The ISME Journal (2010) 4, 611–620

www.nature.com/ismej

ORIGINAL ARTICLE

Developmental microbial ecology of the crop of the folivorous hoatzin

Filipa Godoy-Vitorino[1], Katherine C Goldfarb[2], Eoin L Brodie[2], Maria A Garcia-Amado[3], Fabian Michelangeli[3] and Maria G Domínguez-Bello[1]

[1]*Department of Biology, University of Puerto Rico, San Juan, PR, USA;* [2]*Ecology Department, Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, USA and* [3]*Center of Biophysics and Biochemistry, Instituto Venezolano de Investigaciones Científicas, Caracas, Venezuela*

The hoatzin (*Opisthocomus hoazin*) is a South American strict folivorous bird, with a crop microbial ecosystem that ferments dietary plants. Chicks progressively become independent from the adult-fed regurgitated crop liquids, and we hypothesized that the crop bacterial ecosystem develops through ecological succession mechanisms, as they grow into adults. The aim of this work was to compare the crop bacterial community in hoatzins from three age groups: newly hatched chicks, juveniles and adults by sequencing 16S rRNA genes and using the G2 PhyloChip. Cloning yielded a total of 2123 nearly full-length sequences binned into 294 operational taxonomic units (OTUs) (with $<$97% homology) belonging to 7 phyla, with 91% of novel OTUs. The microarray identified a diverse bacterial community dominated by Firmicutes and Bacteroidetes, with $\sim$1400 taxa grouped in 40 phyla that included those detected by cloning. In comparison with the adult, the hoatzin chick crop had a greater abundance of Flavobacteriaceae, Clostridiaceae and Lachnospiraceae but lacked phyla DSS1, Deferribacteres and Termite group 1, which were mostly present in adults. The overall community structure of the crop of the hoatzin changes with age in a complex manner, probably responding to new niches made available through dietary changes related to the transition from dependent to independent feeding.

The ISME Journal (2010) **4,** 611–620; doi:10.1038/ismej.2009.147; published online 4 February 2010
Subject Category: microbial population and community ecology
Keywords: bacteria; bird; crop; folivore; hoatzin; succession

Introduction

The hoatzin (*Opisthocomus hoazin*) is a South American strict folivorous bird with crop microbial fermentation (Grajal *et al.*, 1989). Like all other vertebrates, the hoatzin lacks enzymes to digest plant cell walls (Yokoe and Yasumasu, 1964) and is reliant upon the crop microorganisms to ferment the plant structural carbohydrates and nourish on the resulting volatile fatty acids (Grajal *et al.*, 1989). The hoatzin crop harbors a diverse microbial community with a high degree of novel bacteria (Godoy-Vitorino *et al.*, 2008), and also contains archaea (Wright *et al.*, 2009), protozoa and fungi (Godoy-Vitorino *et al.*, 2006).

Birds and mammals feed their offspring until they become independent (Clutton-Brock, 1991). The feeding process varies among birds but usually involves the insertion of the parental bill into the open mouth of the chick, depositing fresh foraging materials or partially digested food (Lack, 1940). In the hoatzin, the adult regurgitates a predigested liquid mash of leafy contents into the chicks mouth (Müllner *et al.*, 2004). As the chicks grow into juveniles (at $\sim$7–10 weeks of age), they progressively browse foliage while being still fed by adults, until they become independent flyers (Müllner *et al.*, 2004). Their feeding strategy thus changes from receiving partially fermented liquids from the adults to being progressively more independent, eating intact plant leaves.

Ecological succession refers to time-related changes in community composition (Morin, 1999); although widely studied in the field of plant ecology, its role in the development of microbial organs—colonized by indigenous microbes that have coevolved with and provide a function to the host—is not well known. There are three accepted mechanisms of ecological succession: facilitation, tolerance and inhibition (Connell and Slatyer, 1977). The facilitation model considers that early species modify the environment and prepare the way for later species. The tolerance model suggests that later successional species are neither inhibited nor aided by species of earlier stages, whereas

Correspondence: MG Dominguez-Bello, Department of Biology, University of Puerto Rico, Rio Piedras Campus, PO BOX 23360, San Juan, PR 00931-3360, USA.
E-mail: mgdbello@uprr.pr
Received 25 June 2009; revised 3 December 2009; accepted 3 December 2009; published online 4 February 2010

the inhibition model is assumed when the presence of late species makes the niche unsuitable to early species.

Microbial colonization of the mammalian gut starts during birth and seems to follow a facilitated succession where pioneer bacteria create a reduced environment favorable for the establishment of the anaerobic species that proceed them (Stark and Lee, 1982). In humans, a mix of facultative anaerobes dominate the microbiota in the first weeks after birth and along with developmental changes in the mucosa, the community progresses toward the anaerobic adult-like community (Palmer *et al.*, 2007). In the cow rumen, the complex strict anaerobic community develops only after weaning (Hungate, 1975). The colonization of the digestive tract can be affected by diet (Hidaka *et al.*, 1986; Ito *et al.*, 1990; Donovan and Odle, 1994), mode of delivery (Grönlund *et al.*, 1999) and environmental factors including stress (Bailey *et al.*, 2004; Thompson *et al.*, 2008), and the complex establishment of the organ microbiota can now be studied in due detail with powerful molecular tools. We hypothesized that mechanisms of bacterial succession occur in the crop during the transition from chicks to adults that develop the crop into a more complex and diverse ecosystem. The aim of this study was to compare the structure of the crop bacterial microbiota in the chick, juvenile and adult hoatzins.

Materials and methods

Animals
Three adults (age >60 days), three juveniles (aged 54.7 ± 2.8 days) and three chick hoatzins (aged 3.1 ± 1.2 days) were captured from the wild in the savannas of Cojedes state in Venezuela (68°4′W, 8°82′N), with permits from the Venezuelan Ministry of Environment Number 1224 of 2 April 2007 and from the UPR-IACUC (601-2007). Age estimates were based on a linear regression curve ($Y = 9.61 + 5.63 \times (R = 0.98)$) from a previous field growth study (Domínguez-Bello *et al.*, 1994) relating growth of chicks up to 59 days (*x* axis), as indicated by body mass (*y* axis) (Supplementary Table S1). The three adults (A 1–3), one chick (P1) and one juvenile (J1) were captured in the Tinaco River, whereas two juveniles (J2 and J3) and two chicks (P2 and P3, from the same nest) were captured in the Cojedes River.

