Ocurrence of the antibiotic producing bacterium *Burkholderia* sp. in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*

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Abstract

Fungus garden material from recently established *Atta sexdens rubropilosa* colonies (6–12 months old) was sampled to detect antibiotic producing microorganisms that inhibited the growth of pathogens of insects and of the fungus gardens but did not affect their mutualistic fungus. A bacterium with activity against the entomopathogenic fungus *Beauveria bassiana* was isolated from 56% of the gardens tested (n = 57) and identified from its biochemical profile and from 16S and 23S ribosomal DNA sequences as a member of the genus *Burkholderia*. The ant-associated *Burkholderia* isolates secreted a potent, anti-fungal agent that inhibited germination of conidia of the entomopathogenic fungi *B. bassiana*, *Metarhizium anisopliae*, of the saprophytic *Verticillium lecanii*, and also of a specialist fungus garden *Escovopsis weberi*. Growth of the ant’s mutualist fungus was unaffected.

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1. Introduction

Leaf-cutting ants have long been known to practise a form of agriculture, cultivating a mutualist fungus in a “garden” within their nest. Within this obligate mutualism, the fungus (*Leucoagaricus gongylophorus*, Family Lepiotaecae) supplies the ants with nutrients, while the ants supply plant leaves as a growth substrate and a protected environment for the fungus [1]. Leaf-cutting ants are the dominant herbivores in the Neotropics causing great agricultural losses in South America [2] and no biological control strategy for this pest has so far been successfully achieved.

Attine ants actively manage their fungus gardens, physically removing contaminating fungi [3]. The ants also use chemical control against invading organisms. Compounds secreted by glands on the bodies of the ants may contribute to management of pathogenic fungi [4,5]. Certain fungi, such as *Escovopsis* spp., are highly co-evolved parasites of the ants’ garden fungus [6], and an actinomycete found on the ants’ bodies produces an unidentified antifungal compound that is directed specifically against *Escovopsis* [7].

In this work, we sampled nests of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, a major agricultural pest in Brazil, for the occurrence of antibiotic producing
microorganisms. Beneficial nest-associated organisms may be useful for the control of a variety of invading fungi that would otherwise reduce the productivity of the fungus garden. As ants are also susceptible to fungal diseases [8], we speculate that some nest-associated microorganisms may produce broad-spectrum antibiotics that protect the ants against life-threatening microbial infections.

2. Materials and methods

2.1. Isolation and characterisation of microorganisms

Fungus garden material from 55 individual A. sexdens rubropilosa colonies located in eucalyptus plantations (Rio de Janeiro state, Brazil/21°31’ 174S; 41°14’ 157W) approximately 6 months following establishment, were initially sampled for the presence of culturable microorganisms using Nutrient agar (Oxoid) and the medium described in [9]. At this early stage, the colony has only one chamber with one round mass of fungus garden. Only those colonies with a healthy and well developed fungus (more than 50% of the chamber filled by the fungus) were sampled by taking an aliquot of the part where both the workers and larvae were observed to be present and active. The outer layers of the garden were discarded in order to remove traces of surrounding soil. Samples were immediately transferred to a sterile Petri dish and kept in an aseptic chamber for 12–24 h. Fresh garden samples (1 g) were mixed thoroughly with sterile Tween 80 (0.05%) (1:10). Aliquots of 100 µl of a 10⁻⁴ dilution were transferred to Petri dishes with the above media and incubated at 26 °C for 24 h.

2.2. Plate challenge assays of bacterial isolates versus pathogenic fungi

All the microorganisms obtained were screened using a plate challenge assay for activity against an isolate of the entomopathogenic fungus Beauveria bassiana that is highly pathogenic to A. sexdens rubropilosa (Santos et al., unpublished). A suspension of 10⁶ conidia ml⁻¹ of B. bassiana was spread on Sabouraud dextrose agar (SDA), and the microorganism to be tested was inoculated in the center of the plate. The presence of an inhibition zone after 48 h indicated a positive reaction. Microorganisms that inhibited growth of B. bassiana were further challenged against isolates of the entomopathogenic fungi Metarrhizium anisopliae and Verticillium lecanii and, additionally, against the saprophytes Verticillium lecanii and Trichoderma sp., Aspergillus sp. and the specialized garden parasite Escovopsis weberi. The isolates of B. bassiana and Escovopsis tested were obtained from moribund A. sexdens rubropilosa and their gardens, respectively. M. anisopliae was isolated from Deois incompleta (Homoptera: Cercopidae) and V. lecanii was isolated from an adult aphid (Homoptera: Aphidoidea).

