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Bacterial Associates of Arboreal Ants and Their Putative Functions in an Obligate Ant-Plant Mutualism▽†

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Bacterial communities are highly diverse and have great ecological importance. In the present study, we used an in silico analysis of terminal restriction fragments (tRF) to characterize the bacterial community of the plant ant *Pseudomyrmex ferrugineus*. This species is an obligate inhabitant of *Acacia* myrmecophytes and feeds exclusively on plant-derived food sources. Ants are the dominant insect group in tropical rain forests. Associations of ants with microbes, which contribute particularly to the ants' nitrogen nutrition, could allow these insects to live on mostly or entirely plant-based diets and could thus contribute to the explanation of the high abundances that are reached by tropical ants. We found tRF patterns representing at least 30 prokaryotic taxa, of which the *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Spirochaetes* comprised 93%. Because most bacterial taxa were found in all ant-derived samples studied and because the bacteria detected on the ants' host plant revealed little overlap with this community, we regard our results as reliably representing the bacterial community that is associated with *P. ferrugineus*. Genera with a likely function as ant symbionts were *Burkholderia*, *Pantoea*, *Weissella*, and several members of the *Enterobacteriaceae*. The presence of these and various other groups was confirmed via independent PCR and cultivation approaches. Many of the bacteria that we detected belong to purportedly N-fixing taxa. Bacteria may represent important further partners in ant-plant mutualisms, and their influences on ant nutrition can contribute to the extraordinary abundance and evolutionary success of tropical arboreal ants.

Bacteria are ubiquitous and play important roles in almost every ecological community, including various functions as further partners in the interactions among higher organisms (5, 16, 19, 24, 36, 59). Unfortunately, we lack suitable cultivation protocols for many environmental bacteria, and our knowledge of their distribution, diversity, and ecological function thus remains restricted. Similarly, bacteria such as "*Candidatus* Blochmannia," *Buchnera*, *Rickettsia*, and *Wolbachia* are extremely common intracellular endosymbionts of insects (10, 22, 40, 46, 54), but many further symbionts of insects likely remain to be discovered. Therefore, we need methods for the unbiased description of the entire microbial community of insects in order to gain a more general overview of their effects on the ecology and evolution of their hosts.

Almost the same problems apply to ants. Ants are the dominant insect group and can comprise more than 80% of the arthropods in tropical rain forests (9, 23, 47), but it is not understood how ants can reach this abundance. Strikingly, ants reach much higher biomass values than their supposed prey organisms, a phenomenon termed "Tobin's ant biomass paradoxon" (8, 9, 47). It has been suggested that dominant ant taxa might be "more herbivorous" than originally assumed (8). However, plant-derived food sources appear too low in nitrogen to allow the construction of the ant's bodies, and the question thus remained open how these "vegetarian" ants can obtain balanced alimentation.

One possible explanation would be the existence of ant-microbe mutualisms (8, 59), because many insects depend on symbiotic bacteria for their nutrition (see, e.g., references 5, 36, and 57). *Camponotus* ants harbor intracellular "*Ca.* Blochmannia" symbionts (4), which play an important role in their hosts' nitrogen metabolism (16, 57, 59). Similarly, arboreal *Tetraponera* ants carry gut bacteria that might be able to recycle waste nitrogen or fix atmospheric nitrogen (46, 48). While many symbionts appear to be engaged in a mutualism with their insect host, *Wolbachia* has been reported to parasitize many insects through changing their sex ratios (25). The impact of *Wolbachia* infection in ants, however, remains enigmatic (52). In summary, no generalizations on the interactions between bacteria and ants are possible thus far, because our knowledge of bacteria that are associated with the most successful eusocial insects on earth remains scattered.

The goal of the present study was to obtain an impression of the entire bacterial community that is associated with the ant *Pseudomyrmex ferrugineus*. This species is an obligate plant ant, which defends its host plants against herbivores and encroaching vegetation and feeds exclusively on host-derived food rewards, that is, cellular food bodies and extrafloral nectar (6, 26, 27). Having a very specialized life history and depending on a well-defined type of strictly plant-derived alimentation, this species appeared particularly suitable for discovery of bacteria that could play a role in the success of "vegetarian" arboreal ants. Because larvae feed on food bodies while adult ants

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TABLE 1. Primers used in this work[a]

Primer	Sequence (5′ → 3′)	Reference
27f	GAGAGTTTGATCCTGGCTCAG	51
1494r	CTACGGCTACCTTGTTACGA	51
63f	CAGGCCTAACACATGCAAGTC	34
1387r	GGGCGGWGTGTACAAGGC	34
778r	AGGGTATCTAATCCTGTTTGC	42

[a] Primers for the bacterial 16S rRNA gene used in the present study. Used combinations: 27f + 1494r; 63f + 1387r; 63f + 778r; for tRFLP-PCR, 63f-6-FAM (modified with 6-carboxyfluorescein at 5′ end) + 778r.

appear to feed only on extrafloral nectar, both developmental stages were considered. We used the assignment tool TReFID (42), which employs in silico analysis of terminal restriction fragments (tRF) for the gross characterization of bacterial life in a sample, to answer the following questions: (i) Which bacterial taxa can be found to be associated with this ant species? (ii) Which of the TReFID-based identifications can be confirmed by classical cultivation and sequencing methods? (iii) Does TReFID reveal reproducible results? We finally conducted an extensive literature and database search in order to discuss likely functions of these bacteria in ant ecology and in the obligate mutualism which these ants have established with their specific host plants.

MATERIALS AND METHODS

Study site and organisms. The study was conducted at a site ca. 15 km W of Puerto Escondido in the coastal area of Oaxaca, México (15°55.596′N and 097°09.118′W; elevation, 15 m). *Pseudomyrmex ferrugineus* F. Smith obligatorily inhabits *Acacia* myrmecophytes (27) and feeds exclusively on the plant-derived food rewards (6) to the composition of which it is physiologically adapted (21, 29). The species has never been found nesting outside a myrmecophytic *Acacia* plant (27, 50; personal observations by P. S. Ward and M. Heil). Workers and larvae from three colonies collected directly from *Acacia hindsii* host plants were used for the analysis. Additionally, larvae of a fourth colony were used for seven independent analyses to proof for reproducibility of the TReFID approach.

TReFID software. The software TReFID (tRF identifying program [www.trefid.net]) allows scanning of an environmental sample for the presence of prokaryotic taxa that are present in its source database (15, 42). In short, TReFID applies in silico analysis of tRF obtained from digestions of fluorochrome-labeled PCR products of the 16S rRNA gene with multiple restriction enzymes (see the supplemental material). The current database comprises 22,239 entries from the NCBI database (www.ncbi.nlm.nih.gov). Since information on the taxonomy of many entries of the NCBI database needs revision, the results of the tRF length polymorphism (tRFLP) analyses were counterchecked with the Ribosomal Database Project II (http://rdp.cme.msu.edu/classifier/classifier.jsp) (49) and by a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For validation purposes, this technique was accompanied by conventional cloning and cultivation technologies.

