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Comparison of Airborne Bacterial Communities from a Hog Farm and Spray Field

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Airborne bacteria from hog farms may have detrimental impacts on human health, particularly in terms of antibiotic resistance and pathogen zoonosis. Despite human health risks, very little is known about the composition and diversity of airborne bacteria from hog farms and hog-related spray fields. We used pyrosequencing analysis of 16S rRNA genes to compare airborne bacterial communities in a North Carolina hog farm and lagoon spray field. In addition, we isolated and identified antibiotic-resistant bacteria from both air samples. Based on 16S rRNA gene pyrosequence analysis, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the dominant phyla in airborne bacterial communities from both hog farm and spray field sites. Within the *Firmicutes* genera, *Clostridium* spp. were more abundant in the hog farm, whereas *Staphylococcus* spp. were higher in the spray field. The presence of opportunitic pathogens, including several *Staphylococcus* species and *Propionibacterium acnes*, was detected in both bioaerosol communities based on phylogenetic analysis. The isolation and identification of antibiotic-resistant bacteria from air samples also showed similar results with dominance of *Actinobacteria* and *Proteobacteria* in both hog farm and spray field air. Thus, the existence of opportunistic pathogens and antibiotic resistant bacteria in airborne communities evidences potential health risks to farmers and other residents from swine bioaerosol exposure.

Keywords: Hog, swine, antibiotic resistance, bioaerosol, airborne bacteria

Introduction

In order to keep up with population increases and livestock demands, concentrated animal feeding operations (CAFOs) have become the predominant method for animal production. By concentrating large numbers of animals in confined spaces, there is an increased potential of zoonotic disease and disease transfer [27]. To prevent disease and promote growth, subtherapeutic doses of broad-spectrum antibiotics are routinely administered at continuous low-level doses in animal agriculture [46]. Of particular concern is the swine industry, with an estimated 10.3 million pounds of non-therapeutic antibiotics used annually [24]. Many studies have shown that this sustained, low-level antibiotic use for non-therapeutic purposes selects for high-level resistance to antibiotics in commensal and pathogenic bacteria in swine [1, 2, 5, 10, 30, 45].

One route of transmission for antibiotic-resistant bacteria and disease from CAFOs to humans may be through bioaerosols. Antibiotics used in swine CAFOs may be dispersed through the air [10, 26] from both feed and manure dust [11] as well as spray field application of the waste. Swine waste is commonly disposed of by spreading or spraying effluent over agricultural fields [11], which may generate bioaerosols capable of being transmitted through the air [35]. Gibbs *et al.* [26] found antibiotic-resistant bacteria inside and up to 150 m downwind of a swine facility to be nearly three times that of the upwind site. Swine workers and members of the community living near CAFOs may be directly exposed to antibiotic-resistant

bacteria by inhaling the air from hog farming operations [10]. In addition to antibiotic resistance, bioaerosols from CAFOs may cause other adverse human health effects [13, 14, 49]. Zoonotic pathogens, including *Clostridium difficile*, *Staphylococcus aureus*, and *Streptococcus suis* [41], may transmit to humans *via* direct contact with live animals or bioaerosol exposure. Potential pathogenic bacteria and high levels of mold, yeasts, and bacteria have all been detected in the air of swine CAFOs [13, 15, 16, 29, 37]. Neighbors of swine CAFOs have been shown to have higher incidences of excessive coughing, headaches, sore throats, and diarrhea [47], and swine confinement workers have reported greater incidences of asthma and bronchitis [20, 28, 43, 49] than individuals not associated with swine CAFOs.

Despite the potential impacts on human health and the spread of antibiotic resistance by bioaerosols, much is still unknown regarding the composition and diversity of airborne bacteria from swine CAFOs. Previous studies regarding swine-related bioaerosols have mainly relied on culture-dependent methods to detect and enumerate bacteria present in the air [16, 21]. A more recent study of airborne bacteria in a swine confinement building by Nehme *et al.* [36] demonstrated through the use of quantitative PCR of eubacterial 16S rRNA genes that airborne bacteria were 100 to 1,000 times greater than total culturable bacteria. Pyrosequencing analysis of bioaerosol communities in swine confinement buildings revealed a predominance of *Firmicutes*, with the presence of *Staphylococcus* spp. and *Streptococcus* spp. [29]. However, very few studies have examined the presence of combined zoonotic pathogens and antibiotic-resistant bacteria in bioaerosols of hog farms and spray fields. In this study, we conducted pyrosequencing analysis of 16S rRNA genes to detect the presence of potential zoonotic pathogens in airborne bacterial communities of a North Carolina hog farm and spray field. In addition, antibiotic-resistant airborne bacteria were cultivated and identified to determine if potential pathogens also exhibited antibiotic resistance.

