2004

Plumage as a Habitat for Bacilli

Justine M. Whitaker
College of William & Mary - Arts & Sciences

PLUMAGE AS A HABITAT FOR BACILLI

A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

by

Justine M. Whitaker

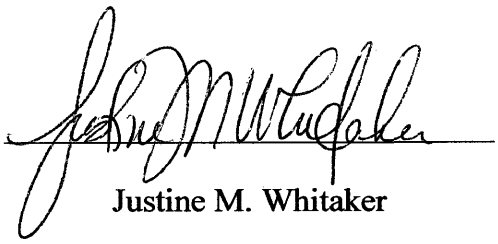
2004

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

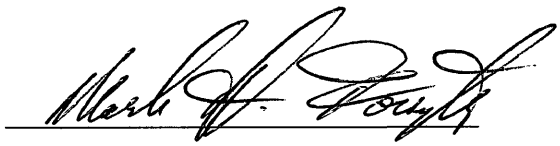
The requirements for the degree of

Master of Arts

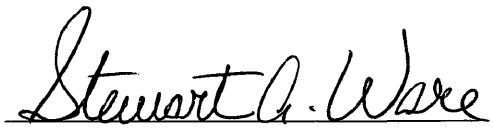


Justine M. Whitaker

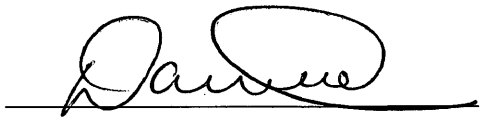
Approved by Committee, March 2004



Mark H. Forsyth



Stewart Ware



Daniel Cristol

To Kevin Croll, whose love of nature inspired me to achieve my goal.

TABLE OF CONTENTS

Acknowlegements.....v

List of Tables.....vi

List of Figures.....vii

Abstract.....viii

Introduction.....1
Evolutionary Response to Feather-degraders.....2
Feather Degradation.....7
Biology of *Bacillus licheniformis*.....9

Objectives.....15

Methods.....16
Sampling.....16
Culturing.....16
Lysis of Isolates.....17
Identification of Isolates.....18
BOXA1R-PCR.....19

Results.....19
Carriage Rate.....19
Identification of Isolates.....19
Mode of Bacterial Transmission.....20
Northern Saw-whet Owls.....21
Gray Catbirds.....22
Yellow-rumped Warblers.....22

Discussion.....23
Carriage Rate.....23
Mode of Bacterial Transmssion.....25

Literature Cited.....36

Vita.....45

ACKNOWLEDGEMENTS

I would like to thank Dr. Mark Forsyth for his knowledge, support, and guidance through the project. I would also like to thank Dr. Daniel Cristol and Dr. Stewart Ware for their input into the design of the project and their thorough proofreading and criticism.

LIST OF TABLES

Table 1. Avian Species Sampled for Keratinolytic Bacteria.....30

Table 2. Percent of Avian Species Colonized with Potentially Keratinolytic Members of the Genus Bacillus.....31

Table 3. Species of Bacillus Isolated from Avian Samples Using Selective and Differential Culture Methods.....32

LIST OF FIGURES

Figure 1 33

Figure 2 34

Figure 3 35

ABSTRACT

Bacillus licheniformis, a soil bacterium capable of degrading feathers in the laboratory, has been isolated from the plumage of wild-caught birds. Microbial degradation may have led to the evolution of avian molt and plumage characteristics. This study focuses on determining the carriage rate of potentially keratinolytic bacilli through culture of 8 avian species from a total population of 567 wild-caught birds. Samples were cultured at 50°C on 7.5% NaCl plates and in 7.5% NaCl nutrient broth as well as at 30°C on skim milk plates to select for the desired bacilli. Potentially keratinolytic bacilli were isolated from 35% of 40 American Redstarts (*Setophaga ruticilla*), 43% of 58 Black-throated Blue Warblers (*Dendroica caerulescens*), 35% of 65 Gray Catbirds *(Dumetella carolinensis*), 21% of 44 Hermit Thrushes (*Catharus guttatus*), 59% of 51 Northern Saw-whet Owls (*Aegolius acadicus*), 58% of 40 Dark-eyed Juncos (*Junco hymenalis*), 27% of 60 White-throated Sparrows (*Zonotrichia albicollis*), and 33% of 103 Yellow-rumped Warblers. The total sample population produced a carriage rate of 38%. Identification of 98 isolates, accomplished by amplifying and sequencing a 900 bp region of the16s *rrnA* gene, indicated that 68 were *Bacillus lichenformis* (69%). At a lower frequency, two other documented keratinolytic bacilli, *Bacillus subtilis and Bacillus pumilis*, were isolated as well as three additional species belonging to the genus *Bacillus, B. megaterium, B. flavothermus, and B.* sp., that have not yet been characterized as keratinolytic. *Kocuria roseus* was also isolated, which has been reported to be keratinolytic.

These results suggest that potentially keratinolytic bacilli are present within the plumage of a large number individuals and that *B.licheniformis* is an important member of the keratinolytic consortium of microbes in plumage. Genetic-fingerprinting analysis performed on isolates identified as *B. lichenformis* isolated from three avian species may suggest that Northern Saw-whet Owls obtain *B. licheniformis* through contamination from the surrounding environment instead of solely through transfer between conspecifics. In contrast, *B. licheniformis* found on Gray Catbirds and Yellow-rumped Warblers was comprised of fewer strains. There was no overwhelming evidence for one method of bacterial transmission between birds.

PLUMAGE AS A MICROHABITAT FOR BACILLI

INTRODUCTION

Plumage maintenance is paramount to avian survival. Flight, protection from the environment (Hood and Healy 1994) and a variety of social behaviors depend upon well-maintained plumage. Although feathers are composed of β-keratin, a highly durable protein, a variety of feather-degrading organisms have been isolated from the plumage of living birds (Noval and Nickerson 1959, Pugh 1964, 1965, Hubalek 1976, Takiouchi et al. 1984, Singh et al. 1997, Burtt and Ichida 1999). However, evolution of adequate avian defense strategies to combat these potential ectoparasites may have ensured that feathers remain in good condition while on a living bird.

By damaging plumage, these feather-degrading symbionts potentially decrease the host's fitness. In response to a decrease in fitness, host species may have evolved mechanisms to reduce damage to their plumage, such as preening, anting, dusting or molt (Hart 1997). It has been proposed (Burtt and Ichida 1999) that *Bacillus licheniformis* may be an important member of a consortium of keratinolytic microbes that have contributed to the evolution of feather shedding and plumage strengthening through increase in melanin content, as well as other traits that might increase fitness in the face of microbial assault on the plumage. Yet, Burtt and Ichida (1999) report a low carriage rate (8%) of keratinolytic bacilli within the plumage of 1,588 birds sampled. This carriage rate may be too low to cause sufficient damage to feathers and result in the evolution of avoidance behaviors to maintain the plumage.

Avian plumage is a home to numerous microbes, some of which are capable of degradation of feathers, therefore an understanding of molt requires an understanding of feather-degrading microbes. As a step toward achieving that goal, I examined the

carriage rate of *Bacillus licheniformis* and related microbes to test the hypothesis that feather-degraders are present on a high proportion of individual birds. I also examined whether the *B. licheniformis* present on an individual bird were of one or many strains to better understand the mode of bacterial transmission between birds. If one strain or a consistent set of strains is present on a species that would suggest transmission between related birds, perhaps occurring in the nest. Understanding if the birds randomly acquire the bacteria from the environment (i.e. contamination) or if the bacteria is passed on by contact of one bird to another related bird (i.e. infection) is critical to determine the potential for coevolution between the birds as hosts and the bacteria as parasites. Infection, as defined in this study, results from passing one strain or a group of strains from one individual to another. Contact is most likely to happen between parent and offspring, female parent and male parent, or conspecifics in competiton for a resource. As these contacts are made, the same strain(s) of bacteria are passed throughout the same species of bird. As these strains are associated with one species of bird, the strains may become adapted to live on specific avian species. Meanwhile, the avian species may become more adept at dealing with those particular strains. The host and symbiont may thus evolve together over the period of time that they are in close contact with each other. By differentiating the strains found on individuals within one avian species compared to those on other avian species, we can determine the likelihood of coevolution.

Evolutionary Response to Feather-degraders

Birds shed their plumage and grow new feathers annually or semi-annually. This process, termed molt, renders them flight-impaired (Swaddle and Witter 1997, Tucker

1991) and bears numerous costs (Payne 1972, Murphy *et al.* 1990, Murphy and King 1992, Lindstrom *et al.* 1993). Molt is an energetically costly endeavor and leaves birds vulnerable to predators, disease and the environment during this period (Cherel *et al.* 1994, Swaddle and Witter 1997). Despite the energetic cost of molt, it occurs at least once, and often twice annually. Birds molt their entire plumage once a year, as well as plumage grown specifically for breeding purposes. The growth and shedding of feathers may be partially explained through the process of sexual selection. Mate choice underlies a process by which favorable aspects, behaviors or resources, are chosen as a signal of mate fitness. The immunocompetence theory of sexual selection proposes that females choose males according to secondary sex characteristics (e.g. bright plumage) because they are honest signals of the mate's health and resistance to disease and parasites (Clayton, D.H. 1991, Hamilton and Zuk 1982). Various examples of sexual selection occur in avian systems and several have been linked to an increased immune efficiency (Linstrom and Lundstrom 2000, Keyser and Hill 1999, Hill 1999) and a small parasite load (Thompson *et al.* 1997, Blanco 1999). If a female can accurately assess the health of the male based upon his parasite load in combination with his ability to produce elaborate ornaments (e.g. large tail on a peacock), she may accordingly choose to mate with him (Hamilton and Zuk 1982).

The immunocompetence theory may help explain the role of feather degrading bacteria in avian biology. Because plumage parasites may cause increased feather destruction, the female may use this to asses the quality of a potential mate. Females may inspect the feathers of a male bird before mating with him, possibly looking for parasite damage (Shresta *et al.* 1997). Feather color and brightness have been documented as

accurate signals of health. These obvious signals can be used by the female to determine the most fit male (Johnston 1981, Keyser and Hill 2000, Lindstrom and Lundstrom 2000, McGraw et al. 2001, Verhulst et al. 1999). Development of choice, as proposed by the immunocompetence theory, causes selective pressures to act against individuals expressing unfavorable traits (Hamilton and Zuk 1982). Those traits that are favorable, such as a strong immune system, are coupled with more obvious characteristics, such as brighter plumage or longer tail feathers because these birds possess the extra energy reserves to produce elaborate characteristics. Invoking the immunocompetence theory, molt may have arisen as a method to rid the males of an unfavorable trait (high parasite loads). New, undamaged feathers may have made the male birds more reproductively successful, leading to the evolution of molt.

