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Variable sensitivity of fungi and bacteria to compounds produced by the metapleural glands of leaf-cutting ants

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Summary. Ants are the only group of insects that have metapleural glands. Secretions of these exocrine glands are known to have antibiotic properties and have been hypothesised to function as a general defence against microbial and fungal infections. Such defences are likely to be particularly important in leaf-cutting ants that need to protect both themselves and their clonal mutualistic fungus against pathogens.

The metapleural gland of the leaf-cutting ant *Acromyrmex octospinosus* produces an array of organic compounds (Ortius-Lechner et al., 2000), suggesting that different compounds may be effective against different kinds of infections. Here we provide a detailed analysis of the sensitivity of two species of bacteria and seven species of fungi (including the mutualistic fungus) to these metapleural gland compounds, grouped in seven classes: acetic acid, short chain acids, medium chain acids, long chain acids, indoleacetic acid, γ-lactones and γ-ketoacids.

All classes of compounds inhibited the growth of at least some of the tested micro-organisms. Cluster analysis produced four groups of micro-organisms differing in their overall sensitivity. Among-cluster differences explained a major part of the total variation in sensitivity (MANOVA), although differences between micro-organisms within clusters were also significant. Fungal hyphae and fungal spores never clustered together, indicating that defence mechanisms against these fungal life stages are fundamentally different. The mutualistic fungus was sensitive to all classes of compounds, which suggests that defence via metapleural gland secretion is under constraint when the protection of the fungus garden is concerned.

Introduction

The continuous interactions between numerous highly related individuals in colonies of social insects facilitate the spread of infectious diseases (Baer and Schmid-Hempel, 1999; Schmid-Hempel, 1998). Most ant species nest in the soil, i.e. in a suitable habitat for free-living micro-organisms that may be infective. One of the exclusive defence mechanisms common to almost all ants is a pair of exocrine glands at the posterolateral end of the mesosoma. These metapleural glands produce secretions that flow out over the body surface where they exert anti-bacterial (Maschwitz et al., 1970; Veal et al., 1992) and anti-fungal (Beattie et al., 1985, 1986; Maschwitz et al., 1970; Nascimento et al., 1996; Schildknecht and Koob, 1971; Poulsen et al., 2002b) properties and from where they possibly spread to other individuals in the nest or to the nest environment (Maschwitz et al., 1970). Metapleural gland secretions are therefore considered to be important for keeping ant colonies free from infections with harmful micro-organisms (Hölldobler and Wilson, 1990, Maschwitz et al., 1970; Poulsen et al., 2002b).

In leaf-cutting ants (Attini, Formicidae) the antibiotic substances produced by the metapleural (and probably other) glands may be of particular significance, because these ants also need to protect a mutualistic fungus against parasites

and competitors (North et al., 1997). Leaf-cutting ants culti-
vate this fungus, the anamorph of which has been named
Leucoagaricus gongylophorus (Möller) Singer (Agaricales:
Basidiomycota) (Chapela et al., 1994; Kreisel, 1972) in
underground nest chambers, where they provide it with leaf
fragments for nutrition and with a stable and non-competitive
habitat. In return the fungus is the major source of nutrition
for the ants, who can not survive without it (Cherrett et al.,
1989; Weber, 1966).

The suggestion that the metapleural gland may be of par-
ticular significance for the fungus garden comes from the
observation that small workers (body length ca. 2 mm),
which are mostly active on the fungus garden, have relative-
ly large metapleural glands compared to large workers (body
length ca 8 mm), which in the course of their life-time
become increasingly involved in tasks outside the nest such
as foraging (Bot and Boomsma, 1996; Bot et al., 2001a; Wet-
terer, 1999; Wilson, 1980). A recent study has estimated that
minor workers have almost seven times as many metapleural
gland cells per unit of biomass as major workers (Bot et al.,
2001b, Poulsen et al., 2002a).

