Inhibition of the cellulolytic activity of *Neocallimastix frontalis* by *Ruminococcus flavefaciens*

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A study was made of the antagonistic effect of *Ruminococcus flavefaciens* on the cellulolytic activity of *Neocallimastix frontalis*. An extracellular factor inhibiting the cellulolytic activity of the fungus was detected in the bacterial supernatant. The antagonistic factor, which precipitated with ammonium sulphate at 40 % saturation, was temperature-sensitive and was destroyed at temperatures above 60 °C. After separation by anion-exchange chromatography, sequential precipitation, dialysis and SDS-PAGE, two protein species of 100 and 24 kDa were identified as being involved in this antagonistic effect. It is not known whether the proteins are two subunits of a single protein or represent two different proteins. The inhibitory factor, which is not a bacterial cellulase, did not affect fungal growth, but it inhibited the activity of the fungal cellulases.

Introduction

Many relationships are known to exist between micro-organisms in the rumen, and various workers have shown that the anaerobic fungi *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis* interact with hydrogen-utilizing bacteria (Bauchop & Mountfort, 1981; Mountfort *et al.*, 1982; Fonty *et al.*, 1988; Joblin *et al.*, 1990; Marvin-Sikkema *et al.*, 1990; Joblin & Williams, 1991; Bernalier *et al.*, 1992). In the presence of methanogens, anaerobic fungi are more effective in degrading cellulose and their metabolism is shifted towards the production of acetate, thereby reducing that of lactate and ethanol. This phenomenon probably results from a transfer of hydrogen between the fungi and the bacteria (Wolin & Miller, 1988). In a more recent study of the interactions between anaerobic fungi and rumen cellulolytic bacteria, *Ruminococcus flavefaciens* was observed to inhibit the ability of *Neo-*

callimastix frontalis to hydrolyse cellulose (Bernalier *et al.*, 1988, 1992). The amount of cellulose degraded in co-cultures of these two micro-organisms was considerably less than in monocultures of the fungus. The aim of the present study was to determine the basis of the inhibitory effect of *R. flavefaciens* on fungal cellulolysis.

Methods

Organisms and culture conditions. *Neocallimastix frontalis* MCH3, which is characterized by a monocentric thallus and polyflagellated zoospores (Orpin, 1975), was isolated in our laboratory from sheep rumen contents, and *Ruminococcus flavefaciens* 007 was kindly provided by Dr C. S. Stewart (Rowett Research Institute, Aberdeen, UK). Fungal and bacterial cultures were prepared and grown under strict anaerobiosis according to the methods of Hungate (1969). The basal medium, as described by Lowe *et al.* (1985) and modified by Gay *et al.* (1989), contained cellulose or cellobiose (0.2 %, w/v) as the sole carbon source. For the preparation of cellulose medium, pieces of Whatman no. 1 filter paper (100 mg) were placed in each tube before the addition of the pre-reduced basal medium (10 ml per tube). The media were dispensed under O₂-free CO₂ in 10 ml volumes into CO₂-filled 16 ml screw-cap Hungate tubes (Bellco Clan). The fungal inoculum consisted of a 1 ml sample of a 48 h culture grown on cellobiose medium, and the bacterial inoculum consisted of 0.5 ml aliquots of a 20 h culture grown on the same medium. All incubations were carried out at 39 °C and all experiments were performed in triplicate. Cellulose degradation in experimental cultures is expressed as a percentage (mean ± SD) of that in control cultures of *N. frontalis* and *R. flavefaciens*.

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Abbreviations: CMC, carboxymethylcellulose; DM, dry matter; HEC, hydroxyethylcellulose.

Dry matter (DM) disappearance. At the end of the period of incubation, the cultures were centrifuged at 1000 *g* for 10 min. The residual cellulose pellets were treated with 1 M-NaOH at 100 °C to remove bacterial and fungal material, then rinsed twice in distilled water and dried to a constant weight at 80 °C.

