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Association between *Pseudonocardia* symbionts and *Atta* leaf-cutting ants suggested by improved isolation methods

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Summary. Fungus-growing ants associate with multiple symbiotic microbes, including Actinobacteria for production of antibiotics. The best studied of these bacteria are within the genus *Pseudonocardia,* which in most fungus-growing ants are conspicuously visible on the external cuticle of workers. However, given that fungus-growing ants in the genus *Atta* do not carry visible Actinobacteria on their cuticle, it is unclear if this genus engages in the symbiosis with *Pseudonocardia*. Here we explore whether improving culturing techniques can allow for successful isolation of *Pseudonocardia* from *Atta cephalotes* leaf-cutting ants. We obtained *Pseudonocardia* from 9 of 11 isolation method/colony component combinations from all 5 colonies intensively sampled. The most efficient technique was bead-beating workers in phosphate buffer solution, then plating the suspension on carboxymethylcellulose medium. Placing these strains in a fungus-growing ant-associated *Pseudonocardia* phylogeny revealed that while some strains grouped with clades of *Pseudonocardia* associated with other genera of fungus-growing ants, a large portion of the isolates fell into two novel phylogenetic clades previously not identified from this ant-microbe symbiosis. Our findings suggest that *Pseudonocardia* may be associated with *Atta* fungus-growing ants, potentially internalized, and that localizing the symbiont and exploring its role is necessary to shed further light on the association. [**Int Microbiol** 2013; 16(1):17-25]

Keywords: *Pseudonocardia* · Actinobacteria · symbiosis · mutualism · *Atta* leaf-cutter ants

Introduction

The fungus-growing ant-microbe symbiosis originated approximately 50 million years ago [28]. Fungus-growing ants farm a specialized fungal cultivar that serves as the ants' primary food source [15]. These fungal gardens host potentially virulent microfungal parasites in the genus *Escovopsis,* which consume the fungal mutualist [8,27]. To help suppress *Escovopsis,* the ants associate with Actinobacteria, which provides protection through antibiotic production. The Actinobacteria genus *Pseudonocardia* has been shown to be consistently present in the association, where it provides defensive antibiotic compounds [4,7,10,23,26]. In addition, other Actinobacteria genera have also been isolated or detected using culture-independent methods [2,14,17], and have been suggested to also contribute to *Escovopsis* suppression [2,14]. The degree of specificity and functional role of these additional Actinobacteria remains to be fully established [cf. 4,5].

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The ant-*Pseudonocardia* association has been documented across most of the phylogenetic diversity of fungus-growing ants (tribe Attini), including the paleo-attine genera *Apterostigma*, *Mycocepurus* and *Myrmicocrypta* (lower attines), and the neo-attine genera *Cyphomyrmex*, *Mycetosoritis*, *Mycetarotes* (lower attines), *Trachymyrmex* and *Acromyrmex* (higher attines) [4,9,10]. *Pseudonocardia* is typically maintained on modified structures on the ant cuticle, and most genera of fungus-growing ants appear to provide the bacteria with nourishment through glandular secretions [9]. In at least two genera, *Sericomyrmex* and *Atta*, the abundance of Actinobacteria on the cuticle of the ants is markedly reduced or completely absent [9], possibly because alternative defenses play a more prominent role in controlling *Escovopsis* [11,13,32]. Despite the absence of visible Actinobacteria symbionts on the cuticle of *Atta* workers, several strains of *Pseudonocardia* that occur in the same clades as symbionts isolated from the other leaf-cutting ant genus *Acromyrmex* have been obtained (clades IV and VI in [4]).

Co-occurrence of *Pseudonocardia* in these phylogenetic clades suggests that *Atta* and *Acromyrmex* maintain the same *Pseudonocardia* species. However, consistent isolations of *Pseudo-nocardia* from *Atta* have proven difficult. This difficulty, in addition to the lack of visible growth of Actinobacteria on workers, makes it uncertain whether *Atta* persistently harbors symbiotic *Pseudonocardia* and even suggests that the Actinobacteria symbiont might have been lost in this ant genus. However, given that microbial symbionts are often fastidious, attempts to improve culturing techniques might determine whether *Pseudonocardia* is consistently present in *Atta*. Here we explore whether different isolation techniques can reliably acquire isolates of *Pseudonocardia* from *Atta*. We conducted targeted isolations for Actinobacteria in association with *Atta cephalotes* applying four methods, including bead-beating, scraping, and washing with and without sonication, to different colony components. To improve our understanding of where *Pseudonocardia* might be located within *Atta cephalotes* colonies, we applied methods to material from both young and mature fungus garden, callow and mature workers, as well as dissected ant infrabuccal pockets.