Sample processing and amplification of procaryotic DNA. Since microbes living on the surfaces of ants can be mutualistic partners of interest (7), we did not sterilize the surfaces of the ants. For every colony, each six larvae and six workers were pooled, and prokaryotic DNA was extracted using the UltraClean Soil DNA kit according to the manufacturer's protocol (MoBio, Solana Beach, CA). Total DNA was used as a template for amplification of the 16S rRNA gene with the universal bacterial primer pairs 27F + 1495R (51) and 63F + 1387R (34) (Table 1). The PCR primer 63f was 5′ labeled with the fluorescent dye 6-carboxyfluorescein (Sigma-Aldrich, Germany).

Hot start PCR utilizing the Peqlab kit (a special kit free of *Escherichia coli* DNA) was carried out in a Peqlab cycler (Peqlab, Erlangen, Germany). The total reaction volume was 25 μl (2.5 μl PCR buffer, 5 μl enhancer solution, 1.5 μl 25 mM $MgCl_2$, 2 μl 25 mM deoxynucleoside triphosphate solution, 1 μl of each primer, 1 μl template DNA, and 11 μl H_2O); the PCR program consisted of 40 cycles (denaturation, 30 s at 94°C; annealing, 30 s at temperatures decreasing stepwise from 66 to 56°C; elongation, 45 s at 72°C). Only those PCR approaches were used for further steps whose corresponding zero sample showed no amplification. All procedures were performed as described previously (42).

The labeled PCR products were purified (QiaQuick spin columns; Qiagen, Hilden, Germany) and partitioned, and aliquots were subjected to restriction digests using up to 13 different enzymes in parallel. The restriction enzymes employed were AluI, Bme1390I, Bsh1236I, BsuRI (HaeIII), Cfr13I, Hin6I, HinfI, MboI, MspI, RsaI, TaiI, TaqI, and TasI (all from Fermentas, St. Leon-Roth, Germany). Digestion with AluI (AG/CT), Bme1390I (CC/NGG), Bsh1236I (CG/CG), Cfr13I (G/GNCC), HaeIII (GG/CC), Hin6I (C/GCG), HinfI (G/ANTC), MboI (/GATC), MspI (C/CGG), or RsaI (GT/AC) was performed at 37°C, and those with TaiI (ACGT/), TacI (T/CGA), and TasI (/AATT) were performed at 65°C. Digests were performed overnight in a total volume of 100 μl (25 μl purified labeled DNA solution, 10 μl restriction enzyme buffer, 1 μl enzyme, and 65 μl H_2O) at the temperatures indicated above. The restriction fragments were precipitated with ethanol and dissolved in 10 μl of deionized water.

Data evaluation with TReFID. Fragment size analysis was performed with an ABI 3730 instrument (Applied Biosystems) by a sequencing company (GENterprice Genomics, Mainz, Germany) using the size standard GeneScan (GS)-500 ROX (Applied Biosystems). Fragment sizes were determined by using the local Southern method, and raw data were handled in the Peak scanner software program, v1.0 (Applied Biosystems). Only fragments of a length between 25 and 500 bp were considered for TReFID analysis, as described earlier (42).

In short, all detected patterns of restriction fragments were compared to all entries of the TReFID database. Closely related bacteria may yield identical tRF patterns and then cannot be distinguished based only on TReFID results. Usually, this resolution limit occurs at the level of congeneric species, seldom at the level of genera that share very similar ribotypes. As a consequence, the score list often contains more entries than there were different tRF patterns (see the supplemental material). Our reports on the diversity of the different bacterial groups detected are therefore only at the individual taxonomic levels that could be reliably defined.

An important difference from other tRFLP approaches is that TReFID detects the presence or absence of tRF patterns, which are specific for a bacterial taxon, but does not quantify peak areas of certain fragment peaks; quantitative conclusions therefore cannot be drawn. However, TReFID allows a relative determination of the bacterial diversity in a sample depending on the number of different taxon-specific tRF patterns.

Culture conditions. To isolate bacteria for cultivation purposes, complete workers and larvae were homogenized in 1 ml sterile phosphate-buffered solution (isotonic, pH 7) and centrifuged at 200 × *g* for 5 min. The same procedure was applied to each three samples of leaves and extrafloral nectaries of *Acacia hindsii* plants, which were included to check for bacteria present in the ants' immediate environment. The supernatant then was plated on plates of agar or Gelrite (Roth, Karlsruhe, Germany) with YEM^+ medium (containing mannitol [10 g/liter] or sacharose [10 g/liter] or glucose [5 g/liter] and fructose [5 g/liter], Bacto-Trypton [0.4 g/liter] [Roth, Karlsruhe], yeast extract [0.4 g/liter] [AppliChem, Darmstadt, Germany], K_2HPO_4 [0.5 g/liter], $MgSO_4 \cdot 7H_2O$ [0.4 g/liter], NaCl [0.1 g/liter], and 1 ml/liter trace element solution SL8 [1]). This medium was chosen because of its transparency and durability, which allowed long-time cultivation of isolates. After differentiation, the isolated colonies could be selected based on their colony shape and/or color in order to obtain many purportedly different isolates for further analysis. All media were supplemented with cycloheximide (1 mg/ml medium) to prevent growth of fungi or protozoa. Plates were incubated at 30°C until colonies were clearly visible. Single colonies were then suspended in distilled water and used directly for PCR amplifications of the 16S rRNA gene using the primers described above.

Test for reproducibility. Total DNA of *P. ferrugineus* larvae of a fourth colony was extracted and a PCR performed as described above. The PCR amplificates were then used as a template for a PCR with fluorochrome-labeled primers in seven independent samples, and the products of each approach were divided into 12 portions for digestion with 12 different restriction enzymes. The tRF data that were obtained from these approaches were then independently analyzed using TReFID.

Nucleotide sequence accession numbers. Accession numbers (GenBank and NCBI; submitted 23 June 2008) of all sequences of bacteria associated with *P. ferrugineus* are as follows: EU841918 to EU841962, EU842025 to EU842031, EU842055 to EU842077, EU842079 to EU842087, and EU842098 to EU842110.

