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RESEARCH ARTICLE

The Bacterial Community Found on the surface Purple Martin (*Progne subis*) Eggs

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Abstract:

Introduction:

The community of microorganisms that lines the surface of avian eggs is the first line of defense against infection by pathogenic bacteria. The protective role of this community is derived from its composition and several studies have focused on identifying the bacterial components. While a diverse group of avian species has been studied, multiple species within the same family have not been independently studied. This depth is necessary to determine the degree of flexibility or plasticity within the community.

Method:

The goal of this study was to identify the bacterial microorganisms found lining the eggshells of an avian species classified within the Hirundinidae family, the Purple Martin (*Progne subis*). Culture-dependent techniques revealed a predominance of *Pseudomonas* before and after clutch completion.

Result:

Interestingly our results correlate with studies involving Pied Flycatchers, House Wrens, and Eurasian Magpies rather than Tree and Violet-Green Swallows.

Conclusion:

Given the variances between Pied Flycatchers, House Wrens, Eurasian Magpies and Purple Martins in regard to breeding habitat, diet, nest construction, and incubation behaviors, we hypothesize that a strong selective force may be provided by uropygial gland secretions or preen oil.

Keywords: Purple Martin, Eggshell Bacterial Community, Uropygial gland secretions, Culture-dependent techniques, *Pseudomonas*, Hirundinidae family.

INTRODUCTION

The community of microorganisms found on the surface of naturally incubated avian eggshells has gained attention over the past ten years because of its role in protecting the egg/embryo from infection by pathogenic bacteria [1]. The porosity of the avian eggshell, while necessary for the exchange of water vapor, oxygen, and carbon dioxide, provides a route for Trans-shell bacterial migration [2]. Eggs have several internal mechanisms to inhibit bacteria, including the maintenance of albumen at a suboptimal pH (9 to 10) for bacterial growth [3] and the presence of antibacterial proteins, such as lysozyme and ovotransferrin [4]. These proteins also have been found in the cuticle, which is the outermost

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proteinaceous layer of the eggshell [5, 6]. This layer may also limit microbial access to pores [7]. However, microbial communities cover the surface of the egg, so they are the first line of defense against infection. The protective role of this community is derived from its composition. Thus, it is important to identify the bacterial components [8].

Bacteria can be identified easily using biochemical and molecular techniques. The difficulty of the task comes from the numerous possible factors that can influence the composition of microbial communities lining avian eggshells. For instance, unincubated eggs harbor more pathogenic bacteria than incubated eggs [1, 9]. However, behaviors vary among avian species. For instance, incubation may be initiated before or after clutch completion. Moreover, males of some species aid in incubation. In addition, numerous environmental factors can influence the composition of the microflora. These factors include temperature of breeding habitat (tropical *vs.* temperate), location of breeding habitat (grassland *vs.* dense forest), nest microclimate (humidity levels), nest construction (open-cup *vs.* cavity), nest materials (grasses, twigs, feathers, green material), and diet. Thus, the first step in understanding the microbial communities lining the eggshell is to identify the bacterial components of closely related and diverse avian species. To date, studies have focused on the Pearly-eyed Thrasher [10], Western Bluebird, Tree Swallow, Violet-Green Swallow [11], Pied Flycatcher [12], House Wren [13], Eurasian Magpie [14], and American Kestrel [15]. This list includes a diverse set of species, but it does not include closely related species within the same family (data for Tree and Violet-Green Swallows were presented together with no discrimination between species). Closely related family members must be examined to test the flexibility or plasticity of the microbial communities lining the eggshell. The goal of this study was to assess the bacterial community found on the surface of Purple Martin (*Progne subis*) eggs. The Purple Martin is the largest of the Swallow family and is unique in comparison to the birds already studied in its colonial nesting habit and onset of incubation at clutch completion.

