerated with a CFU
631 dilution assay. 20 μ g/ml colistin sulfate caused a 3-log reduction in CFUs. Planktonic time-kill assays
632 were used to determine the interaction between nitrite and polymyxins. Subinhibitory concentrations of
633 (B) colistin sulfate (1.5 μ g/ml) and (C) colistimethate (70 μ g/ml) showed increased antimicrobial activity
634 against *P. aeruginosa* strain PA14 when combined with subinhibitory concentrations of acidified sodium
635 nitrite. $p < 0.05$ for polymyxin vs polymyxin + nitrite by two-way ANOVA. (D) Representative cultures
636 showed reduced bacterial growth (turbidity) when both CMS and nitrite are present.

638 **Table 1:** Checkerboard MIC testing under aerobic and anaerobic conditions for 12 isolates of *P.*
639 *aeruginosa*. MIC was determined by agar dilution with pH 6.5 MHB agar. 1% KNO₃ was added for
640 anaerobic growth and anaerobic plates were incubated for 48 hours. Three isolates (33-2, 66-2, and 74-
641 2) grew too poorly under anaerobic conditions to obtain interpretable MICs. The MIC for both colistin
642 sulfate and nitrite are lower under anaerobic conditions. Nitrite and colistin sulfate were synergistic for
643 one strain (PA14) under anaerobic conditions (FIC<0.5).

644
645 **Figure 3: Nitrite and colistimethate additively prevent biotic biofilm formation on primary CF**
646 **human airway epithelial cells.** PAO1 biofilms were grown on primary CF HBE cells. After attachment,
647 developing biofilms were treated with 300mM nitrite, colistimethate 20µg/ml or a combination of the
648 agents. p-values indicated from two-way ANOVA.

649
650 **Figure 4: Increased bacterial killing with nitrite and polymyxins is not due to increased**
651 **intracellular availability of nitrite.** (A) Bacteria were grown in the presence of 15 mM nitrite for 5.5
652 hours, rinsed and exposed to colistimethate in liquid aerobic culture for 60 minutes. Nitrite pretreatment
653 sensitized *P. aeruginosa* to colistimethate in planktonic culture. (B) Planktonic cultures were exposed to
654 polymyxin b (PMB), polymyxin b nonapeptide (PMBN) and 12 mM nitrite for 5.5 hours. No significant
655 difference was seen by one-way ANOVA between PMBN treatment with or without nitrite. PMB + nitrite
656 was significantly lower than PMB or nitrite alone by one-way ANOVA followed by Tukey test.

657
658 **Figure 5: Inhibition of respiration by nitrite and polymyxins** A) Inhibiting respiration with 0.4mM
659 KCN or 5mM NaN₃ increases polymyxin susceptibility of *P. aeruginosa* grown in aerobic LB culture.
660 Colistin sulfate (COL) was used at 1.5µg/ml while colistimethate (CMS) was used at 20µg/ml (both sub
661 inhibitory doses in PA14 under these conditions). (B) Representative tracings of bacterial oxygen
662 consumption measured using a Clark-type electrode in LB with increasing concentrations of nitrite. (C)
663 At low concentrations, both nitrite and CMS inhibit oxygen consumption by 20%. The combination of

664 CMS and nitrite additively blocked oxygen uptake. Brackets show p values <0.05 from one-way ANOVA
665 followed by Tukey test. Data shown as mean +/- SD. (D) Increased killing by nitrite and CMS in
666 combination is not blocked by the addition of the scavenger CPTIO in planktonic culture. (E)
667 Representative tracing of oxygen uptake from a Clark-type electrode. 15mM nitrite decreases oxygen
668 consumption by 50%. Pretreatment with 1mM CPTIO or 15µM hemoglobin did not prevent nitrite
669 induced oxygen consumption blockade.

