'Candidatus Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of Philanthus digger wasps

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Symbiotic interactions with bacteria are essential for the survival and reproduction of many insects. The European beewolf (Philanthus triangulum, Hymenoptera, Crabronidae) engages in a highly specific association with bacteria of the genus Streptomyces that appears to protect beewolf offspring against infection by pathogens. Using transmission and scanning electron microscopy, the bacteria were located in the antennal glands of female wasps, where they form dense cell clusters. Using genetic methods, closely related streptomycetes were found in the antennae of 27 Philanthus species (including two subspecies of P. triangulum from distant localities). In contrast, no endosymbionts could be detected in the antennae of other genera within the subfamily Philanthinae (Aphilanthops, Clypeadon and Cerceris). On the basis of morphological, genetic and ecological data, ‘Candidatus Streptomyces philanthi’ is proposed. 16S rRNA gene sequence data are provided for 28 ecotypes of ‘Candidatus Streptomyces philanthi’ that reside in different host species and subspecies of the genus Philanthus. Primers for the selective amplification of ‘Candidatus Streptomyces philanthi’ and an oligonucleotide probe for specific detection by fluorescence in situ hybridization (FISH) are described.

INTRODUCTION

Many insects have evolved associations with endosymbiotic bacteria that are essential for reproduction or survival of the host (Moran & Baumann, 1994). Most of these bacteria are intracellular symbionts in specialist feeders, e.g. phloem-feeding, blood-sucking or wood-feeding insects (Baumann & Moran, 1997; Priest & Dewar, 2000). Since the diets of these insects lack essential nutrients, they depend on bacteria that are able to synthesize the necessary compounds (Bourtzis & Miller, 2003; Douglas, 1998). In many cases, symbiotic bacteria are transmitted vertically from one generation to the next, resulting in coevolution and cospeciation of hosts and symbionts which is reflected in congruent phylogenies (Bandi et al., 1995; Baumann et al., 1997; Chen et al., 1999; Lo et al., 2003; Moran et al., 1993; Sauer et al., 2000).

The European beewolf (Philanthus triangulum, Hymenoptera, Crabronidae) engages in a unique and highly specific symbiosis with bacteria of the genus Streptomyces (Kaltenpoth et al., 2005). Female beevolves construct nest burrows in sandy soil, hunt honeybees (Apis mellifera), paralyse them by stinging and provision one to five honeybees as larval food in each brood cell (Strohm, 1995; Strohm & Linsenmair, 1995). After feeding on the provisioned prey, larvae spin a cocoon in which they usually overwinter and emerge the following summer (Strohm & Linsenmair, 1995). Since conditions in the brood cells are humid and warm, there is a continuous threat that the female’s investment could be destroyed due to fungal or bacterial infection of the provisions or the immature wasp (Strohm
& Linsenmair, 2001). Recent studies have shown that symbiotic bacteria protect beewolf offspring against fungal infection at the cocoon stage (Kaltenpoth et al., 2005).

The symbionts are cultivated in specialized antennal glands of the beewolf female and are secreted into the brood cell prior to oviposition (Kaltenpoth et al., 2005; Strohm & Linsenmair, 1995). Later, they are taken up by the larva and applied to the outside of the cocoon, where they seem to serve as a protection against fungal infection, presumably by producing antifungal secondary metabolites (Kaltenpoth et al., 2005). A second function of the secretion is to direct the cocoon-spinning of the larva which facilitates its eventual emergence (Strohm & Linsenmair, 1995). The bacteria certainly benefit from the association by obtaining an unoccupied and competition-free ecological niche and a reliable route of transmission into the next generation. They may also receive nutrients from the beewolf (M. Kaltenpoth and E. Strohm, unpublished data). A similar symbiotic relationship for pathogen defence between insects and actinomycetes has been described for leaf-cutter ants (Currie et al., 1999). A species of the family Pseudonocardiaeae protects the ants’ fungus gardens against a parasitic fungus by producing antibiotic substances (Cafaro & Currie, 2005; Currie et al., 1999).

In the present study, we investigated 28 Philanthus species and subspecies and several closely related genera for the presence of endosymbiotic Streptomyces bacteria in their antennae. Ultrastructural and genetic data (16S rRNA gene sequences) are presented that support the description of ‘Candidatus Streptomyces philanthi’, including 28 ecotypes in different host species and subspecies.

