Comparison of growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores during cultivation in a defined medium

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(Received 19 November 1990; revised 22 February 1991; accepted 15 March 1991)

Anaerobic fungi were isolated from rumen fluid of a domestic sheep (*Ovis aries*; a ruminant) and from faeces of five non-ruminants: African elephant (*Loxodonta africana*), black rhinoceros (*Diceros bicornis*), Indian rhinoceros (*Rhinoceros unicornis*), Indian elephant (*Elephas maximus*) and mara (*Dolichotis patagonum*). The anaerobic fungus isolated from the sheep was a *Neocallimastix* species and the isolates from non-ruminants were all species similar to *Piromyces* spp. A defined medium is described which supported growth of all the isolates, and was used to examine growth characteristics of the different strains. For each fungus the lipid phosphate content was determined after growth on cellobiose and the resulting values were used to estimate fungal biomass after growth on solid substrates. The ability of isolates from ruminants and non-ruminants to digest both wheat straw and cellulose was comparable. More than 90% and 60%, respectively, of filter paper cellulose and wheat straw were digested by most strains within 60–78 h. Growth of two fungi, isolated from rumen fluid of a sheep (*Neocallimastix* strain N1) and from faeces of an Indian rhinoceros (*Piromyces* strain R1), on cellobiose was studied in detail. Fungal growth yields on cellobiose were 64.1 g (mol substrate)⁻¹ for N1 and 34.2 g mol⁻¹ for R1. The major fermentation products of both strains were formate, lactate, acetate, ethanol and hydrogen.

Introduction

Anaerobic fungi inhabit defined regions of the alimentary tract of herbivorous mammals. Orpin (1975, 1976, 1977) first recognized that many of the flagellates present in ovine rumen fluid were zoospores of anaerobic fungi. Since then anaerobic fungi have been isolated both from ruminants, e.g. sheep, cattle, macropod marsupials (Bauchop, 1979; Orpin & Joblin, 1988; Barr, 1988), and from faeces of non-ruminants, e.g. horse (Orpin, 1981), elephant, zebra (Milne *et al.*, 1989).

On morphological grounds anaerobic fungi were classified in the Class *Chytridiomycetes* (Heath *et al.*, 1983), but on the basis of the ultrastructural characteristics of the zoospores, they were assigned to the new family *Neocallimasticaceae* (Barr, 1988). Gold *et al.*

(1988) suggested a subdivision of this family into the three genera containing monocentric species – *Neocallimastix*, *Piromyces* (previously *Piromonas*) and *Caecomycetes* (previously *Sphaeromonas*) – which at that time were the only legitimately described genera of anaerobic fungi. Subsequently polycentric species have been described, e.g. *Orpinomyces bovis* (Barr *et al.*, 1989) and *Neocallimastix joyonni* (Breton *et al.*, 1989). Up to now, six anaerobic fungi isolated from foregut fermenters (sheep, cow) have been classified, namely: *Neocallimastix patriciarum* (Orpin & Munn, 1986), *Neocallimastix frontalis* (Heath *et al.*, 1983), *Neocallimastix joyonni* (Breton *et al.*, 1989), *Piromyces communis* (Orpin, 1977), *Caecomycetes communis* (Orpin, 1976, 1988) and *Orpinomyces bovis* (Barr *et al.*, 1989). The latter however does not conform to the proposed generic description. Only one fungal species from a non-ruminant (horse) has been named, *Caecomycetes equi* (Gold *et al.*, 1988).

Abbreviation: DM, dry matter.

Zoospores of anaerobic fungi invade freshly ingested plant tissue (Orpin & Bountiff, 1978), and the vegetative stadia closely associate with, and grow at the expense of, plant particles (Lowe *et al.*, 1987b). The anaerobic fungi produce a range of cell-bound and extracellular polysaccharide-degrading enzymes which participate in the digestion of food particles (Lowe *et al.*, 1987b; Williams & Orpin, 1987). The role of anaerobic fungi in the alimentary tract is not yet clear. The rumen fungi are the initial colonizers of plant fragments (Bauchop, 1979) and pure cultures *in vitro* digest up to 43% of wheat straw on a dry matter basis (Lowe *et al.*, 1987b). Little is known about the digestion of wheat straw and cellulose by anaerobic fungi inhabiting non-ruminant herbivores.

This study was performed to compare the growth characteristics of anaerobic fungi isolated from ruminant and non-ruminant herbivores. A defined medium was developed to study the digestion of filter paper cellulose and wheat straw by newly isolated anaerobic fungi from non-ruminants and other already described species isolated from a ruminant, the sheep.

