nature chemical biology

BRIEF COMMUNICATION

PUBLISHED ONLINE: 28 FEBRUARY 2010; | DOI: 10.1038NCHEMBIO.331

Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring

Johannes Kroiss[1,5], Martin Kaltenpoth[2,5*], Bernd Schneider[3], Maria-Gabriele Schwinger[4], Christian Hertweck[4], Ravi Kumar Maddula[1], Erhard Strohm[2] & Aleš Svatoš[1*]

Beewolf digger wasps cultivate specific symbiotic bacteria (*Streptomyces* spp.) that are incorporated into the larval cocoon for protection against pathogens. We identified the molecular basis of this protective symbiosis in the natural context and demonstrate that the bacteria produce a 'cocktail' of nine antibiotic substances. The complementary action of all symbiont-produced antibiotics confers a potent antimicrobial defense for the wasp larvae that parallels the 'combination prophylaxis' known from human medicine.

Symbioses between eukaryotic organisms and bacteria are ubiquitous from deep sea[1] to rainforest habitats[2] and may even shape entire ecosystems[1]. In insects, most symbionts boost the host's nutrition[3], but some protective mutualists are known to guard the host's nutritional resources from competing microorganisms[2,4,5]. In the unique symbiosis of beewolf digger wasps (*Philanthus* spp., Hymenoptera, Crabronidae), specific actinobacteria ('*Candidatus* Streptomyces philanthi')[6] provide the host itself with protection against pathogens[7]. In the warm and humid underground brood cells of the European beewolf *P. triangulum*, developing larvae and cocoons are constantly threatened by pathogenic fungi and bacteria[8,9] that originate from the surrounding soil or are introduced with the prey (paralyzed honeybee workers[10]). Female beewolves cultivate the symbiotic actinobacteria in unique antennal glands[11] and apply them to the ceiling of the brood cell before oviposition (**Fig. 1a**,**b**). Beewolf larvae take up the symbionts and incorporate them into the cocoon while spinning it. The bacteria significantly enhance the survival probability of the larva in the presence of noxious pathogens[7]. However, the molecular basis of their protective activity has remained elusive.

Here we report on the isolation and identification of nine antibiotic substances from the natural environment (beewolf cocoons) and document their *in situ* spatial distribution using imaging mass spectrometry. Furthermore, we report on the biological activity of individual antibiotic substances and the whole symbiont-produced antibiotic 'cocktail' against a wide range of ubiquitous and entomopathogenic microorganisms.

As a group, the streptomycetes synthesize a huge diversity of antibiotics and other secondary metabolites[12,13], so we hypothesized that the *P. triangulum* symbiont produces one or more such compounds that are responsible for the protective effect to beewolf larvae. We isolated and identified nine antibiotic substances produced by beewolf symbionts from the natural environment by extracting beewolf cocoons from a laboratory population[10] followed by LC-ESI-MS/MS and NMR spectroscopy. This procedure identifies components of the antibiotic defense that occur in the natural symbiotic relationship and is therefore more direct than chemical analyses of compounds produced by isolated microbes in laboratory culture. We identified streptochlorin (**1**) ($C_{11}H_7O\,N_2Cl$; **Fig. 1c** and **Supplementary Results**)[14] and a complex of eight piericidin derivatives from cocoon extracts: piericidin A_1 (**2**; $C_{25}H_{37}O_4N$)[15], piericidin B_1 (**3**; $C_{26}H_{39}O_4N$)[16], glucopiericidin A (**4**; $C_{31}H_{47}O_9N$)[17], piericidin A_5 (**5**; $C_{26}H_{39}O_4N$), piericidin C_1 (**6**; $C_{25}H_{37}O_5N$), 9′-demethyl-piericidin A_1 (**7**; $C_{24}H_{35}O_4N$), piericidin B_5 (**8**; $C_{27}H_{41}O_4N$) and piericidin IT-143-B (**9**; $C_{28}H_{41}O_4N$) (**Fig. 1c**, **Table 1** and **Supplementary Results**). Both streptochlorin and piericidin antibiotics have previously been isolated individually from terrestrial or marine *Streptomyces* spp. strains, but never in combination (**Supplementary Results**). After experimental removal of the symbionts from beewolf brood cells, neither streptochlorin nor any of the piericidines were detectable on the cocoons (**Supplementary Results**). Thus, these compounds must have been produced by the bacterial symbionts. Streptochlorin and several piericidin derivatives have also been identified in the bacteria-containing secretion deposited by the mother beewolf onto the ceiling of the brood cell (**Supplementary Results**).

