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# Symbiotic Bacteria Protect Wasp Larvae from Fungal Infestation

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## Summary

**Symbiotic associations between different organisms are of great importance for evolutionary and ecological processes [1–4]. Bacteria are particularly valuable symbiotic partners owing to their huge diversity of biochemical pathways that may open entirely new ecological niches for higher organisms [1–3]. Here, we report on a unique association between a new *Streptomyces* species and a solitary hunting wasp, the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae). Beewolf females cultivate the *Streptomyces* bacteria in specialized antennal glands and apply them to the brood cell prior to oviposition. The bacteria are taken up by the larva and occur on the walls of the cocoon. Bioassays indicate that the streptomycetes protect the cocoon from fungal infestation and significantly enhance the survival probability of the larva, possibly by producing antibiotics. Behavioral observations strongly suggest a vertical transmission of the bacteria. Two congeneric beewolf species harbor closely related streptomycetes in their antennae, indicating that the association with protective bacteria is widespread among philanthine wasps and might play an important role in other insects as well. This is the first report on the cultivation of bacteria in insect antennae and the first case of a symbiosis involving bacteria of the important antibiotic-producing genus *Streptomyces*.**

## Results and Discussion

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) is a solitary digger wasp that constructs nest burrows in sandy soil. Females hunt honeybees (*Apis mellifera*) [5] and provision one to five prey items as larval food in each brood cell. The larva feeds on the prey and spins a cocoon that is attached with its basal part to the wall of the brood cell. Larvae mostly overwinter and emerge next summer [6, 7]. Because the conditions in the brood cells are humid and warm, there is a continuous threat of fungal or bacterial infestation of the provisions or the immature wasp. To protect the prey against microbes during the feeding period of the larvae, the females embalm the paralyzed honeybees with a cephalic-gland secretion (G.H. et al., unpublished data). However, little was known about how the larva is secured from microbial attack during the 9 month period of diapause in the cocoon.

A promising candidate for such a protective function is a whitish substance that the female secretes into the brood cell in conspicuously large amounts prior to oviposition. The female enters the excavated brood cell and starts to move her body laterally, probably building up a high hemolymph pressure in the antennae [5].The white substance is thus pressed out of specialized antennal glands and appears as white particles on the antennae (Figure 1A; see Movie S1 in the Supplemental Data available with this article online). The female smears these particles on the ceiling of the brood cell. One known function of this secretion is to provide an orientational cue for the emergence of newly eclosed beewolves [5]. However, the unusually large amounts of white substance suggest a second function.

Scanning electron microscopy of newly secreted white substance revealed regularly shaped rod-like and branched structures with a diameter of about 0.5 μm (Figure 1B). Through transmission electron microscopy, these structures were found to be encapsulated in biomembranes, and they sometimes contained circular structures consisting of several layers of membranes. We hypothesized that these structures were bacteria and that those with multiple biomembranes were spores. The overall appearance and the possible occurrence of spores suggested that these bacteria belong to the actinomycetes.

To verify the identity of these bacteria, we used culture-independent molecular techniques. Isolation of DNA from antennae of female beewolves and amplification via polymerase chain reaction (PCR) with actinomycete-specific primers [8, 9] confirmed the presence of actinomycete bacteria. We sequenced about 1300 bp of the 16S rDNA and compared it to known actinomycete sequences. A phylogenetic analysis showed that the bacteria from the beewolf antennae belong to the genus *Streptomyces* (Figure 2). The new type is most closely related to the *S. armeniacus* group (*S. griseocarneum, S. kasugaensis, S. lydicus*, and *S. albulus*). Comparative genetic analyses of the 16S rDNA sequences (700–1320 bp, including the most variable regions) of endosymbionts from 11 *P. triangulum* individuals from four different populations (three in Germany and one in the Ukraine) revealed identical sequences, strongly suggesting that the association between beewolves and *Streptomyces* bacteria is obligate.