MATERIALS AND METHODS

Study Species and Field Site

Purple Martins spend the non-breeding season in Brazil and migrate throughout North America for the breeding season. East of the Rocky Mountains, Purple Martins nest in man-made housing, which typically consists of multi-compartment houses and/or several plastic gourds. Two separate colonies in and around Presque Isle State Park in Erie County, Pennsylvania, were used for this study. The first breeding season for the first colony site was spring/summer 2006. When the colony was first established, the housing consisted of a single T-14 wooden house with four gourds hanging underneath. In 2008, a second T-14 wooden house with four underlying gourds were added to the colony site. In 2012, four additional gourds were added to both T-14 units. One of the wooden houses sampled was from the first T-14 house started in 2006 and 3 wooden houses and 2 plastic gourds were sampled from the additions in 2008. The first breeding season for the second colony site was also in 2006 and housing consisted of an 18-unit gourd rack of artificial gourds. In 2009, a Cedar Suite, which contained six cavities, was added. In 2011, the gourd rack was expanded to hold 24 artificial gourds and 4 gourds were hung from the Cedar Suite. In 2012, a T-14 wooden house and four gourds were added. For this study, one gourd from the original 18-unit gourd rack was sampled and 3 of the wooden houses from the T-14 system added in 2012. All cavities within the wooden houses are roughly 30.5 cm deep and 15.5 cm in width and height. Gourd sizes vary depending on their manufacturer, but have a 25-28 cm radius. Purple Martins began to arrive at the colony sites for the 2013 season beginning in mid-April. At the beginning of each breeding season, needles collected from beneath local white pine, *Pinus strobus,* are placed in the housing to help start nests. An average of 2-7 eggs are laid and females begin incubation upon clutch completion. In the 2013 season, nests were monitored and sampled from 10 May to 8 July. All houses were exclusively used by Purple Martins and the occupancy rate was 100% at the first site and was 94.2% at the second site during this season. At the end of each season, all nests are removed from their cavities and the gourds and nest trays are scraped of all material. All cavities are rinsed with a ten to one bleach and water solution minimize parasites that might overwinter in the housing. Once cleaned, the gourds are removed from the houses and racks and placed in winter storage. The entrances to the wooden houses are blocked and the houses are covered for the winter.

Microbial Sampling

Similar procedures were used as described for swabbing House Wren eggs [13]. In short, gloves were cleaned with 70% ethanol and allowed to air dry before eggs were handled. A stencil used to standardize our sampling zone to a 7- X 11-mm rectangle was sterilized in the same manner. Sterile swabs were dampened with sterile PBS before they were used to sample the egg surface. After swabbing the egg surface, the swab was snapped from its wooden stick and place

in a microfuge tube containing sterile PBS. Samples were stored at 4 °C until they could be processed using culture-dependent techniques.

Bacterial Identification

Samples were processed as previously described to identify the bacterial components [15]. Samples were vortexed for 30 seconds, serially diluted, and spread onto all-purpose, nutrient agar plates. The plates were incubated at 25 °C and 30 °C for 48 h (the majority of the identification were derived from plates incubated at 30 ºC). After incubation, plates containing 30-300 Colony Forming Units (CFUs) were counted and unique bacteria identified by colony morphology were streaked for isolation. A single colony of each unique bacterial type was placed in 50 µL of water and lysed using a freeze-thaw method. Lysed cells were centrifuged and the supernatant containing the DNA was used in a Polymerase Chain Reaction (PCR) to amplify the 16S ribosomal RNA gene. The universal bacterial primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') were used to amplify the genes, and amplification was verified using electrophoresis [16]. The PCR product was sent for sequencing at the Genomics Core Facility at Penn State University Park. Forward and reverse sequences were used to construct a consensus sequence with Geneious software (Biomatters Ltd). Consensus sequences were run through NCBI-BLAST/EzTaxon to identify the genus and species of bacterial isolates.

Statistical Analysis

Bacteria identified are presented in terms of how often they were found on eggs before and after clutch completion. The composition of bacterial communities before and after clutch completion was compared using a two-tailed Fisher's Exact Test in the "vegan" package [17] of the R statistical software program [18]. Bacteria were grouped by genus, family, and phylum, and only those groups in which total prevalence exceeded 10% were analyzed. A p-value of less than 0.05 indicates a difference in prevalence probability. There were 18 eggs sampled before clutch and 41 eggs after clutch for a total of 59 eggs.

Table 1. Number of detections of culturable bacterial species found on eggshells of Purple Martins before and after clutch. From a total of 59 eggs (18 sampled before clutch and 41 sampled after clutch), 66 different bacterial species were identified. The prevalence of bacterial genera, families, and phyla was calculated from the detections recorded for each. A Fisher's exact test comparing the frequency of the most prevalent genera, families, and phyla did not reveal a significant difference before and after clutch completion.