RESULTS

General patterns. The TReFID analysis of *P. ferrugineus* ants indicated the presence of 30 microbial classes (*Acidobacteria*, *Actinobacteria*, *Alphaproteobacteria*, *Anaerolineae*, *Aquifi-*

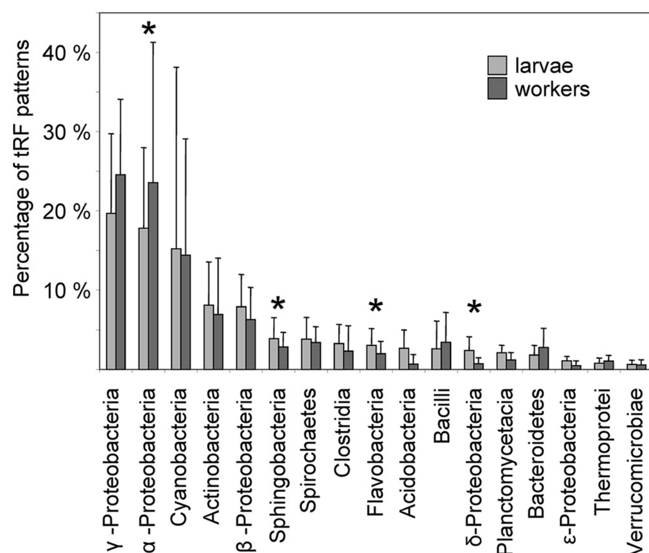

FIG. 1. Bacterial diversity in *Pseudomyrmex ferrugineus*. The relative contributions of tRF patterns (= tRF fingerprints) from different bacterial taxa are depicted separately for larvae ($n = 4$ colonies) and adult workers ($n = 3$ colonies). Statistically significant differences between the relative numbers of tRF patterns of larvae and workers are marked with an asterisk. Statistical evaluation was performed with the Statistica 6 software program (StatSoft) using the Mann-Whitney U test ($P < 0.05$ for *Alphaproteobacteria*, *Sphingobacteria*, *Flavobacteria*, and *Deltaproteobacteria*).

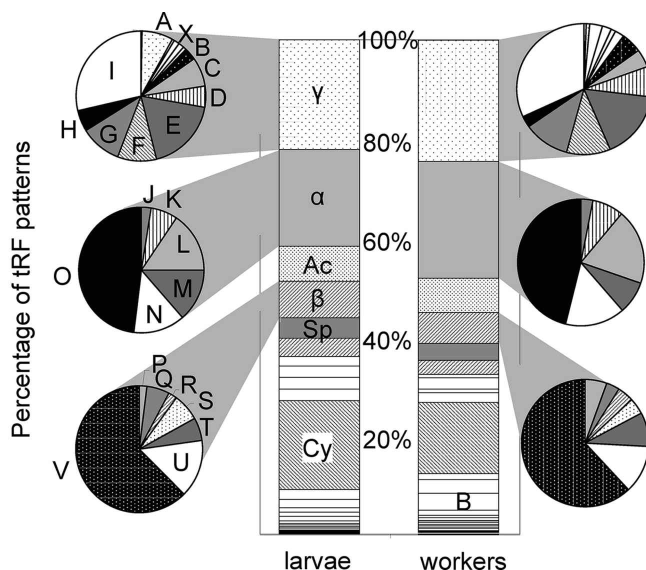

FIG. 2. Bacteria in larvae and workers of *P. ferrugineus*. The major relative contributions of tRF patterns (= tRF fingerprints) from different bacterial taxa are depicted separately for larvae ($n = 4$ colonies) and adult workers ($n = 3$ colonies), and the relative compositions of *Alpha*-, *Beta*-, and *Gammaproteobacteria* in both host life stages are presented. α-, β-, and γ, *Alpha*-, *Beta*-, and *Gammaproteobacteria*; Ac, *Actinobacteria*; B, *Bacilli*; Cy, *Cyanobacteria*; Sp, *Spirochates*; A, *Acidithiobacillales*; B, *Thiotrichales*; C, *Chromatiales*; D, *Xanthomonadales*; E, *Oceanospirillales*; F, *Enterobacteriales*; G, *Alteromonadales*; H, *Pasteurellales*, I, *Pseudomonadales*; J, *Rickettsales*; K, *Caulobacterales*; L, *Sphingomonadales*; M, *Rhodobacterales*; N, *Rhodospirillales*; O, *Rhizobiales*; P, *Nitrosomonadales*; Q, *Procabacteriales*; R, *Hydrogenophilales*; S, *Methylophilales*; T, *Neisseriales*; U, *Rhodocyclales*; V, *Burkholderiales*; X, taxa contributing less than 1% of tRF patterns.

cae, *Bacilli*, *Bacteroidetes*, *Betaproteobacteria*, *Chlorobia*, *Chloroflexi*, *Clostridia*, *Cyanobacteria*, *Deferribacteres*, *Deinococci*, *Deltaproteobacteria*, *Epsilonproteobacteria*, *Flavobacteria*, *Fusobacteria*, *Gammaproteobacteria*, *Gemmatimonadetes*, *Lentisphaerae*, *Mollicutes*, *Nitrospira*, *Planctomycetacia*, *Sphingobacteria*, *Spirochaetes*, *Thermoprotei*, *Thermotogae*, candidate division TM7, and *Verrucomicrobiae*). Eight divisions represented 93% of all tRF patterns that were obtained in the present study. These divisions are *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Firmicutes*, *Planctomycetes*, *Proteobacteria*, and *Spirochaetes* (Fig. 1).

The relative proportions of tRF patterns associated with larvae and adult workers were very similar (Fig. 2) and were dominated by the *Proteobacteria*, which represented some 40% of all patterns, comprising 5 classes and 39 families (Fig. 1 and 2). For both developmental stages, 48% of the diversity of the *Alphaproteobacteria* was represented by the *Rhizobiales* (*Rhizobium* and *Bartonella*), followed by the *Rhodospirillales*, *Rhodobacteriales*, and *Sphingomonadales* (Fig. 2) (each group representing ca. 15% of the *Alphaproteobacteria*). tRF patterns indicating the presence of relatives of the *Rickettsiales* (*Wolbachia*) were detected in all sampled colonies. The *Betaproteobacteria* were dominated by the *Burkholderiales* (ca. 62%), while the most diverse groups within the *Gammaproteobacteria* were the *Pseudomonadales* (30%), *Oceanospirillales* (19%), and the *Enterobacteriales* (10%) (Fig. 2).

From the leaves and nectaries of the host plants, cultivation approaches identified only *Bacillus*, *Sphingomonas*, and *Methylobacterium* species (Table 2). The bacterial community associated with *P. ferrugineus* was thus clearly different from the community of the host organs with which the ants have most intimate contact.

Method validation. Repeated processing and analysis of subsamples revealed a high reproducibility of the tRFLP method (Fig. 3). Two thousand five hundred fifty (47%) of 5,401 tRF patterns found in this sample were identified in all 7 repetitions, and only 11 (0.2%) tRF patterns were found in 3 or

TABLE 2. Bacteria from *Acacia hindsii* host plants

Site(s) of isolation	Closest relative (NCBI BLAST) and accession numbers[a]	Closest relative (% identity[b])
Nectaries	*Bacillus* EU193043 FJ413048	*Bacillus* (100)
Leaves	*Methylobacterium* EU860987 AB299701	*Methylobacterium* (100)
Leaves and nectaries	*Sphingomonas* EU839652 AY894691 EU448284 FJ204417	*Sphingomonas* (100)

[a] GenBank (NCBI) accession no. of most closely related organism is given.
[b] Percent identity with sequences in database of Ribosomal Database Project II (Michigan State University [http://rdp.cme.msu.edu/classifier/classifier.jsp]).

