Antifungals, arthropods and antifungal resistance prevention: lessons from ecological interactions

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Abstract

Arthropods can produce a wide range of antifungal compounds including specialist proteins, cuticular products, venoms and haemolymphs. In spite of this, many arthropod taxa, particularly eusocial insects, make use of additional antifungal compounds derived from their mutualistic association with microbes. Because multiple taxa have evolved such mutualisms it must be assumed that, under certain ecological circumstances, natural selection has favoured them over those relying upon endogenous antifungal compound production. Further, such associations have been shown to persist versus specific pathogenic fungal antagonists for more than 50 million years, suggesting that compounds employed have retained efficacy in spite of the pathogens' capacity to develop resistance. We provide a brief overview of antifungal compounds in the arthropods' armoury, proposing a conceptual model to suggest why their use remains so successful. Fundamental concepts embedded within such a model may suggest strategies by which to reduce the rise of antifungal resistance within the clinical milieu.

1. Introduction

Resistance to antifungal compounds is constantly rising to the point at which it is a critical factor in determining medical policy (Wiederhold 2017). To cope with this crisis, during the last 30 years several techniques have been employed in order to find new antifungals, including genome mining, synthetic biology, and exploring alternative microbial sources, such as marine microbes, and underrepresented taxa (Chevrette et al. 2019 and references within). Despite such efforts, identification and development of new antifungals has showed limited success. During the last decades research has demonstrated that animal taxa such as the Arthropoda have been using antifungals for millions of years. Arthropods produce endogenous antimicrobial compounds or can make use of those produced by bacterial mutualists (Shanchez-Contreras and Vlisidou 2008). Arthropods are highly speciose, occupy many trophic levels within a wide range of heterogeneous ecosystems and offer a wide array of molecules and interactions for research.

As microorganisms and arthropods co-evolved, production of antifungals was influential in defining ecological roles and interactions (Heine et al. 2018). Their secondary metabolites served to enable competition with other arthropods, to resist pathogens and, ultimately, to support growth and reproduction (Rohlfs and Churchill 2011). Thus, the tight regulatory control of antifungal metabolite formation in some model fungi represents an evolved chemical defence system favoured by selection not only against parasites but also animal antagonists (Rohlfs and Churchill 2011). The main use of such antifungals is to improve fitness but how they achieve their effects has not been fully resolved. This is particularly true in case of the apparent lack of development of antifungal resistances by parasitic antagonists.

We propose a conceptual strategy of antifungal use in arthropods employing two main resources: endogenous antifungal peptides and antifungal-producing bacteria. Strategic knowledge acquired via observing these natural systems may offer insights by which to combat not only antifungal resistance but also to prevent development of resistances against antimicrobials in general (Hokken et al. 2019). Current analysis of arthropods and their mutualists offers many specific examples to indicate that such interactions provide an immense reservoir of potential antifungal compounds, but, more importantly,

these systems have much to teach us about fine regulation and long-term strategic employment of such important molecules (Figure 1).

2. Setting the scene: Arthropods use both endogenously and microbially-produced antifungals

This section reviews arthropod and associated microbial antifungal production to contextualise conceptual models put forward in this paper.

2.1 Production of endogenous antifungals by arthropods

Arthropods secrete a wide array of secondary metabolites via their exocrine glands. Some of these secretions are used to communicate with conspecifics (sex, social, etc), others serve as food for developing offspring whilst yet others are used to defend single individuals or their societies from enemies and pathogens, including entomopathogenic fungi and Microsporidia (Schultz and Brady 2008; Mylonakis et al. 2016).

It has been long established that insect outer cuticle forms the first barrier to fungal infection, having either a fungicidal or fungistatic action (Koidsumi 1957, Ortiz et al. 2013). This thin layer is produced by cuticular glands and is composed of a complex mixture of lipids, including abundant straight-chain and methyl-branched, saturated and unsaturated hydrocarbons acting as a primary defence against fungi (Pedrini et al. 2013). Cuticle-degrading enzymes and enzyme-resistant cuticles both evidence the significance of an ongoing arms race between insects and entomopathogenic fungi (Zhang et al 2012, Pedrini et al. 2015).

