ACTINOMYCES RUMINICOLA G10 - THE RUMEN BACTERIUM RECOVERED FROM GLYCEROL ENRICHED CULTIVATION MEDIA

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Abstract: Gradual increasing of glycerol concentration up to 10% using sheep ruminal fluid as an inoculum for in vitro cultivation was accompanied by significant changes in bacterial population as documented by DGGE analysis. The resulting bacterial consortium was composed of three dominant bacteria with *Actinomyces* related bacterium to be predominant. Upon cultivation on media with glycerol as a sole carbon source a single bacterium was cultivated from this consortium. Isolate G10 was found to be anaerobic, Gram-positive rod-shaped bacterium. Phylogenetic analysis based on 16S rRNA gene sequences showed that G10 isolate is related to the *Actinomyces ruminicola* species (97.7% of similarity). The role of rumen actinobacteria is largely unknown and their participation in glycerol utilization (tolerance) has not been described yet. The G10 bacterium and related consortium could be possibly used to improve glycerol tolerance and uptake by ruminants.

Key words: rumen bacteria, sheep, glycerol, *Actinomyces ruminicola*

1. Introduction

Biofuels are nowadays prospective replacement for the classic fossil energy sources which inventory is slowly declining. The most widely produced biofuel is a biodiesel, which is produced from vegetable oils and ethanol produced from plant polysaccharides derived from corn and sugar cane (PATZEK *et al*., 2004). During the biofuels production many secondary products are generated whose disposal is not easy. Such products include glycerol, which occurs mainly in the production of biodiesel from rapeseed and flaxseed oil. Excess glycerol can become environmental problems because it cannot be destroyed naturally in the environment, and its storage and processing is very costly therefore to be should ways to be processed further (ARECHEDERRA and MINTEER, 2008). One of glycerol disposal options is its use as a source of carbon and energy source for the industrial microbiology. Another possibility is the use of glycerol as a feed component for ruminants. The digestive tract of ruminants possesses the unique microbial ecosystem, which allows them to transform the plant food into microbial biomass. In vitro experiments have shown that the addition of glycerol to the diet has no deleterious effect on ruminants. It has been shown that the microorganisms present in the forestomach of ruminants can tolerate elevated concentrations of the glycerol in the feed, but only up to a certain concentration (RÉMOND *et al*., 1993; PARSONS *et al*., 2009; DROUILLARD, 2009; LEE *et al*., 2011).

DOI 10.2478/nbec-2013-0004

The aim of our study was to characterize the effect of glycerol supplementation on composition of rumen microflora *in vitro* and to identify the bacterial species able to grow at high glycerol concentration.

2. Materials and methods

2.1 Origin sample, isolation and identification

The sample of the ruminal fluid was taken from the digestive tract of sheep (Slovenské merino breed) at conventional diet without any glycerol supplementation and analyzed for the presence of cultivable bacteria. To 1 ml of rumen fluid 10 ml of sterile cultivation M2 (HOBSON, 1969) medium were added in anaerobe box-Bactron (Sheldon Manufacturing Inc., USA) under atmosphere formed by an anaerobic gas mixture AMG (Anaerobic mixed gas) consisting of 5% carbon dioxide, 5% hydrogen and 90% nitrogen and cultivated for 48 hours at 37°C. Resulting bacterial consortium was transferred (50µl) every 24 hours to liquid medium M2 with gradually increasing concentration of glycerol (1%, 5%, 7.5%, 10%). The bacterial population from the highest concentration of glycerol was inoculated on M2 agar plates without glycerol and cultivation was conducted under anaerobic conditions for 24-48h. After repeated subcultivations and control for the purity, the isolates were identified by Gram staining and on the basis of colony characteristic.

2.2 DNA isolation and PCR amplification

The total DNA from bacterial cultures was extracted by GenElute™ Bacterial Genomic DNA Kit (SIGMA-ALDRICH, USA). Isolated DNA was used as a template for PCR amplification of 16S rRNA gene fragments. All PCR reactions were performed in a 50 µl PCR mixture containing 1 µl of genomic of DNA, 1 x PCR buffer, 2 mmol/l $MgCl_2$, 1 µl of a 200 µmol/l of each dNTP, 1,25 U Platinum Taq DNA polymerase (Invitrogen, CA USA) and 25 pmol each primer using MJ Mini thermal cycler (Bio-Rad Laboratories, USA).

In the first round of PCR universal primers fD1 (5´-AGA GTT TGA TCC TGG CTC AG-3´) and rP2 (5´-ACG GCT ACC TTG TTA CGA CTT-3´) (WEISBURG *et al*., 1991) were used to amplify a 1500 bp region of 16S rDNA gene. PCR conditions were as follows: 94°C for 5 min followed by 35 cycles of 94°C for 1 min, 52°C for 1 min, 72°C for 1 min 30 sec, 72°C for 5 min. The obtained 16S rDNA fragments were subsequently used as a template for the second round of PCR using specific bacterial primers GC-clamp-968f (5´- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC - 3´) and 1401r (5´- CGG TGT GTA CAA GAC CC – 3´) (NÜBEL *et al*., 1996).

