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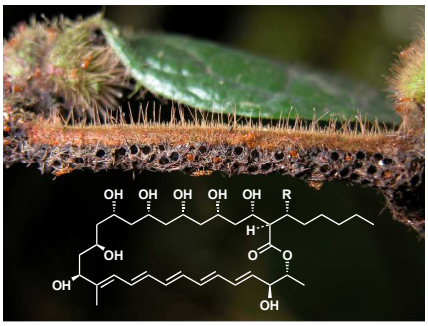

Allomerus **ants ensure that they have sufficient nitrogen in their diet by trapping and consuming insects. In order to construct their traps, like the more extensively studied leaf cutter ants, they employ fungal farming. Pest management within these fungal cultures has been speculated to be due to the ants’ usage of actinomycetes capable of producing antifungal compounds, analogous to the leafcutter ant mutualism. Here we report the first identification of a series of antifungal compounds in this system, the filipins, and their associated biosynthetic genes.**

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Filipins: the first antifungal "weed killers" identified from bacteria isolated from the trap-ant

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Hong Gao [ab], Sabine Grüschow[ab], Jörg Barke[c], Ryan F. Seipke[c], Lionel M. Hill[d], Jérôme Orivel[e], Douglas W. Yu[cf], Matthew Hutchings[c] and Rebecca J.M. Goss[*ab]

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Allomerus **ants ensure that they have sufficient nitrogen in their diet by trapping and consuming other insects. In order to construct their traps, like the more extensively studied leaf cutter ants, they employ fungal farming. Pest management within these fungal cultures has been speculated to be due to the ants' usage of actinomycetes capable of producing antifungal compounds, analogous to the leafcutter ant mutualism. Here we report the first identification of a series of antifungal compounds, the filipins, and their associated biosynthetic genes isolated from a bacterium associated with this system.**

Fig. 1 The galleried trap of the *Allomerus* ants, constructed using a fungal monoculture.

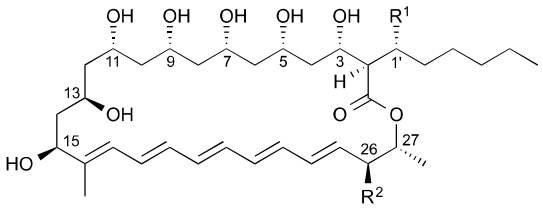
Allomerus (Myrmicinae) ants construct elaborate galleried traps on the stems of their specialist host plants, gluing lengths of cut plant hairs (trichomes) across "pillars" of remaining uncut plant hairs and reinforcing the structure with mycelia of purpose-cultured fungi (Fig. 1). These galleried traps contain regularly spaced holes just large enough for these tiny red ants to stick their heads through. When an insect alights upon the structure, the ants grab the legs to immobilize the prey and stretch it out across the galleried floor, repeatedly stinging it, before moving it to a leaf pouch for dissection[1]. Only mycelia from a single species of sooty mould fungus, from the order Chaetothyriales, have so far been observed in this mutualism; though spores from up to forty-five other fungal species have been detected in the fungal traps and ants' waste, suggesting that the ants are active in maintaining their fungal monoculture[2].

The more extensively studied leafcutter ant system has revealed that the leafcutter ants live in mutualism with bacteria that are capable of generating cocktails of antifungal compounds that, in addition to mechanical maintenance and weeding by the ants, are utilized to maintain a monoculture fungal garden free from parasites and less advantageous fungal species[3-5]. Key antifungal compounds utilized by the leafcutter ants include candicidin, nystatin-like compounds[3] and antimycin[6]. There is urgent need for new compounds for the treatment of drug-resistant fungal pathogens. The ant/fungal/bacterial mutualism systems could potentially yield new and useful compounds.

Recently we isolated and identified seven culturable bacteria (six *Streptomyces* and one *Amycolatopsis*) from the cuticles of worker ants of the species *Allomerus decemarticulatus* and *Allomerus octoarticulatus* and demonstrated that laboratory cultures of four of these strains (FG22, FG23, FG25 and FG26) exhibited antifungal activity[7]. We report here the first identification of a family of antifungal compounds from the trap ant system.

The four bacterial strains were cultured in triplicate in a panel of twelve media with varying carbon and nitrogen sources (see SI, Table S1), and concentrated entire culture broths as well as extracts assayed against the fungus *Candida albicans*. Ethyl acetate extracts of one *Streptomyces* strain, FG26, cultured in M9, a medium containing soluble starch as the sole carbon source, showed potent activity against *C. albicans*. The 16S rDNA sequence of FG26 indicated that the strain was closest to *Streptomyces misionensis* type strain NRRL B-3230 (100% identity)[7], a little studied strain from Argentina[8]. *S. misionensis* had been reported to produce the antifungal tetraene BH890 ($C_{41}H_{74}NO_{16}$, mass=837.033; structure unknown)[9], and we initially postulated that this compound might be responsible for the potent antifungal activity that we observed. However, we were unable to detect this compound in any of our culture conditions.

