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Subterranean termite prophylactic secretions and external antifungal defenses

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ABSTRACT

Termites exploit environments that make them susceptible to infection and rapid disease transmission. Gram-negative bacteria binding proteins (GNBPs) signal the presence of microbes and in some insects directly damage fungal pathogens with β-1,3-glucanase activity. The subterranean termites *Reticulitermes flavipes* and *Reticulitermes virginicus* encounter soil entomopathogenic fungi such as *Metarhizium anisopliae*, which can evade host immune responses after penetrating the cuticle. An external defense that prevents invasion of fungal pathogens could be crucial in termites, allowing them to thrive under high pathogenic pressures. We investigated the role of secreted β-1,3-glucanases in *Reticulitermes* defenses against *M. anisopliae*. Our results show that these termites secrete antifungal β-1,3-glucanases on the cuticle, and the specific inhibition of GNBP associated β-1,3-glucanase activity with D-δ-gluconolactone (GDL) reduces this activity and can cause significant increases in mortality after exposure to *M. anisopliae*. Secreted β-1,3-glucanases appear to be essential in preventing infection by breaking down fungi externally.

1. Introduction

Insects lack a vertebrate-like adaptive immune system and instead rely on physical barriers such as the cuticle, as well as innate cellular and humoral defenses that are activated by the recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs). PRRs include peptidoglycan recognition proteins (PGRPs) that bind peptidoglycans in bacterial cell walls, and Gram-negative bacteria binding proteins (GNBPs) and β-1,3-glucan recognition proteins (BGRPs) that recognize lipopolysaccharides in Gram-negative bacteria outer membranes and β-1,3-glucans in fungal cell walls (Royet, 2004). These PRRs can activate cellular responses, including phagocytosis and encapsulation, as well as pathways leading to the secretion of antimicrobial peptides (AMPs) and reactive oxygen intermediates that are important components of the humoral immune response (Royet, 2004; Strand, 2008). Some PGRPs also exhibit direct bactericidal amidase activity (Mellroth and Steiner, 2006).

GNBPs and BGRPs represent a group of homologous proteins that can activate the prophenoloxidase cascade (Ma and Kanost, 2000; Ochiai and Ashida, 2000; Zhang et al., 2003) as well as the toll and immunodeficiency (IMD) pathways, leading to the production of AMPs (Kim et al., 2000; Warr et al., 2008). These PRRs share sequence homology with bacterial β-1,3-glucanases, and recent work has shown that these molecules duplicated in the ancestor of mollusks and arthropods, diverging into groups that have maintained or lost amino acids essential for β-1,3-glucanase activity (Bragatto et al., 2010). The enzymatically active GNBPs are family 16 glycoside hydrolases (GH16), and contain two active glutamic acid residues in the catalytic cleft (Juncosa et al., 1994). Although these residues have been replaced in many insect GNBPs that act as PRRs, GNBPs and BGRPs that have maintained the active site and exhibit β-1,3-glucanase activity have been identified in several insects, including termites (Bragatto et al., 2010; Bulmer et al., 2009; Genta et al., 2009; Pauchet et al., 2009).

Termites and other social insects live in densely populated colonies that may have highly related individuals in constant close interactions, often sharing fluids through proctodeal and stomodeal trophallaxis. Subterranean termites also nest and forage in soil and decaying wood and may be inbred (Bulmer et al., 2001; Vargo, 2003; Vargo and Husseneder, 2011), making them especially susceptible to the rapid spread of infection throughout a colony (reviewed by Rosengaus et al. (2011)). However, social insects including termites have evolved defenses, ranging from individual innate to social immunity (reviewed by Cremer et al. (2007), Rosengaus et al. (2011)), that have allowed them to become

Abbreviations: PRR, pattern recognition receptor; PAMP, pathogen associated molecular pattern; PGRP, peptidoglycan recognition protein; GNBP, Gram-negative bacteria binding protein; BGRP, β-1,3-glucan recognition protein; AMP, antimicrobial peptide; GH16, family 16 glycoside hydrolases; GDL, D-δ-gluconolactone.

