Inter-annual and body topographic consistency in the plumage bacterial load of Great Tits

Pauli Saag[1, 3], Priit Kilgas[1, 2], Marko Mägi[1], Vallo Tilgar[1], and Raivo Mänd[1]

[1]*Department of Zoology, Institute of Ecology and Earth Sciences, University of Tartu, 46 Vanemuise Street, Tartu, 51014, Estonia*

[2]*Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa*

[3]Corresponding author. Email: pauli.saag@ut.ee

ABSTRACT. Plumage bacteria may play an important role in shaping the life histories of birds. However, to design suitable experiments to examine causal relationships between plumage bacteria and the fitness of host birds, natural variation in plumage bacterial communities needs to be better understood. We examined within-individual consistency of plumage bacterial contamination in Great Tits (*Parus major*), comparing different body regions (ventral vs. dorsal) and comparing contamination between years. Numbers of free-living and attached bacteria and the species richness of feather-degrading bacterial assemblages were studied using flow cytometry and ribosomal intergenic spacer analysis (RISA). Numbers of both types of bacteria were higher on dorsal than on ventral feathers. Numbers of free-living, but not attached, bacteria on the two body regions were highly positively correlated. There was also a strong within-individual correlation between numbers of attached bacteria during the same breeding stages in different years. These results suggest that, despite variation in absolute levels of feather bacterial loads between years and different body regions, sampling individual birds can provide reliable estimates of relative levels of bacterial contamination, as long as sampling time and body region are carefully standardized.

Key words: bacterial communities, cavity breeders, feather-degrading bacteria, microbial influence

Bird plumage is usually colonized by a diverse community of bacteria (e.g., Burtt and Ichida 1999, Bisson et al. 2007, Gunderson 2008, Gunderson et al. 2008), and feather bacteria play an important role in shaping the behavior and life histories of birds (Clayton and Moore 1997, Burtt and Ichida 1999, Muza et al. 2000, Gunderson et al. 2009). Several species of bacteria degrade feathers, potentially reducing thermal insulation (Brush 1965), aerodynamic (Swaddle et al. 1996), or coloration-based communication (Gunderson et al. 2009, Shawkey et al. 2009b) properties of the plumage. In addition, feather bacteria may produce antimicrobial substances and play a role in protecting eggs from being infected by pathogenic bacteria (Gunderson et al. 2008, Shawkey et al. 2009a, Martin-Vivaldi et al. 2010, Peralta-Sanchez et al. 2010). However, some feather-inhabiting bacteria may also be pathogens (Bruce and Drysdale 1994).

Studies of bacterial loads and diversity, and the influence of bacteria on their hosts have been limited by the complex methods required. New opportunities have recently emerged due to the development of molecular and microbiological techniques (Burtt 2009). However, basic information about plumage bacteria is still largely missing. For example, few investigators have examined within-individual temporal variation in plumage bacterial communities (Giraudeau et al. 2010, Saag et al. 2011). Even less is known about variation in plumage bacterial communities in different body regions of birds (Burtt and Ichida 1999, Muza et al. 2000, Bisson et al. 2007). Without such information, investigators cannot be confident that sampling feathers provides reliable estimates of the relative levels of bacterial contamination of different individuals. Thus, our objective was to examine within-individual consistency of plumage bacterial communities of Great Tits (*Parus major*), comparing feathers in different areas and comparing communities over two consecutive years.

METHODS

Our study was conducted near Kilingi-Nõmme (58° 7'N, 25° 5'E) in southwest Estonia during the breeding seasons (late April - June) of 2007 and 2008. The study area covered ~50 km^2 and contained a mosaic of coniferous and deciduous forest. Great Tits nested in wooden nest boxes (11 × 11 × 30 cm). Distances between neighboring nest boxes were 50 to 60 m. Nest boxes were cleaned to remove old nest material before the beginning of each breeding season.

To collect data on the load of attached and free-living types of feather bacteria and feather-degrading bacterial phylotypic richness in the plumage of individual birds, adult tits were captured at their nests in automatic traps during day 7 or 8 of the nestling period of first broods (19-31 May). Sex was determined by the presence or absence of a brood patch. We trapped 34 males and 127 females in 2007, and 84 males and 153 females in 2008. Most of these individuals were also used for parallel studies (Saag et al. 2011). However, we used six females captured in both years to explore inter-annual consistency, and 18 adults (9 males and 9 females, including 8 pairs) captured in 2008 were used for studying body topographic consistency in plumage bacterial loads**.** All captured individuals were banded.