To exclude the possibility of bacterial contamination in the PCR, we designed a specific oligonucleotide probe that perfectly matched a variable region of the 16S rRNA of the putative symbiotic bacteria, while having at least two mismatches with all other *Streptomyces* 16S rRNA sequences in the Ribosomal Database Project

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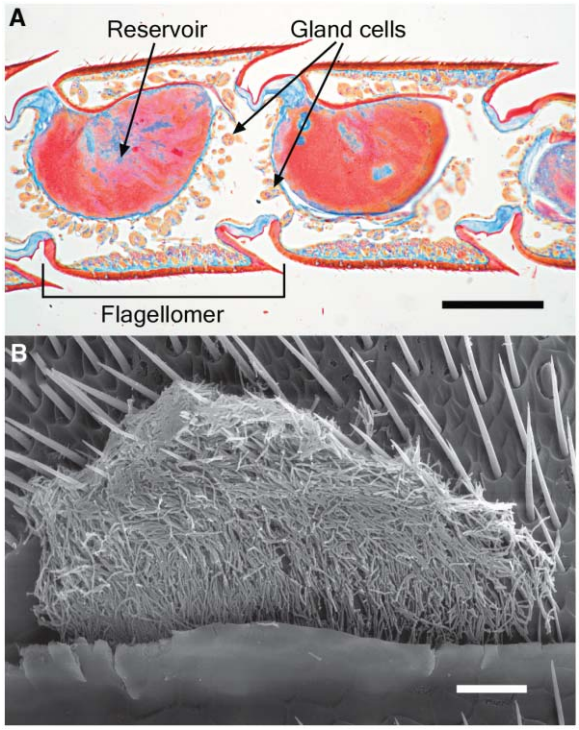


Figure 1. Antennae of Female Beewolves, *Philanthus triangulum*, with Endosymbiotic *Streptomyces*

(A) Light microscopic picture of a semithin section of a female antenna, with endosymbiotic bacteria (red) in the reservoir of antennal glands. The scale bar represents 0.3 mm.

(B) Scanning electron micrograph of a female antenna. White substance is secreted from the opening of the gland at the joint between two flagellomers. The scale bar represents 20 μm.

(RDP II) [10]. The oligonucleotide probe was labeled with a fluorescent dye (Cy3) and used for fluorescence in situ hybridization (FISH). The probe clearly stained large

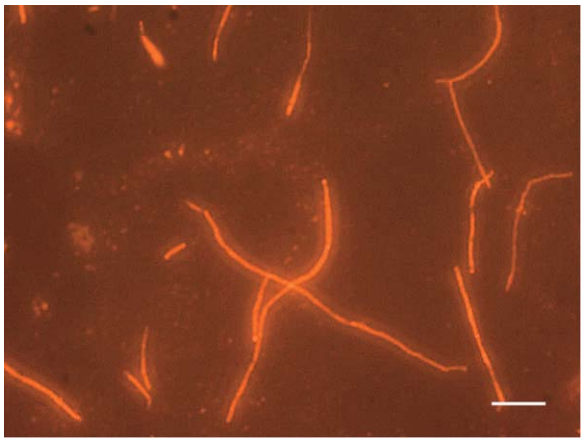

Figure 3. Fluorescence In Situ Hybridization (FISH) of Endosymbiotic *Streptomyces* in the White Substance after Secretion by a Beewolf Female

The scale bar represents 5 μm.

amounts of bacteria present in the white substance (Figure 3) as well as in the antennal glands of female beewolves. Control strains of *Streptomyces aureofaciens* or *Bacillus subtilis* were not stained by the probe, demonstrating the specificity of the probe for the bacterial sequences we obtained by PCR. These results confirm the presence of specialized streptomycete bacteria in the antennae of beewolf females and in the white substance secreted in the brood cells.

Streptomycetes are filamentous high GC Gram-positive soil bacteria belonging to the actinomycetes [11]. The whole group is characterized by the ability to synthesize a huge diversity of antibacterial and antifungal secondary metabolites [11, 12]. In fact, most of the antibiotics used for medical application are produced by *Streptomyces* species [12, 13]. Despite this high potential for producing antibiotics, which would predestine

