UNIVERSITY OF CALIFORNIA

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BACTERIA ISOLATED FROM BATS INHIBIT THE GROWTH OF *PSEUDOGYMNOASCUS DESTRUCTANS*, THE CAUSATIVE AGENT OF WHITE-NOSE SYNDROME

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By

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Bacteria isolated from bats inhibit the growth of *Pseudogymnoascus destructans,* the causative agent of white-nose syndrome

Abstract

Emerging infectious diseases are a key threat to wildlife. Several fungal skin pathogens have recently emerged and caused widespread mortality in vertebrate species, including amphibians, bats, rattlesnakes, and platypus. The importance of the skin microbiome in host-pathogen interactions is increasingly understood to play a large role in determining the course of disease in a host. White-nose syndrome, caused by the fungal skin pathogen *Pseudogymnoascus destructans*, threatens several hibernating bat species with extinction and there are no known effective treatments. We co-cultured bacteria and *P. destructans* from the skin microbiome of four bat species to identify bacterial isolates that might inhibit or kill *P. destructans*. We then conducted two reciprocal challenge experiments *in vitro* with six candidate bacteria (all in the genus *Pseudomonas*) that inhibited *P. destructans* growth across a range of bacterial and fungal concentrations to quantify the effect of these bacteria on the growth of *P. destructans*. All six *Pseudomonas* isolates significantly inhibited growth of *P. destructans* compared to the non-inhibitory control bacteria, and two isolates performed significantly better than others in suppressing *P. destructans* growth for more than 35 days. In both challenge

experiments, the extent of suppression of *P. destructans* growth was dependent on the concentration of *P. destructans* and the initial concentration of the bacterial isolate. These results show that bacteria found naturally occurring on bats can inhibit the growth of *P. destructans* and have promise for development as a biocontrol for bats exposed to white-nose syndrome. In addition, the presence of these bacteria may influence disease outcome among individuals, populations, and species.

ACKNOWLEDGEMENTS

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Introduction

Emerging infectious diseases can have devastating impacts on wildlife and threaten many species with extinction (Langwig et al., 2012; McCallum, 2012; Skerratt et al., 2007; van Riper, van Riper, Goff, & Laird, 1986). With an increase in anthropogenic disturbance and rise in global trade and transportation, the threat posed by wildlife disease is likely to increase (Daszak 2000). Wildlife diseases can be exceedingly challenging to manage due to the need for constant human intervention (Wobeser, 2002). The few successful examples of disease management in wildlife require repeated capture and treatment of individuals, which is costly and only feasible on small scales (Roelle, Miller, Godbey, & Biggins, 2006; Wobeser, 2002). For example, the re-establishment of Black-footed ferrets into their native range required vaccination of adults and young born each year for both plague and canine distemper (Roelle et al., 2006). New approaches that do not require continued intervention are needed in order to reduce the impacts of these devastating diseases (Kilpatrick, 2006).

Several recently emerged wildlife pathogens infect the dermal tissue of their host, and interactions with host skin microbiota could play an important role in the differential disease severity we observe in hosts. Vertebrate skin is an ecosystem composed of different habitats which harbor diverse assemblages of microorganisms (Grice & Segre, 2011). Previously, studies of skin microbiota primarily examined the pathogenic roles of skin microbes, with little attention to the beneficial function that many microorganisms may provide (Cogen, Nizetà, & Gallo, 2008). However,

studies have shown that beneficial bacteria on the skin can provide vital functions to their hosts, including processing of skin proteins, freeing fatty acids to reduce invasion of transient microorganisms, and inhibition of pathogenic microorganisms (Roth & James, 1988). Some bacteria have been developed to reduce the impact of a broad range of diseases, and are termed probiotics (beneficial bacteria; (Schrezenmeir & de Vrese, 2001)).

Probiotics have the potential to provide a long-lasting or permanent solution for managing disease and have the ability to adapt to a changing pathogen pathogen unlike chemical fungicides (Thomas & Willis, 1998). Probiotics are regularly used in the biological control of disease in both aquaculture and agriculture, but have yet to be widely implemented in controlling wildlife disease in part because of perceived risks and lack of demonstrated success (Harris et al., 2009; Harris, James, Lauer, Simon, & Patel, 2006; Irianto & Austin, 2002; Johnson & Stockwell, 1998). Risks associated with augmenting micro-organisms on a host, which can either be ineffective or accidentally harmful, can be minimized by using bacteria that naturally occur in the hosts' environment. Bacteria present on species similar to the target host that exhibit less severe disease symptoms may prove a good source of effective probiotics because these targets would already be competent at colonizing bat skin, and adapting to hibernacula conditions. A successful probiotic might also have motile ability enabling them to use fungal mycelia networks as modes of dispersal, and increasing contact between the bacteria and the fungus (Pion *et al.*, 2013). The ability of motile bacteria to use liquid films forming around fungal hyphae for dispersal

helps them access new microhabitats and nutrient sources (Warmink, Nazir, Corten, & van Elsas, 2011). These same pathways may also increase their ability to invade fungal colonies in the case of an antagonistic relationship.