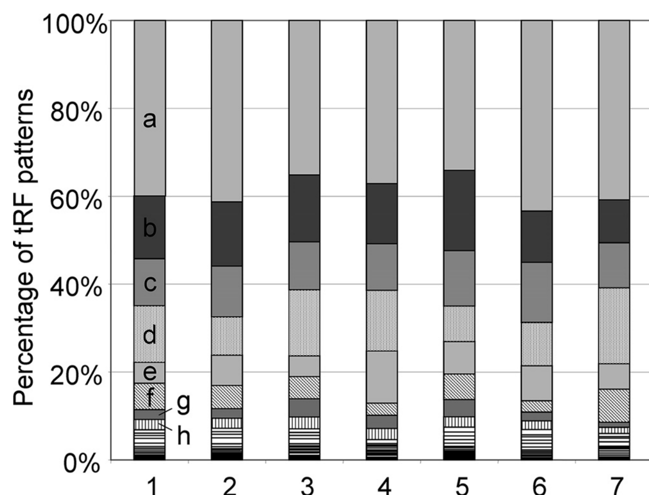

FIG. 3. Reproducibility of TReFID-obtained results. The relative composition of tRF patterns (= tRF fingerprints) of the bacterial community in larvae of *P. ferrugineus* ants is depicted separately for seven independent analyses (1 to 7) of the same original sample. The divisions *Proteobacteria* (a), *Firmicutes* (b), *Bacteroidetes* (c), *Actinobacteria* (d), *Cyanobacteria* (e), *Acidobacteria* (f), *Spirochaetes* (g), and *Planctomycetes* (h) represent about 93% of all detected tRF patterns.

fewer of the parallel samples. The diversity of tRF patterns in all seven repetitions was quantitatively dominated by the *Proteobacteria*, followed by the *Firmicutes*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Acidobacteria*, *Spirochaetes*, and *Planctomycetes* (Fig. 3).

When tests with an artificial mixture of *Escherichia coli*, *Azospirillum brasilense*, and *Rhizobium leguminosarum* were conducted using 10 different restriction enzymes, we obtained 75 tRF patterns specific for *E. coli*, 5 for *A. brasilense*, and 47 for *R. leguminosarum*. Therefore, the method used successfully identified all bacteria present in the sample and revealed no false identification, but the number of specific patterns detected by TReFID for each taxon was not proportional to the abundance of bacteria.

Using standard cultivation approaches, relatives of *Acinetobacter*, *Bacillus*, *Burkholderia*, *Cupriavidus*, *Mesorhizobium*, *Pandoraea*, *Pantoea*, *Serratia*, *Streptomyces*, *Variovorax*, and *Weissella* were confirmed successfully (Table 3). Sequencing of the 16S rRNA gene of cultured microbes added some further genera that were identified using the naive Bayesian rRNA classifier, version 2.0 (49), as *Curtobacterium*, *Erwinia*, and *Streptococcus* (Table 3). In total, 16 of 115 sequences retrieved from sequencing could be assigned to the *Enterobacteriaceae* and 11 to the *Lactobacillales*. All taxa that were identified via sequencing or cultivation were comprised in the TReFID result lists (see Table 3).

DISCUSSION

Symbiotic bacteria exhibit many effects on their insect hosts, including manipulation of their hosts' sex ratio and life cycle (35, 37, 55), dispersal and fecundity (32), host plant choice by herbivorous insects (24), the success of pest species on particular host plants (32), and even host protection from pathogen infection (5, 19). Both the presence and the effects of such bacteria depend, however, on environmental conditions, such as the temperature regime and host nutrition (5, 11, 43), and on the host genotype (10). Because detailed studies of certain preselected taxa of microorganisms can give only a restricted impression of the overall microbial diversity, unbiased and fast characterizations of the gross bacterial communities associated with insects are required to fully understand their ecological and ultimately evolutionary importance. We therefore searched for a rapid and cost-effective method allowing the unbiased detection and preliminary characterization of putative bacterial symbionts of ants.

The bacterial community associated with *P. ferrugineus* turned out to be much more diverse than could have been expected from earlier studies of other ant species (16, 33, 46, 52, 57, 59). Our study revealed tRF pattern indicating the presence of 30 prokaryotic classes, while earlier studies reported 4 (48) or 2 (46) different classes of microorganisms from related ants. Does this result point to artifacts or rather to the presence of a yet-undiscovered biodiversity? When discussing this surprisingly high diversity of tRF patterns, we must recall that little is known about the diversity of environmental bacteria (39), particularly those that are associated with insects. Microorganisms in termites, for instance, have been investigated for a long time, but still an ever-increasing number of microorganisms are newly discovered and described for termites (13, 14, 17, 30, 41, 53). Similarly, our study aimed at getting an impression of an as yet undiscovered diversity of microorganisms.

We analyzed tRF patterns to study the bacterial community associated with *P. ferrugineus*, an obligate plant ant that lives on a strictly host plant-derived diet (6). Several TReFID results were confirmed by classical cultivation and sequencing approaches (Table 3). In seven independent analyses of the same original sample, 47% out of all tRF patterns were identified in all repetitions and only 0.2% of the patterns showed up in fewer than half of the samples, which indicates a high reproducibility of the method chosen (Fig. 3). Moreover, the analysis of an artificially mixed bacterial sample successfully identified all bacteria added and did not reveal false identifications. We therefore regard our results as qualitatively reliable. However, many more tRF patterns representative of *E. coli* and *R. leguminosarum* than of *A. brasilense* were found in the artificial samples, although the same amounts of all three species had been used. The number of specific patterns detected by TReFID for a taxon is thus not proportional to the abundance of bacteria and apparently depends in part on the composition of the database, which in fact contains more entries of different *Escherichia* or *Rhizobium* strains than of *Azospirillum*. Classical diversity indices, such as the Shannon index (45), therefore cannot be applied to data obtained with TReFID because the program was developed as a qualitative identification tool for diverse microbial communities (42).