670
671 **Figure 6: Biofilm cytoostasis is nitric oxide independent.** (A) Nitrite inhibited growth of PA14 in
672 planktonic culture. (B) To confirm that nitrite induced cytoostasis in planktonic cultures is nitric oxide
673 dependent, log-phase oxygenated cultures were grown with nitrite and the nitric oxide scavengers CPTIO
674 or oxyhemoglobin. Growth inhibition was blocked by the addition of either agent. (C) To determine if the
675 prevention of biofilm growth by nitrite requires nitric oxide, static epithelial- *P. aeruginosa* co-cultures
676 were treated with nitrite and the nitric oxide scavenger CPTIO. Growth inhibition was unchanged by the
677 addition of 1mM CPTIO.

678
679 **Supplementary Table 1:** The MIC for colistin sulfate was determined by agar dilution in the presence or
680 absence of 3mM nitrite for 8 *Achromobacter sp.* isolates.

681

682

Table 1.

Strain	Aerobic MIC COL µg/ml	Anaerobic MIC COL µg/ml	Aerobic MIC mM Nitrite	Anaerobic MIC mM Nitrite	Aerobic FIC	Anaerobic FIC
PA14	2	2	16	4	0.75	0.375
PAO1	2	1	16	4	0.75	0.75
31-2	2	0.5	16	4	0.75	0.75
36-3	2	0.5	16	4	0.75	0.75
41-2	4	0.5	8	4	1.5	0.625
47-3	1	0.25	8	2	1	1
60-3	4	1	16	4	0.625	0.75
62-3	1	0.25	8	4	1	1
71-2	4	1	16	4	0.625	0.75
33-2	4	n.d.	16	n.d.	0.75	n.d.
66-2	8	n.d.	8	n.d.	0.625	n.d.
74-2	0.5	n.d.	8	n.d.	1	n.d.

Supplementary Table 1: Nitrite decreases the colistin sulfate MIC of *Achromobacter* isolates.

Isolate #		COL MIC ug/ml	COL MIC with 3mM nitrite
AU19487	<i>A. xylosoxidans</i>	8	2
AU13697	<i>A. ruhlandii</i>	>512	>512
AU2687a	<i>A. xylosoxidans</i>	>512	>512
AU18049	<i>A. xylosoxidans</i>	128	128
AU12168	<i>A. xylosoxidans</i>	128	32
AU12688	<i>A. xylosoxidans</i>	4	2
AU20153	<i>A. dolans</i>	256	256
AU19839	<i>A. xylosoxidans</i>	256	128

Table 1: The MIC for colistin sulfate was determined by agar dilution in the presence or absence of 3mM nitrite for 8 *Achromobacter sp.* isolates.