Probiotics are currently being explored as a management strategy for amphibians suffering from the fungal disease chytridiomycosis, caused by the pathogenic *Batrachochytrium dendrobatidis* chytrid fungus. *Batrachochytrium dendrobatidis* is known to infect over 280 amphibian species, but has highly variable impacts on different species (Kilpatrick et al., 2010; Woodhams et al., 2007). Low mortality from *B. dendrobatidis* infection has been correlated with presence of anti-*B. dendrobatidis* bacteria in several species (Bletz et al., 2013; Harris et al., 2006; Woodhams et al., 2007), and researchers have successfully augmented these bacteria on frogs to reduce infection intensity and mortality caused by *B. dendrobatidis*. However, a probiotic that is beneficial to one species may not be equally effective on all hosts. *Janthiniobacterium lividium*, the bacterium that protected one species of frog, *Rana muscosa*, in California from *B. dendrobatidis* was ineffective in protecting a tropical species, *Atelopus zeteki*, possibly because the skin or environmental conditions of the tropical *Atelopus* was not suitable for the growth of the temperate isolate of *J. lividium* used (Becker et al., 2011). This illustrates a key challenge in developing an effective probiotic - finding a species that will successfully colonize a target host's tissues and persist at a level that reduces impact of a pathogen.

Here, we determine whether bacteria isolated from bats might be potential

probiotics for reducing impacts of the cutaneous fungal disease of bats, white-nose syndrome (WNS). White-nose syndrome first emerged in Howe's Cave, New York, in 2006, and spread quickly, causing precipitous declines in hibernating bats throughout Eastern North America (Blehert et al., 2009; Frick et al., 2010; Langwig et al., 2012). Four species (*Myotis septentrionalis, Myotis sodalis, Myotis lucifugus,* and *Perimyotis subflavus*) have suffered >90% declines in regional populations and one species, *Myotis septentrionalis,* is on a trajectory towards extinction and has recently been proposed by the U.S. Fish and Wildlife Service to be listed under the Endangered Species Act (Langwig et al., 2012). Preliminary investigations of treatments to reduce mortality using fungicides have caused higher mortality in there treated groups than controls, probably as a result of the toxic nature of some chemical fungicides to eukaryotes (Robbins et al., 2011). Currently, there are no management options that have slowed mortality or spread of this disease in North America (Cryan, Meteyer, Boyles, & Blehert, 2013).

WNS is caused by the fungus *Pseudogymnoascus destructans* (Lorch et al., 2011; Warnecke et al., 2012). *Pseudogymnoascus destructans* infects the dermal tissue of bats and grows optimally between 10–14 °C (Verant et al. 2012), similar to the temperature of hibernating bats. *Pseudogymnoascus destructans* infection disrupts bats' physiological processes including heat and water loss and electrolyte balance (Warnecke, Turner, & Bollinger, 2013), and often results in increased arousal frequency in hibernating bats (Reeder et al., 2012; Warnecke et al., 2012). Increased arousal frequency prematurely depletes bats' fat stores, and can result in death

approximately 70-120 days after infection, based on laboratory infection trials (Warnecke et al., 2012, 2013). Bats that are able to survive through hibernation until spring when insect prey become available again, appear to clear infection and fully recover (Meteyer et al., 2011). A probiotic could be an effective treatment to reduce WNS impacts if it could inhibit *P. destructans* growth and delay mortality long enough for bats to survive until spring emergence.

We cultured bacteria from the skin of four species of hibernating bats from eastern North America to determine whether naturally occurring bacterial species might exist within the skin microbiome of bats that could inhibit growth of *P. destructans*. We then quantified the anti-fungal efficacy of these bacteria across a range of concentrations over several weeks to determine if these bacteria might be effective in the treatment and control of *P. destructans* on bats.

Methods:

Sampling, plating and isolating cutaneous microbes

We sampled cutaneous bacteria from hibernating bats in two hibernacula in New York and two in Virginia, by rubbing sterile polyester swabs dipped in sterile water back and forth 5 times along each bat's forearm and muzzle. Swabs were frozen in 20% glycerol stock for later culturing. We swabbed ten individuals from each of four species *Eptesicus fuscus*, *Myotis leibii*, *M. lucifugus*, and *M. sodalis.*

We plated each swab on two types of general media, Reasoner´s 2A agar (R2A) and sabouraud dextrose agar (SDA), and incubated plates at 9°C for three

weeks. We initially classified the bacteria on each plate by morphotype, using color, growth form, and gram staining techniques. We isolated one colony from each morphotype (to reduce repeat sampling of the same isolate) using a sterile inoculating loop and re-plated each isolate on R2A media and grew them for 2-5 days at 9°C. We cryobanked each isolate by sampling from each of these colonies with a sterile inoculating loop, placing the sample in 30% glycerol, and freezing it at -80C for later use.