Leaf-cutting ant colonies have to deal with a large
variety of potentially harmful micro-organisms, some of
which may affect the ants (Hölldobler and Wilson, 1990;
Kermarrec et al., 1986), while others occur in the fungus gar-
den (Craven et al., 1970; Currie et al., 1999a; Fisher et al.,
1996; Kreisel, 1972) and may significantly reduce its pro-
ductivity (Currie et al., 1999a). A general chemical defence
mechanism that inhibits the growth of many different types
of micro-organisms is therefore beneficial for leaf-cutting
ant colonies. The chemical compounds β-hydroxydecanoic
acid and indoleacetic acid have been identified in earlier
studies of the metapleural gland secretion of *Acromyrmex
octopsinosus* (Maschwitz et al., 1970; Nascimento et al.,
1996), and a recent investigation of the chemical composi-
tion of the metapleural gland secretion, by a newly designed
method to analyse highly polar compounds (Maile et al.,
1998), has revealed about 20 additional major compounds
(Ortius-Lechner et al., 2000). These are carbonic com-
pounds, the protein content of the gland has not yet been
investigated. Many of these compounds (short-, medium-
and long chain carboxylic acids, indoleacetic acid) are
known to have general antibiotic, fungistatic and/or fungicid-
al properties (Ortius-Lechner et al., 2000; citations therein).
Lactones and keto-acids, the latter of which form a fairly
large proportion of the metapleural gland secretion (Ortius-
Lechner et al., 2000), have so far not been directly investi-
gated for antibiotic properties. The aim of the present paper
is to investigate to what extent the different classes of
compounds, secreted by the metapleural gland, vary in their
effectiveness against different types and different life
stages of common saprophytic or parasitic soil micro-organ-
isms that may impose problems for attine ants and their
mutualistic fungus.

Materials and methods

Micro-organisms

The various species of bacteria and fungi that were used for this study
are listed in Table 1. General soil bacteria were represented by *Bacillus
subtilis*, a Gram positive coccus and *Pseudomonas stutzeri*, a Gram neg-
ative rod. The entomopathogenic fungi *Metarhizium anisopliae* and
Beauveria bassiana, which have previously been isolated from *Atta*
leaf-cutting ants (Diehl-Fleig et al., 1992; Humber, 1992), are well
known as possible agents for biological control of insect pests (Boucias
and Pendland, 1998). All these fungi and bacteria are common in the soil
micro-habitat where leaf-cutting ants live and are therefore considered
biologically relevant. We also selected two commensal fungi, *Gliocla-
dium virens* and *Aspergillus niger*, because spores of these fungi can be
expected to germinate in fungus gardens where they may thrive as
weeds and may reduce colony productivity. The last category that we
selected included three fungi which were directly isolated from the fun-
gus gardens of our own leaf-cutting ant cultures. The first, *Escovopsis*,
is a specialised fungal parasite that attacks the symbiotic fungus of the
ants (Currie et al., 1999a). The second, *Trichoderma* sp., is an aggres-
sive necrotrophic fungal parasite that is readily isolated from the Attine
habitat, but does not seem to have a clear effect on colony fitness (Cur-
rie et al., 1999a; A.N.M. Bot, unpubl. data). These two fungi were iso-
lated from field colonies of *Acromyrmex octospinosus* just after collec-
tion in Gamboa, Panama in 1998 (Currie et al., 1999a). The third fun-
gus, the mutualistic symbiont of the ants *Leucoagaricus gongylophorus*
(Möller) Singer, was isolated in 1998 according to a technique describ-
ed by Currie et al. (1999a). It originated from a laboratory colony of
Acromyrmex octospinosus collected in 1996 from the same population
in Gamboa and had been kept in the laboratory since.

Escovopsis and *Trichoderma* sp. were obtained from C. Currie and
we isolated the mutualistic symbiont according to Currie et al. (1999a).
All other micro-organisms were purchased from the Deutsche Samm-
lung von Mikroorganismen und Zellkulturen GmbH (see Table 1 for
strain numbers). We selected strains that as much as possible shared the
known optimal growth conditions of the mutualistic fungus (pH ca. 5.00,
temp. 25 °C; Powell and Stradling, 1986). Tests were performed on spore
germination as well as on hyphal growth. The mutualistic fungus does
not produce spores, so that only the sensitivity of the hyphae was tested.

Experimental conditions and tests

Bacteria and fungal hyphae were cultured in 100 ml liquid medium
before they were inoculated and reared on agar plates. We know
(approximately) which quantities of the different compounds are pre-
sent in the secretion behind the bulla of the metapleural gland (Ortius-
Lechner et al., 2000), but not the concentrations of these compounds in
the fungus garden. Local application of compounds on solid agar cul-
tures was thus considered to be the most accurate way to test whether
biologically relevant quantities of these compounds had a measurable
effect. For most species nutrient broth (NB, Difco) was used as liquid
culture medium, but *Escovopsis* and the symbiotic fungus grew better
on potato dextrose broth (PDB, Difco). The liquid cultures of the bacte-
ria and the symbiotic fungus were kept in an incubator at 30 °C. All oth-
er fungal cultures were kept in a 25 °C climate room (see table 1). Fun-
gal spores were obtained from solid medium cultures. The spores were
suspended in sterilised water with Tween 20 for enhanced dispersal
(1 µl in 50 ml). Spore suspensions were diluted until they contained
about 100000 spores per ml.

