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Original article

Effect of *Eubacterium limosum*, a ruminal hydrogenotrophic bacterium, on the degradation and fermentation of cellulose by 3 species of rumen anaerobic fungi

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Summary — The degradation and fermentation of cellulose filter paper were studied in axenic cultures of 3 species of rumen anaerobic fungi, *Neocallimastix frontalis, Piromyces communis* and *Caecomyces communis,* and in cocultures containing 1 of these fungal strains and *Eubacterium limosum*, a hydrogenotrophic rumen bacterial species. When *E limosum* was introduced into fungal cultures a slight decrease in fungal cellulolytic activity was observed. The end products of the fermentation of cellulose found in the cocultures were different from those found in the fungal monocultures. *E limosum* used formate and part of the hydrogen produced by the fungi and probably created a shift in the metabolism of the fungi resulting in a reduction of lactate and ethanol production.

rumen / anaerobic fungi / *Eubacterium limosum* / cellulose degradation / interactions

Résumé — Effet d'*Eubacterium limosum*, bactérie hydrogénotrophe du rumen sur la dégradation et la fermentation de la cellulose par 3 espèces de champignons anaérobies du rumen. *La dégradation et la fermentation de la cellulose de papier filtre ont été étudiées dans des cultures axéniques de 3 espèces de champignons anaérobies du rumen,* Neocallimastix frontalis, Piromyces communis *et* Caecomyces communis, *ainsi que dans des cocultures associant chacune de ces espèces à* Eubacterium limosum, *une espèce bactérienne hydrogénotrophe du rumen. Lorsque* E limosum *est introduit dans les cultures fongiques, on observe une légère diminution de l'activité cellulolytique des champignons. Le profil des métabolites finaux de la fermentation de la cellulose dans les cocultures est différent de celui observé dans les monocultures fongiques.* E limosum *a utilisé le formate et une partie de l'hydrogène produit par les champignons et a vraisemblablement dévié le métabolisme fongique, ce qui a conduit à une réduction de la production de lactate et d'éthanol.*

rumen / champignons anaérobies* / Eubacterium limosum / *dégradation de la cellulose / interactions

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INTRODUCTION

Rumen anaerobic fungi, which are particularly abundant in animals receiving forage-rich diets (Grenet *et al*, 1989), colonize the lignocellulosic tissues that are the most difficult to degrade (Bauchop, 1981). They have the enzymatic equipment necessary to degrade all polysaccharides in plant cell walls, except pectin (Fonty and Joblin, 1991). The hydrolysis of these polymers releases soluble compounds which can be used by non-hydrolytic microorganisms. Likewise, several end products of the fungal cellulose fermentation, such as formate and lactate, can serve as energy sources for other microbial species. For example, the fungi have been observed to interact with methanogenic bacteria (Bauchop and Mountfort, 1981; Fonty *et al*, 1988; Joblin *et al*, 1990; Marvin-Sikkema *et al*, 1990). This type of interaction, in which there is hydrogen transfer, increases the cellulolytic activity of the fungi and shifts their metabolism towards a greater production of acetate, thereby reducing that of formate, lactate and ethanol (Bauchop and Mountfort, 1981; Fonty *et al*, 1988; Marvin-Sikkema *et al*, 1990). The effect of *Selenomonas ruminantium*, another species able to use hydrogen, seems, in contrast, to depend on the strain used and on culture conditions. Marvin-Sikkeman *et al* (1990) observed an increase in fungal hydrolytic activity in the presence of this species, whereas in a study made by Bernalier *et al* (1991), fungal cellulolysis was inhibited in cocultures with *S ruminantium*. There are also complex interactions between the fungi and cellulolytic bacteria. It was observed *in vitro* that *Ruminococcus flavefaciens*, one of the major rumen cellulolytic species, inhibits the cellulolytic activity of *Neocallimastix frontalis* and *Piromyces* (*Piromonas*) *communis* (Bernalier *et al*, 1992, 1993; Stewart *et al*, 1992). The objective of this research was to study the interactions in cellulose degradation between 3 fungal species commonly encountered in the rumen, *N frontalis*, *P communis* and *Caecomyes* (*Sphaeromonas*) *communis*, and the rumen bacterium, *Eubacterium limosum*. Because of its physiological and metabolic characteristics, this bacterial species had the potential to interact with the fungi during cellulolysis; it is able to use various monosaccharides and is a non-methanogenic, hydrogenotrophic bacteria that uses hydrogen to produce acetate (Rode *et al*, 1991).

