Effects of Coumarin and Sparteine on Attachment to Cellulose and Cellulolysis by *Neocallimastix frontalis* RE1

G. MONIELLO,^{1†} A. J. RICHARDSON,² SYLVIA H. DUNCAN,² AND C. S. STEWART^{2*}

*Universita degli Studi di Napoli Federico II, Dipartimento di Scienze Zootechniche, 80137 Naples, Italy,¹
and Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, United Kingdom²*

Received 2 August 1996/Accepted 14 September 1996

The plant secondary metabolites coumarin and sparteine reduced attachment to cellulose, cellulose solubilization, and the proportion of lactate in the fermentation products of the anaerobic fungus *Neocallimastix frontalis* RE1. Neither compound directly inhibited the endoglucanase or lactate dehydrogenase activities of cell extracts of the fungus.

Grazing and browsing ruminants consume graminaceous and leguminous forages and the bark and leaves of trees and shrubs (14) and are thus exposed to a variety of plant secondary metabolites. Cellulolytic ruminal microorganisms in particular may encounter high localized concentrations of such metabolites during plant cell wall degradation (5). Anaerobic fungi, including *Neocallimastix frontalis* and other species, are among the most active degraders of cellulose in this ecosystem (6, 16). Several plant phenolic acids and polyphenolic condensed tannin from birdsfoot trefoil (*Sainfoin*) inhibit the growth and cellulolytic activity of anaerobic fungi (1, 8), and coumarin (2,3-benzopyrone) depresses glucose utilization by *N. frontalis* (2). Here, following a preliminary report (9), the effects of coumarin and the norlupinine alkaloid sparteine (11) on initial attachment to, and solubilization and fermentation of, cellulose by *N. frontalis* are reported.

Attachment to cellulose. *N. frontalis* RE1 (12) was grown at 39°C (used for all incubations) under O₂-free CO₂ in a basal medium (12) containing clarified rumen liquor, Bacto-Casitone, Difco yeast extract, and minerals, with cysteine-HCl as the reductant (final pH, 6.8), contained in glass anaerobic Hungate tubes (16 by 125 mm) (Bellco Glass Inc., Vineland, N.J.). Cellophane strips (10 by 30 mm) (BTL Cellophane, Bridgewater, United Kingdom) were added as the sole carbohydrate before autoclaving (121°C, 15 min). Cultures grown on cellophane for 4 days were shaken by hand; 0.5 ml of the resultant fungal suspension was inoculated into tubes containing the basal medium plus 0.2% glucose as the sole carbohydrate and 0.05% Difco agar. After incubation for 24 h, 0.5 ml of this culture was used to inoculate tubes of basal medium (10 ml) containing D-[U-¹⁴C]glucose (specific activity, 6.8 mCi/mmol) (Sigma) at 2.7 mg per liter as the sole carbohydrate and then incubated for 48 h. After centrifugation at 1,065 × g for 5 min (MSE Minor, fixed-angle rotor), the fungal pellets were washed twice, resuspended in 10 ml of basal medium, and mixed in a vortex mixer for 2 min. This suspension (0.5 ml) was used to inoculate tubes containing pieces (25 by 3 mm, 7.5 mg) of Whatman no. 1 filter paper and 5 ml of basal medium. Sparteine sulfate (Fluka AG) was added to the autoclaved basal medium as an aqueous solution sterilized by filtration (0.2-μm-pore-size membranes). Coumarin (Sigma) was dissolved in dimethyl sulfoxide (DMSO), the results obtained

being expressed relative to those obtained with controls with DMSO (10 μl · ml⁻¹) alone. After incubation for up to 24 h, the cultures were shaken, the supernatants were decanted, and the strips were washed twice with 4 ml of an anaerobic buffer (the minerals from the basal medium with 0.1% [wt/vol] cysteine HCl, pH 6.8) to remove loosely attached or entrapped material. The strips were then placed in vials with 3 ml of scintillation fluid (Optiphase, FSA, Loughborough, United Kingdom) for counting. Radiolabelling of the fungal biomass resulted in ¹⁴C counts of 45,788 ± 8,528 cpm mg⁻¹ (average ± standard deviation [SD], n = 5). Approximately 2.5% of the label was incorporated. Attachment of the fungi to the filter paper strips approached a maximum after around 6 h of incubation (Fig. 1a) and was reduced by the presence of coumarin and sparteine sulfate (Fig. 1b). Light-microscopic examination showed that both fungal rhizoids and zoospores were attached (data not shown).

Cellulose solubilization and fermentation. Cultures pre-grown on cellophane then grown on glucose for 48 h as described above were used to inoculate Hungate tubes containing 30 mg of filter paper strips (Whatman no. 1) and 10 ml of basal medium, with or without coumarin or sparteine. After incubation for 7 days, the residues were washed three times with distilled H₂O and then lyophilized. In a preliminary experiment, the weight loss of paper exceeded cellulose disappearance (estimated colorimetrically [13]) by an average of only 2.9% (n = 48), and so the gravimetric method was used routinely.