Laser desorption/ionization (LDI)-TOF/MS imaging[18] of beewolf cocoons visualized the three major compounds piericidin A_1, piericidin B_1 and streptochlorin on the outer cocoon surface and revealed an almost uniform distribution of these antibiotics (**Fig. 1d**). However, all three compounds are considerably less abundant on the inner side of the cocoon (**Supplementary Results**). Quantification of antibiotics by LC-MS revealed that single cocoons contained 130.5 ± 209.7 µg (mean ± s.d., range 1.67–695.6 µg, median 26.62 µg, n = 12) of all nine antibiotic substances combined, with piericidin A_1 being the major compound (**Table 1** and **Supplementary Results**). The total amount of antibiotics per cocoon as well as the amount of seven out of the nine individual compounds was significantly ($P < 0.05$) correlated with cocoon size (**Supplementary Results**). Although the amount of antibiotics per cocoon was highly variable, the qualitative composition of the antibiotic cocktail was relatively constant (**Supplementary Results**).

The inhibitory effects of crude cocoon extracts and four isolated antibiotic substances (single-cocoon equivalents of streptochlorin, piericidin A_1, piericidin B_1 and glucopiericidin A, isolated as pure compounds by means of preparative HPLC) were assessed in agar diffusion assays (ADAs, **Fig. 2a**) against ten different strains of fungi and bacteria that represent a wide range of ubiquitous mold and putrefactive microbes as well as general and honeybee-specific entomopathogenic microbes (**Fig. 2** and **Supplementary Methods**). The tested strains differed in their susceptibility to the four antibiotics

[1]Research Group Mass Spectrometry, Max Planck Institute for Chemical Ecology, Jena, Germany. [2]Department of Zoology, University of Regensburg, Regensburg, Germany. [3]Research Group Biosynthesis/NMR, Max Planck Institute for Chemical Ecology, Jena, Germany. [4]Leibniz Institute for Natural Product Research and Infection Biology (Hans Knöll Institute), Jena, Germany. [5]Present address: Research Group Insect Symbiosis, Max Planck Institute for Chemical Ecology, Jena, Germany. *e-mail: svatos@ice.mpg.de or mkaltenpoth@ice.mpg.de

