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Survey of Bacteria Associated with White-Nose Syndrome Infected Fluorescing and Nonfluorescing Bats

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Abstract

White-nose syndrome is an emerging disease in Europe and North America, causing significant decline in bat populations. Wing fluorescence is detected in many white-syndrome afflicted bats under UV illumination. Wing pathology in the white-nose infected bats is associated with the fluorescence. Damaged wings significantly reduce thermoregulatory, foraging, and reproductive capacities of bats after hibernation. Thus far, no studies on the fluorescence phenomenon observed in the diseased bats have been done. No surveys of microbes have been performed in white-nose afflicted bats and no comparison studies on changes to the microbiota in diseased animals have been conducted. This study focused on bacteria associated with white-nose syndrome infected bats, collected from fluorescing and nonfluorescing bats. We hypothesized that *Pseudomonas* species such as *P. fluorescens* might be responsible for the fluorescence in white-nose afflicted bats. We also expected that fluorescing and nonfluorescing white-nose syndrome infected bats would have different microbiota. Eighty-two microorganisms were grown in pure culture; fourteen of these were identified. No significant differences were found between the microbiota of fluorescing and nonfluorescing bats. *Pseudomonas fluorescens* along with other *Pseudomonas* species could be responsible for the fluorescence observed on bats with white-nose syndrome.

1. Introduction

Bats play important ecological roles in insect control, plant pollination, seed germination, and forest regeneration [1, 2]. Bat populations have declined drastically since 2006 in Europe and in the northeastern US due to the emergence of white-nose syndrome (WNS) [3, 5, 13-15]. Declining bat populations are likely to have far-reaching ecological consequences [1, 5, 14]; thus, immediate conservation measures need to be taken to minimize the spread of the disease [9, 14].

WNS has been characterized as a condition in hibernating bats [1, 2, 9]. WNS disrupts the hibernating pattern of bats by eroding the epidermal layer of skin (especially around ears and wings), depleting energy reserves, and promoting dehydration [6, 9, 14]. WNS is characterized by the presence of fungal hyphae and abundant conidia on ears, muzzles, forearms, and wing membranes [1, 6, 11]. The fungus also affects hair follicles, connective tissues, sebaceous and apocrine glands [1, 3, 9]. Many white-nose syndrome afflicted bats show fluorescence on the wing membranes, detected under UV light. Wing pathology is associated with the fluorescence in bats with WNS [27]. Damaged wings significantly reduce thermoregulatory, foraging, and reproductive capacities of bats after hibernation [3, 5, 14]. Decreased fat reserves and a suppressed immune system lower bats' survival during and after hibernation and provide ideal conditions for the spread of infection by *Geomyces* fungus [3, 6, 11, 13].

Phylogenetic studies and ribosomal RNA analysis place the fungus associated with WNS in the genus *Geomyces*, specifically in *G. destructans* [1, 2, 6]. The fungus, isolated from hibernating bats grows optimally between 4 ° and 10 ° - 15 °C, with upper limit for growth at about 20 or 24°C [1,6, 15].

The mechanisms of WNS transmission and persistence are still poorly understood [5, 9, 11, 14]. We still do not know if the fungus is primarily responsible for the deaths of bats or if it is a secondary infection. The origin of this fungal disease is still unknown; it is unclear whether the pathogen has recently emerged in Europe or has been there for a long time but not causing massive die-offs in bats [9, 13]. We are not certain if delayed recovery rates are caused by bacterial co-infection in WNS bats [14]. Some researchers declare that neither pathology, nor virology studies conducted revealed any known pathogenic microbes associated with WNS [2, 3, 15]. No studies were conducted on microbiota or the origin of wing fluorescence in WNS-infected bats. Symbiotic relationships, which help to support health and survival of many species, have been mostly ignored in WNS-infected bats [4, 8, 10]. For example, *Pseudomonas* spp. engaged in a mutualistic relationship with its host, provide bacterial protection from pathogens [4]. Human symbiont *Bacteroides fragilis* protects from colitis caused by *Helicobacter hepaticus* (10). Environmentally induced shifts in microbiota (due to changing temperature, pH, etc.) may promote emergence of new diseases (4, 8). Coral bleaching, for example, associated with rising temperature, is interconnected with the loss of mutualistic algae and decreased antibiotic activity of the mutualistic bacteria *Acropora.*