Bacteria, hyphae or spore suspensions (1 ml) were spread out over
the surface of a sterile potato dextrose agar (PDA, Difco) plate with a
pH of 5.6. The plates were subsequently divided into eight equal seg-
ments and a single class of metapleural gland compounds was applied
in the centre of each of these segments. The eighth segment was used for
the total gland suspension, extracted directly from the metapleural
gland. The substance groups were always applied to the plates in the
same order. Of the 20 known major compounds in the metapleural gland

Table 1. Culture medium for bacteria and fungal hyphae or the dilution agent for fungal spores. NB = Nutrient broth; PDB = Potato Dextrose Broth; PDA = Potato Dextrose Agar; H_2O+ = distilled water + TWEEN 20. Incubation time is the time between adding the metapleural gland compounds to the inoculated plates and scoring the results. DSM culture numbers are given in brackets behind the species names

Type		Species	Medium		T (°C)		pH		Incubation time (hours)
			liquid	solid	liquid	solid	liquid	solid	
Bacteria		*Bacillus subtilis* (347)	NB	PDA	30	25	5.6	5.6	24
		Pseudomonas stutzeri (6084)	NB	PDA	30	25	5.6	5.6	48
Fungal hyphae	Insect-pathogenic	*Metarhizium anisopliae* (1490)	NB	PDA	25	25	5.6	5.6	24
		Beauveria bassiana (857)	NB	PDA	25	25	5.6	5.6	24
	Commensal	*Gliocladium virens* (1963)	NB	PDA	25	25	5.6	5.6	24
		Aspergillus niger (1957)	NB	PDA	25	25	5.6	5.6	30
	Leaf-cutting ant related	*Leucoagaricus gongylophorus*	PDB	PDA	30	25	5.6	5.6	48
		Escovopsis spec.	PDB	PDA	25	25	5.6	5.6	24
		Trichoderma spec.	NB	PDA	25	25	5.6	5.6	24
Fungal spores	Insect-pathogenic	*Metarhizium anisopliae* (1490)	H_2O+	PDA	25	25	–	5.6	24
		Beauveria bassiana (857)	H_2O+	PDA	25	25	–	5.6	24
	Commensal	*Gliocladium virens* (1963)	H_2O+	PDA	25	25	–	5.6	24
		Aspergillus niger (1957)	H_2O+	PDA	25	25	–	5.6	24
	Leaf-cutting ant related	*Escovopsis* spec.	H_2O+	PDA	25	25	–	5.6	24
		Trichoderma spec.	H_2O+	PDA	25	25	–	5.6	24

secretion of *Acromyrmex octospinosus*, 17 were purchased from Sigma-Aldrich Co. Ltd. (Gillingham, UK) or synthesised (keto-acids, for method see Ortius-Lechner et al., 2000). Subsequently they were combined into seven functional groups and diluted in the most suitable solvent (see Table 2 for concentrations). A Hamilton µl syringe™ was used for the application of the specified amounts of the compounds onto the plates. The applied quantities corresponded to the maximum amounts ever found in the metapleural gland secretion of a single worker (Ortius-Lechner et al., 2000). Plates were sealed with parafilm and left to incubate in a climate room at 25 °C. For most micro-organisms an incubation time of 24 hours was sufficient, but some slower growing species were incubated longer (Table 1). After incubation, the diameter of the halos in bacterial or fungal mats, caused by the antibiotic action of the applied compounds, were measured. Tests were replicated six times for each micro-organism, in such a way that all substance groups were tested on each of the six plates.

Analysis and statistics

To separate groups of different micro-organisms that showed a similar sensitivity pattern to the organic compounds occurring in the meta-pleural gland secretion, a cluster analysis was performed according to Wald's method (JMP). This method is based on differences in mean squares. To give a further illustration of the respective levels of response-variability at the within-cluster and among-cluster level, a multivariate analysis of variance was performed (MANOVA-SYSTAT), with the organic compounds as dependent variable and clusters and species of micro-organisms within these clusters as factors. In general, the variances in responses of micro-organisms to compounds were low and reasonably homogeneous, although some categories produced only zero responses and thus had no variance at all. However, the levels of significance obtained in the MANOVA were so high that minor deviations from the basal assumptions required for doing this type of test have no effect on the qualitative conclusions that we draw from this analysis.

