Actinomycetes with Antimicrobial Activity Isolated from Paper Wasp (Hymenoptera: Vespidae: Polistinae) Nests

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ABSTRACT   Actinomycetes—a group of antimicrobial producing bacteria—have been successfully cultured and characterized from the nest material of diverse arthropods. Some are symbionts that produce antimicrobial chemicals found to protect nest brood and resources from pathogenic microbes. Others have no known fitness relationship with their associated insects, but have been found to produce antimicrobials in vitro. Consequently, insect nest material is being investigated as a new source of novel antimicrobial producing actinomycetes, which could be harnessed for therapeutic potential. To extend studies of actinomycete-insect associations beyond soil-substrate dwelling insects and wood boring excavators, we conducted a preliminary assessment of the actinomycetes within the nests of the paper wasp, *Polistes dominulus* (Christ). We found that actinomycetes were readily cultured from nest material across multiple invasive *P. dominulus* populations—including members of the genera *Streptomyces*, *Micromonospora*, and *Actinoplanes*. Thirty of these isolates were assayed for antimicrobial activity against the challenge bacteria *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Serratia marcescens*, and *Bacillus subtilis*. Sixty percent of isolates inhibited the growth of at least one challenge strain. This study provides the first assessment of bacteria associated with nests of *P. dominulus*, and the first record of antimicrobial producing actinomycetes isolated from social wasps. We provide a new system to explore nest associated actinomycetes from a ubiquitous and cosmopolitan group of insects.

KEY WORDS   antibiotic, natural products, actinobacteria, *Polistes dominula*

Antimicrobial producing bacteria have been identified in nest material of divergent arthropod lineages. The best studied of these bacteria are actinomycetes (actinobacteria)—a bacterial phylum that produces a wide variety of antimicrobial chemicals (reviewed in Lam 2006). Some of these arthropod bacteria associations include derived symbioses, where the actinomycetes assist in nest or colony hygiene by producing antimicrobial chemicals (reviewed in Seipke et al. 2011a and Kaltenpoth 2009). In addition, recent evidence indicates that arthropod habitats frequently contain a veritable bouquet of diverse, nonsymbiont actinomycete species (e.g., Poulsen et al. 2011, Mueller et al. 2008, Kost et al. 2007, Sen et al. 2009, Promnuan et al. 2009, Hulcr et al. 2011). Such actinomycete associates include both ubiquitous and rare species in the nest material of ants (e.g., Barke et al. 2010, Sen et al. 2009), beetles (Scott et al. 2008. Hulcr et al. 2011,

Grubbs et al. 2011), termites (Visser et al. 2012), bees (Promnuan et al. 2009), and solitary wasps (e.g., Poulsen et al. 2011; Kaltenpoth et al. 2006, 2010). Even a novel actinomycete species, *Actinomadura apis*, was isolated from a honey bee hive (Promnuan et al. 2011).

Many constituents of these associated microbial communities produce antibiotics in vitro, some of which are novel (Poulsen et al. 2011, Mueller et al. 2008). For example, Poulsen et al. (2011) studied solitary mud dauber wasps of the species *Chalybion californicum* and *Sceliphron caementarium*, and isolated at least 15 actinomycete strains of the genus *Streptomyces*. Extensive chemical investigations showed that these symbionts produce eight unique antimicrobials, including the novel antimicrobial sceliphrolactam (Poulsen et al. 2011, Oh et al. 2011).