Most exocrine glands with a known specific defensive function are those of social insects (Wilson and Holldobler 2005). Many ants species' metapleural gland secretions (for example) inhibit not only the growth of various bacteria but also that of some fungi, including entomopathogenic ones (Beattie 1985; Veal, Stokes, and Daggard 1992; Rothberg et al. 2011). Various compounds such as 3-hydroxydecanoic acid, indoleacetic acid and phenylacetic acid secreted by the leafcutter ant, *Acromyrmex octospinosus*, metapleural glands are effective against the parasitic fungus *Escovopsis* but also against their mutualistic fungus (*Leucoagaricus gongillophorus*) (Nascimento et al. 1996; Bot et al. 2002). Hymenopteran venoms can contain antibacterial and antifungal compounds such as

melectin and halictines (Slaninova et al. 2011). For example, ponericins from the venom of the ponerine ant *Pachicondyla gueldi*, can be active against bacteria and yeasts (Orivel et al. 2001), unidentified toxins in the venom of the paper wasp *Polistes flavus* are active against *Candida* and *Aspergillus niger* (Prajapati and Upadhyay 2016), while the venom of *Apis mellifera* and of a sweat bee is active against *Candida* (Ferrell et al. 2015; Lee 2016). The termite *Pseudacanthotermes spiniger* (Silva et al. 2003) produces a compound called termicin, to defend their colonies from pathogenic fungi whilst a small antimicrobial peptide within royal jelly (Jelleine-I) presents potent in vitro and in vivo antifungal activity (Jia et al. 2018).

Antifungal substances are also produced in the haemolymph of non-social insects when induced by a fungal infection. Drosomycin has been extracted from the haemolymph of the fly *Drosophila melanogaster* (Zhang et al. 2009), while the spined soldier bug *Podisus maculiventris* (Hemiptera) produces thanatin (Sinha et al. 2017). The haemolymphs of various Lepidoptera contain antifungal substances such as the gallerimycin from *Galleria mellonella* (Schuhmann et al. 2003).

Arthropods other than insects are known to produce antifungal active substances especially in venom, for example tenecin is an anti-microbial peptide (AMP) found in the venom of the Brazilian yellow scorpion *Tityus serrulatus* (Santussi et al. 2017) and joruin is produced in the haemolymph of the Amazonian pink toe spider *Avicularia juruensis* (Ayroza et al. 2012).

2.2 Arthropod-associated bacteria and symbiotically-produced antifungals

Arthropods' exosymbiotic and endosymbiotic bacteria form co-evolutionary associations ranging from facultative to obligate mutualisms (Chen et al. 2017; Shanchez-Contreras and Vlisidou 2008). They can fulfil a variety of roles including improving nutrient acquisition, facilitating development of resistance to plant secondary metabolites and assisting chemical pollutant and pesticide detoxification (Boucias et al. 2018). Some bacterial symbionts can also produce antifungals evolved to limit replication of the arthropods' fungal antagonists (Holmes et al. 2016).

Fungus farming termites of sub-family *Macrotermitinae* and fungus farming ants of genus *Acromyrmex* exemplify such microorganism-insect associations. Both cultivate specific fungi as

