Special Properties of Polycentric Anaerobic Fungus *Anaeromyces mucronatus*

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Abstract

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The aim of this study was differentiation of polycentric rumen fungus of genera *Anaeromyces* using modern methods of molecular biology. Six polycentric anaerobic fungi isolated from cow, lama and bison were identified as *Anaeromyces* strains and analyzed for variability in internal transcribed spacers and fibrolytic activities. RFLP of both ITS1 and ITS1-4 fragments digested by restriction enzyme *DraI* separated studied strains into two groups with different cleavage profiles. Representatives of each group exhibited different hydrolytic enzyme activities. The largest differences were recorded in production of xylanase, β-endoglucanase and β-glucosidase. The possibility of using RFLP analysis of ITS regions to distinguish fungal isolates has been proved, however the results do not illuminate what kind of distinction is reflected in genetic variability of ITS regions.

Rumen, fungi, anaerobic, cellulose, fiber, endoglucanase

Anaeromyces mucronatus was originally described from a Holstein cow in France (Breton et al. 1990) as the third polycentric anaerobic rumen fungal species besides *Orpinomyces* and *Ruminomyces* genera. However, *Ruminomyces elegans* has been reassigned to *Anaeromyces elegans* in 1993 (Ho et al. 1990, 1993).The morphological differences among polycentric genera *Orpinomyces* and *Anaeromyces* are minimal and may vary under different culture conditions. Moreover, these species tend to lose their ability to produce sporangia in artificial media and therefore pure culture cannot be identified with absolute certainty (Barr et al. 1995).

The development of molecular biological techniques in the last decade has enabled the new approach to the characterization of fungi. Li and Heath (1992) studied relationship of gut fungi based on ITS1 sequences and found that *Anaeromyces* isolates were more distant from other rumen fungi. The whole DNA sequences showed above 80% similarity among *Piromyces*, *Neocallimastix* and *Orpinomyces*, whereas the similarities between *Anaeromyces* and these three genera were only 70%.

Despite the fact that fibrolytic enzymes of anaerobic rumen fungi have been studied intensively, there are no available data describing *Anaeromyces* cellulolytic and xylanolytic activities.

The aim of our experiment was to determine the feasibility of using RFLP analysis of ITS fragment to distinguish among species rumen fungi assigned to *Anaeromyces* genera. In this paper six *Anaeromyces* strains isolated from different animals were studied using RFLP analysis of ITS1 and ITS 1-4 spacer. We present here also for the fist time endoglucanase and xylanase activities of *Anaeromyces* isolates.

Materials and Methods

Organisms and culture conditions

Polycentric anaerobic fungi were isolated by the method of Joblin (1981) from faeces of domestic ruminants

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and herbivores kept in the Prague ZOO. Strain K1 originates from cow, strains LG1 and LG2 from *Lama guanaco*, strains Zu1 and Zu2 from European bison, strain Alp2 originates from *Lama alpaca*. Strains with the same sign (LG or Zu) but different number were isolated from the same animals with the lapse of time. Fungi were maintained anaerobically at 39 °C on the medium M10 (Caldwell and Bryant 1966) enriched by 20% (v/v) of rumen fluid. Glucose (4g/l), cellobiose (4g/l) or microcrystalline cellulose (4g/l) was used as a carbon source. Subculturing of isolates was performed every three days to maintain fungal viability.

Morphological observations and metabolic study

In-two-day cultures, thalli were observed by fluorescence microscopy (Fluoval 2, Carl Zeiss, Jena, F.R.G) using bisbenzimidin (5mg/l) as staining solution. Nuclei fluorescence was examined using transmitted-light microscope fitted with an exciter filter (G 355) and barrier filter (465) as described by Gaillard et al. (1989).

End fermentation products were determined by gas chromatography using column Chromosorb WAW (200 mm, 3 mm ID) and FID detector.

Enzyme assays

Activities of xylanase and β-endoglucanase (CM-cellulase) were measured in the course of reducing sugars release from soluble xylan extracted from oat spelts and carboxymethylcellulose according to the method of Lever (1977). Substrates for an estimation of cellobiohydrolase, β-xylosidase and β-glucosidase activity were 4-nitrophenyl-β-D-cellobioside, 4-nitrophenyl-β-D-xylopyranoside and 4-nitrophenyl-β-D-glucopyranoside (Hodrová et al. 1999)

DNA isolation, PCR and RFLP of ITS fragment

Fungal genomic DNA was extracted using the method of Graham et al. (1994), resuspended in distilled water and stored at -35 °C. ITS 1 region was amplified from genomic DNA by PCR using primers ITS 1 (5`- TCC GTA GGT GAA CCT GCG G –3`) and ITS 2 (5`- GCT GCG TTC TTC ATC GAT GC –3`), ITS 1-4 region was amplified with primers ITS 1 and ITS 4 (5`- TCC TCC GCT TAT TGA TAT GC –3`). The PCR reaction was performed using kit Readymix^{TM} Redtaq^{TM} PCR reaction mix (Sigma, U.S.A). Approximately 50 ng genomic DNA were used as template for each amplification. The temperature conditions were as follows: initial denaturation at 94 ^{o}C for 4 min, followed by 35 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 42 ^{o}C for 30 s and extension at 72 ^{o}C for 1 min. Final step was carried out at 72 ^{o}C for 5 min.