Methods

Source of organisms. Rumen fluid was collected from a fistulated sheep, fed on a grass hay-concentrate diet, at the farm of the University of Nijmegen. Fresh faeces from the non-ruminant animals were gathered into plastic bags from the floor of the mammal enclosure at London Zoo (Regent's Park, London, UK) and the Zoo at Whipsnade (UK). *Neocallimastix patriciarum* (N2), *Piromyces communis* (P1), *Piromyces* sp. (P2), *Caecomyces communis* (S1) (Orpin, 1975, 1976, 1977) isolated from the rumen of sheep and a *Piromyces* sp. (P3) isolated from faeces of a horse (Orpin, 1981) were obtained from the fungal collection of the AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK.

Culture conditions. Two media were used to culture anaerobic fungi: complex medium M1, which contained rumen fluid, and a defined medium M2. Salt solutions A and B were used in medium preparation. Solution A contained (g l⁻¹): KH₂PO₄, 3.0; (NH₄)₂SO₄, 3.0; NaCl, 6.0; MgSO₄, 0.6; CaCl₂, 0.6; solution B contained K₂HPO₄, 3 g l⁻¹.

Complex medium M1 was modified from Kemp *et al.* (1984) and had the following composition: solution A, 150 ml; solution B, 150 ml; centrifuged rumen fluid (Orpin, 1977), 200 ml; Bactocasitone (Difco), 10 g; yeast extract (Oxoid), 2.5 g; NaHCO₃, 6 g; L-cysteine.HCl, 1 g; fructose, 2 g; xylose, 2 g; cellobiose, 2 g; resazurin solution (0.1%, w/v), 1 ml; agar (for solid medium), 8 g; deionized water to 900 ml. The medium was equilibrated with oxygen-free CO₂ and dispensed in 9.0 ml volumes into 16.5 ml screw-top Hungate culture tubes (Bellco Glass). After sealing, the tubes were evacuated and gassed with N₂/CO₂ (80:20, v/v). The medium was then autoclaved for 20 min at 115 °C. After cooling, 0.1% (v/v) vitamin solution 1 was added. Vitamin solution 1 contained (g l⁻¹): thiamin.HCl, 0.10; riboflavin, 0.20; calcium D-pantothenate, 0.60; nicotinic acid, 1.00; nicotinamide, 1.00; folic acid, 0.05; cyanocobalamin, 0.20; biotin, 0.20; pyridoxine.HCl, 0.10; *p*-aminobenzoic acid, 0.01. In isolation procedures 0.1% (v/v) antibiotic solution was added to the medium. It contained chloramphenicol (5 g l⁻¹) and ampicillin (10 g l⁻¹).

The defined medium M2 contained: solution A, 150 ml; solution B, 150 ml; cellobiose, 5 g; NaHCO₃, 6 g; L-cysteine.HCl, 1 g; trace elements solution, 10 ml; haemin solution, 10 ml; resazurin solution (0.1%, w/v),

1 ml; demineralized water to 950 ml. The medium was dispensed in 19.0 ml volumes in 50 ml serum bottles and sealed with butyl rubber stoppers and aluminium crimp caps (D. Prins BV, Schipluiden, The Netherlands). Where specified, the cellobiose was replaced by 0.1 g filter paper cellulose (Whatman no. 1) or wheat straw. The composition of wheat straw was determined by the proximate method of Goering & Van Soest (1970). It contained, on a dry matter basis, 93% organic matter, 47% cellulose, 31% hemicellulose, and 11% lignin. After sterilization of the medium, 0.1% (v/v) vitamin solution 2 was added. The trace element and haemin solutions were prepared as described by Lowe *et al.* (1985). Vitamin solution 2 contained (mg l⁻¹): thiamin.HCl, 5; riboflavin, 5; calcium D-pantothenate, 5; nicotinic acid, 5; folic acid, 2; cyanocobalamin, 1; biotin, 1; pyridoxine.HCl, 10; *p*-aminobenzoic acid, 5. No antibiotics were included in medium M2.

Isolation procedures. Antibiotics solution 1 was included in medium M1 during the isolation procedures until isolates were pure and free from bacteria. Especially the chloramphenicol was shown to be effective in eradication of methanogens and mycoplasmas which can contaminate anaerobic fungal isolates (Kudo *et al.*, 1990).