Total gland suspension

The antibiotic effect of the composite intact metapleural gland secretion, isolated directly from ant workers, was tested as well. However, we were unable to quantify the effects, because it was impossible to get the highly polar metapleural gland secretion out of the glass capillaries in which

Table 2. Single compounds and mixtures (classes of compounds) known to occur in the metapleural gland secretion of *Acromyrmex octospinosus* and used for tests on various bacterial and fungal strains. Compounds were diluted in their respective solvents (hexane and water) and applied amounts were comparable to the maximum amount of the respective compounds detected in a single metapleural gland of an *Acromyrmex* worker (Ortius-Lechner et al., 2000)

Classes of compound (solvent)	Concentration in 1 µl solvent (ng)	Maximal detected quantity per worker (ng)	Amount applied (µl)
Acetic acid (Hexane)	100	2000	20
Short chain acids (Hexane) (C3, C5 and C6)	100	1500	15
Medium chain acids (Hexane) (C8, C9, C10 and C12)	100	250	2.5
Long chain acids (Hexane) (C14, C15 and C16)	100	1500	15
Indoleacetic acid (Water)	500	6000	6
Lactones (Hexane) (γ-Butyro-, Octa- and Decalactone)	100	150	1.5
Keto-acids (Hexane) (4-Oxo-octanoic and -decanoic acid)	500	2000	4

it was collected for application onto the agar plates. The best result was obtained when the ants were held under water and pressure was applied on the bulla of the metapleural gland. The hydrophobic droplets could then be collected together with the surrounding water. This method did not allow the application of standardised quantities of the secretion as the extent of dilution could not be controlled. We can only say that antibiotic effects of the total metapleural gland secretion were almost invariably detected, but refrain from presenting quantitative results.

Results

The cluster analysis revealed four groups of micro-organisms (Fig. 1) with clear differences in sensitivity patterns, but within which species reacted relatively similar to the various antibiotic compounds that were tested (Fig. 2, Table 3). Two of these four groups contained fungal hyphae and two contained fungal spores. There were also marked differences between the two bacterial species. *Pseudomonas stutzeri* clustered within the most sensitive group of fungal spores, whereas *Bacillus subtilis* came out close to the most sensitive fungal hyphae, although this species showed some marked differences from the other members of this group (Fig. 2).

The sensitivity of the hyphae of certain fungi does not always correspond to that of their respective spores. The hyphae of *Escovopsis*, for example, are the least sensitive of all the fungal hyphae that were tested, but its spores cluster with the most sensitive spores. On the other hand, the hyphae of *Aspergillus niger* are sensitive, but the spores of this species are not. The two insect pathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* have both hyphae and spores clustering in the most sensitive groups. Also the two commensal fungi *Gliocladium virens* and *Trichoderma sp.* reacted similar to the spectrum of compounds. Their hyphae and spores belong to the relatively non-sensitive clusters. The

Figure 1. Wald's cluster analysis on the sensitivity data revealed four clusters: sensitive hyphae (1), sensitive spores (2), resistant spores (3) and resistant hyphae (4). Sensitivity decreases from top to bottom. Numbers and alternating open and filled symbols indicate different clusters

mutualistic symbiont of the ants clusters with the most sensitive hyphae.

Differences in the responses of species within clusters are large (MANOVA: $F_{11, 57} = 7.98$; $P = 3.10^{-8}$) (Table 3), but we will mostly concentrate on the even more significant general patterns between clusters, which explain the major part of the variation (MANOVA: $F_{3, 57} = 421.6$; $P = 7.10^{-9}$) (Fig. 2). The cluster containing the non-sensitive fungal hyphae was relatively insensitive to all of the tested compounds. The other three groups reacted similarly to the lactones and keto-acids. Rather large differences between clusters occurred when

Table 3. The effect of the chemical compounds (halo diameter in mm ± SE) on each of the tested micro-organisms. N = 6, unless stated otherwise in brackets