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evolutionarily successful and ecologically dominant. Social immune defenses can include allogrooming and other hygienic behaviors as well as the incorporation of antimicrobials in nest materials.

A selective sweep in *termicins* which code for antifungal peptides in the subterranean termites *Reticulitermes flavipes* and *R. virginicus* suggests that these termites face strong selective pressures on their antifungal defenses (Bulmer et al., 2010). This selection may be driven in large part by the entomopathogenic fungus *Metarhizium anisopliae*, a natural pathogen of *R. flavipes* which is ubiquitous in the soil they exploit (Zoberi, 1995). *M. anisopliae* invades insects by penetrating the cuticle (Gillespie et al., 2000) and can then evade host immune defenses by producing a collagenous coat that prevents hemocyte attachment and masks PAMPs (Wang and St Leger, 2006) and by secreting toxins that interfere with host innate immunity (Vey et al., 2002).

Termite GNBPs also show evidence of a response to selective pressure from pathogens that may include *M. anisopliae* (Bulmer and Crozier, 2006). *GNBPs* duplicated prior to the divergence of termites creating paralogous *GNBP1* and *GNBP2* genes, which have undergone positive selection in a specific lineage associated with a transition to living and foraging in the soil (Bulmer and Crozier, 2006). The isolation and characterization of GNBP2 from the termite *Nasutitermes corniger* showed that these proteins have maintained β-1,3-glucanase activity. The inhibition of GNBP2 β-1,3-glucanase activity by D-δ-gluconolactone (GDL), a monosaccharide closely related to glucose, led to increased mortality from infection by *M. anisopliae* and opportunistic pathogens (Bulmer et al., 2009).

Termite GNBPs may play an important role in external antifungal defenses by breaking down fungal conidia in the nest and on the cuticle (Bulmer et al., 2009). Externalized antifungal defenses have been reported from multiple sources in termites, metapleural glands in ants, and also in amphibian skin secretions (Rollins-Smith et al., 2005; Rosengaus et al., 2011, 2004; Schlüns and Crozier, 2009). Termicins and GNBPs are highly expressed in termite salivary glands (Lamberty et al., 2001; Yuki et al., 2008), and may be secreted onto the cuticle during allogrooming and into building materials during nest and foraging gallery construction. We investigated if these molecules are externalized in subterranean termites and are important in frontline defenses that prevent fungal pathogens from penetrating the cuticle.

2. Materials and methods

2.1. Insect and fungal strain collection and maintenance

Reticulitermes flavipes, R. virginicus, and *M. anisopliae* were collected from Baltimore County, Maryland, in the spring and summer of 2010. Pieces of wood containing dense aggregations of termites were collected and maintained in the lab in dark plastic containers at room temperature with constant moisture. Termite species were identified using intra- and interspecific agonism assays (Polizzi and Forschler, 1998) and mitochondrial rRNA sequences (Bulmer et al., 2010). Local strains of *M. anisopliae* were isolated from soil in close proximity to (<30 cm) each termite collection site as described by Hughes et al. (2004). Briefly, *Tenebrio molitor* larvae were placed in soil samples and monitored daily. Dead larvae were surface sterilized in 70% ethanol and placed on sterile moist filter paper. Conidia were collected from individuals that died of *M. anisopliae* infection, then suspended in 0.1% Tween 80 and stored at 4 °C. Strains were confirmed as *M. anisopliae* var. *anisopliae* with partial sequence of IGS and 16S DNA. Stock strains of the mutant Δ*Mcl1* and its wild type 2575 were kindly provided by Dr. St. Leger at the Department of Entomology, University of Maryland, College Park, Maryland.