The ecological function of many actinomycete antimicrobials remains nebulous. It is clear, however, that arthropod nest material is a valuable source of novel actinomycetes. This is particularly relevant to human health, as the recent increase in antibiotic resistant pathogens necessitates the discovery of novel drugs (Clardy et al. 2006). Because most clinically available antibiotics originate from actinomycetes and fungi (Newman et al. 2003 in Pelaez 2006, reviewed in Lam 2006), the current epidemic of antibiotic resistant pathogens has spawned a renewed interest in identi-
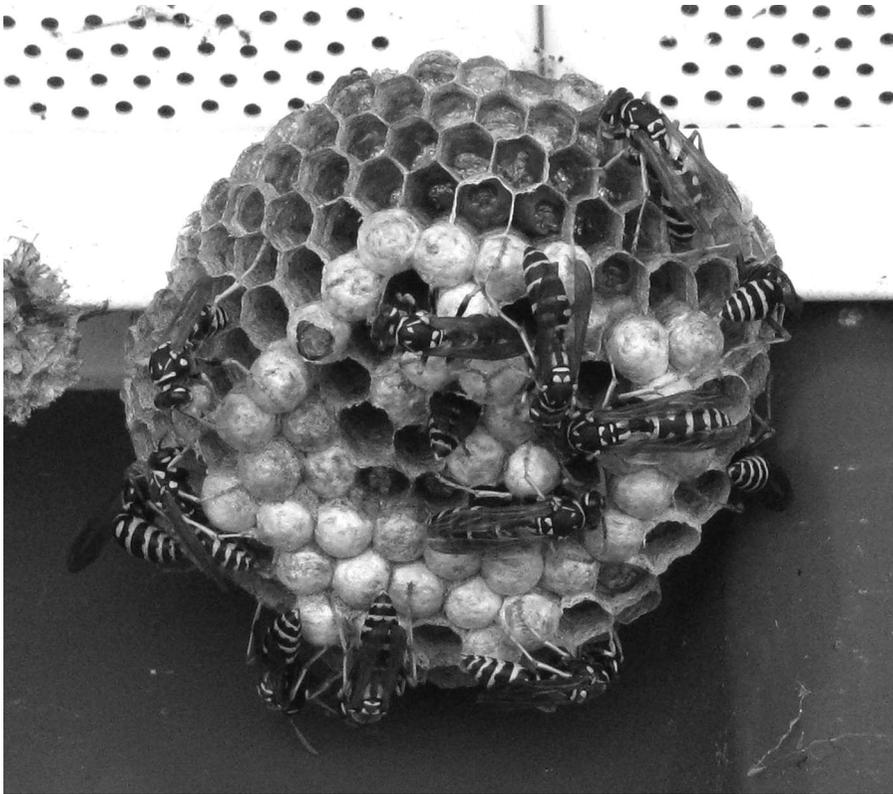
Fig. 1.   *Polistes dominulus* nest in Massachusetts.

fying uncharacterized habitats from which to isolate unique actinomycetes that produce novel antibiotics (Pelaez 2006). Insect nest associates are consequently being investigated as a likely source of such novelty (e.g., Poulsen et al. 2011, Bode 2009). Despite the potential for microbial or chemical novelty, investigations of nest material have been confined to relatively few wood excavating or soil-nest/constructing insects.

Paper wasps of the genus *Polistes* are social, progressively provisioning hymenoptera, which construct open-faced nests out of macerated plant pulp and saliva (Evans and West–Eberhard 1970). One species in particular, *Polistes dominulus* (Fig. 1), is a globally distributed paper wasp that annually constructs nests often associated with anthropogenic structures (reviewed in Liebert et al. 2006). Their proclivity for this accessible habitat, and their status as a successful invader, has contributed to their use as a model system in the fields of behavior, evolution, and invasion ecology (Starks and Turillazzi 2006, reviewed in Liebert et al. 2006). Despite the resultant wealth of information that exists on this species, to date no studies have investigated their associated microbial communities. Indeed, few studies have been conducted on the bacteria of any *Polistes* species that have not solely addressed the *Wolbachia* endosymbiont (Stahlhut et al. 2006, but see Morel and Fouillaud 1992). As part of a preliminary characterization of the fungi within *P.*

dominulus nests in Massachusetts, we recently identified a previously uncharacterized fungal species (Madden et al. 2012), suggesting this may be a habitat that contains novel bacteria as well.

Therefore, we investigated *P. dominulus* nests to provide an initial bacterial characterization of this insect, and to determine if nests of social wasps contain actinomycetes with antimicrobial activity. Eventual novel chemical isolation and structure elucidation traditionally requires culturable isolates. We consequently conducted a preliminary investigation of the actinomycetes associated with the nests of *P. dominulus* using culture dependent methods and assessing in vitro microbial growth inhibition capability. This broadens the studies of actinomycete insect associates to encompass the previously undescribed realm of a globaly distributed, and well-studied insect lineage whose microbiota has previously remained unexplored.