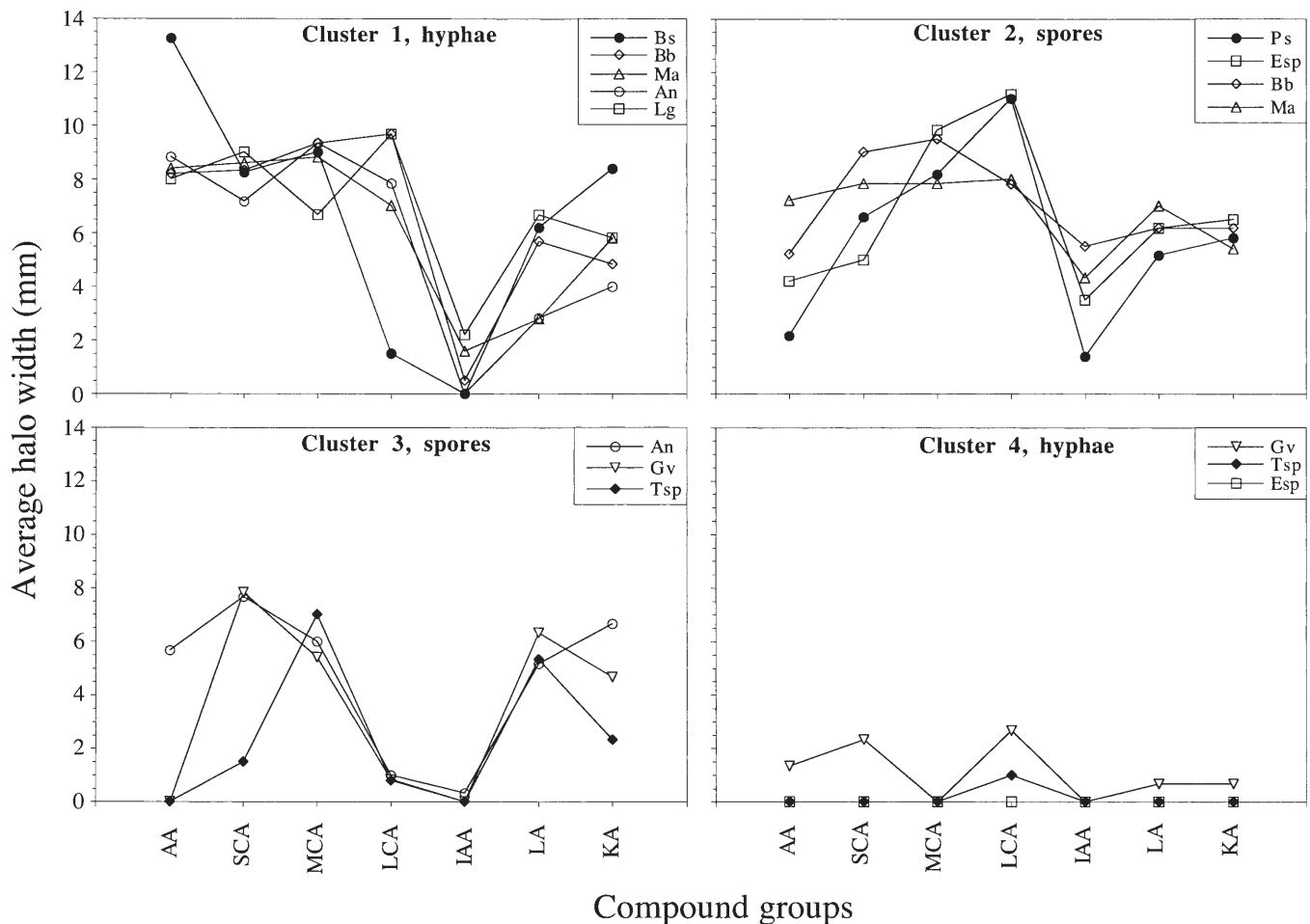|	Acetic acid	Short chain acids	Medium chain acids	Long chain acids	Indoleacetic acid	Lactones	Keto-acids
Bacillus subtilis	13.25 ± 3.5 (4)	8.25 ± 0.92 (5)	9.00 ± 0.26	1.50 ± 1.02 (5)	0.00 ± 0.00	6.20 ± 0.66	8.40 ± 1.43 (5)
Beauveria bassiana	8.20 ± 0.97 (5)	8.33 ± 0.49	9.33 ± 0.42	9.67 ± 0.33	0.50 ± 0.50 (5)	5.67 ± 0.33	4.83 ± 0.70
Leucoagaricus gongylophorus	8.00 ± 0.37	9.00 ± 0.26	6.67 ± 0.33	9.67 ± 0.49	2.20 ± 0.20 (5)	6.67 ± 0.21	5.83 ± 0.40
Metarhizium anisopliae	8.40 ± 0.75 (5)	8.60 ± 0.40 (5)	8.80 ± 1.32 (5)	7.00 ± 0.95 (5)	1.60 ± 1.03 (5)	2.80 ± 0.73 (5)	5.80 ± 0.86 (5)
Aspergillus niger	8.83 ± 0.70	7.17 ± 0.83	9.33 ± 0.67	7.83 ± 0.65	0.00 ± 0.00	2.83 ± 0.31	4.00 ± 0.58
Pseudomonas stutzeri	2.17 ± 1.05	6.60 ± 0.98 (5)	8.17 ± 0.31	11.00 ± 1.00	1.40 ± 0.60 (5)	5.17 ± 0.48	5.83 ± 0.70
Escovopsis spores	4.20 ± 0.80 (5)	5.00 ± 1.26	9.83 ± 0.70	11.17 ± 0.60	3.50 ± 0.62	6.17 ± 0.40	6.50 ± 0.43
Beauveria bassiana spores	5.20 ± 0.58 (5)	9.00 ± 0.86	9.50 ± 0.50	7.80 ± 1.24 (5)	5.50 ± 0.50	6.17 ± 0.31	6.17 ± 0.31
Metarhizium anisopliae spores	7.20 ± 1.20 (5)	7.83 ± 0.60	7.83 ± 0.17	8.00 ± 0.77 (5)	4.33 ± 0.67	7.00 ± 0.55 (5)	5.40 ± 0.93 (5)
Aspergillus niger spores	5.67 ± 0.67	7.67 ± 0.21	6.00 ± 0.82	1.00 ± 0.82	0.33 ± 0.21	5.17 ± 0.48	6.67 ± 0.42
Gliocladium virens spores	0.00 ± 0.00	7.83 ± 0.40	5.40 ± 1.29	0.83 ± 0.65	0.00 ± 0.00	6.33 ± 0.21	4.67 ± 0.33
Trichoderma sp. spores	0.00 ± 0.00	1.50 ± 0.96	7.00 ± 0.45	0.80 ± 0.80 (5)	0.00 ± 0.00	5.33 ± 0.21	2.33 ± 0.80
Gliocladium virens	1.33 ± 1.33	2.33 ± 1.48	0.00 ± 0.00 (5)	2.67 ± 1.71	0.00 ± 0.00	0.67 ± 0.67	0.67 ± 0.67
Trichoderma sp.	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.00 ± 0.63	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Escovopsis	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Figure 2. The sensitivity of each of the tested micro-organisms to the different groups of chemical compounds. Each figure represents one cluster. Micro-organisms tested: Bs = *Bacillus subtilis*; Bb = *Beauveria bassiana*; Ma = *Metarhizium anisopliae*; An = *Apergillus niger*; Lg = *Leucoagaricus gongylyphorus* (mutualistic fungus); Ps = *Pseudomonas stutzeri*; Esp = *Escovopsis* sp.; Tsp = *Trichoderma* sp.; Gv = *Gliocladium virens*. Compounds: AA = acetic acid; SCA = short chain acids; MCA = medium chain acids; LCA = long chain acids; IAA = indoleacetic acid; LA = lactones; KA = keto-acids