Obtaining greater reproductive success does not explain all variations in feather color. Darker plumage on birds, especially those of the same species located in different habitats has been described in the past by Gloger's rule. Gloger proposed that the darkest colored birds tend to live in the warmest, most humid environments and the lightest colored live in the coolest and driest environments (Gloger 1833). Both temperature and relative moisture may affect the degree of avian coloration (James 1970, Aldrich and James 1991). A variety of organisms, including 50 species of North American birds, follow Gloger's coloration cline (Zink and Remsen 1986). Song Saprows (*Melospiza melodia*) living in desert environments tend to be paler than conspecifics living in the humid Pacific Northwest (Burtt 1999). Feathers containing more melanin resist degradation better than those that are pale (Burtt and Ichida 1999, Kose and Moller 1999, Ward *et al.* 2002). Bacteria thrive in moist habitats so Burtt suggests that the avian

species living in humid environments may grow darker feathers to avoid a high degree of degradation. Avian species inhabiting drier habitats may not have invested in a defense mechanism involving melanin because feather-degrading bacteria may not thrive in these arid environments.

Feather Degradation

Feather damage occurs during normal activities performed throughout the life of the bird and causes visible effects such as a frayed appearance at the edge and tips of the feathers and fading of the color of the feather due to UV exposure (Clayton and Tompkins 1995, Davidson *et al.* 1989, Rogers 1990, Van de Wetering and Cooke 2000). Mechanical abrasion occurring during flight is thought to be a major contributor to feather damage. Since the morphological structure contributes greatly to the strength of the feather (Bonser 1995, Corning and Biewener 1998), mechanical abrasions may make the feather more susceptible to further breakdown. Feathers are stronger at the tip than at the proximal portion presumably as the result of selection to reduce damage (Bonser 1995, Corning and Biewener 1998). Pigmentation also adds strength to feathers (Burtt and Ichida 1999), but producing feather colors is costly due to the energy to manufacture the pigment and/or the energy put into searching for the food that contains the desired pigment (Hill 1999).

In addition to costly endeavors such as molting and producing more melanin, some behaviors may serve to maintain the plumage, such as anting and dusting. Anting consists of applying an ant to the base of the feather in an attempt to acquire some of the formic acid from the body of the ant. Formic acid has antimicrobial activity (Hefetz and Blum 1978, Erlich *et al.* 1986, Wenny 1998), and is a chemical irritant to both vertebrates

and invertebrates (Rossini et al. 1997). Formic acid is used as a defense mechanism by one of the 16 subfamilies of ants, Formiciniae (Hefetz and Blum 1978, Bolton 1992, Grimaldi and Agosti 2000). This subfamily of ants includes 48 genera and 3,000 species including some of the more common, ecologically important ants, such as the carpenter (*Camponotus*) and wood ants (*Formica*) (Grimaldi and Agosti 2000). Instead of the ability to sting, as with other ants, the Formiciniae ants are capable of spraying formic acid from a gland that has replaced the pygidial gland (Holldobler and Wilson 1990, Grimaldi and Agosti 2000). Although most of the evidence is anecdotal, by applying the ants to plumage or encouraging the ants to spray them, the birds may utilize the antimicrobial activity of the formic acid to decrease parasite loads.

The behavior referred to as dusting consists of the bird covering iteslf with dry soil in what is commonly interpreted as an attempt to desiccate the feathers (Clayton 1999). The lack of moisture as well as mechanical removal may clear the feathers of parasites, including keratinolytic bacteria that require high humidity.

Preening may also be a way of ridding the plumage of parasites. Preening removes larger organisms such as feather ticks and mites (Clayton 1999). However, these organisms must be within reach of the beak or foot to be removed. Bacteria are orders of magnitude smaller than the ticks and mites and so this behavior is of doubtful importance in decreasing bacterial load.

Parasitic organisms such as feather mites and flies as well as the keratinolytic fungi and bacteria may cause a high degree of damage to the feathers if present within the plumage at high rates and in metabolically active forms. According to one study, *B. licheniformis* is found most commonly on the distal portion of the feather (Muza *et al.*

2000) where it may increase the risk of mechanical breakage during flight (Bonser 1995, Corning and Biewener 1998). Degradation of the proximal (downy) portion of feathers, may conceivably also cause a decrease in fitness. By depleting the amount of air trapped next to a bird's body, the degradation of down may cause the bird to be chilled and weakened. This, in turn, might make it more susceptible to infections by other bacteria and viruses (Clayton 1999) or require it to spend more time staying warm and less time feeding or breeding. An ideal environment for the degradation of downy feathers may be on a rainy summer day. As a bird huddles to avoid rain, it may create a moist, warm habitat for the bacteria within the plumage. Destruction of insulating feathers during summer months may cause adverse effects during winter months if molt did not occur. Molting damaged feathers and parasites before the winter months may provide birds with better insulation to protect them during the winter.

Biology of Bacillus licheniformis

Until 1990, *B. licheniformis* was considered a typical soil bacterium, but Williams et al. (1990) isolated this bacterium from a poultry waste digester and characterized its feather-degrading (keratinolytic) capabilities. A poultry waste digester, utilizing mechanical and chemical degradation, is used to break down all the wastes produced by the harvesting of chickens at poultry production facilities. Feathers are a large component of this waste. Williams et al. (1990) found that this bacterium can utilize feathers as a source for energy, carbon and sulfur. A few species of fungi (Pugh 1964, Hubalek 1976, Salfranek and Goos 1982, Bahuguna and Kushwaha 1989, Rajak et al. 1991), several species of *Streptomyces*, (Noval and Nicherson 1959, Sinha 1991, Kitadokoro *et al.* 1994, Bockle et al. 1995, Chitte et al. 1999, Szabo et al. 2000), *Bacillus*

pumilis (Burtt and Ichida 1999), *Bacillus subtilis* (Evans et al. 2000), a gram-negative bacterium in the family *Vibrionaceae* (Sangali and Brandelli 2000) and one strain of *Escherichia coli* (Lin *et al.* 1995) are also capable of feather degradation. Feathers are mainly composed of a unique form of keratin, which exists in β-pleated sheets, in contrast to the α-helix formation of hair and nails (Pauling and Corey 1951, Rintoul *et al.* 2000). This type of keratin is known to be extremely resistant to degradation in nature (Hood and Healy 1994, Cheng *et al.* 1995, Szabo et al. 2000). However, *B. licheniformis* secretes a protease that targets the β-keratin of feather and rapidly breaks down feathers in the laboratory (Williams et al. 1990, Cheng *et al.* 1995, Lin et al. 1999).

Until recently, interest in the capability of *B. licheniformis* to degrade β-keratin lay primarily in the field of poultry science. As commercial poultry species are harvested, the waste is not easily recycled due to the durability of β-keratin. Recycling is usually by means of chemical breakdown and the result is not a high-quality protein that can be used as a livestock meal (Burtt and Ichida 1999, Ichida et al. 2001). Poultry science researchers strive to understand the means by which feathers are degraded in nature, but a poor understanding of β-keratin degradation prevents this process from being applied for recycling poultry feathers. Other than *B. licheniformis,* no other keratinolytic species has been shown to degrade feathers with the efficiency necessary to recycle the feathers back into livestock feed (Lin *et al* 1995, Kao and Lai 1995, Ichida *et al.* 2001). Chemical methods may either denature the proteins or leave traces of toxic substances in the digested keratin (Hood and Healy 1994). Although other species of bacteria and a few species of fungi are capable of feather degradation, *B. licheniformis* was demonstrated to degrade feathers quickly when grown at optimal temperature, 45°C (Williams et al.

1990, Lin et al. 1992, 1995, 1999, Wang and Shih 1999). This discovery generated further interest in the enzymes produced by *B. licheniformis.*

Bacillus licheniformis is a gram positive, facultatively aerobic bacterium most commonly found in soil environments. It is most closely related to the well-studied bacterium, *Bacillus subtilis*, also a soil bacterium, and the two share many similarities such as temperature range, proteolytic capabilities, and genetic make-up (Duncan et al. 1994). At least 3 species of *Bacillus* are known to degrade β-keratin including *B. licheniformis, B. subtilis* (Evans *et al.* 2000), and *B. pumilis* (Burtt and Ichida 1999). As with other bacillus species found within the soil, *B. licheniformis* has the capability of degrading a variety of different substrates by secreting an assortment of enzymes (Onifade *et al.* 1998, Schmidt *et al.* 1995) many of which have industrial applications (Ferrero et al. 1996, Sonenshein 2000). *Bacillus* spp. secrete proteases used in laundry detergents as well as amylases and isomerases used to produce corn syrup and dextrose (Sonenshein 2000). Although common in the soil, *Bacilli* species can be found in niches that are very specialized such as the gut of arthropods, cattle and humans, or sewage sludge (Sonenshein 2000). In the laboratory, *B. licheniformis* can grow in most media and at temperatures ranging from 32°C to 60°C. *B. licheniformis* can also maintain growth at a pH as high as 9.0. Optimal growth in nutrient broth occurs at 50°C and a pH of 7.5 (Williams et al 1990, Cheng et al. 1995). Resistance to antibiotics is high and the generality of the bacterium makes it well adapted to environments that undergo frequent changes in physical parameters.

An important aspect of *B. licheniformis* that allows it to thrive in the ever-changing soil is its capability to produce endospores. Endospores are metabolically inert

(Makino and Moriyama 2002) and are resistant to dessication, radiation, UV light, chemical treatment, and extreme temperatures (Nicholson et al 2000). When environmental conditions are poor and moisture levels are low, spore-forming bacterial species are more likely to survive than those bacterial species that have no defense against environment changes. Sporulation provides an advantage to bacteria because spores survive harsh conditions that metabolically active, reproducing "vegetative" cells cannot overcome (Gerhardt and Marquis 1989). In the presence of high numbers of bacteria and a limiting carbon source, sporulation will occur. Sporulation is thought to be triggered by low levels of a nutrient source. During sporulation, the cell becomes desiccated, resulting in a loss of 75% of the moisture levels of the vegetative cell, and becomes a small, round, endospore consisting of a core and protective coats (Beaman et al. 1982, Popham et al. 1996, Nicholson et al. 2000 , Driks 2001). The core houses all necessary ingredients for germination. When favorable conditions and germination coincide, the bacteria will proliferate in a more favorable environment with potentially less competitors as they have been removed during the unfavorable environmental conditions. This gives *B. licheniformis* an advantage over the non-spore forming bacteria within the soil. The irreversible process of sporulation (Gonzalez-Pastor *et al.* 2003) can last up to 8 hours, and results in a cell that is incapable of producing enzymes because it is in a metabolically inert state (Sonenshein 2000).