Figure 1
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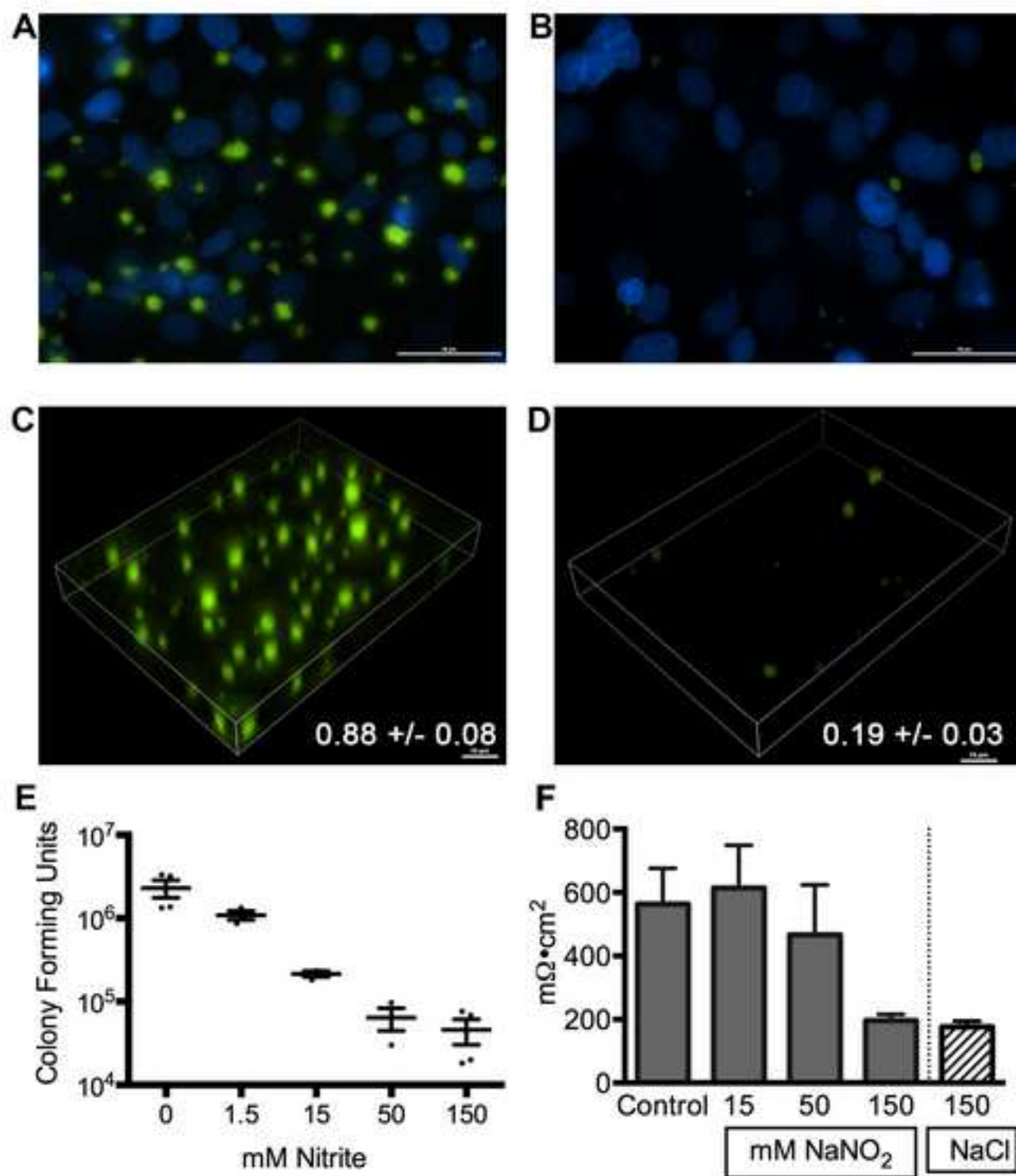