MATERIALS AND METHODS

Microorganisms

N frontalis MCH3 and *P communis* FL were isolated in our laboratory from sheep rumen contents, and *C communis* FG10 from the intestine of a cow (Bernalier *et al*, 1992). E *limosum* 20543 comes from the Deutche Sammlung Mikroorganismen.

Media and growth conditions

The methods used for the preparation of prereduced medium and anaerobic culture techniques were those described by Hungate (1969). Basal medium was that of Lowe *et al* (1985) as modified by Gay *et al* (1989). Complete media contained either cellobiose, glucose (0.5% wt/vol) or filter paper (Watman N°1) as a carbon and energy source. For the preparation of cellulose medium, pieces of filter paper (100 mg) were added to each tube before the addition of the prereduced basal medium (10 ml/tube). The culture media were kept under O_2-free CO_2 and dispensed in 10 or 100 ml volumes into CO_2-filled 16-ml screw-cap Hungate tubes (Bellco Glass Inc, Vineland, NJ), or into 125-ml serum bottles with rubber septa and sterilized at 120°C for 20 mn.

The fungi were maintained at 39°C by subculturing on filter paper every 3–4 d. Fungal ino-

cula for cellulose degradation in monocultures or cocultures were obtained as follows: fungi were grown on a cellobiose medium in serum bottles for 48 h, and the cultures were then filtered in an anaerobic glove-box through a 50 µm mesh nylon filter. The filtrate was centrifuged for 10 min at 1 000 *g*, and the resulting pellet resuspended in 5 ml sugar-free medium. The zoospores were then counted in a haemacytometer cell. The fungal monocultures and the cocultures were inoculated with 1 ml of a suspension of zoospores containing, on average, 10^3 to 10^4 zoospores. Bacterial inocula were composed of 0.5 ml of a 24-h-old culture on glucose medium in Hungate tubes.

Cocultures were performed by inoculating the bacterium 2 d after the fungus. For each incubation time, the experiments were carried out in triplicate, using 3 tubes of fungal monoculture.

Analytical methods

Cellulose degradation was determined by measuring the dry weight (DW) of the filter paper remaining in the tubes. The tubes were centrifuged for 15 min at 1 000 *g*. The pellet was treated with 1 ml of 1M NaOH, at 100°C for 10 min, and then rinsed with distilled water to discard any adhering microorganisms. Afterwards, it was dried at 80°C for 72 h and weighed. The supernatant was frozen and kept at –20°C before analysis of the end products of fermentation.

Volatile fatty acids, alcohol, hydrogen and carbon dioxide were analyzed by gas chromatography (Jouany, 1982). The volume of headspace gas was estimated by using gas-tight syringes. Formate and lactate were determined according to the Boehringer method (Boehringer Mannheim, France SA). The amount of reducing sugars remaining in the cultures was determining by the Miller method (Miller, 1959). All the analyses were carried out after 2, 4, 6, 8, 10 and 12 d of incubation.

RESULTS

E limosum exerted the same effect on all 3 fungal cultures. The addition of the bacterium to 48-h cultures of *N frontalis*, *P communis* or *C communis* decreased the amount of cellulose degraded by about 5–6% (fig 1). After *E limosum* was inoculated into fungal cultures, although cellulolysis continued, the reducing sugars no longer accumulated, which indicates that they were used by the bacterium (fig 2). (The evolution of the concentration of reducing sugars from cellulose followed the same pattern in all 3 cocultures, consequently only one example is shown in this paper, that of *N frontalis–E limosum*.)

In monoculture, whatever the fungal species, the end products of cellulose fermentation were formate, acetate, lactate, ethanol, hydrogen and CO_2. At the end of the period of incubation, formate remained undetected in all 3 cocultures (table I). Lactate, hydrogen and ethanol were found in lower concentrations than in the fungal monocultures. In contrast, acetate and CO_2 were present in greater concentrations. Butyrate, which is an end product of *E limosum,* was produced at 17 mM/100 mM fermented hexose units (table I). The kinetic analysis of the end products of cellulose fermentation (fig 3) showed that formate, formed by the fungi, decreased after the introduction of *E limosum*, which indicates the utilization of this metabolite by the bacterium. Once *E limosum* was inoculated, lactate and ethanol no longer accumulated in the cocultures, which is probably an indication of a change in the metabolism of the fungus. In addition, *E limosum* is able to utilize the hydrogen produced by the fungi, but in the coculture did so only to a limited extent, since the decrease in the concentration of the gas was minor (table I).