Captures were carried out in the early morning—after the hoatzin peak of feeding activity—by shooting individual birds roosting in the tree branches. Animals were immediately dissected *in situ* and the crop contents sealed in their posterior and anterior ends, and stored in 95% ethanol at room temperature. DNA was extracted from the samples within the following 5 weeks.

DNA extraction
Crop contents from chicks had a thick liquid and sand-like texture, juveniles had progressively more solid particles and the adults had intact leaves and stems. DNA was extracted from ~200 mg of bulk crop contents from each animal, after using sterilized filter paper to absorb off the alcohol, using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA, USA). We modified the first step of the extraction by adding 0.5 g of sterile 0.1-mm-diameter zirconium beads (Biospec Products, Bartlesville, OK, USA), and 1 ml buffer ASL (Qiagen) to each 200 mg of crop contents, and homogenizing (5000 r.p.m. for 2 min at room temperature) in a bead beater (Biospec Products). DNA samples were stored frozen (−20 °C) until use.

Amplification of 16S rRNA genes
PCR was performed using universal bacterial primers 27F (5′-AGRGTTTGATCMTGGCTCAG) and 1492R (5′-GGTTACCTTGTTACGACTT) (Lane, 1991). The PCR mixtures contained 25 μl of PCR Master Mix (Promega, Madison, WI, USA) ~50 ng of DNA template, and 10 pmol of each primer in a 50 μl reaction. For each sample, eight replicate PCR amplifications were performed, with a range of annealing temperatures from 48 to 58 °C, with an initial denaturation at 95 °C (3 min), followed by 25 cycles of denaturation at 95 °C (30 s), annealing (30 s), extension at 72 °C (2 min) and a final extension at 72 °C (10 min). The multiple PCR products for each sample were verified for correct product formation by electrophoresis on a 2% agarose gel. The PCR products were pooled and purified with a PCR purification kit (Qiagen), and used for both clone library construction and hybridization onto the 16S rRNA gene microarray.

Clone libraries
The PCR purified 16S rRNA products were ligated to the pGEM-T-Easy vector (Promega) and transformed into *Escherichia coli* XL1-Blue competent cells (Stratagene, La Jolla, CA, USA). Sequencing of plasmid inserts was carried out using the PCR primers (27F and 1492R) as well as a reverse primer 27R (5′-CGACAICCATGCAICACCT) corresponding to position 1064 of the *E. coli* 16S rDNA (Gao *et al.*, 2007), in an ABI 3730xl instrument (Applied Biosystems, Foster City, CA, USA).

DNA microarray
We used the PhyloChip (G2Chip), a 16S rRNA gene microarray developed by the Lawrence Berkeley National Laboratory and synthesized by Affymetrix (Santa Clara, CA, USA). The array has 506 944 probes arranged in 712 rows and columns representing ~8400 bacterial taxa, with at least one order of magnitude of sensitivity higher than that of a clone

library with hundreds of clones (DeSantis *et al.*, 2007). Each chip has additional probes that serve as controls: (1) targeting amplicons of prokaryotic and eukaryotic metabolic genes (added just before fragmentation) to test simultaneously fragmentation, biotinylation, hybridization, washing, staining and scanning; and (2) prelabeled oligonucleotides added into the hybridization mix to account for variation in hybridization, washing, staining and scanning.

The mix of the pooled PCRs from the crop samples and control amplicons was fragmented to 50–200 bp using DNase I (0.02 U mg^{-1} DNA; Invitrogen, Carlsbad, CA, USA) and One-Phor-All buffer (GE Healthcare Biosciences, Piscataway, NJ, USA). Biotin labeling was performed with deoxyribonucleotransferase (Promega) as per the manufacturer's instructions. Denaturing occurred later at 99 °C for 5 min and hybridization onto the PhyloChip occurred overnight at 48 °C at 60 r.p.m. The arrays were subsequently washed, stained and scanned. Detailed methodology is specified elsewhere (Brodie *et al.*, 2006). Scanning of the arrays was performed using a GeneArray Scanner (Affymetrix) and probe intensities were treated as previously reported (Brodie *et al.*, 2006). Positive probe pairs met two criteria: (1) fluorescence of the perfectly matched probe was at least 1.3 times greater than the intensity of the control (mismatch probe); and (2) the value of the difference between perfectly matched probe and mismatch probe intensities was at least 130 times greater than the squared noise value. The value of the positive fraction was calculated for each probe set as the number of positive probe pairs divided by the total number of probe pairs in a probe set. A positive fraction equal to or greater than 0.90 was used to denote presence/absence of an operational taxonomic unit (OTU)/taxon.

Analytical methods

Sequence analyses. Sequences were trimmed for quality and edited using Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA). We used Greengenes (DeSantis *et al.*, 2006a) to perform multiple sequence alignments with NAST (DeSantis *et al.*, 2006b) as well as to classify the clones according to both the Hugenholtz and G2 chip taxonomies (as of 19 November 2008). Hoatzin crop 16S rRNA sequences from all nine individuals and their closest neighbors in Greengenes were then imported to ARB (Ludwig *et al.*, 2004). Chimeras (checked with Bellerophon, version 3 (Huber *et al.*, 2004)) ($n = 970$) and sequences corresponding to plant chloroplasts ($n = 152$) were excluded from the analysis. These sequence data have been submitted to the GenBank database under accession numbers FJ832164–FJ834286.

Phylogenetic analyses and diversity estimates. For the richness and coverage estimations, sequences were binned into OTUs using a 97% cutoff level in DOTUR (Schloss and Handelsman, 2005) (with sequence hypervariable regions masked using the lanemaskPH (Hugenholtz, 2002) in ARB (Ludwig *et al.*, 2004)). One representative sequence per OTU was randomly selected for calculating a phylogenetic tree using the Neighbor-joining algorithm (Saitou and Nei, 1987) in ARB (Ludwig *et al.*, 2004). OTU abundance values were used to create a heatmap for interindividual comparisons (TreeView, http://rana.lbl.gov). OTUs were computed in DOTUR (Schloss and Handelsman, 2005) and the rarefaction curves of observed and estimated (Chao1, C_{ACE}) richness were carried out with 50 randomizations using EstimateS (Colwell, 2004). The Diversity indices of Shannon (1948) and Simpson (1949) were estimated in DOTUR, as well as the Pielou evenness index (Pielou, 1966). Good's coverage index (Good, 1953) was estimated using singleton sequences obtained from the analyses in DOTUR.