The rumen fungi were isolated from serial dilutions (10⁻¹–10⁻⁵) of strained rumen fluid in M1 broth without substrate. Subsequently, 1.0 ml of each dilution was inoculated in Hungate tubes with molten M1 agar at 44 °C. After 3 d incubation at 39 °C, individual colonies from dilution tubes with fewer than 20 colonies were transferred with a sterile Pasteur pipette to M1 broth. After 2 d the culture fluid was again diluted and the procedure was repeated until the isolates were pure and free of bacteria.

Anaerobic fungi from non-ruminants were isolated from collected faecal samples by cultivation in M1 broth in Hungate tubes. The fungi were isolated from the culture fluid after 3 d incubation at 39 °C using the procedures described for isolations from strained rumen fluid.

Culture conditions. Stock cultures were maintained on 0.1 g milled wheat straw in 19 ml medium M2. Cultures were inoculated with 1 ml of culture fluid (containing zoospores), and subcultured every 3–4 d. Stock cultures were preserved anaerobically at –80 °C or in liquid nitrogen in medium M2 with 5% (v/v) dimethylsulphoxide (DMSO) as cryoprotectant. The DMSO (0.25 ml) was added anaerobically to 5 ml of 3 d-old cultures of anaerobic fungi and the cultures were subsequently stored at –80 °C. For storage over longer periods (more than 6 months) cultures were placed in liquid nitrogen after 1 d at –80 °C. Frozen cultures were resuscitated by rapid thawing at 39 °C and inoculating in fresh medium M2 with cellobiose as substrate.

Inocula (5%, v/v) for growth experiments on cellobiose, wheat straw and cellulose were cultured for 3 d at 39 °C in medium M2 with cellobiose as substrate. Growth of the fungi was monitored by measuring hydrogen production. After growth had stopped, triplicate cultures were harvested and fermentation products, fungal biomass and substrate utilization were determined.

Sample collection. Cultures were harvested on glass-fibre filters (grade C, Whatman), washed twice with 20 ml deionized water, and freeze-dried overnight. The dried filters with fungus and substrate residue were stored at –20 °C. Samples of the culture fluid (filtrate) were stored at –20 °C for later analysis of water-soluble sugars and fermentation products. The biomass produced by growth on soluble sugars was determined from dry weight measurements. Growth of the fungus on wheat straw and filter paper cellulose was estimated from the lipid phosphate content. Phosphate release by perchloric acid digestion of the phospholipid extract was correlated with the fungal dry matter (DM) (White *et al.*, 1979). The lipid phosphate content of wheat straw cultures was corrected for the background lipid phosphate level of wheat straw [0.034 mg phosphate (g DM)⁻¹]. The correlation of biomass and lipid phosphate content was determined for each fungus after growth on cellobiose in medium M2.

Determination of digestion of wheat straw and cellulose. The extent of digestion of cellulose and wheat straw was calculated by subtracting the estimated fungal biomass from the total mass of the fraction collected after growth. For wheat straw, values were also corrected for dry weight losses due to non-microbial solubilization of the material which occurred during preparation and incubation of the medium ($15.1\% \pm 0.5\%$).

Analytical procedures. Formic acid was determined by the colorimetric method of Sleat & Mah (1984). Other fermentation products were determined by GLC using the method of Teunissen *et al.* (1989) with a Hewlett Packard HP 5890A gas chromatograph. The column was packed with GP 10%-SP1200 on Chromosorb WAW/1% H_3PO_4 (Supelco). Hydrogen in 0.5 ml of head-space gas samples was quantified with a Hewlett Packard HP 5890A gas chromatograph fitted with a thermal conductivity detector and a column packed with 80–100 mesh Porapak Q (Supelco). Ethane was used as internal standard and hydrogen as external standard.

Soluble sugars were quantified by enzymic methods. Glucose was determined using glucose oxidase and peroxidase (Bergmeyer & Bernt, 1974). The same assay was used to determine cellobiose after enzymic hydrolysis with β -D-glucoside glucohydrolase (EC 3.2.1.21) (Russell & Baldwin, 1978).

Results

Isolation of anaerobic fungi from rumen fluid and faeces

Anaerobic fungi could be isolated, using medium M1, from faeces of six of the ten species of animal surveyed (Table 1). The fungi could also be isolated from faeces stored for up to 8 h in a plastic bag at room temperature (longer storage periods were not tested). All the isolates grew in medium M1 in which the carbohydrates were replaced by wheat straw as substrate. One fungus was isolated from the rumen fluid of a sheep; this species formed monocentric sporangia borne on an extensive network of branched rhizoids; the zoospores possessed up to 20 flagella. All fungi isolated from faeces of the non-ruminants produced zoospores with not more than four flagella (generally one or two), and monocentric sporangia borne on an extensively branched rhizoid.