Materials and methods

Ant colonies. Most isolation attempts were done using five *Atta cephalotes* colonies collected in Gamboa, Panama in 2003 and 2005. These colonies (Pan03MB, Pan03BB, CC031208-10, AL050513-21, AL050513-22) had been housed at the University of Wisconsin-Madison for between 2 and 7 years at the time of isolation. Colonies were maintained at 24 °C in the dark to mimic underground conditions, with overhead lights only illuminated periodically. Ant colonies were housed in plastic containers, with each colony kept in a large outer container (28 cm-high × 40 cm-wide × 56 cm-long) accommodating one smaller plastic container for refuse material (dump) and one to five smaller plastic containers (ranging from 3.0 cm-high by 7.5 cm-wide × 7.5 cm-long to 11 cm-high by 19.5 cm-long by 12.5 cm-wide) enclosing the colony fungus gardens. Each container had a 1 cm diameter hole drilled to allow ants to move in and out. Mineral oil was regularly applied to the top 4 cm of each outer container to prevent the ants from escaping. The ants were provisioned with maple (*Acer* sp.) and oak (*Quercus* sp.) leaves (frozen in the winter months and fresh during summer) three times per week. Leaves were supplemented with oatmeal, rice and cornmeal. Wet cotton balls were applied to the outer box to increase humidity. After initially determining what methods of isolation were most likely to be successful (see below), we increased the sampling to other laboratory colonies (*A. cephalotes* JS090511-01 from Panama; AP061022-01 from Costa Rica; EC090907-05 and MP090907-15 from Peru; *A. colombica* MP010708 from Panama; *Atta* sp. EC090827-04 and UP08 from Peru).

Isolations. To compare the yield and efficiency of various Actinobacteria isolation techniques, we tested 4 different methods on 1 to 5 colony components, using 1 or 2 types of media and material from five *A. cephalotes* colonies including callow and media workers, infrabuccal pockets dissected from media workers, as well as young and mature garden. The isolation methods included washing, scraping, homogenization, sonication, and bead-beating (see below and Table 1 for details). We replicated each method five times for each of five colonies, for a minimum of 25 Petri plates per method employed. In total, we conducted targeted isolations on 375 plates. For methods generating liquid inocula, plates were allowed to dry before being wrapped with parafilm.

Inocula were plated onto either chitin or carboxymethylcellulose (CMC) minimal nutrient media. Chitin medium is selective for Actinobacteria and we made it by autoclaving 15 g agar, 3 g chitin, 0.575 g K_2HPO_4, 0.375 g $MgSO_4 \cdot 7H_2O$, 0.275 g KH_2PO_4, 7.5 mg $FeSO_4 \cdot 7H_2O$, 0.75 mg $MnCl_2 \cdot 4H_2O$, 0.75 mg $ZnSO_4 \cdot 7H_2O$ in 750 ml H_2O for 30 min. On this medium, Actinobacteria appear white and faintly fuzzy, and grow flat on the surface. CMC media consisted of carboxymethylcellulose (5 g/l) and agar (15 g/l) as the sole nutrient sources. Once bacteria were observed on minimal media, individual cultures were transferred to richer yeast malt extract agar (YMEA, 4 g yeast extract, 10 g malt extract, 4 g dextrose, 20 g agar, 1-liter H_2O). Antifungals (20 ml/l nystatin and 0.05 g/l cycloheximide) were added to media [3,33].

Actinobacteria derived from washes were obtained by aseptically placing workers in 1 ml of autoclaved Milli-Q water inside an autoclave-sterilized 1.5 ml microcentrifuge tube, which was vortexed for 30 s. One hundred µl of this suspension were plated onto each of five plates. Using this washing technique, we sampled medium-sized workers (three per replicate), callow workers (two per replicate, identified by their lighter color), dissected infrabuccal pockets, and small pieces of top (youngest) or middle (older) garden material. Media workers and middle fungus garden were sonicated for 45 s prior to plating.

'Scraping' isolations were done by rubbing the surface of workers with a sterilized bent micro-spatula under a dissecting microscope. This method has been employed to isolate visible *Pseudonocardia* from the cuticle of workers from other fungus-growing ant genera [3]. Bacteria inocula from the tool surface were transferred to chitin plates. This method was conducted on the cuticle of medium-sized workers. Infrabuccal pocket material was collected by dissecting out pockets, then using a sterile isolation loop to spread the pocket content directly onto chitin plates [18].

Bead-beating (Mini-Beadbeater-96, BioSpec products [http://www.biospec.com]) was performed on single medium-sized ants placed in 1.5-ml microcentrifuge tubes containing 500 µl phosphate buffer solution (PBS). We used PBS for bead-beating following Medina-Rivera (2008 Masters thesis, University of Puerto Rico-Mayaguez), who successfully used this solution to isolate Actinobacteria from a non-fungus-growing ant, *Odontomachus ruginodis*. Ants were bead-beaten for two and a half min, after which 100 µl of the suspension were pipetted onto either chitin or CMC plates.