Many *Pseudomonas* species that produce yellow-green fluorescence (fluorescence similar to the fluorescent pigment found on the wings of WNS-infected bats) have optimal growth at 4^{o}C and around 20^{o}C [18,19]. Many fluorescing Pseudomonads produce antimicrobial and antifungal compounds, effective against some plant or animal diseases [17, 20, 23-26]. For example, phenanazines, blue-green metabolites produced by some fluorescent pseudomonads as an adaptation to a biofilm lifestyle, are natural antibiotics [26]. Some *Pseudomonas* spp. exhibit in *vitro* activity against fungal pathogens causing dermatophytosis [25].

We hypothesized that *Pseudomonas* species such as *P. fluorescens* might be responsible for the fluorescence in WNS bats. We also expected that fluorescing and nonfluorescing WNS-infected bats would have different microbiota. We conducted this study to find out the origin of fluorescence in WNS bats and to survey microorganisms that might be associated with WNS.

2. Materials and Methods

Microbe samples were provided by John Gumbs, principal researcher for the Bats Research Center in Shohola, Pa. Samples were taken from bats in deep torpor, with the exception of a few specimens from a euthermic bat. Fifteen dry and wet tissue samples included microbes from ears, wings, forewings, and forearms.

We added 1 ml of 0.8 % sterile saline to dry samples and 4 ml to wet samples. Then, we plated 100 microliters of undiluted samples on S1 agar[7] without antibiotics and on blood agar (tryptic soy agar with 5 % defibrinated sheep blood). We preserved the original samples in 20 % glycerol and placed them in a freezer at -80 ^{o}C. We incubated 4 plates of each bat sample: one of each agar at room temperature and one of each agar at 4°C. We incubated samples for 2 weeks and then examined all 60 plates under 366 nm UV light.

Since multiple colonies were present on the initial plates, we subcultured the microorganisms and replated them several times in order to obtain pure cultures. Fluorescence was monitored on a weekly basis. We also gram-stained the fluorescent cultures (freshly prepared air-dried slides were stained with a crystal violet reagent for 1 minute and Gram's iodine for 1 minute; then, the slides were decolorized with 95 % ethanol and counterstained with safranin for about 1 minute. We washed the slides with a gentle stream of tap water in between the staining procedures[28]).

DNA was isolated from the pure cultures using the fast DNA Spin kit (MP Biomedicals) following the procedures provided. Gel electrophoresis (1.5 % agarose gel in TBE buffer) and Nano Drop spectrophotometry (ND-1000, NanoDrop Technologies, Inc., Wilmington, DE) were performed to verify successful DNA isolation. We then amplified small subunit rRNA genes by polymerase chain reaction (PCR) using BIO-RAD thermocycler, Hot StarTaq polymerase, and 50 µl volume of PCR mixture in each tube (DNA was denatured at 95^{0}C for 15 minute and for 1 minute at 94^{0}C; 2 oligonucleotide primers were annealed at 53^{0}C for 45 seconds; DNA synthesis continued for 2 minutes at 72^{0}C; the cycle was repeated 34 times). We subjected the PCR products to 50-minute electrophoresis, using 10 µl of each sample and 1.5 % agarose gel in TBE buffer. Afterwards, DNA sequencing was performed using universal primer U1R[16] and 10 µl of the PCR product as template. We analyzed BLAST results of the 14 samples to identify fluorescing—as well as nonfluorescing—cultures based on comparisons of DNA sequences [28].

3. Results

Out of the initial 60 cultivation plates, 37 had visible growth. The majority of the 23 plates that did not have any visible microbial growth were inoculated with the dry samples. There were five initial culture plates that had visible yellow-greenish fluorescence under 366 nm UV light (the fluorescence pigment looked bluish-purple in photos; figure 1). The fluorescence pigment produced by our cultivated samples looked similar to the fluorescence produced by *P. fluorescens* (Figure 1).

