Antiamoebins, myrocin B and the basis of antifungal antibiosis in the coprophilous fungus *Stilbella erythrocephala* (syn. *S. fimetaria*)

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Abstract

Antiamoebins I, III and XVI as well as several others in minor amounts were produced by four strains of the coprophilous fungus *Stilbella erythrocephala* (syn. *S. fimetaria*) in its natural substrate and in liquid culture. The total antiamoebin concentration in dung was 126–624 $\mu g\,g^{-1}$ fresh weight, with minimum inhibitory concentrations against most other coprophilous fungi being at or below 100 $\mu g\,mL^{-1}$. Myrocin B, not previously described from *S. erythrocephala*, was also produced, but only at low, nonfungicidal levels ($< 5.3\,\mu g\,g^{-1}$). No other antifungal substances were detected. It is concluded that antiamoebins are responsible for antibiosis in dung colonized by *S. erythrocephala*.

Introduction

The dung of herbivores is colonized by a rich diversity of fungi, many of which show specific adaptations to their habitat in the form of long-range spore discharge, the ability to withstand passage through the digestive tract of herbivores, and a high degree of competitiveness against other fungi (Webster, 1970). Prominent examples of antagonistic interactions between coprophilous fungi are biotrophic or necrotrophic mycoparasitism, and inhibition upon hyphal contact (Dix & Webster, 1995). Another possible strategy is inhibition by antibiosis, i.e. the secretion of antifungal substances by a fungus in order to keep potential competitors at bay. So far, the concept of antibiosis in coprophilous fungi has been based on indirect evidence provided by their ability to synthesize biologically active secondary metabolites in pure culture (Gloer, 1995), sometimes accompanied by ecological observations in which the fungus in question displaced or excluded other species from its colonized dung patch. Unfortunately, data on the production of active metabolites in dung are sparse. To our knowledge, only one report on this topic has been published, in which the concentrations of antifungal sordarins in dung were shown to be insufficient to account for the observed competitive advantage of its producer, *Podospora pleiospora*, over other coprophilous fungi (Weber *et al.*, 2005).

The classical case of a coprophilous fungus assumed to defend its substrate by antibiosis is *Stilbella erythrocephala*, which is sometimes synonymized with *S. fimetaria* (Seifert *et al.*, 1983; Seifert, 1985). This is an asexual (conidial) species which colonizes individual rabbit pellets and is conspicuous in its exclusion of other fungi (Fig. 1). Pure cultures of *S. erythrocephala* have been shown to produce the antifungal antiamoebins, a group of peptaibol-type compounds (Sasaki *et al.*, 1971; Singh & Webster, 1973; Jaworski & Brückner, 2000). Peptaibols are linear oligopeptides which self-assemble into oligomers forming pores or channels across the plasma membrane of their target organisms (Chugh & Wallace, 2001). They are produced as families of closely related peptides differing in only one or a few amino acids, and often showing similar biological activities (Goulard *et al.*, 1995). Typically, peptaibols have a wide spectrum of activity against bacteria, fungi, nematodes and mammalian cells (for references, see Degenkolb *et al.*, 2003).

In the work reported here, we have quantified the production of the antifungal metabolites myrocin B and antiamoebins (Fig. 2) by *S. erythrocephala* in pure culture and in artificially inoculated as well as naturally colonized