Table 1. Actinobacteria and *Pseudonocardia* isolated from *Atta cephalotes* colonies

					Total Actinobacteria		*Pseudonocardia* only	
	Liquid	Media	Colony component	#	CFUs Mean ± SE	Success rate	CFUs Mean ± SE	Success rate
Wash	H_20	Chitin	Media callow worker	2	0 ± 0	0	0 ± 0	0
			Media adult worker	3	16.4 ± 10.1	80 %	6.0 ± 2.8	60 %
			Worker infrabuccal pocket	1	48.6 ± 17.4	100 %	0 ± 0	0
			Young garden	2	1.0 ± 0.6	40 %	0.4 ± 0.4	20 %
			Mature garden	2	1.2 ± 0.5	60 %	0.4 ± 0.4	20 %
Wash & Sonication	H_20	Chitin	Media adult worker	3	11.2 ± 7.7	60 %	0.8 ± 0.4	20 %
			Mature garden	2	32.4 ± 27.9	40 %	0.2 ± 0.2	20 %
Scraping	–	Chitin	Media adult worker	1	16.2 ± 11.6	100 %	0.4 ± 0.2	40 %
			Media worker infrabuccal pocket	1	32.2 ± 11.3	80 %	0.4 ± 0.2	40 %
Bead-beating	PBS	Chitin	Media adult worker	1	40.1 ± 20.4	100 %	4.3 ± 3.3	40 %
		CMC	Media adult worker	1	35.8 ± 24.9	100 %	7.4 ± 5.1	80 %

An overview of the four Actinobacteria isolation methods (coupled with colony component variation) and their results. Techniques fall into four main categories: washing, washing with sonication, scraping, and beat-beating. Within each technique the liquid used (water or PBS) and growth media used (chitin or CMC) are specified. The number (of individuals or pieces) and type of colony components are also specified including whole callow and media workers, infrabuccal pockets isolated from media workers, as well as young and mature garden. The number of each type of CFU counts and standard errors are reported per plate per colony, based on an average of five plates from each of five colonies, for the total Actinobacteria as well as for *Pseudonocardia* specifically. Isolation success rate of Actinobacteria and *Pseudonocardia* represent the proportion of the colonies yielding at least one CFU of total Actinobacteria and *Pseudonocardia* in particular in five isolation attempts per colony.

To evaluate whether Actinobacteria could have been introduced to the colonies on lab-provisioned leaves, we isolated Actinobacteria from leaf material. These isolations targeted both bacteria from leaf surfaces and endophytic Actinobacteria from surface-sterilized leaves. Two to three leaves were collected from each of two oak and two maple trees routinely used to feed the ants in Madison, WI, for a total of five leaves per species and 10 leaves in total. Leaves were placed in sterile tubes and transported back to the lab. We cut leaves into 0.5-cm^2 pieces and performed four types of targeted isolations. One set of untreated leaves was blotted directly onto chitin plates to isolate external bacteria. A second set was homogenized for 20 s with a sterile plastic pestle in 1.5-ml Eppendorf tubes containing 500 µl autoclaved Milli-Q water. One hundred µl of liquid were then plated onto chitin plates, spread and allowed to dry before wrapping with parafilm. A third set of 0.5-cm^2 pieces were surface sterilized by submersion in 70 % ethanol for 2 min. Pieces were then submerged in 1 % bleach containing 0.001 % Tween 20 for 1 minute. Leaves were subsequently rinsed in 1 ml autoclaved Milli-Q water for at least 10 s. The bleach wash was repeated three times. Finally, these surface-sterilized pieces were homogenized and plated as described above. A fourth set of 0.5-cm^2 pieces were surface sterilized and blotted directly onto chitin plates to assess the efficiency of surface sterilization.

For all isolation attempts performed, plates were incubated for 3–5 weeks at room temperature (ca. 22 °C) before bacterial colonies with Actinobacteria morphology (filamentous, dry and spiky or dusty in appearance) were picked and transferred to YMEA. We allowed colonies to grow for 1–4 weeks, and subsequently confirmed morphology-based species identifications for all *Pseudonocardia*-like colonies using 16S rDNA sequencing (see below). To compare and quantify the efficiency of Actinobacteria and *Pseudonocardia* isolation, we counted the number of colony forming units (CFUs) produced on plates. We further calculated the proportion of colonies from which we were able to successfully isolate Actinobacteria and *Pseudonocardia* for each method. Additional *Pseudonocardia* isolated from lab colonies were included in tables and the phylogenetic analysis but not in the method efficacy analysis.

DNA sequencing and phylogenetic analysis. Once bacteria were obtained in axenic culture, we extracted DNA using a standard cetyltrimethylammonium bromide (CTAB) protocol [3,25]. DNA was quantified using a NanoDrop photospectrometer (Thermo-Fisher) and diluted to 50 ng/µl in tris-ethylenediaminetetraacetic acid (TE) buffer. We then amplified 16S rRNA using universal primers (27f 5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r

5′-TACGGYTACCTTGTTACGACTT-3′, [17b]) and nuclear Elongation Factor-Tu (EF-Tu 5′-CACGACAAGTCCCGAACCT-3′ and 5′-AGTTG TTGAAGAACGGGGTG-3′ [5]). Amplicons were sequenced using the dideoxyterminator method (Big Dye terminator mix; Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl DNA Analyzer at the University of Wisconsin Biotechnology Center (Madison, WI, USA) following a cleaning using the CleanSeq magnetic bead protocol (Agencourt Biosciences, Beverly, MA, USA).