Identification of bacteria with anti-P. destructans properties

We determined whether each isolate could inhibit the growth of *P. destructans* using a challenge protocol adapted from the National Committee for Clinical Laboratory Standards (Clinical Laboratory Standards Institute, 2006). We made a 1.7×10^7 conidia/mL *P. destructans* solution (quantified using a hemocytometer) by flooding a mature plate (3+ weeks growth) of *P. destructans* in 20ml of 1X $PBST_{20}$. We submersed colonies for 5 minutes in solution, and then gently rubbed them with a sterile inoculating loop to free the conidia. The supernatant was drawn off and placed into a 50ml falcon tube and vortexed to homogenize the solution. We inoculated each 90mm plate with 200ul of the *P. destructans* solution and allowed it to air dry for 10 minutes to fix the conidia to the surface. We then added bacteria from a growing culture using pinpoint inoculation at three equally spaced points on top of the dried *P. destructans* solution. Bacteria cultures were grown from frozen stock 24 hours earlier on SDA. We placed the plates into incubators at 9-10°C, which is within the range

that bats hibernate (Storm & Boyles, 2010), and monitored growth every 14 days. We noted any bacteria that produced a zone of inhibition (a visible reduction of *P. destructans* growth surrounding the bacterial colony) and included them in subsequent challenge experiments described below.

In addition to the six bacterial isolates from bats that showed *P. destructans* inhibition, we included a *Pseudomonas fluorescens* isolate A506 commonly used in biocontrol of agricultural fungal pathogens (Lindow, 1985), and two types of controls, 1) a sham inoculation with 20% sterile glycerol stock, and 2) two bacterial isolates from bats in the genus *Chryseobacterium* and *Sphingomonas* (both gram-negative rod shaped bacteria) that are not known to produce anti-fungal compounds (CLSI, 2006).

Identification and Phylogenetic analysis of bacterial isolates

We identified bacterial isolates using PCR amplification and DNA sequencing. We obtained DNA for PCR by suspending a small amount of a bacterial colony in 100 µl of sterile deionized water (SDW) and lysed the cells at 95°C (10 min). Universal bacterial 16S rRNA gene primers were used to amplify the ~1.5 kb 16S rRNA gene fragment (Edwards et al. 1989). We added the following to each PCR template: 1 µl of crude lysate DNA template, 1.5 µl of each 0.6 µM forward and reverse primer, and 5 µl of Taq 5X MM (NEB) at 1X concentration, which contains 1.5 mM MgCl2, 2mM dNTPs, and PCR buffer. Reaction volumes were made up to 25 µl with SDW. The 16S rRNA gene sequences were compared with known

sequences in the EMBL database using MEGA BLAST (BLASTN 2.1.1, (Altschup, Gish, Miller, Meyers, & Lipman, 1990)) to identify the most similar sequence alignment. We used *Pseudomonas fluorescens* isolate *Pf*A506 to assure proper alignment of our sequences, and eliminated all positions with less than 95% site coverage. There were a total of 1333 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011).

The evolutionary history of our isolates was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Hasegawa, Kishino, & Yano, 1985). Initial tree(s) for heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.0611)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 18.2445% sites). Analyses were conducted in MEGA5 (Tamura et al., 2011).

Challenge experiments: Inhibition of P. destructans by growth suppression or bacterial invasion

We performed two types of challenge experiments to determine whether bacterial isolates could invade media colonized by *P. destructans* or suppress the

growth of a colony of *P.destructans*. In the invasion experiment, we determined the ability of each bacterial isolate to grow on lawns of different starting concentrations of *P. destructans*, and to produce a zone of inhibition in which *P. destructans* was either unable to grow or had growth halted adjacent to the bacteria colony. This experiment was designed to mimic a case where a probiotic would be applied to a bat that is already infected with *P. destructans*. A successful probiotic would be able to grow and produce a wide zone of inhibition. In the second inhibition experiment, we measured the growth of *P. destructans* on a lawn of different starting concentrations of each bacterial isolate. This experiment was designed to mimic a situation where the probiotic has already established on bat skin prior to *P. destructans* exposure. A successful probiotic would suppress the growth of *P. destructan*s.

In the invasion experiment, we quantified the growth of each bacterial isolate in media inoculated with four *P. destructans* concentrations (10^7, 10^6, 10^5, and 10^4 conidia/ml). We prepared the media by inoculating 200 μl of *P. destructans* stock in $PBST_{20}$ and spread this solution evenly across a petri dish with SDA media. After the *P. destructans* solution was dry and conidia fixed to the plate we used a pipette to inoculate the plates with 0.1 μl of a 10^8 cfu/ml solution of a given bacterial isolate. The bacterial solution was prepared by suspending whole colonies in 30% glycerol, and using an inoculating loop to suspend the colony into solution, We replicated each treatment nine times and grew cultures at 9°C for 37 days. We quantified the zone of inhibition (the area where *P. destructans* growth was not visible or arrested) by measuring the distance from the edge of the bacterial colony to the edge of the visible

P. destructans growth every other day (CLSI Clinical Laboratory Standards Institute, 2006). We also examined the zones of inhibition microscopically to characterize the effects of the bacteria on the growth of the *P. destructans* (Figure 1f,g).