**METHODS**

**Specimens.** Specimens of 27 Philanthus species, including two subspecies of *P. triangulum*, two *Cerceris* species, *Aphlanchophos frigidus* and two *Clypeadon* species, were collected in Germany, Greece, Oman, South Africa, Ukraine and the USA (Table 1). The South African specimens were identified by comparison with voucher specimens in the collection of the Albany Museum in Grahamstown, South Africa, and the South African Museum, Cape Town, South Africa. The US species were identified according to Bohart & Grissell (1975) and Ferguson (1983a, b). Because males lack the relevant glands (Strohm & Linsenmair, 1995) and the endosymbiotic bacteria have so far only been found in females (M. Kaltenpoth, unpublished data), only antennae from female specimens were used for electron microscopy and genetic analyses.

**Electron microscopy.** For scanning electron microscopy (SEM), specimens were fixed in alcoholic Bouin’s fixative for 3 h and dehydrated in a graded acetone series. The specimens were then critical-point dried (CPD 030; BAL-TEC), sputtered with Pt/Pd (SCD 005; BAL-TEC) and examined with a digital scanning electron microscope (DSM 962; Zeiss). To investigate their interior ultrastructure, preserved antennae were cut with a razor blade before sputtering. Specimens for transmission electron microscopy (TEM) were fixed for 2 h in a cold solution of 2% glutaraldehyde, 2.5% formaldehyde and 5% sucrose buffered in 50 mM sodium cacodylate, pH 7.2. After post-fixation in 2% OsO4 and dehydration in an ethanol series, the specimens were embedded in Epon 812. Ultrathin sections of about 70 nm thickness (MT-7000 microtome; RMC; 45° diamond knife) were stained with 2% uranyl acetate and Reynolds’ lead citrate. Electron micrographs were taken with a transmission electron microscope (EM10; Zeiss) at 80 kV.

**DNA extraction, PCR and sequencing.** DNA was extracted from whole beewolf antennae according to a standard phenol/chloroform extraction protocol (Sambrook et al., 1989). The following primer pairs were used for the amplification of Streptomyces 16S rRNA gene: fD1 (forward) (Weisburg et al., 1991) and StreP (reverse) (Rintala et al., 2001), Act-S20 (forward) (Stach et al., 2003) and rP2 (reverse) (Weisburg et al., 1991). While primers fD1 and rP2 can be used to amplify a wide range of eubacterial 16S RNA, the combination with StreP and Act-S20 ensured that the PCR was specific for actinomycete 16S RNA. PCR amplification was performed on Eppendorf Mastercyler in a total reaction volume of 25 μl containing 2 μl template, 1 x PCR buffer (10 mM Tris/HCl, pH 8.8; 50 mM KCl; 0.08% NP-40), 2.5 mM MgCl2, 240 μM dNTPs, 20 pmol each primer and 1 U Taq DNA polymerase (MBI Fermentas). Cycle parameters were as follows: 3 min at 94°C, followed by 32 cycles of 94°C for 40 s, 65°C for 1 min and 72°C for 1 min, and a final extension time of 4 min at 72°C. For sequencing, the following primers were used: fD1 (forward), Act-S20 (forward), Act-A19 (reverse) (Stach et al., 2003), StreP (reverse), rP2 (reverse).

