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ORIGINAL PAPER

Non-specific association between filamentous bacteria and fungus-growing ants

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Abstract Fungus-growing ants and their fungal cultivar form a highly evolved mutualism that is negatively affected by the specialized parasitic fungus *Escovopsis*. Filamentous *Pseudonocardia* bacteria occurring on the cuticle of attine ants have been proposed to form a mutualistic interaction with these ants in which they are vertically transmitted (i.e. from parent to offspring colonies). Given a strictly vertical transmission of *Pseudonocardia*, the evolutionary theory predicts a reduced genetic variability of symbionts among ant lineages. The aim of this study was to verify whether actinomycetes, which occur on *Acromyrmex octospinosus* leaf-cutting ants, meet this expectation by comparing their genotypic variability with restriction fragment length polymorphisms. Multiple actinomycete strains could be isolated from both individual ant workers and colonies (one to seven strains per colony). The colony specificity of actinomycete communities was high: Only 15% of all strains were isolated from more than one colony, and just 5% were present in both populations investigated. Partial sequencing of 16S ribosomal deoxyribonucleic acid of two of the isolated strains assigned both of them to the genus *Streptomyces*. Actinomycetes could also be isolated from workers of the two non-attine ant species *Myrmica rugulosa* and *Lasius flavus*. Sixty-two percent of the strains derived from attine ants and 80% of the strains isolated from non-attine ants inhibited the growth of *Escovopsis*. Our data suggest that the association between attine ants and their actinomycete symbionts is less specific then previously thought. Soil-dwelling actinomycetes may have been dynamically recruited from the environment (horizontal transmission), probably reflecting an adaptation to a diverse community of microbial pathogens.

Keywords Actinomycetes · Leaf-cutting ants · *Acromyrmex octospinosus* · *Escovopsis* · *Streptomyces* · Tripartite mutualism

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Introduction

Mutualisms are prominent features of most biotic communities and are profoundly influential at all levels of biological organization (Boucher 1988; Douglas 1994). Although mutualisms can be simply defined as reciprocally beneficial relationships between organisms, they range from diffuse and indirect interactions to highly integrated and coevolved associations between pairs of species.

The interaction between fungus-growing ants (Hymenoptera: Formicidae: Attini) and their fungal cultivars (Agaricales: mostly Lepiotaceae: Leucocoprineae) is a classical example of a highly evolved mutualism (Weber

1966; Martin 1970; Chapela et al. 1994). The ants provide the fungus with fresh substrate and protection against competitors and pathogens (Bass and Cherrett 1994; North et al. 1997), and virgin ant queens carry their mother's symbiont when leaving their colony to mate and disperse (von Ihering 1898). As a reward, the fungus produces nutrient-rich bodies (gongylidia), which the workers harvest as a sole food source for their larvae and the queen. This mutualistic interaction has become enormously successful: *Atta* colonies, for example, may contain several millions of individuals and remove up to 12.5% of the total leaf area available in their foraging areas (Wirth et al. 2003).

Being propagated asexually as a clonal monoculture, the fungus gardens are under permanent pressure of parasitism by microorganisms that are competitively superior to the fungus cultivated by the ants (Weber 1966). Indeed, an intensive survey of non-mutualistic fungi associated with fungus-growing ant gardens revealed a relatively frequent contamination with alien fungi (Currie et al. 1999a). The most common alien fungus isolated during this study was a highly specialized garden parasite of the genus *Escovopsis* (Ascomycota: anamorphic Hypocreales), which was shown to have potential detrimental effects on the health of the fungal gardens and consequently on the survival of the ant colony.