As metabolically inert spores, bacillus species cannot proliferate in a particular niche. Non-sporulating bacteria may take advantage of a quick change back to favorable conditions and thus have an advantage over competitors locked in the process of sporulation. Germination, the process of returning from a spore form to the vegetative

state, is not as well characterized as sporulation, but may be induced by particular nutrients, such as alanine (Foster and Johnstone 1989, Nicholson et al. 2000, Makino and Moriyama 2002), and is rapid in comparison to sporulation (Atrih et al. 1996, Driks 2001). Returning to the vegetative state would be detrimental if the cells germinate at an inopportune time, such as when environmental conditions are still disadvantageous or when the niche is full due to the proliferation of non-sporulating bacteria. Sporulation may benefit keratinolytic organisms that are incidentally transferred from the usual habitat of soil to the plumage of a bird. If plumage, as microhabitat, does not promote a thriving ecosystem for keratinolytic bacilli, the bacteria may survive within the plumage by sporulating. When more hospitable conditions arise, such as plumage becoming damp, or birds molting, which would carry bacteria back to the preferable environment of the soil, the bacteria may germinate and begin degrading the plumage.

Although degradation of keratin by bacilli during residence in the plumage of wild birds is a possibility, sporulation due to adverse conditions may be equally likely. Keratinase secreted through the membrane of bacteria diffuses out into the environment. A release of nutrients may occur during degradation of β-keratin, but the bacteria that released the keratinase may not be close enough to the spot of degradation to benefit from the enzymatic event. To coordinate gene expression and avoid inappropriate synthesis of enzymes, many bacteria use a process known as quorum sensing to determine the relative population of related bacteria in the area (Fuqua and Winans 1994, Schneider et al. 2002, Dunny and Winans 1999). As the population increases, the bacteria detect the population density due to secreted pheromones. When population levels reach a critical threshold density (quorum), gene expression may be induced to secrete a particular enzyme

(Schneider et al. 2002). Therefore, if only a small population of bacteria is transferred from the soil to the plumage of a bird, keratin degradation may not occur. Sporulation may be a more likely event since a readily available food source may not be present within the plumage upon initial colonization.

Along with sporulation, the ability of gram-positive bacteria to secrete proteins directly into the environment via the general secretory pathway, makes *B. licheniformis* more adept at living within the plumage. This pathway is sufficient to secrete proteins directly into the environment because gram positive bacteria, unlike gram-negative bacteria, have only to translocate secreted materials across a single lipid bilayer. A nascent protein to be exported is targeted for export by an amino-terminal signal sequence, which possesses a cleavage site such that it can be removed after translocation across the cytoplasmic membrane (Harper and Silhavy 2001). The Sec protein group, which is necessary to transport the protein across the membrane, is made up of both soluble proteins and cytoplasmic membrane proteins. One Sec protein (SecA) binds the protein to be exported while SecB chaperones the protein destined for export to the membrane and keeps the protein from folding within the cytoplasm, a requirement for secretion. The Sec proteins also aid in the initial contact of the proteins with the membrane as well as insertion into the membrane. ATP hydrolysis is used repeatedly to move the protein through the membrane and into the external environment. Signal peptidase cleaves the protein, thus freeing the translocated mature protein into the external environment (Harper et al. 2001).

B. licheniformis secretes a proteolytic enzyme, keratinase, that is capable of degrading β-keratin, the main constituent of feathers (Williams *et al.* 1990). Two other species

within the genus *Bacillus* known to degrade β-keratin are *B. subtilis,* and *B. pumilis* (Burtt and Ichida 1999). The optimal temperature for keratin hydrolysis is 45°C and *B. licheniformis* most efficiently utilizes feathers as a source of nutrients in an aerobic environment although some degradation may occur in anaerobic conditions (Williams *et al.* 1990). The enzyme functions in a range of temperatures similar to the range for the bacterium (45°- 60°C), as well as in an anaerobic environment (Lin *et al.* 1996). Keratinase is not capable of breaking down all substrates and some soluble proteins will actually halt the production of keratinase and *B. licheniformis* will secrete a protease that targets the most available substrate (Ferrero *et al.* 1996, Lin et al. 1999, Heineken and O'Conner 1972). This control mechanism allows for the degradation of alternative substrates and implies that *B. licheniformis* is not restricted solely to plumage, but is perhaps an ecological generalist.

Keratinase is also structurally similar to a previously characterized protein, subtilisin Carlsberg (Evans et al. 2000), consisting of a peptide chain of 274 residues (Smith et al. 1996). Subtilisins, naturally and industrially produced have been shown to degrade the keratin of feathers. There is a difference of only a single amino acid between the Carlsberg gene from a variety of *B. subtilis* strains and the kerA gene from strains of *B. licheniformis* (Evans et al. 2000). *B. licheniformis* has been documented to produce antimicrobial agents (Jacobs 1985, Lebbadi et al. 1994, and can kill several other gram-positive competitors, such as *B. megaterium* (Lebbadi et al. 1994) and *B. subtilis* in the environment by forming pores in the membrane (Breukink *et al.* 1999, Stein et al. 2002). The ability to kill closely related bacteria in a new environment, such as may be the case when soil microbes are transferred to dry plumage of a bird, would benefit a keratinolytic

organism. If the keratinolytic organisms present within avian plumage are degrading β-keratin, the ability of *B. licheniformis* to kill competitors may give it an advantage in a potentially crowded niche.

Objectives

As stated earlier, Burtt and Ichida (1999) proposed that keratinolytic bacteria may be degrading feathers within the plumage and could have caused the evolution of avoidance behaviors, such as molt. Based on this hypothesis, I predicted that a colonization rate greater than 8% would be required to significantly affect the evolution of molt, pigmentation, or behavioral changes. My first objective was to determine if the previous estimate of 8% colonization rate of birds by keratinolytic bacilli was accurate (Burtt and Ichida 1999).

My second objective was to determine the mode of bacterial transmission by *B. licheniformis* between hosts. Genetic-fingerprinting of *B. licheniformis* strains cultured from wild-caught birds was used to study the genetic diversity found within the *B. licheniformis* strains of each of three avian species. Repetitive extragenic palindromic polymerase chain reaction (REP-PCR) has been used to differentiate strains of a variety of bacillus species (Brumlik *et al.* 2001, Herman and Heyndrickx 2000) and differentiates *B. licheniformis* isolates well. This procedure will help differentiate between colonization of birds by *B. licheniformis* resulting from contamination or resulting from infection.

METHODS

Sampling

To determine that carriage rate of bacillus species within the plumage of wild-caught birds, 594 birds were mist netted during routine bird banding operations of the Coastal Virginia Wildlife Observatory in Kiptopeke State Park, Virginia between 5 September, 2001 and 18 March, 2002. These were sampled by rubbing a sterile cotton-tipped applicator through the feathers. The applicators were saturated in isotonic saline and sterilized by autoclaving (120°C/15 PSI, 20 minutes) before sampling. Two consecutive samples were taken from the same individual bird with separate applicators. The cotton-tipped applicator was continually rotated during sampling to ensure optimal contact between the applicator and feathers. The applicator was rubbed through the feathers of the right side of the head, over the top of the head and through the feathers of the left side of the head. The applicator was then rubbed down the ventral side, through the tail and across the rump. The applicators used in sampling were then stored frozen at -80°C until they were cultured.

Culturing

A. Differential Culturing (Isolation of Proteolytic Bacteria)

To determine if bacteria capable of secreting proteases, such as keratinase, were present on each individual bird, one applicator from each bird was applied to a nutrient agar medium containing 1% skim milk (SMM) and incubated at 37°C for 24 hours. All growth showing evidence of proteolytic activity, defined as a visible zone of clearing around the colony indicating proteolytic cleavage of casein, was then transferred from the

SMM to high salt medium (7.5% NaCl Nutrient Agar) and incubated at 50°C to assess the halotolerance and thermotolerance of each proteolytic isolate (see below).

B. Selective Culturing (Isolation of Halotolerant, Mildly Thermophilic Bacteria)

To select for bacteria that can tolerate high salt, the applicator was also used to inoculate nutrient media containing 7.5% NaCl. This inoculated high salt medium (HSM) was incubated at 50°C in a humidified chamber for up to 7 days to select for mildly thermophillic bacteria. This selective step of the culturing process eliminated the growth of a wide variety of bacteria, especially any gram-negative bacteria that may have resulted from fecal contamination.

After inoculation of SMM and HSM, the tip of the applicator was cut off and placed into a 15 ml glass tube containing 5 ml of high salt nutrient broth (7.5% NaCl w/v). The growth from the HSM was also transferred to the plates containing skim milk and incubated at 37°C to determine the proteolytic capabilities of the thermophillic, halotolerant isolates.

The colonies capable of casein hydrolysis (proteolytic) that could tolerate the high salt environment at high temperatures (50°C) were then gram stained to determine morphology and gram reaction status. Those determined to be gram-positive proteolytic, halotolerant, thermophillic bacilli were considered to be potentially keratinolytic and stored on nutrient broth agar slants at room temperature.

Lysis of Isolates

To obtain genomic DNA (gDNA) for further species identification, isolates of thermophilic, halotolerant, proteolytic bacilli were grown in 10 ml Luria-Bertani (LB) media and incubated overnight at 37°C with aerobic aeration and then lysed with the

following method. The pelleted cells were resuspended in 300 µl of 10mM Tris-HCl (pH 8.0) and incubated for 48 hrs with 10% lysozyme at 55°C. The lysis solution was then incubated at 100°C for 10 min. The lysed isolates were then centrifuged at 14,000 x g for 5 min and the supernatant was transferred to a sterile tube. Polymerase chain reaction (PCR) was performed using 5 µl of the supernatant as template (see details below).

Identification of Isolates

Isolates were identified to ensure that the selective and differential culture scheme used was efficient at isolating potentially keratinolytic bacilli. A highly conserved section (~900 bp) of the 16S *rrnA* was amplified from lysates by polymerase chain reaction (PCR). The 50 µl reaction consisted of 1x Reaction Buffer (Promega), 2 mM Mg^{2+}, 0.2 mM each dATP, dCTP, dGTP, dTTP, 400 ng of reverse primer (5'CCCGGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3'), 400 ng of forward primer (5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3'), and 2.5 U Taq DNA polymerase (Promega). The thermal cycling conditions used were 25 cycles of 94°C for 30 seconds, 54°C for 30 seconds, 72°C for 1.5 minutes.