For the selective amplification of *Philanthus* endosymbionts, the following forward primers were designed on the basis of the 16S rRNA gene sequences of the endosymbiotic Streptomyces and reference strains from the GenBank database: StreP phi fwd1, 5’-TACCGATGCC-ATGTGTGAG-3’; StreP phi fwd2, 5’-TATGACTACGAGCCT- TGG-3’; StreP phi fwd3, 5’-CATGGTTTGTTGGAAGCC-3’; StreP phi fwd4, 5’-GTTGTTGGAAGCTCTGAC-3’ [binding to nucleotide positions 177–196, 170–188, 184–203 and 192–209, respectively, following the *Streptomyces ambifaciens* nomenclature (Pernodet et al., 1989)]. The forward primers StreP phi fwd1–4 were used in combination with the general actinomyces reverse primer Act-A19. Temperature gradient PCRs were performed for all primer combinations and two Mg++ concentrations were used to adjust the stringency of the reaction (1.5 and 2.5 mM). Final PCR conditions were the same as described above, except that 1.5 mM MgCl2 was used for StreP phi fwd4/Act-A19. The annealing temperature was set at 65°C for Strept phi fwd2/Act-A19 and to 68°C for the three other primer combinations. DNA extracts from the antennae of 27 *Philanthus* species and one subspecies, two *Cerceris* species, *Aphlanchophos frigidus* and two *Clypeadon* species (Table 1) were used as templates. Extracted DNA from cultures of *Streptomyces rimosus* DSM 40260T, *Streptomyces aureofaciens* DSM 40631 and *Streptomyces venezuelae* DSM 40230T was included to assess the specificity of the primers for *Philanthus* endosymbiont DNA.

**Fluorescence in situ hybridization (FISH).** The general eubacterial probe EUB 338 (Amann et al., 1990) and the specific oligonucleotide probe SPT 177 (5’-Cy3-CACCAACACTATGCGCCGTA-3’) (Kaltenpoth et al., 2005) were used for FISH. *S. ambifaciens* DSM 40631, *S. venezuelae* DSM 40230T, *S. rimosus* DSM 40260T and *Bacillus subtilis* DSM 402 served as negative controls for the specific probe. The SPT 177 probe is complementary to positions 177–196 of the *P. triangulum* endosymbiont 16S RNA gene sequence (S. ambifaciens nomenclature; Pernodet et al., 1989). Secretions of the white substance from beewolf females were harvested from brood cells and spread onto six-field microscope slides. Fixation and hybridization were carried out as described previously (Grimm et al., 1998), with minor modifications: the hybridization buffer contained only 50 ng labelled probe and samples were incubated for 90 min at 45°C for hybridization. For hybridization within the antennae, fresh antennae from female beewolves were cut into sections with a razor
blade and glued onto microscope slides. Fixation and pre-treatment of the samples was performed following a previously described protocol (Sauer et al., 2002). Hybridization was carried out as for the bacterial samples, but with 3 h incubation with the labelled probe.

**Phylogenetic analysis.** BioEdit 7.0.4.1 software was used to assemble and align sequences and to calculate DNA distances with the DNADIST 3.5c algorithm by Joseph Felsenstein. The alignment was checked by eye and arbitrary alignment regions were excluded.

### Table 1. Occurrence of endosymbiotic *Streptomyces* bacteria in antennae of philanthine wasps (Hymenoptera, Crabronidae, Philanthinae) and amplification with the specific primers Strep_phil_fwd1–4 in combination with the general actinomycete primer Act-A19

To assess the specificity of the primers, the DNA of three cultivated *Streptomyces* species was included in the PCRs. + + +, Strong amplification; +, weak amplification; −, no amplification; Y, symbionts present; N, symbionts not present; NA, not applicable; SA, South Africa; KZN, KwaZulu Natal; WCP, Western Cape Province; ECP, Eastern Cape Province. Standard two-letter abbreviations are used for US States.

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from further analysis. The aligned sequences were imported into PAUP 4.0. Phylogenetic trees were constructed based on 1324 bp of 16S rRNA gene sequences in a full heuristic search with tree bisection and reconnection (TBR) branch swapping and 10 random addition sequence replicates, saving no more than 100 trees with a score ≥ 100 per replicate. Gaps were treated as a fifth character state and Arthrobacter globiformis DSM 20124T was defined as the outgroup. Using the same settings, bootstrap values were obtained from a search with 1000 replicates.

RESULTS

Localization of endosymbionts

Scanning electron micrographs of the antennal surface of P. triangulum, Philanthus loefflingi and Philanthus fuscipennis females revealed that the bacteria are present at the openings of the antennal glands from which they are secreted into the brood cell (Kaltenpoth et al., 2005) (Fig. 1). The appearance of symbiotic bacteria on the outer surface of the antennae is probably due to accidental compressions of the antennae prior to or during preservation; under natural conditions they are unlikely to be found on the antennal surface, except during the secretion process into the brood cell.