Only those bacteria isolated more frequently and displaying strong antifungal activity against B. bassiana and the other fungi tested were characterized and identified using standard procedures [10]. Gram-negative bacteria were identified using API 20NE kit (bioMérieux).

2.3. Bacterial identification using multiplex PCR and sequencing

The PCR methods used, as well as the three sets of primers, were the same as described in [11], except that the annealing temperature was 60 °C for 30 s. Amplification was carried out in a thermal cycler (PTC-100/MJ Research, Inc.). Products from the PCR reaction was separated using electrophoresis (1.5% agarose gel) and the molecular weights were determined by comparison with a 100 bp DNA ladder (Life Technologies).

DNA sequencing was carried out in two ways: (a) 16S rRNA gene (rDNA) sequences were obtained after PCR [12] and TOPO TA cloning (Invitrogen); (b) 23S rRNA gene (rDNA) sequences were obtained directly from PCR products. The product obtained with the multiplex PCR was recovered and purified from the agarose gel using DEAE membranes according to [13,14] and sequenced using the same primers used in the multiplex PCR. Sequencing was carried out on an ABI Prism 377 DNA sequencer (Applied Biosystems). Sequences were analyzed using tools available from the ribosomal database project, Center for Microbial Ecology, Michigan State University (http://rdp.cme.msu.edu/html/).

2.4. Field sampling for the presence of Burkholderia sp. in ant colonies

In a further study, a total of 57 additional nests were sampled from an eucalyptus plantation located in the North-west of Rio de Janeiro State (21°31’ 174S; 41°14’ 157W) and from pasture land in the North of Minas Gerais State (20°39’ 04 S; 42°51’ 24W). This fieldwork was carried out to recover the main bacterium identified (Burkholderia sp.), using the selective medium Trypan Blue + Tetracycline [15]. Pseudomonas Agar Base (Oxoid CM559) was also used with the selective supplement CFC (Oxoid SR103E). The multiplex PCR method described above was used to confirm the identification of the isolates.

In order to assess the occurrence of Burkholderia sp. on the bodies of ants and also on leaf fragments taken into the colony, ten ants were collected from each of the fungus gardens and foraging trail and put on Petri plates with the above selective media. After 5 h the
insects were removed aseptically and the plates incubated as described above. Twenty leaf fragments were collected from the foragers near the nest entrance. The fragments were then transferred, individually, to polyethylene microcentrifuge tubes containing 500 μl of Tween 80 (0.05%). Aliquots of 100 μl were then transferred to the selective media described above.

3. Results

Initially, 62 bacterial isolates with some inhibitory activity were obtained during plate challenge assays against the entomopathogenic fungus *B. bassiana*. Three main isolates displaying antifungal activity were identified using API 20NE kits as members of the genus *Burkholderia* by their biochemical profile.

Sequences of PCR fragments of the 16S and 23S rDNA from *Burkholderia* sp. isolates showed strong homology with database sequences from members of the *B. cepacia* complex. Sequences generated in this work were deposited in the GenBank database under Accession Nos. AY314741 (*Burkholderia* sp. partial 23S rRNA gene sequence, isolate 2b) and AY314742 (*Burkholderia* sp. partial 16S rRNA gene sequence, isolate 2b).

The isolates of *Burkholderia* sp. strongly inhibited the growth not only of *B. bassiana* but also of *E. weberi*, *M. anisopliae*, *V. lecanii* (Fig. 1), *Trichoderma* sp. and *Aspergillus* sp. (not shown). In contrast, this bacterium did not inhibit the growth of the ants’ own garden fungus *L. gongylophorus*, which grew normally when plated together with the bacterial colony, eventually overgrowing it (not shown). Isolates of *Burkholderia* sp. were found in fungus garden samples from 10 out of 18 (55.56%) and 22 out of 39 (56.41%) *A. sexdens rubropilos* nests sampled in Rio de Janeiro and Minas Gerais, respectively. The average number of colony forming unit (CFU) per gram of fresh sample was $8.7 \times 10^5$ (±3.6) and $9.7 \times 10^5$ (±6.4) in Rio de Janeiro and Minas Gerais state, respectively. Isolates of *Burkholderia* sp. were also found in the soil surrounding the ants’ fungus chamber in 10 out of 15 (66.67%) samples, with $3.3 \times 10^5$ (±1.6) CFU per gram of soil. *Burkholderia* sp. was never isolated from leaf material carried by foraging ants, nor from the bodies of ants returning to the colony.