Materials and Methods

Nest Collection. Mature, active *P. dominulus* nests were aseptically removed at the top of their nest pedicel and stored at 4°C. Nests were identified as *P. dominulus* and determined to be active if at the time of collection (August) they were found to have live *P. dominulus* residents on them at night. In the laboratory, all active individuals, larvae, pupae, and eggs

were aseptically removed from the nest. Nests were visually inspected and any found to be parasitized by brood parasitoids (Madden et al. 2010) were removed from the study. Nests were collected from three geographically distinct regions in Eastern Massachusetts in the towns of Waltham ($n = 4$), Easton ($n = 3$), and Medford ($n = 5$). These sites were determined to be geographically isolated because they were separated by a distance exceeding the foraging range of this species (Ugolini and Cannicci 1996). They were chosen to reduce the probability that one insect had visited, and thereby contaminated, all nests collected because of potential low nest fidelity (Sumner et al. 2007).

Microorganism Isolation. For each nest, nest material (including paper, pedicel, meconium, and honey if present) was aseptically mechanically homogenized with forceps, and placed in a conical tube with a $1\times$ phosphate buffered saline solution diluted to contain 5 mM phosphate. Tubes were vortexed and the resulting homogenate was diluted in the same solvent and plated in 1% Luria–Bertani (Miller) medium (15% bacto agar) (BD [Becton, Dickinson and Company], Sparks Glencoe, MD), Actinomycete Isolation medium (BD), and a low nutrient medium: 1% Skim Milk and 18% Gellan Gum (GELZAN CM, Sigma–Aldrich, Milwaukee, WI). Plates were incubated at ambient room temperature ($\approx 22°C$) and bacterial colonies were streaked for purification as they grew (continuously after ≈ 72 h) (Supp. Table 1 [online only]). Colonies exhibiting morphologies indicative of actinomycetes were selected for further purification.

Molecular Analysis. Genomic DNA was extracted from cultured microorganisms using the UltraClean microbial DNA extraction kit (MoBio, Carlsbad, CA) per the manufacturer's instructions, including a 10 min heating step at 70°C to enhance lysis. A large fragment of the 16S rRNA gene was amplified, as there is enough variability in this fragment to allow for determinative identity to the genus level in this phylum (Stackebrandt et al. 1997). The fragment was amplified using the universal eubacterial primers 27F and 1492R (Heuer et al. 1997, Eden et al. 1991). The polymerase chain reaction (PCR) cocktail contained: 5 μl of $5\times$ Green GoTaq Reaction Buffer (Promega, Madison, WI), 5 μl template DNA, 11.95 μl water, 1.5 μl of 25 mM MgCl$_2$ (Promega), 0.15 μl (100 pM/μl) of each primer, 1 μl of dNTP Mix (10 mM) (Promega), and 0.25 μl GoTaq Polymerase (50 U/μl) (Promega) for a total reaction volume of 25 μl. PCR was conducted using a thermocycler (model 2700, GeneAmp PCR System, Applied Biosystems, San Francisco, CA) with the cycling parameters of 95°C for 5 min, then 30 cycles of 95°C for 1 min, 54°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. Amplified products of the estimated fragment length were confirmed by running them on a 1.5% agarose electrophoresis gel and visualizing with ethidium bromide stain.

Successfully amplified samples were purified and sequenced by MacrogenUSA (Rockville, MD) using the amplification primers. Sequence ends were trimmed and a consensus contiguous sequence was created using DNA Baser Software (Heracles Software, Germany). Resulting sequences were compared with those in the GenBank sequence repository using the BLAST algorithm, and highest matching identity was recorded for probable identification. A phylogenetic tree was constructed using the parameters described by Liu et al. (2009). Briefly, MEGA v4.0 (Tamura et al. 2007) was used for both the construction of the multiple alignment (using ClustalW and the IUB DNA weight matrix) and the phylogenetic trees using the neighbor-joining tree-making algorithms (Saitou and Nei 1987). Pairwise and evolutionary distances for the neighbor-joining algorithms were estimated using the Kimura two-parameter model (with both transitions and transversions included) (Kimura 1980), with positions containing gaps or missing data excluded (complete deletion option).

Strain sequences from Haeder et al. (2009) and other high homology strains, as determined by BLAST analysis, were included in the multiple alignments for comparison. Sequences were trimmed to $\approx 1,300$ bp before alignment to reduce false distance analyses. The bacterial 16S rRNA gene sequence of *Bifidobacterium longum* subsp. *suis* (AB437360), downloaded from the GenBank database, was used as an outgroup. The resultant tree was evaluated using bootstrap analysis (Felsenstein 1985) based on 1,000 replicates.