Careful activity-guided fractionation of our culture extract revealed the presence of a series of compounds with potent antifungal activity. LC-MSMS analysis was consistent with these compounds being three components of the filipin complex (Sigma-Aldrich, St. Louis, MO, USA), a mixture of related compounds, filipins I to IV (Fig. 2). The major filipin that was detected in the FG26 extract corresponds to filipin III by accurate mass (calculated for $C_{35}H_{58}O_{11}Na$ $[M+Na]^+$ 677.3871, found 677.3872, error 0.1 ppm, Fig. S1, S2), fragmentation pattern (Fig. S6), co-elution with the commercial standard filipin complex (Fig. S5) and UV spectrum (Fig. S7). Similarly, two minor metabolites consistent with filipins II (calculated for $C_{35}H_{58}O_{10}Na$ $[M+Na]^+$ 661.3922, found 661.3922, error -0.01 ppm, Fig. S4) and IV (calculated for $C_{35}H_{58}O_{11}Na$ $[M+Na]^+$ 677.3871, found 677.3871, error -0.01 ppm, Fig. S3) were also identified. The relative retention times of filipins II (20 .9 min), III (18.0 min), and IV (18.9 min) produced by FG26 match those previously reported[10], and these data in combination with detailed gene sequence analysis are strongly indicative that these compounds are identical. The Argentinian *S. misionensis* had also been reported to generate a further antifungal, misionin, for which there was little characterisation. Published UV absorbance data for this compound, however, would be consistent with it being a filipin analogue[8].



Filipin I ($R^1 = R^2 = H$)
Filipin II ($R^1 = H$, $R^2 = OH$)
Filipin III ($R^1 = R^2 = OH$)

Fig. 2 Filipin complex. Filipin IV is thought to be an epimer of Filipin III at the 1' or 3 position[11].

To further verify the identification of the first antifungals from a bacterium isolated from the *Allomerus* system, we sought the genes responsible for the biosynthesis of these compounds. Filipin biosynthesis has previously been reported in *Streptomyces avermitilis*, and the genes encoding the biosynthetic pathway identified[12]. Investigation of the FG26 genome sequence revealed a biosynthetic cluster, with four open reading frames encoding modular polyketide synthases with a very high level of similarity to the genes previously identified as encoding filipin biosynthesis, supporting our findings that the bacterium produced filipin complex.

The filipin gene cluster identified from FG26 has the same ordering as that of *S. avermitilis* (Fig. 3). Both gene clusters encode 13 proteins, and their functions and identities are illustrated in Table 1. Sequence identities at the protein level are high, ranging from 83-92%. The identification of the filipin gene cluster supports the proposed ability of FG26 to produce filipins.

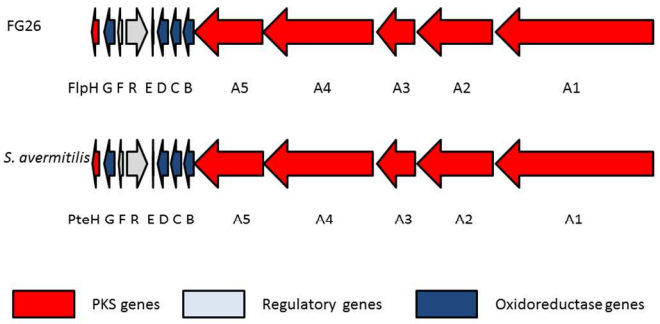


Fig. 3 Gene cluster of the *Streptomyces* FG26 filipins biosynthetic gene cluster, compared to the filipin biosynthetic cluster from *S. avermitilis*. The clusters encode for 13 proteins, from FlpA1 to FlpH.

Table 1. Proposed functions of proteins encoded by the filipins biosynthetic gene cluster, based on protein sequence identity. PKS: polyketide synthase.

FG26 Protein	Proposed function	*Streptomyces avermitilis* MA-4680 protein	Protein Identity (%)
FlpA1	PKS	PteA1	84
FlpA2	PKS	PteA2	85
FlpA3	PKS	PteA3	84
FlpA4	PKS	PteA4	84
FlpA5	PKS	PteA5	85
FlpB	Putative dehydrogenase	PteB	92
FlpC	Cytochrome P450 hydroxylase	PteC	91
FlpD	Cytochrome P450 hydroxylase	PteD	88
FlpE	Ferredoxin	PteE	87
FlpR	DnrI/RedD/AfsR-family transcriptional regulator	PteR	78
FlpF	LuxR family transcriptional regulator	PteF	88