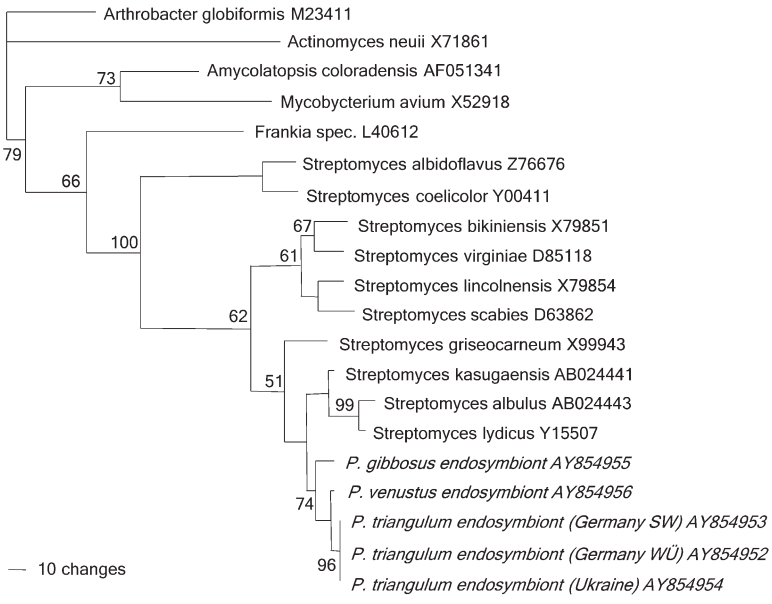


Figure 2. Phylogenetic Position of Beewolf Endosymbionts within the Actinomycetes

First of three equally parsimonious trees from a full heuristic search with TBR branch swapping and random addition sequence (100 replicates). Analysis is based on 1324 bp of 16S rDNA, with 219 characters being parsimony informative. *A. globiformis* was defined as the outgroup. Values at the nodes represent bootstrap values from 1000 replicates. GenBank accession numbers are given behind the species names. *P. triangulum* specimens were collected at three different locations: in Schweinfurt (Germany SW), in Würzburg (Germany WÜ), and in the Ukraine.

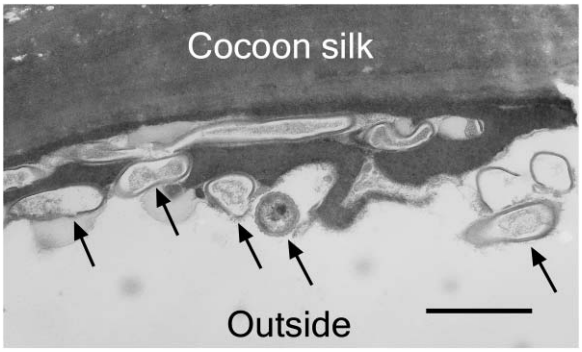


Figure 4. Transmission Electron Micrograph of a Cocoon Cross-Section with Bacteria on the Outside (Arrows)
The scale bar represents 1 μm.

streptomycetes as symbionts of other organisms, this is—to our knowledge—the first description of a mutualistic interaction between streptomycetes and animals, and there are only a few known examples of symbioses with actinomycetes. The best-studied animal-actinomycete symbiosis is that of leaf-cutter ants and actinomycete bacteria of the family Pseudonocardiaceae [14–17]. These ants tend fungus gardens in their nests for nutrition, and they carry the bacteria on specific regions of their cuticle [16]. The actinomycetes produce compounds that specifically inhibit the growth of a specialized parasitic fungus of the fungus gardens [15, 16]. This association is widespread among attine ants, and the vertical mode of transmission points to a long coevolutionary relationship between the symbionts [16].

Beewolves face a high risk of fungal and bacterial infestation in the brood cell, especially during the first days after cocoon spinning, because fungi develop on the remains of the honeybee prey and may also infest the cocoon (unpublished data). We hypothesized that—as in the attine ants—the beewolf endosymbionts may function as producers of antibiotics and protect the larva against pathogen attack. We examined cocoons for the presence of the antennal bacteria with the FISH method described above as well as transmission electron microscopy. The endosymbiotic streptomycetes were present in large numbers on the walls of the cocoon (Figure 4). In fresh cocoons (1–3 weeks old), bacterial cells were conspicuously longer and covered the walls of the cocoons in higher density than in 1-year-old cocoons from which the progeny had already emerged. We hypothesized that the short cells on old cocoons were metabolically inactive spores. Thus, if the bacteria protect the cocoons from bacterial or fungal infestations, old cocoons should be more susceptible than fresh ones. Bioassays confirmed this hypothesis. Fungal growth was significantly delayed on fresh cocoons as compared to 1-year-old cocoons. This was true for the part where the cocoon is attached to the brood cell ($p = 0.0041$) and even more pronounced for the rest of the cocoon ($p = 0.0013$). Additionally, the development of fungal conidia was significantly delayed or even completely inhibited ($p = 0.0005$) (Gehan-Wilcoxon tests). The effects in fresh cocoons were independent of the presence of a larva (Gehan-Wilcoxon test, $p > 0.10$ for all comparisons).