exposed to acetic acid and the short-, medium- and long-chain fatty acids. The two clusters of spores show the same pattern of sensitivity towards acetic acid, short chain acids and medium chain acids. The largest difference between these two groups of spores is in the sensitivity towards long chain acids, where the non-sensitive spores are least sensitive and the sensitive spores most sensitive of all clusters. The sensitive hyphae follow a reverse pattern of sensitivity from that of the spores, reacting strongly towards acetic acid and the short chain acids and being relatively insensitive towards the long chain acids.

Discussion

Overall patterns of sensitivity

Different micro-organisms reacted differently to each of the chemical compounds or classes of compounds that are produced by the metapleural gland of *Acromyrmex* leaf-cutting

ants. Four broad groups of micro-organisms can be distinguished based on differences in their overall sensitivity patterns. These are two groups of hyphae and two groups of spores. Spores and hyphae from the same fungus species reacted differently to the (classes of) organic compounds, but spores of different species were often similar in their reaction patterns and the same holds true for hyphae.

The variation in sensitivity between different micro-organisms or between the life stages of the same micro-organism implies that the ants may be able to target a specific group of potential pathogens by adjusting the relative concentrations of their metapleural gland compounds. This type of context-dependent expression of a generalised defence mechanism would most likely occur across workers of different caste or age.