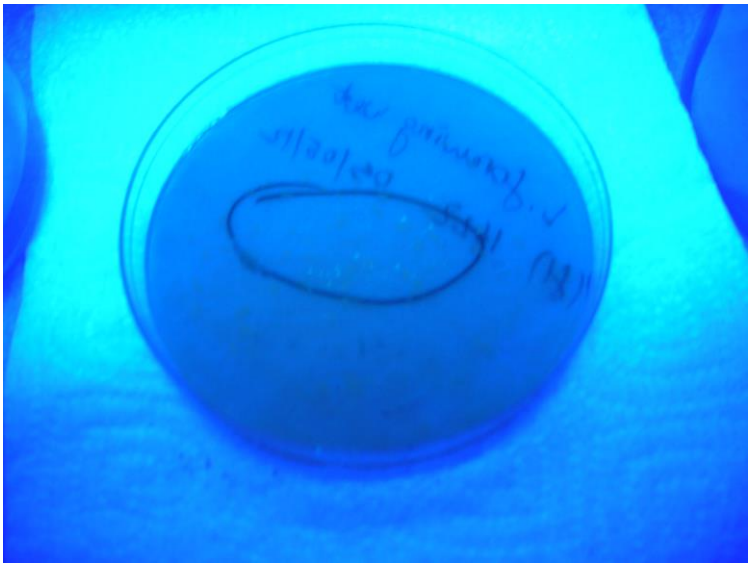

Figure 1 Fluorescing culture under 366 nm UV light, taken from the left forewing of a fluorescing bat (wet sample #31) grown on S1 medium at room temperature. The fluorescent yellow-greenish pigment appears bluish on the picture.

Four of the five fluorescing cultures, cultivated at room temperature, came from two right forewing samples, left forearm (L.Fa.), and right wing (R.W.) of fluorescing bats; meanwhile, one fluorescing culture originated from the right forewing (R.F.) of a nonfluorescing bat, cultivated at 4^0C. The initial culture plates had a variety of fluorescing colonies on them (Figure 2). All fluorescing colonies were flat or slightly raised, circular, shiny, ranging from 0.5 to 7 mm in diameter. The fluorescence appeared and disappeared, as well as migrated to other parts of the initial culture plates. Fluorescence color disappeared from some plates within a month, from others within a few months. However, one of the initial plates that came from a left forewing (sample #5) retained fluorescence and a bright yellow-green color of the medium for about 6 months; unfortunately, we could not identify this sample. Many of the initial cultures that did not seem to fluoresce came from both fluorescing and nonfluorescing bats.