A fresh pair of examination gloves was used each time a new bird was handled. Within 30 sec after capture, ~5 yellow ventral feathers were plucked from the right side of each bird's chest using forceps cleaned in 96% ethanol and placed in dry clean disposable microtubes (feathers from each body region were placed in different tubes) for assessing the number of bacteria on feathers. In 2008, but not in 2007, the same number of green dorsal feathers (from the center of the back) was also collected from 18 adults and handled in the same manner. In both years, another sample of five feathers (from the same body regions) was collected from each bird to determine the species richness of feather-degrading bacterial assemblages.

Microtubes were immediately stored at 4°C and transported to the laboratory within a few days (1-7 days), where they were stored at -80°C prior to analysis. In the lab, 1 mL of phosphate buffered saline (PBS) solution pH 7.2 was added to each tube, which was then vortexed for 1 min. Free-living bacteria were thus washed out from the feathers and collected in the PBS solution. To remove attached bacteria, feathers were sonicated for 10 min in 1 mL of a detergent solution containing 2.5% polyethylene glycol 6000 and 0.1% sodium deoxycholate (Lucas et al. 2003). Free-living and attached bacteria samples were stored at -80° C and subsequently counted separately. Direct counts were performed for free-living and attached bacteria using a flow cytometry machine (BD LSR II, BD Biosciences, Franklin Lakes, NJ) calibrated to detect only bacterium-sized tagged particles. For tagging, DNA-binding dye SYBR Green (Bio-Rad, Hercules, CA) was used. The number of feathers in each sample was recorded to calculate bacterial number per feather (Lucas et al. 2005). Free-living bacteria were only counted in 2007 because many samples from 2008 were lost due to human error.

To determine species richness of feather-degrading bacteria, sampled feathers were covered with buffer solution (1.5 mL of PBS). Feather-degrading bacterial assemblages were enriched in this buffer by incubating them for 30 days at 26°C in the dark without using a shaker (following Lucas et al. 2005). Because feathers were the only source of carbon in the enrichment media, only bacteria capable of degrading keratin were promoted. Certain non-keratin-degrading species may have occurred at extremely high densities before enrichment of samples and their DNA could have been detected by the analysis even after the enrichment procedures. However, given the low mean number of phylotypes per bird (Saag et al. 2011), the probability that such species rather than feather-degrading species were detected in later analyses is low. The ribosomal intergenic spacer analysis (RISA) method was used to analyze the structure of feather-degrading assemblages obtained in the enrichment cultures (Ranjard

et al. 2000b). Each RISA band is assumed to correspond to one bacterial species and will be referred to as a phylotype (Muyzer et al. 1993, Stach et al. 2003). Thus, the number of bands corresponds to bacterial assemblage richness (Ranjard et al. 2000a).

To carry out the RISA, DNA was extracted from bacterial cultures with 200 µL extraction buffer (10 mM Tris HCl, 1% SDS, 2 mM EDTA, 400 mM NaCl, and 0.4 mg/mL proteinase K). After 5 hrs incubation at 37°C, 150 µL of 5 M NaCl were added and samples were vortexed. DNA was purified two times with pure chloroform. DNA was precipitated in absolute alcohol and re-suspended in 100 µL of TE buffer pH 8.0 (10 mM Tris HCl, 1 mM EDTA).

The 16S−23S rRNA intergenic spacer was amplified using the primers S-D-Bact-1522-b-S-20 and L-D-Bact-132-a-A-18 (Ranjard et al. 2000a). Polymerase chain reaction (PCR) was conducted in 10 µl with ~10 ng of crude DNA, 1 × Taq buffer (Fermentas International, Glen Burnie, MD), 2.5 mM $MgCl_2$, 200 µM dNTPs, 5 µM of each primer and 0.5 U (units) of Taq polymerase (Fermentas International). Amplification was performed as follows: 94°C for 3 min, 35 cycles at 94°C for 1 min, 55°C for 30 sec and 72°C for 1 min, and finally 72°C for 5 min (Ranjard et al. 2000a). Amplified products were separated using electrophoresis on a 3% agarose gel for 1 hr at 140 V. Band profiles were photographed and aligned by eye (Adobe Photoshop, Adobe Systems, San Jose, CA). Bacterial richness was estimated for each sample as the total number of phylotypes.