Workers that are on the fungus garden are exposed to different micro-organisms than workers that go out foraging and it is the latter category that may pick up new infections and bring them to the fungus garden. It is also the foragers that are most likely to come in contact with insect pathogenic

fungi. On the other hand, workers that are on the fungus garden have to control contaminants that come in with the collected leaf material either before of after germination, and they should achieve these tasks without harming the mutualistic fungus. Large *Acromyrmex* workers start their lives working on the fungus garden, but gradually move on to tasks outside the nest (Hölldobler and Wilson, 1990; Wilson, 1980). Whether the ants can regulate the expression of the general metapleural gland defence system to optimally meet caste specific and age specific challenges is subject to further investigation (D. Ortius-Lechner, R. Maile, H.C. Petersen and J.J. Boomsma, in prep.).

Specific sensitivity reactions of the tested micro-organisms

The mutualistic fungus belongs to the most sensitive group of fungal hyphae (Fig. 1), but our results on this may not be entirely conclusive, because the mutualist probably has the largest disadvantage when grown on PDA, whereas it has the largest advantage when grown by the ants in the fungus garden. However, we can not exclude that concentrations of metapleural gland compounds that exceed specific threshold values may be damaging for the mutualistic fungus. Further research on the specific interaction between metapleural gland secretion and maintenance of the fungus garden will be needed to clarify this point.

The hyphae of the non-mutualistic fungus *Escovopsis* were unaffected by the metapleural gland compounds. *Escovopsis* is a specialised parasite of ant fungus gardens and has a distinct negative effect on the fitness of a colony (Currie et al., 1999a). Occasionally, *Escovopsis* can completely overgrow fungus gardens and cause the death of a colony (Currie et al., 1999a). The insensitivity of this fungal parasite might be a specific adaptation to evade the general defence mechanism of the ants. However, this is not necessarily the case, because other fungi (*Trichoderma* sp. and *Gliocladium virens*) which are not adapted to living in fungus gardens and are unlikely to be adapted to the metapleural gland compounds, are equally insensitive. A more likely explanation is, therefore, that the ants do not need the metapleural gland secretion to be effective, because they maintain a separate defence mechanism against *Escovopsis* in the form of *Actinomycete* bacteria that grow on their cuticle and produce specific antibiotic substances that reduce the growth of *Escovopsis* hyphae (Currie et al., 1999b; 2002).

Insensitivity of some fungi can potentially be explained by the way in which they grow. All insensitive fungi tested have very long and fast growing hyphae, which are not dependent on the local growth patch for nutrition, whereas most of the sensitive fungi spread more slowly over the medium. *Aspergillus niger*, however, grows rapidly. Its spores belong to the least sensitive ones, but its hyphae seem to actively avoid high concentrations of metapleural gland substances.

Two more fungal species with sensitive hyphae are the entomopathogenic fungi *Metarhizium anisopliae* and *Beau-*veria bassiana*. However, as was earlier suggested by Beattie et al. (1986), the suppression of hyphal growth of these fungi is probably of little importance as a direct defence mechanism, because the hyphae grow in the hemolymph of the ants, out of reach of the metapleural gland substances. When these fungi manage to grow hyphae, other defence mechanisms, such as the innate immune system (Boucias and Pendland, 1998) or inducible antibiotic peptides in the haemolymph (Mackintosh et al., 1998) are decisive for survival of the host. However, the metapleural gland substances may reduce the quantities of free living hyphae of entomopathogentic fungi in the ant habitat (Hajek and Leger, 1994) and they are obviously of high significance for the inhibition of spore germination of these fungi. We conclude that the only contaminant fungal hyphae suppressed by the metapleural gland compounds are those of *Aspergillus niger* and that the metapleural gland compounds may thus generally be of more importance for the protection against fungal spores and bacteria than for the growth suppression of fungal hyphae.

Function of the specific classes of metapleural gland compounds

This study presents the first evidence for antibiotic properties of γ-keto-acids. Even though the quantities that are found in individual leaf-cutting ants are small, a clear and strong antibiotic effect of these substances was detected for all fungal spores and for the cluster with the most sensitive hyphae. The group of sensitive fungal spores is particularly sensitive with respect to indoleacetic acid, confirming previous investigations by do Nascimento et al. (1996). Since the group of sensitive spore micro-organisms includes the insect pathogenic fungi and since the mutualistic fungus is also relatively sensitive to indoleacetic acid, this compound may be of most importance for defences away from the fungus garden.