Antimicrobial Production Assay. The ability of the actinomycete isolates to inhibit bacterial growth was assessed using a modified agar cross-streak method (Waksman et al. 1965). This method involves two challenge bacteria being streaked perpendicularly to one another. After incubation, antimicrobial activity is assessed as the presence of a clear, no growth zone between the putative antimicrobial producer, and the challenge species. Isolated and control actinomycete strains were challenged with a broad spectrum of bacterial species including *Escherichia coli*, the clinically relevant *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus* (methicillin sensitive), and *Bacillus subtilis.* A hybrid assay medium containing 50% Luria–Bertani (Miller) medium (BD) and 17% bacto agar (BD) was used because the majority of isolated strains grew on this medium well, as did the more fastidious challenge bacteria. Assay media plates were prepared at a depth of 3 mm. Isolated actinomycetes were streaked to confluency over half of the plate from axenic, mature cultures grown on the same medium. Plates containing the growing isolated actinomycetes were incubated for three days at 30°C to allow for robust growth.

Challenge bacteria were grown in 50% Luria–Bertani (Miller) broth (BD) at 37°C to exponential phase. They were subsequently diluted to the McFarland 0.5 turbidity standard in the same broth to control for cell concentration (McFarland 1907). A sterile cotton swab was dipped into the liquid culture for 2 s (to allow for saturation) and then pressed against the side of the tube to remove excess liquid. The challenge organism was cross-streaked on the plates, perpendicularly to the actinomycete growth. Plates that had not been inoculated, were streaked with the challenge organism in the same manner to serve as negative controls. *Streptomyces griseus*, a
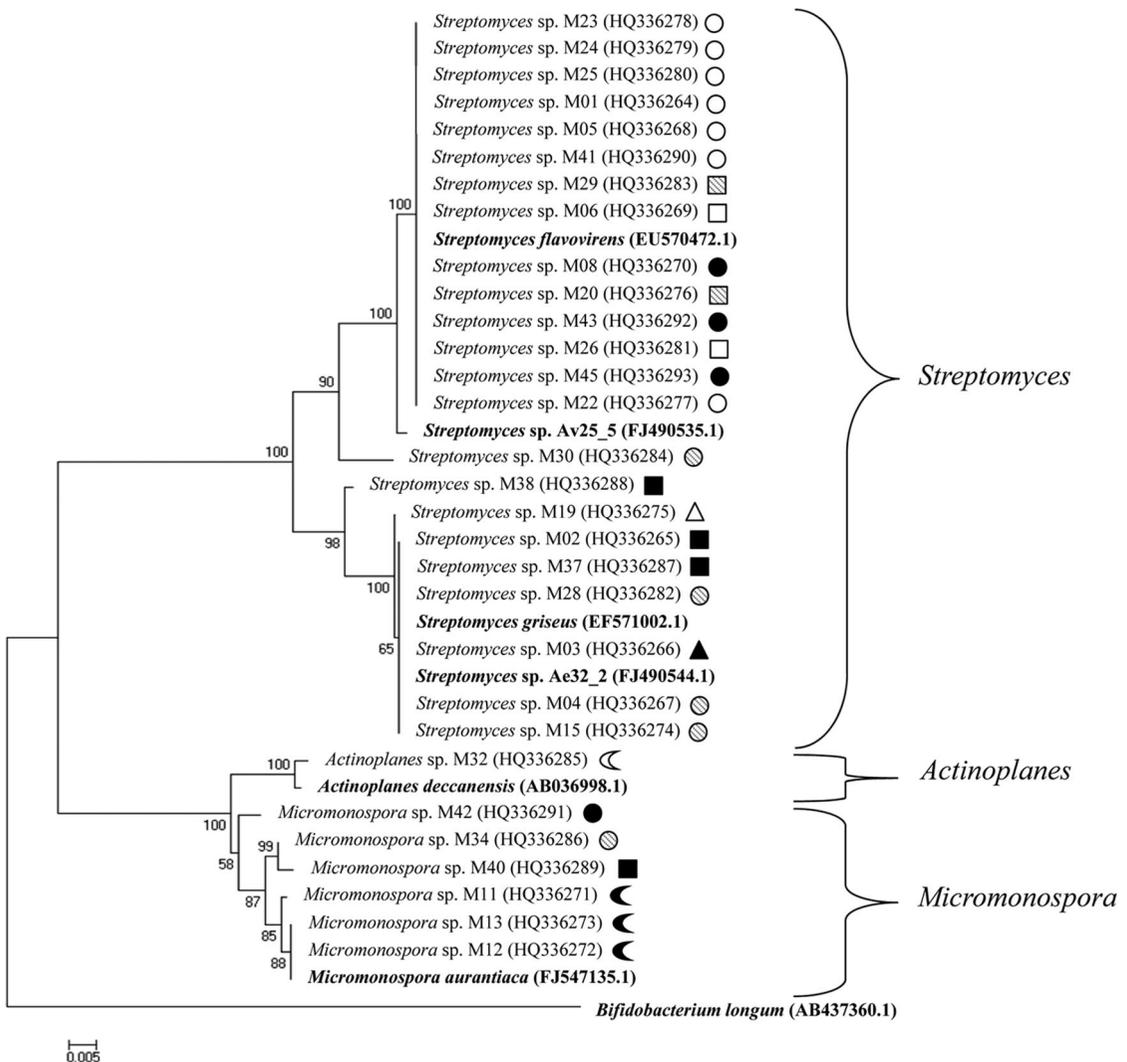
Fig. 2. Neighbor-joining phylogenetic tree based on partial 16S rDNA gene sequences, showing the relationships among actinomycetes isolated from *P. dominulus* nests. Accession numbers are given in parentheses. *Bifidobacterium longum* was used as an outgroup. Neighbor-joining algorithms were applied using the software package MEGA v4.0 (Tamura et al. 2007). Bootstrap percentages (based on 1,000 replications) are shown at branch points. Bar represents 0.005 substitutions per nucleotide position. Symbols next to strains indicate the three populations sampled. Symbol shades indicate the three populations sampled (black, Medford; striped, Easton; white, Waltham), and symbol shapes indicate unique nests within each population.