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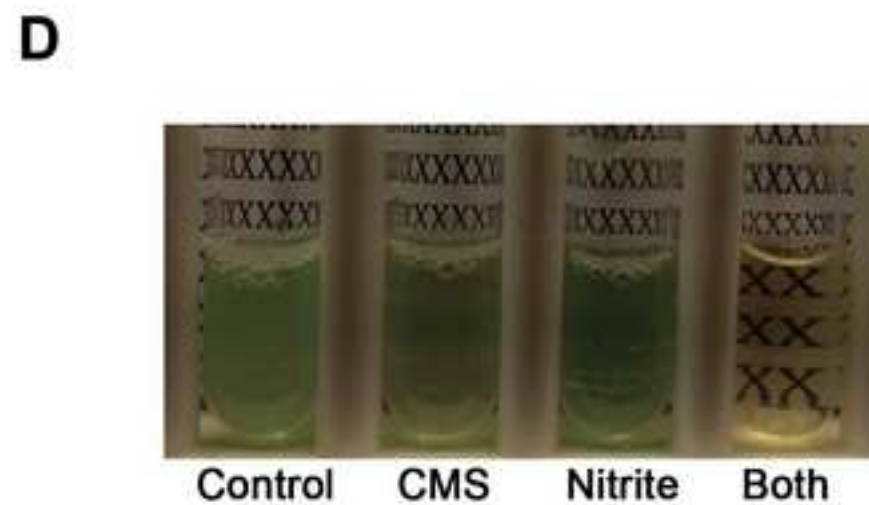