Materials and Methods

Pyrosequencing Analysis of Airborne Bacterial Communities from a Hog Farm and Spray Field

Bioaerosol samplings were conducted at two locations: (1) a hog farm and (2) an adjacent, recently sprayed field located in Burgaw, North Carolina (Fig. 1). The hog farm site was located next to a hog lagoon and approximately 5 m away from a hog house. The spray field sample site was located approximately

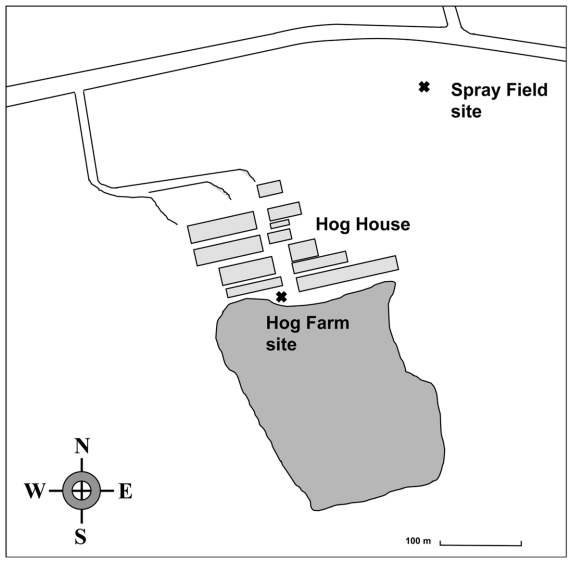

Fig. 1. Location of hog farm and spray field bioaerosol and air isolate sample collections.

300 m from the sampled hog farm and sprayed with hog lagoon waste 24 h prior to sampling. Metrological conditions during the day of sampling were obtained from the North Carolina Climate Retrieval and Observations Network of the Southeast Database (http://www.nc-climate.ncsu.edu/cronos). The temperature was 87°C with a relative humidity of 42%, and the wind direction was from the Southeast (138°) with a wind speed of 8.1 mph.

Duplicate samplings at each site were conducted 2 m above ground level. Air from the hog farm or spray field site was vacuumed for 10 min through a 0.22 µm nitrocellulose filter (45 mm diameter; PALL Corporation, New York, NY, USA) using a Gast vacuum pump (Sterilich, Kent, WA, USA) with a flow rate of 34 liters per minute. The filters were stored at –20°C prior to DNA extraction. DNA extraction using the PowerSoil DNA Kit (Mo-bio Laboratories, Inc., Carlsbad, CA, USA) was conducted on half filters from each sample, following the manufacturer's protocol. All samples were disrupted using Thermo Savant Fast Prep FP 120 Cell Disrupter (Qbiogene Inc. Carlsbad CA, USA). The concentration of DNA was quantified using the Qubit DNA quantitation assay (Life Technologies, Grand Island, NY, USA) following the manufacturer's instructions. A total of 6.9 and 5.1 ng of DNA were obtained from the hog farm and spray field filters, respectively. Triplicate PCRs (20 µl volume) were conducted for each sample with 1.5 ng of DNA template and primers 27F and 338R [3], which were modified to include an 8 bp barcode (reverse primers) and adapter sequence for the 454 Genomic Sequencer Junior (454 Life Sciences, Branford, CT, USA). PCRs were carried out using Phusion taq (New England Biolabs, Ipswich, MA, USA). The PCR cycle was as follows: 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were

Table 1. Comparison of richness, diversity, and coverage of airborne bacterial communities.

	Sequences	OTUs[a]	Chao1[a]	Shannon[a]	Coverage[a]
Hog farm air	2,173	441	729.1	7.173	0.91
Spray field air	1,494	379	633.1	7.291	0.89

[a]OTUs were determined with 97% sequence similarity cutoff.

run on 1.0% agarose using gel electrophoresis and gene cleaned following the protocol for the UltraClean GelSpin DNA Purification Kit (Mo-Bio, Carlsbad, CA, USA). The purified amplicons were used as templates for 454 pyrosequencing following the manufacturer's instruction.