The presence of sparteine sulfate reduced the rate and extent of filter paper solubilization (Fig. 2a); about 3% was solubilized upon autoclaving (Fig. 2a has been corrected for this). Capillary gas chromatography analysis (10) showed that sparteine, especially at higher concentrations, markedly decreased the concentration of lactate produced relative to the concentration of other products (Fig. 2b). Measurements by gas chromatography (12) showed that 10-ml cultures of strain RE1 grown on cellulose for 7 days produced 0.35 ml of H₂ in the gas phase. The presence of 5 or 10 mM sparteine reduced H₂ production to 0.25 and 0.1 ml, respectively.

The presence of coumarin also depressed cellulose solubilization and fermentation, and 10 mM coumarin depressed the amount of lactate formed relative to that of acetate (Table 1).

Glucose disappearance. Estimation (2) of the disappearance of glucose from basal medium amended with 0.2% (wt/vol) glucose showed that, after 48 h of incubation, strain RE1

* Corresponding author. Phone: 44 1224 716654. Fax: 44 1224 716687. Electronic mail address: css@rri.sari.ac.uk.

† Present address: Istituto di Produzioni Animali, Universita degli Studi di Sassari, 07100 Sassari, Italy.

FIG. 1. (a) Time course of attachment of ^{14}C -labelled *N. frontalis* to filter paper. Average of three observations; bars represent SD. (b) Effect of coumarin (■) and sparteine sulfate (○) on attachment of radiolabelled *N. frontalis* to filter paper after 6 h of incubation. Average of eight observations; bars represent SD.

removed approximately $70\% \pm 7\%$ of the glucose from controls; the presence of 5 or 10 mM sparteine reduced glucose disappearance to $46\% \pm 3\%$ and $35\% \pm 5\%$, respectively (average $\pm$ SD, $n = 3$). This is a less marked effect than that reported with coumarin (2).

Fungal lactate dehydrogenase and endoglucanase activities. Strain RE1 was grown on glucose (as described above) without added plant metabolites. Cell extracts were made and enzyme assays were performed as previously described (7). Both NADH- and NADPH-dependent lactate dehydrogenase activities were detected (specific activities, 195 and 28 $\text{nmol} \cdot \text{min}^{-1} \cdot \text{mg}$ of protein $^{-1}$, respectively), but neither was affected by the presence of up to 10 mM sparteine or 2 mM coumarin in the assay mixtures (data not shown) (the UV absorbance of coumarin prevented its use in these tests at higher concentrations). Upon incubation with carboxymethyl-cellulose (15), the cell extract released 277 nmol of reducing sugar $\cdot \text{h}^{-1} \cdot \text{mg}$ of protein $^{-1}$, and the presence of up to 10 mM sparteine or coumarin did not reduce this activity (data not shown). In contrast, condensed tannins strongly inhibit the endoglucanase activity of the same strain (8), as does methyl-cellulose, which inhibits fungal attachment (3).

Fermentation of plant metabolites. Strain RE1 was incubated for 7 days in basal medium with added coumarin or sparteine sulfate as the sole substrate. The fungal biomass was recovered by centrifugation (as described above), washed three times with distilled H_2O , and lyophilized. Although small amounts of products were formed in the basal medium without addition of plant metabolites, these two compounds clearly did not themselves support growth or fermentation (data not shown).

Although other compounds appear to influence the colonization of cellulosic substrates by anaerobic fungi (1, 3, 8), this is the first study in which effects on initial attachment have been studied specifically. This study also highlights potential effects on fungal fermentation products. Understanding how different metabolites may affect functionally important processes could provide bioassays which, together with chemical analyses, assist in the detection of such compounds in plant material or extracts. The importance of sparteine and coumarin in ruminal microbial ecology will be dependent on their concentrations in the biofilm at the plant cell wall-microbial interface (1, 4), which remain unknown.

FIG. 2. Effect of sparteine sulfate on (a) loss in weight of filter paper (■, control; ○, 2.5 mM; ▲, 5.0 mM; ◇, 10 mM) and (b) fermentation products (■, formate; ○, acetate; ▲, lactate; ◇, succinate; □, ethanol) during incubation with *N. frontalis*. Averages of three observations; bars represent SD.