Growth of anaerobic fungi on cellobiose in the complex medium M1 and the defined medium M2

The anaerobic fungi were isolated and cultivated in complex medium M1. A defined medium in which all isolated fungi grew well was obtained by omitting the yeast extract, Bactocastone and rumen fluid from medium M1. Although haemin was necessary for optimal growth, vitamin solution 2, which contained 100–1000 times less vitamins than vitamin solution 1, ensured good growth of the fungi. All the newly isolated strains grew in medium M2 with cellobiose or wheat straw as substrate. Most of the strains from the fungal collection of the AFRC Institute could be grown in this medium. However, *Piromyces* strain P3 and *Caecomycetes communis* strain S1 failed to grow in medium M2.

Growth on cellobiose in defined medium M2 and determination of the lipid phosphate content of the different strains

The different strains were cultured on cellobiose in medium M2 and growth was monitored by measurement of H_2 production. After H_2 production ceased the fungi were harvested and the lipid phosphate content was determined for each strain (Table 2). At this time significant autolysis of the fungi occurred (see Fig. 1). *Piromyces* strains E1 (isolated from African elephant) and R3 and R4 (isolated from an Indian rhinoceros) grew slowly and the amount of fungal biomass formed was relatively low. The *Neocallimastix* strain isolated from the sheep rumen (N1) and the AFRC sheep rumen isolates (*Neocallimastix* strain N2 and *Piromyces* strains P1 and P2) produced more hydrogen and yielded more fungal DM than the isolates from the hindgut fermenters. The lipid phosphate content of each fungus was calculated and varied between 1.97 and 3.51 mg lipid phosphate per g fungal DM. The lipid phosphate content

Table 1. Isolation of anaerobic fungi from the faeces and rumen fluid of herbivorous mammals

Anaerobic fungi were not isolated from faecal samples of the following animals: African wood elephant (*Loxodonta cyclotis*), white rhinoceros (*Ceratotherium simum*), hippopotamus (*Hippopotamus amphibius*) and pygmy hippopotamus (*Choeropsis liberiensis*).

Animal	Place of isolation	Major diet	Code	Fungal type
African elephant (<i>Loxodonta africana</i>)	Whipsnade	Clover hay, concentrate	E1	<i>Piromyces</i>
Indian elephant (<i>Elephas maximus</i>)	London Zoo	Clover hay, concentrate, vegetables	E2	<i>Piromyces</i>
Indian elephant (<i>Elephas maximus</i>)	Whipsnade	Clover hay, concentrate, vegetables	E3	<i>Piromyces</i>
Black rhinoceros (<i>Diceros bicornis</i>)	London Zoo	Clover hay, concentrate	R1	<i>Piromyces</i>
Black rhinoceros (<i>Diceros bicornis</i>)	Whipsnade	Clover hay, concentrate, vegetables	R2	<i>Piromyces</i>
Indian rhinoceros (<i>Rhinoceros unicornis</i>)*	Whipsnade	Clover hay, concentrate, vegetable	R3/R4	<i>Piromyces</i>
Mara (<i>Dolichotis patagonum</i>)	Whipsnade	Grass, hay	M1	<i>Piromyces</i>
Sheep† (<i>Ovis aries</i>)	Nijmegen	Grass, hay	N1	<i>Neocallimastix</i>

* Isolated from faeces from one animal.

† A ruminant; all other animals are non-ruminants.

Table 2. Hydrogen production, biomass, and lipid phosphate content of different strains of anaerobic fungi after growth in defined medium M2 with cellobiose as substrate