Bioinformatics and Phylogenetic Analyses

Raw sequences were initially demultiplexed using the Quantitative Insights Into Microbial Ecology (QIIME) package [9] to select for high-quality sequences and to assign the selected sequences to two bins based on barcode sequences. Binned sequences were then denoised using Acacia [7], followed by chimera removal using USEARCH 6.0 [22]. After initial processing, Operational Taxonomic Units (OTUs) for each sequence library were determined based on sequence similarity with a minimum coverage of 99% and a minimum identity of 97% using QIIME [9]. A representative sequence from each OTU was selected and used for taxonomic identification by comparing the sequences in the Greengenes database [19]. Species richness and diversity of the two bioaerosol communities were calculated using the taxonomic assignments. Sequence coverage was calculated using Good's Coverage Estimator. The relative abundance of each genus was calculated by dividing the number of assigned sequences in each genus by the total number of sequences. Genera with more than 1% relative abundance were considered as dominant taxa in the two bioaerosol communities.

Among the dominant taxa, the sequences assigned to the genera of *Clostridium, Propionibacterium,* and *Staphylococcus* were selected for phylogenetic analysis to identify the presence of potential zoonotic pathogens. MEGA (ver. 6.0; http://www.megasoftware.net; [42]) was used to align OTU reference sequences with reference 16S rRNA sequences found in the GenBank database (http://www.ncbi.nlm.nih.gov/). The neighbor-joining method [40] with the Kimura 2-parameter [32] was used to construct a phylogenetic tree of 16S rRNA genes. Bootstrap analysis [23] of 1,000 repetitions was used to estimate the reliability of the phylogenetic reconstruction with a 50% support threshold.

Cultivation and Identification of Antibiotic-Resistant Bacteria

Exposure to antibiotic plates for airborne bacterial sampling was conducted at the same two locations where bioaerosol sampling was performed: (1) hog farm and (2) spray field (Fig. 1). At each site, duplicate Luria-Bertani plates containing 100 mg/ml of kanamycin (LBK) and commercial methicillin-resistant *Staphylococcus aureus* (MRSA) plates containing oxacillin (Bacto) were exposed to the air for 10 min. The number of bacteria resistant to kanamycin or oxacillin was counted after 48 h incubation at 37°C.

Thirty-seven isolates resistant to either oxacillin or kanamycin were randomly selected and grown in LB broth for 24 h at 37°C. Direct amplification of 16S rRNA genes was conducted using GoTaq (Promega, Madison, WI, USA) master mix with universal 16S rRNA gene primers 27F and 685R [3] using the PCR cycle described above. The PCR products were directly sequenced using 685R primer with Big Dye Terminator v1.1 following the manufacturer's instruction (Applied Biosystems, Carlsbad CA, USA). Taxonomic identification of 16S rRNA sequences was achieved based on a BLAST search at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/). Sequences obtained from this study were deposited under the accession numbers SRR1460715 and KM067995 to KM068031.

Results

Comparison of Composition and Diversity of Airborne Bacteria in a Hog Farm and Spray Field

A total of 3,667 16S rRNA high-quality, cleaned, and trimmed sequences was obtained from the hog farm air (2,364) and spray field air (1,522) (Table 1). Based on 97% sequence identity, 441 and 379 OTUs were found in the bioaerosol communities of hog farm and spray field, respectively. Higher richness of airborne bacterial community was found in the hog farm air samples, but diversity was

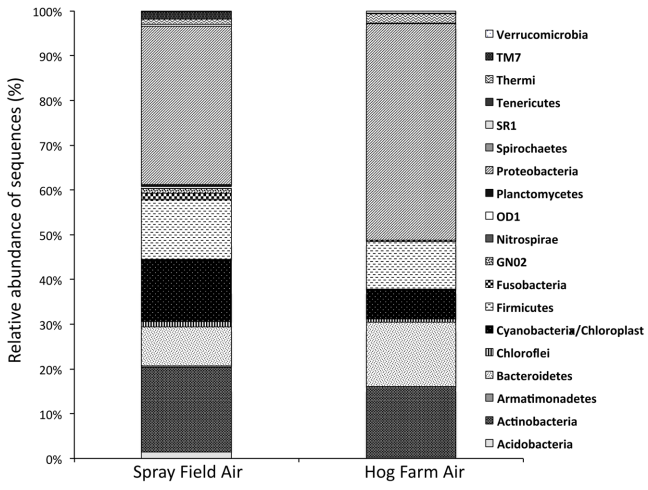

Fig. 2. Airborne bacterial community profile of phyla identified in hog farm and spray field bioaerosols.