When a flagellomere was cut open, filamentous bacteria were clearly visible in large numbers within the gland reservoir (Fig. 2a), where they formed a dense cluster of cells (Fig. 2b). Transmission electron micrographs confirmed the presence of endosymbiotic bacteria within the antennal gland reservoir and suggest that the endosymbionts constitute the main component of the antennal gland content in female beewolves (Fig. 3). The bacteria showed a filamentous morphology with long and sometimes branched cells and were embedded in a matrix containing numerous vesicles in the gland reservoir. Bacterial cells were 0.38–0.62 μm wide and highly variable in length (5–20 μm).

The bacteria were clearly stained by the specific fluorescent probe SPT 177 both within female beewolf antennae and in the antennal gland secretion after it had been applied to the brood cell (Fig. 4). Reference strains of S. aureofaciens, S. venezuelae, S. rimosus and B. subtilis were not stained by the probe. The general eubacterial probe EUB 338 gave positive results in all cases.

Distribution of symbionts among philanthine wasps

All 28 Philanthus species, including the two subspecies of P. triangulum, yielded amplicons of the expected length in at least three of the four PCRs with the specific 16S rRNA

Fig. 1. SEM image of an antenna of a female European beewolf (P. triangulum) with symbiotic Streptomyces bacteria being secreted from the antennal glands. Bar, 100 μm.

Fig. 2. SEM image of the interior of an antennal segment of a female P. loefflingi. (a) Longitudinal section of a flagellomere. The reservoir of the antennal gland is indicated by arrows. (b) Symbiotic Streptomyces bacteria forming a dense cluster within the antennal gland. Bars, 200 μm (a) and 10 μm (b).
primers Strep_phil_fwd1–4 in combination with the general actinomycete primer Act-A19 (Stach et al., 2003) (Table 1). One species, Philanthus psyche, generally yielded only weak amplicons and failed to amplify altogether in one of the four specific PCRs. Philanthus crabroniformis and Philanthus lepidus each also yielded no amplicons in one of the PCRs, but gave strong amplicons in all other PCRs.

Specimens of the other wasp genera of the subfamily Philantinae (Aphilanthops, Clypeadon and Cerceris) yielded no amplicons in any of the specific PCRs. In PCRs with general actinomycete primers (Act-S20 and Act-A19), antennal DNA from Aphilanthops, Clypeadon and Cerceris yielded no, or very weak, amplicons. The sequences obtained from the weak amplicons were not closely related to the Philanthus endosymbionts and were probably due to contamination of the antennae from the surrounding soil during the life of the digger wasps within subterranean nests (data not shown). Thus, symbiosis with bacteria of the genus Streptomyces seems to be widespread among wasps of the genus Philanthus, but appears to be absent in other genera of the subfamily.

Streptomyces control strains yielded no amplicons in most of the PCRs, demonstrating the specificity of the primers for the Philanthus endosymbionts. However, Strep_phil_fwd2/Act-A19 did amplify the 16S rRNA gene of S. rimosus DSM 40260T, a close relative of the Philanthus symbionts (Fig. 5) which shares around 98·0 to 98·5 % of its 16S rRNA gene sequence. Control PCRs with general actinomycete 16S rRNA primers (Act-S20/Act-A19) resulted in strong ampli-
cons for all of the Streptomyces strains, showing that the lack of amplification in the specific PCRs was not due to general problems with the template DNA.

**Phylogenetic position of ‘Candidatus Streptomyces philanthi’**

The partial 16S rRNA gene sequences from the endosymbionts of 27 Philanthus species and one subspecies grouped together in a monophyletic clade within the genus Streptom
cyes (Fig. 5). The phylogenetic analysis indicates that the symbionts belong to the Streptomyces armeniacus group, the closest relatives being Streptomyces kasugaensis and Streptomyces sapporonensis, with a mean sequence divergence of about 1·1 and 1·2 %, respectively. The similarity among the endosymbionts of the 28 different Philanthus taxa was relatively high, ranging from 98·9 to 100·0 % 16S rRNA gene sequence similarity.

Almost complete 16S rRNA gene sequences for the 28 ecotypes of ‘Candidatus Streptomyces philanthi’ have been

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**Fig. 3.** TEM image of a cross-section through the antennal gland of a female P. triangulum. Some endosymbiotic Streptomyces are indicated by arrows. Bar, 1 μm.