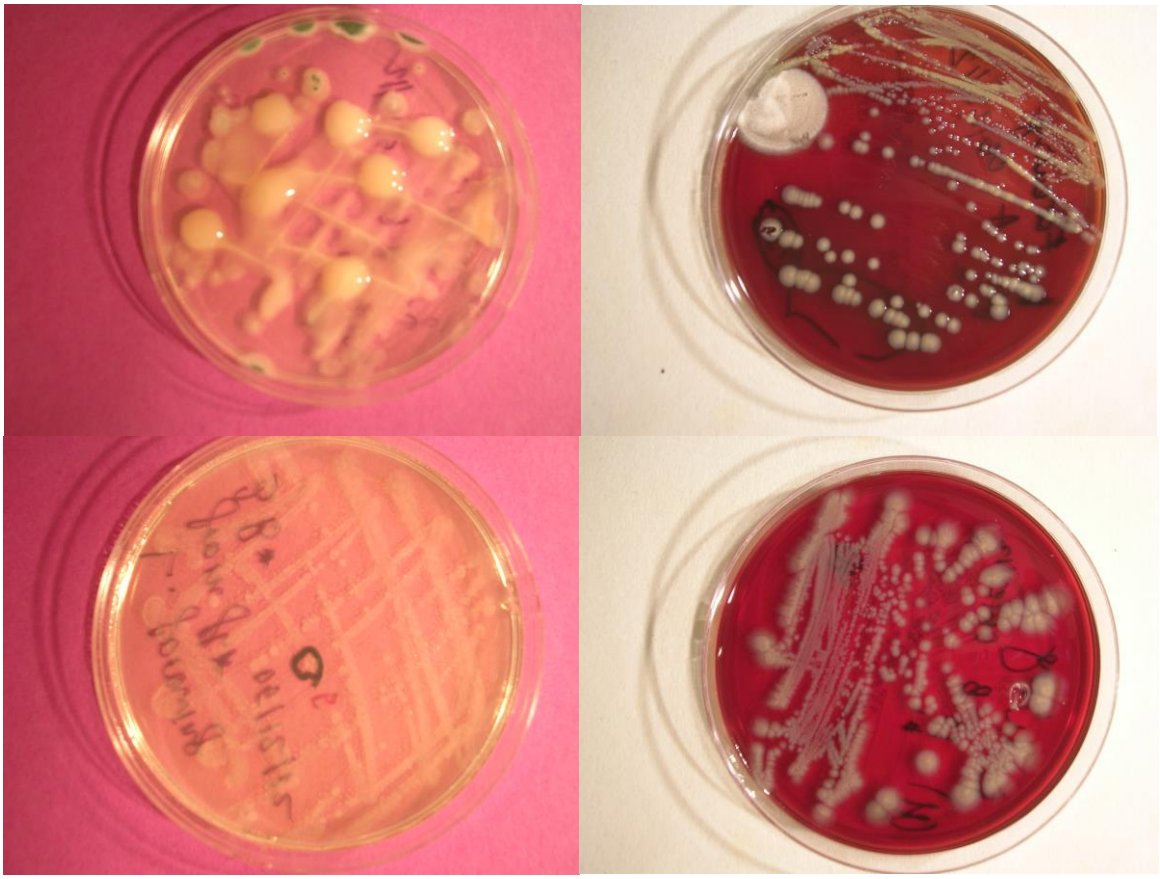

Figure 2 Initial culture plates having intermixed fluorescing and nonflourescing colonies (upper left and right; bottom right); one intial plate with yellow-green color of the medium having a variety of fluorescing colonies (lower left).

From 37 initial plates we subcultured about 250 different microorganisms. However many subcultured organisms failed to grow successfully in pure culture. Subsequently, out of 250 replated microorganisms, we were able to successfully cultivate only 82 pure cultures, out of which 15 were fluorescent, including 5 samples from the nonfluorescing bat. The gram-staining procedure of some fluorescening cultures revealed presence of gram-negative rods (Figure 3). Many of the 15 fluorescent microbes lost their fluorescence within a couple of months.

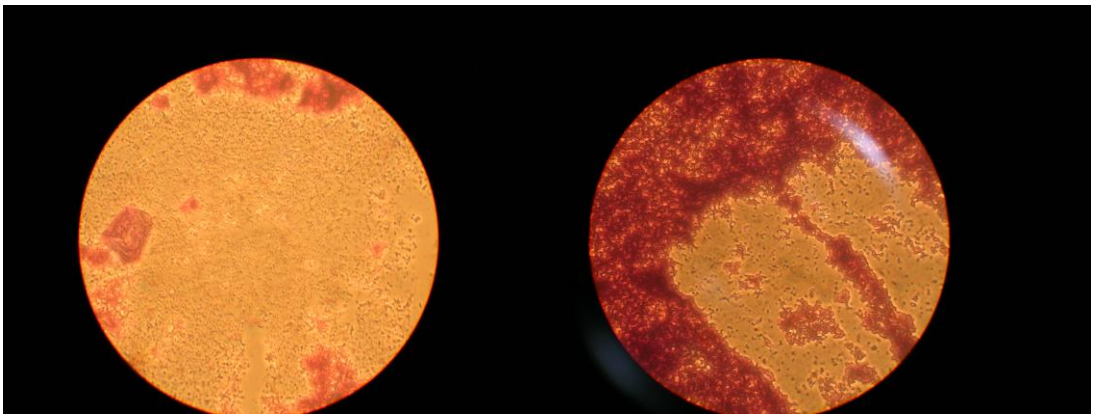

Figure 3 Gram-staining results of two cultures, fluorescing under 366 nm UV light (samples # 10, 50), isolated from left forewings of fluorescent bats. Pink rods represent gram-negative bacteria.