We edited sequences using Sequencher 4.5 (Gene Codes Corporation) to assemble the forward and reverse sequences, then reconciled differences and removed automated calling errors as required. In cases of uncertainty, samples were re-sequenced. To identify each isolate to the genus level, we used BLASTn nucleotide sequences against the non-redundant nucleotide collection using megablast [20]. We screened all 16S sequences for possible chimeras using Bellerophon [16] through the Greengenes database with the default parameters. We found no chimeric sequences.

To generate a phylogeny for all *Pseudonocardia* isolates, we aligned sequences from the EF-Tu gene using Clustal X [http://www.clustal.org/], checked the alignment in MacClade 4.07 [19], and thereafter used Mega 5.0 [30] to generate a maximum likelihood phylogeny (Tamura-Nei assuming uniform rates and using all sites, sequences were truncated such that all were the same length) and bootstrapped 1000 times. The analysis included 38 strains previously isolated and sequenced from other colonies of fungus-growing ants (14 from leaf-cutting ants: 5 in genus *Atta*, and 9 in the genus *Acromyrmex*) as well as 16 species of free-living (non-ant-associated) *Pseudonocardia*. We rooted the tree using two species of *Streptomyces*. All generated sequences are available at GenBank under accession numbers JQ731834–85.

Results and Discussion

We were consistently able to isolate Actinobacteria from *Atta cephalotes* colonies using the different methods employed including washing, washing with sonication, scraping, and bead-beating (Table 1). On average, across all isolation method-colony component combinations for the five ant colonies tested, we obtained 20.6±6.3 (Mean ± SE) Actinobacterial CFUs per plate. We also isolated *Pseudonocardia* from all *Atta* colonies, with an average of 2.86 ± 1.47 CFUs of *Pseudonocardia* per plate across all isolation methods and the five ant colonies sampled. 16S sequencing of these Actinobacteria revealed some phylogenetic diversity: seven *Streptomyces,* two *Nocardioides*, and seventeen *Pseudonocardia* isolates (Table 2). Best-BLAST hits for the *Nocardioides* and *Streptomyces* isolated here correspond to strains obtained from a wide variety of habitats, including soil, ocean and other environments. While some insect-associated samples have similar 16S rRNA genes sequences (in one case, up to 100 % identity with a single termite gut-associated bacterial sequence that shares an equivalent level of identity with 57 other environmentally associated sequences), none of the strains found here had an identical 16S rRNA gene sequence to those previously reported from leaf-cutting ants [e.g., 2,14,29]. The phylogenetic distance between leaf-cutting ant-associated strains of *Nocardioides* and *Streptomyces* found here suggests a lack of specificity, and that the obtained isolates could be allochthonous.

Bead-beating was the most successful method for Actinobacteria isolation, generating an average of 40.1 ± 24.0 (Mean ± SE) CFUs per plate on chitin medium and 35.8 ± 24.9 CFUs per plate on CMC medium. Washing also resulted in large numbers of Actinobacteria, including an average of 16.4 ± 10.1 CFUs from washed workers. The number of Actinobacterial CFUs isolated for each of the 11 method/colony component combinations was significantly different from one another by a Kruskal-Wallis rank sum test (df =10, p = 0.008). Bead-beating, worker scraping, and infrabuccal pocket washes all resulted in the isolation of Actinobacteria, while worker washes and infrabuccal pocket scrapes were less efficient, yielding Actinobacteria from only 4 of the 5 colonies. The most efficient methods for *Pseudonocardia* isolation involved direct processing of worker ants, while fungus garden isolations were less successful. Isolates of *Pseudonocardia* were never obtained from leaves provided to the colonies, despite this bacterial genus including endophytic species [6,22]. Collectively, the findings suggest that *Atta* colonies harbor *Pseudonocardia* and that the bacterium is associated with colony workers. This is congruent with the finding that the majority of the diversity of fungus-growing ants maintains Actinobacteria on the cuticle [cf. 9].

Previous work has had limited success isolating *Pseudonocardia* using maceration and vortexing methods, but *Pseudonocardia* had previously been obtained from five *Atta* colonies [4,33]. For *Pseudonocardia* isolations, nine of our eleven isolation method/colony component combinations resulted in at least one isolate (Table 1). Bead-beating of workers was the most efficient method for obtaining *Pseudonocardia*, yielding 7.4 ± 5.1 and 4.3 ± 3.3 CFUs per plate on CMC and chitin, respectively. Bead-beating isolated significantly more CFUs of *Pseudonocardia* than the other methods tested here by the Wilcoxon Rank sum test (W = 127, p-value = 0.016). This method was not successful across all colonies, recovering *Pseudonocardia* from only 4 out of 5 colonies tested, however bead-beating represents a substantial improvement over previously employed techniques. Worker washing was the only other method that resulted in substantial *Pseudonocardia* CFUs, with an average of 6 ± 2.8 per plate. A comparison of CFUs isolated by this method against all others pooled by the Wilcoxon Rank sum test approached, but did not reach, statistical significance (W = 68, $P = 0.056$). We were able to obtain *Pseudonocardia* from 10 additional lab colonies applying these methods (see Table 2, Fig. 1).