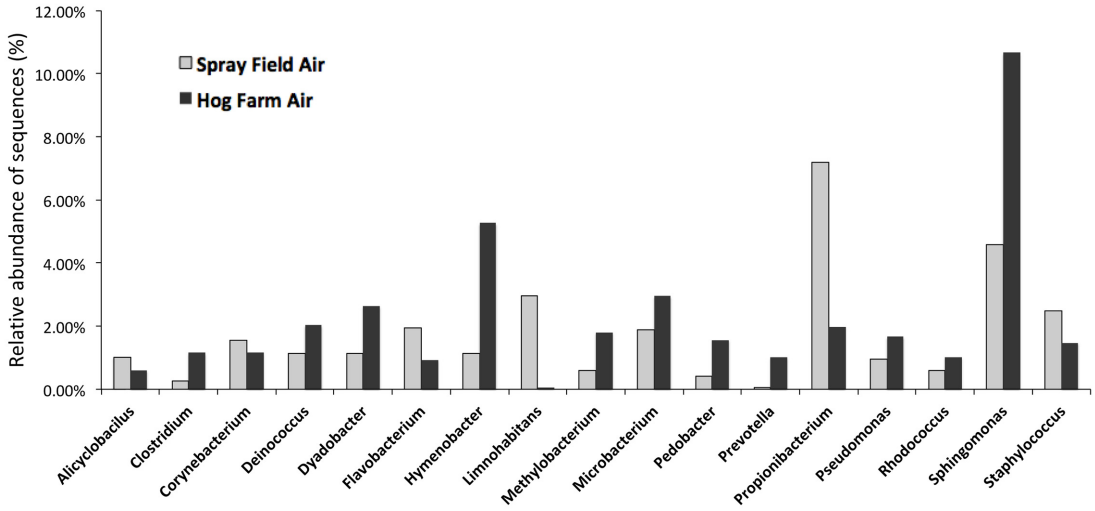

Fig. 3. Comparison of dominant genera between two bioaerosol communities.
Dominant genera were defined as genera having a 1.0% or greater relative abundance, based on total number of sequences.

higher in the spray field air samples (Table 1). The hog farm bioaerosol community was composed of 16 different phyla, whereas 17 phyla were present in the spray field community (Fig. 2). *Proteobacteria* was the predominant phylum with 48.3% and 35.3% of percent abundance in the bioaerosol communities of the hog farm and spray field, respectively. *Actinobacteria* was the second most abundant phylum, representing 15.7% abundance in hog farm air and 18.8% in spray field air. *Bacteroidetes* accounted for 14.3% of the sequences in the hog farm air and 8.7% of the sequences found in spray field air, whereas *Firmicutes* accounted for 10.4% and 11.6% of airborne communities in the hog farm and spray field, respectively.

Combined, both bioaerosol communities had a total of 17 dominant bacterial taxa at the genus level, based on more than 1% of relative abundance in the 16S rRNA gene pyrosequences (Fig. 3). *Cornybacterium*, *Microbacterium*, *Propionibacterium*, and *Rhodococcus* were the dominant genera of *Actinobacteria*, whereas dominant genera of the *Bacteroidetes* phylum consisted of *Dyadobacter*, *Flavobacterim*, *Hymenobacter*, *Pedobacter*, and *Prevotella*. Four genera, *Limonohabitans*, *Methylobacterium*, *Pseudomonas*, and *Sphingomonas*, were dominant taxa in the *Proteobacteria* phylum. *Alicyclobacillus*, *Clostridium*, and *Staphylococcus* were the dominant genera of phylum *Firmicutes*, with *Clostridium* spp. being more abundant in the hog farm air and *Staphylococcus* spp. higher in the spray field air (Fig. 3). Of all the genera represented, the most abundant genus in hog farm bioaerosols was *Sphingomonas*, whereas *Propionibacterium* was the most dominant taxa in spray field bioaerosols