**Fig. 4.** FISH of antennal Streptomyces endosymbionts. Phase-contrast micrograph of symbiotic bacteria in the antennal gland secretion of a female beewolf (a) and of a negative control strain of Streptomyces rimosus DSM 40260T (b). (c, d) Epifluorescence micrographs of the same areas after staining with the specific Cy3-labelled probe SPT 177. Bar, 10 μm.
deposited in the GenBank database with accession numbers DQ375779–DQ375806. The accession numbers for specific ecotypes are shown in Fig. 5 and Table 1.

**DISCUSSION**

Endosymbiotic bacteria of insects are usually localized in the gut or reside within specialized host cells, so-called mycetocytes or bacteriocytes, which often form dedicated organ-like structures or are associated with the mid-gut epithelium (Baumann & Moran, 1997; Buchner, 1921; Ishikawa, 2003; Moran & Telang, 1998). The Philanthus–Streptomyces association represents the first case of endosymbiotic bacteria being localized in insect antennae. Correspondingly, the specialized antennal glands harbouring the symbionts have so far only been found in species of the genus Philanthus and appear to be absent even in closely related genera of philanthine wasps (E. Strohm, unpublished data). As is the case with many other endosymbiotic bacteria, attempts to cultivate the Philanthus symbionts using standard cultivation techniques and media were not successful (see Supplementary material in IJSEM Online).
The endosymbionts are present in the antennal gland reservoir of Philanthus females in large numbers and they can be detected by SEM, TEM, FISH (with a specific oligonucleotide probe) and by PCRs with specific primers. Genetic analyses of the 16S rRNA gene sequences of endosymbionts from the antennae of different bee species revealed that all species investigated so far harbour Streptomyces bacteria and that the Philanthus endosymbionts appear to represent a monophyletic clade within the genus Streptomyces. The antennal endosymbionts share an average of 98.8–98.9% 16S rRNA gene sequence with their closest relatives, S. kasugaensis and S. sapporonus. Despite this high sequence similarity, we propose the name ‘Candidatus Streptomyces philan thi’ for the endosymbionts of Philanthus species because they are clearly separated from other species by their unique ecological niche. Several studies have shown that 16S rRNA gene sequence similarity alone is often inappropriate for the distinction of two species and the general rule of 3% 16S rRNA gene sequence divergence between species tends to greatly underestimate the number of species (Cohan, 2002; Konstantinidis & Tiedje, 2005), as has been recently demonstrated for a number of Streptomyces groups (Liu et al., 2005; Manfio et al., 2003; Sembiring et al., 2000). Therefore, it is desirable to include ecological characteristics in the description of novel species (Cohan, 2002; Konstantinidis & Tiedje, 2005). Among Philanthus endosymbionts, 16S rRNA gene sequence similarity is relatively high (98.9–100.0%). We propose that the endosymbionts represent a single species with different ecotypes that are separated by their ecological niches (i.e. their host species).

The high degree of 16S rRNA gene sequence similarity among Philanthus endosymbionts suggests that they are transmitted vertically from mother to offspring, as has been described for many other endosymbiotic bacteria (Aksoy et al., 1997; Clark et al., 2000, 2001; Ishikawa, 2003; Moran & Baumann, 2000; Sauer et al., 2000). Alternatively, the bacteria may be taken up from the environment with certain mechanisms preventing the uptake of non-symbiotic bacteria, a transmission route that has been demonstrated for the symbionts of the squid Euprymna scolopes (McFall-Ngai & Ruby, 1991; Nishiguchi, 2002; Nyholm et al., 2000; Nyholm & McFall-Ngai, 2004). The following evidence points to vertical transmission of the bacteria from mother to offspring in Philanthus. (i) the bacteria are secreted into the brood cell and later taken up by the larva and (ii) a female larva that was reared in the absence of the white substance in its brood cell apparently lacked the symbiotic bacteria as an adult (Kaltenpoth et al., 2005). However, further studies on the phylogenetic relationships of bee species and their endosymbionts are needed to confirm vertical transmission and to determine whether horizontal transfer of symbionts between Philanthus species (e.g. via chrysidid parasitoids, interspecific nest usurpation or nest reuse) may have played a role in the evolution of the symbiosis.