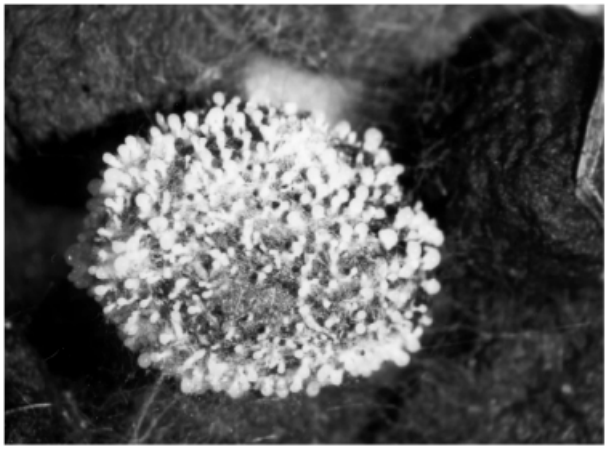


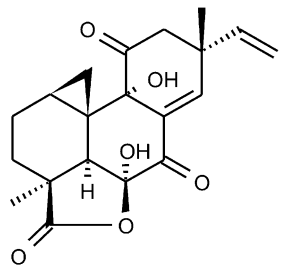
Fig. 1. Exclusive colonization behaviour of *Stilbella erythrocephala* on rabbit pellets collected from the field (Exeter, UK). The pellet showing a dense stand of synnemata of *S. erythrocephala* has remained uncolonized by other fungi.

dung. The suggestion that this fungus uses antibiosis as a strategy for habitat conquest is discussed in the light of the activity of antiamoebins, at concentrations measured in colonized dung, against a wide range of coprophilous fungi.

Materials and methods

Producing organisms

Four strains of *Stilbella erythrocephala* were isolated from dung samples by streaking out conidial drops produced from synnemata onto 2% malt extract agar augmented with 200 mg L^{-1} each of penicillin G and streptomycin sulphate. Strain D99026 was isolated from rabbit dung (Stonebarrow, Devon, UK), D01024 from rabbit dung (Concepción, Chile), D03001 from rabbit dung (Exeter, Devon, UK), and D03012 from the dung of a tortoise (*Testudo hermanni*) kept outdoors as a pet (Winnweiler, Pfalz, Germany). Pure sporulating cultures of all strains are maintained in the Culture Collection of the Department of Biotechnology, University of Kaiserslautern. In order to confirm species identification, the ITS1-5.8S-ITS2 sequences of the ribosomal RNA gene cluster of the four strains were compared. DNA amplification by PCR using the primer pair ITS5 and ITS4 (White *et al.*, 1990), DNA purification, and sequencing were carried out as described previously (Köpcke *et al.*, 2002; Schwarz *et al.*, 2004).



Antiamoebins

I CH_3CO-Phe Aib Aib Aib D-Iva Gly Leu Aib Aib Hyp Gln D-Iva Hyp Aib Pro Phe-OH

III CH_3CO-Phe Aib Aib Aib Aib Gly Leu Aib Aib Hyp Gln D-Iva Hyp Aib Pro Phe-OH

XVI CH_3CO-Leu Aib Aib Aib D-Iva Gly Leu Aib Aib Hyp Gln D-Iva Hyp Aib Pro Phe-OH

Fig. 2. Structures of myrocin B and antiamoebins I, III and XVI.

Cultivation of *S. erythrocephala*

Stilbella erythrocephala was cultivated in a range of liquid media, with the highest antifungal activity obtained in 5 L shaken flasks (120 r.p.m., 22 °C) containing 2 L double-strength (4%) malt extract. Culture samples (100 mL) of all four strains were taken daily, and the mycelium was separated by filtration. The culture fluid was extracted with 100 mL ethyl acetate, and the mycelium with 100 mL ethanol/acetone (1 : 1, v/v). After evaporation to dryness under reduced pressure, the residues were redissolved in methanol to give 10 mg mL^{-1}, and 10 μL aliquots on filter paper disks (6 mm diameter) were tested for antifungal activity by incubation for 24 h at 37 °C on YM agar plates (4 g yeast extract, 4 g glucose, 10 g malt extract, 20 g agar, 1 L tap water) containing cell suspensions of *Nematospora coryli* ATCC10647 (Anke *et al.*, 1989), and by analytical methods described below. The concentrations of glucose and maltose were determined enzymatically (Anon, 1989). All reagents were from Roche Diagnostics (Mannheim, Germany) except for α-glucosidase and triethanolamine (both from Sigma, St Louis, MO, USA).

