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INHIBITION OF THE FUNGAL PATHOGEN *METARHIZIUM ANISOPLIAE* IN THE ALIMENTARY TRACTS OF FIVE TERMITE (ISOPTERA) SPECIES

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There are more than 2,600 described species of termites, from 7 families, and they are adapted to a wide range of habitats resulting in high diversity in morphology, physiology, behavior, nesting ecology, and associated microbial community (Abe et al. 2000). Sociality is thought to be a key factor in their ecological success and diversity, but how termites evolved to resist the increased pressure from pathogens as they moved from the solitary lifestyle of the wood roach-like ancestor towards eusociality remains poorly understood (Fefferman et al. 2007). Chouvenc and Su (2010) showed that the subterranean termite *Reticulitermes flavipes* (Kollar) has the capacity to prevent epizootics of the entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin due to synergy among defense mechanisms. Chouvenc et al. (2009a) described *in vivo* the antifungal activity of the alimentary tract in *R. flavipes* and showed that after grooming and ingestion of conidia of *M. anisopliae*, germination did not occur in the alimentary tract of living termites. In dead termites, this antifungal activity remained strong at least 2 d *post mortem* throughout all gut regions.

We hypothesized that gut antifungal activity is an ancestral feature and was maintained during the radiation of the isopteran lineage. To test this, we extended our observations to determine if this mechanism exists in 5 additional termite species from 5 different families. Histological preparation was identical to Chouvenc et al. (2009a) and was performed on selected specimens described in Chouvenc et al. (2009c). Histological analysis included 25 *Hodotermopsis sjoestedti* Holmgren (Termopsidae), 8 *Hodotermes mossambicus* (Hagen) (Hodotermitidae), 13 *Kalotermes flavicollis* (Fabricius) (Kalotermitidae), 10 *Prorhinotermes canalifrons* (Sjöestedt) (Rhinotermitidae), and 2 *Nasutitermes voeltzkowi* (Wasmann) (Termitidae). The presence or absence of conidia in the gut was recorded in all specimens, and the presence of fungal hyphae was examined in healthy, moribund, and dead specimens. Full histological observations for each species are available in the supplemental information.

After exposure to a solution of *M. anisopliae* conidia, grooming was performed by the termites and conidia were subsequently found in the alimentary tracts of the groomers for all 5 termite species (Fig. 1A). For each species, conidia in the alimentary tract did not germinate, as previously described in *R. flavipes* (Chouvenc et al. 2009a). In all the specimens that were fixed after their death (usually 2-5 d after fungal exposure) for histological preparation, the fungal hyphae quickly spread in the hemocoel, the muscles (Fig. 1B) and the fat body within the 24 h *post mortem*, but did not readily invade the salivary glands. The fungus did not penetrate the salivary glands until at least after 1 d *post mortem*. In addition, *M. anisopliae* hyphae did not penetrate the gut lumen until at least after 2 d *post mortem*. We confirmed that the fungistatic activity of the alimentary tract was present in all tested species and that this activity remained strong in the alimentary tract at least 2 d *post mortem* (Figs. 1C and 1D), although the salivary glands were already invaded by the fungus at this time (Fig. 1E). Therefore, all conidia found in the digestive tract of live termites did not germinate and the fungistatic activity of the gut in all termite species remained active for several days after the death of the termites, as previously showed in *R. flavipes* (Chouvenc et al. 2009a). We suggest that gut fungistatic activity is an ancestral trait that has been conserved during the radiation of the Isoptera. However, we previously showed that all termites studied had a variable mortality rate when exposed to *M. anisopliae* (Chouvenc et al. 2009c). The present results suggest that this difference of susceptibility among species may depend on other physiological factors, such as the cellular immunity (Chouvenc et al. 2009b), or other behavioral and physiological factors (Cremer et al. 2007). It has been suggested that part of this fungistatic activity is due to the production of antimicrobial peptides in the salivary glands (Lamberty et al. 2001; Bulmer & Crozier 2004), but our results showed that in all species the salivary glands were invaded by the fungus much