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

Strain	Incubation time (h)	Hydrogen production (mmol l ⁻¹)†	Fungal DM (g l ⁻¹)	Lipid phosphate (mg l ⁻¹)	Lipid phosphate content [mg PO ₄ ³⁻ (g DM) ⁻¹]
E1	92	7.11 $\pm$ 0.80	0.24 $\pm$ 0.02	0.72 $\pm$ 0.06	2.94 $\pm$ 0.30
E2	120	5.82 $\pm$ 0.74	0.24 $\pm$ 0.01	0.70 $\pm$ 0.02	2.92 $\pm$ 0.14
E3	92	4.98 $\pm$ 0.42	0.39 $\pm$ 0.02	1.09 $\pm$ 0.05	2.79 $\pm$ 0.18
M1	90	6.75 $\pm$ 0.38	0.33 $\pm$ 0.02	0.95 $\pm$ 0.10	2.88 $\pm$ 0.03
N1	90	9.11 $\pm$ 0.21	0.57 $\pm$ 0.00	1.40 $\pm$ 0.01	2.46 $\pm$ 0.04
N2*	92	9.04 $\pm$ 0.16	0.52 $\pm$ 0.02	1.36 $\pm$ 0.15	2.60 $\pm$ 0.39
P1*	120	8.63 $\pm$ 0.26	0.45 $\pm$ 0.01	1.06 $\pm$ 0.04	2.32 $\pm$ 0.06
P2*	90	8.10 $\pm$ 0.02	0.40 $\pm$ 0.01	1.05 $\pm$ 0.07	2.60 $\pm$ 0.11
R1	92	8.11 $\pm$ 0.03	0.37 $\pm$ 0.01	0.95 $\pm$ 0.06	2.58 $\pm$ 0.22
R2	144	5.28 $\pm$ 0.63	0.40 $\pm$ 0.03	0.79 $\pm$ 0.09	1.97 $\pm$ 0.26
R3	166	5.38 $\pm$ 0.98	0.26 $\pm$ 0.05	0.79 $\pm$ 0.04	3.06 $\pm$ 0.26
R4	148	5.21 $\pm$ 0.40	0.25 $\pm$ 0.01	0.84 $\pm$ 0.04	3.36 $\pm$ 0.29

* Strains from the fungal collection of the AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, UK; N2 is *Neocallimastix patriciarum*; P1 is *Piromyces communis*; P2 is *Piromyces* sp.

† Hydrogen production measured in the head space was normalized to the volume of liquid in the culture bottles.

Table 3. Growth and digestion of cellulose and wheat straw by several anaerobic fungi after cultivation in defined medium M2

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

Strain*	Wheat straw				Filter paper			
	Incubation time (h)	Period of growth (h)†	Fungal DM (g l ⁻¹)‡	Digestion (%)	Incubation time (h)	Period of growth (h)†	Fungal DM (g l ⁻¹)‡	Digestion (%)
E1	168	148	0.11 $\pm$ 0.01	41 $\pm$ 2	164	145	0.05 $\pm$ 0.02	17 $\pm$ 2
E2	144	105	0.23 $\pm$ 0.02	68 $\pm$ 0	90	60	0.23 $\pm$ 0.01	96 $\pm$ 1
E3	92	78	0.20 $\pm$ 0.03	60 $\pm$ 1	92	60	0.20 $\pm$ 0.02	97 $\pm$ 0
M1	95	54	0.22 $\pm$ 0.02	52 $\pm$ 3	90	60	0.33 $\pm$ 0.01	90 $\pm$ 2
N1	95	78	0.36 $\pm$ 0.06	68 $\pm$ 2	95	60	0.50 $\pm$ 0.05	97 $\pm$ 1
N2	92	78	0.45 $\pm$ 0.09	64 $\pm$ 3	164	65	0.50 $\pm$ 0.09	95 $\pm$ 1
P1	120	114	0.27 $\pm$ 0.03	61 $\pm$ 1	120	90	0.43 $\pm$ 0.03	96 $\pm$ 1
P2	144	115	0.09 $\pm$ 0.05	30 $\pm$ 3	144	136	0.17 $\pm$ 0.05	38 $\pm$ 1
R1	95	78	0.22 $\pm$ 0.02	65 $\pm$ 1	94	60	0.33 $\pm$ 0.02	94 $\pm$ 1
R2	144	140	0.18 $\pm$ 0.01	62 $\pm$ 1	240	150	0.43 $\pm$ 0.05	88 $\pm$ 4
R3	168	130	0.16 $\pm$ 0.01	60 $\pm$ 1	168	120	0.16 $\pm$ 0.03	53 $\pm$ 4
R4	168	135	0.15 $\pm$ 0.03	53 $\pm$ 4	168	122	0.21 $\pm$ 0.02	61 $\pm$ 5

* Strains are listed in Table 1 and in the first footnote of Table 2.

† The period of growth was estimated from the H₂ production curves.

‡ Estimated from lipid phosphate contents of residues.

of *Neocallimastix* strain N1 and *Piromyces* strain R1 remained constant during the period of fast growth and autolysis.