FlpG	Putative oxidase	PteG	92
FlpH	Thioesterase	PteH	80

In modular PKSs, the KR (ketoreductase) domains are responsible for the stereospecific formation of β-hydroxyacyl thioester intermediates.[13] Bioinformatic analysis of KR domains has shown that conserved signature motifs correlate with the stereochemistry of the alcohol, and have thus been classified into A-type or B-type KR domains (Fig. 4).[14] Comparative examination of these signature motifs in the newly identified FG26 filipin KR domains with those of the *S. avermitilis* homologues was indicative that the FG26 filipins and the *S. avermitilis* filipins shared the same stereochemistry. Of the 13 KR domains in the filipin biosynthetic gene cluster, KR1 and KR7–KR13 are responsible for the alcohol stereochemistry at positions 27, 15, 13, 11, 9, 7, 5, and 3, respectively (Fig. 2). KR1 and KR7 have the conserved LDD motif of B-type KR domains, whereas the remaining six KR domains lack the LDD motif but contain the conserved tryptophan in the second motif indicative of A-type KR domains (Fig. 4B). These assignments match the observed stereochemistry in filipins I-III; most importantly, they perfectly match the KR types of the *S. avermitilis*.[15] The 26- and 1'-OH groups in filipin III are installed by the P450 enzymes PteC and PteD, respectively. PteC has been crystallised with its substrate filipin I.[16] Structural alignment of PteC and the FG26 homologue FlpC shows all substrate binding residues to be conserved (Fig. S9), which is a strong indication that the substrate positioning in FlpC and the stereochemical outcome of the hydroxylation will be identical to PteC. The sequence identity between PteD and FlpD is somewhat lower than that for the PteC/FlpC pair (88 *vs* 91 %, respectively) and no ligand-bound structure is available for PteD.[16] This makes the prediction of substrate binding for FlpD less reliable. However, given the high sequence identity of all filipin biosynthetic genes and the conserved organisation of the biosynthetic cluster compared to *S. avermitilis,* in combination with LC-MSMS data and known shifts in retention times, even for filipin stereomers (filipin III and filipin IV), the assignment of the prevalent filipin produced by the ant symbiont FG26 as filipin III is very likely.

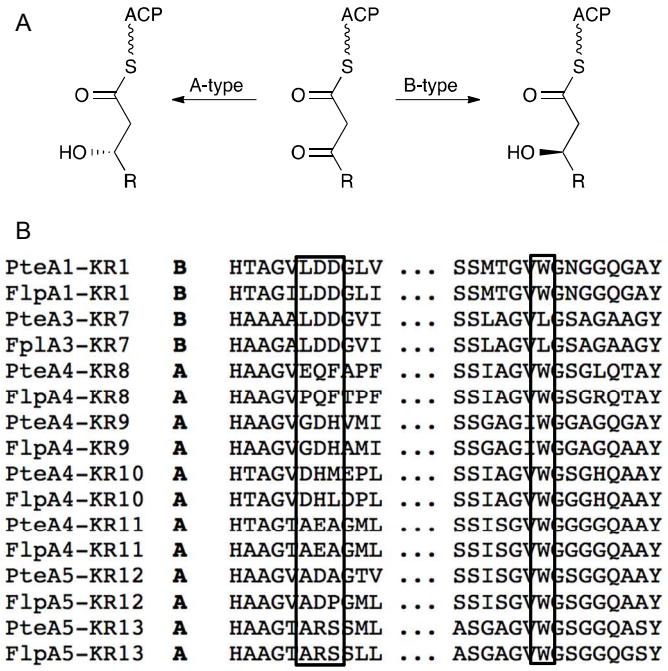


Fig. 4 KR domain specificity. A: stereochemical outcome of A- and B-type KR domains. B: signature motifs of KR domains in the filipin PKS from FG26 (Flp) and *S. avermitilis* (Pte); diagnostic residues are boxed, the complete sequence alignment is provided in Fig. S8.

Conclusions

Several antifungal compounds have now been reported from symbiont actinomycetes of the leafcutting ants *Acromyrmex*, including polyene antibiotics such as candicidin[6,17] and nystatin P13, and other antibiotics such as antimycin[6,18], actinomycin and valinomycin[16]. Few investigations have so far been carried out of any other ant-fungus symbioses. Here we report initial investigations into identifying compounds associated with the ant *Allomerus*. In this work we reveal that the *Allomerus*-associated actinomycete, *Streptomyces* FG26, is capable of generating a suite of filipins. Further *in vivo* experimental work will be needed to test the hypothesis that the filipins produced by this putative ant symbiont indeed confer a fitness benefit on *Allomerus*.

Notes and references

[a] *School of Chemistry, University of St. Andrews, St. Andrews, Fife, UK KY16 9ST.*
[b] *School of Chemistry, University of East Anglia, Norwich Research Park, Norwich, Norfolk, UK NR4 7TJ.*
[c] *School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, Norfolk, UK NR4 7TJ.*
[d] *John Innes Centre, Norwich Research Park, Norwich, UK NR4 7UH.*
[e] *CNRS, UMR Ecologie des Forêts de Guyane, Campus Agronomique, BP 316, 97379 Kourou Cedex, France*
[f] *State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Kunming, Yunnan, P. R. China 650223.*

† Electronic Supplementary Information (ESI) available: [Culturing conditions, extractions, bioassays and LC-MS analysis are reported here]. See DOI: 10.1039/c000000x/

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