To examine the effect of potential competitors on the production of antifungal substances in liquid culture, cells of the yeast *Sporobolomyces roseus* D99040 (Basidiomycota) or spores of the coprophilous filamentous fungi *Mucor mucedo* D99041 (Zygomycota) or *Penicillium claviforme* D99028 (Ascomycota) were added to 5-day-old cultures of *S. erythrocephala* D99026 at a final concentration of 5×10^5 mL^{-1}. Samples were taken as above. For an examination of metabolite production in the natural substrate, batches of 50 sterilized rabbit pellets were briefly rolled over the surface of a sporulating agar plate, and incubated for 14 days on moist sea sand. In addition, naturally colonized pellets were collected from the Exeter site. All dung samples were lyophilized and extracted in 100 mL methanol/acetone (1 : 1, v/v) for 3 h, followed by evaporation of the solvents and re-extraction of the residue in water and ethyl acetate (50 mL each). The resulting organic phase was re-evaporated, and the crude extract thus obtained was dissolved in acetonitrile.

Purification and identification of antifungal metabolites

The filtrate (7.75 L) of a 9-day-old culture of *S. erythrocephala* D99026 was extracted with an equal volume of ethyl acetate, and the crude extract obtained (2.2 g) was separated by silica gel column chromatography (150 × 25 mm; Kieselgel 60, particle size 63–200 μm; Merck, Darmstadt, Germany). A fraction enriched in myrocin B (231 mg) was eluted with cyclohexane/ethyl acetate (1 : 1). Final purification of myrocin B was achieved with a Jasco modular HPLC system consisting of two pumps (PU-1586), the multiwavelength detector UV-1570 M and a Hibar LiChrosorb RP-18 column (250 × 25 mm, 7 μm particle size; Merck). Using isocratic conditions (water/methanol 1 : 1), 8.4 mg myrocin B eluted at 12.5 min at a flow rate of 30 mL min^{-1} at 22 °C (method 1). Because of its instability in methanol, pure myrocin B was kept dry or dissolved in acetonitrile from this time-point onwards.

From a 10 mg subsample of the antiamoebin-enriched fraction (58 mg) eluting from the silica gel column in ethyl acetate/methanol (1 : 1), antiamoebins I (2.2 mg) and XVI (0.5 mg) were purified with an Agilent Series 1100 HPLC instrument fitted with a Zorbax Eclipse XDB C8 column (150 × 4.6 mm; 5 μm particle size; Agilent, Waldbronn, Germany). Good semipreparative separation of the major antiamoebin peaks was obtained at a flow rate of 3 mL min^{-1} in the isocratic mode with 0.1% aqueous formic acid (60%) and acetonitrile (40%) at 50 °C (method 2). Similar chromatographic conditions were used to generate samples for quadrupole time-of-flight (Q-TOF) analysis, except for a reduction of the flow rate to 1 mL min^{-1} and of the temperature to 40 °C, to obtain an improved separation of the minor antiamoebin peaks. Fractions (250 μL) were collected in a 96-well microtitre plate (Sarstedt, Nümbrecht, Germany) (method 3).

The mass and UV spectra of myrocin B and antiamoebins were analyzed with a Hewlett-Packard Series 1100LC-MSD instrument fitted with a Zorbax Eclipse XDB C8 column (100 × 3 mm; 3.5 μm particle size; Agilent), and run in the isocratic mode at 63% aqueous solvent (0.1% formic acid) and 37% acetonitrile, at 33 °C and a flow rate of 0.6 mL min^{-1}. Atmospheric pressure-electrospray ionization (API-ES) was applied at a fragmentor voltage of 140 V, capillary voltages of 4600 and 5200 V in the ionization-positive and -negative modes (respectively), a nebulizer pressure of 60 psi and a drying gas flow of 12 L min^{-1} at 350 °C (method 4).

The IR spectrum of myrocin B was recorded with a Perkin Elmer One spectrometer (Perkin Elmer, Beaconsfield, UK). For ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectroscopy, a Bruker DRX500 spectrometer (Bruker, Karlsruhe, Germany) with an inverse multinuclear 5 mm probehead equipped with a shielded gradient coil was used. Spectra were recorded in $CDCl_3$. ^{1}H-^{1}H COSY (chemical shift correlated spectroscopy), HMQC (heteronuclear multiple quantum correlation) and HMBC (heteronuclear multiple bond correlation) experiments were recorded with gradient enhancements using sine-shaped gradient pulses.