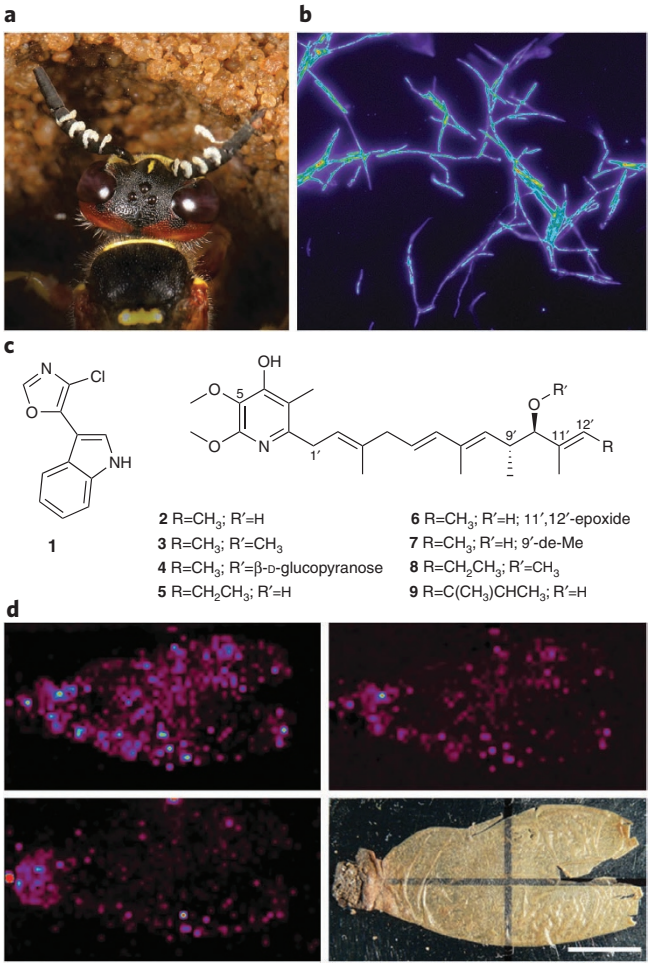

Figure 1 | Wasp symbionts from female antennae produce an antibiotic cocktail on the larval cocoon. (**a**) Female European beewolf secreting symbiotic streptomycetes (white areas) from antennal glands onto the brood cell ceiling (brood cell covered with a glass plate in an observation cage). (**b**) Fluorescence *in situ* hybridization (FISH) micrograph of beewolf symbionts in the antennal gland secretion using the specific Cy3-labeled probe SPT177 (in pseudocolors)[7,8]. (**c**) Antibiotic substances isolated from beewolf cocoons: streptochlorin (left) and eight piericidin derivatives (right). (**d**) Mass spectrometric imaging (LDI-TOF/MS) of the three most abundant antibiotic substances on a beewolf cocoon. Ion intensity maps of piericidin A_1 (*m/z* 454 ± 0.5 $[M+K]^+$) (upper left), piericidin B_1 (*m/z* 468 ± 0.5 $[M+K]^+$) (upper right) and streptochlorin (*m/z* 219 ± 0.5 $[M+H]^+$) (lower left). Intensities of ions in the imaged spots are color coded using a heat map; black corresponds to 0 and red corresponds to 255 counts. Lower right: photograph of the cocoon used for LDI-TOF/MS imaging mounted on a MALDI target plate (scale bar, 5 mm).

(**Fig. 2b** and **Supplementary Results**). While the inhibition of most strains was strongest for the most abundant antibiotic, piericidin A_1, some strains were more effectively inhibited by either piericidin B_1 or streptochlorin. Inhibition by single-cocoon equivalents of glucopiericidin A (the isolated substance with the lowest abundance on beewolf cocoons) was generally low. However, the growth of all strains was strongly inhibited by the complete spectrum of antibiotics present in crude cocoon methanol extracts, indicating a complementary action of all substances.

The dose-response relationship and the minimum inhibitory amount of piericidin A_1 for all pathogenic strains was determined in ADAs. The minimum inhibitory amount for the tested strains ranged from 0.1 µg to 10 µg (0.24–24 nmol), with most strains being clearly inhibited by 1 µg of piericidin A_1 (**Fig. 2c** and **Supplementary Results**). The minimum inhibitory amount was always well below the median and even below the first quartile of the amount of piericidin A_1 on individual cocoons (**Fig. 2c**, bottom).

Table 1 | Absolute and relative abundance of antibiotic substances on beewolf cocoons

Compound	Amount per cocoon (µg)	Amount per cocoon (nmol)	Proportion (%)
Streptochlorin	0.90 ± 0.84	4.13 ± 3.83	1.57 ± 1.65
Piericidin A_1	83.37 ± 141.60	200.9 ± 341.2	63.07 ± 6,70
Piericidin B_1	36.77 ± 56.97	85.71 ± 132.8	26.38 ± 8.43
Glucopiericidin A	0.26 ± 0.37	0.44 ± 0.63	0.63 ± 0.72
Piericidin A_5	7.27 ± 12.07	16.94 ± 28.15	4.39 ± 1.46
Piericidin C_1	1.25 ± 0.78	2.91 ± 1.82	3.79 ± 2.98
De-Me-pieridicin A_1	0.12 ± 0.24	0.30 ± 0.59	0.05 ± 0.05
Piericidin B_5	0.37 ± 0.69	0.83 ± 1.55	0.10 ± 0.19
IT-143-B	0.15 ± 0.28	0.32 ± 0.61	0.04 ± 0.06

Values are mean ± s.d.