Growth of the anaerobic fungi on wheat straw and filter paper cellulose in defined medium M2

The ability of the different strains to grow on wheat straw and filter paper cellulose was examined. Wheat

straw and filter paper cellulose digestion correlated with hydrogen production (data not shown). For each fungus the duration of growth was estimated from hydrogen production curves. Fungal growth and digestion of filter paper cellulose and wheat straw was determined after hydrogen production stopped (Table 3). All values were corrected for DM losses due to non-microbial solubilization during preparation and incubation of the medium. Most strains digested more than 60% of the wheat straw,

Fig. 1. (a, b) Growth of *Neocallimastix* strain N1 (a) and *Piromyces* strain R1 (b) in defined medium M2 containing 0.5% (w/v) cellobiose as substrate. $\square$, Fungal dry matter; $\blacklozenge$, cellobiose; $\bullet$, glucose. (c, d) Formation of end-products by strains N1 (c) and R1 (d) during growth on cellobiose in defined medium M2. $\circ$, Formate; $\bullet$, lactate; $\blacktriangle$, acetate; $\blacksquare$, ethanol; $\square$, hydrogen. Hydrogen was measured in the headspace and normalized to the volume of liquid in the culture bottles. Each value represents the mean of three replicates. Bars in (a) and (b) represent the SD. ($n = 3$); in (c) and (d) the standard deviations were all less than 10% of the mean.

but *Piromyces* strains E1 and P2 digested only 41% and 30%, respectively. After a lag period between 6 and 38 h, the period during which growth on wheat straw occurred varied between 54 and 148 h. Strains digesting wheat straw most rapidly were *Neocallimastix* strains N1 and N2, and *Piromyces* strains E2 and R1.

All strains were able to use filter paper as a substrate. The differences between the strains that were observed during the digestion of wheat straw followed the same pattern with filter paper cellulose, and were even more pronounced. Most strains digested the substrate almost completely, although the period of growth of the different strains varied between 60 and 145 h. As found

with wheat straw, *Piromyces* strains E1 and P2 digested less filter paper cellulose than the other isolates. The strains digesting more than 90% of the cellulose within a growth period of approximately 60 h were *Neocallimastix* strain N1 and *Piromyces* strains E2, E3 and R1.

Growth on cellobiose in defined medium M2

The growth of *Neocallimastix* strain N1 (isolated from a ruminant) and *Piromyces* strain R1 (isolated from a non-ruminant) was studied in more detail. Growth of the two strains in defined medium M2 with cellobiose as substrate was followed during a time-course study (Fig. 1a, b).

After a lag period of 8 h *Neocallimastix* strain N1 started to grow and fungal dry matter increased to a maximum of 0.82 g l^{-1} 48 h after inoculation (Fig. 1a). During this period 91% of the cellobiose was used and the growth yield was $64.1 \text{ g (mol substrate)}^{-1}$. During the period of fast growth (8–48 h) a small amount of glucose (0.16 g l^{-1}) temporarily accumulated. After exhaustion of the substrate the fungal DM decreased rapidly to approximately 70% of the maximal value at 76 h. From this time on the fungal DM remained stable.

After a short initial lag period, the fungal dry weight of *Piromyces* strain R1 increased rapidly between 21 and 52 h and reached a maximum of 0.49 g l^{-1} at 52 h (Fig. 1b). During this period of fast growth 98% of the cellobiose was used and the growth yield was $34.2 \text{ g (mol substrate)}^{-1}$. Glucose accumulated transiently in this period to a maximum of 0.43 g l^{-1} after 52 h; by 100 h most of the glucose had been used and only 0.08 g l^{-1} could be detected in the medium. By 65 h after inoculation the cellobiose had been utilized and the fungal DM of strain R1 had fallen to, and thereafter remained at, approximately 70% of the maximal value.

Fermentation products

Growth of both fungi on cellobiose resulted in a mixed acid type of fermentation with formate, acetate, lactate, ethanol and hydrogen as end-products (Fig. 1c, d). No C_3 – C_6 volatile fatty acids or C_1 – C_5 alcohols other than ethanol could be detected.

Formic and lactic acid were the major end-products of strain N1, reaching 24.0 and 21.6 mM, respectively, 75 h after inoculation (Fig. 1c). During the same period, acetic acid and ethanol increased to 14.1 and 7.8 mM, respectively. Hydrogen, normalized to the volume of liquid in the culture bottles, increased to 9.7 mM.

The fermentation of cellobiose by *Piromyces* strain R1 produced formic acid as the major end-product (22.9 mM) (Fig. 1d). Lactic and acetic acids were formed in equimolar amounts, reaching 14.9 and 14.4 mM, respectively, 76 h after inoculation. During the period of growth ethanol increased to 8.6 mM and hydrogen to 7.5 mM.