Inhibitory factor produced by *R. flavefaciens*. After 4 d of incubation (t_4), the fungal and the bacterial monocultures were centrifuged at 1000 *g* for 10 min in their culture tubes to harvest non-degraded filter paper and micro-organisms attached to cellulose fibres. The bacterial supernatant was centrifuged again at 3000 *g* for 10 min to remove the bacterial cells. The fungal and bacterial supernatants were then sterilized by passage through a 0.2 µm mesh Millipore filter in an anaerobic glove box. The fungal supernatant was added to the non-degraded bacterial cellulose pellet and the bacterial supernatant was added to the non-degraded fungal cellulose pellet. These cultures were then incubated for 4 d (t_8) and the dry matter (DM) disappearance of cellulose and the fungal and bacterial biomasses were determined. The fungal and bacterial biomasses were assayed by measuring the protein contents according to Bradford (1976).

A series of control cultures was made by suspending fungal and bacterial pellets with their own supernatants, i.e. the cultures were reconstituted. In addition, for all experiments, *N. frontalis* and *R. flavefaciens* were cultured on filter paper as controls and incubated for 8 d.

Influence of various treatments of the bacterial supernatant on the interaction between *N. frontalis* and *R. flavefaciens*. To study the effect of the age of the bacterial supernatant on the fungal cellulose degradation, the supernatant of *R. flavefaciens* was taken after 2, 4 or 6 d of culture and added to a 4-d-old fungal cellulose pellet. The cultures were then incubated for 4 d. To study the effect of the volume of the bacterial supernatant exchanged, 1, 5, 8 or 10 ml of this supernatant, representing 10, 50, 80 and 100%, respectively, of the total supernatant, were exchanged after 4 d of incubation. To study the effect of temperature, bacterial supernatants collected from the 4-d-old cultures of *R. flavefaciens* were heated for 15 min at 50, 60, 80 and 100 °C and then mixed with fungal cellulose pellets and incubated for 4 d. In all these studies, only the DM disappearance of filter paper was measured at the end of the period of incubation (t_8).

Adsorption of cellulases. To remove the fungal and bacterial cellulases, the supernatants were treated with a mixture of equal amounts of different celluloses: Sigmacell 50 and 100, Avicel, cellulose fibre, and ground filter paper (for the effectiveness of this treatment, see Results). One hundred milligrams of this cellulose mixture was added to each of the supernatants from 4-d-old cultures and incubated for 15 min with shaking. After incubation, the tubes were centrifuged at 700 *g* for 10 min to precipitate the cellulose. They were centrifuged twice more, after which the endoglucanase activity of the supernatants was measured by viscosimetry with hydroxyethylcellulose (HEC) as substrate (Gaudet, 1983). The treated supernatants of the fungal and bacterial cultures were exchanged as described above. After 4 d of incubation (t_8), the DM disappearance of the filter paper was determined.

Determination of the biochemical nature of the bacterial factor. Proteins in the supernatants of bacterial cultures were precipitated with ammonium sulphate at 40 or 60% saturation. After centrifugation at 7000 *g* for 15 min, the bacterial proteins were suspended in 0.5 ml 0.02 M-Tris/HCl pH 8.0 (buffer T) and their concentrations were assayed spectrophotometrically using bicinchoninic acid (BCA) reagent (Pierce-Amersham). The proteins precipitated by ammonium sulphate were then added to a 4-d-old fungal culture. The proteins from the culture supernatants in which the cellulases had been partially absorbed on celluloses were treated in the same manner. Proteins from an uninoculated medium were also collected and added to the fungal

cultures. DM measurements were made 4 d (t_8) after the addition of the bacterial proteins to the fungal cultures. A control culture was made by adding to a 4-d-old fungal culture the same concentration of ammonium sulphate as that used in the assay cultures.