In order to characterize antiamoebins, samples collected in a 96-well plate as described above were dried and dissolved in 20–50 μL 0.1% formic acid/methanol (1 : 1, v/v). Samples from individual wells were loaded into nano electrospray needles and measured with a Micromass Q-TOF2 mass spectrometer at a source temperature of 60 °C, an ESI capillary voltage of 800 V, a cone voltage of 45 V and a variable collision energy (5–50 eV). Molecular ions and singly charged fragments obtained from in-source fragmentation were used for elucidation of antiamoebin sequences (Suwan *et al.*, 2000).

Quantification of myrocin B and antiamoebins

Crude dung extracts and supernatant fluid extracts of liquid cultures obtained from triplicate samples were adjusted to a concentration of 5 mg mL^{-1} and analyzed for the presence of myrocin B and antiamoebins with the 1100LC-MSD instrument as described above, but fitted with a Zorbax Eclipse XDB C8 column (Agilent; 50 × 2.1 mm; 3.5 μm particle size). For myrocin B, isocratic separation was achieved with 0.1% aqueous formic acid (75%) and acetonitrile (25%) at a flow rate of 0.6 mL min^{-1} at 50 °C, scanning for qualifier signals at *m/z* 313.2 and 357.1 (signal 1, negative ionization), and using the positive ionization signal 2 at *m/z* 341.1 for quantification (method 5). Extracts were measured separately for antiamoebins, eluting with 0.1% aqueous formic acid (63%) and acetonitrile (37%) at 0.6 mL min^{-1} and 50 °C, and using the signals at *m/z* 1641.0, 1655.0 and 1669.0 (signal 3, negative ionization) and 1607.0, 1621.0 and 1635.0 (signal 4, negative ionization) as quantification signals (method 6). Calibration curves were established with purified myrocin B and antiamoebins I and XVI. The latter two produced identical calibration curves under our conditions, indicating ionization signals of similar strength as expected of such similar compounds. Therefore, other antiamoebins in our extracts were quantified against the calibration curves established for antiamoebins I (signal 3) and XVI (signal 4), correcting for elution time-dependent peak broadening.

Antifungal activities of myrocin B and antiamoebins

The effect of pure myrocin B, antiamoebin I and the antiamoebin-enriched silica gel fraction on the germination and growth of coprophilous fungi and a range of yeasts and

bacteria (see Table 2) was determined as the minimum inhibitory concentration (MIC) in 96-well microtitre plates (see Weber *et al.*, 2005). Additionally, the effect of a combination of myrocin B and either antiamoebin I or the antiamoebin mixture, each at half its MIC, was tested with the same test organisms. To each well, the appropriate amount of myrocin B and/or antiamoebins was added, followed by evaporation of the solvent and addition of 200 μL of a cell or spore suspension at a density of 10^5 propagules per mL. *Chaetomium bostrychodes*, *Podospora pleiospora* and *Thelebolus nanus* were prepared as mycelial suspensions. Coprophilous fungi were suspended in dung extract prepared by soaking 125 g air-dry cow dung in 1 L distilled water followed by filtration and autoclaving. Other fungi were prepared in liquid YM medium and bacteria in NB medium containing 8 g nutrient broth (Oxoid, Basingstoke, UK) and 5 g NaCl L^{-1} tap water.

Results

Identification of the producing strains

All four isolates were identified as *Stilbella erythrocephala* on the basis of their white synnematal stalk bearing an orange-coloured conidial drop (Seifert *et al.*, 1983; Ellis & Ellis, 1998). Conidia were ovoid to ellipsoid, smooth-walled, hyaline, and measured 3.5–5.5 × 2–3 μm. Species identity of our four isolates was confirmed by a comparison of their ITS1-5.8S-ITS2 sequences, all of which comprised 604 nt (including the primers ITS5 and ITS4) and were identical except for that of D03012, which showed a single base change (A → T) at position 526. A preliminary examination of the overall metabolite spectra in crude culture filtrate extracts also indicated the close similarity of all four strains (N.-A. Lehr, unpublished results), as did their production of the same types of antifungal metabolites. The complete ITS sequence of strain D99026 has been deposited in GenBank (accession number AY952467).