			Number of Detections	
Phylum	**Family**	**Genus/Species**	**Before**	**After**
Actinobacteria	Microbacteriaceae	*Curtobacterium flaccumfaciens*	1	0
Alpha-proteobacteria	Brucellaceae	*Pseudochrobactrum kiredjianiae*	0	2
Alpha-proteobacteria	Sphingomonadaceae	*Sphingomonas ginsenosidivorax*	0	1
Alpha-proteobacteria	Sphingomonadaceae	*Sphingomonas mucosissima*	1	0
Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium anhuiense*	0	1
Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium kitahiroshimense*	0	3
Bacteroidetes	Sphingobacteriaceae	*Sphingobacterium shayense*	0	1
Beta-proteobacteria	Alcaligenaceae	*Achromobacter xylosoxidans*	1	0
Beta-proteobacteria	Comamonadaceae	*Variovorax boronicumulans*	0	1
Beta-proteobacteria	Comamonadaceae	*Variovorax ginsengisoli*	0	1
Beta-proteobacteria	Neisseriaceae	*Prolinoborus fasciculus*	0	1
Beta-proteobacteria	Oxalobacteraceae	*Duganella zoogloeoides*	0	1
Firmicutes	Staphylococcaceae	*Staphylococcus epidermidis*	1	3
Gamma-proteobacteria	Enterobacteriaceae	*Citrobacter freundii*	0	1
Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter amnigenus*	0	2
Gamma-proteobacteria	Enterobacteriaceae	*Enterobacter ludwigii*	1	1
Gamma-proteobacteria	Erwiniaceae	*Erwinia billingiae*	0	3
Gamma-proteobacteria	Erwiniaceae	*Pantoa eucalypti*	0	1
Gamma-proteobacteria	Erwiniaceae	*Pantoea rodasii*	0	1
Gamma-proteobacteria	Erwiniaceae	*Pantoea vagans*	0	1
Gamma-proteobacteria	Moraxellaceae	*Acinetobacter calcoaceticus*	0	1
Gamma-proteobacteria	Moraxellaceae	*Acinetobacter lwoffi*	0	2

(Table 1) contd.....

			Number of Detections	
Phylum	**Family**	**Genus/Species**	**Before**	**After**
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas abietaniphila*	0	5
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas alcaligenes*	0	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas arsenicoxydans*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas avellanae*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas baetica*	0	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas brenneri*	1	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas canabina*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas cedrina*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas chlororaphis*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas cichorii*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas ficuserectae*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas graminis*	1	4
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas helmanticensis*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas hunanensis*	1	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas japonica*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas jessenii*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas koreensis*	1	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas libanensis*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas lurida*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas lutea*	1	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas migulae*	1	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas mohnii*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas moorei*	2	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas oryzihabitans*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas parafulva*	1	0
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas plecoglossicida*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas poae*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas prosekii*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas putida*	2	10
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas reinekei*	2	3
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas rhizophaerae*	1	6
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas rhodesiae*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas saponiphila*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas simiae*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas syringae*	1	5
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas tremae*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas trivialis*	0	2
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas vancouverensis*	0	1
Gamma-proteobacteria	Pseudomonadaceae	*Pseudomonas xanthomarina*	0	2
Gamma-proteobacteria	Rhodanobacteraceae	*Luteibacter anthropi*	0	1
Gamma-proteobacteria	Xanthomonadaceae	*Stenotrophomonas maltophilia*	0	1
Gamma-proteobacteria	Xanthomonadaceae	*Stenotrophomonas rhizophila*	7	15
Gamma-proteobacteria	Xanthomonadaceae	*Xanthomonas gardneri*	0	1
Gamma-proteobacteria	Xanthomonadaceae	*Xanthomonas vesicatoria*	0	1