Our study shows that the actinobacterial symbionts cultivated in the antennae of European beewolf females produce a cocktail of different piericidines and streptochlorin. These antibiotics provide the larval cocoon with a broad-spectrum defense against a diverse range of potentially lethal fungi and bacteria. Notably, the antibiotic compounds occur in much higher concentrations on the outer surface of the cocoon than on the inner surface, even though the cocoon is very thin. This gradient may be established during spinning, as the larva appears to incorporate the majority of the bacteria into the cocoon silk early on in the spinning process, so the number of remaining symbionts is low when the larva is finishing the inner surface of the cocoon[7]. Such a non-uniform distribution of antibiotics might serve both to enhance their effectiveness against microbes attacking the cocoon from outside and to minimize any deleterious effects these compounds might have on the developing prepupa.

Previous studies of protective symbioses have elucidated the chemistries and activities of candidate antibiotics isolated from bacteria on culture plates—that is, away from their hosts[4,5,19]. Here, we isolated and identified symbiont-produced antibiotics directly from the natural environment (beewolf cocoons) and visualized their distributions *in situ* using LDI-TOF/MS imaging. Since the potential roles of such antibiotics in the natural setting remain controversial[20,21], precisely locating their sites of production and deployment contributes to the elucidation of their ecological relevance.

Fungus-growing ants and bark beetles represent prime examples of insect protective symbioses: the symbiotic fungi cultivated by both taxa for their own nourishment are heavily threatened by specific pathogenic or competing microorganisms. To protect their fungal cultivars against these harmful microbes, some taxa have evolved a secondary protective symbiosis with actinomycete bacteria[2,5]. The symbionts have been reported to produce single antibiotic substances that specifically inhibit the growth of coevolved and highly specialized pathogens or competitors, but do not interfere with the development of the fungus gardens[4,5,19]. In the beewolf-*Streptomyces* symbiosis, however, a cocktail of symbiont-produced antibiotics protects the host itself against infestation by a diverse range of probably opportunistic microbial pathogens. Such a protection by antibiotic substances may be especially important for beewolf larvae because the behavioral defenses used by social insects[22] are not available to solitary wasp larvae that spend many months alone and immobilized. Additionally, the virulence of pathogens in solitary insects is generally expected to be higher as compared to social species[23], and it may be even further increased by competition of multiple pathogens, which likely occurs in the beewolf brood cell.