The PCR products were digested with enzyme *DraI* (at 37 °C for 2 h) and restriction fragments were resolved in TBE buffer on 3% agarose gel with ethidium bromide at 40V for 4 h. The pUC18 *MspI* Digest (Sigma, U.S.A) ladder was used as molecular weight standard.

Results and Discussion

Characterization of isolates

All six strains K1, LG1, LG2, Zu1, Zu2 and Alp2 observed by fluorescence microscopy after staining with bisbenzimide exhibited extensive highly branched thallus (Plate I, Fig. 1) and large hyphae with constrictions (Fig. 2) typical for *Anaeromyces* genera. Both figures show multinucleate filamentous rhizomycelium with exogenous development of sporangia. The production of zoospores has not been observed in this study.

No differences among studied strains were measured in production of volatile fatty acids. All six strains had similar mixed-acid fermentation profiles where acetate was the main end product regardless of utilized substrates (glucose, cellobiose or cellulose).

Restriction polymorphism of the intergenic regions

Digestion of the ITS fragments by restriction endonuclease could distinguish among studied *Anaeromyces* isolates. Fig. 3 (Plate II) shows that splitting of ITS1 as well as ITS1-4 spacers by enzyme *DraI* produced the same cleavage profiles for strains Zu1, LG1 and K1 different from restriction pattern of strains Zu2, LG2 and Alp2. The approximate length of ITS restriction fragments is summarized in Table 1. These results demonstrate that PCR-generated RFLP analysis of the intergenic rDNA region is a reliable technique to distinguish among isolates of rumen fungi of the same genera, however our results do not answer a question which differences these analysis reflect. Study of larger number of strains belonging to *Anaeromyces* genera of different geographical origin is necessary to succeed to solve this problem.

Table 1
RFLP profiles resulting from *DraI* digestion of ITS fragments

	ITS 1		ITS 1-4	
Strains	LG1, Zu1, K1	LG2, Zu2, Alp2	LG1, Zu1, K1	LG2, Zu2, Alp2
Band sizes (bp)	175, 62, 30	105, 62, 50, 30	237, 188, 108, 80	230, 102, 63, 40

Fibrolytic activities

Selected isolates were tested for their fibrolytic activities (Table 2). Representative of first group was isolate Zu1 and from the second group were chosen isolates Alp2 and LG2. Second group was characterized by high β-endoglucanase, xylanase and β-glucosidase activity while isolate Zu1 showed higher activities of cellobiohydrolase and β-xylosidase. Obtained results contribute to enzyme characterization of polycentric anaerobic fungi reported by Ho et al. (1994) and open a scope for reflection of probability that both groups of Anaeromyces isolates have different strategy for fiber degradation. The first group is focused on oligosaccharides and the second one on plant structural polysaccharides. Both groups can cooperate in fiber degradation in the rumen environment.

Table 2
Hydrolytic activities of *Anaeomyces* isolates. Fungi were clustered to groups according to their genetic properties.

	Group1	Group2	
	Zu1	Alp2	LG2
	μg saccharide·ml^{-1}·h^{-1}		
β-endoglucanase	32.6 ± 1.2	124.5 ± 4.5	286.0 ± 6.9
Xylanase	37.0 ± 2.2	166.3 ± 4.8	237.4 ± 3.7
Cellobiohydolase	29.6 ± 0.9	6.8 ± 0.8	14.9 ± 1.1
β-glucosidase	1.0 ± 0.6	140.7 ± 4.6	107.4 ± 6.2
β-xylosidase	135.1 ± 5.1	80.9 ± 3.3	88.0 ± 2.5

Rozdílné vlastnosti izolátů polycentrické anaerobní houby *Anaeromyces mucronatus*

Šest anaerobních polycentrických hub bylo izolováno z tráveniny krávy, lamy a bizona. Tyto izoláty byly identifikovány jako kmeny rodu *Anaeromyces.* U všech kmenů byla analyzována podobnost ITS (intergenic transcribed spacer) fragmentů a stanovena fibrolytická aktivita. Na základě RFLP (restriction fragment length polymorphism) ITS fragmentů štěpených restrikční endonukleasou *Dra I* byly testované kmeny rozděleny do dvou skupin. Zástupci obou skupin vykazovaly rozdílné aktivity hydrolytických enzymů. Největší rozdíly byly pozorovány v aktivitách xylanasy, β-endoglukanasy a β-glukosidasy. Bylo znovu prokázáno, že ITS fragmenty mohou být využity pro taxonomické účely a aspoň jedna skupina našich izolátů představuje nový druh anaerobní houby.