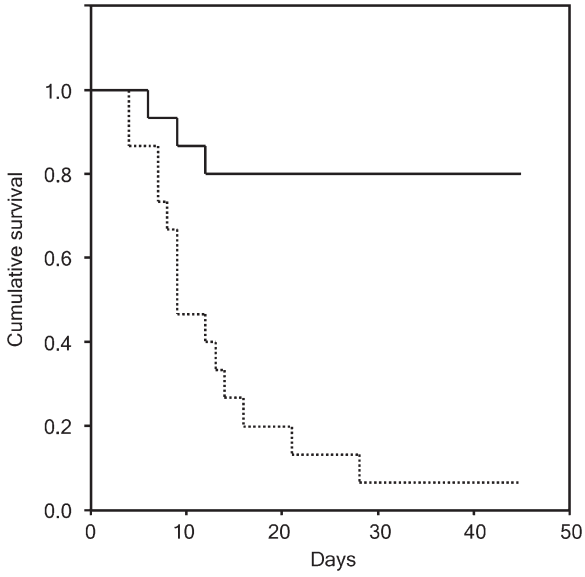


Figure 5. Cumulative Survival of Larvae with (Solid Line) and without (Dotted Line) White Substance in the Brood Cell
The experiment was terminated after 45 days.

In a second series of bioassays, we examined the importance of the white substance for the actual survival of larvae in the brood cells. Larvae had a dramatically reduced survival probability when they had no access to the white substance (Figure 5; Gehan-Wilcoxon test, $Z = 3.401$, $p = 0.00067$). Only 1 out of 15 individuals that had no access to the white substance survived until emergence (6.7%), whereas 15 of the 18 control individuals with white substance (83.3%) successfully emerged or survived as larvae until the end of the experiment (45 days). The experiment was terminated after 45 days because the most critical phase after cocoon spinning was over, and the surviving larvae had either emerged or entered diapause for overwintering. Taken together, the results of the bioassays strongly support the hypothesis that the *Streptomyces* bacteria protect the cocoon from fungus infestation and thereby increase the survival probability of beewolf larvae.

An important question is how beewolf females acquire the antennal bacteria. A priori, there are two alternatives: Females might opportunistically take up the bacteria from the environment, or they may inherit them from their mother [18]. Observations of larvae searching for and apparently ingesting parts of the white substance in the brood cell before spinning the cocoon suggest a vertical transmission of the bacteria from mother to daughters (see Movie S2). Further evidence for vertical transfer is provided by one beewolf female that survived until adulthood in the absence of white substance. The female failed to construct any brood cells during her entire lifetime, and PCR-based attempts to detect endosymbionts in the antennae yielded no amplicons, strongly suggesting that this female did not harbor endosymbiotic *Streptomyces* bacteria in her antennae.

The complexity of the association including the occurrence of unique glands, uptake of the bacteria by the larva, application to the cocoon, and a probably vertical

transmission make it unlikely that this association is limited to *P. triangulum*. Therefore, we examined two congeneric species for the presence of antennal symbionts: *P. venustus*, from southern Europe, and *P. gibbosus*, from North America. We found streptomycetes in the antennae of both species, and comparative 16S rDNA sequence analysis revealed that they are very closely related to the endosymbionts of *P. triangulum*. In fact, the endosymbionts of the three *Philanthus* species form a monophyletic clade within the genus *Streptomyces* (Figure 2). These results point to an early origin of the beewolf-*Streptomyces* mutualism, possibly during the formation of the genus *Philanthus*. Further studies on the phylogenies of both hosts and symbionts are necessary to illuminate the coevolutionary patterns and to investigate whether horizontal transfer has occurred during the evolutionary history of the symbiosis.

Soil-nesting hymenoptera and other ground-dwelling arthropods generally face a high risk of bacterial and fungal infestation of the provisions and the progeny from the surrounding soil. Therefore, one would expect high selection pressures to act on the evolution of protective mechanisms against pathogen attack. The cultivation of antibiotic-producing bacteria in specialized organs might represent a key invention to cope with the threat of pathogen infestation. So far, this is the only study providing evidence for a symbiosis between a ground-nesting wasp and protective bacteria, but associations of this kind may be much more widespread and might have played a crucial role in the evolution of ground-nesting behavior. Furthermore, assuming that the protection against microbes is mediated by chemicals, the study of actinomycete-insect associations may provide knowledge on novel antimicrobial compounds. Because the antibiotics involved should not harm their eukaryotic hosts, they might be of particular value for medical use.