The PCR amplicon was purified using the Qiagen PCR purification kit by manufacturers suggested protocol. A 20 µl Fluorescent Dye Terminator sequencing reaction (Big-Dye Terminator, ABI PRISM) was performed on the purified 16S-PCR product using 500 ng of the PCR amplicon, 1x Ready Reaction mix, and 200 ng of the reverse primer. The following thermal cycling conditions were used: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 4 minutes. The reaction was then purified using a DTR Gel Filtration Cartridge kit (Edge Biosystems). Complete sequencing reactions were processed in an ABI 3100 Avant Gene Sequencer.

BOXA1R-PCR

BOX-PCR, a method of Repetitive Extragenic Palindromic Polymerase Chain Reacion (REP-PCR), was used to differentiate strains of *B. licheniformis*. BOX-PCR utilizes a single primer to produce a strain specific array of bands by amplifying the sequences between repetitive extragenic repeats conserved throughout most bacterial genomes. The 50 µl reaction consisted of 1x Reaction Buffer (Promega), 2 mM Mg^{2+}, 0.2 mM each dATP, dCTP, dGTP, dTTP, 400 ng of primer (5′CTACGGCAAGGCGACGCTGA3′) (Van Belkum et al. 1996), and 2.5 U Taq polymerase (Promega). The thermal cycling conditions used were 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1.5 minutes. The strain specific banding patterns were visualized on 5% neutral polyacrylamide gels stained with ethidium bromide. Polyacrylamide gel electrophoresis (PAGE) is a method of separating nucleic acids by fragment length with high resolution using a constant voltage in a vertical electrophoresis chamber.

RESULTS

Carriage Rate

This portion of the study focused on determining the carriage rate of mildly thermophillic, halotolerant, proteolytic bacilli through culture of a sampling base of eight wild-caught avian species of 40 or more individuals (Table 1). These characteristics were chosen because they are characteristics of known keratinolytic species of bacteria. We hypothesized that these phenotypic characteristics, together, may serve to select for keratinolytic bacilli. Each individual sampled was cultured at 50°C on 7.5% NaCl plates

and in 7.5% NaCl nutrient broth as well as at 30°C on skim milk plates to select for the putative keratinolytic species. The total sample population, including all eight avian species, produced a carriage rate of 38% (n= 461) for potentially keratinolytic bacteria. The carriage rates of the various avian species did not differ statistically (Table 2). Although a previous study suggested that ground-dwelling birds are more likely to be colonized by *B. licheniformis* (Burtt and Ichida 1999a), our results did not support that idea. Instead, our sample, which included ground-dwellers, arboreal species and a bird of prey, suggest that all of the avian species we sampled were colonized to similar extents by potentially keratinolytic bacilli (χ^2=2.58,df=1,P=0.11).

Identification of Isolates

To determine whether our selective and differential culture scheme was effective in isolating potentially keratinolytic bacilli, we identified 98 out of approximately 500 isolates to the species level. Identification of the isolates, accomplished by amplifying and sequencing a ~900 bp portion of the 16s *rrnA* genes, indicated that 68 isolates (69%) were *B. lichenformis*. About 92% of isolates were determined to be keratinolytic bacillus (Table 3) species. The keratinolytic capabilities of these species have been demonstrated previously (Burtt and Ichida 1999, Kim *et al.* 2001, Suh and Lee 2001). Our results demonstrate the effective nature of our differential and selective culturing scheme as well as the efficacy of casein hydrolysis as a surrogate for keratin hydrolysis. Differential and selective culturing was based on our hypothesis that *B. licheniformis* may have been a minor component of a keratinolytic bacterial microbiota of avian plumage. However, the identification of those isolates revealed that the majority of mildly thermophilic, halotolerant, proteolytic Bacilli found within the plumage were *B. licheniformis*. We also

isolated, albeit at a much lower frequency, two congeneric bacterial species known to degrade keratin, *Bacillus subtilis* (Suh and Lee 2001) and *Bacillus pumilis* (Burtt and Ichida 1999). Also isolated were three additional species that have not been characterized as keratinolytic: *Bacillus megaterium*, *Bacillus flavothermus, Virgibacillus* sp. (Table 3). Finally, we isolated one gram positive coccus, *Kocuria roseus*, which has been characterized as keratinolytic (Vidal *et al.* 2000). The 98 isolates that were sequenced consisted of 6 species: *Bacillus licheniformis, Bacillus megaterium, Bacillus pumilis, Bacillus subtilis, Bacillus flavothermus, Virgibacillus* sp., and *Bacillus* sp. (Table 3).

Mode of Bacterial Transmission

Northern Saw-Whet Owls

Seventeen isolates from Northern Saw-Whet Owls, were identified as *B. licheniformis* (Table 3) via 16s *rrnA* sequencing. Fifteen of those 17 isolates were used in genetic-fingerprinting. BOXA1R-PCR is a repetitive extragenic polymorphic polymerase chain reaction (REP-PCR), which amplifies the regions of DNA found between commonly repeated sequences found throughout all gram-negative and most gram-positive bacteria (Van Belkum *et al.* 1996). BOXA1R-PCR produced complex and easily identifiable banding patterns for all 15 isolates. Eight distinct strains were identified from the 15 isolates cultured from nine individual birds. Seven of the 15 isolates were isolated from the same bird and produced four distinct amplicon array patterns suggesting that four genetically distinct strains of *B. licheniformis* were isolated from one bird. The remaining eight isolates were isolated from eight different birds. Of the isolates found on

different birds, four of the eight isolates produced identical band patterns, indicating that these four isolates are genetically indistinguishable strains of *B. licheniformis* (Fig 1).

Gray Catbirds

Inoculation of Gray Catbird samples produced 27 isolates of *B. licheniformis*, as determined by 16s *rrnA* sequencing (Table 3). Twenty of those 27 isolates were used in BOXA1R-PCR. The 20 isolates were grouped into six strain types that are genetically indistinguishable. In some cases, the same strain was cultured from multiple birds (Fig 2). Culturing of four of the birds (labeled 15156, 15190, 12894, 12878, Fig 2) produced two isolates per bird, of which one pair comprised strains that were not identical (labeled 12894-1and 12894-2, Fig 2). The other three sets were comprised of two strains that were identical to one another. The overall genetic diversity of the Gray Catbird isolates appeared lower than that found on Northern Saw-whet Owls.

Yellow-rumped Warblers

Strains of *B. licheniformis* isolated from Yellow-rumped Warblers yielded results similar to those obtained from the Gray-Catbird. Twenty isolates were determined to be *B. licheniformis* (Table 3). Of the 6 isolates of *Bacillus licheniformis,* examined by BOXA1R-PCR, 3 distinct strains were identified (Fig 3). Two isolates were cultured from one bird (labeled 31391, Fig 3) and determined to be the genotypically indistinguishable. Cultures of two isolates from two different birds, one labeled 31395 and a second labeled 31388 (Fig 3), were determined to be the same strain based on DNA fingerprinting.

DISCUSSION

Carriage Rate

The 8% carriage rate determined by Burtt and Ichida (1999) may be inadequate to generate sufficient damage to the plumage to contribute to the evolution of avoidance behaviors such as molt and dusting. I hypothesized that the carriage rate of keratinolytic bacilli within the plumage of birds is higher than 8%. To test this hypothesis, I developed a selective and differential culture scheme that isolated all mildly thermophilic, halotolerant, proteolytic, gram-positive (potentially keratinolytic) bacilli from samples of migratory birds. This culture scheme isolated potentially keratinolytic bacilli from the plumage of 38% of the 8 avian species sampled. Further investigation into the identification of the isolates demonstrated that *B. licheniformis* was the dominant member of the keratinolytic consortium present within the plumage, which demonstrates that our culture scheme was effective in culturing keratinolytic bacilli.

The carriage rates among wild-caught avian species ranged from 21% to 59%, but frequencies did not differ significantly from one another, including when I compared ground-dwellers to all other species. Each species used to determine carriage rate consisted of 40 or more individuals, so if a "medium" or "large" difference in frequency had existed (as defined by Cohen 1988), I had a >80% chance of detecting it in my statistical comparison. I would not have detected "small" differences, as defined by Cohen (1988) because the statistical power was too low (e.g., I would have needed ~200 birds to achieve power = 0.80 for small diferences). Because I had sufficient power for these comparisons, I conclude that any difference in carriage rate between species is quite small and probably of little biological significance.

The carriage rate study I performed was different than that performed by Burtt and Ichida (1999). Site location, the time of year that samples were taken, sampling technique and culture technique varied between the two studies. Any one of these differences may have resulted in the higher carriage rate that I reported.

Burtt and Ichida sampled from May 18, 1993 to December 7, 1996 at locations in Ohio, Massachusetts, and Manitoba. My samples were collected between September 5, 2001 and March 18, 2002. Sampling in Burtt's study was accomplished by rubbing 3 separate dacron-tipped applicators through the feathers. Each applicator was used to sample a different area of the plumage. My sampling was performed using one swab to obtain bacteria from the entire bird. Burtt and Ichida (1999) cultured keratinolytic bacilli in modified nutrient broth (7.5% NaCl) at 50°C. Our culture scheme consisted of using two types of media and incubation temperatures. In addition to applying the applicator to high salt agar incubated at 50°C, I also struck the applicator across skim milk media (SMM), which was incubated at 37°C. The addition of the SMM allowed some bacteria to grow that may otherwise not have grown when removed from the bird and cultured under harsh conditions (high salt, mildly-thermophilic). The differences in location, sampling and culturing methods may explain the higher carriage rate found in this study.

Although we have detected high carriage rates, our method of culture fails to distinguish between vegetative cells and spores. The prediction that a higher carriage rate results in increased damage to feathers may be incorrect because of the ability of bacilli to sporulate. Our culture methods may have detected bacilli that were in spore form within the plumage and awaiting molt or the bird's demise to initiate germination and feather degradation on the ground.

We found no trend in carriage rate of keratinoltyic species according to feeding guild as was suggested in the study performed by Burtt and Ichida (1999). Samples taken from the Northern Saw-whet Owl produced a 58% carriage rate and those from the Dark-eyed Junco produced a carriage rate of 59%. These two avian species have very different natural histories. While the owl spend the majority of the day in trees, feeds on mice, and nests in cavities, the Dark-eyed Junco is a ground-feeder and nester. Clearly, the carriage rate data did not show a trend of carriage rate correlating with feeding guild as was reported by Burtt and Ichida (1999).