We were able to identify 14 samples out of the 82 pure cultures. The best matches for each of sequence analyzed are shown in Table 1. A majority of the identified microorganisms belong to the genus *Pseudomonas.* We compared microbes isolated from the forewings of fluorescing and nonfluorescing WNS-infected bats. A variety of *Pseudomonas* species were present in both kinds of samples, with no significant differences in the top sequence hits, with the exception that *Klebsiella* sp. was found in a fluorescent bat; meanwhile, *Rhodococcus* sp. originated from a nonfluorescent specimen (Tables 1, 2). Top hits in Table 1 represent best microorganisms matches to our query sequences, based on the criteria described below. Query cover is the percentage of query covered by alignment to the database sequence. Max score represents the highest alignment score from that database sequence; meanwhile, total score refers to the total alignment scores from all alignment segments. E value is expected value of all alignments from that database sequence; it describes the number of hits one can "expect" to see by chance when searching a database of a particular size. The lower the E-value, or the closer it is to zero, the more "significant" the match is. The Max ident is the highest percent identity of all query-subject alignments [29, 30].

The Max ident, the query cover, max score and total scores are very high for the majority of our top matches, meaning that our microbial species sequenced belong to the species matched or are closely related to them.

Table 1. Best matches for the 14 samples obtained from fluorescing and nonfluorescing WNS-infected bats.

Sequence Analyzed	Top Hits: Genus	Top Hits: Species	Query Length	Max Score	Total Score	Query Cover	E Value	Max Ident	Original Sample Number
SSUrRNA1	*Pseudomonas*	N/A	772	1369	1369	96%	$1e^{-179}$	99%	1
	Pseudomonas	*fragi*							
	Pseudomonas	*psychrophila*							
SSUrRNA2	*Pseudomonas*	N/A	774	1358	1358	96%	$1e^{-179}$	99 %	2
SSUrRNA4	*Pseudomonas*	*cedrina*	768	1345	1345	97%	$1e^{-179}$	99%	9
	Pseudomonas	*fluorescens*							
	Pseudomonas	*azotoformans*							
SSUrRNA6	*Pseudomonas*	N/A	736	1291	1291	95%	$1e^{-179}$	99%	13
	Pseudomonas	*psychrophila*							
SSUrRNA7	*Klebsiella*	*oxytoca*	735	1258	1258	93%	$1e^{-179}$	99%	15
	Klebsiella sp.	N/A							
SSUrRNA8	*Pseudomonas*	*cedrina*	733	1306	1306	96%	$1e^{-179}$	99%	22
	Pseudomonas	*N/A*							
SSUrRNA9	*Pseudomonas*	N/A	769	1339	1339	96%	$1e^{-179}$	99%	35
	Pseudomonas	*fragi*							
SSUrRNA10	*Pseudomonas*	N/A	766	1349	1349	96%	$1e^{-179}$	99%	38
	Pseudomonas	*azotoformans*							
SSUrRNA11	*Pseudomonas*	N/A	766	1367	1367	96%	$1e^{-179}$	99%	50
	Pseudomonas	*cedrina*							
SSUrRNA12	*Pseudomonas*	N/A	769	1360	1360	97%	$1e^{-179}$	99%	53
	Pseudomonas	*cedrina*							
	Pseudomonas	*fluorescens*							
SSUrRNA13	*Pseudomonas*	N/A	775	1354	1354	96%	$1e^{-179}$	99%	63
	Pseudomonas	*psychrophila*							
SSUrRNA14	*Pseudomonas*	N/A	765	1332	1332	96%	$1e^{-179}$	99%	10
	Pseudomonas	*cedrina*							
SSUrRNA15	*Rhodococcus*	*erythropolis*	762	1070	1070	86%	$1e^{-179}$	95%	75
	Rhodococcus	N/A							
SSUrRNA16	*Pseudomonas*	N/A	766	1282	1282	92%	$1e^{-179}$	99%	81
	Pseudomonas	*cedrina*							