Moran et al. (1993) estimated an evolutionary age of 160–280 million years for the symbiosis between aphids and their endosymbiont Buchnera aphidicola and Bandi et al. (1995) dated the origin of the association of cockroaches and termites with bacteria of the Flavobacterium–Bacteroides group to about 135–250 million years ago. Under the assumption of strictly vertical transmission of the symbionts, the low 16S rRNA gene sequence divergence among the endosymbionts of Philanthus wasps suggests that the symbiosis is of relatively recent origin. Assuming a mean rate of 0.008–0.02 substitutions per site per 50 million years (Bandi et al., 1994; Moran et al., 1993; Ochman & Wilson, 1987), the maximum sequence divergence of 1.07% indicates that the origin of the symbiosis between bees and streptomycetes dates back about 26–67 million years. Taking into account that all Philanthus species investigated so far harbour the symbiotic bacteria, the association with bacteria probably evolved at around the time of origin of the genus Philanthus.

The evolution of specialized antennal glands in Philanthus females may have represented a key invention and evolutionary preadaptation for a symbiosis with Streptomyces bacteria. Strohm & Linsenmair (1995) demonstrated that the antennal gland secretion serves a second function by providing directional information to the beewolf larva that is necessary later for successful emergence. Thus, we hypothesize that the antennal glands originally evolved in the context of directing cocoon-spinning and emergence and that they might have been secondarily invaded by Streptomyces bacteria from the surrounding soil. In the beginning, the bacteria may have been commensals, or even parasites, in the antennal glands. In a sequence of evolutionary steps, including the uptake of the bacteria by the larva and their application to the cocoon, the antimicrobial activity of the streptomycetes might have been subsequently exploited by the beewolf hosts to protect their offspring against pathogen infection. Further studies are needed to investigate how related genera of ground-nesting digger wasps cope with the threat of pathogenic soil micro-organisms infecting their progeny.

**Description of ‘Candidatus Streptomyces philanthi’**

‘Candidatus Streptomyces philan thi’ [phi.lan’thi. N.L. n. Philanthus (Hymenoptera, Crabronidae) the generic name of the host organism; N.L. gen. n. philan thi of Philanthus, referring to the association with digger wasps of the genus Philanthus].

The reference strain is ‘Candidatus Streptomyces philant hi triangulum’.

Uncultured, Gram-positive, non-motile, possibly sporulating, filamentous bacteria with sometimes branched cells that can be assigned to the genus Streptomyces on the basis of their 16S rRNA gene sequence. A detailed description of the methods used in an attempt to cultivate the endosymbionts can be found as supplementary material in IJSEM Online. Cells are 0.38–0.62 μm wide and of highly variable length.
(5–20 μm). The bacteria live as symbionts within specialized antennal glands of female digger wasps of the genus Philanthus. They are secreted into the brood cells, taken up by the larva and applied to the cocoon, where they appear to protect the beeewolf offspring against fungal infection (Kaltenpoth et al., 2005). Bacteria of different Philanthus species differ in their 16S rRNA gene sequence, but sequence divergence is relatively low (0–1 %). We propose that endosymbionts of different Philanthus species should be treated as ecotypes of ‘Candidatus Streptomyces philanthi’ and named according to the host species. The 16S rRNA gene sequences of all ecotypes found so far can be amplified selectively by the specific forward primer Strep_phil_fwd3 (5’-CATGGTTRGTGGAAGAGC-3’ in combination with the general actinomycete reverse primer Act-A19 (Stach et al., 2003). The ecotype ‘Candidatus Streptomyces philanthi triangulum’ can be stained with the fluorescent probe SPT 177 (5’-Cy3-CACCAACCATGCCATCGGTA-3’) (Kaltenpoth et al., 2005).

[(Streptomyces) NC; G+; F; NAS (GenBank accession number DQ375802), oligonucleotide sequence of unique region of the 16S rRNA gene is 5’-TACCGATCGCATGTTGGTG-3’; S (Philanthus, antennal glands); M]. Kaltenpoth et al., this study.

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