Table 2. Actinobacteria sequences

Colony Code (Isolate)	Taxon	Location	Method	Closest 16S BLAST hit (GenBank Accession number)	GenBank #
N/A (A)	*Q. pal*	US	blot-Chi	*Streptomyces glauciniger* strain FXJ14 (AY314782.1)	JQ731859
N/A (B)	*Q. pal*	US	blot-Chi	*Streptomyces* sp. I08A-01824 (GU550598.1)	JQ731860
N/A (C)	*Q. pal*	US	blot-Chi	*Streptomyces owasiensis*, strain: NBRC 13832 (AB184515.1)	JQ731861
N/A (C)	*Acer*	US	blot-Chi	*Streptomyces halstedii* strain G8A-5 16S (CP002993.1)	JQ731864
MP010708F-1	*A. col*	Panama	fungal garden wash-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ768367
AL050513-21(C)	*A. cep*	Panama	fungal garden wash/sonicate-H_2O/Chi	*Streptomyces drozdowiczii* strain GYB24 (JQ342930.1)	JQ731836
MB03(B)	*A. cep*	Panama	garden wash-H_2O/Chi	*Pseudonocardia* sp. CC011205-08 (EU928998.1)	JQ731855
N/A (A)	*Acer*	US	homogenize-H_2O/Chi	*Streptomyces* sp. 163005 (GU263862.1)	JQ731862
N/A (B)	*Acer*	US	homogenize-H_2O/Chi	*Streptomyces* sp. JW1 (EU906929.1)	JQ731863
MB03(B)	*A. cep*	Panama	new garden wash-H_2O/Chi	Actinobacterium ZXY009 (JN049458.1)	JQ731851
AL050513-22(A)	*A. cep*	Panama	worker e beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731838
AP061022-01(A)	*A. cep*	CR	worker bead-beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731840
EC090907-05	*A. cep*	Peru	worker bead-beat-H_2O/Chi	*Pseudonocardia* sp. TFS 1235 (EF216360.1)	JQ731845
MP090907-15	*A. cep*	Peru	worker bead-beat-H_2O/Chi	*Pseudonocardia* sp. YIM 45505 (NR_043742.1)	JQ731846
UP08	*Atta* sp.	Peru	worker bead-beat-PBS/Chi	*Pseudonocardia* sp. FXJ3.021 (JN683673.1)	JQ731857
JS090511-01	*A. cep*	Panama	worker bead-beat-PBS/Chi	*Pseudonocardia* sp. JS020524-10_B1F6 (EU928990.1)	JQ731858
AL050513-21	*A. cep*	Panama	worker bead-beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731837
BB03(A)	*A. cep*	Panama	worker bead-beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731848
MB03(A)	*A. cep*	Panama	worker bead-beat-PBS/Chi & PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731854
EC090827-04	*A. cep*	Peru	worker bead-beat-PBS/CMC	*Pseudonocardia* sp. MVT7 (EU931094.1)	JQ731844
BB03(B)	*A. cep*	Panama	worker bead-beat-PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731849
BB03	*A. cep*	Panama	worker infrabuccal pocket scrape-Chi	*Streptomyces* sp. 172624 (EF550508.1)	JQ731847
AL050513-21(B)	*A. cep*	Panama	worker infrabuccal pocket wash-H_2O/Chi	*Streptomyces* sp. RS-2011-I28 (HE617241.1)	JQ731835
AL050513-21(A)	*A. cep*	Panama	worker scrape-Chi	*Streptomyces* sp. JS520 (JQ288109.1)	JQ731834
MB03	*A. cep*	Panama	worker wash & wash/sonicate-H_2O/Chi	*Nocardioides albus* (AF004997.1)	JQ731853
CC031208-10(A)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardia* sp. GB7 (EF451805.1)	JQ731842
CC031208-10(B)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardiaceae* isolate SR 244a (X87314.1)	JQ731843
MB03(C)	*A. cep*	Panama	worker wash-H_2O/Chi	*Pseudonocardia* sp. GB7 (EF451805.1)	JQ731856
AL050513-22(B)	*A. cep*	Panama	worker wash, worker bead-beat-PBS/CMC	*Pseudonocardia* sp. BMWB1 (FJ948119.2)	JQ731839
CC031208-10	*A. cep*	Panama	worker wash/sonicate-H_2O/Chi	*Nocardioides albus* (AF004997.1)	JQ731841
MB03(C)	*A. cep*	Panama	worker wash/sonicate-H_2O/Chi	*Streptomyces* sp. 10213 (FJ262955.1)	JQ731852
MB03(A)	*A. cep*	Panama	worker wash/sonicate-H_2O/Cih	*Streptomyces* sp. Ank245 (HQ662223.1)	JQ731850

Table of 16S rDNA sequences acquired from *Atta* sp. colonies and leaf material. Abbreviations are *A. cep*: *Atta cephalotes*; *A. col*: *Atta colombia; Acer*: *Acer* sp.; *Q. pal*: *Quercus palustris*; Chi: Chitin; CMC: carboxymethylcellulose; CR: Costa Rica. In the far let column, (A), (B) and (C) indicate different isolates from the same colony.