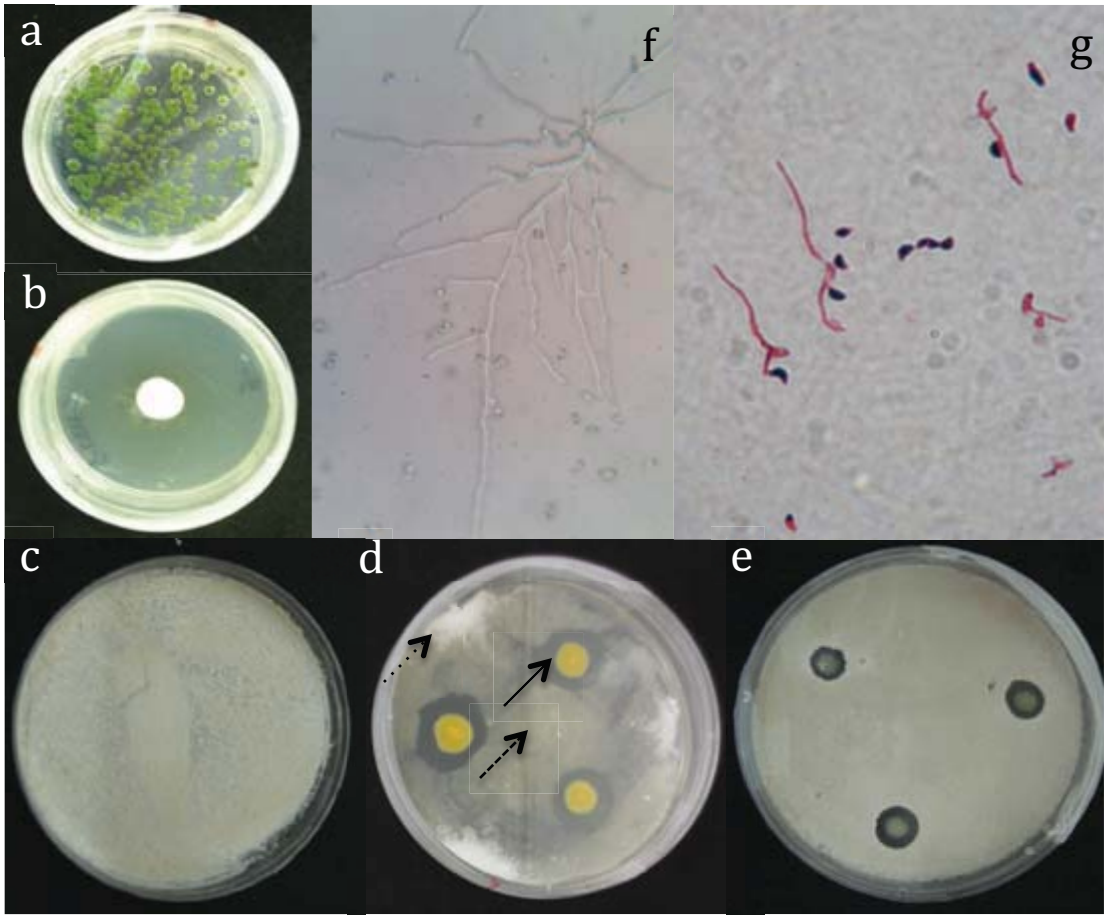

Figure 1: Plates Showing the Inhibition of P. destructans. (a) Suppression of *P. destructans* colonies by a lawn of 10^4 cfu/ml bacteria (PF2) showing no visible P. destructans growth. Compared to the (b) control plate showing optimal *P. destructans* colony growth at day 43. (d) Zones of inhibition produced by one of the top performing *P. fluorescens* isolates (PF2) compared to the sham inoculated control (c) and the widely used strain of *P. fluorescens* for control of plant pathogens *Pf*A506 (e) (PF7). (d) There are two distinct zones of inhibition produced by the top performing strain The first zone indicated by the black solid arrow, where the conidia were suspended immediately upon germinations (g) and the second zone indicated by the dashed line where *P. destructans* began to produce a mycelia network before its growth was arrested (f). And the white growth indicated by the dotted line if where P. destructans is growing optimally. Within the first zone, indicated by the dark ring

surrounding the yellow bacteria colony (PF2), the bacteria was able to arrest the conidia growth almost immediately upon germination (g) which can be seen by the small hyphael extension (pink) from the conidia (purple). Outside of this first zone the growth of *P. destructans* was still arrested (d), but the hyphae growth was much more extensive (f).

Finally, we performed an experiment to determine if anti-fungal compounds were produced by the bacteria in the in the initial culture. We drew off cell-free supernatant after centrifuging a culture of fresh bacteria inoculated in lysogeny broth both by itself, and co-cultured with *P. destructans* and inoculated three plates with 50μL of the supernatant on a lawn of *P. destructans*. In both cases, supernatant had no effects on growth of *P. destructans*, which grew uniformly across the plates.