The reaction of micro-organisms to acetic acid and fatty acids is highly variable. This may mean that there is potential for differential and context specific use of these compounds. It is also possible that the antibiotic properties of these acids are just a secondary effect and that they primarily have an additive role in the antibiotic function of other compounds. A combined function of all the acids in the secretion may be to maintain a low pH in the fungus garden. Kreisel (1972) showed that a few days after all ants are removed from the fungus garden, the pH gradually increases and the garden is quickly overgrown by opportunistic fungi and bacteria. Acetic acid and fatty acids are general metabolites of many micro-organisms and most *Bacillus* species, including *Bacillus subtilis*, can break down acetic acid to CO_2 and H_2O (Hanson et al., 1963; 1964). The relatively strong sensitivity of the bacteria to this compound is therefore somewhat surprising. A large part of the antibiotic effect of acetic acid and fatty acids in our tests is thus likely to be due to the general acidic conditions that they help to maintain.

Anti-bacterial defence and quorum sensing

Various growth and virulence aspects of bacteria are cell-density dependent and are regulated by between-cell signalling with low molecular weight molecules. This type of regulation is called quorum sensing. Gram negative bacteria often use acylated homoserine lactones for between-cell signalling, whereas gram positive bacteria mostly use peptides (Dunny and Leaonard, 1997; Dunny and Winans, 1999). Some of the compounds that have been identified in the metapleural gland substances could possibly be involved in interfering with this form of bacterial communication. γ-Butyro-lactones are known to be involved in quorum sensing in *Streptomycete* bacteria where they induce the production of secondary compounds (Horinouchi, 1999). The octo- and deca-lactones that are produced by the ants are similar to the homoserine lactones that are involved in quorum sensing in many gram-negative bacteria, but they lack an amino group that makes the original signalling molecules hydrophilic and thus suitable as diffusible signalling molecules. The octo- and deca-lactones that are produced by the ants may therefore not be as effective in interfering with bacterial communication, but they may also perform better on a cuticular wax layer. In addition to the lactones, the metapleural glands are known to produce proteins, of which the identity is yet unknown (Nascimento et al., 1996), but which may interfere with quorum sensing in gram positive bacteria.

In our tests the ant-produced lactones are not specifically inhibiting the growth of gram negative bacteria. They prevented the germination of all fungal spores and the growth of fungal hyphae, gram negative and gram-positive bacteria. Because of this lack of specificity no conclusions can be drawn about the exact function of the lactones and we can thus not reject the hypothesis that the ant-produced lactones are just general antibiotic compounds that have nothing to do with quorum sensing. Leaf-cutting ants are not the only insects that use lactones as an antiseptic as halictid bees have been recorded to use lactones in order to preserve pollen stores (Blum, 1981).

In general, the role of bacteria in the ant-fungus mutualism has not been intensively studied, but other systems are known where eukaryotic organisms protect themselves against bacteria by producing secondary compounds that interfere with the quorum sensing system of their pathogens (Kjellerberg et al., 1997). The identification of specific bacterial symbionts additional to the mutualistic *Streptomycete* bacteria (Currie et al., 1999b; C.R. Currie, A.N.M. Bot and J.J. Boomsma, submitted) will help in determining the exact role of ant-produced compounds against bacteria.

The overall significance of the metapleural gland

The metapleural gland is not the only form of defence against micro-organisms that leaf-cutting ants possess. Other glands, like the mandibular gland, may contribute additional antibiotic defences (North et al., 1997) and there is also some evidence for defensive abilities of the mutualistic fungus itself (Hervey and Nair, 1979; Nair and Hervey, 1978). In addition, as already mentioned above, *Streptomycete* bacteria that live on the cuticle of the ants produce substances that specifically reduce the growth of the parasitic fungus *Escovopsis* (Currie et al., 1999b; C.R. Currie, A.N.M. Bot and J.J. Boomsma, submitted). Finally, the ants have advanced hygienic behaviours (Bot et al., 2001), which they are able to adapt to the presence or absence of *Escovopsis* (Hart, Bot and Brown, 2002). In spite of these other possible defence mechanisms, our present results confirm that the metapleural gland is a powerful general and potentially flexible base-line defence against microbial challenges of the leaf-cutting ant-fungus system. It is a defence system unique for and shared by all ants, except for a few isolated cases in which the gland was secondary lost (Hölldobler and Engel-Siegel, 1984). The results obtained by Ortius-Lechner et al. (2000), Poulsen et al. (2002b) and in the present study clearly show that the metapleural gland defence in *Acromyrmex* leaf-cutting ants is both elaborate and effective.

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