colony food source and their bacterial mutualists produce antifungals to protect the cultivar. Some 30 million years ago *Macrotermitinae* termites (of which there are some 350 species) evolved the cultivation of basidiomycetes within the genus *Termitomyces* as their primary food source (Otani et al. 2014; Lever et al. 2015; Aanen et al. 2002). *Termitomyces* is subject to parasitism by opportunistic fungi belonging to the genus *Pseudoxylaria* spp. and competition from fungi such as *Trichoderma* or *Beauveria* (Um et al. 2013; Otani et al. 2019; Katariya et al. 2017; Katariya, Ramesh, and Borges 2018). It is likely that the *Macrotermitinae* employ multiple strategies to control such antagonists and production of antifungals is one of them (Katariya et al. 2017; Um et al. 2013). So far, seven prokaryotic phyla have been identified in the *Macrotermitinae*'s gut flora (Otani et al. 2014). Among them *Bacillus* strains are dominant and can produce antifungals. An initial liquid chromatography and mass spectrometry (LC/MS) analysis of an extract of the *Bacillus* strains cultures revealed a major secondary metabolite: bacillaene, a polyene polyketide, common to all strains, which inhibits the growth of *Pseudoxylaria*, *Trichoderma*, *Coriolopsis*, *Umbelopsis* and *Fusarium* in a dose-dependent manner (Um et al. 2013). Fungus-growing termites also support *Streptomyces* which produce the antifungal natalamycin (Kim et al. 2014). The *Streptomyces* strain associated with fungus-growing termites also produces additional antibiotics: microtermolides A and B (Carr et al. 2012).

A similar association occurs in leafcutter ant, *Acromyrmex* spp. colonies. *Acromyrmex* cultivate a fungal mutualist, *Leucoagaricus gongylophorus* as their sole source of nutrition and support *Pseudonocardia* bacteria within their metapleural glands (Heine et al. 2018; Holmes et al. 2016). The *L. gongylophorus* cultivar is parasitized by another fungus: *Escovopsis* (Schultz and Brady 2008; Yek, Boomsma, and Poulsen 2012). The *Pseudonocardia* synthesize different variants of the broad-spectrum polyene antifungal nystatin P1 to control *Escovopsis* (Holmes et al. 2016). In addition, *Pseudonocardia* associated with the attines *Apterostigma dentigerum* and *Trachymyrmex cornetzi* have recently been found to produce novel cyclic depsipeptide compounds called gerumycins A-C, (Holmes et al. 2016). The gerumycins are slightly smaller versions of dentigerumycin, a cyclic depsipeptide that, at micromolar concentrations, also selectively inhibits *Escovopsis* (Sit et al. 2015) without affecting the ants' fungal cultivar (Oh et al. 2009). In contrast, purified gerumycin A did not exhibit significant antifungal activity *in vitro* up to 1 mM against a dentigerumycin-sensitive strain,

and phenotypic screening of the gerumycin-producing bacteria against *Escovopsis* did not display marked activity, indicating that dentigerumycin is at least three orders of magnitude more potent than the gerumycins at suppressing *Escovopsis* (Sit et al. 2015). Such differences in potency may form the basis of a strategy inhibiting development of resistance wherein different antifungal variants may be effective against different species of *Escovopsis* and do not act as general purpose antifungals (Baym, Stone, and Kishony 2016).

Streptomyces are commonly found in insect microbiomes: southern pine beetle (*Dendroctonus frontalis*) exhibits mutualism with *Streptomyces*, strains of which produce a number of secondary metabolites including frontalamide A, frontalamide B, and mycangimycin (Scott et al. 2008; Blodgett et al. 2010). Mycangimycin inhibits the beetles' antagonistic fungus *Ophiostoma minus* and has potent inhibitory activity against *Plasmodium falciparum*, whilst frontalamides have general antifungal activity (Scott et al. 2008; Blodgett et al. 2010; Baniecki, Wirth, and Clardy 2007). *Streptomyces* spp. are also associated with the solitary wasps, *Sceliphron caementarium*, and *Chalybion californicum*, providing antibacterial and antifungal chemical protection to their larvae via production of streptochlorin, and a variety of piericidin analogues (Poulsen et al. 2011). The antifungal compound sceliphrolactam was isolated from *Streptomyces* associated with the mud dauber wasp *Sceliphron caementarium* (Poulsen et al. 2011). The compound is a polyene macrocyclic lactam displaying antifungal activity against amphotericin B-resistant *Candida albicans* (Oh et al. 2011).