In the suppression experiment, we determined the ability of each bacterial isolate to prevent growth of *P. destructans* across a series of six bacterial concentrations. Each bacterial isolate was plated from cryobanked glycerol stock onto a Petri dish with SDA media and allowed to incubate for two days at 9°C before being added to a 30% sterile glycerol solution. We standardized the concentration of each isolate by making serial ten-fold dilutions of the culturing stock and then counting the number of colony forming units (cfu)/ ml the bacterial glycerol solution was frozen at -20°C while calculating the cfu/ml. Each stock was standardized to the same concentration of $7.5x10^7$ cfu/ml using 30% glycerol. We plated 50 μl of each bacterium at each dilution on SDA in 60 mm Petri dishes. For bacterial concentrations 10^6, 10^2, and 10^1 cfu/ml we used three replicates per treatment and for concentrations 10^5, 10^4, and 10^3 cfu/ml we used five replicates. For the control plate

we added 50 μl of sterile 30% glycerol solution to the plates and then inoculated with *P. destructans*. We measured the diameter of the *P. destructans* colony every other day for 14 days, and then once every seven days for a total of 42 days.

Bacteria motility and preferential movement towards P. destructans:

We conducted a bacterial motility experiment to assess whether the wild-type *Pseudomonas* isolates from bats were able to use flagellated movement, and whether there is chemotaxis towards *P. destructans*. We made a 0.3% agar SDA media and a sterile inoculating loop was dipped into a $7.5x10^7$ cfu/ml of bacterial solution and then stabbed 2 in. into the soft agar. This was repeated for all nine bacterial isolates in Table 1. To determine whether or not the bacteria preferentially moved towards *P. destructans*, we repeated the same methods described as above, but included a small colony of Pd that was punched into one side of the tube. The tubes were incubated for 1 week at 10°C and then stabs were inspected for signs of bacteria movement away from the initial stab and whether there was preferential movement towards the *P. destructans* stab.

Results:

Bacteria with anti-P. destructans properties

We isolated 133 bacterial morphotypes from the 40 bats we swabbed**.** Four isolates from *E. fuscus* (from 3 bats) and two isolates from two separate *M. lucifugus* showed inhibition of *P. destructans* growth in standard challenge assays (Table 1).

UCSC Microbial Id	*Graph ID*	*Bat Species*	*Collection County*	*Collection Date*	*Bacteria ID*	**Motile**
CHR_NYWWO1C	*CHR*	*M.lucifugus*	Ulster, NY	08 April 2012	*Chryseobacterium sp.*	No
SPH_MP0022B	*SPH2*	*E. fuscus*	Bath, VA	29 March 2012	*Sphingomonas sp.*	No
PS_MP0014B	*PF1*	*E. fuscus*	Bath, VA	28 March 2012	*P. fluorescens*	Yes
PS_MP0022D	*PF2*	*E. fuscus*	Bath, VA	29 March 2012	*P. fluorescens*	Yes
PS_MYLUc	*PF3*	*M. lucifugus*	Highland, VA	19 March 2012	*P. fluorescens*	Yes
PS_KL12VA650C	*PF4*	*M. lucifugus*	Bath, VA	29 March 2012	*P. fluorescens*	Yes
PS_MP0050A	*PF5*	*E. fuscus*	Albany, NY	January 2009	*P. fluorescens*	Yes
PS_MP0022A	*PA6*	*E. fuscus*	Bath, VA	29 March 2012	*P. abietaniphila*	Yes
PS_ PfA506	*PF7*	NA	NA	NA	*P. fluorescens A506*	Yes

All six bacteria were in the genus *Pseudomonas*, with five of the six isolates belonging to the *Pseudomonas fluorescens* group and the other isolate (PA6) being most closely related to *Pseudomonas abietaniphila* (HF952541) (Figure 5).

Figure 5. Molecular Phylogenetic anaylsis of 16S rRNA using Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site the scale bar equals 0.02. The analysis involved 31 nucleotide sequences. *Pseudomonas* isolates from *M. lucifugus* and *E. fuscus* were compared to other known isolates in the *Pseudomonas fluorescens* group. (I am still working on the tree and need to include the bootstrap support values.)

Bacterial colony growth and invasion of P. destructans

In the invasion experiment, all bacteria initially grew rapidly and then plateaued in size, with growth continuing for longer at lower initial concentrations of Pd in the media (Figure 2).