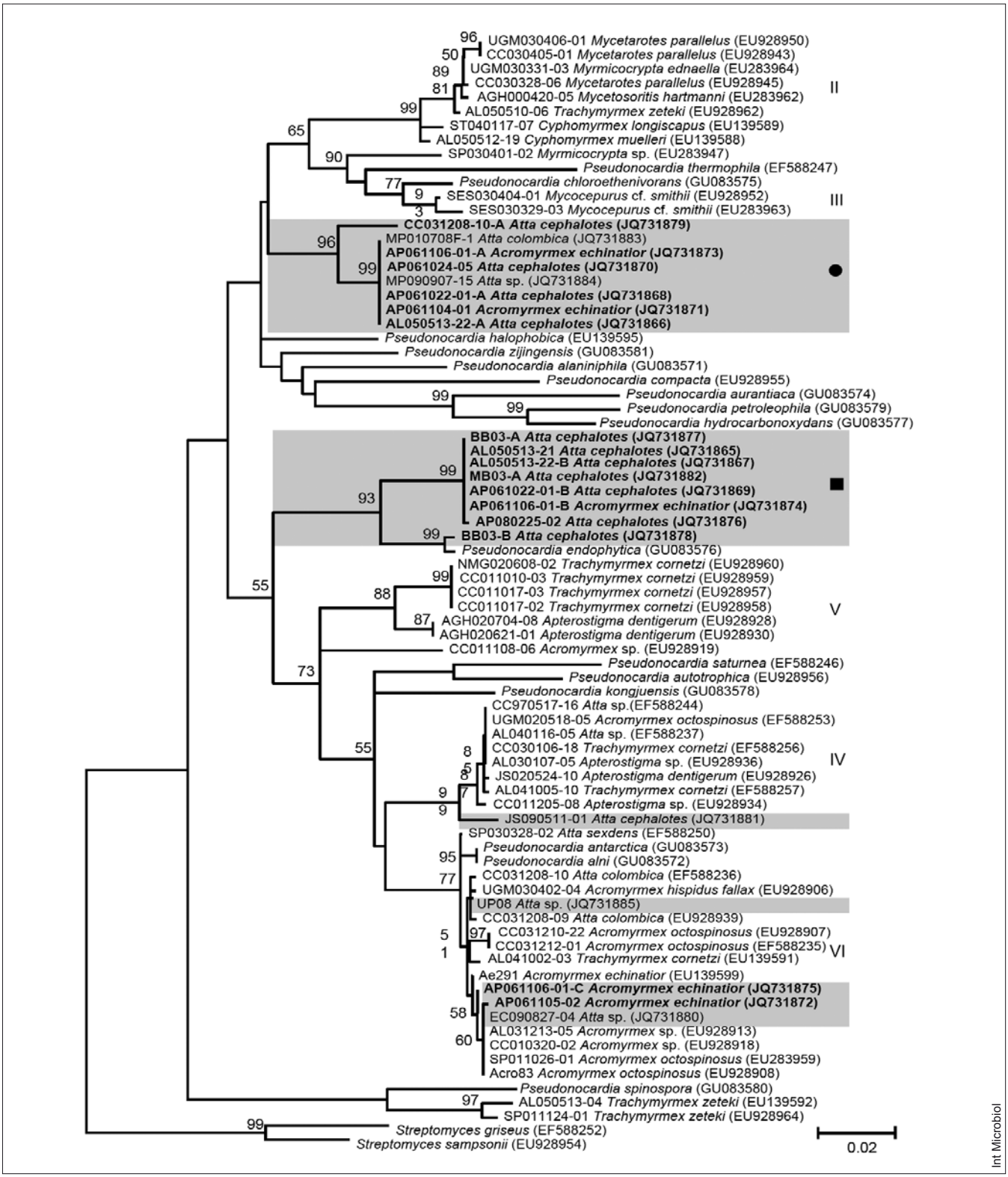

Fig. 1. Elongation factor-Tu phylogeny of *Pseudonocardia* isolates from *Atta* and *Acromyrmex* generated in this study, along with other symbionts from across the diversity of fungus-growing ants. *Pseudonocardia* strains are labeled with the colony code and species of the ant from which they were isolated. Grey boxes highlight strains isolated as a part of this study, with strains isolated from colonies housed in the laboratory long-term in bold. Some *Atta*-associated strains of *Pseudonocardia* isolated in this study fall into clades with other leaf-cutting ant-associated *Pseudonocardia* (clades IV and VI, cf. 4); however, this study also identified two previously undescribed clades of ant-associated *Pseudonocardia* (indicated with circle and square).