Identity of antifungal metabolites

Liquid cultures of *S. erythrocephala* grown in 4% malt extract showed a high antifungal activity in the supernatant fluid, whereas mycelial samples had little or no activity. The secretion of antifungal metabolites into the culture fluid correlated with the onset of the stationary growth phase and the disappearance of glucose and maltose from the medium (Fig. 3). The fermentation curves were similar in all four strains (not shown). A complex mixture of antiamoebins was produced (Fig. 4), and these were identified (in order of decreasing concentration) as antiamoebins I > III > XVI (see Fig. 2) > others (IV, VI, VIII, XII, XIII, XIV, XV). Although the technique employed could not distinguish

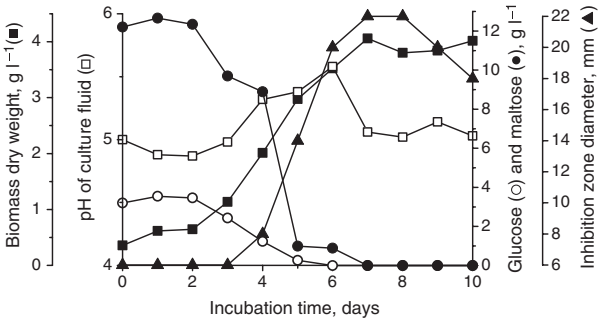


Fig. 3. Representative fermentation curve of *Stilbella erythrocephala* D99026 in shaking-flask culture in 4% malt extract. The levels of glucose and maltose, pH of the culture fluid, biomass dry weight and inhibition zone diameter of 10 μg crude extract against *N. coryli* are shown.

between isobaric amino acids (e.g. leucine/isoleucine, valine/isovaline) or chirality (D and L forms), we assigned these features on the basis of the detailed study published previously for antiamoebins from the same species (Jaworski & Brückner, 2000). Traces of several new forms, all representing minor variations at positions known to be microheterogeneous in antiamoebins (Jaworski & Brückner, 2000), were also detected (L. Antelo, unpublished data).

An antifungal metabolite with different chromatographic properties was purified and identified as the diterpene myrocin B (Fig. 2) based on NMR spectroscopic measurements which were in perfect agreement with published data (Hsu *et al.*, 1988). Like the antiamoebins, myrocin B was

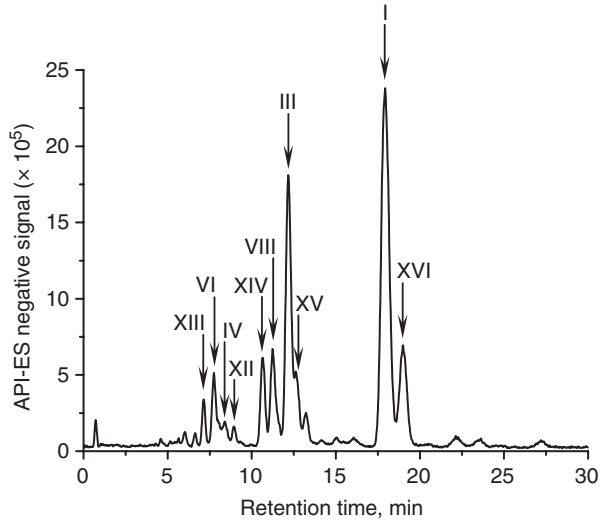


Fig. 4. The spectrum of antiamoebins produced by *Stilbella erythrocephala* D99026 as shown in the negative ionization mode, scanning for *m/z* 1500–1800 (method 4). Anti-amoebins identified both by their molecular ion and by Q-TOF2 sequencing are indicated by their numbers. Several new antiamoebins were detected among the unlabelled peaks (not shown).