DISCUSSION

This study has reported a new example of interactions *in vitro* between rumen anaer-

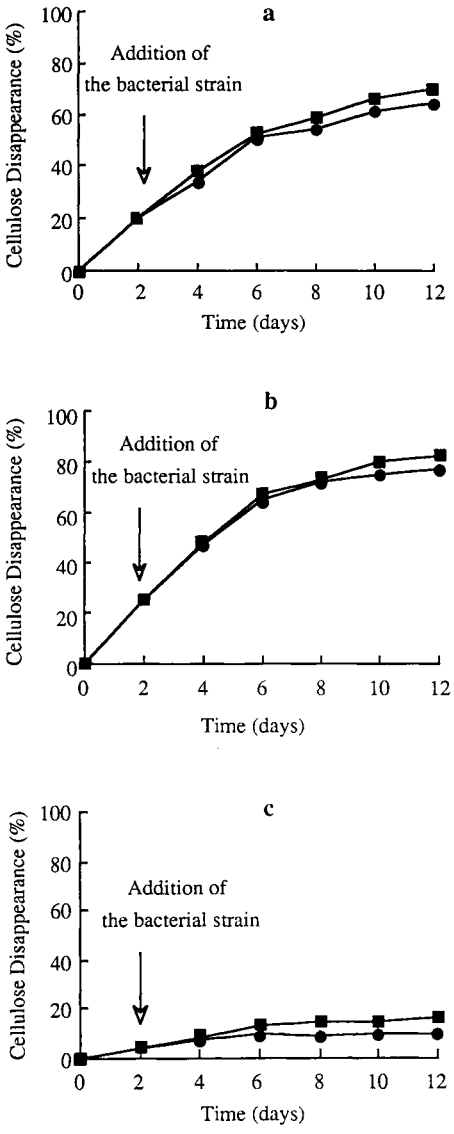

Fig 1. Kinetics of filter-paper degradation by the rumen anaerobic fungi in monocultures or cocultures with *E limosum*. a) (■—■) *N frontalis* MCH3, (●—●) *N frontalis* MCH3 + *E limosum* 20543; b) (■—■) *P communis* FL, (●—●) *P communis* FL + *E limosum* 20543; c) (■—■) *P communis* FG10, (●—●) *C communis* FG10 + *E limosum* 20543. Each point represents the mean of 3 cultures. Standard deviations were less than 5% of the means.

obic fungi and another hydrogenotrophic bacterial species. *E limosum* produced a small decrease in fungal hydrolytic activity. However, unlike methanogenic bacteria (Fonty and Joblin, 1991), and in some instances *Selenomonas ruminantium* (Marvin-Sikkema *et al,* 1990), *E limosum* did not produce an increase in the amount of cellulose degraded or in the rate of cellulolysis, but caused a slight inhibition of cellulolytic activity in all 3 fungal species. This effect was observed both when the bacterium was inoculated 2 d after the beginning of fungal culture, and also when the 2 microorganisms were inoculated simultaneously (Morvan B, Fonty G, Doré J, unpublished results). This difference of effect can be explained by the fact that *E limosum* did not totally metabolize the hydrogen produced by the fungi. In addition, it used the sugars released by the hydrolysis of cellulose, thereby depriving the fungi of their energy source, and probably slowing their growth. Unlike *Methanobrevibacter ruminantium*, which uses only hydrogen and formate (Stewart and Bryant, 1988), *E limosum* is a versatile species with a complex metabolism (Stewart and Bryant,

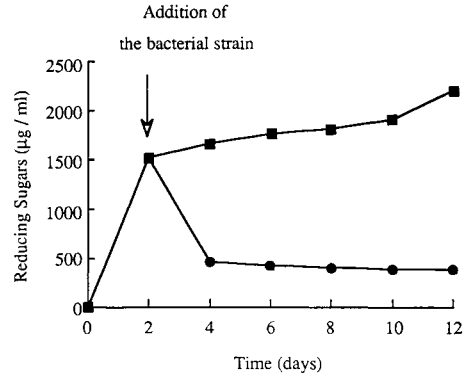

Fig 2. Evolution of the concentration of reducing sugars released from cellulose by *N frontalis* MCH3 in monoculture (■—■) and in coculture with *E limosum* 20543 (●—●). Each point represents the mean of 3 culltures. Standard deviations were less than 5% of the means.