Mode of Bacterial Transmission

After concluding that the carriage rate of *B. licheniformis* by wild birds may be higher than a previous report suggested, I next investigated the genetic diversity of the *B. licheniformis* cultured from three avian species. I hypothesized that if *B. licheniformis* is growing vegetatively in the plumage of birds and degrading feathers, then we would see evidence of a clonal population of *B. licheniformis* indicative of an infection. A clonal population (infection) may result from vertical transfer of *B. licheniformis*, i.e. from parent to offspring, or horizontally from adult to adult. The presence of a clonal population of isolates from the same bird, or members of the same species might suggest an initial acquisition and subsequent spread throughout the plumage and other avian conspecifics.

The alternative mechanism would be contamination, which is the random acquisition of *B. licheniformis* isolates through contact with the environment. In the case of contamination, a bird or species of bird may acquire a heterogeneous population of *B. licheniformis* isolates through repeated contact with a variety of sources including, but

not limited to, the soil, vegetation and predators. The natural history of the bird may determine the variety of substrates from which *B. licheniformis* could be obtained. I utilized BOXA1R-PCR, a method of REP-PCR, to examine the genetic diversity of *B. licheniformis* isolates from the plumage of wild-caught birds. Each of the 98 isolates used in the genetic-fingerprinting assay were identified as *B. licheniformis.* This genetic diversity analysis did not provide conclusive evidence for either infection or contamination.

Although we hypothesized that there would be evidence for infection, clonal populations were only seen in the *B. licheniformis* isolates cultured from Gray Catbirds and Yellow-rumped Warblers. There is a dominant amplicon array pattern found within the Gray Catbird isolates (Fig 2). This pattern can also be found in the isolates of *B. licheniformis* cultured from Yellow-rumped Warblers (Fig 3). This dominant amplicon array pattern may be evidence of infection. However, the sample size of the Yellow-rumped Warbler isolates is low and further analysis would be necessary to determine if infection is the mode of bacterial transmission in Gray Catbirds or Yellow-rumped Warblers.

The isolates from Northern Saw-whet Owls appeared to be more genetically diverse than the isolates from either passerine species analyzed. One individual owl carried at least seven genetically distinct strains. However, the same *B. licheniformis* pattern could be found on four different owls, which contradicts the evidence for a heterogeneous population found on the one individual. The data for all three avian species does not provide conclusive support for either the theory of infection or contamination. Further research is needed to test this hypothesis properly.

Although researchers have historically believed that most infectious diseases were the result of a clonal infection, recent evidence suggests otherwise (Lord et al. 1999, Read *et al.* 2000, Thompson 2000). Genotyping studies such as those performed on *Plasmodium falciparum*, the etiologic agent of malaria, have provided evidence that more than one genetically distinct population may be thriving within a diseased organism (Daubersies *et al.* 1996, Mercereau-Puijalon 1996) and that those clones are competing with one another (Snounou *et al.* 1996, Taylor *et al.* 1997, Read *et al.* 2002). Competition between these genetically distinct populations occurs as they attempt to utilize the same nutrient source. Competitive interaction results in evolution of beneficial traits and may influence disease severity. The on-going process of strains competing with other strains may result in a high degree of damage to a host (Read *et al.* 2001).

These same theories may be applied to the host-parasite interactions between bird and kertinolytic bacteria. Diversity of *B. licheniformis* strains was found on the Northern Saw-whet Owls sampled in this study and although the genetic-fingerprinting data suggests lower diversity of isolates found on both Gray Catbirds and Yellow-rumped Warblers, these avian species still had a variety of *B. lichenformis* isolates within their plumage. One strain could monopolize the plumage and become highly adapted to this niche, because fewer measures are needed for competition. However, if a variety of strains of *B. licheniformis* are competing with each other within the plumage of birds, they may out-compete each other by becoming better adapted to conditions within the plumage, such as secreting a more efficient protease. Ecological studies of avian parasites have shown that ectoparasites that are transferred from parents to offspring (vertically), are often cause less damage to the host than those passed horizontally

(Anderson and May 1982, Ewald 1983, Clayton and Tompkins 1995, Tompkins *et al.* 1996). These horizontally-passed parasites may show greater diversity than those passed vertically. This may also be the case with bacteria, resulting in a benign infection of bacteria passed between conspecifics.

Although this study provides evidence that keratinolytic bacilli are present within the plumage, it does not address whether bacteria are growing vegetatively while in the plumage. At least three scenarios may be hypothesized when a bird becomes colonized with *B. licheniformis*. The first model is that, although capable of degrading feathers, *Bacillus* species may sporulate while within the plumage and germinate again when the conditions are closer to optimal for growth on such a substrate. These favorable conditions may exist when the bird is living, but there is a possibility that this habitat may not be suitable until the bird is no longer living or the feather has been molted. The plumage may be unfavorable a majority of the time since it is often kept dry and the body temperature of a bird is about 40°C, which is lower than the documented optimal temperature (45°C) for feather degradation by *B.* licheniformis (Lin et al. 1992). However, once the feathers have reached the soil, the habitat may be prime for keratinolysis due to increased moisture or a more optimal temperature that may be achieved in an environment where temperatures rise due to the metabolic action of a large number of biomass degrading bacteria, as occurs in compost heaps. The bacteria may also be incidental "hitch-hikers" that sporulate once in the plumage. As the bird comes into contact with the soil again through various behaviors such as feeding or dusting, the bacterial spore may be transferred from the plumage back to the soil having never secreted keratinase or degraded a feather. The bacterium has thus been transferred to a

new portion of soil, possibly from a crowded or unfavorable habitat to a less crowded or more favorable habitat. A third possible theory is that *B. licheniformis* may grow vegetatively within the plumage of live birds, resulting in damage to the feathers of a living bird. This last theory would involve implications for both avian and microbial ecology. The current study cannot address which of these competing hypotheses may be correct, but paves the way for such experiments by establishing that numerous strains of *B. licheniformis* are present on each avian species and that keratinolytic bacteria are present on more than one-third of birds across a variety of species.

Further culturing data including species with more diverse natural histories such as waterfowl, predatory birds, and passerines would provide more information as to the specificity of *B. licheniformis* isolates to avian hosts. Developing an efficient and quick molecular method to detect keratinolytic organisms, such as PCR, may also provide more conclusive data on the colonization rate of these organisms within the plumage. A more complete inventory of keratinolytic bacteria may be accomplished if selective and differential methods are developed to isolate gram-negative bacteria, gram-positive cocci, and fungi capable of feather degradation. Isolating other potentially keratinolytic microbes, such as *Micrococcus* species, may reveal that other microbes are also widespread on wild-caught birds and have an equally important role in feather degradation. Finally, because Bacillus were abundant within the plumage of wild-caught birds, determining the potential for sporulation of keratinolytic bacilli after arrival within the plumage, would provide information as to the possibility of selection pressures by feather-degrading microbes on avian behaviors involving plumage maintanence.

Table 1. Avian Species Sampled for Keratinolytic Bacteria

Those 8 species in bold typing were used to culture for carriage rate of mildly thermophilic, halotolerant, proteolytic bacilli. These species were selected due to the higher number of individuals sampled per species ($n \geq 40$).

Yellow-rumped Warbler	103
Gray Catbird	65
White-throated Sparrow	60
Black-throated-Blue Warbler	58
Northern Saw-whet Owl	51
Hermit Thrush	44
American Redstart	40
Dark-eyed Junco	40
Black-and-White Warbler	19
Magnolia Warbler	19
Song Sparrow	14
Field Sparrow	12
Swamp Sparrow	5
Chipping Sparrow	4
Palm Warbler	7
Ruby-crowned Kinglet	4
Blue Jay	3
Cardinal	3
Sharp-shinned Hawk	3
Tufted Titmouse	3
Northern Mockingbird	2
Swainson's Thrush	2
Veery	2
Gray-cheeked Thrush	1
Northern Water-thrush	1
Ovenbird	1
Red-breasted Nuthatch	1
Total	567

Table 2. Percent of Avian Species Colonized with Potentially Keratinolytic Members of the Genus Bacillus.

Mildly thermophilic, halotolerant, proteolytic Bacillus species were cultured from each avian sample. The percent colonization rates of the various avian species were not significantly different from one another (df=7, $\chi^2 = 7.815$, $P > 0.05$).

Avian Species	Colonization Rate	Habitat
Northern saw-whet Owl	59% (n=51)	Aboreal
Dark-eyed Junco	58% (n=40)	Ground
Black-throated Blue Warbler	43% (n=58)	Aboreal
Gray Catbird	35% (n=65)	Ground
American Redstart	35% (n=40)	Aboreal
Yellow-rumped Warbler	33% (n=103)	Aboreal
White-throated Sparrow	27% (n=60)	Ground
Hermit Thrush	21% (n=44)	Ground

Table 3. Species of Bacillus Isolated from Avian Samples Using Selective and Differential Culture Methods.

Isolates of potentially keratinolytic Bacillus spp. cultured through selective and differential culture techniques were identified to the species level through sequencing of a 900 bp portion of the 16s *rrnA*.

Avian Species	**Total**	***B. l.*[1]**	***B. m.*[2]**	***B. p.*[3]**	***B. s.*[4]**	***B. spp.*[5]**	***B. f.*[6]**	**V. spp.[7]**
Northern Saw-whet Owl	27	17	5	1	0	4	0	0
Gray Catbird	31	27	0	1	0	3	0	0
Yellow-rumped Warbler	25	20	1	2	2	0	0	0
Dark-eyed Junco	15	4	0	7	2	0	1	1
Total	98	68(69%)	6(6%)	11(11%)	4(4%)	7(7%)	1(1%)	1(1%)

[1]*Bacillus licheniformis*, [2]*Bacillus megaterium*, [3]*Bacillus pumilis*, [4]*Bacillus subtilis*, [5]Bacilli that cannot be identified at the species level, [6]*Bacillus flavothermus*, [7]An a isolate of the genus *Virgibacillus* that cannot be identified at the species level.