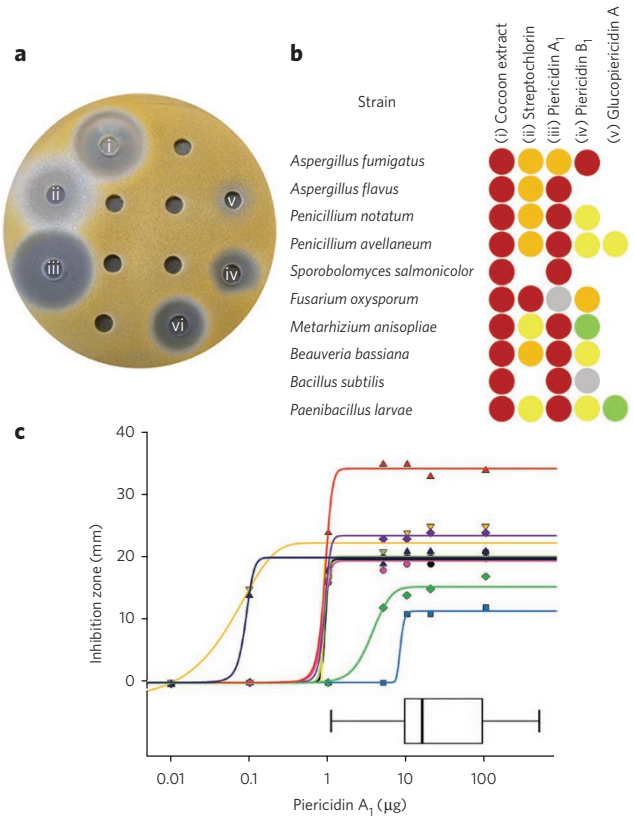

Figure 2 | Biological activity of antibiotic substances produced by beewolf symbionts. (**a**) Representative picture of an agar-diffusion assay with single-cocoon equivalents of crude beewolf cocoon extract (i) and four individual antibiotic substances (ii, streptochlorin; iii, piericidin A_1; iv, piericidin B_1; v, glucopiericidin A) as well as a positive control (vi, nystatin) against *Penicillium avellaneum*. (**b**) Inhibition of ten strains of ubiquitous and entomopathogenic fungi and bacteria by antibiotic substances produced by beewolf symbionts (red, maximum inhibition zone; orange, 76–99%; yellow, 51–75%; green, 26–50%; gray, 1–25% of maximum inhibition zone). (**c**) Dose-response curve for ten fungal and bacterial pathogens for piericidin A_1 (*Aspergillus fumigatus*, black circle; *Aspergillus flavus*, purple rhombus; *Penicillium notatum*, pink circle; *Penicillium avellaneum*, orange inverted triangle; *Sporobolomyces salmonicolor*, light green inverted triangle; *Fusarium oxysporum*, yellow square; *Metarhizium anisopliae*, red triangle; *Beauveria bassiana*, dark blue triangle; *Bacillus subtilis*, blue square; *Paenibacillus larvae*, green rhombus). Bottom of **c**: amount of piericidin A_1 present on single cocoons (the bold line represents the median, the box comprises the interquartile range and the bars indicate minimum and maximum values).

The mixture of antibiotics from beewolf cocoons strongly inhibited all tested microbes. This suggests that beewolf symbionts use a defensive strategy against bacterial and fungal pathogens that corresponds to the 'combination therapy' or 'combination prophylaxis' increasingly used in human medicine[24]. Such a treatment exploits the complementary or synergistic action of two or more antibiotics. It results in a higher efficacy against a broader spectrum of pathogens and is known to prevent pathogens from developing resistance against the antibiotic substances[23]. Both of these advantages are important factors for the beewolf symbiosis since beewolf larvae are prone to infection with a broad and probably unpredictable spectrum of pathogens during a hibernation of up to nine months. Thus, a combination prophylaxis with a diverse set of antibiotics probably represents a key innovation that provides an effective long-term defense against microbial attack while preventing an evolutionary arms race by coevolving pathogens.

In summary, the beewolf symbiosis differs from other protective symbioses with regard to both the evolutionary history of the interacting organisms and the ecological conditions of pathogen infection and virulence. We conclude that the acquisition of a bacterial symbiont capable of producing a whole spectrum of antibiotics constitutes a breakthrough in the evolution of these ground-nesting insects, and that protective symbioses with actinobacteria might constitute a general theme in the ecology and evolution of many arthropod species[25].

Received 28 August 2009; accepted 22 January 2010; published online 28 February 2010

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Acknowledgments

We acknowledge financial support from the Max Planck Society (J.K., B.S., R.K.M. and A.S.), the German Research Foundation Deutsche Forschungsgemeinschaft (J.K., M.K. and E.S.), the Volkswagen Foundation (M.K.), the International Leibniz Research School for Microbial and Biomolecular Interactions (M.-G.S. and C.H.) and the Jena School for Microbial Communication (M.-G.S. and C.H.). We thank K. Roeser-Mueller, T. Engl and K. Angermeier (all at the University of Regensburg) for providing beewolf cocoons; J. Doubský (Max Planck Institute for Chemical Ecology) for the camalexin standard; and J. Seger for valuable comments on the manuscript.

Author contributions

J.K., A.S. and M.K. conceived of the study. J.K. and A.S. isolated, identified and quantified the antibiotic substances and performed the imaging mass spectrometry. J.K., A.S., M.K. and E.S. wrote the manuscript. B.S. carried out the NMR experiments. M.-G.S. and C.H. performed the biological activity experiments. R.K.M. conducted the MS-MS experiments. M.K. and E.S. carried out the GC-MS experiments.

Competing financial interests

The authors declare no competing financial interests.

Additional information

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