The cycling conditions were 94°C for 5 min, 9 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min; 14 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min followed by final extension at 72°C for 10 min. PCR products were detected by 1.2% agarose gel electrophoresis containing ethidium bromide.

2.3 Sequence and phylogenetic analysis

Amplified PCR fragments (either amplified 16S rRNA of G10 isolate or DGGE band) were purified by purification kit (Wizard SV Gel and PCR Clean-Up system, Promega) cloned into plasmid vector (InsTAclone PCR Cloning Kit, Thermo Scientific) and subsequently sequenced using Sanger didexy sequencing method using plasmid specific primers at GATC Biotech sequencing facility (GATC Biotech AG, Konstanz, Deutschland). 16S rRNA sequences were subsequently subjected to BlastN analysis at http://www.ncbi.nlm.nih.gov. The sequences of type strains of *Actinomyces* spp. were downloaded from GenBank database, aligned using clustalw algorithm, and phylogenetic tree was constructed using minimum-evolution method. For all phylogenetic analyses software MEGA 5 (TAMURA *et al*., 2011) was used.

The sequence of 16S rRNA gene of G10 isolate was deposited in the GenBank database under the accession number KC866613.

2.4 DGGE analysis

PCR products generated with GC-clamp-968f and 1401r primers were subjected to DGGE analysis. DGGE was performed using DCodeTM Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA USA). The round II PCR reaction products in a total volume of 45 µl were loaded onto 8% (w/v) polyacrylamide gel (40% Acrylamide-Bis 37.5:1) in 1 x TAE (40 mM Tris, 20 mM acetate, 1mM EDTA) containing a linear denaturing gradient ranging from 30 - 60% denaturant (100% denaturant solution consists of 7 M urea and 40% formamide). Electrophoresis was run for 17h at a constant voltage of 50V and a temperature 60°C. After electrophoresis, the gel was incubated for 20 min in ethidium bromide (0.5 µg/ml), rinsed for 20 min in distilled water, and photographed with UV transillumination using a Gel Logic Imaging System (Carestream, NY USA).

3. Results and discussion

The rumen fluid used in our work came from the rumen of sheep (Slovenske merino breed) on regular diet (forage to concentrate ratio 70:30). By cultivation in the liquid M2 cultivation medium in the presence of increased concentration of glycerol (1%, 5%, 7.5%, 10%) substantial changes in the composition of bacterial population were observed using DGGE method (Fig. 1). Variability of inoculum continually decreased and dominant species practically disappeared and were replaced by bacterial strains undetectable in original inoculum. Gradually increased concentrations of glycerol significantly decreased variability of population and bacterial consortium growing at 10% glycerol consisted of three dominant bacterial species. No significant changes in the composition of bacterial community were observed upon repeated subculturing at 10% glycerol (data not shown). Dominant species represented by band marked G10 (Fig. 1) represented approximately 34% of the total of bacterial population.

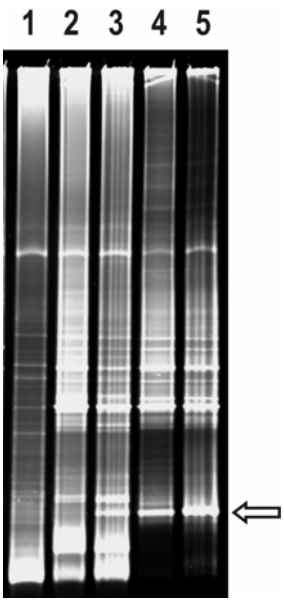

Fig. 1. DGGE analysis of ruminal fluid sheep inoculum growing in the presence of increasing glycerol concentrations. 1 - mixed bacterial culture from the rumen of sheep; 2 - bacterial culture growing at 1% glycerol; 3 - bacterial culture growing at 5% glycerol; 4 - bacterial culture growing at 7.5% glycerol; 5 - bacterial culture growing at 10% glycerol. The arrow indicates the band corresponding to G10 isolate.

Sequence analysis of this band showed that this band shows the highest similarity to *Actinomyces ruminicola* (96%). Bacterial consortium growing at 10% glycerol was tested for the presence of cultivable bacteria. Cultivation on M2 agar without glycerol yielded one type of isolate only. By Gram staining and on the basis of cell morphology, the isolate was identified as Gram-positive and rod-shaped bacterium. The isolate was designated as G10 and subsequently characterized. The complete identity was observed between sequence of G10 DGGE band and 16S rRNA sequence of G10 isolate. Phylogenetic analysis showed that 16S rRNA sequence of G10 isolate reveals the highest similarity (97.7% at 16S rRNA level) to *A. ruminicola*, indicating that G10 isolate is possibly a representative of a new, yet undescribed anaerobic rumen bacterium belonging to the *Actinomyces* genus. Phylogenetic tree showed that our sequence form together with *A. ruminicola* species a separate branch within *A. dentalis* cluster (Fig. 2). Based on this comparison our sequences belong to the *A. ruminicola* species.