Overall, this study isolated 20 additional strains of *Pseudonocardia* (4 confirmed by 16S rDNA gene sequencing, 3 confirmed by Ef-Tu gene sequencing and 13 confirmed by both) from a total of 15 ant colonies from three *Atta* species. Because 16S rRNA genes alone provide limited species-level phylogenetic resolution, to further elucidate potential specificity we generated a phylogeny of the *Pseudonocardia* isolates based on EF-Tu (Fig. 1). Three *Pseudonocardia* isolates grouped with isolates from *Acromyrmex* leaf-cutting ants (one in clade IV, isolated by bead-beating with PBS onto chitin, and two in clade VI isolated by bead-beating with PBS onto chitin and CMC, Fig. 1). We also recovered 13 *Pseudonocardia* strains from two clades that had not previously been identified in associations with fungus-growing ants, which might represent hitherto unknown diversity within the symbiosis (Fig. 1, clades labeled with circle and square, respectively).

A concurrent study employing bead-beating with *Acromyrmex* ants identified sequences mapping to these novel clades from three *Acromyrmex* leaf-cutter ants (unpublished data, sequences included in Fig. 1; *Acromyrmex echinatior* colonies AP061104-01, AP061105-02, and AP061106-01 from Costa Rica). The clustering of these ant-associated strains suggests a specific association between *Atta* and *Pseudonocardia*. Given the different isolation methods (e.g., bead-beating, washing with sonication) it is possible that the *Pseudonocardia* originated from inside the ants, and not on the external surface or in structures associated with cuticle. Additional isolations and phylogenetic studies will be needed to substantiate the presence of these novel genotypes and further explore the extent and role of this relationship.

We did not isolate *Pseudonocardia* from oak and maple leaves blotted on chitin media, but did obtain CFUs of other Actinobacteria: an average of 0.4 ± 0.28 (mean ± SE) (0.5 ± 0.47 from oak, and 0.3 ± 0.28 from maple). Isolations by homogenization resulted in an average of 0 CFUs of Actinobacteria from oak, and 0.8 ± 0.76 from maple. Sequencing of the strains recovered from these isolations revealed only *Streptomyces*. No Actinobacteria colonies were isolated on CMC medium. The Actinobacteria isolation rate from leaves was therefore low overall, with only a single leaf out of the ten resulting in CFUs. This indicates that ant-associated *Pseudonocardia* do not appear to be present on provisioned leaf material, suggesting their presence in the symbiosis, but we cannot rule out alternative sources of introduction of the bacteria to ant workers.

Our limited isolation success from certain colony components concurs with the culture-dependent findings by Mueller and colleagues [21]. Both studies found little to no *Pseudonocardia* from the majority of *Atta* colony components, including fungal gardens and the ants' infrabuccal pockets. Further, *Pseudonocardia* has not been detected in a recent community metagenomic study of *Atta cephalotes* garden [1], suggesting that it is unlikely to reside in this component of the system. Neither Mueller et al. [21], Aylward et al. [1], nor this study found convincing evidence of garden or other environmental sources for *Pseudonocardia* in the *Atta* leaf-cutting ant symbiosis. We found the highest isolation rates directly from whole ants, suggesting that if the association of *Pseudonocardia* is maintained in *Atta*, it is specifically with ant workers, but not in the infrabuccal pocket.

Atta colonies include large work forces of highly polymorphic ants, in addition to age-dependent division of labor [15]. In other genera of attine ants, *Pseudonocardia* are present on a variety of cuticular structures [9], and might be the symbiont is present on inaccessible parts of the ant cuticle. It is furthermore conceivable that only some worker castes associate with *Pseudo-nocardia* and employ its antifungal compound(s), as is the case in the less polymorphic genus *Acromyrmex*, where mainly major workers carry *Pseudonocardia* [7,24]. Collectively, this suggests that age- or caste dependent within-colony composition of the bacterial symbiont population could make it harder to obtain without considerable targeted effort to screen across a wide range of ages and castes.

It is well known that many symbiotic microbes are extremely difficult to culture in isolation, and even when these microbes can grow in culture, they are often extremely fastidious, requiring a specific combination of growth conditions and nutrients normally provisioned by the host to meet their physiological needs [12]. Thus, one possible reason for previous attempts to isolate *Pseudonocardia* from *Atta* having had limited success is not an absence of the symbionts in these hosts, but rather that the strains associated with *Atta* are more fastidious. A considerable effort allowed us to isolate strains of *Pseudonocardia* from 15 *Atta* colonies; this suggests the presence of *Pseudonocardia* in the association, but the importance in defense remains to be established. Currie and Stuart [11] have shown that *Atta* ants employ fungus grooming at increased levels in response to pathogens, and Fernández-Marín and colleagues [13] went on to demonstrate that *Atta* ants increase the levels of metapleural gland grooming behavior when challenged with *Escovopsis*. These findings indicate that *Atta* ants may have a low need to utilize *Pseudonocardia* for defense. Consequently, although our work supports that there might still be an association between *Pseudonocardia* and *Atta*, further work is needed to understand the role and impact of the bacteria on *Atta* colonies.