RESULTS

Purple Martin eggs from ten different nests were swabbed before and after clutch completion to determine if there were any noticeable differences in the bacterial composition before the onset of continuous incubation which occurs upon clutch completion with Purple Martins. A total of 161 bacterial taxa belonging to four different phyla (Actinobacteria, Bacteroidetes, Firmicutes, and the alpha, beta, and gamma subphyla of Proteobacteria) and 18 genera were identified Table (**1**). Bacteria within the Gamma-proteobacteria subphylum had an overall prevalence of 94.92% (100% prevalence before clutch and 92.68% after clutch completion). The prevalence of bacteria in the Bacteroidetes, Firmicutes, Alpha-proteobacteria, and Beta-proteobacteria was just above 5% for each (6.78%). The Actinobacteria

phylum had a prevalence of 1.69%. Three families within the Gamma-proteobacteria phylum had a prevalence greater than 10% including Erwiniaceae (10.17%), Pseudomonadaceae (91.53%), and Xanthomonadaceae (40.68%). Bacteria within Pseudomonadaceae and Xanthomonadaceae had a prevalence above 10% before and after clutch; however, bacteria within the Erwiniaceae family were not found on any eggs before clutch. Three bacterial families had a prevalence of 6.87% including Enterobacteriaceae, Sphingobacteriaceae, and Xanthomonadaceae. The remaining bacterial families including Alcaligenaceae, Brucellaceae, Comamonadaceae, Microbacteriaceae, Moraxellaceae, Neisseriaceae, Oxalobacteriaceae, Rhodanobacteriaceae, and Sphingomonadaceae were below 4%. Only two genera had a prevalence greater than 10% both before and after clutch completion, *Pseudomonas* (88.89% and 92.98%) and *Stenotrophomonas* (38.89% and 42.11%). With the exception of *Duganella*, *Prolinoborus*, and *Citrobacter* all bacteria genera identified in this study had been identified previously in the eggshell microflora of another avian species. Frequencies of the most prevalent bacterial phyla, families, and genera did not change throughout incubation (Fisher's exact test, all $P > 0.164$, $N = 59$).

DISCUSSION

The predominant genus before and after clutch completion was *Pseudomonas*. This genus comprises a large and diverse group of microorganisms found in terrestrial, freshwater, and marine environments [19]. Predominance of *Pseudomonas* has also been observed in nests of Blue and Great Tits [20], the plumage of Eastern Bluebirds [21] and American Redstarts [22], and on the surface of Pied Flycatcher [8], House Wren [13], and Eurasian Magpie [14] eggs. The predominance of *Pseudomonas* may stem from their ability to produce antibiotic substances referred to as pyocins [23], which can provide them with a competitive advantage over other bacteria. Pyocins have broad-spectrum capabilities and can inhibit members of the pathogenic Enterobacteriaceae family [24]. Thus, pyocins may also protect the eggs. Moreover, the Enterobacteriaceae are not discussed as a significant components of bacterial communities on plumage, nests and egg surfaces in which *Pseudomonas* is a predominant member. Further insight into understanding the maintenance of the *Pseudomonas* would be to examine the antibacterial power of *Pseudomonas* against indicator strains as described by Ruiz-Rodriguez *et al*. [25]. While *Pseudomonas* may play an important role in maintaining the eggshell microflora, studies have shown that a few species within the *Pseudomonas* genus can undergo trans-shell migration and cause spoilage in chicken eggs [2, 26]. However, the pathogenic role of *Pseudomonas* has not been well studied in passerine populations. Ruiz-de-Castaneda *et al*. [8] noted that the hatching success of Pied-Flycatcher eggs was not affected by a predominance of *Pseudomonas* within the bacterial eggshell community. A benefit to the maintenance of a portion of the population may be to expose nestlings to the pathogen so antibodies can be generated early and protect individuals into adulthood [20].

Our bacterial findings were similar to those of other studies examining bacterial communities lining the eggshells of Pied Flycatcher [8], House Wren [13], and Eurasian Magpies [14]. However, our findings differ from those in a study by Wang *et al*. [11] examining the microbial eggshell communities of Tree and Violet-Green Swallows, which are in the same family as Purple Martins. Wang *et al*. [11] found a predominance of *Bacillus*, *Staphylococcus*, *Arthrobacter*, and *Sporosarcina*, whereas we found predominantly *Pseudomonas* and low relative abundance of *Staphylococcus*. In both studies, bacteria were cultured on all-purpose agar, isolated based on colony morphology and identified by amplifying the 16S rRNA gene and comparing to a gene database. Thus, one possible procedural explanation for this discrepancy could be a bias imposed by the specific primers used to identify the bacterial isolates. This explanation seems unlikely because the same forward primer was used and the reverse primer sequence overlapped with the exception of three bases. In addition, the same primers were used to study the microflora on American Kestrel eggs, and bacteria in the genera *Bacillus*, *Staphylococcus*, *Arthrobacter*, and *Sporosarcina* were detected [13, 15]. Thus, the difference in bacterial composition between Purple Martin (this study) and Tree and Violet-Green swallow [11] is not a procedural artefact. We hypothesize that the bacterial communities found on eggshells varies among these species and that the selective pressures differ between Purple Martins and Tree and Violet-Green swallows despite their phylogentic relationships.