Mode of action of the bacterial factor. To study the effect of the factor on the growth of *N. frontalis*, the fungus was cultured on medium containing 0.2% glucose. The bacterial proteins collected from a filter paper culture supernatant after precipitation with 40% saturated ammonium sulphate solution were added to 17 h fungal cultures. After 72 h incubation, the fungal biomass was determined by protein assay according to the method of Bradford (1976) as described above. Cultures of *N. frontalis* 17 and 72 h old were used as controls. To study the effect of the bacterial factor on the fungal cellulases, the fungal proteins were precipitated from culture supernatants with 40% saturated ammonium sulphate solution and concentrated by ultrafiltration-centrifugation (Centricon 10 microconcentrators, cut-off 10 kDa, Amicon). Electrophoresis of these proteins was performed with a non-denaturing polyacrylamide gel containing 1% (w/v) carboxymethylcellulose (CMC) (Schwartz *et al.*, 1987). After migration, the gel was incubated overnight at 39 °C in 50 mM-K₂HPO₄ buffer (pH 7.0) in the absence or presence of bacterial supernatant. The gel was then stained for 30 min in a 0.1% solution of Congo Red and rinsed with 1 M-NaCl.

Synthesis and secretion of the bacterial factor. The inhibitory effect of a supernatant from *R. flavefaciens* culture obtained from basal medium containing 0.2% cellobiose was measured. The bacterial cultures in exponential or stationary phase were centrifuged at 3000 *g* for 15 min, and the supernatants recovered and filtered through 0.2 µm Millipore filters. The supernatants were mixed with 4-d-old fungal pellets grown on filter paper medium. These cultures were incubated for 4 d and the DM disappearance of cellulose was determined.

Partial purification of the bacterial factor. Filtered supernatants from bacterial cultures were subjected to chromatographic analysis in an anion-exchange column (Millipore, DEAE Memsep 1000) equilibrated with buffer T. The non-retained fraction was collected and the proteins retained in the column were eluted with different concentrations of NaCl (0.25, 0.5, 0.75 or 1.0 M) in buffer T. The elution of the proteins was followed at 280 nm. The protein fractions recovered were precipitated with ammonium sulphate at 60% saturation and then redissolved in buffer T. The effectiveness of each fraction in inhibiting fungal cellulolysis was determined by measuring the amount of residual substrate 4 d after addition of the protein pool to a 4-d-old fungal culture grown on basal medium. Subsequently, the fractions were analysed by SDS-PAGE in a 12% (w/v) polyacrylamide gel according to Laemmli (1970). After migration, the electrophoresis gels were stained with silver (Oakley, 1980).

Medium fractionation by precipitation. After adsorption of the cellulases onto celluloses the supernatants from 4-d-old *R. flavefaciens* cultures were treated sequentially with ammonium sulphate at 20, 40 and 70% saturation. The precipitated proteins were added to buffer T, concentrated by ultrafiltration and analysed by electrophoresis (SDS-PAGE). The inhibitory activity of each protein fraction against fungal cellulolysis was also determined by adding the proteins to 4-d-old fungal cultures and measuring the DM disappearance of cellulose after 4 d incubation (t_8).

Medium fractionation by dialysis. The supernatants of *R. flavefaciens* were treated with the cellulose mixture and dialysed on an ultrafiltration membrane having a 50 kDa cut-off against 20 mM-KH₂PO₄ (pH 7.0). The proteins retained in the supernatant were then precipitated with ammonium sulphate at 60% saturation and concentrated by ultrafiltration. The protein fraction collected was first analysed by SDS-PAGE and then added to a 4-d-old fungal culture to study its effect on fungal cellulolysis.