known producer of the antibiotic streptomycin, was used as a positive control. Growth inhibition was measured after bacterial plates were incubated at 37°C for 18 h. Assays were performed in triplicate and inhibition was scored as a clear zone of growth inhibition (+) or no clear zone of growth inhibition (−). Results were averaged across replicates.

Results

Actinomycete Isolation. Bacterial isolates with macroscopic colony morphologies consistent with actinomycetes were isolated from 12 of the 15 collected *P. dominulus* nests, and from all three sites sampled (Fig. 2). Of the 39 strains isolated, 30 were haphazardly

chosen for sequencing. BLAST searches of the sequenced 16S rRNA gene fragments within GenBank revealed high similarity with bacterial sequences from three different actinomycete genera: *Streptomyces*, *Micromonospora*, and *Actinoplanes*.

Six bacterial strains with a high sequence similarity (≥98% 16S rRNA gene) to *Micromonospora* spp. were isolated from four nests at two field site locations (Fig. 2). A strain with a nearest sequence homology to *Actinoplanes deccanensis* (99%) was isolated from one nest, at one location (Fig. 2).

Most of the isolated strains sequenced (77%) were *Streptomyces*. These strains were isolated from eight nests and from all three field sites (Fig. 2). Subsequent amplification and sequencing of the 16S rDNA re-

Table 1. Antibiotic activity of actinomycetes isolated from the nests of *Polistes dominulus*