Phylogenetic Analysis of *Clostridium* spp., *Propionibacterium* spp., and *Staphylococcus* spp. in Bioaerosol Communities

Among the dominant genera, *Clostridium* and *Staphylococcus* are known to contain two potential zoonotic pathogens, C. *difficile* and *S. aureus*, respectively [41]. Phylogenetic analysis was conducted with the dereplicated bioaerosol sequences assigned to these two genera (Fig. 4). Most sequences of *Clostridium* spp. in the hog farm and spray field bioaerosols were closely related to *C. butyricum* and *C. disporicum,* whereas only one hog farm sequence was most closely related to *C. chartatabidum* (Fig. 4). None of the sequences shared more than 97% sequence identity with *C. difficile*. Airborne *Staphylococcus* spp. in common between the hog farm and spray field were identified or closely related to *S. hominis* and *S. saccharolyticus* (Fig. 4). Individually, in hog farm air bioaerosols, the presence of *S. cohnii* and *S. warneri* was detected, whereas in the spray field bioaerosols, *S. auricularis* and *S. epidermidis* were found. None of the sequences identified were closely related to *S. aureus*.

The sequences assigned to the genus *Propionibacterium* were also included in the phylogenetic analysis to idenitify the presence of *P. acnes*, an opportunistic human pathogen. Both bioaerosol communities contained sequences closely related to *P. acnes* (Fig. 4). A total of 105 sequences in the spray field air and 20 sequences in hog farm air were closely related to *P. acnes*.

Enumeration and Identification of Antibiotic-Resistant Bacteria

Both the oxacillin and kanamycin plates exposed to hog

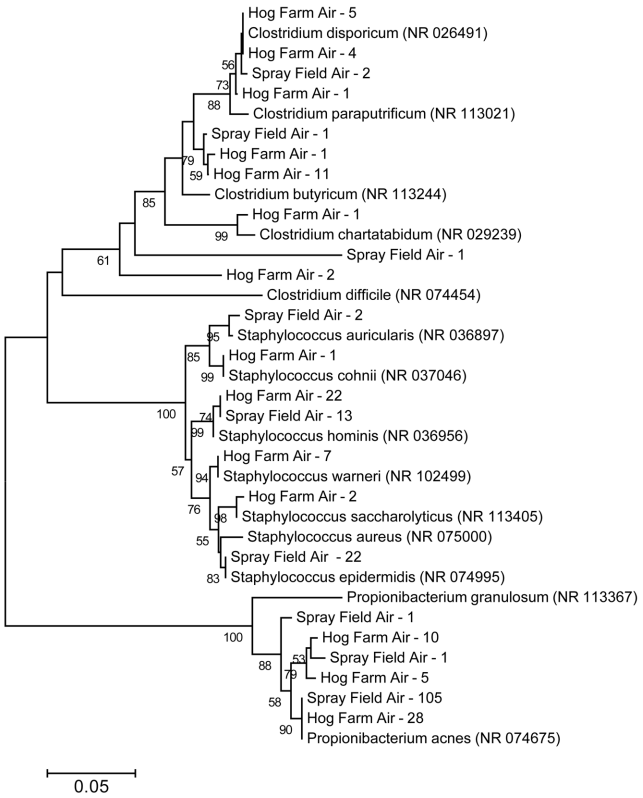

Fig. 4. Phylogenetic tree showing the relationship between 16S rRNA OTU representative gene sequences derived from hog farm and spray field bioaerosols and GenBank reference sequences from genera *Clostridium*, *Propionibacterium*, and *Staphylococcus*.

Bioaerosol OTU representative sequences are based on 97% identity, and numbers following the OTU indicate the number of corresponding sequences.