Table 1. Quantification of myrocin B and antiamoebins in liquid culture and in dung

	Myrocin B		Antiamoebins	
	Liquid culture ($\mu g\,mL^{-1}$)	Dung ($\mu g\,g^{-1}$ fresh weight)	Liquid culture ($\mu g\,mL^{-1}$)	Dung ($\mu g\,g^{-1}$ fresh weight)
D99026	1.756 ± 0.655	0 ± 0	17.25 ± 4.263	125.6 ± 10.24
D01024	0.003 ± 0.001	2.291 ± 1.099	9.524 ± 2.684	623.6 ± 250.9
D03001	0.451 ± 0.021	4.100 ± 2.232	14.30 ± 0.522	541.2 ± 83.55
D03012	0.011 ± 0.002	5.294 ± 4.011	8.257 ± 1.277	576.3 ± 176.2
Wild dung	–	0.505 ± 0.352	–	236.0 ± 117.5

Values are given as mean $\pm$ SD ($n = 3$).

produced by all four *S. erythrocephala* strains, although at highly variable concentrations (Table 1).

Biological activities

The antimicrobial activities of myrocin B and antiamoebin I are given in Table 2. Both compounds showed a weak to moderate activity against bacteria, yeasts and filamentous coprophilous fungi at concentrations of 10–200 $\mu g\,mL^{-1}$. The activity of the partially-purified antiamoebin mixture (not shown) from *S. erythrocephala* D99026 was identical to that determined for antiamoebin I (Table 2). *Stilbella erythrocephala* itself was unaffected by the highest concentrations of myrocin B ($100\,\mu g\,mL^{-1}$) and antiamoebin I ($200\,\mu g\,mL^{-1}$) tested. With the exception of *Podospora pleiospora* and *Enterobacter dissolvens*, all other test organisms were inhibited at or below 100 μg antiamoebin I mL^{-1}. When myrocin B and antiamoebin I were applied in combination at concentrations half as high as their individual MIC values, no inhibition was observed against most test organisms (Table 2), indicating that these two substances did not act synergistically at subtoxic concentrations.

Quantification of myrocin B and antiamoebins

Although quantification of myrocin B was reproducible for each strain under a given set of culture conditions, the production of this compound was highly variable between

Table 2. Minimum inhibitory concentrations (MIC) of myrocin B (Myr. B) and antiamoebin I (Ant. I), given as $\mu g\,mL^{-1}$

		MIC		Myr. B+Ant. I	
Test organism[a]	Medium[b]	Myr. B	Ant. I	Concentration	Effect
Bacteria					
Bacillus brevis ATCC9999	NB[a]	10	100	5+50	+
B. subtilis ATCC6633	NB	10	100	5+50	+
Sarcina lutea ATCC381	NB	10	25	5+12.5	+
Enterobacter dissolvens LMG2683	NB	> 100	200	100+100	+
Yeasts					
Nematospora coryli ATCC10647	YM	10	100	5+50	–
Saccharomyces cerevisiae TEA011[b]	YM	10	100	5+50	+
Schizosaccharomyces octosporus TEA012	YM	25	100	12.5+50	+
Sporobolomyces roseus D99040[b]	DE	50	50	25+25	+
Coprophilous fungi					
Arthrobotrys superba D01021	DE	100	100	50+50	+
Chaetomium bostrychodes D01006	DE	100	100	50+50	+
Coprinopsis radiata D04007	DE	100	50	50+25	–
Mucor mucedo D99041	DE	100	50	50+25	+
Penicillium claviforme TEA087	DE	> 100	100	100+50	+
Podospora pleiospora D01035	DE	> 100	200	100+100	+
Sordaria lappae D04001	DE	> 100	50	100+25	+
Stilbella erythrocephala D99026	DE	> 100	> 200	100+200	+
Thelebolus nanus D99059	DE	100	50	50+25	+

[a]All strains with accession numbers containing the letters D or TEA were obtained from the Culture Collection, Department of Biotechnology, University of Kaiserslautern.

[b]Test organisms were incubated in nutrient broth (NB), yeast-malt medium (YM) or dung extract (DE).