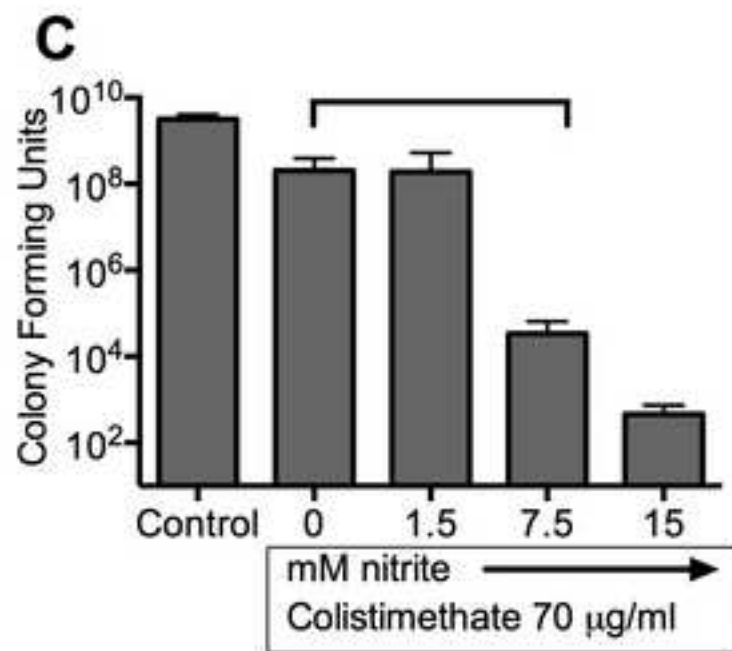
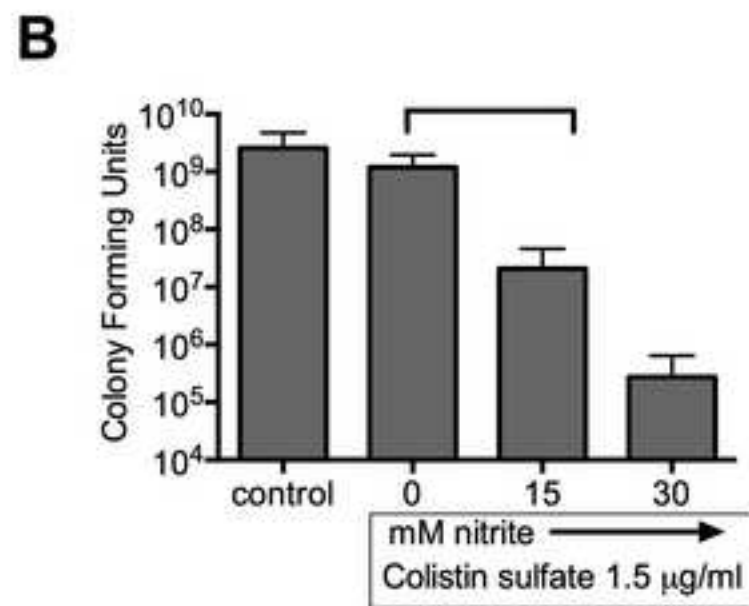
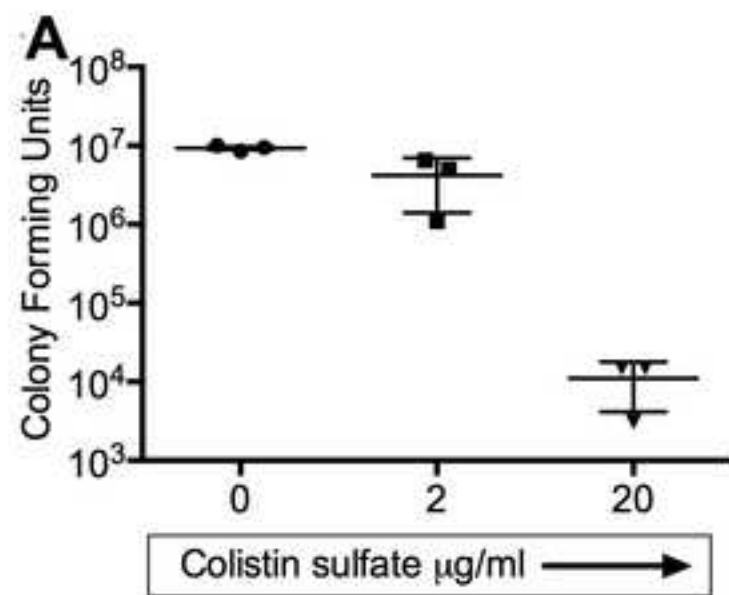