farm air had greater than 300 colonies. The kanamycin plate exposed to spray field air had greater than 300 colonies, whereas the oxacillin plate had 25 colonies. Randomly selected 37 isolates were taxonomically classified based on 16S rRNA sequence analysis (Table 2). All of the isolates were identified within the dominant phyla *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, and *Proteobacteria*. The majority of isolates, 10 each from the hog farm and spray field, were assigned to phylum *Proteobacteria.* All of the proteobacterial isolates in hog farm bioaerosols were *Pantoea* spp., whereas the proteobacterial isolates of spray field bioaerosols were identified to be *Cronobacter* spp., *Halomonas* spp., *Pantoea* spp., *Pseudomonas* spp., and *Sphingobacterium* spp. A total of 15 isolates (8 from the hog farm and 7 from the spray field) belonged to phylum *Actinobacteria*. *Corynebacterium spp.* were the dominant actinobacterial isolates in the hog farm air, but six different genera were found in the spray field bioaerosols. Only one *Staphylococcus* isolate in the *Firmicutes* phylum was found in the hog farm, whereas one *Chryseobacterium* spp. in *Bacteroidetes* was isolated from the spray field air sample. Comparing 16S rRNA pyrosequences and antibiotic-resistant isolates, the members of phyla *Actinobacteria* and *Proteobacteria* were the predominant airborne bacterial communities found in both hog farm and spray field air (Fig. 2 and Table 2).

Discussion

The airborne bacterial communities (Fig. 2) and antibiotic isolates of both the hog farm and spray field were somewhat similar in composition, with *Proteobacteria, Actinobacteria, Bacteroides,* and *Firmicutes* being among the most abundant taxa in both communities, suggesting a similar origin of bioaerosols. These results are comparable to other bioaerosol studies that have found *Proteobacteria, Actinobacteria, Bacteroides*, and *Firmicutes* to be abundant in bioaersol samples [29, 38]. However, the findings from our study were not consistent with other bioaerosol studies involving swine farms in which gut-related bacteria from phylum *Firmicutes* were dominant [29, 36]. Less than 14% of the bioaerosol pyrosequences from the hog farm or spray field (Fig. 2) and only one isolate from the spray field were grouped within phylum *Firmicutes*. The differences in dominance between *Firmicutes* and other taxa among the air samples may be due to different locations of bioaerosol sampling. The samples for this study were collected outdoors, whereas Nehme *et al*. [36] and Hong *et al*. [29] collected air samples within swine confinement buildings. Bioaerosols closely associated with gut bacteria may be highly concentrated within swine buildings but become quickly diluted or dispersed upon contact with open air. In addition, factors such as temperature and seasonality may influence different bacterial dominance [25]. A study conducted by Ravva *et al*. [38] showed that bioaerosol communities from two different dairies varied widely in terms of relative abundance of communities, based on environmental conditions and wind direction. Despite both dairies producing a large amount of manure, one bioaerosol communitiy was dominated by *Firmicutes* and the other by *Proteobacteria* [38].

In addition to several similarities, there were also some distinct differences in bioaerosol communities and antibiotic-resistant isolates between the hog farm and spray field locations. *Sphingomonas* sequences were more abundant within the hog farm bioaerosol communties, whereas *Propionibacterium*

Table 2. Taxonomic identification of antibiotic-resistant bacterial isolates.

Isolate		Closest match in bacterial species	Identity	Coverage
Hog farm air	HLA-9H	*Cellulosimicrobium funkei*	99%	100%
	HLA-11B	*Corynebacterium argentoratense*	94%	94%
	HLA-11D	*Corynebacterium argentoratense*	87%	87%
	HLA-11H	*Corynebacterium argentoratense*	97%	97%
	HLA-9E	*Corynebacterium argentoratense*	97%	97%
	HLA-11F	*Corynebacterium singulare*	91%	91%
	HLA-L3-1	*Curtobacterium plantarum*	99%	100%
	HLA-9A	*Kocuria rhizophila*	99%	100%
	HLA-L4-1	*Pantoea agglomerans*	99%	99%
	HLA-L2-2	*Pantoea ananatis*	98%	98%
	HLA-L4-2	*Pantoea cypripedii*	98%	98%
	HLA-L6-2	*Pantoea cypripedii*	98%	98%
	HLA-L1-1	*Pantoea vagans*	99%	100%
	HLA-L2-1	*Pantoea vagans*	100%	100%
	HLA-L5-1	*Pantoea vagans*	99%	99%
	HLA-L5-2	*Pantoea vagans*	99%	99%
	HLA-L6-1	*Pantoea vagans*	99%	99%
	HLA-L7-2	*Pantoea vagans*	99%	99%
	HLA-11C	*Staphylococcus epidermidis*	99%	100%
Sprayfield air	SFA-7D	*Agrococcus lahaulensis*	95%	95%
	SFA-2A	*Arthrobacter arilaitensis*	98%	98%
	SFA-1F	*Arthrobacter mysorens*	99%	99%
	SFA-8G	*Cellulomonas hominis*	99%	99%
	SFA-7H	*Chryseobacterium haifense*	90%	90%
	SFA-SF1-1	*Cronobacter zurichensis*	99%	99%
	SFA-1D	*Curtobacterium plantarum*	89%	89%
	SFA-2C	*Halomonas zhanjiangensis*	99%	99%
	SFA-7C	*Leifsonia kafniensis*	98%	98%
	SFA-7A	*Microbacterium paraoxydans*	99%	100%
	SFA-SF3-1	*Pantoea agglomerans*	99%	99%
	SFA-SF8-1	*Pantoea agglomerans*	95%	95%
	SFA-2H	*Pantoea vagans*	98%	98%
	SFA-1E	*Pseudomonas argentinensis*	97%	97%
	SFA-2D	*Pseudomonas argentinensis*	100%	100%
	SFA-2E	*Pseudomonas punonensis*	99%	99%
	SFA-SF4-2	*Pseudomonas punonensis*	99%	99%
	SFA-7F	*Sphingobacterium alimentarium*	92%	92%