2.2. GNBP amino acid alignment

Amino acid sequences of the catalytic cleft and flanking regions of *N. corniger* (*Ncr*) GNBP1 and 2 (Bulmer et al., 2009) and *Spodoptera frugiperda* (*Sf*) SLam (Bragatto et al., 2010), which have been shown to exhibit β-1,3-glucanase activity, were aligned with sequences for *R. flavipes* (*Rf*) and *R. virginicus* (*Rv*) GNBP1 and 2 (Bulmer et al., 2010). To characterize homology of GNBP1 and 2 within species, a 329-residue region without the leader peptide was compared using BLAST. (GenBank IDs: *Ncr1*, JF683377; *Ncr2*, JF683378; *Sf*, EF641300; *Rf1*, JF683373; *Rf2*, JF683375; *Rv1*, JF683374; *Rv2*, JF683376).

2.3. Termite extracts, cuticular washes, and nest material sampling

For crude extracts, two workers were cold-immobilized on ice and then homogenized in 10 μl of 100 mM sodium acetate (pH 5.0) with QIAshredder columns (QIAGEN). For gutted crude extracts, the hindgut containing the bacterial and protozoan symbiont community was first removed, leaving the foregut and salivary gland reservoirs intact, and then prepared as described for crude extracts. Salivary glands and reservoirs of two termites were extracted and vortexed in 10 μl of 100 mM sodium acetate (pH 5.0). To standardize for differences between species in cuticular washes, surface area was calculated using Meeh's formula, where surface area = $kW^{2/3}$ (W is mass (g) and k is the species constant which is 12 for termites (Sponsler and Appeal, 1990)). For each colony of termites, the average mass of workers used for cuticular washes was used to calculate surface area, and five workers of *R. flavipes* and seven workers of *R. virginicus* were used for each wash. Termites were extracted from nests and placed in Petri dishes lined with moist filter paper for 24 h to allow for allogrooming and the removal of nest debris and microbes. Cold-immobilized workers were placed in 20.5 μl of 0.1% Tween 80 per cm^2 of surface area, gently agitated for 10 s, and 20 μl of wash was extracted. Washes used in antifungal assays were filter sterilized with 0.22 μm Ultrafree filters (Millipore), although later we determined that filter sterilization was not necessary to avoid microbial contamination. Termite cuticles appear to be free of microbes that readily grow on potato dextrose agar (PDA) supplemented with ampicillin after termites have been maintained for 24 h in sterile petri dishes. Crude extract, gutted crude extract, and salivary gland and reservoir contents were kept on ice and used in β-1,3-glucanase activity assays. Cuticular washes were used for β-1,3-glucanase activity and antifungal assays.

Nest materials were also prepared for β-1,3-glucanase assays. One hundred milligram of foraging gallery material from active laboratory-maintained termite colonies as well as foraging gallery material constructed from 500 mg of soil processed by 100 *R. flavipes* workers for 5 days were washed with 500 μl of 0.1% Tween 80, then centrifuged three times for 1 min at 16,000 *g*, and the supernatant was concentrated to 20 μl with an Ultracel-10 filter (Amicon). A 3 mm by 3 mm piece of filter paper that had been processed by 12 *R. flavipes* workers for 48 h was soaked in 15 μl of 0.1% Tween 80 for 1 min, and the liquid and paper were then separated in a QIAshredder column. Unprocessed filter paper and sterile soil soaked in 0.1% Tween 80 were used as controls.

2.4. β-1,3-Glucanase activity assays

β-1,3-Glucanase activity was detected using a gel electrophoresis assay (Kalix and Buchenauer, 1995). Briefly, samples were run on polyacrylamide gels containing Carboxymethyl Curdlan Remazol Brilliant Blue (Loewe Biochemica) and incubated in 100 mM sodium acetate (pH 5.0, optimal pH) for 24 h. β-1,3-Glucanase activity results in the digestion of curdlan (a β-1,3-glucan polymer)