Removal of ants from *Escovopsis*-contaminated fungus gardens led to rapid overgrowth of the garden by this fungus (Currie et al. 1999a; Rodrigues et al. 2005), thus clearly assigning the responsibility for maintaining the health of the fungal mutualist to the ant partner. This interesting finding immediately raises the question of how attine ants manage to protect their fungal gardens from invading microbes. Among all possible mechanisms suggested to explain this observation (for review, see Poulsen and Currie 2006), one is particularly appealing: Certain areas of the cuticle of fungus-growing ants are coated with whitish filamentous structures that occur on genus-specific regions of the ventral surface of ants (i.e. under forelegs or on laterocervical plates of propleura). Currie et al. (1999b, 2003) interpreted this whitish coating as a thick growth of filamentous bacteria of the genus *Pseudonocardia* (initially misidentified as *Streptomyces* sp.). These bacteria belong to the order Actinomycetales, a group that is well known for producing many important antibiotics (Waksman and Lechevalier 1962) giving rise to the hypothesis that the filamentous bacteria may produce bioactive compounds, which could help to maintain hygiene within fungal gardens. Furthermore, foundress queens carried the bacterium from the natal nest during their mating flight on their cuticle, whereas they were absent on males, thereby suggesting a vertical transmission of the bacterium (from parent to offspring colony; Currie et al. 1999b). All these findings suggest a co-evolutionary involvement of the actinomycete bacteria into the tripartite symbiosis between fungus-growing ants, their fungal cultivar and the garden parasite *Escovopsis*.

For an exclusively vertical transmission of *Pseudonocardia* symbionts, the evolutionary theory predicts *symbiont sorting* by drift: Transmission of symbionts from parent to offspring colonies leads to random losses of symbionts resulting in the long term to a single genotype of symbiont per host individual (Douglas 1995; Wilkinson and Sherratt 2001).

The aim of this study was to verify whether this prediction of genetic uniformity was met for seven colonies of leaf-cutting ants (*Acromyrmex octospinosus*) from two distant populations in French Guiana. To further explore whether ants may also newly acquire bacterial symbionts from their environment, we verified whether actinomycetes that do inhibit the growth of the garden parasite *Escovopsis* can also be isolated from ant species that do not grow fungi. In particular, we focused on the following questions: (1) Are multiple genotypes of actinomycetes present on individual ants? (2) What is the level of actinomycete diversity within ant colonies? (3) How similar are actinomycete assemblages when comparing colonies from two distant populations? (4) What is the taxonomic identity of the actinomycetes present on the ants? (5) Do actinomycetes also occur on non-attine ants (i.e. ants that do not grow fungi)? (6) Do all isolated actinomycete strains inhibit the growth of the specialized garden parasite *Escovopsis*?

Materials and methods

Sampling of ants

A. octospinosus colonies were collected from two ant populations adjacent to the research station of Paracou in the lowland moist forest of French Guiana (FG; 5°18′N, 52°53′W) in March 2000. Three colonies were obtained from a population at a roadside, which was characterized by secondary vegetation growing on white sand (i.e. population 1). Four colonies were collected at a loamy undisturbed *Curatella americana* savanna (i.e. population 2) at a distance of approximately 4.5 km beeline from the first site. Between 150 and 250 workers and fungi (approx. 300 ml) of the seven colonies were excavated in FG and transported to Germany within 5 days where the bacteria were isolated from their cuticles. The colonies were kept at approx. 25°C and at a high humidity (approx. 90% relative humidity), which was achieved by placing wetted clay inside the transport boxes. All *Acromyrmex* workers used for the isolation were nearly entirely covered with a whitish coating including the thorax, gaster and parts of the legs

(see Fig. 2A *A. octospinosus* in Currie et al. 2006), which supposedly consists of *Pseudonocardia* symbionts.

Individuals from the temperate non-attine ant species *Lasius flavus* and *Myrmica rugulosa* were collected from three adjacent colonies in the private garden of M. Redenbach close to the University of Kaiserslautern (49°27′N, 7°33′E).

Bacterial isolation

Workers of all size classes of seven *A. octospinosus* colonies (n=157) were carefully pressed on the dorsal alitrunk with a sterile toothpick to touch the surface of the agar plates with their laterocervical plate and streaked ventrally over plates containing soya agar (Kieser et al. 2000). After incubation of the agar plates for at least 3 days at 30°C, emerging filamentous growing colonies were transferred to a new agar plate. Selection of strains was based on colony and microscopic morphology, which allowed confident identification of isolates as actinomycetes (Smibert and Krieg 1994). Colonies were repeatedly sub-cultivated until pure cultures of the corresponding bacterial strains were obtained. Isolation of bacteria from temperate ant species was performed similarly using six and seven individuals of *M. rugulosa* and *L. flavus*, respectively. All purified bacterial strains were preserved as spore suspensions at −80°C. Categorizing all *A. octospinosus* ants used for the isolation into three size classes and testing the probability with which actinomycetes could be isolated from them indicated no effect of ant size on abundance of strains (Pearson's Chi-squared test: χ^2=0.0629, df=2, P=0.969).