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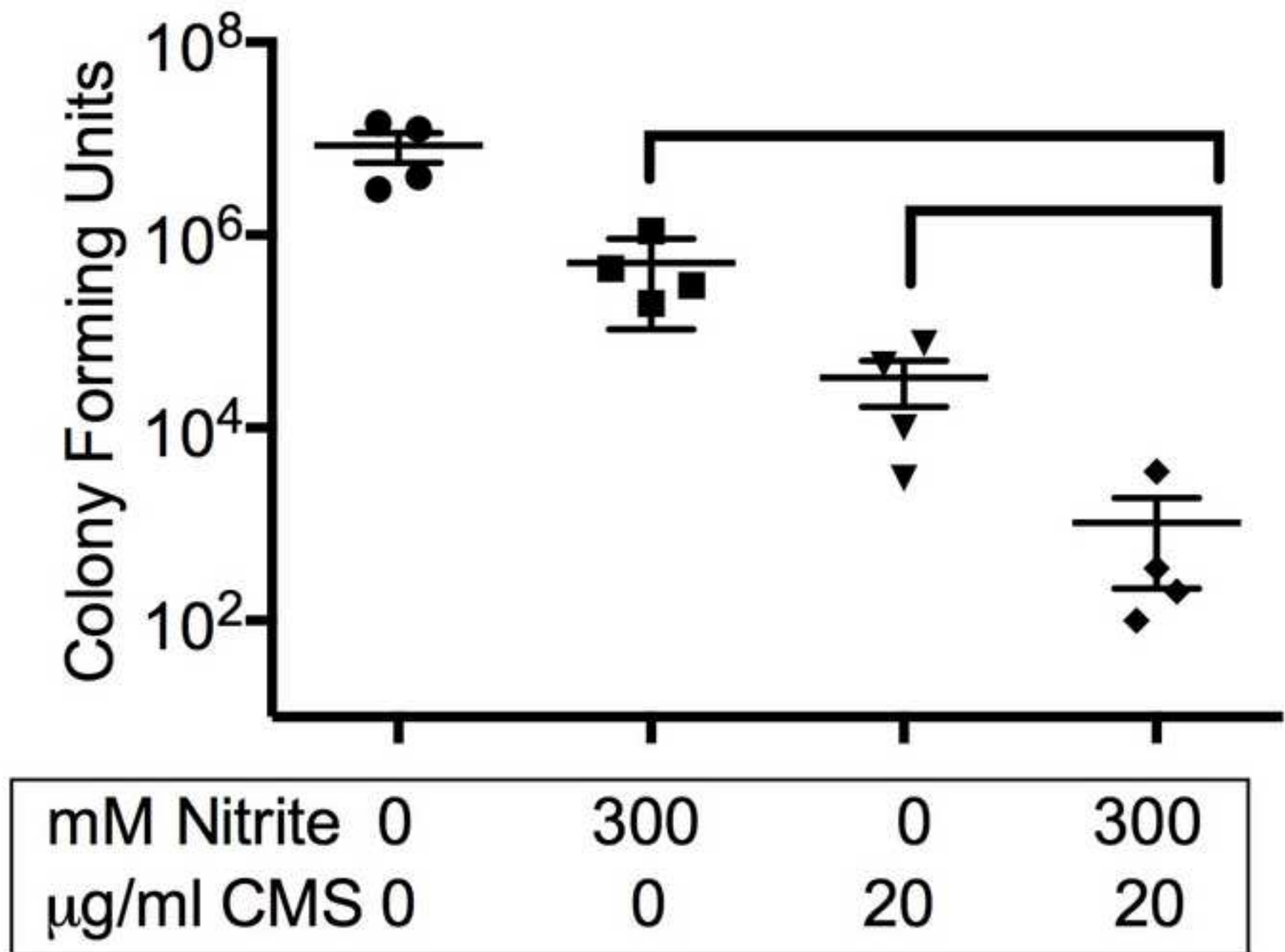
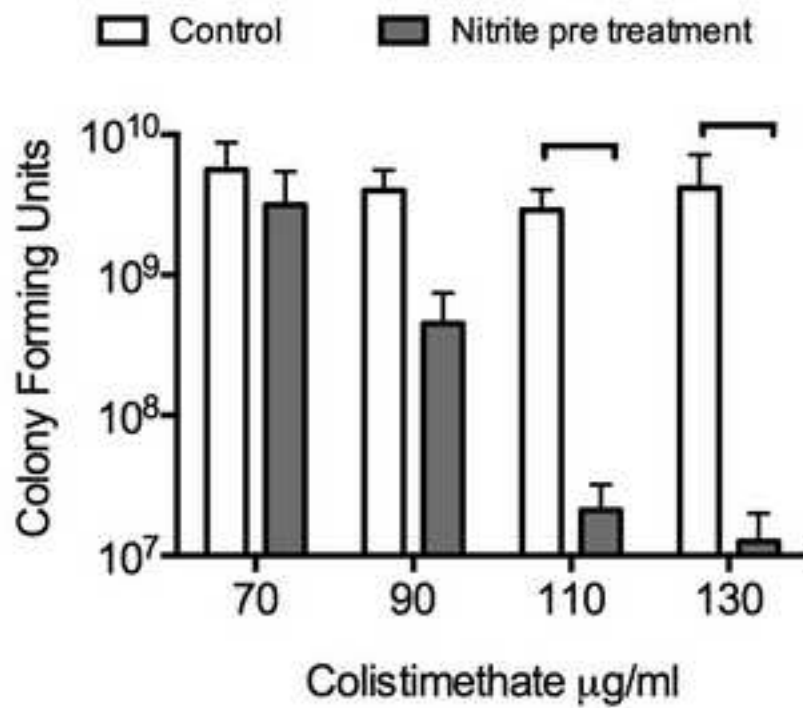