Results

Inhibitory factor produced by *R. flavefaciens*

When added to *N. frontalis*, the 3000 g supernatant prepared from an *R. flavefaciens* culture inhibited the extent of cellulose degradation (Fig. 1). In contrast, the fungal supernatant stimulated bacterial cellulolysis: 30% more cellulose was degraded by bacterial cultures to which fungal supernatants had been added (Fig. 1). After 8 d incubation, the biomasses of the fungal and bacterial cultures whose supernatants had been exchanged were comparable to those of their respective controls (Table 1). This shows that the exchange of supernatants did not affect the growth of either micro-organism. To determine whether the increase in bacterial cellulolysis was due to the presence of fungal cellulases in the exchange supernatant, the latter was incubated at 39 °C in the presence of a mixture of celluloses. The endoglucanase activity of the supernatants was then measured by viscosimetry and was found to have only a quarter of the activity of the supernatants of unadsorbed controls ($9 \pm 2\%$ of decrease in the viscosity of an HEC solution versus $36 \pm 2\%$), showing that the cellulose mixture

Fig. 1. Kinetics of filter paper degradation in cultures of *N. frontalis* (■, □) and *R. flavefaciens* (▲, △). Filled symbols, control cultures; open symbols, after the supernatant exchange. Each experimental value represents the mean of three assays. The SD values were < 5% of the mean and are not indicated on the figure.

Table 1. Growth of *N. frontalis* and *R. flavefaciens* in control cultures and in cultures whose supernatants were exchanged after 4 d of incubation

Culture age (d)	Biomass ($\mu\text{g protein ml}^{-1}$)*			
	<i>N. frontalis</i>		<i>R. flavefaciens</i>	
	Control	After exchange	Control	After exchange
4	78.8 $\pm$ 9.7	—	100.6 $\pm$ 1.8	—
8	91.6 $\pm$ 1.8	94.6 $\pm$ 7.6	108.7 $\pm$ 9.9	108.3 $\pm$ 9.4

* Each value represents mean $\pm$ SD ($n = 3$).

treatment resulted in good adsorption of cellulases. When treated with cellulose, the fungal supernatant lost much of its effect on cellulose degradation in the bacterial culture (an increase of $2 \pm 1.9\%$ compared with $24.7 \pm 1.8\%$ with untreated supernatant). Hence, the removal of fungal cellulases by adsorption depresses the cellulolytic activity of the supernatant.

Influence of various treatments of the bacterial supernatant on the interaction between *N. frontalis* and *R. flavefaciens*

The inhibitory effect of the bacterial supernatant on the cellulolytic activity of the fungus was the same for exchanges of supernatants made after 2, 4 or 6 d of culture. The bacterium inhibited the cellulolysis of *N. frontalis* even when only a small volume of supernatant was transferred: 1 ml of supernatant was sufficient to achieve maximum inhibition of fungal cellulolysis (Table 2). The extent of cellulose degradation increased slightly with the increase in the volume of the bacterial supernatant. The antagonistic effect of the bacterial supernatant on the cellulolytic activity of the fungus was only slightly affected after it had been heated at 50 °C, but was completely destroyed at 60, 80 and 100 °C. In controls, the cellulolytic ability of *N. frontalis* was not affected after resuspension in its own heated supernatant.

After successive incubations of the bacterial supernatants with mixtures of celluloses, most of the extracellular cellulases produced by *R. flavefaciens* were removed from the supernatant, e.g. the endoglucanase activity of these supernatants was only a third of that of non-adsorbed controls ($9.3 \pm 1.5\%$ decrease in the viscosity of an HEC solution versus $31.2 \pm 0.9\%$). Treatment with a mixture of celluloses did not alter the effect of the bacterial supernatant on the fungal activity ($69.7 \pm 4.4\%$ of cellulose degraded compared with $70.9 \pm 1.4\%$).

Biochemical nature of the factor

The proteins in the bacterial supernatant reduced the amount of cellulose degraded by fungal cultures (Table 3). The greatest inhibitory effect of these proteins was observed when they were precipitated with ammonium sulphate at 60% saturation (Table 3). The proteins from a bacterial supernatant treated with a mixture of celluloses were also capable of decreasing fungal cellulolysis. The inhibitory activity of these proteins was slightly greater than that of both the total bacterial supernatant and the proteins separated from it (Table 3). The proteins from uninoculated culture medium did not decrease fungal cellulolysis (Table 3). Therefore the antagonistic effect on fungal cellulolysis of the proteins

Table 2. Effect of the amount of *R. flavefaciens* supernatant exchanged on cellulose degradation by *N. frontalis*

The bacterial supernatant was added to the *N. frontalis* culture after 4 d. Cellulose degradation was measured 4 d later.