RFLP-PFGE analysis

Yeast extract–malt extract (YEME) liquid medium was inoculated with purified spore suspension of isolated actinomycete strains and incubated at 30°C for 4 days (Kieser et al. 2000). Mycelium was collected by centrifugation, and deoxyribonucleic acid (DNA) for pulsed-field gel electrophoresis (PFGE) was isolated and handled as described (Redenbach et al. 1996). PFGE blocks were restricted with *Vsp* I and applied to 1% agarose gels in a Bio-Rad CHEF System. Two programs were used to resolve a maximum number of macrorestriction fragments: program 1: ramping 30–60 s, 150 V for 36 h at 14°C and program 2: ramping 90–180 s, 150 V for 36 h at 14°C.

To compare the communities of strains isolated from two individual ant colonies, we calculated Sørensen's index of similarity, SI (%)=$[(2G)\times(C_1+C_2)^{-1}]\times 100$ where G is the number of strains in common and C_1 and C_2 are the total number of strains isolated from the two colonies to be compared (Mühlenberg 1993).

Sequence analysis

Two actinomycete strains were randomly selected for partial sequencing of their 16S ribosomal DNA (rDNA): strain 2 from colony P1 C1 and strain 5 from colony P1 C2 (Fig. 1). YEME liquid medium (50 ml) was inoculated with their purified spore suspension and incubated at 30°C for 4 days (Kieser et al. 2000). DNA was isolated using procedure 1 described by Hopwood et al. 1985, and the resulting pellet was dissolved in 500 µl Tris–ethylenediamine tetraacetic acid.

The sequences for the PCR primers selected corresponded to the conserved region of the *S. ambofaciens* rDNA sequence (nucleotide positions 1–500; Pernodet et al. 1989). The sequences of the synthesized oligonucleotides used for PCR were: sense primer 5′-TCACGGAGAGTTTGATCCTG-3′ and anti-sense primer 5′-GCGGCTGCTGGCACGTAGTT-3′. Bacterial DNA was diluted 1:1, 1:100, 1:500 and 1:1,000, and each dilution was used for polymerase chain reaction (PCR). For the PCR, 100 pmol of each primer was mixed with

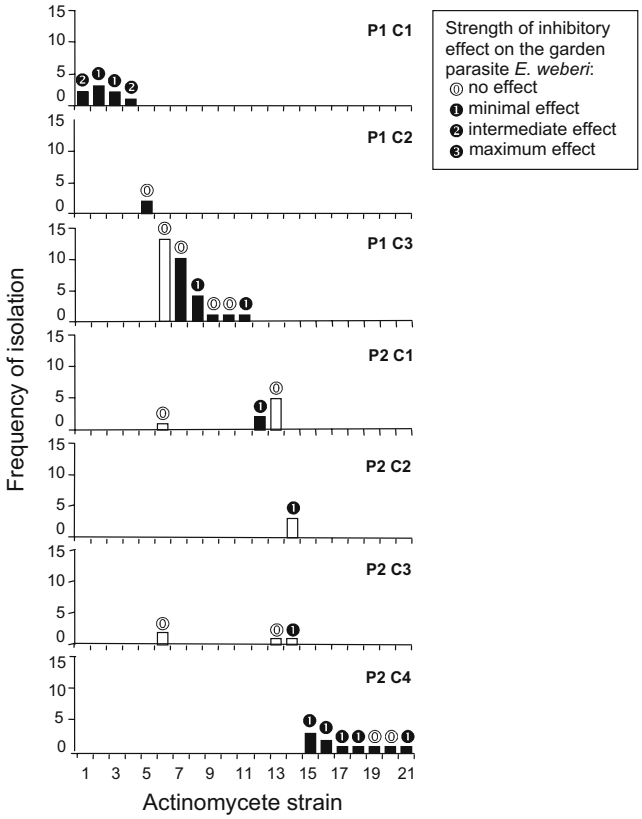

Fig. 1 Frequency at which different actinomycete strains were isolated from each of seven *A. octospinosus* colonies (*C*) out of two populations (*P*). Bacterial strains were differentiated with RFLP-PFGE analysis. Sample sizes of ants used for isolation per ant colony were 20–23 in P1 and 20–26 in P2. *Black bars* represent genotypes present in just one ant colony; *white bars* are those isolated from more than one ant colony. *Circles above bars* indicate the inhibitory effect of the respective strains on the growth of the specialized garden parasite *Escovopsis weberi* (for details on inhibition classes, see legend of Fig. 2)