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References

1. Aylward FO, Burnum KE, Scott JJ, et al. (2012) Metagenomic and metaproteomic insights into bacterial communities in leaf-cutter ant fungus garden. ISME J 6:1688-1701
2. Barke J, Seipke RF, Grüschow S, Heavens D, Drou N, Bibb MJ, Goss RJ, Yu DW, Hutchings MI (2010) A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*. BMC Biol 8:109-118
3. Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. Can J Microbiol 51:441-446
4. Cafaro MJ, Poulsen M, Little AEF, Price SL, Gerardo NM, Wong B, Stuart AE, Larget B, Abbot P, Currie CR (2011) Specificity in the symbiotic association between fungus-growing ants and protective *Pseudonocardia* bacteria. Proc R Soc B 278:1814-822
5. Caldera EJ, Currie CR (2012) The population structure of antibiotic-producing bacterial symbionts of *Apterostigma dentigerum* ants: Impacts of coevolution and multipartite symbiosis. Am Nat 180:604-617
6. Chen HH, Qin S, Li J, Zhang YQ, Xu LH, Jiang CL, Kim CJ, Li WJ (2009) *Pseudonocardia endophytica* sp. nov., isolated from the pharmaceutical plant *Lobelia clavata*. Int J Syst Evol Microbiol 59:559-563
7. Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. Oikos 101:91-102
8. Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. Proc Natl Acad Sci USA 96:7998-8002
9. Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81-83
10. Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704
11. Currie CR, Stuart AE (2001) Weeding and grooming of pathogens in agriculture by ants. Proc R Soc B 268:1033-1039
12. Darby AC, Welburn SC (2003) Symbiont culture. In: Bourtzis K, Miller TA (eds) Insect symbiosis. (vol 2) CRC Press, Boca Raton, FL, pp 141-155
13. Fernández-Marín H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT (2009) Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. Proc R Soc B 276:2263-2269
14. Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. Proc Natl Acad Sci USA 106:4742-4746
15. Hölldobler B, Wilson EO (1990) The Ants. Belknap Press of Harvard, Cambridge, MA, USA
16. Huber T, Faulkner G, Hugenholtz P (2004) Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 20:2317-2319
17. Kost C, Lakatos T, Böttcher I, Arendholz W-R, Redenbach M, Wirth R (2007) Non-specific association between filamentous bacteria and fungus-growing ants. Naturwissenschaften 94:821-828

17b. Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, UK, pp 115-175

18. Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: fungus-growing ants combine specialized behaviours and microbial symbionts to protect their fungus gardens. Biol Lett 2:12-16
19. Maddison DR, Maddison WP (2005) MacClade 4 release version 4.07 for OSX. Sinauer Associates, Inc., Sunderland, MA, USA
20. Morgulis A, Coulouris G, Raytselis Y, Madden TL, Agarwala R, Schaffer AA (2008) Database indexing for production MegaBLAST searches. Bioinformatics 24:1757-1764
21. Mueller UG, Dash D, Rabeling C, Rodrigues A (2008) Coevolution between Attine ants and Actinomycete bacteria: A reevaluation. Evolution 62:2894-2912
22. Nimnoi P, Pongsilp N, Lumyong S (2009) Endophytic actinomycetes isolated from *Aquilaria crassna* Pierre ex Lec and screening of plant growth promoters production. World J Microbiol Biotechnol 26:193-203
23. Oh D-C, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: a bacterial mediator of an ant-fungus symbiosis. Nat Chem Biol 5:391-393
24. Poulsen M, Bot ANM, Currie CR, Boomsma JJ (2002) Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in *Acromyrmex* leaf-cutting ants. Insectes Soc 49:15-19
25. Poulsen M, Cafaro MJ, Boomsma JJ, Currie CR (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. Mol Ecol 14:3597-3604
26. Poulsen M, Cafaro MJ, Erhardt DP, Little AEF, Gerardo NM, Tebbets B, Klein BS, Currie CR (2010) Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. Environm Microbiol Reports 2:534-540
27. Reynolds HT, Currie CR (2004) Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. Mycologia 96:955-959
28. Schultz TR, Brady SG (2008) Major evolutionary transitions in ant agriculture. Proc Natl Acad Sci USA 105:5435-5440
29. Sen R, Ishak HD, Estrada D, Dowd SE, Hong E, Mueller UG (2009) Generalized antifungal activity and 454-screening of *Pseudonocardia*

and *Amycolatopsis* bacteria in nests of fungus-growing ants. Proc Natl Acad Sci USA 106:17805-17810

30. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599
31. Van Borm S, Billen J, Boomsma JJ (2002) The diversity of microorganisms associated with *Acromyrmex* leafcutter ants. BMC Evol Biol 2:9-19
32. Yek SH, Boomsma JJ, Poulsen M (2012) Towards a better understanding of the evolution of specialized parasites of fungus-growing ant crops. Psyche: A Journal of Entomology Vol 2012:1-10
33. Zhang MM, Poulsen M, Currie CR (2007) Symbiont recognition of mutualistic bacteria by *Acromyrmex* leaf-cutting ants. ISME J 1:313-320