Amount of supernatant exchanged (% of final volume)	Cellulose degraded by <i>N. frontalis</i> (% of that degraded in control)*
0	100.0 ± 0.0
10	52.7 ± 2.3
50	55.5 ± 2.4
80	56.5 ± 2.6
100	62.1 ± 0.9

* Each value represents the mean ± SD ($n = 3$).

Table 3. Effect on the degradation of cellulose by *N. frontalis* of the addition of proteins precipitated from the supernatant of *R. flavefaciens* culture, proteins precipitated from the sterile culture medium, and the bacterial supernatant treated with a mixture of celluloses

Cellulose degradation was measured 4 d after the addition of proteins.

Addition	Cellulose degraded by <i>N. frontalis</i> (% of that degraded in control culture)*
Total bacterial supernatant	72.1 ± 3.7
Bacterial supernatant proteins precipitated by	
40% saturated $(\text{NH}_4)_2\text{SO}_4$	87.1 ± 2.8
60% saturated $(\text{NH}_4)_2\text{SO}_4$	76.1 ± 4.5
Proteins from an uninoculated medium precipitated by 40% saturated $(\text{NH}_4)_2\text{SO}_4$	96.5 ± 1.7
Proteins from a bacterial supernatant treated with cellulose and precipitated by 40% saturated $(\text{NH}_4)_2\text{SO}_4$	62.9 ± 3.5

* Each value represents the mean ± SD ($n = 3$).

from the bacterial supernatants was not due to a component of the culture medium. In the control culture, the addition of 33 mM-ammonium sulphate (corresponding to the concentration in a 60% saturated solution) did not reduce the cellulolytic activity of the fungus.

Mode of action of the factor

The results in Table 1 suggest that the bacterium had no antagonistic effect on fungal growth. To confirm this, *N. frontalis* was cultured on glucose medium in the absence and presence of bacterial proteins. The addition of bacterial proteins to a 20-h-old *N. frontalis* culture did not affect its growth, since after 72 h, the concentration

Fig. 2. DEAE-Mensep 1000 ion-exchange chromatography (DEAE) of *R. flavefaciens* culture supernatant. The proteins were eluted by stepwise addition of NaCl and the A_{280} measured (■). The inhibitory activity of each protein fraction was determined by measuring the amount of cellulose (DW) degraded by *N. frontalis* after the addition of these fractions and is expressed as the percentage of degradation (mg DW disappeared) in the control fungal culture that had undergone supernatant exchange with *R. flavefaciens* (●).

of fungal proteins found was the same in the presence and absence of the bacterial factor.

The cellulolytic activity of the fungus was examined by zymogram analysis. When no bacterial supernatant was added to the gel incubation buffer, the fungal cellulases had a high hydrolytic activity against CMC embedded in the gel (large zone of clearing on the gel). The addition of bacterial supernatant to the incubation buffer strongly inhibited the CMCase activity of *N. frontalis* (no zone of cellulolysis on the gel).

Secretion of the factor by the bacterium

The effectiveness of the inhibition of the bacterial supernatant was the same whether cellobiose or cellulose was used as the carbon source for bacterial growth. Supernatants from exponential-phase bacterial cultures grown on cellobiose had a slightly lower inhibitory activity than supernatants from stationary-phase cultures.

Partial purification of the inhibitory protein secreted by *R. flavefaciens*

The supernatants of *R. flavefaciens* cultures were passed through an anion-exchange column. The two protein peaks obtained by elution with 0.25 and 1 M-NaCl were quantitatively the highest and represented the fractions containing the inhibiting factor (Fig. 2). The two other salt concentrations eluted only a small amount of proteins and these had no effect on fungal cellulolysis (Fig. 2). The specific activity of the proteins eluted with 1 M-NaCl was greater than that of proteins eluted with

Table 4. Effect on the cellulose degradation by *N. frontalis* of protein fractions from the supernatant of an *R. flavefaciens* culture

The fractions were obtained by elution from a DEAE column (see Methods). Cellulose degradation was measured 4 d after the addition of proteins.