200 μM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$ and 1 U of *Taq* polymerase (Fermentas) to a total volume of 50 μl. After a first denaturation step (96°C for 5 min), the reaction mix was amplified in 30 cycles of denaturation (for 30 s at 96°C), annealing (for 45 s at 60°C) and extension (for 1 min at 72°C) in a PCT-100 thermocycler (MJ Research). The amplified PCR products were phenol-chloroform-purified and ligated in a pBluescript II KS-vector (Stratagene). For this, vector DNA was restricted with *Eco*RV and incubated with *Taq* polymerase (1 U μg^{-1} plasmid in a total volume of 20 μl) and 2 mM deoxythymidine triphosphate for 2 h at 70°C under standard buffer conditions (50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$ and 200 μg μl^{-1} bovine serum albumin). Subsequently, vector DNA was phenol–chloroform purified, and EtOH was precipitated. The vector DNA was then mixed with the PCR products in a 1:2 ratio (total volume 10 μl) and incubated in the presence of 1.5 U T_4 DNA ligase (Fermentas), 2 μl adenosine triphosphate (5 mM) and 1× ligation buffer for 14 h at 14°C. The resulting plasmids were transformed by electroporation into competent cells of *Escherichia coli* XL-1 Blue (Stratagen), and recombinant plasmids were identified by blue–white screening (Maniatis et al. 1989). Positive recombinant clones were screened by PCR using the T_3 (5′-AATTAACCCTCACTAAAGGG-3′) and T_7 primer sequence in the vector (5′-GTAATACGACTCACTATAGGGC-3′). Positively identified clones were sequenced using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit with 7-deaza-deoxyguanosine triphosphate (Amersham Life Science) following standard protocols of the manufacturer. Gels were electrophoresed on a 41-cm-length gel, and fluorescent DNA sequencing fragments were detected using a LI-COR Model 4000 automated DNA sequencer. Seven positive clones of strains 2 and 8 of strain 5 were sequenced, and the resulting sequences were aligned with Clustal X (Thompson et al. 1997). The consensus sequences were used in a basic local alignment search tool (BLAST) search to determine the taxonomic identity of the two strains.

Inhibition assays

Escovopsis weberi (CBS 810.71) was obtained from the Centraalbureau voor Schimmelcultures (The Netherlands). Purified actinomycete spore suspensions (7 μl) were inoculated in the centre of a plate (8.5 cm diameter) with soya medium generating circular lawns with a maximal diameter of 1 cm. Plates were then incubated for 48 h at 30°C to develop aerial mycelium and sporulation of actinomycetes. Subsequently, the mycelium of *Escovopsis*, which had been grown on soya medium for at least 7 days at 20°C, was point inoculated at the edge of the plate (3.6 cm from the actinomycetes lawn) and incubated for 5 days at 20°C. Every strain was challenged five times against *Escovopsis*. Control plates without actinomycete lawns were treated likewise. After 5 days, the distance between the outer rim of the actinomycete colony and the *Escovopsis* inoculum was measured, and the values were averaged for the five replicates. The resulting value was used to assign each strain to one of the following four classes of inhibition: 0= no effect (fungus overgrows actinomycete), 1=minimal effect (area of inhibition<18 mm), 2=intermediate effect (area of inhibition≥18 mm) and 3=maximum inhibitory effect (no fungal growth).

Results

RFLP-PFGE analysis of bacterial diversity on attine ants

A total of 157 worker ants were streaked ventrally over the agar medium to isolate the bacteria present on their laterocervical plate. Although exoskeleton parts of the investigated ants were conspicuously covered with the whitish coating as described previously (Fig. 2A *A. octospinosus* in Currie et al. 2006), the smearing of only 58% of these ants resulted in the development of mycelium-forming bacteria on the agar plates. From these 92 ant smearings, a total of 63 bacterial strains could be isolated to pure culture. Subsequent PFGE fingerprinting was used to differentiate the isolated actinomycete strains at the genomic level. This allowed us to distinguish 21 unique PFGE patterns, each corresponding to individual bacterial strains (Fig. 1). Rarefaction curves of the cumulative number of actinomycete strains detected versus the number of ant colonies investigated did not reach an asymptote (not shown), indicating an incomplete assessment of the bacterial community at the study site (Gotelli and Colwell 2001).