Table 2. Colony morphology of the 14 cultures identified by sequence

Original Sample Number	Sample From	Fluorescence under UV Light	Agar Type	Plate Color Change	Colony Appearance	Inoculation Temperature
1	R.W. from fluorescent bat	No	S1	No	Flat, circular with undulate edges, clear to milkish, shiny, 7mm	4^0 C
2	R.W. from fluorescent bat	No	S1	No	Flat, circular with regular edges, clear, 3mm	Room temp
9	L.F. from fluorescent bat	Yes	S1	Yes, green-yellowish plate	Flat, circular with irregular edges, milkfish-yellowish, shiny, 5mm	Room temp
10	L.F. from fluorescent bat	Yes	S1	Yes, bright yellowish plate	Flat, circular with irregular edges, clear, shiny, 2mm	Room temp
13	L.E. from fluorescent bat	No	Blood	No	Slightly raised, circular, grayish-clear, 1.5mm	Room temp
15	R.F. from fluorescent bat	No	S1	No	Raised, irregular shape and edges, yellowish, 1-2mm, distinct smell	Room temp
22	R.F. from Non-fluorescent bat	Yes	S1	No	Somewhat raised, circular with filamentous edges, beige, shiny, 10mm	Room temp
35	R.W. from fluorescent bat	No	S1	No	Flat, irregular shape with undulate edges, grayish, shiny, 25mm	Room temp
38	L.F. from fluorescent bat	Yes	S1	No	Raised center, circular with irregular edges, yellowish, 9mm	Room temp
50	L.F. from fluorescent bat	Yes	S1	Yes, yellow-green	Raised, circular with irregular edges, bright yellow, 2.5mm	Room temp
53	R.F. from Non-fluorescent bat	Yes	S1	No	Raised center, circular with undulate edges, yellow-grayish with clear shiny edges, 7mm	4^o C
63	R.F. from Non-fluorescent bat	No	S1	No	Slightly raised, irregular shape, milkfish, dull, 2 by 5mm	4^o C
75	R.F. from Non-fluorescent bat	No	S1	No	Raised, irregular shape with undulate edges, beige	4^o C
81	R.F. from Non-fluorescent bat	Yes	S1	Yes, yellow-green	Flat, circular with irregular edges, yellow-green, less than 1mm	4^o C

4. Discussion

Thus far, no surveys of microbes and no studies on the origin of fluorescence in the WNS bats have been conducted. Isolating and identifying total microbiota presents a challenge. Some microbial species grow poorly in laboratory conditions. Failure to grow many of the initial cultures from the dry specimens may be due to death of microbes in the time between collection and plating. Out of 250 microorganisms subcultured, we were able to grow 82 pure cultures and identify 14 samples (Table 1).

A majority of the microbes identified exhibited optimal growth at room temperature; meanwhile, a few others such as *P. psychrophila*, *P. cedrina*, and *P. fragi* were able to grow at 4 ^{0}C. *P. psychrophila* is a facultative psychrophilic bacterium, closely related to such Pseudomonads as *P. lundensis*, *P. fragi*, and *P. fluorescence*. *Pseudomonas* spp. are also gram-negative, non-sporeforming rods[18]. These observations are consistent with the cultivation of many *Pseudomonas* species, which we identified in our samples [12, 17, 18, 19, 20]. We have discovered no new species (sequences similarities were within 99%). We did not compare our sequences to each other, but according to the slight differences in the sequences, we can conclude that we have found various species of *Pseudomonas* and species from two other genera (Table 1). There is a chance that differences among the Pseudomonads are not real due to sequencing error(s). Possible approach to examine whether DNA sequence differences are real is to sequence them using a different primer.