Classification of the cloned OTUs. To compare the PhyloChip with cloned sequences, we carried out the classification of cloned OTUs using both the G2Chip and Hugenholtz taxonomies at each level of taxonomic resolution: Phylum 80%, Class 85%, Order 90%, Family 92%, Subfamily 94%, OTU 97% (DeSantis *et al.*, 2007). Cloned OTUs without representatives on the PhyloChip were those with a similarity to nearest G2Chip OTU below each of the above-mentioned thresholds (as of 19 November 2008). The relatedness of the hoatzin crop OTUs to known SSU *rRNA* gene sequences was determined by BLAST comparisons to the Greengenes database.

Bacterial community composition analyses using the weighted UniFrac distance metric. We compared the bacterial communities from the nine crops (chicks, juveniles and adults) using UniFrac (Lozupone and Knight, 2005). UniFrac community analyses were based on an ARB neighbor-joining tree of all 16S rRNA gene sequences. To determine whether the individuals or age groups were significantly different from each other, we performed the UniFrac Significance that tests whether the sequences in the tree are significantly different from each other. To cluster samples with related bacterial communities, we clustered environments and performed jackknife analysis for node support. We also performed Principal Components Analyses to study sample distribution along axes of variation.

Cluster analyses of PhyloChip microarray data. Taxa that significantly changed with age were determined by analysis of variance with *P*-values corrected for multiple observations by the Holm procedure, a little less stringent than Bonferroni. A representative sequence of each taxon was exported from Greengenes (DeSantis *et al.*, 2006a), and imported to ARB for neighbor-joining tree construction. A matrix with

relative abundance of each taxon in each sample was also prepared. Hierarchical cluster analyses and heatmap construction were carried out using *Cluster* (Eisen *et al.*, 1998) and TreeView (http://rana.lbl.gov/EisenSoftware.htm).

Results

Comparisons of cloning and DNA microarray

A total of 2123 sequences were obtained corresponding to 236 ± 48 cloned sequences per animal and 707 ± 37 sequences per age group (Table 1). The 2123 sequences were binned into 294 OTUs and Chao1 estimated a richness of 534 OTUs. There were approximately 130 OTUs per age group, highly evenly distributed (Table 1). Observed rarefaction curves (Supplementary Figure S1) were lower than the estimated ones for all age groups both at 97% and 80% cutoff levels, and non-asymptotic, likely being an underestimation due to insufficient clone sampling. The collector's curves at 80% cutoff show that this microbial ecosystem may bear a high number of phyla (~25–30) (Supplementary Figure S1). Cloning and sequencing of crop bacteria did not result in age-related differences for observed ($P = 0.47$) or estimated ($P = 0.9$) bacterial richness in the crop. About 56% of the cloned OTUs had >97% identity to hoatzin crop bacteria from our previous study (Godoy-Vitorino *et al.*, 2008). The OTUs, whose best match is a rumen bacterium (18% of the total), had 16S *rRNA* sequence identities ranging from 95% to 99% mainly with clone bacteria found in the bovine rumen (Supplementary Table S2).

Since cloning usually under samples true diversity in a complex ecosystem, retrieving only dominant taxa, we used a DNA microarray—the G2 PhyloChip (Brodie *et al.*, 2006)—as an additional tool to study the crop bacterial community structure. The microarray detected as many as 40 phyla (including the 7 detected by cloning) (Figure 1), and consistently detected more taxa in each of the three age groups (Table 2, Supplementary Table S3), confirming the Chao1 predictions of greater richness than observed by cloning (Figure 1, Supplementary Table S3). The PhyloChip-based OTU richness was 10.51 ± 0.7-fold higher than that predicted by cloning and sequencing (Table 2). Some of the Phyla only detected by the PhyloChip include Deferribacteres and DSS1, which were exclusively present in juveniles and adults, and the Termite group 1 that was only found in adults (Supplementary Table S3).

The microarray also detected phyla of putatively thermophilic microorganisms, such as the Aquificae, Thermodesulfobacteria, Coprothermobacteria

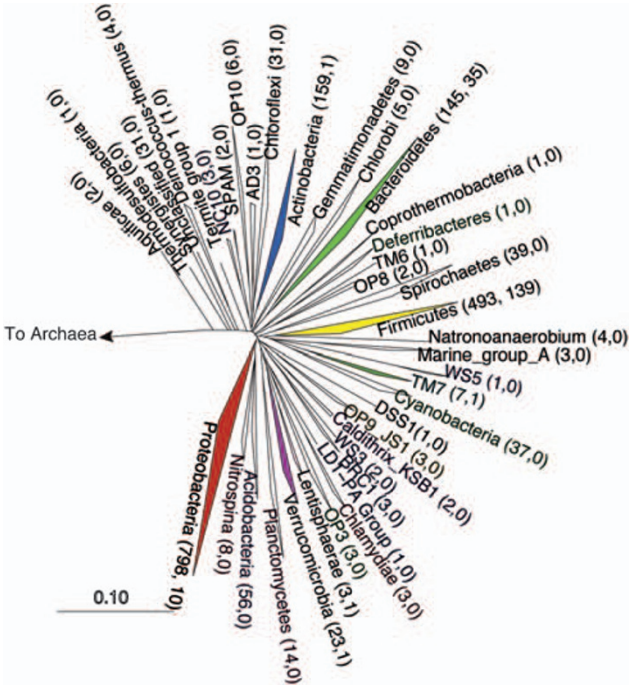

Figure 1 Neighbor-joining tree of the 40 phyla detected by the DNA microarray. The area of the branch wedges is proportional to the number of operational taxonomic units (OTUs; from a total of 1742), and colored wedges show the 7 phyla that were also detected by cloning. In parentheses are the numbers of OTUs per phylum found in the DNA microarray and clone libraries, respectively.

Table 1 Clone library-based estimates of diversity in birds of different age groups

Matrix	*N Sequences*	*Singletons*	*Good's index*[a]	*Observed richness (95% CI)*	*Predicted richness (95% CI)*	*Diversity*		*Evenness*[d]
						Shannon[b]	*Simpson*[c]	
Chicks	792	53	0.93	127 (113.6–140.3)	195 (160.9–266.7)	4.03	0.03	0.83
Juveniles	692	71	0.89	148 (133.3–162.6)	234 (194.5–305.8)	4.16	0.02	0.83
Adults	639	60	0.90	136 (122.7–149.2)	201 (170.2–260.2)	4.06	0.03	0.82
Pool[e]	2123	139	0.93	294	534	4.66	0.018	0.80

[a]Coverage: sum of probabilities of observed classes calculated as $(1–(n/N)) \times 100$, where n is the number of singleton sequences and N is the total number of sequences (Good, 1953).
[b]Takes into account the number and evenness of species. The higher the diversity, the higher the Shannon–Weaver index (Shannon, 1948; Chao, 2003).
[c]Estimates the probability that two randomly selected individuals belong to the same species (Simpson, 1949)
[d]Measure of evenly distributed abundance (ranging from 0 (uneven) to 1 (even); (Pielou, 1966)).
[e]Pooled samples (considering all sequences from the three age groups).