Screening for novel antimicrobials produced by actinobacteria, revealed a kanchanamycin-producing actinomycete with antifungal activity isolated from the head of *Lasius fuliginosus L.* (Ye et al. 2017). Similarly, another actinomycete, isolated from the head of the Japanese carpenter ant *Camponotus japonicas* exhibits specific antifungal activity against the plant-pathogens *Phytophthora infestans* and *Corynespora cassiicola* (Bai et al. 2016; Bowen et al. 2018; Izbiańska et al. 2019). Even entomopathogenic fungi can produce antifungal peptides to combat their own fungal antagonists; conidial cell walls of the insect pathogen fungus, *Beauveria bassiana*, express and release an antifungal peptide (BbAFP1) into surrounding microenvironments, inhibiting growth of other, competing fungi (Tong et al. 2020).

3. An antifungals arms race: mix to evolve, evolve to mix.

Complex organisms' main defence against pathogens is their immune system. Antifungal molecules are integral components of the innate immune system in many taxa. Mammalian antifungal peptides such as defensins, protegrins, histatins, lactoferricins as well as antifungal peptides produced by birds, amphibians and insects all play pivotal roles in fighting fungal pathogens (Neelabh, Singh, and Rani 2016; Hegedüs and Marx 2013).

This being so, it begs a question; if such organisms have evolved to produce their own antifungal compounds why have some arthropods, notably those associated with specific fungal mutualists, evolved further mutualisms with bacteria that provide their hosts with additional antifungal compounds? The answer may lie in the development of resistances by their fungal antagonists. Antifungal compounds, mainly peptides or proteins have been proposed as a primitive mechanism of immunology (Hegedüs and Marx 2013) and there are no doubts about their potency, but small changes in fungal antagonists' epitope can inhibit or eliminate their efficacy. Thus, in such cases, how does participation in such mutualistic associations avoid development of antifungal resistances, whilst possession of integral antifungal peptides alone does not?

Attine ants (tribe: Attini) provide a useful model by which to examine these questions. To counter the threat of pathogenic infection of their garden fungus, the attines have multiple strategies including a tripartite mutualistic relationship within which they host antibiotic-producing bacteria on their bodies (Barke et al. 2010). Many of these bacteria have coevolved with their hosts, producing antifungals to inhibit parasitic fungi (*Escovopsis* spp. and allied taxa) whilst in return, the ants feed them via unique exocrine glands within elaborate cuticular crypts that also offer the bacteria their favoured microclimate (Currie et al. 2006). For the attine:cultivar association to have persisted for 50 million years in the face of *Escovopsis* parasitism, it suggests that any resistance *Escovopsis* evolves to antifungals employed against it must be countered by a similar flexibility in antifungal innovation on the part of the multi-partite mutualists. It is this flexibility, essential in a fast moving, co-evolutionary conflict between mutualists and parasite, that the bacteria provide. In the example of the attine cultivar, comparing molecular structures of different gerumycins and dentigerumycin variations