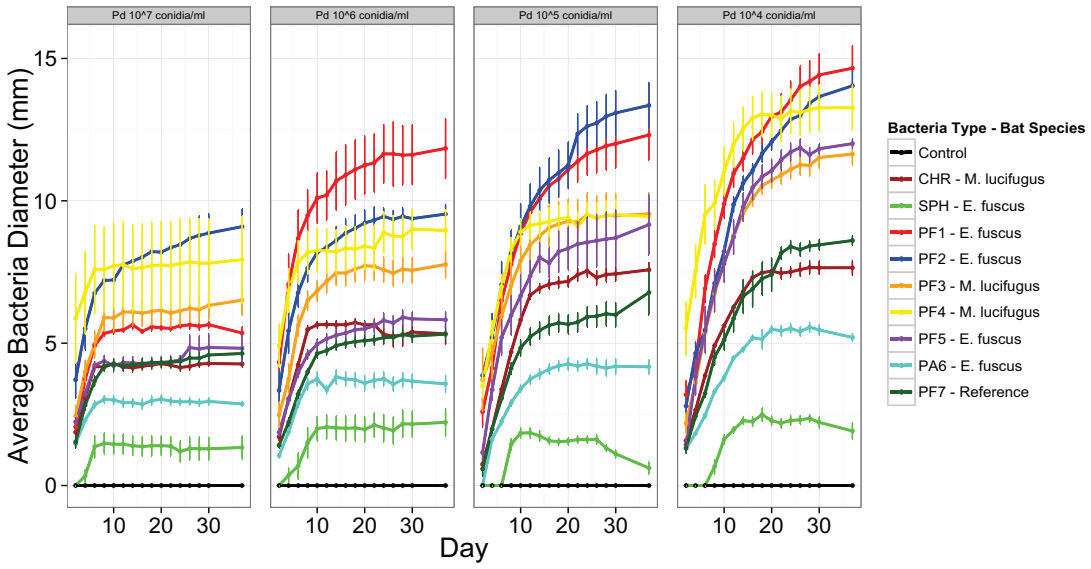

Figure 2: Bacterial colony size during invasion experiment. Colony size produced by nine bacterial isolates grown on plates inoculated with four different concentrations of *Pseudogymnoascus destructans* with fungal concentrations decreasing from left to right. CHR and SPH are isolates in the genus *Chryseobacterium* and *Sphingomonas* that are not known to produce antifungal compounds. The Control is a sham inoculation of 30% glycerol stock. PF1-7 and PA6 are bacterial isolates in the genus *Pseudomonas*.

Some bacterial isolates grew to form much larger colonies than others, with PF1, PF2, and PF4 frequently forming the largest colonies. The size of bacterial colonies increased with decreasing concentrations of *P. destructans*.

We were first able to visually detect the zones of inhibition between days 9 and 11 when there was enough *P. destructans* growth to make the zones apparent. At this time the zones of inhibition already differed significantly among bacterial isolates and concentrations and differences among isolates differed among concentrations (Mixed effects models with day as a random effect: a model with bacteria type interacting with *P. destructans* concentration as fixed effects was highly preferred over models with the two fixed effects additively (ΔAIC = 225.6), or models with just concentration (ΔAIC = 4103.8), just bacteria type (ΔAIC = 254.7), or a null intercept model with just day as a random effect (ΔAIC = 4109.9); Figure 3).

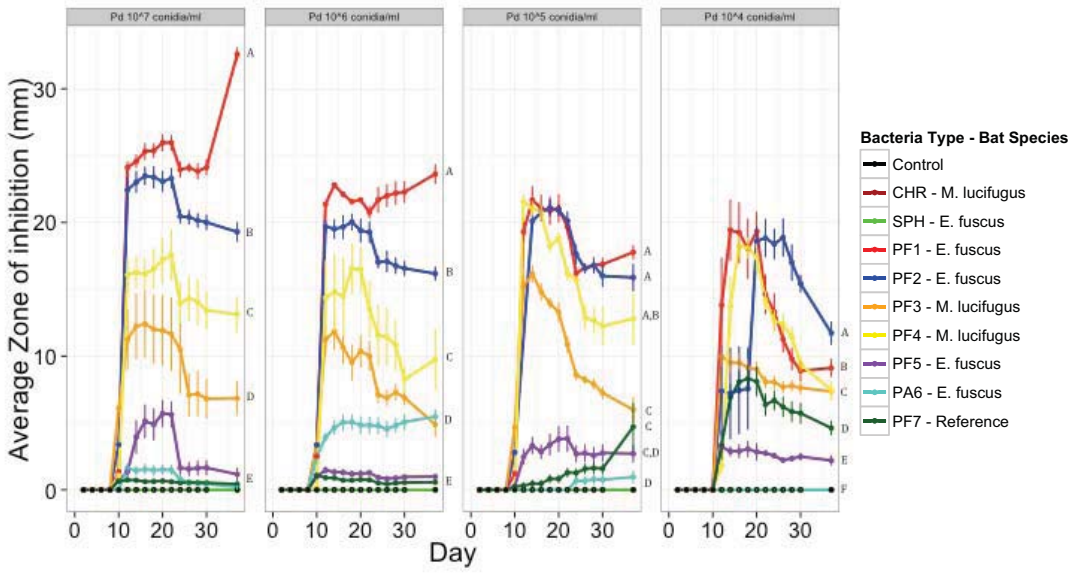

Figure 3: *Pseudogymnoascus destructans* invasion experiment.

Width of zones of inhibition produced by bacterial isolates when inoculated on a plate with four *P. destructans* concentrations. Zones of inhibition could not be visualized until *P. destructans* growth was visible on day 9-11 depending on the concentration of *P. destructans*. Lines denoted by the same letter did not differ significantly on the last day of the experiment (Table S2). CHR and SPH are isolates in the genus *Chryseobacterium* and *Sphingomonas* that are not known to produce antifungal compounds. CONTROL is an inoculation of 30% glycerol stock. PF1-5,7 and PA6 are isolates in the genus *Pseudomonas*.