Table 2 Number of distinct bacterial taxonomic groups detected by the DNA microarray and by clone libraries in the crops of chicks, juveniles and adults

Age group	*Taxonomic level*	*Array*	*Clone*	*Ratio array:clone*
Chicks[a]	Phylum	37	4	9.25
	Class	47	6	6.71
	Order	90	10	8.18
	Family	147	17	7.74
	Subfamily	305	20	13.86
	OTU	1438	127[b]	11.33
Juveniles[c]	Phylum	39	4	7.80
	Class	49	10	4.45
	Order	90	13	6.43
	Family	152	21	6.08
	Subfamily	313	24	11.18
	OTU	1521	148[b]	10.29
Adults[d]	Phylum	40	5	8.00
	Class	49	11	4.08
	Order	88	13	6.29
	Family	148	22	5.92
	Subfamily	306	24	10.93
	OTU	1351	136[b]	9.94

Abbreviation: OUT, operational taxonomic unit.
Taxonomical classification of the clone libraries was carried out according to the G2 Chip taxonomy in Greengenes.
[a]The number of OTUs per individual chicks (1–3) were respectively, 1243, 1156 and 1136.
[b]The number of OTUs in clone libraries corresponds to a 97% OTU cutoff.
[c]The number of OTUs per individual juveniles (1–3) were respectively, 1052, 1133 and 1379.
[d]The number of OTUs per individual adults (1–3) were respectively, 987, 1166 and 1038.

and Caldithrix (Figure 1, Supplementary Table S3). Despite its higher sensitivity, the PhyloChip did not detect many cloned bacteria at shallow phylogenetic levels (for example, genera and OTU in contrast to deeper levels such as phylum); however, a concordance between cloning and DNA microarray was observed from OTU to phylum levels (Supplementary Figure S2). Few (~7) of the cloned OTUs classified as Firmicutes and Verrucomicrobia had DNAML identity values below 80% with the G2Chip taxonomy as well as ~72% DNAML identity with Unclassified bacteria (OTU 2400) according to the Hugenholtz taxonomy, suggesting these could be novel phyla. There were ~60% of the cloned families and ~97% of the OTUs with low DNAML homologies to existing PhyloChip OTUs, and of these ~27% and 91%, respectively, were novel (Supplementary Figure S2).

Age-related changes in the crop bacterial community structure

Cloned bacterial sequences evidenced age group differences in the crop bacterial composition. A neighbor-joining tree of the 294 cloned OTUs (Figure 2a) shows 7 phyla detected: Firmicutes, Bacteroidetes (most abundant phyla), followed by Proteobacteria, Actinobacteria, Verrucomicrobia, Lentisphaerae and TM7 (Supplementary Figure S3). Firmicutes were dominated by the class Clostridia (~58%), mostly due to high representation of family Lachnospiraceae and in the Bacteroidetes, class Bacteroidetes (~20%) was dominated by family Prevotellaceae (Supplementary Table S4). As shown in the heatmap, there was a high interindividual variation with only four OTUs abundant in all nine individuals (Figure 2a (arrows)), corresponding to families Rikenellaceae and Bacteroidaceae within phylum Bacteroidetes, and to *Butyrivibrio fibrisolvens* and an uncultured Lachnospiraceae within phylum Firmicutes. There were four types of age-changing bacterial patterns: (1) OTUs that appear in adults (highlighted in pink), (2) OTUs that disappear with age (absent in adults) and highlighted in yellow, (3) OTUs that were absent in juveniles and highlighted with a blue square and (4) OTUs predominant in juveniles, highlighted in green. Some of the OTUs that appear in adults belonged to Coriobacteraceae (Actinobacteria), Desulfovibrionaceae (Proteobacteria), Erysipelotrichaceae and Acidaminococcaceae (Firmicutes) and OTUs in the phyla Lentisphaerae and Verrucomicrobia. The group of OTUs that disappear with age mostly belonged to Firmicutes (specifically some Erysipelotrichaceae and Lachnospiraceae). OTUs absent in juveniles belonged to families Lachnospiraceae and phylum Lentisphaerae. The OTUs that were more abundant in juveniles belonged to Succinivibrionaceae and Desulfovibrionaceae (Proteobacteria) as well as Acidaminococcaceae (Firmicutes) (Figure 2a).

The hierarchical cluster analyses of the 95 analysis of variance-determined age-varying taxa identified 5 primary response groups (Figure 2b): Group 1 comprised OTUs most abundant in chicks (highlighted in yellow); Group 2 comprised OTUs less abundant in juveniles (highlighted blue); Group 3 comprised OTUs absent in chicks (highlighted in pink); Group 4 comprised OTUs most abundant in juveniles (highlighted in orange) and Group 5 comprised OTUs mostly absent in adults (highlighted in green). Although Group 1 was composed of 13 OTUs in 4 Phyla and included exclusive OTUs of Cellulomonadaceae (Actinobacteria), Flavobacteriaceae (Bacteroidetes) and Methylobacteraceae (Proteobacteria), Group 2 had 4 OTUs in 2 phyla with no exclusive families (Supplementary Table S5). Group 3 had 18 OTUs in 8 phyla including Erysopelotrichaceae (Firmicutes), NC10 and Verrucomicrobia (Supplementary Table S5). Group 4 had 21 OTUs in 4 phyla, such as Coriobacteraceae (Actinobacteraceae), Flexibacteraceae (Bacteroidetes) and Acetobacteraceae (Proteobacteria) (Supplementary Table S5). Group 5 had 39 OTUs in 6 phyla such as Alteromonadaceae (Proteobacteria), Leuconostocaceae (Firmicutes), Dermabacteraceae (Actinobacteria) and Synergistes.