Among all *A. octospinosus* workers of which more than one bacterial colony could be isolated to pure cultures (n= 21), eight ants were bearing two and one ant was even bearing three strains that differed in their PFGE pattern. The number of actinomycete strains isolated per ant colony ranged from one to seven with a high variability of their abundance (Fig. 1). The majority of all strains (85%) were isolated from single colonies, indicating a high colony specificity of bacterial strains. The similarity of the bacterial communities isolated from the two ant populations was low. Sørensen's index of similarity ranged from 0 to 25% among ant colonies. While the total strain richness of bacterial communities was equally high in the two populations (11 strains each), there was less than 5% of similarity between them (i.e. one common strain; Fig. 1).

Partial sequencing of 16S rDNA of two selected strains

Two actinomycete strains were randomly selected for sequencing: strain 2 from colony P1 C1 and strain 5 from

colony P1 C2 (Fig. 1). Strain 2 shared a 96% sequence identity with *Streptomyces* sp. EF-93, *Streptomyces setonii*, and *Streptomyces caviscabies* strain ATCC51928 and strain 5 showed a nucleotide identity of 95% to the same three species. Consequently, both isolates most likely belonged to the genus *Streptomyces*.

Inhibition assays with bacterial strains of attine origin

The inhibitory effect of the isolated actinomycete strains on the growth of the specialized fungus garden parasite was tested in plate inhibition assays. Strains representing a unique PFGE profile were challenged against *E. weberi* on agar plates with soya medium. In 62% of the bioassays, the tested actinomycete strain inhibited growth of *Escovopsis* (Fig. 2, inhibition classes 1–3). The strength of the inhibitory effect, however, differed largely among strains (Fig. 1). Particularly interesting was the observation that none of the 21 strains tested inhibited the growth of *Escovopsis* completely (Fig. 2, inhibition class 3). Moreover, the three most frequently isolated strains (i.e. strains 6, 7 and 13) did not inhibit the growth of *Escovopsis* at all (Fig. 1, inhibition class 0).

Isolation of bacteria from non-attine ants

The same bacterial isolation procedure as applied previously to attine ants was used to isolate actinomycetes from two temperate ant species. In the case of *M. rugulosa*, actinomycetes could be isolated from six of seven ants (86%) and from two of six ants (33%) of *L. flavus*. In total, two different strains of mycelium-forming bacteria could be isolated from *L. flavus* and 17 strains from *M. rugulosa*.

Inhibition assays with bacterial strains of non-attine origin

In addition, the actinomycete strains derived from temperate ant species (*M. rugulosa* and *L. flavus*) were tested for their capability to suppress the growth of the garden parasite *E. weberi*. More than 80% of the selected strains inhibited the growth of the ascomycete *E. weberi* (Fig. 2). The strength of inhibition, however, varied considerably among strains. In contrast to the strains isolated from *A. octospinosus*, three strains derived from *M. rugulosa* showed a maximal inhibitory effect. However, there was no significant difference in the frequency distributions of the observed inhibitory effects when strains derived from attine ants were compared with those isolated from non-attine ants (Yates-corrected χ^2=6.05, *df*=3, *P*=0.11).

Discussion

The aim of this study was to investigate the specificity of the interaction between leaf-cutting ants of the species *A. octospinosus* and their actinomycete symbionts. Multiple strains of actinomycetes could be isolated from individual ant workers. The level of actinomycete diversity within ant colonies was relatively high, ranging from one to seven bacterial strains per colony. Likewise, the colony specificity of the actinomycete communities was high: Only 15% of all strains could be isolated from more than one colony, and just 5% was present in both populations investigated. Two of the isolated strains could be assigned to the actinomycete genus *Streptomyces*. Moreover, actinomycetes could also be isolated from workers of the two non-attine ant species *M. rugulosa* and *L. flavus* from the temperate zone. Sixty-two percent of the strains derived from attine ants and 80% of