The effects of a combination of myrocin B and antiamoebin I, each at the subinhibitory concentrations indicated, are shown as inhibition (–) or growth (+).

the four strains of *S. erythrocephala*, and between liquid cultivation and growth on dung (Table 1). Strikingly, strain D99026, the most prolific producer in pure culture (1.8 $\mu g\,mL^{-1}$), failed to produce myrocin B at detectable levels when inoculated onto dung. The rabbit pellets used for our inoculation studies had a water-holding capacity of 400% relative to their dry weight. If, for comparison with liquid culture conditions, we approximate that fully hydrated dung has a density of $1\,g\,mL^{-1}$, *S. erythrocephala* strains D01024, D03001 and D03012 produced higher levels of myrocin B in their natural substrate (2.3–5.3 $\mu g\,mL^{-1}$) than in liquid culture. Myrocin B was also detected in dung collected from the wild (0.5 $\mu g\,mL^{-1}$).

The production of antiamoebins by *S. erythrocephala* was less variable than that of myrocin B. In liquid culture, the four strains produced between 8.3 and 17.3 μg antiamoebins mL^{-1}, whereas concentrations in the range 126–624 $\mu g\,mL^{-1}$ were measured in artificially inoculated dung (Table 1), again assuming a density of $1\,g\,mL^{-1}$ for fully hydrated dung. The composition of antiamoebins was relatively similar in all four strains, and in any one strain between growth in liquid culture and on artificially inoculated dung, with antiamoebins I, III and XVI together contributing 67.8–98.2% of the total antiamoebin concentration measured (Fig. 5). An average of 236 μg antiamoebins mL^{-1} was determined for colonized dung collected in the field (Table 1).

No significant effect on the production of antiamoebins or myrocin B was observed when *S. erythrocephala* D99026 was grown in liquid culture together with the potential competitors *Mucor mucedo*, *Penicillium claviforme* or *Sporobolomyces roseus*, as compared to the pure culture control (not shown).

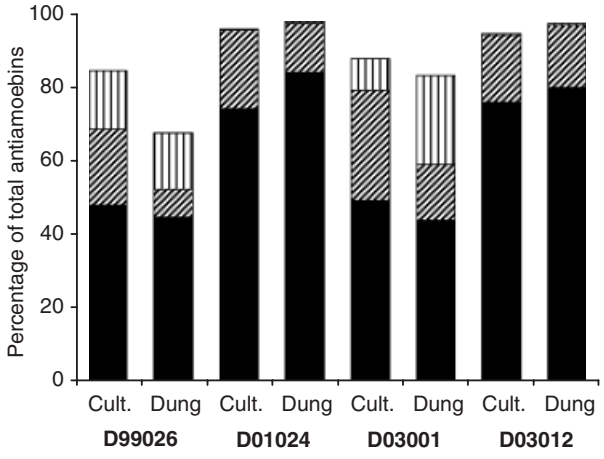


Fig. 5. Relative abundance of antiamoebins I (black area), III (oblique lines) and XVI (vertical lines) in all four *S. erythrocephala* strains grown in pure culture (Cult.) and on artificially inoculated dung ($n = 3$).

Discussion

The present study has shown that *Stilbella erythrocephala* produced antiamoebins in its natural substrate at concentrations well above the threshold concentration shown previously (Sasaki *et al.*, 1971; Schirmböck *et al.*, 1994) and in the current report to be required for inhibiting most competing coprophilous fungi as well as other fungi and bacteria. In contrast, even the highest levels of myrocin B measured in colonized dung were below those causing antifungal effects. *Stilbella erythrocephala* itself was resistant to its own metabolites even at the highest concentrations tested. These results strongly support the hypothesis that the exclusive colonization of individual rabbit pellets by *S. erythrocephala* (Fig. 1), which is commonly observed in nature or if field-collected dung samples are incubated in the laboratory, is due to antiamoebin-based antibiosis. The case is strengthened because similar antiamoebin concentrations were measured in dung colonized by four different strains of *S. erythrocephala*, and in naturally colonized rabbit pellets collected in the field. This is the first such report for coprophilous fungi, and it is significant because the mere demonstration of antifungal metabolites in pure culture is clearly insufficient to support assumptions of antibiosis in natural situations, as shown for myrocin B in *S. erythrocephala* (present report) and for sordarins in *Podospora pleiospora* (Weber *et al.*, 2005). Indeed, even the antiamoebin concentrations produced in pure culture by all four *S. erythrocephala* strains would have been too low to inhibit most competing fungi. The quantification of fungal metabolites in the natural substrate, although technically challenging, should therefore be a critical component in studies of their biological roles, which are the subject of ongoing debates (Zähner *et al.*, 1983; Demain & Fang, 2000). Few of the many antifungal metabolites isolated from pure cultures of saprotrophic fungi have been quantified in the natural substrate, a well-known exception being the fungicidal strobilurins and related metabolites, which are produced by a range of basidiomycetes in pure culture as well as rotting wood and litter (Engler *et al.*, 1998).