197 produced by different *Pseudonocardia* associated with two different attine genera (Sit et al. 2015)
198 suggests pathways via which closely related symbiotic bacteria acquire the capacity to produce novel
199 molecules with new functions. Their analysis revealed very different biosynthetic architectures and
200 they posit these result from chromosomal incorporation of disparate plasmid-borne genomic islands,
201 acquired via horizontal gene transfer, leading to bacterial biosynthesis of varying antifungal
202 molecules with virtually identical core structures (Sit et al. 2015). In this example each effective core
203 forms a foundation for several different antifungal variants with different efficacies. Thus natural
204 selection favours a combination of enhanced genetic variants available for rapid evolutionary
205 selection to retard the development of antifungal resistances (Bergstrom, Lo, and Lipsitch 2004;
206 Baym, Stone, and Kishony 2016). We speculate that in order to synthesise an effective variability of
207 mixed antifungals, both on short and on long evolutionary timescales, bacteria are better weapons
208 compared with the relatively slow genetic variation/selection rates possible within arthropods.
209 Nevertheless, perhaps further emphasising the magnitude of microbial challenge insects face, their
210 endogenous antifungal peptides already display a remarkable evolutionary plasticity, originating from
211 gene duplication, subsequent diversification, and *de novo* creation from non-coding sequences
212 (Mylonakis et al. 2016). Horizontal gene transfer is relatively rare in metazoa (Nakabachi 2015) so
213 specific antifungal peptide families have been identified clustered within single insect orders and
214 restricted taxonomic groups, reflecting specific evolutionary adaptation (Mylonakis et al. 2016).
215 Therefore, the antifungal peptides are less plastic when compared the antifungals synthesized from
216 bacterial antifungal gene clusters. In addition, bacterial mutualists, with plastic haploid genomes, offer
217 faster mutation rates and frequent employment of horizontal gene transfer, whilst, by comparison, *n*-
218 ploid arthropod reproduction/selection is slower in securing and expressing effective changes.
219 This is particularly the case in eusocial arthropods such as attines ants. Comprising up to several
220 million individuals harvesting vegetation to feed their cultivars, such colonies might be classed as
221 'super-organisms' (Hölldobler and Edward 2009) peculiarly vulnerable to the threat parasitic fungi
222 present. Workers spend much of their time foraging implying continual contact with genetically-
223 varied spores of fungal strains pathogenic to their mutualistic fungus cultivar (Poulsen et al. 2002). In
224 this scenario, *Escovopsis* strains are potentially variable via recruitment (Poulsen et al. 2010) as well

as via their innate ability to offer genetic differentiation (De Mana et al. 2016). Their cultivar is genetically homogenous (Kooij et al. 2015) and the colony is long-lived, so potentially parasitic fungi have years to adapt to it. The colony is slow to reproduce, although one colony may survive many years and can produce many alates a year, it may require five years or more before it is capable of their production and can never gain the equivalent benefits of multiple offspring/multiple generation breeding strategies that short-lived insects enjoy (Keller and Genoud 1997). Other factors are also influential: multi-mated queens notwithstanding, workers possess relative high genetic homogeneity (Holzer, Keller, and Chapuisat 2009), limiting the range of endogenous antifungals any one colony can produce whilst living underground in humid, fungus-friendly environments encourages invasion by other competing/parasitic fungi (Pie, Rosengaus, and Traniello 2004).

Thus, such eusocial insect colonies experience many of the disadvantages of a long-lived complex organism's long-term interactions with pathogens without the benefit of its more advanced, adaptive, 'memory-driven' immune system (Gross et al. 2009). Bacteria and fungi have been antagonists for millennia and have evolved sophisticated compound spectra by which to inhibit/destroy each other so it is entirely understandable that some eusocial insects, depending upon long-term mutualistic relationships with fungi, would exploit antifungal-producing bacteria as a form of colonial/'super-organismal' 'immune system' (Penick et al. 2018). Thus, in lieu of rapid reproduction providing continual variation in immunity or a system of adaptive immunity, the attines (and others of their eusocial ilk) exploit bacteria as anti-pathogenic defence systems to the extent that they are dependent upon them.

The virtue of these mutualistic bacteria, with *Pseudonocardia* prominent amongst them, is that they are genetically-specialised to offer continual production of varied self-similar but non-repeating antifungal compound assemblages (Pathak, Kett, and Marvasi 2019). By so doing they produce stochastically-varying anti-fungal conditions to which parasitic fungi cannot respond with sufficient rapidity to 'outwit'; a 'Red Queen environment' to keep them evolutionarily outmanoeuvred.

It is therefore important to find out whether single-drug–resistance steps would be selected for or against in a multidrug environment. We speculate that mixtures of bacterial antifungal variants would help ants' antifungal peptides retain their efficacy, delaying the parasite's antifungal resistance. The