sequences were more abundant in the spray field (Fig. 3). *Sphingomonas* spp. may be a predominant member of swine bioaerosol [12] and *Propionibacterium* spp., specifically sequences closely related to *P. acnes* found in this study, have been found to be ubiquitous in outdoor bioaerosols [18]. Among the antibiotic-resistant isolates unique to each environment, *Corynebacterium* was the most isolated bacteria from hog farm air, and *Pseudomonas* was the most isolated bacteria from spray field air. These differences in community structure and antibiotic isolates suggest that there are

different bacterial origins to the bioaersol communities at each location in addition to an overall general community. These unique communities may be affected by physical factors such as wind direction and dispersal patterns.

With regard to human health, *Staphylococcus* and *Clostridium* are two genera of bacteria that contain potential zoonotic pathogens [41]. Within the genus *Staphylococcus*, most species are generally believed to be ubiquitous in bioaerosol samples of minimally impacted environments, such as offices and nursing homes [39], but have also been described from clone libraries obtained from pig gastrointestinal tracts [33] and may indicate pig origin. Among the related *Staphylococcus* bioaerosol sequences found in the hog farm and spray field bioaerosol communities, *S. auricularis*, *S. cohnii, S. epidermidis, S. hominis, S. warneri,* and *S. saccharolyticus* are all considered to be potential opportunistic pathogens associated with human or animal skin flora (Fig. 4). In addition, one antibiotic isolate from the hog farm air identified with the potential opportunistic pathogen *Staphylococcus epidermis* (Table 2).

In contrast to *Staphylococcus*, *Clostridum* spp. in bioaerosols are commonly associated with fecal matter [12, 36] and are abundant in pig bioaerosols [29]. The majority of uncultured *Clostridium* sequences and those related to C. *disporicum* and *C. butyricum* were similar between hog farm and spray field communities (Fig. 4). Both *C. disporicum* and *C. butyricum* have been found to be major phylotypes in liquid swine manure [34]. Only one sequence, related to *C. chartatabidum*, isolated from rumen contents [31], was unique to hog farm sequences. In addition to zoonotic pathogens, sequences closely related to the human opportunistic pathogen *P. acnes* [8] were found in this study. However, *P. acnes* is commonly found on human skin [44] and is ubiquitous in air samples from different environments [18], suggesting that the presence of this bacterium in our air samples does not necessarily indicate hog farm origin.

The transmission of airborne, antibiotic-resistant pathogens is also of significant concern regarding human health. Airborne antibiotic-resistant bacteria from CAFOs may be a mediator of dispersing antibiotic resistance to surrounding environments and capable of transmitting antibiotic resistance to other human disease-causing pathogenic bacteria. Of the antibiotic-resistant bacteria isolated from hog farm and spray field air, none of the isolates from this study were known to be obligate or zoonotic pathogens. However, some of the isolates, in addition to *S. epidermis* described above, may be considered to be opportunistic human pathogens, such as *P. agglomerans* [17] isolated from both hog farm and spray field air. A broader and more in-depth airborne study would need to be conducted in order to assess the overall health risks from antibiotic-resistant pathogens in hog farm and spray field bioaerosols.

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