Comparison of growth in complex medium M1 and defined medium M2 with cellobiose as substrate

The omission of the complex compounds and the more diluted vitamin solution had no effect on the growth yield of either *Neocallimastix* strain N1 or *Piromyces* strain R1. The fermentation pattern was slightly influenced by the omission of the complex compounds. In medium M1 *Neocallimastix* strain N1 produced 90% more ethanol, 30% more hydrogen and 15% less formate whereas

Piromyces strain R1 grown in medium M1 produced 200% more ethanol, 20% more hydrogen and 15% less formate than when grown in medium M2. Acetate and lactate production was the same in both media.

Discussion

This study describes the isolation of anaerobic fungi from several herbivores, ruminant as well as non-ruminant. Failure to isolate anaerobic fungi from four of the ten surveyed animal species may have been because the faeces were too old rather than because these animals lacked anaerobic fungi. Milne *et al.* (1989) showed that no fungi could be isolated from sheep faecal pellets after storage for 1 d under the conditions used in this study. All of the anaerobic fungi isolated from non-ruminants were *Piromyces*-like organisms, i.e. the vegetative stage consisted of a monocentric sporangium with branched rhizoid; the released zoospores possessed not more than four flagella (Kudo *et al.*, 1990). The isolation of a fungal species of the genus *Caecomyces* from a non-ruminant (horse) has been reported previously (Gold *et al.*, 1988). The fungus we isolated from the rumen of a sheep was of the *Neocallimastix* type: it formed monocentric sporangia and an extensive network of branched rhizoids; the zoospores possessed numerous flagella (Heath *et al.*, 1983; Orpin & Munn, 1986). All *Neocallimastix* species so far described have been isolated from ruminants: from sheep (Orpin, 1975; Heath *et al.*, 1983; Lowe *et al.*, 1985; Phillips & Gordon, 1989), cattle (Orpin, 1988), reindeer, camel and antelope (Orpin & Joblin, 1988; Milne *et al.*, 1989).

Defined media have great advantages over complex media. For cultivation of anaerobic fungi, complex media containing rumen fluid, yeast extract, protein digests, etc., have been described (Bauchop, 1979; Kemp *et al.*, 1984; Phillips & Gordon, 1989), but until now only two defined media have been described (Lowe *et al.*, 1985; Orpin & Greenwood, 1986). Many of the new isolates grew slowly in the medium of Lowe *et al.* (1985) and several isolates did not grow in the medium of Orpin & Greenwood (1986) (data not shown). The major advantage of defined medium M2 was that it ensured good growth of all the new isolates. However, a *Piromyces* species (P3) and *Caecomyces communis* (S1) from the AFRC collection failed to grow. These strains have been cultivated for a long time in a complex medium (Kemp *et al.*, 1984) and may have become dependent on complex nutrient factors. Haemin was necessary for optimal growth of the anaerobic fungi, as was found for *Neocallimastix patriciarum* by Orpin & Greenwood (1986). Compared to the defined medium of Lowe *et al.* (1985), defined medium M2 contains no

volatile fatty acids, a 25- to 40-fold lower concentration of vitamins, and a buffer composition which is normally used for the cultivation of rumen micro-organisms. Defined medium M2 differs from the defined medium of Orpin & Greenwood (1986) in that trace elements and more vitamins were added.

Several methods can be used to determine growth of micro-organisms on solid substrates. Hydrogen and formate production can be used to determine anaerobic fungal growth (Pearce & Bauchop, 1985; Lowe *et al.*, 1987a); however comparative growth yield measurements cannot be made as the formation of these end-products is substrate dependent. The lipid phosphate content of micro-organisms has been used to determine the mass of micro-organisms during growth on solid substrates or in natural environments (White *et al.*, 1979). The lipid phosphate content of the anaerobic fungi studied here varied between 1.97 and 3.36 mg phosphate per g DM. Comparison with literature values is hampered because it is unclear if values are expressed on basis of lipid phosphorus or lipid phosphate (Kemp *et al.*, 1984; Orpin & Greenwood, 1986). Direct biomass estimations of fungi grown with wheat straw as a substrate are always influenced by residual substrate. Values for wheat straw and cellulose digestion were adjusted on the assumption that the lipid phosphate contents of fungi grown on soluble and insoluble substrates are the same.

The degradation of cellulose and wheat straw by rumen anaerobic fungi has been reported before (Bauchop, 1979; Pearce & Bauchop, 1985; Lowe *et al.*, 1987b; Gordon & Phillips, 1989). Anaerobic fungi were isolated on cellulose and wheat straw by Milne *et al.* (1989), but digestion of these substrates was not quantified. All the anaerobic fungi tested in this study, isolated from both ruminants and non-ruminants, were able to use cellulose and wheat straw. The strains with the highest capacity for cellulose and wheat straw digestion were isolated from sheep, Indian elephant, and black rhinoceros. Only two of the tested strains showed low cellulose and wheat straw digestion. This study demonstrated that anaerobic fungi from ruminants and non-ruminants have similar abilities to degrade cellulose and wheat straw.