Table I. End products of cellulose fermentation by 3 rumen anaerobic fungi in monoculture or coculture with *Eubacterium limosum*[a].

	mmol products / 100 mmol hexose units fermented						
Fermentation products	N frontalis *MCH3*[b]	P communis *FL*[b]	C communis *FG10*[b]	E limosum *20543*[c]	N frontalis *MCH3* + E limosum *20543*[b]	P communis *FL* + E limosum *20543*[b]	C communis *FG10* + E limosum *20543*[b]
Formate	71.3	75.7	84.2	0.0	0.0	0.0	0.0
Acetate	45.5	36.5	60.7	85.3	67.0	69.6	126.1
Butyrate	0.0	0.0	0.0	49.4	17.0	17.4	17.1
Lactate	45.5	44.2	56.0	0.0	23.1	14.8	12.2
Ethanol	45.0	49.3	27.0	0.0	14.4	3.5	0.0
H_2	41.1	47.4	59.3	0.0	29.3	35.3	59.5
CO_2	34.7	34.2	37.6	90.0	102.1	101.4	157.4
%C recovery	70.5	69.0	77.5	76.4	63.7	60.7	80.7

[a] Fermentation products were analyzed after 12 d of incubation. The figures represent the mean number for 3 different experiments; [b] hexose units from cellulose; [c] hexose units = glucose; the percentage of C recovery does not take into account the cell carbon.

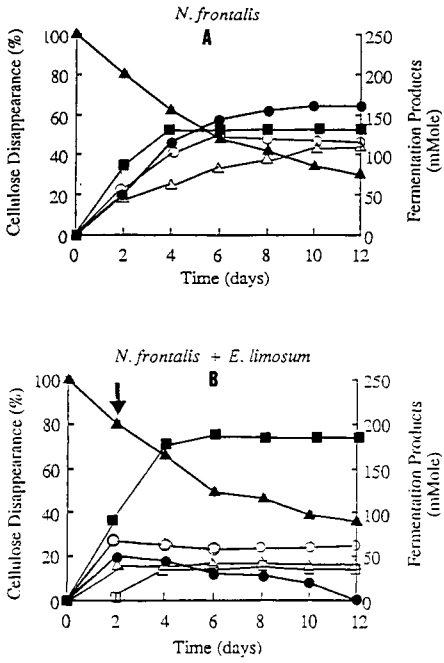

Fig 3. Kinetics of filter paper degradation (▲) and production of formate (●), acetate (■), lactate (○), ethanol (Δ) and butyrate (□) in (a) the monoculture of *N frontalis* MCH3 and (b) the coculture *N frontalis* MCH3–*E limosum* 20543. (→) = Addition of the bacterial strain. Each point represents the mean of 3 cultures.

1988). It is capable of obtaining its energy from a wide variety of substrates produced by the fungi, such as hydrogen, formate and the sugars released from the hydrolysis of cellulose. It is not known which strategy *E limosum* adopts in the use of these different compounds, but it is probably governed by catabolite repression. Diauxie was observed when this species was cultivated in the presence of glucose and H_2/CO_2 (Genthner and Bryant, 1982, 1987). Therefore, in cocultures with fungi, *E limosum* will preferentially metabolize glucose. Since *E limosum* may potentially use glucose, formate and hydrogen, it is difficult to compare the fermentative profiles of the cocultures and those of the bacterial monocultures made on glucose. The end products of the fermentation of *E limosum* differ according to the substrate it degrades. With formate and hydrogen, Loubière *et al* (1987) observed a homoacetic fermentation. The fermentation of glucose leads to the formation of acetate and butyrate (Genthner and Bryant, 1987). Lactate and ethanol concentrations were lower in cocultures than in fungal monocultures. Since *E limosum* is not known to use these compounds, the difference can only be explained by a decrease in their production by the fungi. This observation, associated with the increase in acetate concentration in the cocultures, might be the result of a shift in fungal metabolism owing to the effect of *E limosum*. However the respective part of the 2 microorganisms in acetate production in the cocultures is not known. The mechanism involved may be similar to that described by Rode *et al* (1981) in cocultures of *Lachnospira multiparus* and *E limosum*, in which the absence of ethanol was due to interspecies hydrogen transfer. In cocultures with fungi, *E limosum*, by using hydrogen, may allow the reoxidation of reduced coenzymes (NADH) by H_2 production, thereby leading to the synthesis of acetate, rather than by ethanol and lactate production (Wolin and Miller, 1988). However, the shift observed in the fungal metabolism with *E limosum* is less pronounced that than observed with methanogens (Bauchop and Mountfort, 1981; Fonty *et al*, 1988; Bernalier *et al*, 1991).

This new example of interactions illustrates the complexity of the relationships between microorganisms in the rumen ecosystem. *In vivo*, *E limosum* is in competition with methanogens for the use of H_2/CO_2 and formate. However, since methanogenic bacteria have a greater affinity for hydrogen, and since the utilization of hy-

drogen by *E limosum* is repressed in the presence of glucose, the competition favors the methanogenic bacteria. However, the ability of *E limosum* to tolerate the low pH levels and high osmolarity found in the rumen with certain diets may enhance its competitiveness (Genthner and Bryant, 1987).

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