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A



B

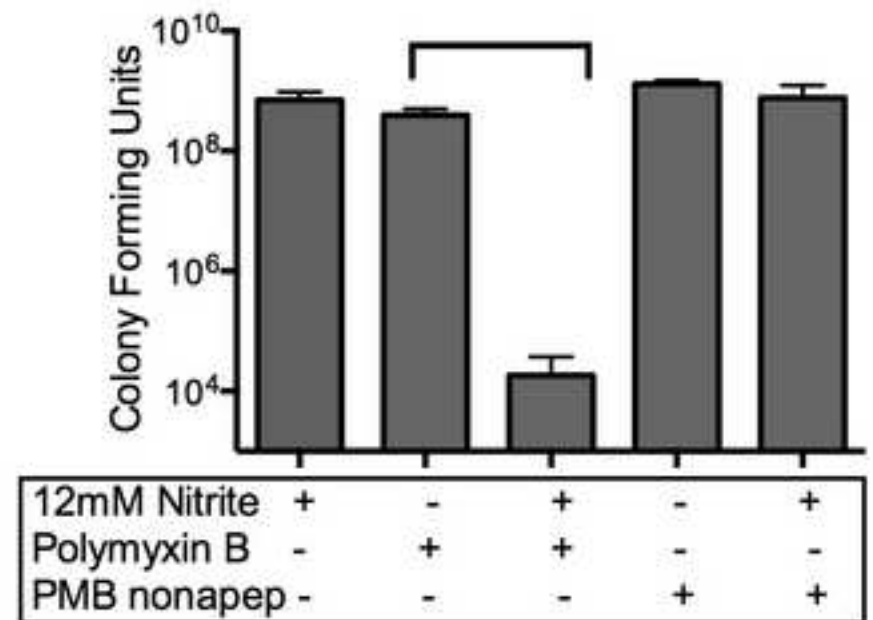


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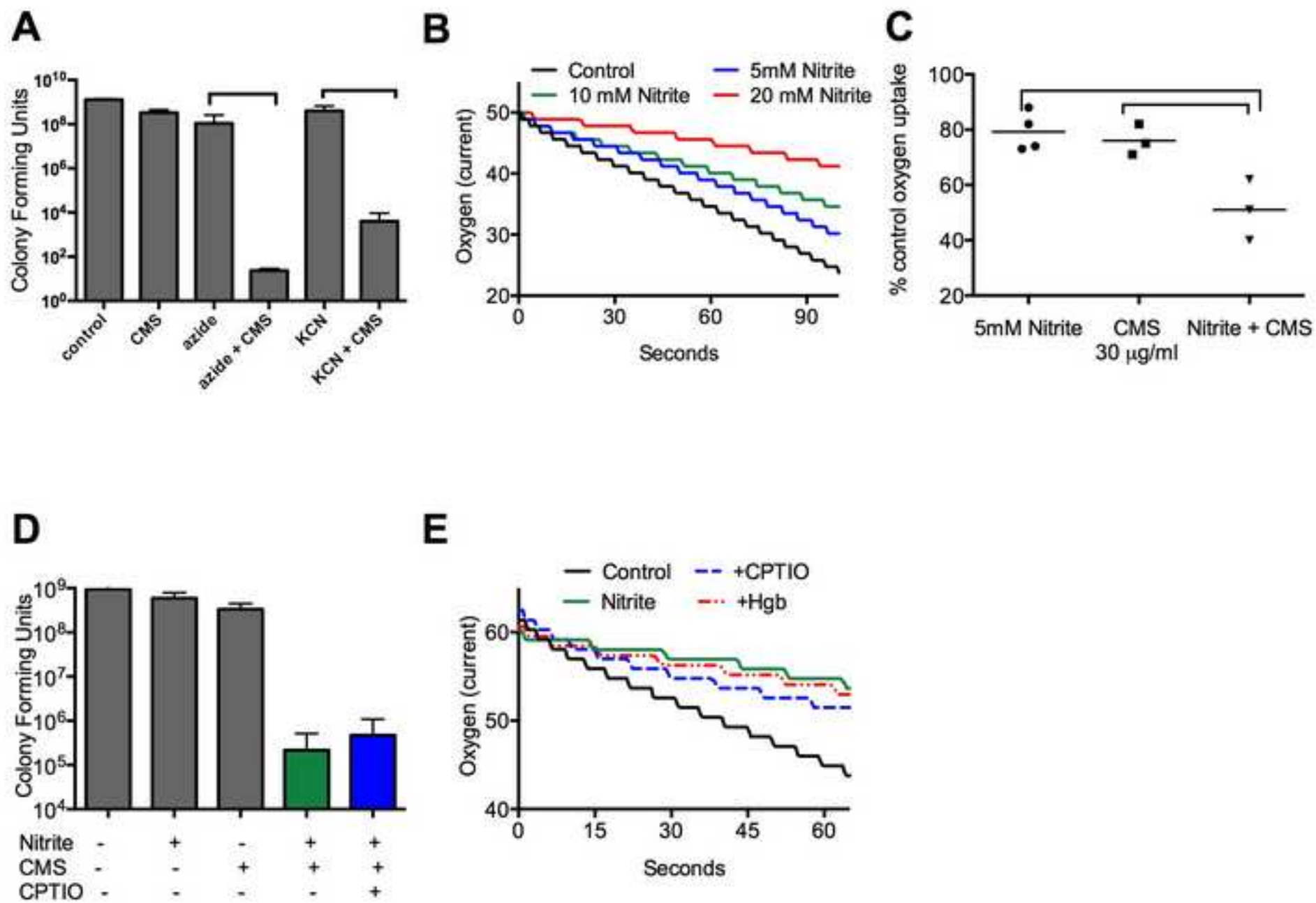