The bacterial associates of *P. ferrugineus* were dominated by the three groups of *Proteobacteria* (*Alpha*-, *Beta*-, and *Gammaproteobacteria*), which comprise most of the known symbionts of ants ("*Ca.* Blochmannia," *Gammaproteobacteria*; *Wolbachia*, *Alphaproteobacteria*; gut symbionts of *Tetraponera*, *Alpha*-, *Beta*-, and *Gammaproteobacteria*). Several genera have already been reported as symbionts of other insects (Table 4),

TABLE 3. Congruency among bacterial taxa identified with TReFID with those confirmed by classical approaches[a]

Class	Order	Genus	TReFID score (%)[b]	Confirmed by classical approach via:	
				PCR	Cultivation
Acidobacteria	*Acidobacteriales*		100		
Actinobacteria	*Actinomycetales*	*Actinomyces*	94		
		Corynebacterium	100		
		Curtobacterium	83	*Curtobacterium*	
		Mycobacterium	94		
		Streptomyces	100		*Streptomyces*
	Bifidobacteriales	*Bifidobacterium*	100		
		Scardovia	95		
	Coriobacteriales		100		
	Rubrobacterales		100		
Alphaproteobacteria	*Rhizobiales*	*Afipia*	100		
		Azorhizobium	100		
		Bartonella	100		
		Bosea	91		
		Bradyrhizobium	100		
		Devosia	95		
		Ensifer	100		
		Fulvimarina	95		
		Hyphomicrobium	91		
		Mesorhizobium	93		*Mesorhizobium*
		Ochrobactrum	100		
	Rhodobacteriales	*Paracoccus*	95		
	Sphingomonadales		100		
	Rhodospirillales	*Acetobacter*	100		
		Azospirillum	90		
		Stella	100		
		Swaminathania	100		
	Caulobacteriales	*Brevundimonas*	100		
		Caulobacter	100		
		Phenylobacterium	90		
	Rickettsiales	*Anaplasma*	95		
		Neorickettsia	89		No cultivation methods available
		Rickettsia	100		
		Wolbachia	100		
Betaproteobacteria	*Burkholderiales*	*Achromobacter*	100		
		Acidovorax	100		
		Alicycliphilus	93		
		Burkholderia	100	*Burkholderia*	*Burkholderia*
		Comamonas	100		
		Cupriavidus	100	Unclassified *Burkholderiales*	*Cupravidus*
		Dechloromonas	100		
		Delftia	100		
		Herbaspirillum	100		
		Janthinobacterium	100		
		Limnobacter	100		
		Massilia	100		
		Pandoraea	95		*Pandoraea*
		Variovorax	88	*Variovorax*	*Variovorax*
					Unclassified Incertae sedis 5
	Rhodocyclales		100		
	Neisseriales	*Laribacter*	100		
Gammaproteobacteria	*Pseudomonadales*	*Acinetobacter*	100	Unclassified *Gammaproteobacteria*	*Acinetobacter*
		Pseudomonas	100		
	Oceanospirillales				
	Xanthomonadales	*Zooshikella*	100		
	Enterobacteriales	*Buchnera*	90	Unclassified *Enterobacteriaceae*	
		Buttiauxella	100		
		Citrobacter	90		
		Dickeya	100		
		Enterobacter	91		

Continued on following page

TABLE 3—*Continued*

Class	Order	Genus	TReFID score (%)[b]	Confirmed by classical approach via:	
				PCR	Cultivation
		Erwinia	85	*Erwinia*	
		Morganella	91		
		Pantoea	91	*Pantoea*	*Pantoea*
		Serratia	95	*Serratia*	*Serratia*
		Shewanella	100		
		Sodalis	86		
	Alteromonadales	*Alteromonas*	100		
		Shewanella	100		
	Chromatiales	*Allochromatium*	100		
	Aeromonadales	*Anaerobiospirillum*	100		
Deltaproteobacteria	*Bdellovibrionales*	*Bdellovibrio*	100		
	Desulfovibrionales	*Bilophila*	100		
Firmicutes	*Bacillales*	*Bacillus*	100	*Bacillus*	*Bacillus*
		Kurthia	100		
		Listeria	100		
		Paenibacillus	100		
	Lactobacillales	*Enterococcus*	100		
		Geobacillus	95		
		Lactobacillus	100		
		Leuconostoc	100		
		Streptococcus	100	*Streptococcus*	
		Symbiobacterium	89		
		Weissella	83	*Weissella*	*Weissella*
Bacterioidetes	*Bacteroidales*		100		Hard to cultivate
Cyanobacteria	Family 1.1		100		Hard to cultivate
	Family 3.1	*Arthrospira*	100		
		Oscillatoria	100		
	Family 4.1	*Nostoc*	100		
		Aphanizomenon	100		
		Cylindrospermum	100		
Spirochaetes	*Spirochaetales*	*Borrelia*	100		Hard to cultivate
		Brachyspira	100		
		Brevinema	100		
		Leptospira	100		
		Treponema	100		

[a] The listed TReFID results (class, order, and genus) comprise 94% of all tRFLP patterns that could be found in the samples; classical approaches applied for the detection of bacteria in environmental samples were a PCR with universal bacterial primers and cultivation. Thirteen out of 86 genera that had been identified with TReFID could be detected with the classical approaches, while all genera found in the classical approaches also showed up in the TReFID results.

[b] The TReFID score refers to the percent similarity of the tRFLP pattern detected with patterns published in the database.

while others are generally regarded as typical symbionts of plants rather than animals (see Table S1 in the supplemental material) (*Azorhizobium*, *Bradyrhizobium*, *Devosia*, *Ensifer*, and *Mesorhizobium* [*Rhizobiales*], *Swaminathania* [*Rhodospiralles*], and *Acidovorax* [*Burkholderiales*]) or they are usually considered free-living terrestrial or marine bacteria (Table 4) (*Fulvimarina* and *Hyphomicrobium* [*Rhizobiales*] and *Acetobacter* [*Rhodospirillales*]). However, only three of these taxa could be cultivated from samples taken from the surfaces of the *Acacia* host plants (Table 2), although the same media allowed the cultivation of many more bacteria from the ant samples (Table 3). We therefore conclude that the majority of the bacteria that we identified exclusively in the ant-derived samples indeed are regularly associated with *P. ferrugineus* rather than representing environmental contaminations.

We found almost no overlap between bacteria associated with the ants and those that could be cultivated from plant-derived samples. This observation opens the question of where the ants obtain the bacteria. Extrafloral nectar of *Acacia* myrmecophytes possesses highly active pathogenesis-related proteins, such as chitinases, glucanases, and peroxidases, which keep this nectar virtually free of microbes (18). The extrafloral nectar can therefore be excluded as a major source of bacteria. All larvae, workers, and reproductive individuals in an ant colony are continuously exchanging gut content via trophallaxis (23). Horizontal (that is, among members of the same generation) and vertical (that is, transgenerational) transfers of bacteria within an ant colony are thus highly likely. This assumption is in line with the observation that samples of larvae and adult workers revealed very similar bacterial communities (Fig. 1 and 2), although the two stages feed on different food sources. A direct and continuous ingestion of food-derived bacteria therefore cannot explain the patterns found in the present study. However, future studies will have to examine in

TABLE 4. Bacteria found in *Pseudomyrmex ferrugineus* that have been described previously as symbionts of other ants[a]