We confirmed our hypothesis that fluorescence came from *Pseudomonas spp*., but fluorescent pigment was not produced by a single species such as *P. fluorescens. P. cedrina*, for example, produces similar yellow-greenish glow and changes the plate color to bright yellow-green as does *P. fluorescens* [12, 18]. Four of our cultures that caused plate color change during cultivation showed *P. cedrina* as one of the top hits for the sequences subjected to blast analysis (Tables 1, 2) [30]. Therefore, fluorescence could have been produced by *P. fluorescens, P. cedrina*, or any other fluorescent Pseudomonads. Genus *Pseudomonas* is one of the genera that has the largest number of species, among which are 29 fluorescent Pseudomonads that produce various fluorescent pigments [17,18]. We do believe that *Pseudomonas* species are the source of fluorescence on the WNS-infected bats. In addition to the blast results, the fluorescence of microbes cultivated in our lab is comparable to the fluorescence seen on the hibernating WNS-infected bats, and to that of Pseudomonads [7,12,17,18].

We concluded that the fluorescence phenomenon was not limited to the microorganisms isolated only from the fluorescing bats. We had a few cultures (in both initial plating and pure culture) that produced fluorescent pigments and were identified as *Pseudomonas* species, but which came from the nonfluorescing bat. We also observed that many samples taken from the fluorescing bats did not give rise to any fluorescent microorganisms. Fluorescence may be dependent on metabolites produced by *Pseudomonas* species in different growth phases (some in stationary, some in rapid growth phase). Nutrients, such as iron**,** may serve as limiting factors. For example, the water-soluble pigment responsible for fluorescence of *P. fluorescens* is produced only when iron concentration is low [12].

For the comparison study of the microbiota from fluorescing and nonfluorescing bats, we decided to examine the samples taken from a comparable body part; we compared microbes isolated from the forewings of WNS bats. We could not confirm our hypothesis that the microbiota of fluorescing and nonfluorescing bats would differ, since our results did not show any significantly different microbial species. The only difference was in the presence of *Rhodococcus* nonvirulent strand in a nonfluorescing bat and *Klebsiella* species, commonly giving rise to urinary and respiratory infections, in a fluorescing bat [21, 22]. We cannot make any definitive conclusions based on these two differences. More microbes need to be surveyed in order to examine the differences in the microbiota of fluorescing and nonflourescing bats. Physiological differences between the two kinds of bats may explain the differences in fluorescence.

We may have identified mostly *Pseudomonas* strains because we used media that promote growth of Pseudomonads. However, the numerous numbers of Pseudomonads might also be associated with the WNS and potential antimicrobial properties of Pseudomonads. Fluorescent Pseudomonads are known to control several diseases caused by soil borne pathogens [20, 23]. Antimicrobial activity is correlated with the density and competition of Pseudomonads with other microorganisms [17, 20]. This fact can also account for the dominant number of *Pseudomonas* spp. that we isolated and identified from WNS bats. There is also a variety of antifungal metabolites produced by such fluorescent Pseudomonads as *P. fluorescens* and *P. aeruginosa* [17, 20, 23-26]. Fluorescent Pseudomonads produce such antifungal and antimicrobial secondary metabolites as 2,4-diacetylphloroglucinol, hydrogen cyanide, iron-chelating sidephores, and extracellular lytic enzymes [23-25] . For example, an antifungal metabolite produced by *P. fluorescens* inhibits growth of *Piriculaiia oryzae* and *Rhizoctonia solani* [20]. Another antimicrobial, a diffusible bioactive product, suppresses a plant fungus *Pythium ultimum* in *vitro* [17]. There might be

also a lack of correlation between in *vitro* and in *vivo* antimicrobial activity of Pseudomonads due to competitiveness and ecological fitness of a biological species[17, 24].

Thus far, no surveys of microbes have been performed in WNS bats and no comparison studies on changes to the microbiota in diseased animals have been conducted. Unfortunately, we could not confirm that microbiota of fluorescing and nonfluorescing diseased bats differed. More studies need to be conducted. One of the goals for future research would be to compare microorganisms found in healthy bats with the microbes isolated from WNS-infected bats. The observations that fluorescence is associated with infection of bats by *Geomyces destructans* [27] and that similar Pseudomonads were found on fluorescing bats in this study suggest that infection by *G. destructans* may alter the physiology of *Pseudomonas* species resulting in production of fluorescent compounds.

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