Two bacterial isolates, PF1 and PF2, showed greater zones of inhibition across most concentrations of *P. destructans* at the end of the experiment (Table S2). Three isolates (PF1, PF2, and PF7) established two zones, one where growth of *P. destructans* was suspended immediately upon germination (Figure 1g), and another outside of this zone where growth was still arrested, but after the mycelial mat had begun to develop (Figure 1f). Zones of inhibition on the last day of the experiment (day 37) increased with increasing concentrations of *P. destructans* for the *Pseudomonas* isolates showing the strongest inhibition (Figure 3; PF1, PF2, and PF4; all concentration slopes were significantly negative, all p-values <0.03). For the other four *Pseudomonas* isolates the zone of inhibition was either variable across concentrations (Figure 3; PF3, PF5, PA6) or increased with decreasing *P. destructans* concentration (PF7; concentration coeff. 1.72 ± 0.64, $p = 0.008$). The two top performing isolates, PF1 and PF2, out performed the reference *P. fluorescens* strains (PF7 (*PfA506*)) at all concentrations with at least two fold differences in performance (Figure 3). The two bacteria used as controls (*Chryseobacterium sp. and Sphingomonas sp.)* and the sham inoculated control showed no zones of inhibition (Figure 3).

Suppression of P. destructans colonies by a series of bacterial concentrations

In the suppression experiment, *P. destructans* grew optimally in the absence of bacteria, and on media with low concentrations of the control bacteria (Figure 4).

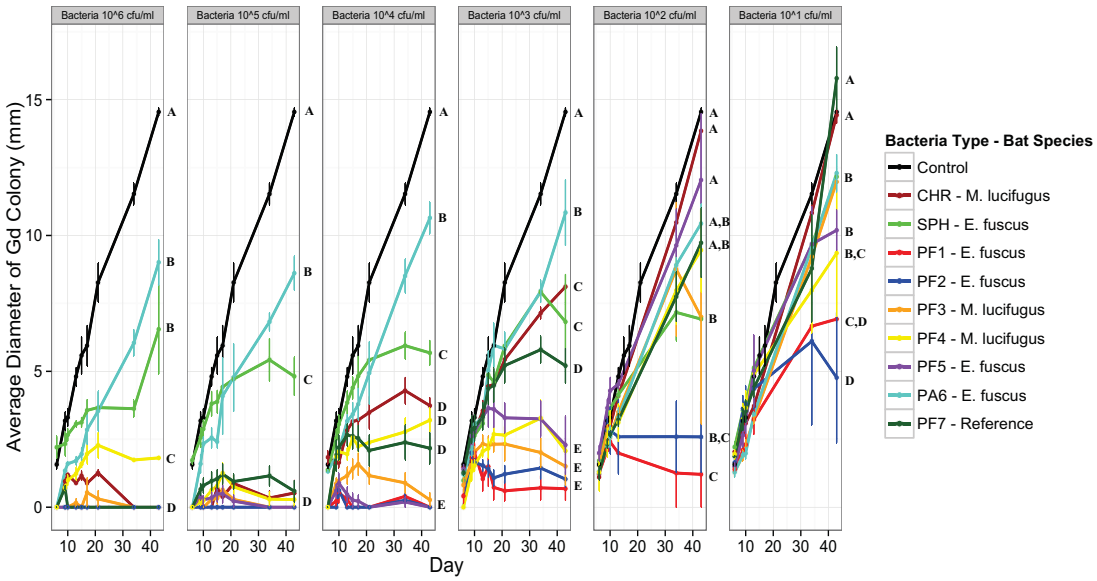

Figure 4: *Pseudogymnoascus destructans* suppression experiment. *Pseudogymnoascus destructans* colony size grown on plates with nine bacteria at six different concentrations (highest to lowest, left to right). Lines denoted by the same letter did not differ significantly on the last day of the experiment. CHR and SPH are isolates in the genus *Chryseobacterium* and *Sphingomonas* that are not known to produce antifungal compounds. The Control is a sham inoculation of 30% glycerol stock. PF1-7 and PA6 are isolates in the genus *Pseudomonas*.

The size of the *P. destructans* colonies differed between bacterial isolates and concentrations and the effect of bacterial isolate varied among concentrations (Mixed effects models with day as a random effect: a model with bacteria type interacting

with concentration as fixed effects was highly preferred over models with the two fixed effects additively (ΔAIC = 198.4), or models with just concentration (ΔAIC = 1261.9), just bacteria type (ΔAIC = 689.1), or a null intercept model with just day as a random effect (ΔAIC = 1612.7); Figure 4). At the three highest bacterial concentrations (10^6-10^4 cfu/ml), isolates PF1, PF2, and PF5 completely suppressed *P. destructans* growth, which had no visible growth on the plate for the duration of the experiment (day 42) (Table S3). At the three lowest concentrations of the bacterial lawn, two *Pseudomonas* isolates, PF1 and PF2 performed significantly better than most other isolates in reducing *P. destructans* growth (Figure 4; Table S3) and on plates with these isolates *P. destructans* did not grow between days 10 and 30. At the highest concentration (10^6 cells/ml), all bacterial lawns, including the two control bacteria, reduced growth of *P. destructans.* As the starting concentration of the bacterial lawn decreased, fewer isolates significantly reduced the growth of *P. destructans*. In both experiments, isolates PF1 and PF2 had maximum reduction of mycelial growth across most concentrations, regardless of the way the isolates and *P. destructans* were co-cultured.