4. Discussion

*Burkholderia* sp., a bacterium with antifungal activity was frequently isolated from the nests of leaf cutting ants. The prevalence of this bacterium within the fungal garden of young colonies suggests that the consortium of microorganisms involved in the ant–fungal garden is even more complex than previously envisaged. To our knowledge, this is the first study to report an association of *Burkholderia* sp. with leaf-cutting ants.

![Fig. 1. Antifungal activity of ant-associated *Burkholderia* sp. (isolate 2b) against the entomopathogens: (a) *Metarhizium anisopliae*, (b) *Verticillium lecanii*, (c) *Beauveria bassiana*, (d) against the specific garden parasite *Escovopsis weberi*.](https://academic.oup.com/femsle/article-abstract/239/2/319/623082)
*B. cepacia* is a Gram-negative soil bacterium commonly associated with plants, although certain members of the complex are also opportunistic human pathogens [16,17]. Bacteria of the *B. cepacia* complex are well known to produce antifungal compounds [18] and have been proposed as biocontrol agents for soil-borne plant pathogens [19]. PCR of 16S and 23S rDNA genes clearly identified the ant-associated isolates as members of the *B. cepacia* complex. Because taxonomy of the *B. cepacia* complex is complicated, and because additional phylogenetic analysis is necessary to position them in the correct group of the *B. cepacia* complex, we prefer for the present to describe the ant-associated bacteria simply as *Burkholderia* sp.

The nests of leaf-cutting ants sampled were from recently established colonies at a soil depth of approximately 30–40 cm. The soil where leaf-cutting ants establish their nests has been shown to possess around ten times more microorganisms than the soil surrounding mature colonies [20]. Consequently, a wide range of potential intruders surround young ant colonies. The presence of *Burkholderia* sp. in young nests of *A. sexdens rubropilosa*, may play an important role in their defence against pathogenic microorganisms.

The selective medium TB-T may not be the most appropriate tool to assess the prevalence of *Burkholderia* in ant nests, although it was originally designed for environmental samples [15,21,22]. According to [15,23] TB-T is highly selective based on its efficiency of recovering *B. cepacia* strains from low dilutions of soil samples (10^5–10^6). The fact that *Burkholderia* sp. was found in only 56% of the nests sampled, may indicate that the bacterium is not always present, but equally it is likely that the isolation method used may underestimate the true rate of incidence. The presence of the same antibiotic-producing *Burkholderia* in the soil immediately adjacent to the fungus garden suggests that these bacteria could be selectively acquired by the ants from the environment.

Another ant, *Tetraponera* (a distant relative of leaf-cutting ants) has previously been found to be associated with a *Burkholderia* sp. together with four other bacterial endosymbionts [24]. All the five bacterial symbionts of *Tetraponera* are related to root-nodule bacteria. *B. cepacia* is also well known to be found in the rhizosphere of plants [17]. Accordingly, both ant species might have acquired two different groups of *Burkholderia* from soil; one with ability to recycle nitrogen and another with ability to produce broad spectrum antibiotic compounds.

Our results demonstrate a further layer of complexity in the relationship between attine ants and their mutualist fungi. It is now evident that there is a consortium of microorganisms present in the leaf-cutting ant nest. An actinomycete found only on the bodies of ants can specifically defend garden fungi against *Escovopsis* [7]. The actinomycete’s antifungal agent, however, lacks activity against generalist saprotrophic fungi, and does not affect entomopathogenic fungi. Other microorganisms present in leaf-cutting ant nests, such as the *Burkholderia* sp. described here, may also participate in broad-spectrum garden defence, as well as playing a protective role against fungal diseases.

Our work may have implications for biological control strategies to be developed for leaf-cutting ants. Any biological agent to be used against these insects, apart from overcoming the behavioural defences of the ants themselves [25,26], must also be able to withstand the antibiotic compounds produced by the microorganisms that are associated with the ants.

Thus, attine ants may have developed multiple biological sources of antibiotics, active against microbial agents of diseases and competitors of their mutualist fungus, 45–65 million years [1] before the origins of human agriculture or medicine.

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