Treatment	Cellulose degraded by <i>N. frontalis</i> (% of that degraded in control)	Specific activity [mg dry wt cellulose degraded (mg added protein) ⁻¹]
Exchange of		
Total supernatant	72.8 ± 3.2	90.6
Non-retained fraction	102 ± 5.1	ND
Addition of proteins eluted by		
0.25 M-NaCl	90.8 ± 4.1	230
0.50 M-NaCl	100.0 ± 1.9	ND
0.75 M-NaCl	114.4 ± 3.8	ND
1 M-NaCl	92.9 ± 1.7	645.5

ND, Not determined.

0.25 M-NaCl (Table 4). Hence the activity of the antagonistic factor seemed to be greater in the second peak. The bacterial proteins recovered from the anion-exchange column came from six supernatants and, since protein from a single supernatant was sufficient to produce maximum inhibitory activity, no increased inhibition was expected by using protein from six supernatants. Therefore it was possible to compare the inhibitory effects produced by culture supernatants with that of proteins eluted from the column. While the transfer of the bacterial supernatant to the fungal culture resulted in a 27% decrease in the cellulose degraded, the addition of the two protein fractions (0.25 and 1 M) produced a decrease of only 16.3%. It is possible that some inhibitory proteins were lost during purification, since a protein yield of 72% was obtained. However, the decreased effectiveness of the inhibitory proteins may

also have been due to alterations in their activity during the different treatments.

Electrophoretic analysis of the proteins from a bacterial supernatant showed four major protein bands, corresponding to proteins with molecular masses of 100, 65, 45 and 24 kDa (Fig. 3, lane 2). The electrophoretic pattern of the bacterial supernatant treated with a mixture of celluloses showed a sharp decrease in the number of protein bands compared to the untreated supernatant (Fig. 3, lane 3); this is explained by the cellulose treatment eliminating a large number of extracellular cellulases. However, three major protein bands (100, 65 and 24 kDa) were not eliminated by the treatment and might therefore have been involved in the inhibitory effect of *R. flavefaciens* on the cellulolysis by the fungus. The inhibitory activity of the cellulose-treated supernatant could only have been due to the action of one or all three of the proteins. The disappearance of the 45 kDa major protein band from the cellulose-treated supernatants suggests that this protein corresponds to one of the bacterial hydrolases. Analysis of the proteins in the non-retained fraction in the anion-exchange column showed the presence of many protein bands, including two major bands corresponding to molecular masses of 65 and 45 kDa (Fig. 3, lane 4). The absence of any inhibitory activity of this protein pool against *N. frontalis* (Table 4) indicates that the 65 and 45 kDa proteins of the bacterial supernatant were not involved in the inhibitory effect. In contrast, the protein bands corresponding to molecular masses of 100 and 24 kDa, which were absent from the non-retained fraction, were found in large quantities in the protein pool eluted with 0.25 M-NaCl (Fig. 3, lane 5). Since this protein fraction, like the cellulose-treated bacterial supernatant, had inhibitory activity against the fungal cellulolysis, one of the two major protein bands, or both together, might correspond to the factor involved in the inhibition of the cellulolytic activity of *N. frontalis*.

Fig. 3. SDS-PAGE analysis of protein fractions. Lanes: 1, uninoculated medium; 2, supernatant of *R. flavefaciens* culture on filter paper; 3, supernatant of *R. flavefaciens* culture after treatment with a mixture of celluloses; 4, fraction of the bacterial supernatant not retained on the ion-exchange column; 5, fractions of the bacterial supernatant eluted from the anion-exchange column with 0.25 M-NaCl; 6–8, fraction of the bacterial supernatant precipitated with $(\text{NH}_4)_2\text{SO}_4$ at (6) 20%, (7) 40% and (8) 70% saturation; 9–10, fraction of the bacterial supernatant eluted from the anion-exchange column with (9) 0.75 M-NaCl and (10) 0.5 M-NaCl; 11, fractions of the bacterial supernatant retained by an ultrafiltration membrane having a cut-off of 50 kDa. The sizes of the bands were determined by comparing their position with those of standard proteins.