Bacteria motility and chemotaxis

All seven *Pseudomonas* isolates showed signs of motility but there was no observed chemotaxis towards *P. destructans* colonies. We observed two of the *Pseudomonas* isolates, PF1 and PF2, dispersing along *P. destructans* hyphae. The two control bacteria showed no signs of motility (Table 1).

Discussion:

As the threat of emerging infectious disease grows with increased global travel and trade (Daszak et al., 2000), new ways of managing wildlife disease must be considered (Kilpatrick, 2006). Traditionally, fungal pathogens have been managed using chemical fungicides (Knight et al., 1997), but toxicity, effects on non-target organisms, and application challenges makes it difficult for the broad scale use on wildlife fungal pathogens (Robbins et al., 2011). Our results suggest that bacteria isolated from the same or related hosts could be useful in reducing pathogen growth, and augmenting these bacteria may reduce disease severity for fungal diseases like WNS.

The results from the two sets of experiments demonstrate that bacteria cultured from bats can inhibit the growth of *P. destructans* through both suppression and invasion. Our results suggest that augmentation prior to *P. destructans* exposure could prevent colonization, whereas bacterial augmentation after exposure could displace *P. destructans*. Applying these bacteria to bats could reduce disease severity, but only if they would persist on bat skin at high enough concentrations to limit *P. destructans* growth below levels that cause lethal disease. The inhibitory effects of the bacteria on *P. destructans* lasted for the full 40 days of our experiments, which suggests they might help bats to survive hibernation with WNS.

The bacteria we isolated from bats, *Pseudomonas fluorescens*, is ubiquitous in the environment and is well known to have anti-fungal properties (Rainey &

Travisano, 1998). *Pseudomonas fluorescens* has been detected on several mammals (including bats), as well as amphibians, fish, and plants (Gram et al. 1999, Grice et al., 2008, Culp et al. 2007, Zanowiak et al. 2013,). Members of the *P. fluorescens* group are known to produce a suite of antifungal compounds that can inhibit many plant fungal pathogens (Bangera and Thomashow 1999) as well as the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (Brucker, Baylor, & Walters, 2008). Some strains in the *P. fluorescens* group are also capable of producing mycolysing enzymes that can colonize the mycelia and conidia of fungi rendering them no longer viable (Diby et al. 2005). All of our *P. fluorescens* isolates showed motility, which would allow it to use the mycelial networks of fungal colonies to aid in dispersal and colonization (Warmink et al., 2011). All of these attributes make *P. fluorescens* an ideal candidate biological control agent for reducing infection intensity and increasing survival of bats exposed to *P. destructans*.

Whether these antifungal bacteria that naturally occur on bat skin could partially explain differences in mortality from WNS among populations and species is currently unknown. The isolates with strongest inhibitory properties were cultured from *E. fuscus*, which has lower mortality from WNS compared to other species (Langwig et al., 2012). However, we also isolated two strains of *P. fluorescens* (PF3 and PF4) that showed moderate *P. destructans* inhibition from *M. lucifugus,* a species that has suffered severe mortality from WNS (Frick et al., 2010). Future research is needed to determine the relative abundance, distribution, and inhibitory ability of *P.*

fluorescens on wild bats and whether presence and abundance of *P. fluorescens* on bats is related to differential disease severity and impacts on species and populations.

Our results present evidence for the potential of probiotic bacteria to reduce the growth of *P. destructans*, the cause of WNS. Next steps in developing a probiotic for WNS should include testing *in vivo* one or more of the *P. fluorescens* strains that we isolated against *P. destructans* using a bat species such as *M. lucifugus*, *M. septentrionalis*, or *Perimyotis subflavus* that suffers high disease mortality from WNS (Langwig et al., 2012). Studies with live hibernating bats will determine whether interactions observed in vitro have functional significance in disease outcomes for bat species currently threatened by WNS.

List of supplemental tables:

Table S1: BLAST analysis of 16S rRNA sequence from the National Center of Biological Information database with six strains isolated from bats (*M. lucifugus* and *E. fuscus*).

Table S2: Coefficients for linear models of the influence of nine bacterial treatments and a control on the radius of the zones of inhibition of *P. destructans* produced by bacteria at four different concentrations of P. destructans on day 37 for the data shown in Figure 3.

Table S3: Coefficients for linear models of the influence of nine bacterial isolates on the diameter of *P. destructans* colonies for each bacterial concentration on day 43 for the data shown in Figure 4.

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