Statistical analysis was performed using Statistica 8.0 (Statsoft, Tulsa, OK). Bacterial numbers were ln-transformed prior to analysis to satisfy the assumption of normality. The Pearson correlation coefficient *r* was used to describe within-individual correlation of bacterial variables between ventral and dorsal body regions and between years. Because there were eight breeding pairs among the 18 adults used for the correlation analysis between body regions, we also controlled each result for a possible effect of being paired. For this we used

GLM models with the corresponding bacterial variable in one body region as a dependent variable, nest as a random factor and bacterial variable in other body region as a covariate. Paired *t*-tests were used to compare bacterial numbers and richness between body regions (ventral or dorsal) and between years. The possible effects of adult sex and nesting habitat type on these results were also tested by re-analyzing the same data using repeated-measures GLM, with sex and body region included in the model as repeated measures and habitat type and its interaction with body region as factors. All tests were two-tailed.

RESULTS

Numbers of free-living, but not attached, bacteria on dorsal and ventral feathers of Great Tits were positively correlated, and the positive correlation in the richness of feather-degrading bacterial communities between these areas was marginally non-significant ($P = 0.07$; Table 1, Fig. 1). Similar results were obtained after controlling for the effect of adults being paired (number of attached bacteria, $P = 0.9$; number of free-living bacteria, $P = 0.018$; phylotypic richness of feather-degrading bacteria, $P = 0.6$; the effect of being paired on all bacterial variables (numbers of attached and free-living bacteria and phylotypic richness) was not significant, all $P > 0.05$).

Numbers of both attached and free-living bacteria were significantly higher on dorsal than ventral feathers, but the richness of feather-degrading bacterial communities on dorsal and ventral feathers did not differ (Table 1). Similar results were obtained after controlling for the effect of sex and habitat type (density of attached bacteria, $P = 0.013$; density of free-living bacteria, $P = 0.005$; phylotypic richness of feather-degrading bacteria, $P = 0.3$; in all models, the main effect of sex and the interactive effect of habitat on all bacterial variables was not significant, all $P > 0.05$).

For six females captured in two consecutive years, we found a significant positive correlation between numbers of attached bacteria on ventral feathers between years (Fig. 2). In addition, the number of attached bacteria on feathers was generally higher in 2008 than 2007, but the difference was not significant ($t_5 = 2.2$, $P = 0.08$). The mean number of bacterial phylotypes for the same individuals was not correlated between years ($P = 0.15$).

DISCUSSION

We found a significant correlation between numbers of free-living bacteria on the ventral and dorsal feathers of Great Tits. Similarly, Gunderson et al. (2009) found a strong correlation between bacterial numbers on different body parts of the same Eastern Bluebirds (*Sialia sialis*). Such results are not surprising given that bacterial loads are known to be correlated even between members of breeding pairs (Lucas et al. 2005, Gunderson et al. 2009, Saag et al. 2011), with pair-mates likely infecting each other with bacteria via common nests and broods (Saag et al. 2011). If bacteria can pass between members of a pair, spreading from one body region to another would also be expected.

Compared with free-living bacteria, the correlation between different body regions in the number of attached bacteria in our study was not significant. Similarly, Saag et al. (2011) found that the correlation between levels of contamination of pair-mates was much weaker for attached than free-living bacteria. Thus, the propensity for spreading by attached bacteria appears to be more limited than that of free-living bacteria. Also former studies have demonstrated that free-living bacteria are overall more labile, while attachment provides a more stable environment (see Selje and Simon 2003 for references).

We also found a significant positive correlation between attached bacterial numbers of individual female Great Tits over two successive years. Studying the same population of Great Tits, Saag et al. (2011) found that numbers of attached bacteria on feathers were higher

in 2008 than 2007. These results indicate that knowing the bacterial numbers on feathers of an individual in one year does not allow accurate estimation of the absolute bacterial numbers on its feathers in the next year. Rather, it allows prediction of the rank or magnitude of bacterial load compared with other individuals because this appears to remain fairly constant between years. However, these data also indicate that bacteria loads of individuals are not the result of unpredictable contingencies, but reflect something about individual birds that remains constant over time. Unfortunately, our data do not allow to determine if this is related to individual differences in body condition (Clayton 1999), preening behavior (Walther and Clayton 2005), uropygial oil production or its composition (Martin-Vivaldi et al. 2009, Møller et al. 2009), properties of nesting areas, or some other factor. Further study is needed involving larger samples and more variables.