Fig. 2 Inhibitory effect of actinomycete strains isolated from attine (*Acromyrmex octospinosus*, *n*=21) and non-attine ants (*M. rugulosa*, *n*=17 and *L. flavus*, *n*=2) on the growth of the specialized garden parasite *Escovopsis weberi*. The inhibitory effect of each strain was categorized as 0=no effect (fungus overgrows actinomycete), 1=minimal effect (area of inhibition<18 mm), 2=intermediate effect (area of inhibition≥18 mm) and 3=maximum inhibitory effect (no fungal growth). The categorization of each strain was based on its average effect within five independently replicated inhibition assays. The two strains isolated from *L. flavus* showed an inhibitory effect of class 2. *Below*, example bioassays are displayed, which have been performed on potato dextrose agar (Ranzoni 1968) for a better visual contrast between fungus and agar. Bioassays shown represent strains 10, 3, 1 as well as a strain isolated from *M. rugulosa* (from *left* to *right*)

the strains isolated from non-attine ants inhibited the growth of the specialized garden parasite *Escovopsis*.

Our assessment of the actinomycete diversity among ant colonies of *A. octospinosus* did not meet our expectation of a reduced richness of bacterial strains because of symbiont sorting (Douglas 1995; Wilkinson and Sherratt 2001). In contrast, our survey revealed a highly diverse community of genotypes. Rarefaction curves of the isolated actinomycete strains plotted against sampled colonies did not reach a stationary plateau, indicating an incomplete assessment of the bacterial community: Increasing the sample size would have resulted in an even higher number of isolated strains. Moreover, in some cases, more than one bacterial strain could be isolated from an individual ant.

Symbionts living in close association with their host should be selected for some horizontal transmission to reduce competition with close relatives and colonize new hosts (Hamilton and May 1977; Frank 1997). While the maintenance of single symbiont strains of fungi within attine fungus gardens largely corresponds to theoretical expectations (Frank 2003; Poulsen and Boomsma 2005), our finding of a rather non-specific distribution of actinomycete strains among ant colonies is inconsistent with a purely vertical transmission of the symbiont bacteria by foundress queens, in which case, a single genotype of symbiont per host individual would be expected (Douglas 1995; Wilkinson and Sherratt 2001). Instead, our findings may rather be explained by a de novo acquisition of bacteria from the environment (horizontal transmission), which may include the surrounding soil, other ant colonies or a mixture of both as potential sources for the actinomycetes.

In case of the fungal symbiont, the ants are expected to have evolved efficient control mechanisms to retain their resident fungus in a state of genetic homogeneity: From their perspective, costs resulting from a decreased productivity or even death of the fungal garden because of competition with an introduced cultivar may be much higher than is the potential gain from a new cultivar (Bot et al. 2001; Poulsen and Boomsma 2005). The situation may be different for the associated actinomycetes. Given their involvement in the protection of the fungal gardens against a diverse spectrum of both opportunistic and highly specialized pathogens (Currie et al. 1999a; Rodrigues et al. 2005), genotypic diversity among these bacterial strains may provide the ants with multiple secondary compounds, thereby increasing the chance of targeting possible invaders of the fungal gardens. The strength of the inhibitory effect on the growth of *E. weberi* varied considerably between different bacterial isolates from attine ants (Fig. 2). This variability in combination with the diversity of strains, which are present in a single ant colony, may reflect a variety of alternative responses, because different pathogens require different methods of elimination.

This conclusion, however, immediately raises the question whether ants do have any regulatory mechanisms to control the composition of the bacterial community present on their surface. According to recent observations, fungus-growing ants such as *A. octospinosus* support associated bacteria by specialized exocrine glands and cuticular crypts (Currie et al. 2006). Any bacterium occurring on the ant's surface should compete with a resident mutualist such as *Pseudonocardia* for these resources. Observations of *A. octospinosus* workers in the field, which are nearly completely covered with a whitish coating including the thorax, abdomen and parts of the legs (Kost and Wirth, personal observation; Fig. 2A in Currie et al. 2006) suggest the likely presence of highly efficient filter mechanisms in these ants that stabilize specific mutualisms with suitable symbionts.