Reported classification (reference)	Genus (accession no.)	Related genera found in *P. ferrugineus* with TReFID[b]	Ribosomal classifier value (%)[c]	TReFID score (%)[d]
Tetraponera (48)	***Rhizobium*** (AF459798)	***Rhizobium***	100	77–100
		Azorhizobium	100	90–100
		Bradyrhizobium	100	Up to 92
		*Mesorhizobium****	100	Up to 92
		Sinorhizobium	94	83
	Methylobacterium (AF459799)	***Methylobacterium***	100	78–100
		Microvirga	95	80–90
Tetraponera (48)	***Burkholderia*** (AF459796)	***Burkholderia******	100	80–100
		Achromobacterium	100	78–100
		Acidovorax	100	78–100
Atta (44)		*Alcaligenes*	100	Up to 95
		Aquabacterium	100	77–100
		Bordetella	100	Up to 95
		Caenibacterium	100	Up to 95
		Chitinimonas	100	80–100
		Collimonas	100	80–100
		*Cupriavidus****	100	77–100
		Delftia	100	77–100
		*Herbaspirillum****	100	77–100
		Janthinobacterium	100	83–100
		*Pandoraea****	100	Up to 95
		Ralstonia	100	Up to 91
Tetraponera (48)	***Pseudomonas*** (AF459797)	***Pseudomonas***	100	80
		Cellvibrio	100	80–100
	Flavobacterium (AF459795)	***Flavobacterium***	100	78–100
		Aequorivita	100	Up to 91
		Bergeyella	100	Up to 95
		Capnocytophaga	100	77–100
		Cellulophaga	100	Up to 90
		Chryseobacterium	100	77–100
		Elizabethkingia	100	Up to 86
Tetraponera, *Dolichoderus* (46)	***Bartonella***	***Bartonella***	100	80–100
	Sodalis	***Sodalis***	100	78–83
	Enterobacter	***Enterobacter******	100	Up to 85
	Pantoea	***Pantoea******	100	82
		Buchnera	100	78–90
		"*Ca.* Blochmannia"	94	79
		Citrobacter	100	Up to 90
		*Erwinia****	100	Up to 85
		Escherichia	100	80–90
		Klebsiella	100	78–91
		Kluyvera	100	78–91
		Morganella	100	80–91
		Salmonella	100	78–88
		*Serratia****	100	78–95
	Wolbachia	***Wolbachia***	100	Up to 100
		Rickettsia	100	78–100
	Bacillus	***Bacillus******	100	77–100
		Exiguobacterium	100	78–94
		Geobacillus	100	77–90
		Ureibacillus	100	86–100
	Leptospira	***Leptospira***	100	79–100
		Leptonema	100	85
	Brevibacterium	***Brevibacterium***	100	78–100
Acromyrmex (56)	***Pseudonocardia***	***Pseudonocardia***	100	Up to 88

[a] Genera reported from other bacterium-insect symbioses appear in bold letters; closely related genera detected in *P. ferrugineus* are additionally listed when they were highly supported.

[b] Genera marked with three asterisks were independently confirmed by classical cultivation methods.

[c] Percent identity with sequences in database Ribosomal Database Project II (Michigan State University [http://rdp.cme.msu.edu/classifier/classifier.jsp]).

[d] The TReFID score refers to the percent similarity of the tRFLP pattern detected with patterns published in the database.

detail whether the high bacterial diversity that we encountered in association with *P. ferrugineus* is indeed transmitted from one generation to the other, as has been described for (endo)symbiotic bacteria (59).

While several taxa represented typical intestinal bacteria (*Enterobacteria*), we detected seven taxa of potentially N-fixing organisms (*Rhizobiales*, *Burkholderiales*, *Pseudomonadales*, *Spirochaeta*, *Cyanobacteria*, and the two genera of the *Gammaproteobacteria*, *Pantoea* and *Serratia*), which comprised a large part of the overall bacterial diversity (Fig. 2). The last two genera were identified by tRFLP, sequencing, and cultivation (Table 3) and thus were beyond doubt present in or on *P. ferrugineus*.

N-fixing gut bacteria or N-recycling intracellular bacteria can be an important general source of diet supplementation for arthropods that feed on nitrogen-poor food sources (2, 31, 36). For example, termites can feed exclusively on wood or cellulose only due to their association with microorganisms, some of which (*Treponema* and *Pantoea*) are capable of nitrogen fixation (3, 38). Interestingly, *Pantoea* has already been found in the gut flora of *Tetraponera pilosa* (46), and its presence in the *P. ferrugineus* samples was confirmed via tRFLP, 16S rRNA sequencing, and cultivation (Table 3). Little further knowledge exists for ants, but carpenter ants (*Camponotus*) harbor intracellular bacterial symbionts (4), which apparently function as an N-recycling system (58). These "*Ca.* Blochmannia" bacteria are capable of synthesizing essential amino acids and play a role in their host's nitrogen metabolism (16, 57, 59). Similarly, aphids are able to live exclusively on amino acid-deficient phloem sap because they are associated with N-recycling *Buchnera* bacteria that provide their host with essential amino acids (12), and tRF patterns very similar to those of *Buchnera* were detected in all of our samples. N recycling and N fixation by symbiotic bacteria have been suggested as a general explanation for the tremendous ecological success of arboreal ants, since δ-^{15}N ratios of ants from Amazonia and Borneo demonstrated that many arboreal species obtain little nitrogen through predation (8). Although final experimental evidence for this hypothesis is lacking, our findings demonstrate that putatively N-fixing bacteria in ants can be much more common and taxonomically diverse than has been known so far.

We decided not to sterilize the surface of the ants used here, since important bacterial symbionts of fungus-growing ants were reported to live on the exoskeleton (7). Although typical gut bacteria comprise a large part of the microorganisms that were identified in the present study, it is not possible to decide which percentage of the bacteria that we have found to be associated with *P. ferrugineus* represents endosymbionts. However, all samples displayed a very similar composition of the bacterial community, demonstrating that most of these bacteria are commonly associated with these ants and that they likely represent symbionts. Nevertheless, some results, such as the presence of cyanobacteria, remain enigmatic and require further investigation of the putative role of these bacteria in the life of ants.

Which other functions might be fulfilled by the bacterial associates of *P. ferrugineus*? All samples tested positive for infection with *Rickettsiales*-like bacteria. *Burkholderia* (confirmed by all three attempts; Table 3) was isolated from fungus gardens of the leaf cutting ant *Atta sexdens rubropilosa* and found to produce antibiotics (44) and was also found in *Tetraponera* ants (48). Interestingly, patterns of bacterial genera found in lower termites and in our present study were strikingly similar: even some uncommon groups, such as the *Bacteroidales*, methanogenic *Archaea*, and methanotrophic bacteria, were found in termites (see Table S1 in the supplemental material) and in *P. ferrugineus* samples. At least some of these bacteria appear related to fermentation processes in the insect gut, since we detected members of *Lactobacillales*, *Enterobacteriales*, *Bacillales*, *Bifidobacteriales*, and *Clostridiales*. Members of *Lactobacillales* and *Enterobacteriales* are also well known from other hymenopterans, such as honey bees (28).