Numbers of feather bacteria associated with Great Tits in our study were higher on the dorsal than the ventral parts of their bodies. Significant variation in feather bacterial loads among different body regions of birds has also been reported in previous studies (Burtt and Ichida 1999, Bisson et al. 2007). However, Burtt and Ichida (1999) reported that bacterial loads in several species of birds tended to be higher on ventral than dorsal feathers. This result seems more intuitive than ours because the main source of bacteria is presumed to be the soil, and ventral feathers would come into closer contact with the ground and other contaminated substrates (Burtt and Ichida 1999). Moreover, the fact that sunlight inhibits bacterial growth (Saranathan and Burtt 2007) should also decrease dorsal bacterial densities. However, the yellow chest in Great Tits is probably used in signalling to conspecifics (Hõrak et al. 2001) and dirt accumulation can reduce their plumage coloration intensity (Surmacki and Nowakowski 2007). Therefore, Great Tits may preen feathers on their chests more than those on their backs. In addition, Burtt and Ichida (1999) examined the presence or absence of feather-degrading bacilli (mainly *Bacillus licheniformis*), which represent only a fraction

of the entire diversity of feather bacteria. In contrast, we used non-selective methods to estimate the total abundance of all types of feather-inhabiting bacteria. Our results thus suggest that the difference between ventral and dorsal body regions reported by Burtt and Ichida (1999) may not apply for all types of bacteria, and that factors other than contact with the ground may contribute to differences among body regions in the abundance of feather bacteria.

Further study is needed, involving larger samples and more species, to test the generality of our results. However, despite variation in absolute levels of feather bacterial loads between years and different body regions, our results suggest that sampling feathers can provide reliable estimates of similarities or differences among individuals in their relative levels of bacterial contamination if sampling time and body region are standardized.

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Table 1. Plumage bacterial communities found on different body regions (dorsal and ventral) of Great Tits in southwest Estonia in 2008.

Variable	Median or mean		*N*	Difference between body regions		Correlation between body regions	
	Dorsal	Ventral		*t*	*P*	*r*	*P*
Number of attached bacteria per feather[a]	2.2×10^4	1.0×10^4	18	2.2	**0.038**	0.25	0.31
Number of free-living bacteria per feather[a]	3.9×10^4	8.9×10^3	18	4.4	**<0.001**	0.63	**0.005**
Bacterial phylotypic richness[b]	1.67±1.37	1.83±1.47	18	0.5	0.64	0.44	0.07

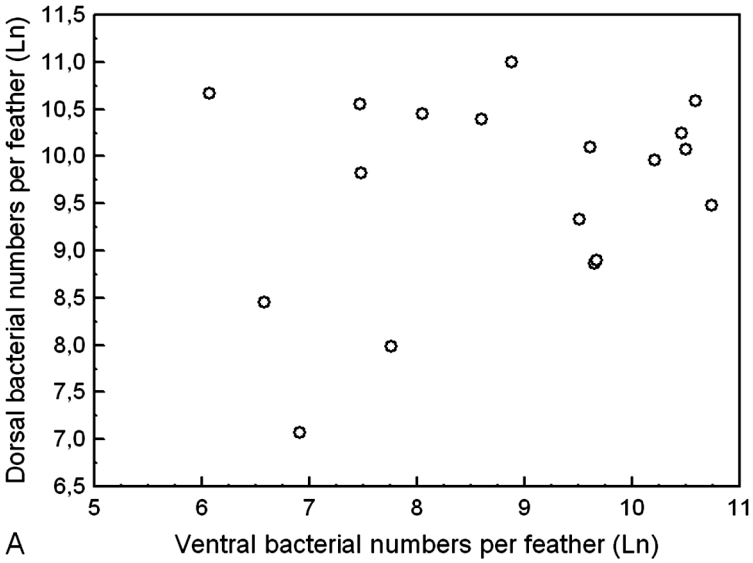
[a]Medians (bacterial cells per feather)

[b]Means ± 1 SD (bacterial phylotypes per bird)

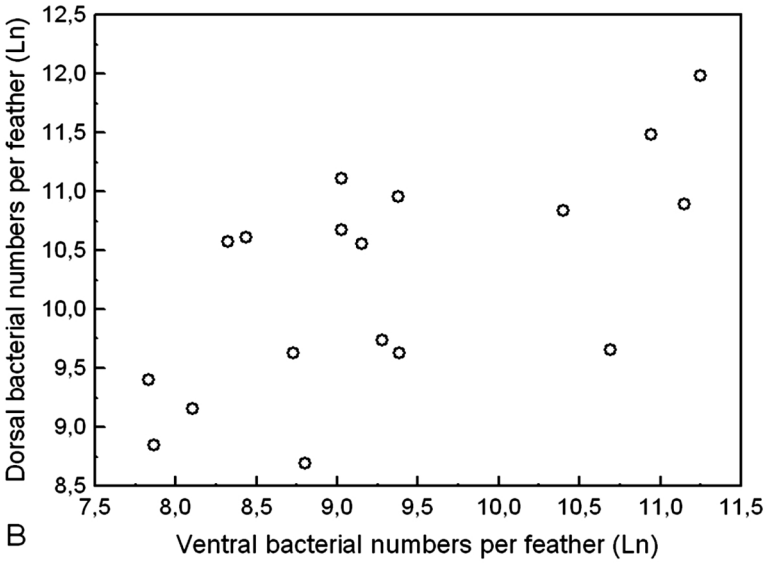
Figure legends

Fig. 1. Parameters of feather bacterial assemblages on the ventral and dorsal body regions of the same individual Great Tits (N = 18). A) numbers of attached bacteria (ln transformed), B) numbers of free-living bacteria (ln transformed), and C) number of bacterial phylotypes.