first important assumption is that antibiotic interactions can change with the acquisition of particular mutations (leading to resistance) (Baym, Stone, and Kishony 2016). In Figure 2, three models are proposed. In the Induced Synergy Model (Figure 2 A) ants' antifungal peptides act in synergy with the bacterial antifungal mixtures. In this model the parasite may develop an antifungal peptide-resistance allele which, whilst conferring resistance to the antifungal peptide, also changes its interaction with the bacterial antifungal mixture, making the resistant parasite more sensitive to the overall treatment. This principle has been established in other contexts, such as in *Escherichia coli* and cell lung cancer lines resistant to chemotherapeutics (Wood et al. 2014). Efficacy persistence of bacterial antifungal mixtures is greater than that of individual compounds, so that complexes of antifungal peptides isolated from maggots of *Calliphoridae* flies prevent development of resistance better than their individual component small molecules and peptides (Chernysh, Gordya, and Suborova 2015). The second model (Figure 2 B) shows the collateral sensitivity which occurs without co-application of the bacterial antifungal and the antifungal peptide. Mutant alleles conferring resistance to antifungal peptides induce susceptibility to the bacterial antifungal (Baym, Stone, and Kishony 2016). In the third model (Figure 2, C), the two molecules interact, and the sensitive microorganisms can grow at high concentrations of bacterial antifungal when the peptide antifungal is also present. However, the efficacy of bacterial antifungals is reduced due to the evolution of resistance to co-applied antifungal peptide. In all these contexts cycling of bacterial antifungal *mixtures* may prevent the parasite's escape towards resistance. Thus, the rapidity of bacterial antibiotic evolutionary rate does not solely rely on antibiotic cycling. Cycling utilises a recurring series of antibiotics, but antibiotic production by *Pseudonocardia* (or other microorganisms) is unlikely to exhibit a cyclic development, rather it produces unpredictable, non-repetitive compound variants over time (Pathak, Kett, and Marvasi 2019).

This interaction may be considered a Chase Red Queen (CRQ) scenario, in which local directional selection drives coevolutionary chases between exploiter (bacteria) and victim (arthropods' fungal parasites) phenotypes (Brockhurst et al. 2014). CRQ dynamics generally occur when interactions have a complex genetic basis; in this case the acquisition, exchange and recombination of genes related to antifungal synthesis by bacteria. This results in a chase in multiple ways. In the attine-

cultivar scenario both the pathogen and host cover the same role: hosts are under selection to increase phenotypic distance through de novo evolution of novelty, while exploiters are under selection to reduce phenotypic distance (Brockhurst et al. 2014). In the attine-cultivar the CRQ imposes a coevolution process comprising a continual series of selective sweeps, which reduce genetic diversity within populations but that drive divergence between populations. The extent to which this operates in arthropod-bacterial mutualisms should be clarified in further experiments assessing genetic diversity of the microbiome across nests and metagenomics and metatassonomic diversity (Lozupone et al. 2007). Sustained cycles of coevolutionary chase may occur through phenotype space whereby the direction and intensity of selection vary according to the relative locations of the species in phenotype space (Brockhurst et al. 2014).

CAPTIONS

Figure 1. Mechanisms preventing development of antifungal resistance. In this example, ants can release a range of both endogenous and bacterial antifungals.

Bacteria can exploit genetic changes resulting from horizontal gene transfer, gene rearrangement, mutation and haploidy plus rapid reproduction to produce quickly changing antifungal mixtures.

Ants do not reproduce as fast as bacteria, have much lower population numbers and more homogenous genes. They can, however, produce a range of antimicrobial peptides (AMPs) with antifungal activity to act as an effective first defence.

Figure 2. Strategies for preventing development of antifungal resistance. The models are particular cases from those proposed by Baym et al. (2016). In this context the two key players are AMPs produced by insects and antifungals produced by bacteria. (A) In a synergistic antagonistic interaction acquisition of resistance makes the mutant more sensitive to the combination of the antimicrobial peptide and bacterial antifungal. (B) In the collateral sensitivity hypothesis, which occurs without co-application, acquired resistance to AMPs induce susceptibility of the bacterial antifungal thus allowing selection against resistance. (C) In a suppressive interaction strategy, due to

molecular interaction of the bacterial antifungals and antifungal peptides, efficacy of bacterial antifungals is reduced as the resistance to antimicrobial peptide evolves. Figure modified from Baym et al. (2016).

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