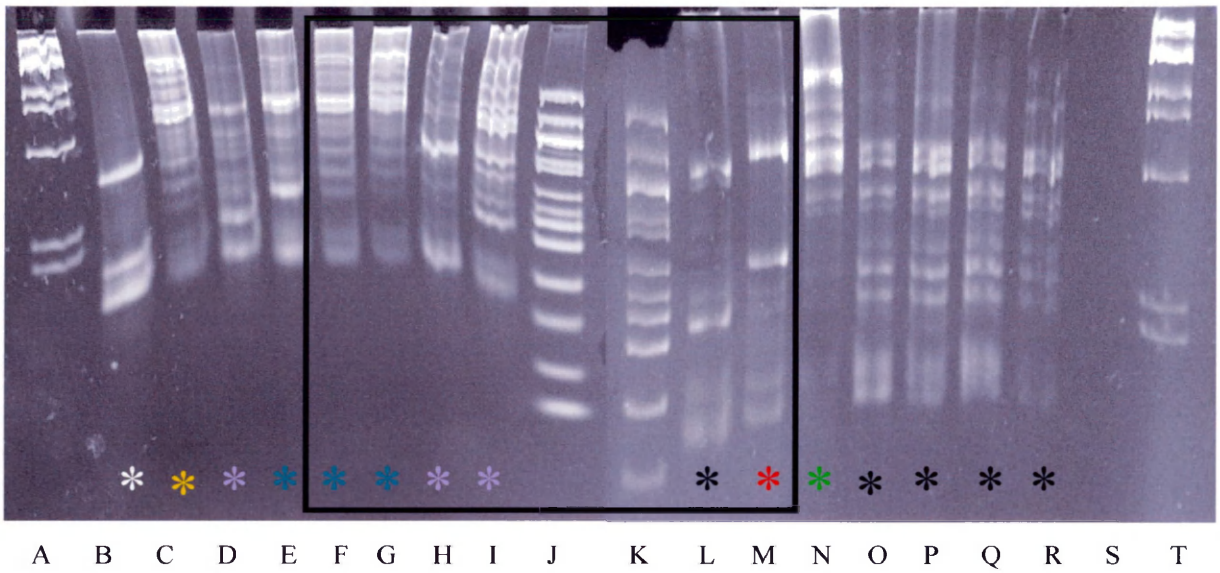


Figure 1

BOX-A1R PCR analysis of a subset of *B. licheniformis* isolates from Northern Saw-whet Owls. Each isolate was isolated via the selective and differential media developed during the current study (see Methods) and identified as *B. licheniformis* via sequencing of a 900 bp section of the16s *rrnA* gene. The BOXA1R- PCR was run on a 5% polyacrylamide gel. The box that encloses the 7 lanes F,G, H, I, L, and M indicates that those lanes are isolates from the same bird and all of the other isolates are each from different birds. A. 1 kB DNA ladder B. 89080-39 C. 89083-31 D. 89084-44 E. 89088-2 F. 89088-5 G. 89088-8 H. 89088-10 I. 89088-11 J. 100 bp DNA ladder K. 100 bp ladder L. 89088-14 M. 89088-15. N. 89091-35 O. 54859-27 P. 25475-30 Q. 57529 R. 57519-33 S. Blank T. 100 bp DNA ladder.

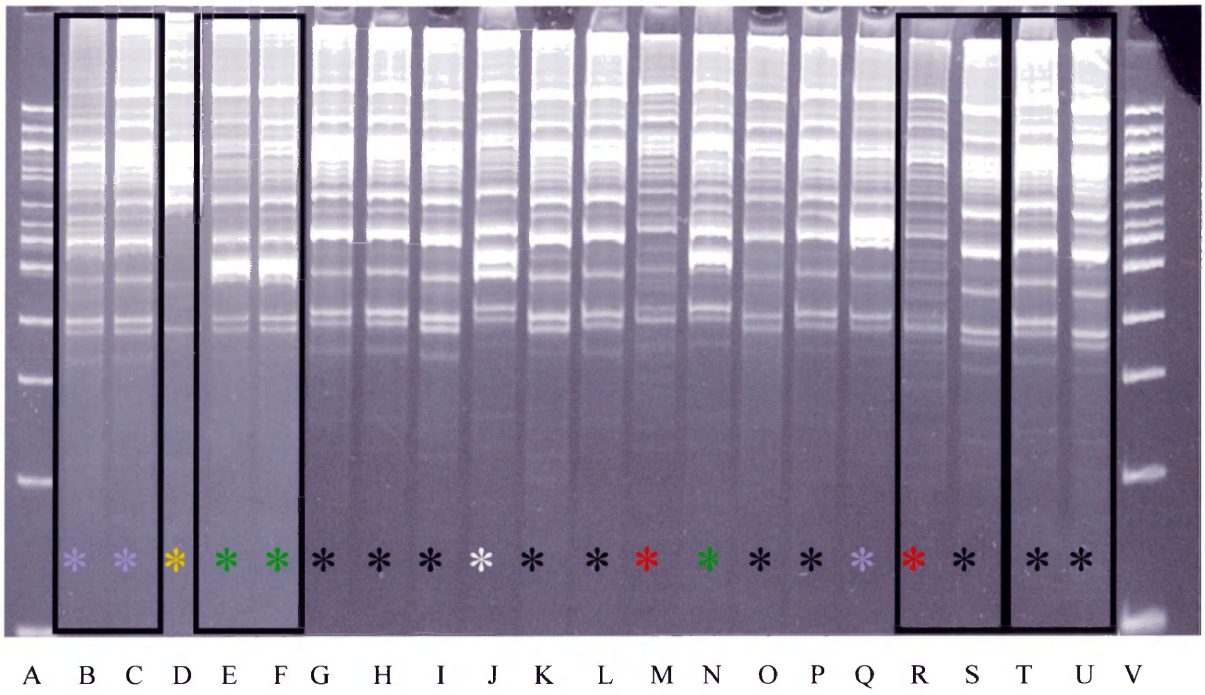


Figure 2

BOX-A1R PCR analysis of a subset of *B. licheniformis* isolates from Gray Catbirds. Each isolate was isolated through selective and differential media and identified as *B. licheniformis* via sequencing of a 900 bp section of the16s *rrnA* gene. The BOXA1R-PCR was run on a 5% polyacrylamide gel. Each colored * represents strains that are genetically distinct. Each box encloses two strains that were isolated from the same individual.

A. 100 bp ladder B. 15156-1 C. 15156-2 D. 15091 E. 15190-1 F. 15190-2 G. 15153 H. 15138 I. 12938 J. 12937 K. 12929 L. 12928 M. 12927 N. 12917 O. 12898 P. 12896 Q. 12894-1 R. 12894-2 S. 12893 T. 12878-1 U. 12878-3 V. 100 bp ladder.

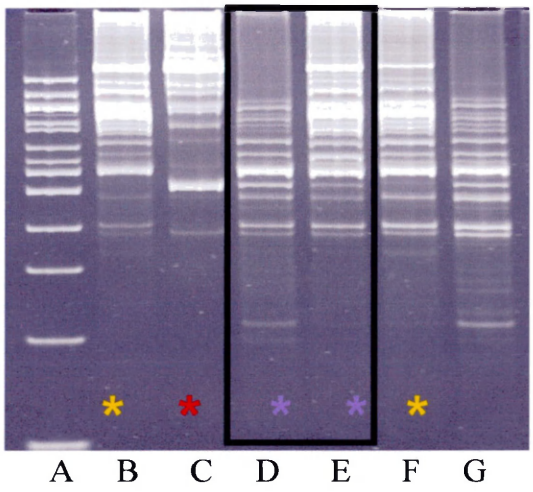


Figure 3

BOX-A1R PCR analysis of a subset of *B. licheniformis* isolates from Yellow-rumped Warblers. Each isolate was isolated through selective and differential media and identified as *B. licheniformis* via sequencing of a 900 bp portion of the16s *rrnA* gene. The BOXA1R- PCR was run on a 5% polyacrylamide gel. Each colored * represents strains that are genetically distinct. The box encloses two strains that were isolated from the same individual.

A. 100 bp ladder B. 31388 C. 31389 D. 31391-1 E. 31391-2 F. 13195 G. 31833

LITERATURE CITED

Aldrich, J.W., F.C. James. 1991. *Ecogeographic variation in the American robin (Turdus migratorius).* Auk. 108: 230-49.

Alsop, F.J. III. 2001. Smithsonian Handbooks: Birds of North America. Smithsonian Press, NY.

Anderson, R.M. and R.M. May. 1981. *The Population Dynamics of Microparasites and Their Invertebrate Hosts.* Philosophical Transactions of the Royal Society B. 291: 451-524.

Atrih, A., P. Zollner, G. Allmaier. 1996. *Structural analysis of B. subtilis 168 endospore peptidoglycan and its role during differentiation.* Journal of Bacteriology. 178: 6173-83.

Bahuguna, S., R.K.S. Kushwaha. 1989. *Hair perforation by keratinophilic fungi.* Mycoses. 32: 340-3.

Beaman, T.C., J.T. Greenamyre, T.R. Corner, H.S. Pankratz, P. Gerhardt. 1982. *Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio.* Journal of Bacteriology. 150: 870-7.

Blanco, G., J. Seoane, J. de la Puente. 1999. *Showiness, Non-Parasitic Symbionts and Nutritional Condition in a Passerine Bird.* Annual Zoological Fennici. 36: 83-91.

Blanco, G., Jose L. Tella, Jaime Potti. 2001. *Feather mites on birds: costs of parasitism or conditional outcomes?* Journal of Avian Biology. 32: 271-4.

Bockle, B., B. Galunksky, R. Muller. 1995. *Characterization of keratinolytic serine proteinase from S. pactum DSM 40530.* Applied and Environemental Microbiology. 61: 3705-10.

Bolton, B. 1992. Identification Guide to the Ant Genera of the World. Harvard U. Press, Cambridge, MA.

Bonser, R.H.C. 1995. *Melanin and the abrasion resistance of feathers.* Condor. 97: 590-1.

Breukink, E., I. Wiedermann, C. van Kraaij, O.P. Kuipers, H-G. Sahl, B. de Kruijff. Dec. 1999. *Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic.* Science. 5448: 2361-4.

Brumlik, M.J., July 2001. U. Szymajda, D. Zakowska, X. Liang, R.J. Redkar, G. Patra,

V.G. Del Vecchio. *Use of Long0Range Repetitive Element Polymorphism-PCR to Differentiate Bacillus anthracis Strains.* Applied and Environmental Microbiology. 67: 3021-28.

Burtt, E.H. Jr. 1999. *Rules to Bird by: Gloger's and Allen's.* Birding. 31: 362-5.

Burtt, E.H. Jr., J.M. Ichida. 1999. *Occurrence of Feather-Degrading Bacilli in the Plumage of Birds.* The Auk. 116: 364-72.

Cherel, Y., J-B. Charrassin, E. Challet. 1994. *Energy and protein requirements for molt in the king penguin Aptenodytes patagonicus.* American Journal of Physiology. 266: R1182-8.

Cheng, S-W., H-M. Hu, S-W. Shen, H. Takagi, M. Asano, Y-C. Tsai. 1995. *Production and Characterization of Keratinase of a Feather-degrading Bacillus licheniformis PWD-1.* Bioscience, Biotechnology, Biochemistry. 59: 2239-43.