Fig. 2. Correlation between numbers of attached bacteria on the feathers of female Great Tits (N = 6) in 2007 and 2008.


Dorsal bacterial numbers per feather (Ln)
11,5
11,0
10,5
10,0
9,5
9,0
8,5
8,0
7,5
7,0
6,5
5
6
7
8
9
10
11
Ventral bacterial numbers per feather (Ln)

A


Dorsal bacterial numbers per feather (Ln)
12,5
12,0
11,5
11,0
10,5
10,0
9,5
9,0
8,5
7,5
8,0
8,5
9,0
9,5
10,0
10,5
11,0
11,5
Ventral bacterial numbers per feather (Ln)

B

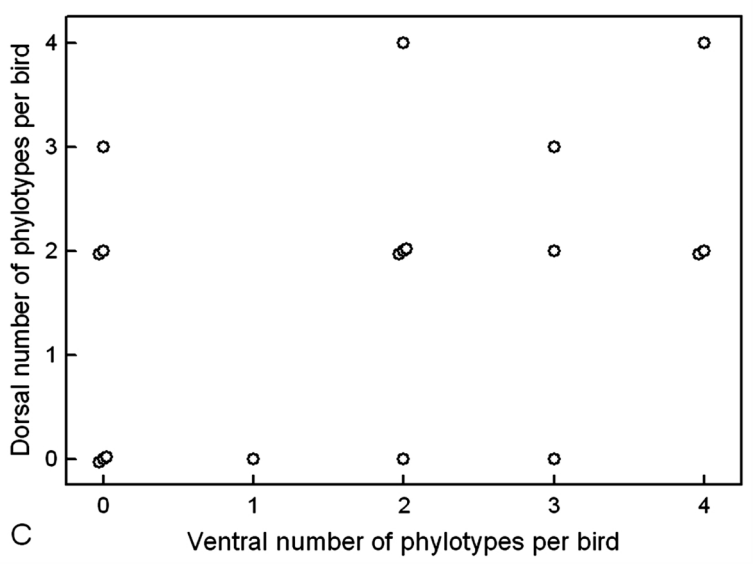

Dorsal number of phylotypes per bird
Ventral number of phylotypes per bird
0
1
2
3
4

Fig. 1.

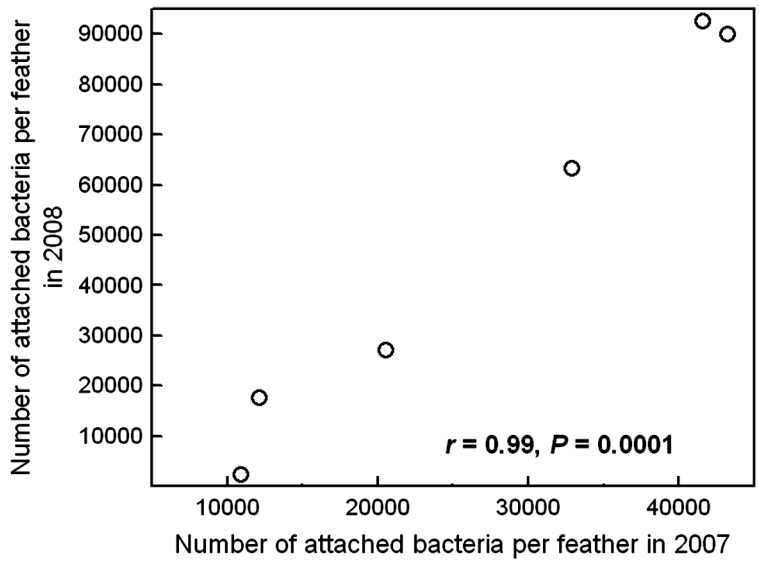

Number of attached bacteria per feather in 2008
Number of attached bacteria per feather in 2007
r = 0.99, P = 0.0001
10000
20000
30000
40000
50000
60000
70000
80000
90000

Fig. 2.