Although $\leq 97\%$ of cellulose was digested by the anaerobic fungi only $\leq 68\%$ of wheat straw was digested. This is probably due to the high lignin content of the wheat straw used (11%). The maximum wheat straw digestion of 68% observed is considerably higher than the 43–45% DM loss previously reported for *Neocallimastix* species (Gordon & Phillips, 1989; Lowe *et al.*, 1987b). The low substrate loading used in this study (0.05%, w/v) compared to that (0.1%) in both other studies may account for this. Furthermore, wheat straw degradation is likely to be higher than the reported loss of

DM due to formation of fungal DM, but this cannot account completely for the difference. Strains with low cellulolytic capacity also had a low wheat straw digestion capacity. However, the digestion of wheat straw by these strains was higher than the digestion of filter paper cellulose; this may indicate that wheat straw hemicellulose is more easily degraded than cellulose.

Growth yields of the two fungi studied in detail were equal in medium M1 and medium M2, suggesting that the Bactocasitone, yeast extract and rumen fluid contain no compounds which contribute to growth yields. *Piromyces* strain R1 is the first anaerobic fungus isolated from a non-ruminant whose fermentation pattern has been described. Its major fermentation products were similar to those formed by *Neocallimastix* strain N1 and other rumen anaerobic fungi grown on carbohydrates (Orpin & Munn, 1986; Lowe *et al.*, 1987a).

The growth of *Neocallimastix* strain N1 and *Piromyces* strain R1 on cellobiose in medium M2 showed a pattern which was typical for anaerobic rumen fungi (Lowe *et al.*, 1987a; Phillips & Gordon, 1989). The growth of both fungi appeared to be linear during the entire growth phase. For most fungi, initial growth is exponential and usually followed by a longer period of declining growth rate which can appear as linear growth (Griffen, 1981). To answer the question if this is also the case for the isolates examined here, more frequent sampling and synchronization of growth are required. The autolysis of the fungi after substrate exhaustion that we observed was also found for other anaerobic fungi (Lowe *et al.*, 1987a; Phillips & Gordon, 1989).

Although the growth yield of *Neocallimastix* strain N1 was double that of *Piromyces* strain R1, the accumulation of fermentation products was nearly identical. Although succinate and malate could only be detected in trace amounts for both strains (data not shown), unidentified end-products or the accumulation of storage carbohydrates, as was shown to occur during growth on cellobiose (Phillips & Gordon, 1989), may have caused the observed difference in growth yield. The lower growth yield of strain R1 is comparable to the yields reported for *Neocallimastix* and *Piromyces* species (Phillips & Gordon, 1989).

During the fast growth of the two tested anaerobic fungi on cellobiose small amounts of glucose were found to accumulate transiently, presumably because the rate of sugar release by extracellular enzyme action was greater than the rate of utilization for growth. β -Glucosidase activity has been found in the medium during cultivation of anaerobic fungi on several substrates including cellulose, cellobiose, glucose and wheat straw (Pearce & Bauchop, 1985; Gordon & Phillips, 1989; Lowe *et al.*, 1987b). It is not known if all cellobiose is hydrolysed extracellularly or partly hydrolysed extra-

cellularly and partly transported across the membrane and hydrolysed intracellularly.

The contribution of anaerobic fungi to forage fibre digestion *in vivo* is not yet clear. Orpin & Bountiff (1978), supported by Bauchop (1979), suggest that anaerobic fungi are the initial colonizers of forage fibre in the rumen. *In vivo* anaerobic rumen fungi may represent up to 8% of the microbial biomass (Orpin, 1983/1984). Since they are very active in the digestion of a wide range of cell wall polymers (Lowe *et al.*, 1987*b*; Williams & Orpin, 1987) it is likely that they have a significant role in the rumen. This study has demonstrated that pure cultures of anaerobic fungi from foregut-fermenting and, in particular, hindgut-fermenting herbivores, are fibrolytic and able to degrade cellulose and wheat straw *in vitro*. The fungal biomass in non-ruminants *in vivo* and its contribution to forage fibre digestion in the animal remain to be studied.

This research was supported by grants from the Technology Foundation (STW) and the Ministry of Economic Affairs of The Netherlands.

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