Our data implies that similar selective influences must be present within Purple Martins, Pied-Flycatchers and House Wrens because of the predominance of *Pseudomonas* within the bacterial eggshell communties [8, 13, 14]. Differences in breeding habitat, diet, nest construction, and incubation behaviors have been tried in previous studies to explain differences in bacterial profiles associated with avian species [27, 28], but it is hard to align the above avian species as similar based on these factors and different from other avian species such as American Kestrels and Tree and Violet-Green Swallows that have different bacterial communities lining their eggshell. Thus, a stronger selective force may be provided by another broader but perhaps less flexible mechanism, such as uropygial gland secretions or preen

oil.

The majority of dominant bacterial genera lining the eggshell of all avian species studied thus far have been associated with feather degradation or found on plumage. *Pseudomonas* and *Stenotrophomonas* (predominant genera in the present study) and three of the four most common genera identified on the surface of Tree and Violet-Green Swallow eggshells (*Bacillus*, *Arthrobacter*, and *Staphylococcus*) have been linked to feather degradation [29, 10]. *Microbacterium*, the most common genus isolated from American Kestrel eggs [30], and the four main genera found on the surface of Pied-Flycatcher (*Acinetobacter*, *Enterococcus*, *Ochrobactrum*, and *Pseudomonas*) and House Wren eggs (*Burkholderia*, *Pseudomonas*, *Staphylococcus*, and *Stenotrophomonas*) also have been linked to feather degradation [21, 29, 31, 32]. In *in vitro* studies, preen oil inhibited feather-degrading bacteria and other cultivable bacteria isolated from plumage, suggesting preen oil has inherent antimicrobial properties [33, 34]. However, these *in vitro* results have not been corroborated in *in vivo* studies [35, 36].

The composition of preen oils differ among avian species. Long-chain esters that have the same molecular weight are common among species, but the use and ratios of acids and alcohols in the preen oil differ among species [37]. Thus, it is feasible that preen oil has common functions, such as waterproofing, in all species, but has specific roles, such as regulating bacterial loads, that vary based on the physical properties achieved when the acid and alcohol composition is altered [37]. Females change the composition of their preen oil before incubation and maintain the new composition throughout the incubation period [38]. For example, in Hoopoe females, the size of the uropygial gland and secretions increase dramatically when incubation begins [38]. Hoopoe eggs are pale blue when laid but turn brown within a few days, most likely because of preening of the egg or its intimate association with the mother's feathers, which contain the oil [39]. Thus, studies should be undertaken to determine the composition and antibacterial activity of preen oil collected from Purple Martins and the other avian species that have a predominance of *Pseudomonas* on their eggshells.

CONCLUSION

Consistent trends appear to exist in the bacterial communities found lining avian eggshells. *Pseudomonas* predominated in the eggshell microflora of Purple Martins, Pied-Flyatchers, House Wrens, and Eurasian Magpies, whereas bacteria within the Actinobacteria and Firmicutes phyla predominated in the eggshell microflora of other avian species, such as Tree and Violet-Green Swallows. Even closely related avian species may have different eggshell bacterial communities, and the differences do not appear to be directly related to breeding habitat, diet, nest construction, or incubation behaviors. We propose that feathers and preen oil are important in selecting bacteria that can colonize the egg. Further differences in the composition of the microbial communities may be regulated by antibiotic production within the bacterial community and may be controlled by temperature variances caused by female attentiveness during the incubation period. Subtle underlying changes within the microbial communities may be attributed to variances in nesting habitat, nest construction, and diet. Additional studies are necessary to test these hypotheses and further examine the interesting relationship between the avian egg, the bacterial communities maintained on the surface of the egg, and pathogenic bacteria.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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