Antiamoebins and other peptaibols appear to be confined to a limited range of ascomycetes and their associated conidial forms, with a strong emphasis on species belonging to the Pyrenomycetes, including *Trichoderma*, *Sepedonium*, *Acremonium* and *Paecilomyces* (see Degenkolb *et al.*, 2003). Given that the taxonomic placement of *S. erythrocephala* is as yet unclear, we performed a GenBank search with the ITS sequence of strain D99026, which revealed only two close matches (99.6% identity in 544 nt overlap). These were the soil-inhabiting species *Emericellopsis synnematicola* (AY632665) and *E. salmosynnemata* (AY632666), both of which have been placed in the Bionectriaceae (Hypocreales, Pyrenomycetes) by Zuccaro *et al.* (2004). The assignment of

S. erythrocephala to this group is supported by a detailed report of the presence of antiamoebins in all three species, with antiamoebins I and III being the major ones in *Emericellopsis* spp. and I, III and XVI in *S. erythrocephala* (Jaworski & Brückner, 2000), as also observed in the present work.

The second type of compound isolated in the present work, myrocin B, has not been reported previously for *S. erythrocephala*. Myrocin B and the related compound myrocin C were described originally from *Myrothecium* spp. (Hsu *et al.*, 1988; Nakagawa *et al.*, 1989), a genus which, like *Stilbella*, is currently being accommodated in the Hypocreales (Seifert & Samuels, 1997). Myrocin B most probably has the same mode of action as myrocin C which has been proposed to be converted into an unstable intermediate reacting with DNA (Duclohier *et al.*, 1998; Wolkenberg & Boger, 2002). In contrast, antiamoebins act primarily by forming pores or channels in lipid bilayer membranes (Duclohier *et al.*, 1998; Chugh & Wallace, 2001; O'Reilly & Wallace, 2003). It is attractive to speculate that myrocin B and antiamoebins may act synergistically under certain circumstances, as has been reported for the concerted action of secreted cell wall-degrading enzymes and peptaibols of *Trichoderma harzianum* against other fungi (Lorito *et al.*, 1996). However, the low and erratic production of myrocin B by *S. erythrocephala* does not implicate it in any role in the natural substrate, and our inhibition tests with combinations of myrocin B and antiamoebins also failed to show clear-cut synergistic effects.

Most fungi producing peptaibols are isolated from soil, decaying plant remains, or as parasites of mushroom fruitbodies, i.e. from highly competitive habitats (Lorito *et al.*, 1996; Jaworski & Brückner, 2000, 2001; Degenkolb *et al.*, 2003; Wilhelm *et al.*, 2004). The coprophilous habitat of *S. erythrocephala* is similarly competitive. In fact, dung may be especially suitable for fungi defending their habitat by means of nitrogen-containing metabolites such as peptaibols, as it is known to be a nitrogen-rich substrate. This feature may explain the surprisingly high accumulation of antiamoebins in dung colonized by all four *S. erythrocephala* strains, as compared to their liquid cultures. Further work should aim at suppressing nonribosomal peptide synthase activity in *S. erythrocephala* by inhibitors or deletion of its putative gene known to be present (N.-A. Lehr, unpublished observations), and at examining whether this might compromise the competitive ability of this fungus on dung. It would also be interesting to quantify the production of peptaibols by noncoprophilous fungi in their respective natural habitats. The presence of these substances in species from a narrow taxonomic range, but capable of conquering a wide diversity of competitive habitats, indicates their broader ecophysiological role.

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