Strain homology[a]	Strain	Gram negative			Gram positive	
		Escherichia coli	*Pseudomonas aeruginosa*	*Serratia marcescens*	*Bacillus subtilis*	*Staphylococcus aureus*
Streptomyces flavovirens species complex	M23	−	−	−	−	−
	M24	−	−	−	−	−
	M25	+	−	−	−	−
	M01	−	−	−	−	−
	M05	−	−	−	−	−
	M41	−	−	−	−	−
	M29	+	−	−	+	+
	M06	−	−	−	−	−
	M08	−	−	−	−	−
	M20	−	−	−	−	−
	M43	−	−	−	−	−
	M26	−	−	−	+	−
	M45	−	−	−	−	−
	M22	−	−	−	−	−
	M30	+	−	−	+	−
Streptomyces griseus species complex	M38	−	+	−	+	+
	M19	−	−	−	−	−
	M02	−	−	−	+	+
	M37	−	−	−	+	+
	M28	−	−	−	+	−
	S. griseus	+	−	+	+	+
	M03	−	−	−	+	+
	M04	−	−	−	+	+
	M15	−	+	−	+	−
Actinoplanes sp.	M32	−	−	−	+	+
Micromonospora spp.	M42	−	−	−	−	+
	M34	−	−	−	+	+
	M40	+	−	−	−	−
	M11	−	−	−	+	+
	M13	−	−	−	+	+
	M12	−	−	−	+	+

Antibiotic activity was determined using a modified agar cross-streak assay on 50% Luria–Bertani (Miller) agar plates ($n = 3$). Results for a known antibiotic producing strain of *Streptomyces griseus* are included for comparison ($n = 8$). Strains are arranged as they appear on the phylogenetic tree (Fig. 2).

[a] There was a ≥98% 16S rRNA gene fragment sequence similarity.

vealed that these actinomycete strains formed two well-supported clades. One cluster included strains exhibiting a high degree of 16S rRNA gene sequence similarity (98–100%) to a strain of *S. griseus* and isolates from other insect nest material (Fig. 2). This includes *Streptomyces* sp. Ae32_2, which is an antifungal producing actinomycete isolated from Panamanian leaf-cutter ant fungus garden nests of *Acromyrmex echinatior* (Haeder et al. 2009) (Fig. 2). These strains were isolated from four nests collected from all three locations sampled (Fig. 2).

A second cluster of actinomycete strains was isolated from five *P. dominulus* nests, from all three locations sampled (Fig. 2). These strains had a high degree of sequence similarity (≥99%) to *Streptomyces flavovirens*, and to a second isolated ectosymbiont cultured by Haeder et al. (2009)— *Streptomyces* sp. Av25_5. Haeder et al. (2009) isolated this strain from the nest of the Panamanian leaf-cutter ant *Acromyrmex volcanus* (Fig. 2).

Antimicrobial Activity. Of the 30 strains isolated and confirmed to be actinomycetes, 60% exhibited antimicrobial activity against one of the five challenge strains: *E. coli*, *P. aeruginosa*, *S. marcescens*, *S. aureus*, and *B. subtilis*. Antibiotic activity appeared to be predicted by phylogenetic relatedness (Table 1), although low sample sizes and the inability to detect unique, nonreplicate strains prohibited statistical testing of this hypothesis. Fifty-three percent of the strains showed growth inhibition activity against at least one of the gram-positive strains in the panel (Table 1). Notably, strains with a higher sequence similarity to *S. griseus*, *Micromonospora* spp., and *A. deccanensis*, inhibited the growth of the gram positives in the panel (*B. subtilis* and *S. aureus*) (Table 1).

Twenty percent of the confirmed actinomycete isolates displayed antimicrobial activity against the gram negatives included in the challenge panel (Table 1). Six of the strains with a high degree of homology to *Streptomyces* spp. and *Micromonospora* spp. inhibited *E. coli* (Table 1). Only two strains (with a high sequence similarity to *S. griseus* and a *Micromonospora* sp.), out of the total tested, exhibited any growth inhibition of *P. aeruginosa* (Table 1). None of the 30 isolates inhibited the growth of *S. marcescens* (Table 1).

Discussion

This is the first assessment to reveal that diverse actinomycetes can be readily isolated from the nest material of a paper nest building vespid. The 30 se-

quenced actinomycetes isolated from these nests belong to the two most bioactively-rich actinomycete families: Streptomycetaceae and Micromonosporaceae (reviewed in Berdy 2005), including the three genera, *Streptomyces, Micromonospora,* and *Actinoplanes* (Fig. 2). While *Streptomyces,* and arguably *Micromonospora,* are common soil microbes (Williams and Wellington 1982 in El-Tarabily and Sivasithamparam 2006, but see review by Lazzarini et al. 2001), *Actinoplanes* is a rare actinomycete genus (Yi-Quing et al. 2008). It is particularly surprising that we were able to isolate a strain of this genus, as we were not conducting a comprehensive study of the total culturable actinomycetes within this habitat. To the authors' knowledge, this is the first strain of this genus isolated from insect nest material.