## Experimental Procedures

### PCR and Sequencing

Bacterial DNA was extracted from whole beewolf antennae according to a standard phenol-chloroform extraction protocol. The following primer pairs were used for amplification of *Streptomyces*: fD1 (forward) [19] and StrepF (reverse) [8] and Act-S20 (forward) [9] and rP2 (reverse) [19]. PCR amplification was performed on Eppendorf Mastercyclers in a total reaction volume of 25 μl containing 4 μl of template, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, and 0.08% Nonidet P40), 2.5 mM $MgCl_2$, 240 μM dNTPs, 20 pmol of each primer, and 1 U of Taq DNA polymerase (MBI Fermentas). Cycle parameters were as follows: 3 min at 94°C, and then 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, we used the following primers: fD1 (forward), Act-S20 (forward), Act-A19 (reverse) [9], StrepF (reverse), and rP2 (reverse). Sequencing was carried out on a Beckmann-Coulter CEQ 2000 XL sequencer.

### Phylogenetic Analysis

Partial 16S rDNA sequences of the endosymbionts and representative actinomycete genera from the GenBank database (accession numbers are given in Figure 2) were aligned in ClustalX 1.83 with the default settings and imported into PAUP 4.0. Phylogenetic trees were constructed on the basis of 1324 bp of 16S rDNA in a full heuristic search with tree bisection and reconnection (TBR) branch swapping and random addition sequence (100 replicates). Bootstrap values were obtained from a search with 1000 replicates.

### FISH

The following species-specific oligonucleotide probe was designed for the endosymbiont by comparison with known sequences in the RDP II: 5′-Cy3-CACCAACCATGCGATCGGTA-3′ (positions 176–196, *Streptomyces ambofaciens* nomenclature [20]). The unspecific eubacterial probe EUB 338 was used as a positive control [21]. Secretions of the white substance from beewolf females were harvested and spread onto six-field microscope slides. Fixation and hybridization were carried out as described previously [22], with minor modifications: Hybridization buffer contained only 50 ng of the labeled probe, and samples were incubated for 90 min at 45°C for hybridization. For hybridization within the antennae, fresh female antennae were cut into thin sections with a razor blade and glued onto microscope slides. Fixation and pretreatment of the samples were done according to the protocol of Sauer et al. [23]. Hybridization was carried out as for the bacterial samples, but with 3 hr of incubation with the labeled probe.

### Fungal Infestation Bioassays with Beewolf Cocoons

Paper towels were placed in eight petri dishes and moistened with 3 ml distilled water. Three cocoons were placed in each petri dish: an empty 1-year-old cocoon, a fresh cocoon with larva (1–3 weeks old), and a fresh cocoon from which the larva had been removed. Petri dishes were kept in a closed box at room temperature to keep moisture approximately constant. Fungal growth was recorded daily under a Wild Heerbrugg M3B dissecting scope with 40× magnification. Usually, fungi started to grow at the basal part of the cocoon where it had been attached to the brood cell. Therefore, fungal growth was recorded separately for the attachment site and the rest of the cocoon. The time until first appearance of fungi, the time until fungi completely covered the attachment site or the whole cocoon, and the time until conidia formation were compared among groups with survival analyses (Gehan-Wilcoxon tests; software: Bias 8.05).

### Survival of Larvae with and without White Substance

Newly provisioned brood cells in the nesting cages of seven females were assigned randomly to two different groups: with (control group) and without (experimental group) white substance. In cells of the experimental group, the glass covering the brood cells in the observation cages was lifted, and a microscope cover slip was introduced between the brood cell and the glass cover. Thus, the white substance that is applied to the ceiling of the brood cell was covered, and the larva had no access to the white substance. In control cells, the glass cover was also lifted, but no cover slip was introduced, so the white substance was freely accessible to the larva. Survival of the larvae was checked daily for all brood cells and compared between groups with survival analysis (Gehan-Wilcoxon test; software: Bias 8.05). Larvae that survived until the end of the experiment (45 days) and individuals that emerged successfully from the cocoon were included as censored data.

### Supplemental Data

Two supplemental movies are available at http://www.current-biology.com/cgi/content/full/15/5/475/DC1/.

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## Accession Numbers

Partial 16S rDNA sequences from *Streptomyces* endosymbionts of *Philanthus triangulum* (from three different populations: Würzburg, Germany; Schweinfurt, Germany; and the Ukraine), *P. venustus*, and *P. gibbosus* are available at GenBank (http://www.ncbi.nlm.nih.gov/) with the accession numbers AY854952–AY854956.