Chitte, R.R., V.K. Nalawade, S. Dey. 1999. *Keratinolytic Activity from the Broth of a Feather-Degrading Thermophilic Streptomyces thermoviolaceus Strain SD8.* Letters in Applied Microbiology. 28: 131-6.

Clayton, D.H. 1991. *The Influence of Parasites on Host Sexual Selection.* Parasitology. 7: 329-34.

Clayton, D. H. 1999. *Feather-Busting Bacteria.* The Auk. 116: 302-4.

Clayton, D.H., D.M. Tompkins. 1995. *Comparative Effects of Mites and Lice on the Reproductive Success of Rock Doves (Columa livia).* Parasitology. 110: 195-206.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences. 2nd Ed. Lawrence Erlbaum Associate Press; NJ.

Corning, W.R., A.A. Biewener. 1998. *In Vivo Strains in Pigeons flight Feather Shafts: Implications for Structural Design.* The Journal of Experimental Biology. 201: 3057-65.

Daubersies, P., S. Sallenave-Sales, S. Magne, J.F. Trape, H. Contamin, T. Fandeur, C. Roger, O. Mercereau-Puijalon, P. Druilhe. 1996. *Rapid Turnover of Plasmodium falciparum Populations in Asymptomatic Individuals Living in a High Transmission Area.* American Journal of Tropical Medicine. 54:18.

Davidson, W., E.B. Shotts, J. Teska, D.W. Moreland. 1989. *Feather Damage Due to Mycotic Infections in Wild Turkeys.* Journal of Wildife Disease. 25: 534-9.

Driks, A. 2001. *Proteins of the spore core and coat.* In: Sonenshein, A.L., J.A. Hock, R. Losick. (eds) B. subtilis and its Closest Relatives: From Genes to Cells.

Washington D.C., American Society for Microbiology. 537-48.

Duncan, K., N. Ferguson, K. Kimura. 1994. *Fine-Scale Genetic and Phenotypic Structure in Natural Populations of Bacillus subtilis and Bacillus licheniformis: Implications for Bacterial Evolution and Speciation.* Evolution. 48: 2002-25.

Dunny, G.M. and S.C. Winans. 1999. *Cell-cell Signaling in Bacteria.* American Society for Microbiology. Washington D.C.

Erlich, P.R., D.S. Dobkin, D. Wheye. 1986. *The Adaptive Significance of Anting.* The Auk: 103: 835.

Evans, K.L., J. Crowder, E. S. Miller. 2000. *Subtilisins of Bacillus spp. Hydrolyze Keratin and Allow Gowth on Feathers.* Canadian Journal of Microbiology. 46: 1004-11.

Ewald, P.W. 1983. *Host-parasite Relations, Vectors, and the Evolution of Disease Severity.* Annual Review of Ecology and Systematics. 14: 465-85.

Ferrero, M.A., G.R. Castro, C.M. Abate, M.D. Baigori, F. Sineriz. 1996. *Thermostable Alkaline Proteases of Bacillus licheniformis MIR 29: Isolation, Production and Characterization.* Applied Microbiology and Biotechnology. 45: 327-32.

Foster, S.J., K. Johnstone. March 1989. *The trigger mechanism of bacterial spore germination.* Regulation of Prokaryotic Development, Structural and Functional Analysis of Bacterial Sporulation and Germination. Pp. 89-108.

Fuqua, W.C, S.C. Winans, E.P. Greenberg. 1994. *Quorum Sensing in Bacteria: The LuxR-LuxI Family of Cell Density-Responsive Transcriptional Regulators.* Journal of Bacteriology. 176: 269-75.

Gerhardt, P., R.E. Marquis. Mar. 1988. *Spore thermoresistance mechanisms.* Regulation of Procaryotic Development. Structural and Functional Analysis of Bacterial Sporulation and Germination. Pp. 43-63.

Gloger, C.L. 1833 *Das Abandern der Vogel Durch.* Eingluss des Klines. Breslau

Gonzalez-Pastor, Jose E., Errett C. Hobbs, Richard Losick. 25 July 2003. *Cannibalism by Sporulating Bacteria.* Science. 301: 510-13.

Grimaldi, D., D. Agosti. 2000. *A Formicine in New Jersey Cretaceous Amber (Hymenoptera: Formicidae) and Early Evolution of the Ants.* Proceedings of the National Academy of Science. 97: 13678-83.

Hamilton, W.D., M. Zuk. 1982. *Heritable true fitness and bright birds: role for parasites?* Science. 218: 384-87.

Harper, J.R., T.J. Silhavy. 2001. *Germ Warfare: The Mechanisms of Virulence Factor Delivery.* Principles of Bacterial Pathogenesis. Academic Press; NY. pgs.43-61.

Hart, B.L. *Behavioral defense* - In: Clayton, D.H. and Moore, J. (eds). *Host-Parasite Evolution.* General Principles of Avian Models. Oxford U. Press, Oxford. Pp. 59-77.

Hefetz, A., M.S. Blum. 1978. *Biosynthesis of formic acid by the poison glands of formicine ants.* Biochim. Biophys. Acta. 1; 543: 484-96.

Heineken, F.G., and R.J. O'Conner. 1972. *Continuous Culture Studies on the Biosynthesis of Alkaline Protease, Neutral Protease and χ-amylase by Bacillus subtilis NRRL-B3411.* Journal of Genetics and Microbiology. 73: 35-44.

Herman, L., M. Heyndrickx. 2000. *The Presence of Intragenically Located REP-like Elements in Bacillus sporothermodurans is Sufficient for REP-PCR Typing.* Research in Microbiology. 151: 255-61.

Hill, G.E. 1999. *Is There an Immunological Cost to Carotenoid-based Ornamental Coloration?* The American Naturalist. 154: 589-95.

Holldobler, B., E.O. Wilson. 1990. The Ants. Belknap, Cambridge, MA.

Hood, C.M. and M.G. Healy. 1994 *Bioconversion of waste keratins: wool and feathers.* Resources, Conservation and Recycling. 11: 179-88.

Hubalek, Z. 1976. *Interspecific Affinity Among Keratinolytic Fungi Associated with Birds.* Folia Parasitology. 23: 267-72.

Ichida, J.M., L. Krizova, C.A. LeFevre, H.M. Keener, D.L. Elwell, E.H. Burtt, Jr. . *Bacterial Inoculum Enhances Keratin Degradation and Biofilm Formation in Poultry Compost.* Journal of Microbiological Methods. 47: 199-208.

Jacobs, M., M. Eliasson, M. Uhlen, J-I. Flock. 1985. *Cloning, Sequencing and Expression of Subtilisin Carlsberg from Bacillus licheniformis.* Nucleic Acid Research. 13: 8913-27.

James, E.C. 1970. *Geographic Size Variation of Birds and its Relationship to Climate.* Ecology. 51: 365-90.

Johnston, R. 1981. *Seasonal Variation in Plumage Color in House Sparrows.* Journal of Field Ornithology. 52: 127-33.

Kao, M-M., H-Y. Lai. 1995. *The study of the selection of feather-degrading microorganisms.* Journal of the Chinese Institute of Environmental Engineering. 5: 37-43.

Keyser, A.J., G.E. Hill. 2000. *Structurally Based Plumage Coloration is an Honest Signal of Quality in Male Blue Grosbeaks.* Behavioral Ecology. 11: 202-9.

Kim, J.M., W.J. Lim, H.J. Suh. *Feather-degrading Bacillus species from Poultry Waste.* Processes in Biochemistry. 37: 287-91.

Kitadokoro, K., H. Tsuzuki, E. Nakamura, T. Sato, H. Teraoka. 1994. *Purification, characterization, primary structure, crystallization and preliminary crystallographic study of a serine proteinase from Streptomyces fradiae ATCC 14544.* European Journal of Biochemistry. 220: 55-61.

Kose, M., A.P. Moller. 1999. *Sexual Selection, Feather Breakage and Parasites: the Importance of White Spots in the Tail of the Barn Swallow (Hirundo rustica)* Behavioral Ecology and Sociobiology. 45: 430-6.

Lebbadi, M., A. Galvez, E. Valdivia, M. Martinez-Bueno, M. Maqueda. 1994. *Biological Activity of Amoebicin m4-A from Bacillus liceniformis M-4.* Anitmicrobial Agents and Chemotherapy. 38: 1820-3.

Lin, X., C-G. Lee, E.S. Casale, J.C.H. Shih. 1992. *Purification and Characterization of a Keratinase from a Feather-Degrading Bacillus licheniformis Strain.* Applied and Environmental Microbiology. 58: 3271-5.

Lin, X., D.W. Lelemen, E.S. Miller, J.C. H. Shih. 1995. *Nucleotide Sequence and Expression of kerA, the Gene Encoding a Keratinolytic Protease of Bacillus licheniformis PWD-1.* Applied and Environmental Microbiology. 61: 1469-74.

Lin, X, G.D. Inglis, L.J. Yanke, K-J Cheng. 1999. *Selection and Characterization of Feather-Degrading Bacteria from Canola Meal Compost.* Journal of Industrial Microbiology and Biotechnology. 23: 149-53.

Lindstrom, A., G. H. Visser, S. Daan. 1993. *The Energetic Cost of Feather Synthesis is Proportional to Basal Metabolic Rate.* Physiological Zoology. 66: 490-10.

Linstrom, K., and J. Lundstrom. 2000. *Male Greenfinches (Carduelis chloris) with Brighter Ornaments Have Higher Virus Infection Clearance Rate.* Behavioral Ecology and Sociobiology. 48: 44-51.

Lord, C.C., B. Barnard, K. Day, J.W. Hargrove, J.J. McNamara, R.E. Paul, K. Trenhome, M.E. Woolhouse. 1999. *Aggrgation and Distribution of Strains in Mircrparasites.* Transactions of the Royal Society of London. 354: 799.

Makino, S., R. Mriyama. 2002. *Hydrolysis of Cortex Peptidoglycan During Bacterial Spore Germination.* Medical Science Monit. 8: RA119-27.

McGraw, K.J., A.M. Stoehr, P.M. Nolan and G.E. Hill. 2001. *Plumage Redness Predicts Breeding Onset and Reproductive Success in the House Finch: a Validation of Darwin's Theory.* Journal of Avian Biology. 32: 90-4.

Mercereau-Puijalon, O. 1996. *Revisiting Host/Parasite Interactions: Molecular analysis of Parasites Collected During Longitudinal and Cross-sectional Surveys in Humans.* Parasite Immunology. 18: 173.

Murphy, M.E. and J.R. King. 1992. *Energy and Nutrient Use During Moult by White-Crowned Sparrows Zonotichia leucophrys gambelii.* Ornis. Scandanavia. 23: 304-13.