Table 5. Effect on the cellulose degradation by *N. frontalis* of the addition of the protein fraction of the supernatant of an *R. flavefaciens* culture, treated with a mixture of celluloses

The protein fraction was either precipitated sequentially by $(\text{NH}_4)_2\text{SO}_4$ or dialysed and then precipitated with $(\text{NH}_4)_2\text{SO}_4$. Cellulose degradation was measured 4 d after the addition of proteins.

Treatment of bacterial supernatant added	Cellulose degraded by <i>N. frontalis</i> (% of that degraded in control cultures)*
Cellulose-treated	78.6 ± 1.8
Fractionation of proteins by sequential precipitation with	
20 % saturated $(\text{NH}_4)_2\text{SO}_4$	83.2 ± 1.8
40 % saturated $(\text{NH}_4)_2\text{SO}_4$	75.3 ± 2.1
70 % saturated $(\text{NH}_4)_2\text{SO}_4$	94.4 ± 1.7
Dialysis and precipitation with	
60 % saturated $(\text{NH}_4)_2\text{SO}_4$	81.4 ± 8.4

* Each value represents the mean ± SD ($n = 3$).

Sequential precipitation and dialysis of the bacterial supernatant

To determine whether the expression of inhibitory activity required the presence of one or both of the 100 and 24 kDa proteins, the proteins were separated by two different techniques: sequential ammonium sulphate precipitation, and dialysis.

The greatest inhibition of fungal cellulolysis was observed with the protein fraction precipitated at 40 % ammonium sulphate saturation. The bacterial proteins precipitated at 20 % ammonium sulphate saturation also had an inhibitory effect, but those precipitated at 70 % saturation had only a slight inhibitory effect (Table 5). The electrophoretic profiles of the proteins precipitated at the three successive ammonium sulphate concentrations showed the presence of two major protein bands of 100 and 24 kDa (Fig. 3, lanes 6–8). The presence of these two protein bands suggested the existence of two proteins of very different molecular masses. These bands could also correspond to two subunits of the same protein, since electrophoresis was carried out in denaturing conditions. This protein might have been precipitated by the successive concentrations of ammonium sulphate, which would explain why its inhibitory activity was observed in the three fractions.

When proteins from a cellulose-treated bacterial supernatant were dialysed with a membrane having a cut-off of 50 kDa and then added to a 4-d-old fungal culture, there was a decrease in the cellulolytic activity of the fungus (Table 5). The factor responsible for the inhibitory effect of the bacterial supernatant was there-

fore present in the protein fraction that was retained. The analysis of this fraction by electrophoresis in denaturing conditions showed a sharp decrease in the number of protein bands compared with the protein pattern obtained for a supernatant adsorbed to cellulose but not dialysed. However, the major protein bands of 100 and 24 kDa were still present (Fig. 3, lane 11). The technique used was thus not able to separate these two protein bands, both of which may be involved in the inhibitory effect.

Discussion

This study confirms the existence of an interaction between *N. frontalis* and the cellulolytic bacterium *R. flavefaciens* in the degradation of cellulose observed previously (Bernalier *et al.*, 1988, 1992) and demonstrates the nature of the factor involved in the inhibition of fungal cellulolysis by *R. flavefaciens*.