TABLE 1. Effect of coumarin on cellulose disappearance and the formation of fermentation products by *N. frontalis* RE1^a

Coumarin (mM)	% wt loss of filter paper	Final product concn (mM) $\pm$ SD				
		Formate	Acetate	Lactate	Succinate	Ethanol
0	87.7 $\pm$ 2.5	15.1 $\pm$ 0.8	12.7 $\pm$ 2.6	20.3 $\pm$ 3.5	1.6 $\pm$ 0.4	4.5 $\pm$ 0.2
1	87.7 $\pm$ 0.5	15.7 $\pm$ 1.0	11.8 $\pm$ 1.9	18.0 $\pm$ 2.4	1.1 $\pm$ 0.1	4.6 $\pm$ 0.2
2.5	85.5 $\pm$ 0.9	13.7 $\pm$ 1.0	8.0 $\pm$ 2.1	16.0 $\pm$ 1.7	1.0 $\pm$ 0.1	5.6 $\pm$ 0.8
5.0	82.9 $\pm$ 1.9	14.0 $\pm$ 1.3	9.9 $\pm$ 0.7	13.9 $\pm$ 2.4	0.8 $\pm$ 0.1	3.9 $\pm$ 0.3
10.0	51.4 $\pm$ 2.4	11.7 $\pm$ 0.2	9.5 $\pm$ 0.7	11.0 $\pm$ 0.5	0.6 $\pm$ 0.1	3.6 $\pm$ 0.2

^a Incubation was for 7 days. Average of four determinations $\pm$ SD.

We thank T. Acamovic for helpful advice.

The Rowett Research Institute is funded by the Scottish Office of Agriculture, Environment and Fisheries Department. We thank the Banco di Napoli for a grant to G.M. and the European Union (ERBTS3/CT930211) for financial support.

REFERENCES

1. Akin, D. E., and L. L. Rigsby. 1985. Influence of phenolic acids on rumen fungi. *Agron. J.* **77**:180–182.
2. Cansunar, E., A. J. Richardson, G. Wallace, and C. S. Stewart. 1990. Effect of coumarin on glucose uptake by anaerobic rumen fungi in the presence and absence of *Methanobrevibacter smithii*. *FEMS Microbiol. Lett.* **70**:157–160.
3. Cheng, K.-J., H. Kudo, S. H. Duncan, A. Mesbah, C. S. Stewart, A. Bernalier, G. Fonty, and J. W. Costerton. 1991. Prevention of fungal colonisation and digestion of cellulose by the addition of methylcellulose. *Can. J. Microbiol.* **37**:484–487.
4. Cheng, K.-J., C. S. Stewart, D. Dinsdale, and J. W. Costerton. 1983/84. Electron microscopy of bacteria involved in the digestion of plant cell walls. *Anim. Feed Sci. Technol.* **10**:93–120.
5. Chesson, A., C. S. Stewart, and R. J. Wallace. 1982. Influence of plant phenolic acids on growth and cellulolytic activity of rumen bacteria. *Appl. Environ. Microbiol.* **44**:597–603.
6. Gordon, G. L. R., and M. W. Phillips. 1993. Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and in vivo fibre digestion. *Lett. Appl. Microbiol.* **17**:220–223.
7. Marvin-Sikkema, F. D., A. J. M. Driessen, J. C. Gottschal, and R. A. Prins. 1994. Metabolic energy generation in hydrogenosomes of the anaerobic fungus *Neocallimastix*: evidence for a functional relationship with mitochondria. *Mycol. Res.* **98**:205–212.
8. McAllister, T. A., H. D. Bae, L. J. Yanke, K.-J. Cheng, and A. Muir. 1994. Effect of condensed tannins from birdsfoot trefoil on endoglucanase activity and the digestion of cellulose filter paper by anaerobic fungi. *Can. J. Microbiol.* **40**:298–305.
9. Moniello, G., A. J. Richardson, and C. S. Stewart. 1995. Effects of the plant metabolites saponin, coumarin and sparteine on attachment, cellulolysis, and fermentation of glucose by the anaerobic fungus *Neocallimastix frontalis* strain RE1, abstr. I-50, p. 326. In Abstracts of the 95th General Meeting of the American Society for Microbiology 1995. American Society for Microbiology, Washington, D.C.
10. Richardson, A. J., A. G. Calder, C. S. Stewart, and A. Smith. 1989. Simultaneous determination of volatile and non-volatile acidic fermentation products of anaerobes by capillary gas chromatography. *Lett. Appl. Microbiol.* **9**:5–8.
11. Saito, K., S. Takamatsu, S. Ohmiya, H. Otomatsu, M. Yasuda, Y. Kano, and I. Murakoshi. 1987. Lupin alkaloids from the seeds of *Thermopsis lupinoides*. *Phytochemistry* **27**:3715–3716.
12. Stewart, C. S., and A. J. Richardson. 1989. Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. *J. Appl. Bacteriol.* **66**:85–93.
13. Updegraff, D. M. 1969. Semi micro determination of cellulose in biological materials. *Anal. Biochem.* **32**:420–424.
14. Van Soest, P. J. 1994. Nutritional ecology of the ruminant, p. 93–107, 196–229. Cornell University Press, Ithaca.
15. Williams, A. G., and C. G. Orpin. 1987. Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi grown on a range of carbohydrate substrates. *Can. J. Microbiol.* **33**:418–426.
16. Wubah, D. A., D. E. Akin, and W. S. Bornemann. 1993. Biology, fiber-degradation, and enzymology of anaerobic zoospore fungi. *Crit. Rev. Microbiol.* **19**:99–115.