The genus *Actinomyces* consists of a heterogeneous group of anaerobic and facultative anaerobic, asporogenous, Gram-positive, non-acid-fast, rod-shaped organisms. Of about 40 species of this genus occur worldwide as commensals and/or pathogens of man and other animals (HANSEN *et al*., 2009). *A. ruminicola* species was originally isolated from the rumen of cattle. Bacteria of *A. ruminicola* species hydrolyse xylan and starch, ferment some mono-, di- and oligosaccharides and

produce formic, acetic and lactic acids as end fermentation products of glucose (AN *et al*., 2006). The role of rumen actinobacteria or *A. ruminicola* is largely unknown and their participation in glycerol utilization (tolerance) has not been described yet.

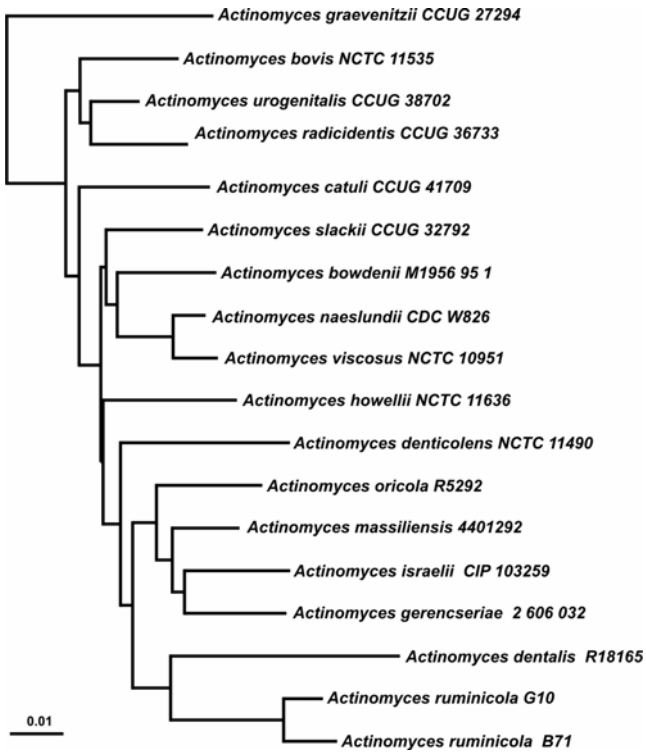

Fig. 2. Phylogenetic tree showing phylogenetic relatedness of *A. ruminicola* G10 isolate based on 16S ribosomal RNA sequence comparison. The scale bar represents 0.01 substitutions per nucleotide site.

The G10 bacterium and related consortium could be possibly used to improve glycerol tolerance and uptake by ruminants. Research has shown that feeding glycerol up to a rate of 10% of the daily dietary dry matter has no effect on feed intake or performance in finishing beef cattle or lactating dairy cows (KRUEGER *et al*., 2010). However, in most experiments fermentation capacity of the whole rumen microbial population was accessed without attempts to analyze the possible changes in rumen microbial population composition. Predominant glycerol utilizing bacteria are not known and in feeding experiments in vivo it was shown that facultatively anaerobic bacteria appear to play little part in glycerol fermentation in the sheep rumen. Amongst the most important members of the glycerol-fermenting flora are strict anaerobes of the group *Selenomonas ruminantium* var. *lactilytica* (HOBSON and MANN, 1961). ABU *et al*., (2011) showed that glycerol substitution had no effects on pH, ammonia nitrogen concentration, and dry matter digestibility *in vitro* fermentations using cow rumen fluid as an inoculum. The numbers of *Butyrivibrio*

fibrisolvens, *Clostridium proteoclasticum*, and *S. ruminantium* decreased under high level glycerol concentration diet but no differences for *Ruminococcus albus* and *Succinivibrio dextrinosolvens* among diets with different glycerol concentrations were observed. Authors suggest that substituting corn with glycerol at low level (up to 4%) had no adverse effects on fermentation, digestion or ruminal bacteria population. Higher substitution levels, however, may adversely affect rumen fermentation through reducing fiber digestion, acetate production and bacterial populations. Other experiments, both *in vitro* and *in vivo*, will be necessary to understand the effect of glycerol supplementation on rumen microbial population.

4. Conclusions

A bacterial consortium able to grow in the presence of 10% glycerol was prepared from the rumen sheep inoculum. The consortium is composed of 3 bacterial species from which single only, *Actinomyces ruminicola* G10 is cultivable under *in vitro* conditions.

Acknowledgements: This publication is the result of the project No. 26220220152 implementation supported by the Research & Development Operational Programme funded by the ERDF.

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