When the supernatant of *N. frontalis* was transferred to a culture of *R. flavefaciens*, the amount of cellulose degraded in the bacterial culture rose by 25 %. This increase in cellulolysis was probably due to the activity of the fungal cellulases supplied by the supernatant of *N. frontalis*. The glucose released by the cellulolytic enzymes was not used by the strain of *R. flavefaciens* 007 studied (A. Bernalier, unpublished results), which explains why there was no stimulation of the bacterial growth. In contrast, the transfer of a small volume of a supernatant of a culture of *R. flavefaciens* to a culture of *N. frontalis* inhibited the cellulolytic activity of the latter (approximately 30 % less cellulose was degraded compared to untreated fungal culture). The fungal biomass was not influenced because when the bacterial supernatant was added, the fungal growth was almost at its highest.

The inhibition of fungal activity is caused by an extracellular protein released by the cellulolytic bacterium. Although the inhibitory factor was only partly characterized, we identified two proteins with molecular masses of 100 and 24 kDa which may have been involved in the antagonistic effect. The fact that the attempts to separate these two protein bands were unsuccessful suggests that the inhibitory factor might be a protein composed of two subunits. The inhibitory activity observed in the two major protein pools recovered from the anion-exchange column (fractions eluted with 0.25 and 1 M-NaCl) was probably due to the protein being progressively eluted as the salt concentration was increased. The weak inhibitory effect of the protein fractions may have been caused by the loss of a part of these proteins during purification or by a decrease in their activities in the course of the different treatments. However, the existence of two different protein fractions (eluted at 0.25 and 1 M-NaCl) having antagonistic effects

against fungal cellulolytic activity suggests that there were two distinct inhibitory proteins, able to act separately but achieving maximum activity when associated. Electrophoretic analysis of the protein pattern of the fraction eluted with 1 M-NaCl should indicate whether the inhibiting factor was the same as that in the 0.25 M fraction. Unfortunately, the amount of protein eluted with 1 M-NaCl was insufficient both to check the inhibitory effect and to do the electrophoretic analysis. The presence of the 100 and 24 kDa proteins in the fraction eluted with 0.25 M-NaCl is consistent with the possibility that the inhibitory protein is made up of two subunits. However, it is also possible that each of the suspected subunits comes from a different protein. If this were the case they would have molecular masses of 50 kDa or more since they were not separated by dialysis in which a membrane with a cut-off equal to this molecular mass was used.

The cellulase activity of *N. frontalis* was greatly inhibited by the presence of the bacterial protein, as evidenced by the zymogram analyses, but fungal growth was not affected. The bacterial supernatant in the incubation buffer of the gel inhibited fungal cellulases.

The mechanism by which the bacterial protein inhibits fungal cellulases was not elucidated. The fact that the extent of the inhibition of the cellulolytic activity of *N. frontalis* was the same after adsorption of the cellulases of *R. flavefaciens* on a mixture of celluloses clearly indicates that the inhibitory bacterial protein was not a cellulase. Since only a small amount of the bacterial supernatant was required to produce a great inhibitory effect, the factor could be an enzyme that acts on the fungal cellulase complex. However, this enzyme is probably not a protease since *R. flavefaciens* is not a proteolytic species (Stewart & Bryant, 1988). The inhibitory protein may have homologies with one of the enzymes that take part in the formation of the fungal cellulase complex and thereby prevent the formation of a functional hydrolytic complex, or it may have a closer affinity for one of the enzymes of the complex and specifically inhibit it. The action of this inhibiting factor on the cellulolysis of other cellulolytic micro-organisms (anaerobic bacteria or aerobic fungi) should be studied to determine the specificity of the protein for cellulases and to provide information on its mode of action. Little is known about the factors capable of inhibiting the cellulases of rumen anaerobic fungi and work has focused mainly on metals such as iron and mercury (Barichievich & Calza, 1990).

Our *in vitro* study shows that a dominant bacterial species of the rumen is capable of decreasing the cellulolytic activity of a rumen fungus that is very effective in degrading cellulose (Hebraud & Fèvre, 1988; Gordon & Phillips, 1989; Bernalier *et al.*, 1992).

Gnotobiotic lambs harbouring a defined cellulolytic flora (Fonty & Gouet, 1989) could be a suitable animal model to determine if this interaction occurs in the rumen.

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