Vertical transmission ensures that the offspring of the host is always provided with symbionts, thereby avoiding the costs of searching for suitable heterospecifics (Wilkinson and Sherratt 2001). Actinomycetes, however, are soil-dwelling microorganisms that occur ubiquitously in tropical and temperate soils (Fernandez and Szabó 1978; Rodrigues and Drozdowicz 1978; Wang et al. 1999; Lee and Hwang 2002). This factor could facilitate the acquisition of suitable actinomycete symbionts from the environment. Indeed, two of the strains we isolated belonged to the genus *Streptomyces*, a large group of soil-dwelling organisms known for their production of secondary compounds with antifungal and antibacterial properties (Goodfellow and Cross 1984). Moreover, we also isolated actinomycetes from the cuticle of two temperate ant species. These strains inhibited the growth of the specialized garden parasite *E. weberi* (Fig. 2) to a similar extent as did strains isolated from attine ants, supporting the hypothesis of a dynamic recruitment of suitable actinomycetes from the soil. Although an acquisition of actinomycetes from the surrounding soil is the most likely scenario for the observed high diversity of bacterial symbionts, other sources such as neighbouring conspecific ant colonies or a mixture of horizontal and vertical transmission cannot be ruled out.

A previous study by Poulsen et al. 2005 on the specificity of the association between actinomycete bacteria and two sympatric species of leaf-cutting ants did not find evidence for genetic variation among *Pseudonocardia* symbionts. Comparing the sequences of the gene Elongation Factor-Tu (*EF-Tu*) from putative bacterial mutualists derived from colonies of *Acromyrmex echinatior* (n=16) and *A. octospinosus* (n=18) revealed neither within- nor between-colonies variability of bacterial genotypes. Even on the between-species level, this analysis indicated a shared pool of *Pseudonocardia* symbionts. As already mentioned by the authors, the observed lack of sequence divergence may reflect a limited variation in the sequence analysed. Because of the more integrative nature of the

restriction fragment length polymorphism (RFLP)-PFGE analysis used in this study, we assume an increased discriminatory power between strains than the *EF-Tu* gene (Bidet et al. 2000). The sequences analysed by Poulsen et al. (2005) formed two unexpected clades during phylogenetic analysis, which the authors interpret as resulting from allopatric speciation of two distinct *Acromyrmex* clades and subsequent restoration of sympatry within evolutionary time. Assuming a low variability of the *EF-Tu* gene among actinomycete strains, the most parsimonious way in which the results of Poulsen et al. can be interpreted in the light of our data is a dynamic recruitment of actinomycetes strains from the environment.

This interpretation is further supported by a study in which actinomycete bacteria were isolated from workers originating from 71 colonies of *Acromyrmex* sp. (Cafaro and Currie 2005). A phylogenetic analysis with 16S rDNA did not resolve the sequences analysed as monophyletic, again indicating that ants acquired actinomycetes on multiple occasions.

The fact that we detected actinomycete bacteria also on the cuticle of ants that do not grow fungi sheds a new light on the evolutionary origin of ant–actinomycete interactions: The presence of fungicide-producing bacteria may also be beneficial to non-attine ants. Because of their high relatedness among individuals, the frequency of amicable interactions (trophallaxis and grooming) between nestmate ants and their predominantly subterraneous way of life, ants should be particularly favourable hosts for entomopathogenic fungi (Wheeler 1910). However, relatively few such parasites are known from social insects (Schmid-Hempel 1998; Hughes et al. 2004). Possible explanations for this observation could be the extensive grooming behaviour (Farish 1972) or the secretion of antibiotic compounds from exocrine glands by the ants (Maschwitz 1974; Poulsen et al. 2002). Additionally, a diverse community of actinomycetes present on the ants' cuticle, which produce secondary compounds with fungicide bioactivity, could protect ants from entomopathogens. If actinomycete bacteria also fulfil a protective function in ant species that do not grow fungi and whether this association served as a basis for the closer evolutionary interaction between actinomycetes and attine ants need to be further explored.

Our finding of a non-specific association between leaf-cutting ants and their symbiotic actinomycetes opens the door to a wealth of interesting questions. Most pressing among them is whether ants do have any regulatory mechanisms to control the community of bacteria present on their surface. The benefit attine ants gain from the interaction with their actinomycete partners likely depends on factors such as the density and composition of the community of pathogens threatening the fungal gardens and the ants on one side and the identity/community of the actinomycetes present on the ant on the other side. Focussing on the interplay between these factors in future studies will provide further insights into this fascinating tripartite mutualism and help to better understand symbioses in general.

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