Qualitative differences in crop bacteria of the different age groups were confirmed by principal component and cluster analyses in UniFrac (Figure 3, Supplementary Figures S4 and S5). The

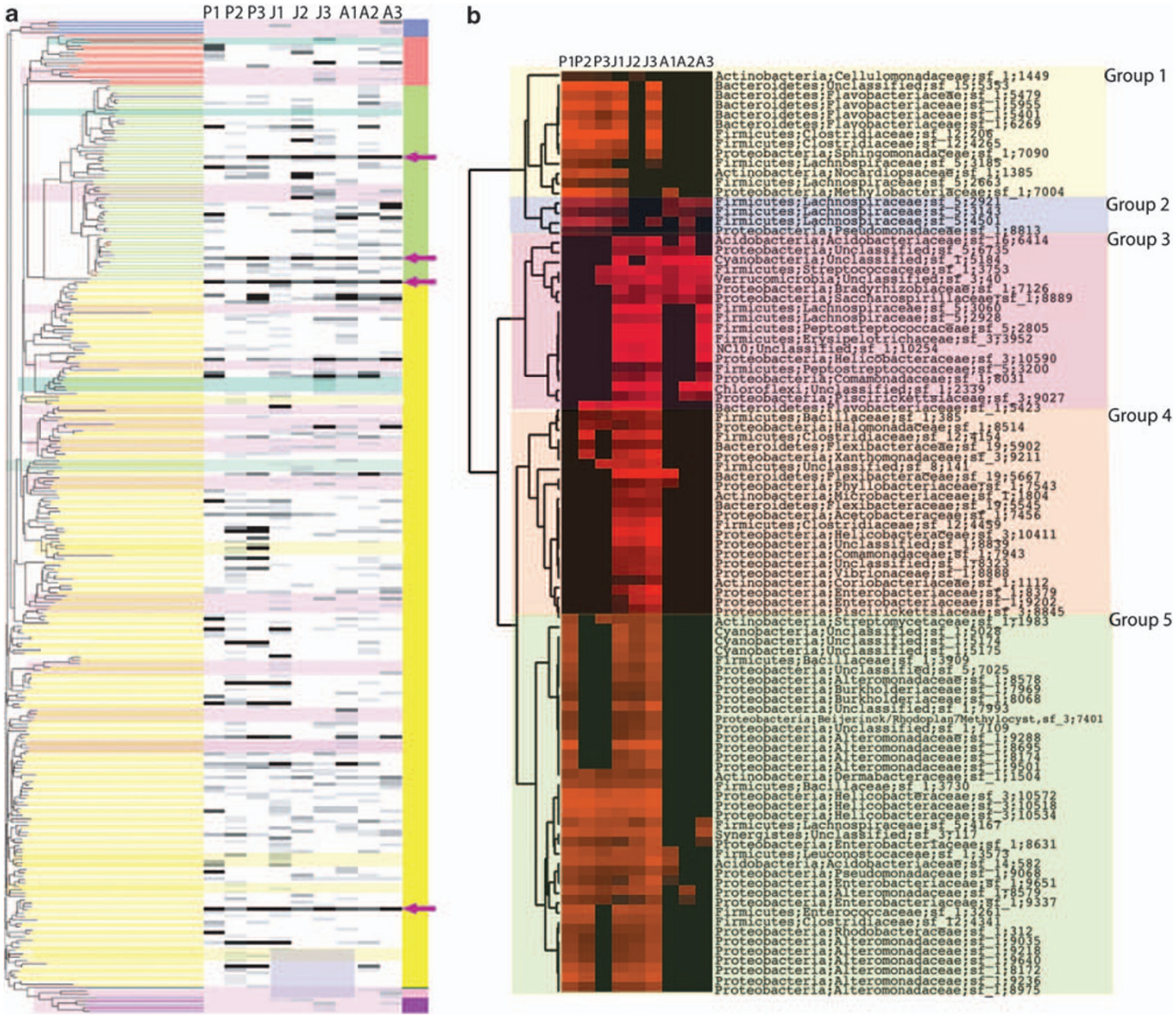

Figure 2 Age-related changes in the crop bacterial community structure. (**a**) Neighbor-joining tree and heatmap of 294 clone-based bacterial operational taxonomic units (OTUs) from the crop. Phyla are color-coded both in the tree branches and in the side vertical bars: blue for Actinobacteria, red for Proteobacteria, green for Bacteroidetes, yellow for Firmicutes, dark green for TM7, pink for Lentisphaerae and violet for Verrucomicrobia. Colored bars on the right-side Heatmap columns are animals (chicks (P), juveniles (J), and adults (A)) and rows are OTUs shown by relative clone abundance (white = 0%; black = 100%). (**b**) Hierarchical clustering and heatmap of 95 differentially selected OTUs found in the DNA microarray for the three age groups. The selected taxa appearing in this figure were based on analysis of variance (Holm—FWER corrected, $P<0.05$) representing the OTUs that significantly changed with age. Taxa with similar abundance distributions across samples are clustered together on the *y* axis with a color gradient from black to red representing increasing DNA microarray hybridization intensity.

UniFrac analysis of cloned sequences separated chicks from both juveniles and adults (Figure 3), which was confirmed by the UniFrac Significance test ($P<0.05$). Chicks 2 and 3, which shared the same nest, form a very well-supported node (Figure 3 and Supplementary Figure S5). Nonetheless the juveniles' microbiota is indeed intermediate, and an individual juvenile shows greater similarity to chicks whereas the other two are more similar to adults (Figure 3, Supplementary Figures S4 and S5).

To summarize, in comparison with cloning, the PhyloChip detected a much higher bacterial richness at deeper phylogenetic levels (Phylum) but novel bacteria were only detected by cloning. There was a concordance in both cloning and PhyloChip showing that the chicks possess a different crop bacterial community structure than especially that of the adults, with juveniles scattered between these groups. The major differences were that chicks lacked Verrucomicrobia, some Erysopelotrichaceae, NC10, Deferribacteres and Desulfovibrionaceae and had higher abundance of some Flavobacteriaceae, Synergistes and certain Proteobacteria.