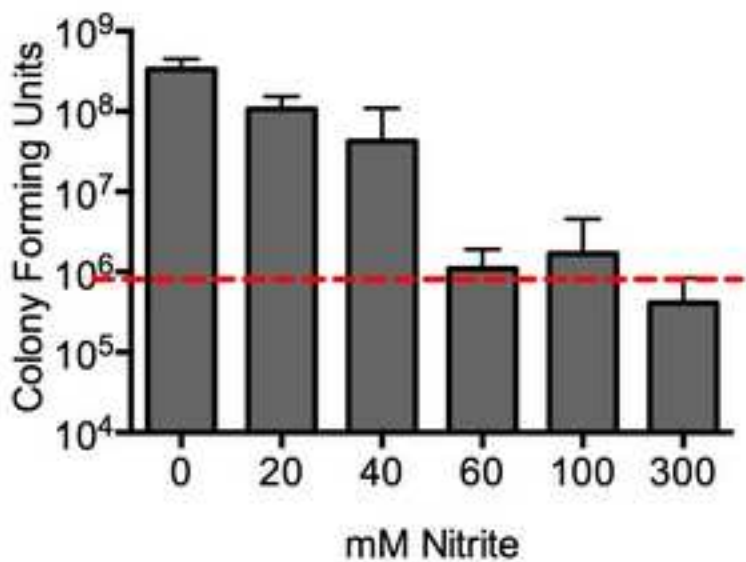
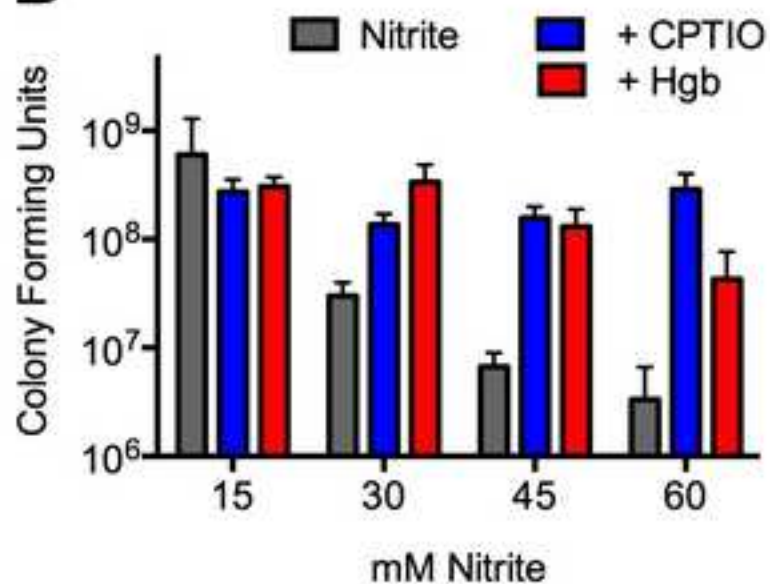


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A



B



C