Conclusions. We are just starting to realize the importance of associations among bacteria and ants, and much more has to be done before we can understand the roles that bacteria play in the life of ants, which represent the most successful eusocial insects, or in the mutualisms of ants with higher organisms. Unbiased investigation of the entire bacterial flora of ants—or other arthropods—appears to be an important step toward the discovery of as yet unknown bacteria that fulfill this or other ecologically important functions.

TReFID turned out to be a highly suitable approach to obtain a first insight into the high diversity of the bacterial community which was found to be regularly associated with the ant *P. ferrugineus*. Both the high overall diversity detected and the occurrence of various potentially N-fixing taxa point to important and manifold roles of these bacteria in the life style of *P. ferrugineus* as an obligate plant ant. Apparently, we must widen the current concept of ant-plant mutualisms (20) to include bacterial partners, which might alter the ants' physiological capacities and thereby significantly affect their life-style as obligate plant ants and specialized "vegetarians."

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REFERENCES

1. **Bast, E.** 1999. Mikrobiologische Methoden. Spektrum Akademischer Verlag, Heidelberg, Germany.
2. **Behar, A., B. Yuval, and E. Jurkevitch.** 2005. Enterobacteria-mediated nitrogen fixation in natural populations of the fruit fly *Ceratitis capitata*. Mol. Ecol. **14:**2637–2643.
3. **Benemann, J. R.** 1973. Nitrogen-fixation in termites. Science **181:**164–165.
4. **Buchner, P.** 1965. Endosymbiosis of animals with plant microorganisms. J. Wiley & Sons, New York, NY.
5. **Chandler, S. M., T. L. Wilkinson, and A. E. Douglas.** 2008. Impact of plant nutrients on the relationship between a herbivorous insect and its symbiotic bacteria. Proc. R. Soc. Lond. B **275:**565–570.
6. **Clement, L. W., S. Köppen, W. A. Brand, and M. Heil.** 2008. Strategies of a parasite of the ant-Acacia mutualisms. Behav. Ecol. Sociobiol. **26:**953–962.
7. **Currie, C. R., M. Poulsen, J. Mendenhall, J. J. Boomsma, and J. Billen.** 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science **311:**81–83.
8. **Davidson, D. W., S. C. Cook, R. R. Snelling, and T. H. Chua.** 2003. Explaining the abundance of ants in lowland tropical rainforest canopies. Science **300:**969–972.
9. **Davidson, D. W., and L. Patrell-Kim.** 1996. Tropical arboreal ants: why so abundant? p. 127–140. *In* A. C. Gibson (ed.), Neotropical biodiversity and conservation. Mildred E. Mathias Botanical Garden, University of California, Los Angeles.
10. **Dean, M. D.** 2006. A *Wolbachia*-associated fitness benefit depends on genetic background in *Drosophila simulans*. Proc. R. Soc. Lond. B **273:**1415–1420.
11. **de Vries, E. J., G. Jacobs, M. W. Sabelis, S. B. J. Menken, and J. A. J.**

Breeuwer. 2004. Diet-dependent effects of gut bacteria on their insect host: the symbiosis of *Erwinia* sp and western flower thrips. Proc. R. Soc. Lond. B **271:**2171–2178.