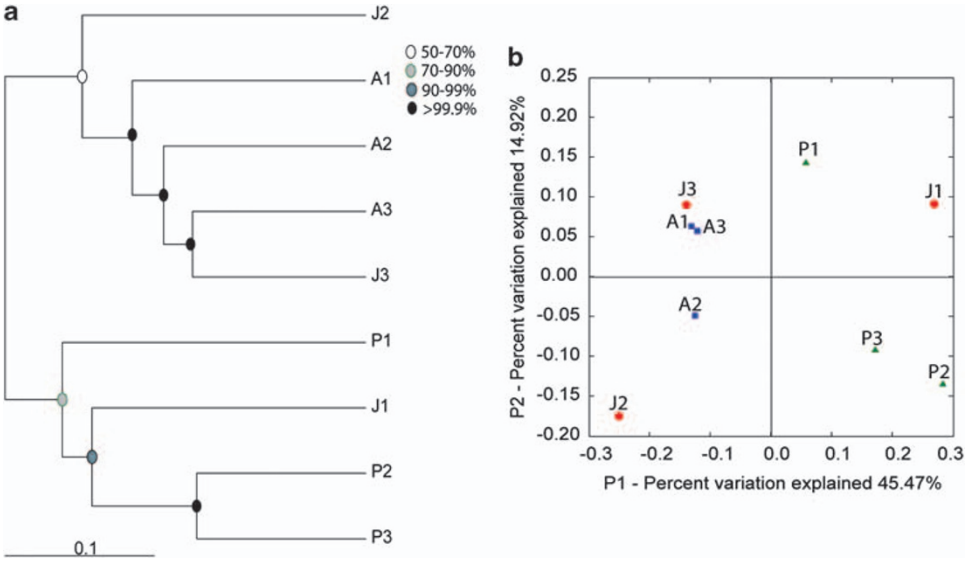

Figure 3 UniFrac community analyses of cloned sequences from the three age groups. (**a**) Cloning-based clustering of each of the nine individuals. (**b**) Principal Component Analyses of the bacterial community of clones from the nine animals. Jackknife node supports are represented by the colored circles. Note that A corresponds to adults, J to juveniles and P to chicks.

Discussion

This is the first molecular study on succession of bacterial populations in the crop of a bird, and it shows a complex pattern of dynamic shifts in the microbiota. The microarray approach revealed an unprecedented diversity with ~1400 OTUs grouped in 40 phyla (7 of which were also detected by cloning) in comparison, with previous studies on the human gut (14 phyla (Andersson *et al.*, 2008)), cow rumen (12 phyla (Tajima *et al.*, 2001; Ozutsumi *et al.*, 2005; Karnati *et al.*, 2007)) chicken ceca (5 phyla (Lan *et al.*, 2002; Zhu *et al.*, 2002)) and other gastrointestinal tracts of several mammalian (overall 22 phyla (Ley *et al.*, 2008)). The high number of phyla was partially confirmed by the rarefaction's curves at 80% sequence homology and the high OTU-level richness was also confirmed by the estimated values previously published (Godoy-Vitorino *et al.*, 2008). Because the chip fails to detect novel diversity detected by cloning, adopting both techniques simultaneously is beneficial.

In this study, most of the cloned sequences had low homology to the PhyloChip OTUs, but were similar to the hoatzin sequences previous reported (Godoy-Vitorino *et al.*, 2008). Some (18%) of the crop clones resembled rumen bacteria, but only 8% had $\geqslant$95% BLAST identity with bacterial sequences from the database. No members of the rumen cellulolytic Fibrobacteria phylum were identified. The apparent absence of certain rumen cellulolytic bacteria, and the discovery of many novel rumen-related sequences raise the possibility that these unclassified sequences may include cellulolytic bacteria. Cloning has unveiled the high degree of novelty in relation with the previously described microbiota of fermentative organs in other vertebrates.

The dominance of Bacteroidetes and Firmicutes, as observed in the hoatzin crop, is a feature typical of the vertebrate gut, both in mammals (Ley *et al.*, 2008) and in the ceca of chickens (Lan *et al.*, 2002) or turkeys (Scupham *et al.*, 2008). Interestingly, the microarray detected several phyla with relatively high number of taxa including Spirochaetes, Synergistes or Acidobacteria and some thermophilic bacteria, never reported before in a vertebrate gut, such as Aquificae, Coprothermobacteria, Thermodesulfobacteria and Caldithrix. Recent work by us using 454 pyrosequencing of 16S rDNA amplicons (pyrotags), confirmed some of the phyla—only detected here by the PhyloChip—such as Spirochaetes, Synergistes, Acidobacteria, Chloroflexi and Planctomyces (unpublished data). The use of pyrotags is becoming more popular and allows an in-depth perspective on the diversity of microbial communities.

Both cloning and the DNA microarray methods show that the chick's crop community is significantly different from that of the adult hoatzin, with the juveniles being intermediate. This maybe related to the fact that the juveniles in the fledging state have both parental and independent feeding, which in turn lead to the detection of bacteria already present in the chicks and to the development of certain bacteria that will remain in the adult crop.

Functional changes in the crop are expected to be driven mostly by (1) changes in dietary substrate availability, from liquid and small particles to solid leafy pieces; (2) changes in the crop environment by microbes that colonize the crop and (3) changes in the host physiology and host response to microbes.

Bacterial changes in the rumen occur under different diets (Tajima *et al.*, 2001) as well as in relation to solid or liquid digesta fractions (Rodriguez *et al.*, 2000), which supports our findings of a different community structure in chicks receiving mostly liquid fraction from the adults. The observation that sibling chicks cluster together, and apart from non-siblings, further supports that dietary substrate and genetics are key in shaping the community structure of the crop. In fact in the case of humans, twins possess a strikingly similar microbiota composition (Palmer *et al.*, 2007).

Age-related bacterial changes are evidenced by the late appearance of Deferribacteres and Termite group 1, and disappearance of pioneer bacteria such as Clostridiaceae and Synergistes. These changes are consistent with facilitation and inhibition mechanisms of succession and are likely related with differences in dietary resource availability and the animal's developmental physiology. In this scenario, bacteria from the liquid fraction change the local conditions, opening niches to be occupied by new species while loss of niches and competition lead to the disappearance of other species, similarly to what occurs during biofilm development (Jackson *et al.*, 2001). The continuous presence of niches throughout time and the persistence of species from chicks to adults, such as Streptococcaceae or Spirochaetes, is consistent with the tolerance mechanism. Most likely, the bacterial populations develop through these succession mechanisms into a more stable ecosystem in the adults, more robust in the face of perturbations (Little *et al.*, 2008). In addition, there are age-related physical and chemical changes driven by the animal's diet and physiology that allow the permanence of the residents of the community. We expected that crop bacterial richness would increase with age, but instead, there was a slight peak of richness in the juvenile's crop. This might be explained by the juvenile's double feeding strategy of receiving a continuous liquid culture from adults plus browsing for leaves independently.