Murphy, M.E., J.R. King, and T.G. Taruscio. 1990. *Amino Acid Composition of Feather Barbs and Rachises in Three Species of Pygoscelid Penjuins: Nutritional Implicaitons.* Condor. 92: 913-21.

Muza, M.M., E.H. Burtt, Jr., J.M. Ichida. 2000. *Distribution of Bacteria on Feathers of Some Eastern North American Birds.* Wilson Bulletin. 112: 432-5.

Nicholson, W.L., N. Munakata, G. Horneck, H.J. Melosh, P. Setlow. 2000. *Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments.* Microbiology and Molecular Biology Reviews. 64: 548-72.

Noval, J.J., and W.J. Nickerson. 1959. *Decomposition of naitve keratin by S. fradiae.* Journal of Bacteriology. 77: 251-63.

Onifade, A. A., N.A. Al-Sane, A.A. Al-Musallam, S. Al-Zarban. 1998. *A Review: Potentials for Biotechnological Applications of Keratin-degrading Microorganisms and Their Enzymes For Nutritional Improvement of Feathers and Other Keratins As Livestock Feed Resources.* Bioresource Technology. 66:1-11.

Pauling, L., R.B. Corey. 1951. *The Structure of Feather Rachis Keratin.* Proceedings of the National Academy of Science, USA. 37: 256-61.

Payne, R.B. 1972. *Mechanics and Control of Moult. In* Avian Biology. Vol. 2. D.S. Farner and J.R. King. Eds. Academic Press, New York. Pp. 103-55.

Popham, D.L., J. Helin, C.E. Costello, P. Setlow. 1996. *Muramic lactam in peptidoglycan of Bacillus subtilis spores is required for spore outgrowth but not for spore dehydration or heat resistance.* Proceedings of the National Academy of Science of the United States of America. 93: 15405-10.

Pugh, G.J.F. 1964. *Dispersal of Arthroderma curreyi by birds and its role in the soil.* Sabouraudia. 3: 275-8.

Pugh, G.J.F. 1965. *Cellulolytic and keratinophilic fungi recorded on birds.*

Sabouraudia. 4: 85-91.

Rajak, R.C., H. Malviya, H. Deshpande, S.K. Hasija. 1991. *Production and comparative characterization of keratinase from Absidia cylindrospora and Rhizomucor pusillus.* Indian Journal of Microbiology. 31: 243-50.

Read, A.F and L.H. Taylor. 2000. Molecular Epidemiology of Infectious Diseases. R.C.A. Thompson, ed. Arnold; London: pp. 59-75.

Read, A.F and L.H. Taylor. 11 May 2001. *The Ecology of GeneticallyDiverse Infections.* Science. 292: 1099-102.

Read, A.F., M.J. Mackinnon, M.A. Anwar, L.H. Taylor. 2002. Virulence Management: The Adaptive Dynamics of Pathogen-Host Interactions. U. Dieckmann, J.A.J. Metz, M.W. Sabelis, K. Sigmund, Eds. Cambridge University Press; Cambridge.

Rintoul, L, E.A. Carter, S.D. Stewart, P.M. Fredericks. 2000. *Keratin Orientation in Wool and Feathers by Polarized Raman Spectroscopy.* Biopolymers(Biospectoscopy). 57: 19-28.

Rogers, D. I. 1990. *Use of Feather Abrasion in Moult Studies.* Corella: Journal of the Australian Bird Study Association. 14: 141-7.

Rossini, C., A.B. Attygalle, A. Gonzalez, S.R. Smedley, M. Eisner, J. Meinwald, T. Eisner. June 1997. *Defensive production of formic acid (80%) by a carabid beetle (Galarita lecontei).* Proceedings of the National Academy of Sciences. 94: 6792-7.

Safranek, W.W., R.D. Goos. 1982. *Degradation of Wool by Saprotrophic Fungi.* Canadian Journal of Microbiology. 28: 137-40.

Sangali, S., A. Brandelli. 2000. *Isolation and Characterization of a Novel Feather-Degrading Bacterial Strain.* Applied Biochemistry and Biotechnology. 87: 17-32.

Schmidt, B.F., L. Woodhouse, R.M. Adams, T. Ward, S.E. Mainzer, P.J. Lad. 1995. *Alkalophilic Bacillus sp. Strain LG12 Has a Series of Serine Protease Genes.* Applied and Environmental Microbiology. 61: 4490-93.

Schneider, K.B., T.M. Palmer, and A.D. Grossman. 2002. *Characterization of comQ and comX, Two Genes Required for Production of ComX Pheromone in Bacillus subtilis.* Journal of Bacteriology. 184: 410-19.

Shresta, S., J.R. Smyth, Jr., G.F. Erf. 1997. *Profiles of Pulp Infiltrating Lymphocytes at Variaous Times Throughout Feather Regeneration in Smyth Line Chickens with Vitiligo.* Autoimmunity. 25: 193-201.

Singh, C.J. and B.G. Singh. 1995. *Characterization of Extracellular Proteolytic Enzymes of C. tropicum CF 34 and its Role in Keratin Degradation.* Indian Journal of Microbiology. 35: 311-14.

Sinha, U., S.A. Wolz, P.J. Lad. 1991. *Two new extracellular serine proteases from Streptomyces fradiae.* International Journal of Biochemistry. 23: 979-84.

Smith, T., I. Felger , M. Tanner, H.P. Beck. 1999. *Premunition in Plasmodium faciparum infection: insights from the epidemiology of multiple infections.* Transceedings of the Royal Society for Tropical Medicine and Hygiene. 93: 59-64.

Snounou, G., W. Jarra, S. Viriyakodol, J.C. Wood, K.N. Brown. 1996. *Use of a DNA Probe to Analyse the Dynamics of Infection with Rodent Malaria parasites Confirms that Parasite Clearance During Crisis is Predominantly Strain- and Species-Specific.* Annals Tropical Medicine. 54: 18.

Sonenshein, A.L. 2000. *Endospore-Forming Bacteria: An Overview.* Prokaryotic Development: Chapter 6. American Society for Microbiology; D.C.: pgs. 133-46.

Stein, T., S. Borchert, P. Kiesau, S. Heinzmann, S. Klein, M. Helfrich, K. Entian. Apr. 2002. *Dual control of subtilin biosythesis and immunity in Bacillus subtilis.* Molecular Microbiology. 44: 403-16.

Suh, H.J., H.K. Lee. February 2001 *Characterization of a keratinolytic serine protease from Bacillus subtilis KS-1.* Journal of Protein Chemistry. 20: 165-9.

Swaddle, J.P., M.S. Witter. 1997. *The Effects of Molt on the Flight Performance, Body Mass, and Behavior of European Starlings (Sturnis vulgaris): An Experimental Approach.* Canadian Journal of Zoology. 75: 1135-46.

Szabo, I. , A. Benedek, I. Mihaly Szabo, Gy. Barabas. 2000. *Feather Degrading with a Thermotolerant Streptomyces gramificaciens strain.* World Journal of Microbiology and Biotechnology. 16: 253-5.

Tackiouchi, I., D. Higuchi, Y. Sei, M. Koga. 1982. *Isolation of an Extracellular Protease Keratinase from Microsporum canis.* Sabouradia. 20: 281-3.

Taylor, L.H., D. Walliker, A.F. Read. 1997. *Mixed-genotype Infections of Malaria Parasites: Within-host Dynamics and Transmission Success of Competing Clones.* Proceedings of the Royal Society, London. 264: 927.

Thompson, C., N. Hillgarth, M. Leu, H.E. McClure. *High Parasite Load in House Finches (Carpodacus mexicanus) is Correlated with Reduced Expression of a Secually Selected Trait.* The American Naturalist. 149: 270-94.

Thompson, R.C.A., Ed. 2000. Molecular Epidemiology of Infectious Diseases. Arnold; London.

Tompkins, D.M., T. Jones and D.H. Clayton. *Effect of Vertically Transmitted Ectoparasites on the Reproductive Success of Swifts (Apus apus).* Functional Ecology. 10: 733-40.

Tucker, V.A. 1991. *The Effect of Moulting on the Gliding Performance of a Harris' Hawk, Parabuteo unicinctus.* Auk. 108: 108-13.

Van Belkum, A., M. Sluijter, R. de Groot, H. Verbrugh, and P. W. M. Hermans. 1996. *Novel BOX repeat PCR assay for high-resolution typing of Streptococcus pneumoniae* strains. Journal of Clinical Microbiology. 34: 1176-9.

Van de Wetering, D., F. Cooke. 2000. *Body Weight and Feather Growth of Male Barrow's Goldeneye During Wing Molt.* The Condor. 102: 228-31.

Verhulst, S., S.J. Dieleman, H.K. Parmentier. April 1999. *A Tradeoff Between Immunocompetence and Sexual Ornamentation in Domestic Fowl.* Proceedings of the National Academy of Science, USA. 96: 4478-81.

Vidal, L, P. Christen, M.N. Coello. 2000. *Feather Degradation by Kocuria rosea in Submerged culture.* World Journal of Microbiology. 16: 551-4.

Wang, J-J., J.C.H. Shih. 1999. *Fementation Production of Keratinase from Bacillus licheniformis PWD-1 and a Recombinant B. subtilis FDB-29.* Journal of Industrial Mircrobiology and Biotechnology. 22: 608-16.

Ward, J.M., J.D. Blount, G.D. Ruxton, D.C. Houston. 2002. *The Adaptive Significance of Dark Plumage for Birds in Desert Environments.* Ardea. 90: 311-23.

Wenny, D. 1998. *Three-striped Warbler (Baileuterus tristriatus) "Anting" with a Caterpillar.* Wilson Bulletins. 110: 128-31.

Williams, C.M., C.S. Richter, J.M. MacKenzie, Jr., Jason C.H. Shih. 1990. *Isolation, Identification, and Characterization of a Feather-Degrading Bacterium.* Applied and Environmental Microbiology. 56: 1509-15.

Zink, R.M. and J.V. Remsen Jr. 1986. *Evolutionary Processes and Patterns of Geographic Variation in Birds.* Current Ornithology. 4: 1-69.

VITA

Justine Whitaker was born June 19, 1978 in Virginia Beach, Virginia. She obtained her Bachelor of Science degree from Longwood University in Farmville, Virginia in May of 2000. As a senior project, she developed an amphibian monitoring program in Farmville to monitor population levels of aquatic and terrestrial salamanders, as well as frogs. On August 18, 2003, Justine began working for the University of Maryland Center for Environmental Science/Chesapeake Biological Laboratory in invertebrate immunology.