Under our assay conditions, the antimicrobial activity among isolated strains was variable, even among closely related isolates (Table 1). This is consistent with other studies on intraspecific differences within streptomycete species (Antony–Babu et al. 2008, Hulcr et al. 2011). Our isolates displayed predominately gram-positive antagonism; however, it is not clear if this activity arose from one, or multiple antimicrobial compounds. *Streptomyces* spp. are capable of producing over 100,000 different antibiotics (Watve et al. 2001), with some strains producing multiple antimicrobials (Seipke et al. 2011b). This is true even under in vivo conditions, where a cocktail of antimicrobials is expected to assist in nest hygiene (Kroiss et al. 2010). Because of such complexity, further studies relating to extracts from our isolates, produced under numerous culture conditions, would need to be conducted to understand the mechanism behind the bioactivity displayed in our assays.

Most of the strains we isolated belong to the genus *Streptomyces,* consistent with similar studies investigating nest-associated insect material. This includes those studies relating to leaf-cutter ants (Haeder et al. 2009, Barke et al. 2010, Zucchi et al. 2011, Kost et al. 2007), wood boring beetles (Hulcr et al. 2011, Scott et al. 2008, Grubbs et al. 2011), honey and stingless bees (Promnuan et al. 2009, Gilliam and Prest 1987), solitary bees (Batra et al. 1973, Inglis et al. 1993), digger wasps (Kaltenpoth et al. 2006), mud dauber wasps (Kumar et al. 2011a,b; Poulsen et al. 2011), and termites (e.g., Bignell et al. 1991, Visser et al. 2012). Furthermore, a study by Ruddick and Williams (1972), suggests that spores of *Streptomyces* spp. are associated with the cuticle of many arthropods. Therefore, it is not surprising that strepomycetes are often found in nest material. The abundance of streptomycetes that we found may also be reflective of the fact that they are abundant in many environmental habitats—including plant material such as that used by *P. dominulus* to construct nests (see references in Williams et al. 1989). It should be noted though, that the perceived unique ubiquity of this genus in insect material when compared with other actinomycetes may be a byproduct of standard culturing practices, which select for their fast and dominant growth (Manteca and Sanchez 2009).

The *Streptomyces* spp. portion of our isolates formed two well-supported clusters in our phylogenetic tree, with similar strains being isolated across nests and across *P. dominulus* populations (Fig. 2). Many of these strains produced antimicrobial activity in vitro (Table 1), and are similar to strains identified in analogous screens of actinomycetes of various insects— such as the *Streptomyces* spp. identified by Haeder et al. (2009) in association with leaf cutter ants (Fig. 2). Therefore, it is tempting to speculate a fitness relationship between these microbes and the wasps, particularly as paper wasps are expected to face a similar assortment of microbial pathogens to those insects in established symbioses (Jeanne 1996). However, it is likely that at least some of the actinomycetes within the *P. dominulus* nest are dormant spores. This rationale is based partially on the knowledge that nests of *P. dominulus* are known to be collection points for local heavy metal particles from combustion engines (Urbini et al. 2006) and therefore may be traps for aerially dispersed spores as well.

While relatively little is known about how paper wasps control nest hygiene (but see Turillazzi et al. 2004, Turillazzi 2006), Hoggard et al. (2011) found that paper nest building wasps produce active cuticular antimicrobial compounds. This may preclude the need for a symbiont to produce such compounds. Our data appear to be consistent with other studies relating to actinomycetes of other insect nest substrates, suggesting arthropod nest material is a habitat for culturable, allochthonous actinomycetes that include *Streptomyces* as well as other, more rare genera (e.g., Promnuan et al. 2009, Inglis et al. 1993, Haeder et al. 2009, Bignell et al. 1991), regardless of potential symbiotic associations.

Future studies characterizing the antimicrobial activity witnessed in our studies will be necessary to determine if the actinomycetes isolated within this study produce novel chemistry. By further targeting the full diversity of the microbial community associated with these wasps, we will be able to better understand how these wasps maintain nest hygiene, and what microbes may impact their fitness. This work is thus an important first step in exploring a new system for the study of arthropod associated actinomycetes.

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