At the light of the neutral theory of ecology the crop patterns of diversity and abundance with age may also occur as a result of stochastic events (Chave, 2004). It is possible that there are several population guilds in the crop—groups of species that use similar resources in similar ways, and the differences among these guilds likely matter to the assembly, stability and resilience of communities to disturbance (Hubbell, 2005).

The functional significance of age-related community changes can be understood by the functional insight that other techniques such as metagenomics may bring (Kunin *et al.*, 2008). However, some information can be inferred from the bacterial taxa phylogenies. Among bacteria that appear late in the succession, Deferribacteres are important in the degradation of amino acids under anaerobic conditions (Godon *et al.*, 1997), suggesting that the redox potential in the chick crop may be too high (not reduced enough) to support such strict anaerobes. Termite group 1 represent a deep branch in the 16S rRNA gene (Hugenholtz *et al.*, 1998) comprising bacteria associated with flagellate protists in wood-feeding insects (Stingl *et al.*, 2005), probably here these bacteria are associated with crop ciliate protozoa contributing to the degradation of the secondary plant cell-wall compounds. Among other bacterial phyla with members involved in fiber degradation, Lentisphaerae and Verrucomicrobia are linked with cellobiose degradation (Zoetendal *et al.*, 2003), which require a low redox potential likely explaining the low abundance of these taxa in the hatchling crop. The Spirochaetes, despite their low abundance in the crop, have been shown to be involved in the degradation of fiber in the termite hindgut (Warnecke *et al.*, 2007).

To summarize, the age-related changes of the crop bacterial community are complex and may involve several successional and other ecological mechanisms, responding to the opening of new niches driven by dietary and bird developmental changes. The functional relevance of the age-specific bacterial groups remains to be elucidated and would benefit from a metagenomics approach.

Acknowledgements

This work was supported by grants from NSF: IOS 0716911, DDIG 0709840, CREST HRD0206200 and UPR Grant FIPI 8-80314. Part of this work was performed at Lawrence Berkeley National Laboratory under the auspices of the University of California—Contract Number DE-AC02-05CH11231. We thank Todd DeSantis for developing and assisting with the G2 chip taxonomy classifier and Humberto Ortiz for helping with python scripts including the BLAST parser. The Logistic field support from Hato Mataclara personnel José, Juan and Antonio González, and the issuing of permits by the Venezuelan Ministry of Environment are deeply appreciated.

References

Andersson AF, Lindberg M, Jakobsson H, Backhed F, Nyren P, Engstrand L. (2008). Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE* **3**: e2836.

Bailey MT, Lubach GR, Coe CL. (2004). Prenatal stress alters bacterial colonization of the gut in infant monkeys. *J Pediatr Gastroenterol Nutr* **38**: 414–421.

Brodie EL, Desantis TZ, Joyner DC, Baek SM, Larsen JT, Andersen GL *et al.* (2006). Application of a high-density oligonucleotide microarray approach to study bacterial popultion dynamics during uranium reduction and reoxidation. *Appl Environ Microbiol* **72**: 6288–6298.

Chao A, Shen T-J. (2003). Nonparametric estimation of Shannon's diversity index when there are unseen species in sample. *Environ Ecol Stat* **10**: 429–443.