Acknowledgements

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Plate I
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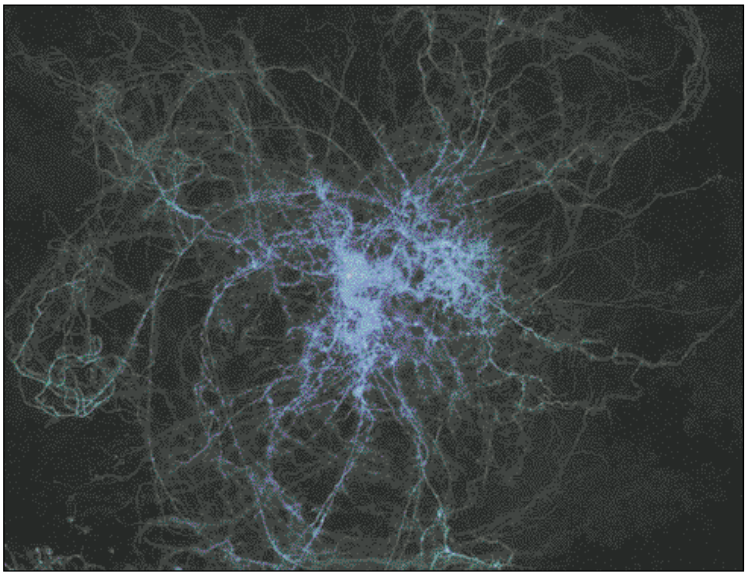

Fig. 1. Negative photograph of *Anaeromyces* sp., Zu1 branched mycelium. Nuclei were stained with bisbenzimidin.

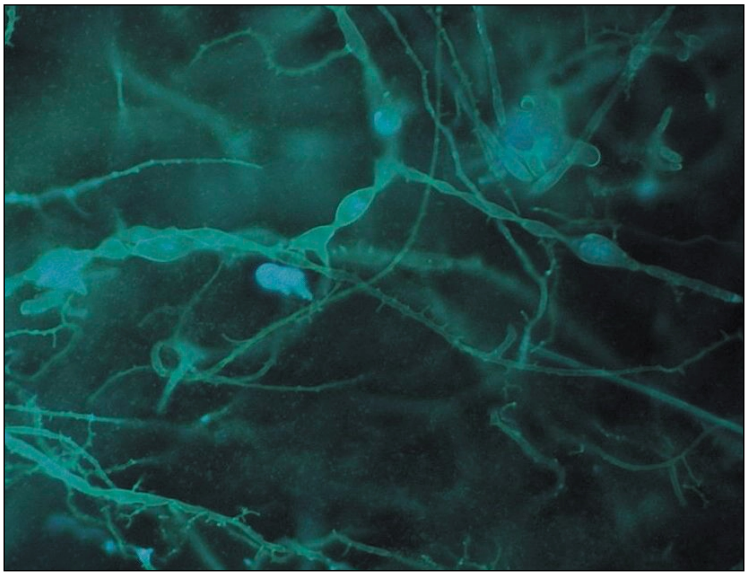

Fig. 2. Negative photograph of *Anaeromyces* sp., Zu2. mycelium with developed constrictions.

Plate II

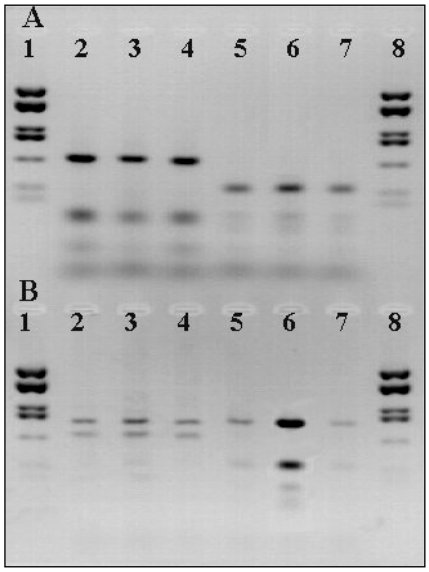

Fig. 3. Gel electrophoresis of ITS RFLP analysis. A – ITS 1, B –ITS 1-4; 2 – LG1, 3 – Zu1, 4 – K1, 5 – LG2, 6 – Zu2, 7 – Alp2; 1 and 8 – DNA ladder (pUC18 *MspI* Digest contains the following visible fragments: 501, 489, 404, 353, 242, 190 and 147pb).