12. **Douglas, A. E.** 2006. Phloem-sap feeding by animals: problems and solutions. J. Exp. Bot. **57:**747–754.
13. **Droge, S., J. Frohlich, R. Radek, and H. König.** 2006. *Spirochaeta coccoides* sp. nov., a novel coccoid spirochete from the hindgut of the termite *Neotermes castaneus*. Appl. Environ. Microbiol. **72:**392–397.
14. **Droge, S., R. Rachel, R. Radek, and H. König.** 2008. *Treponema isoptericolens* sp nov., a novel spirochaete from the hindgut of the termite *Incisitermes tabogae*. Int. J. Syst. Evol. Microbiol. **58:**1079–1083.
15. **Eilmus, S., C. Roesch, and H. Bothe.** 2007. Prokaryotic life in a potash-polluted marsh with emphasis on N-metabolizing microorganisms. Environ. Pollut. **146:**478–491.
16. **Feldhaar, H., J. Straka, M. Krischke, K. Berthold, S. Stoll, M. J. Mueller, and R. Gross.** 2007. Nutritional upgrading for omnivorous carpenter ants by the endosymbiont *Blochmannia*. BMC Biol. **5:**48.
17. **Frohlich, J., C. Koustiane, P. Kampfer, R. Rossello-Mora, M. Valens, M. Berchtold, T. Kuhnigk, H. Hertel, D. K. Maheshwari, and H. König.** 2007. Occurrence of rhizobia in the gut of the higher termite *Nasutitermes nigriceps*. Syst. Appl. Microbiol. **30:**68–74.
18. **González-Teuber, M., S. Eilmus, A. Muck, A. Svatos, and M. Heil.** 2009. Pathogenesis-related proteins protect extrafloral nectar from microbial infestation. Plant J. **58:**464–473.
19. **Haine, E. R.** 2008. Symbiont-mediated protection. Proc. R. Soc. Lond. B **275:**353–361.
20. **Heil, M., and D. McKey.** 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu. Rev. Ecol. Evol. Syst. **34:**425–453.
21. **Heil, M., J. Rattke, and W. Boland.** 2005. Post-secretory hydrolysis of nectar sucrose and specialization in ant/plant mutualism. Science **308:**560–563.
22. **Hilgenboecker, K., P. Hammerstein, P. Schlattmann, A. Telschow, and J. H. Werren.** 2008. How many species are infected with Wolbachia? A statistical analysis of current data. FEMS Microbiol. Lett. **281:**215–220.
23. **Hölldobler, B., and E. O. Wilson.** 1990. The ants. Springer, Berlin, Germany.
24. **Hosokawa, T., Y. Kikuchi, M. Shimada, and T. Fukatsu.** 2007. Obligate symbiont involved in pest status of host insect. Proc. R. Soc. Lond. B **274:**1979–1984.
25. **Hurst, G. D. D., L. D. Hurst, and M. E. N. Majerus.** 1993. Problems and paradigms: altering sex ratios: the games microbes play. BioEssays **15:**695–697.
26. **Janzen, D. H.** 1967. Interaction of the bull's-horn acacia (Acacia cornigera L.) with an ant inhabitant (Pseudomyrmex ferruginea F. Smith) in eastern Mexico. Kans. Univ. Sci. Bull. **47:**315–558.
27. **Janzen, D. H.** 1974. Swollen-thorn acacias of Central America, vol. 13. Smithsonian Institution Press, Washington, DC.
28. **Kacaniova, M., R. Chlebo, M. Kopernicky, and A. Trakovicka.** 2004. Microflora of the honeybee gastrointestinal tract. Folia Microbiol. **49:**169–171.
29. **Kautz, S., H. T. Lumbsch, P. S. Ward, and M. Heil.** 2009. How to prevent cheating: a digestive specialization ties mutualistic plant-ants to their ant-plant partners. Evolution **63:**839–853.
30. **Kohler, T., U. Stingl, K. Meuser, and A. Brune.** 2008. Novel lineages of planctomycetes densely colonize the alkaline gut of soil-feeding termites (*Cubitermes* spp.). Environ. Microbiol. **10:**1260–1270.
31. **Kuranouchi, T., T. Nakamura, S. Shimamura, H. Kojima, K. Goka, K. Okabe, and A. Mochizuki.** 2006. Nitrogen fixation in the stag beetle, *Dorcus* (*Macrodorcus*) *rectus* (Motschulsky) (Col., Lucanidae). J. Appl. Entomol. **130:**471–472.
32. **Leonardo, T. E., and E. B. Mondor.** 2006. Symbiont modifies host life-history traits that affect gene flow. Proc. R. Soc. Lond. B **273:**1079–1084.
33. **Li, H. W., F. Medina, S. B. Vinson, and C. J. Coates.** 2005. Isolation, characterization, and molecular identification of bacteria from the red imported fire ant (*Solenopsis invicta*) midgut. J. Invertebr. Pathol. **89:**203–209.
34. **Marchesi, J. R., T. Sato, A. J. Weightman, T. A. Martin, J. C. Fry, S. J. Hiom, and W. G. Wade.** 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. Appl. Environ. Microbiol. **64:**795–799.
35. **Nakanishi, K., M. Hoshino, M. Nakai, and Y. Kunimi.** 2008. Novel RNA sequences associated with late male killing in *Homona magnanima*. Proc. R. Soc. Lond. B **275:**1249–1254.
36. **Nardi, J. B., R. I. Mackie, and J. O. Dawson.** 2002. Could microbial symbionts of arthropod guts contribute significantly to nitrogen fixation in terrestrial ecosystems? J. Insect Physiol. **48:**751–763.
37. **Negri, I., M. Pellecchia, P. J. Mazzoglio, A. Patetta, and A. Alma.** 2006. Feminizing Wolbachia in Zyginidia pullula (Insecta, Hemiptera), a leafhopper with an XX/XO sex-determination system. Proc. R. Soc. Lond. B **273:**2409–2416.
38. **Ohkuma, M., S. Noda, and T. Kudo.** 1999. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl. Environ. Microbiol. **65:**4926–4934.
39. **Pace, N. R.** 1997. A molecular view of microbial diversity and the biosphere. Science **276:**734–740.
40. **Perlman, S. J., M. S. Hunter, and E. Zchori-Fein.** 2006. The emerging diversity of *Rickettsia*. Proc. R. Soc. Lond. B **273:**2097–2106.
41. **Pittman, G. W., S. M. Brumbley, P. G. Allsopp, and S. L. O'Neil.** 2008. "Endomicrobia" and other bacteria associated with the hindgut of *Dermolepida albohirtum* larvae. Appl. Environ. Microbiol. **74:**762–767.
42. **Roesch, C., and H. Bothe.** 2005. Improved assessment of denitrifying, nitrogen-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. Appl. Environ. Microbiol. **71:**2026–2035.
43. **Russell, J. A., and N. A. Moran.** 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc. R. Soc. Lond. B **273:**603–610.
44. **Santos, A. V., R. J. Dillon, V. M. Dillon, S. E. Reynolds, and R. I. Samuels.** 2004. Occurrence of the antibiotic producing bacterium *Burkholderia* sp in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. FEMS Microbiol. Lett. **239:**319–323.
45. **Shannon, C. E.** 1948. A mathematical theory of communication. Bell Syst. Tech. J. **27:**379–423, 623–656.
46. **Stoll, S., J. Gadau, R. Gross, and H. Feldhaar.** 2007. Bacterial microbiota associated with ants of the genus *Tetraponera*. Biol. J. Linn. Soc. **90:**399–412.
47. **Tobin, J. E.** 1995. Ecology and diversity of tropical forest canopy ants, p. 129–147. *In* M. D. Lowman and N. M. Nadkarni (ed.), Forest canopies. Academic Press, San Diego, CA.
48. **Van Borm, S., A. Buschinger, J. J. Boomsma, and J. Billen.** 2002. *Tetraponera* ants have gut symbionts related to nitrogen-fixing root-nodule bacteria. Proc. R. Soc. Lond. B **269:**2023–2027.
49. **Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73:**5261–5267.
50. **Ward, P. S.** 1993. Systematic studies on *Pseudomyrmex* acacia-ants (Hymenoptera: Formicidae: Pseudomyrmecinae). J. Hymenopt. Res. **2:**117–168.
51. **Weisburg, W. G., S. M. Barns, D. A. Pelletier, and D. J. Lane.** 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. **173:**697–703.
52. **Wenseleers, T., L. Sundstrom, and J. Billen.** 2002. Deleterious *Wolbachia* in the ant *Formica truncorum*. Proc. R. Soc. Lond. B **269:**623–629.
53. **Wenzel, M., I. Schonig, M. Berchtold, P. Kampfer, and K. König.** 2002. Aerobic and facultatively anaerobic cellulolytic bacteria from the gut of the termite *Zootermopsis angusticollis*. J. Appl. Microbiol. **92:**32–40.
54. **Werren, J. H., D. Windsor, and L. R. Guo.** 1995. Distribution of Wolbachia among neotropical arthropods. Proc. R. Soc. Lond. B **262:**197–204.
55. **Wolschin, F., B. Holldobler, R. Gross, and E. Zientz.** 2004. Replication of the endosymbiotic bacterium *Blochmannia floridanus* is correlated with the developmental and reproductive stages of its ant host. Appl. Environ. Microbiol. **70:**4096–4102.
56. **Zhang, M. M., M. Poulsen, and C. R. Currie.** 2007. Symbiont recognition of mutualistic bacteria by *Acromyrmex* leaf-cutting ants. ISME J. **1:**313–320.
57. **Zientz, E., N. Beyaert, R. Gross, and H. Feldhaar.** 2006. Relevance of the endosymbiosis of Blochmannia floridanus and carpenter ants at different stages of the life cycle of the host. Schriftenr. Vegetationskd. **72:**6027–6033.
58. **Zientz, E., T. Dandekar, and R. Gross.** 2004. Metabolic interdependence of obligate intracellular bacteria and their insect hosts. Microbiol. Mol. Biol. Rev. **68:**745–770.
59. **Zientz, E., H. Feldhaar, S. Stoll, and R. Gross.** 2005. Insights into the microbial world associated with ants. Arch. Microbiol. **184:**199–206.