Chave J. (2004). Neutral theory and community ecology. *Ecol Lett* **7**: 241–253.
Clutton-Brock TH. (1991). *The Evolution of Parental Care*. Princeton University Press: Princeton, NJ, USA.
Colwell RK. (2004). EstimateS: Statistical Estimation of Species Richness and Shared Species from Samples. *Version 7 (*http://viceroy.eeb.uconn.edu/estimates; permanent URLwww.purl.oclc.org/estimates*)*..
Connell JH, Slatyer RO. (1977). Mechanisms of succession in natural communities and their role in community stability and organization. *Am Nat* **111**: 1119–1144.
DeSantis TZ, Brodie EL, Moberg JP, Zubieta IX, Piceno YM, Andersen GL. (2007). High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb Ecol* **53**: 371–383.
DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K *et al.* (2006a). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol* **72**: 5069–5072.
DeSantis Jr TZ, Hugenholtz P, Keller K, Brodie EL, Larsen N, Piceno YM *et al.* (2006b). NAST: a multiple sequence alignment server for comparative analysis of 16S rRNA genes. *Nucleic Acids Res* **34**: W394–W399.
Domínguez-Bello MG, Michelangeli F, Ruiz MC, Garcia A, Rodriguez E. (1994). Ecology of the folivorous hoatzin (*Opisthocomus hoazin*) on the Venezuelan plains. *Auk* **111**: 643–651.
Donovan SM, Odle J. (1994). Growth factors in milk as mediators of infant development. *Annu Rev Nutr* **14**: 147–167.
Eisen MB, Spellman PT, Brown PO, Botstein D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* **95**: 14863–14868.
Gao Z, Tseng CH, Pei Z, Blaser MJ. (2007). Molecular analysis of human forearm superficial skin bacterial biota. *Proc Natl Acad Sci USA* **104**: 2927–2932.
Godon JJ, Zumstein E, Dabert P, Habouzit F, Moletta R. (1997). Molecular microbial diversity of an anaerobic digestor as determined by small-subunit rDNA sequence analysis. *Appl Environ Microbiol* **63**: 2802–2813.
Godoy-Vitorino F, Gao Z, Pei Z, Ley R, Gordon J, Hackstein J *et al.* (2006). *11th International Symposium on Microbial Ecology: The Hidden Powers—Microbial Communities in Action. Ecology (ISME).* Vienna, Austria, pp 121.
Godoy-Vitorino F, Ley RE, Gao Z, Pei Z, Ortiz-Zuazaga H, Pericchi LR *et al.* (2008). Bacterial community in the crop of the hoatzin, a neotropical folivorous flying bird. *Appl Environ Microbiol* **74**: 5905–5912.
Good I. (1953). The population frequencies of species and the estimation of population parameters. *Biometrika* **40**: 237–264.
Grajal A, Strahl SD, Parra R, Dominguez MG, Neher A. (1989). Foregut fermentation in the Hoatzin, a neotropical leaf-eating bird. *Science* **127**: 1131–1134.
Grönlund MM, Lehtonen OP, Eerola E. (1999). Fecal microflora in healthy infants born by different methods of delivery: permanent changes in intestinal flora after Cesarean delivery. *J Pediatr Gastroenterol Nutr* **28**: 19–25.
Hidaka H, Eida T, Takizawa T, Tokunaga T, Yashiro T. (1986). Effects of fructooligosaccharides on intestinal flora and human health. *Bifidobacteria Microflora* **5**: 37–50.
Hubbell SP. (2005). Neutral theory in community ecology and the hypothesis of functional equivalence. *Funct Ecol* **19**: 166–172.
Huber T, Faulkner G, Hugenholtz P. (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* **20**: 2317–2319.
Hugenholtz P. (2002). Exploring prokaryotic diversity in the genomic era. *Genome Biol* **3**: Reviews0003.
Hugenholtz P, Goebel B, Pace N. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**: 4765–4774.
Hungate RE. (1975). The rumen microbial ecosystem. *Annu Rev Ecol Syst* **6**: 39–66.
Ito M, Deguchi Y, Miyamori A. (1990). Effect of administration of galacto-oligosaccharides on the human faecal microflora, stool, weight and abdominal sensation. *Microbiol Ecol Health Dis* **3**: 285–292.
Jackson C, Churchill P, Roden E. (2001). Successional changes in bacterial assemblage structure during epilithic biofilm development. *Ecology* **82**: 555–566.
Karnati SK, Sylvester JT, Noftsger SM, Yu Z, St-Pierre NR, Firkins JL. (2007). Assessment of ruminal bacterial populations and protozoal generation time in cows fed different methionine sources. *J Dairy Sci* **90**: 798–809.
Kunin V, Copeland A, Lapidus A, Mavromatis K, Hugenholtz P. (2008). A bioinformatician's guide to metagenomics. *Microbiol Mol Biol Rev* **72**: 557–578, Table of Contents.
Lack D. (1940). Courtship feeding in birds. *Auk* **57**: 169–178.
Lan PT, Hayashi H, Sakamoto M, Benno Y. (2002). Phylogenetic analysis of cecal microbiota in chicken by the use of 16S rDNA clone libraries. *Microbiol Immunol* **46**: 371–382.
Lane DJ. (1991). *16S/23S rRNA sequencing*. Wiley: London, pp 115–175.
Ley RE, Hamady M, Lozupone C, Turnbaugh PJ, Ramey RR, Bircher JS *et al.* (2008). Evolution of mammals and their gut microbes. *Science* **320**: 1647–1651.
Little AE, Robinson CJ, Peterson SB, Raffa KF, Handelsman J. (2008). Rules of engagement: interspecies interactions that regulate microbial communities. *Annu Rev Microbiol* **62**: 375–401.
Lozupone C, Knight R. (2005). UniFrac: a new phylogenetic method for comparing microbial communities. *Appl Environ Microbiol* **71**: 8228–8235.
Ludwig W, Strunk O, Westram R, Richter L, Meier H, Yadhukumar A *et al.* (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
Morin PJ. (1999). *Community Ecology*. Wiley-Blackwell: Malden, MA, USA, pp 424.
Müllner A, Linsenmair KE, Wikelski M. (2004). Exposure to ecotourism reduces survival and affects hormonal stress response in Hoatzin chicks (*Opisthocomus hoazin*). *Biol Conserv* **118**: 549–558.
Ozutsumi Y, Hayashi H, Sakamoto M, Itabashi H, Benno Y. (2005). Culture-independent analysis of fecal microbiota in cattle. *Biosci Biotechnol Biochem* **69**: 1793–1797.
Palmer C, Bik EM, Digiulio DB, Relman DA, Brown PO. (2007). Development of the human infant intestinal microbiota. *PLoS Biol* **5**: e177.
Pielou EC. (1966). The measurement of diversity in different types of biological collections. *J Theor Biol* **13**: 131–144.

Rodriguez CA, Gonzalez J, Alvir MR, Repetto JL, Centeno C, Lamrani F. (2000). Composition of bacteria harvested from the liquid and solid fractions of the rumen of sheep as influenced by feed intake. *Br J Nutr* **84**: 369–376.

Saitou N, Nei M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**: 406–425.

Schloss P, Handelsman J. (2005). Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl Environ Microbiol* **71**: 1501–1506.

Scupham J, Patton TG, Bent E, Bayles DO. (2008). Comparison of the cecal microbiota of domestic and wild turkeys. *Microb Ecol* **56**: 322–331.

Shannon CE. (1948). A mathematical theory of communication. *Bell Syst Tech J* **27**: 379–423, 623–656.

Simpson EH. (1949). Measurement of diversity. *Nature* **163**: 688–689.

Stark PL, Lee A. (1982). The microbial ecology of the large bowel of breastfed and formula-fed infants during the first year of life. *J Med Microbiol* **15**: 189–203.

Stingl U, Radek R, Yang H, Brune A. (2005). 'Endomicrobia': cytoplasmic symbionts of termite gut protozoa form a separate phylum of prokaryotes. *Appl Environ Microbiol* **71**: 1473–1479.

Tajima K, Aminov RI, Nagamine T, Matsui H, Nakamura M, Benno Y. (2001). Diet-dependent shifts in the bacterial population of the rumen revealed with real-time PCR. *Appl Environ Microbiol* **67**: 2766–2774.

Thompson CL, Wang B, Holmes AJ. (2008). The immediate environment during postnatal development has long-term impact on gut community structure in pigs. *ISME J* **2**: 739–748.

Warnecke F, Luginbuhl P, Ivanova N, Ghassemian M, Richardson TH, Stege JT *et al.* (2007). Metagenomic and functional analysis of hindgut microbiota of a wood-feeding higher termite. *Nature* **450**: 560–565.

Wright AD, Northwood KS, Obispo NE. (2009). Rumen-like methanogens identified from the crop of the folivorous South American bird, the hoatzin (*Opisthocomus hoazin*). *ISME J* **3**: 1120–1126.

Yokoe Y, Yasumasu IC. (1964). The distribution of cellulase in invertebrates. *Comp Biochem Physiol* **13**: 323–338.

Zhu XY, Zhong T, Pandya Y, Joerger RD. (2002). 16S rRNA-based analysis of microbiota from the cecum of broiler chickens. *Appl Environ Microbiol* **68**: 124–137.

Zoetendal EG, Plugge CM, Akkermans AD, de Vos WM. (2003). *Victivallis vadensis* gen. nov., sp. nov., a sugar-fermenting anaerobe from human faeces. *Int J Syst Evol Microbiol* **53**: 211–215.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)