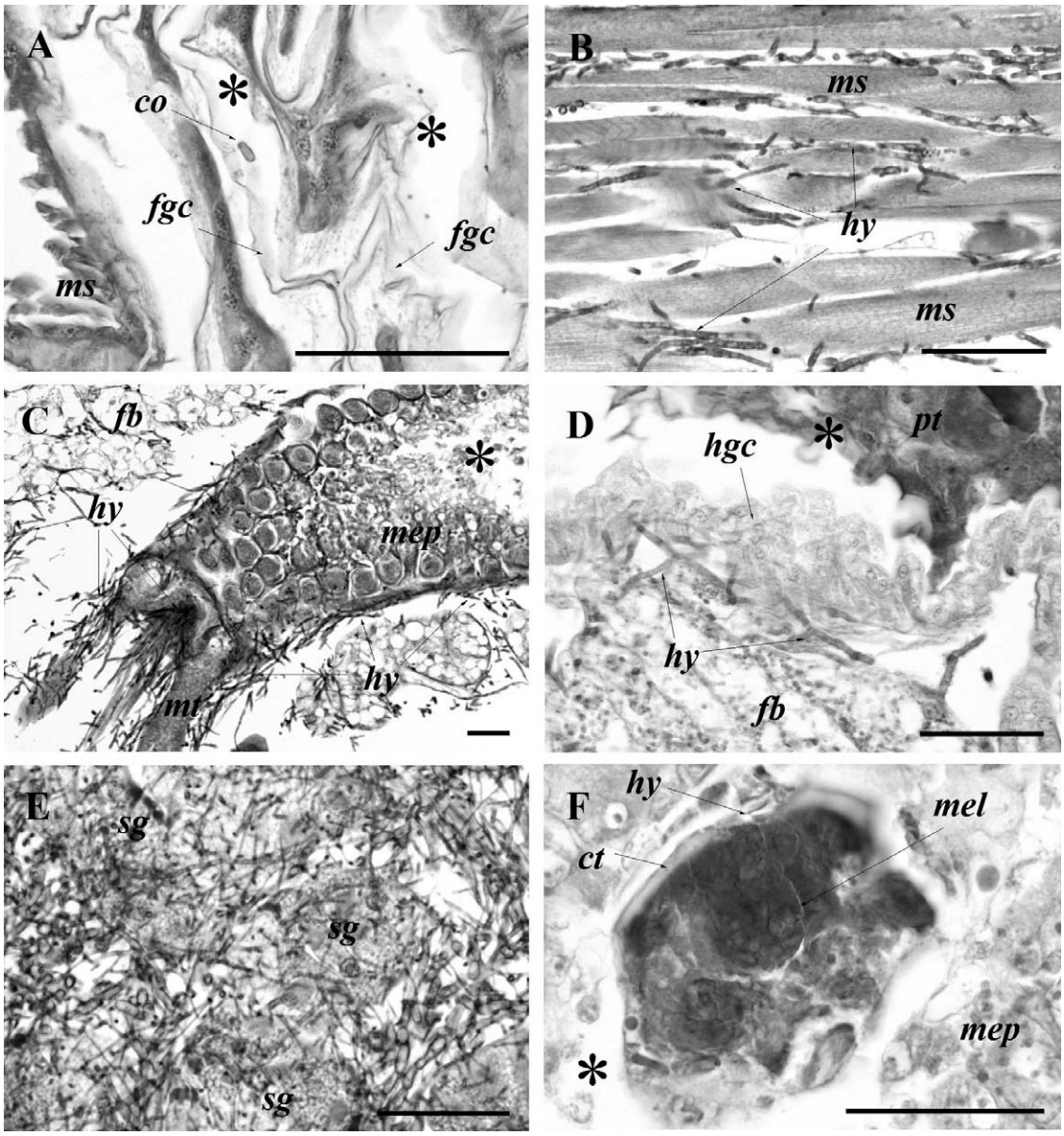

Fig. 1. Observation of *Metarhizium anisopliae* in termites alimentary tract of 6 species after histological preparation. (A) *Nasutitermes voeltzkowi*, ungerminated conidium in cuticular foldings of the crop (foregut). (B) *Hodotermes mossambicus*, growth of hyphae in the hemocoel and in the muscles at 1 d *post mortem*. (C) *Hodotermopsis sjoestedti*, growth of hyphal bodies in the muscles around the midgut but absence of penetration of the midgut lumen by the fungus at 1 d *post mortem*. (D) *Hodotermopsis sjoestedti*, beginning of penetration by the fungus of the hindgut lumen at 3 d *post mortem*. (E) *Prorhinotermes canalifrons*, invasion of the salivary glands at 2 d *post mortem*. (F) *Kalotermes flavicolis*, fragment of a cellular encapsulation from the external cuticle of a cannibalized nestmate in the midgut. *co* = conidia, *ct* = external cuticle, *fb* = fat body, *fgc* = foregut cuticle, *hgc* = hindgut cuticle, *hy* = hyphae, *mel* = melanization, *mep* = midgut epithelium, *mt* = malpighian tubule, *ms* = muscle, *pt* = protozoan, *sg* = salivary gland, * = gut lumen. The scale bars represent 50 µm.

faster than the gut lumen, indicating that the antimicrobial compounds contained in the salivary glands may have a limited activity inside the digestive tract, or that the fungistatic activity of the gut is due to a range of various compounds.

Finally, we observed in the midgut of a *K. flavicollis* specimen the presence of a fragment of external cuticle with a cellular encapsulation (Fig. 1F), as described by Chouvenc et al. (2009b). This observation suggests that the termite performed

necrophagy on a dead nestmate that has been previously infected by *M. anisopliae*, and that the passage through the gut lumen did not allow this ingested fungal particles to grow while in the alimentary tract. Therefore, cannibalism of an infected cadaver may aid in preventing *M. anisopliae* from completing its life cycle in a termite group. Grooming and necrophagy in association with gut antifungal activity provides an environment that reduces the chances for the fungus to produce an epizootic into the termite nest. This mechanism appeared to be conserved in all tested species from 5 different families.

Summary

We previously showed that in the subterranean termite *Reticulitermes flavipes* conidia of *Metarhizium anisopliae* did not germinate in the alimentary tract of living termites and the antifungal activity remained for 2 d *post mortem* in the gut. We have confirmed that this mechanism is common throughout the termite phylogeny by extending our observations to 5 additional species, from 5 different families. Grooming and ingestion of microorganisms from the cuticle of nestmate was found to be consistent in all the species and none of the conidia found in the alimentary tracts germinated.

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