O. Berezina, V. V. Zverlov, S. Rief, R. Einspanier, W. H. Schwarz

Institute of Molecular Genetics RAS, Kurchatov Sq. 123182 Moscow, Russia, Institute of Physiology, Technische Universität München, Weihenstephaner Berg 3 and Research Group Microbial Biotechnology, TUM, Am Hochanger 4, D-85350 Freising-Weihenstephan, Germany
e-mail: zverlov@hotmail.com

Bacterial diversity in the rumen of cows fed transgenic maize

Aims and objectives

Comparison and characterization of microflora from the rumen of cows fed with silage of two maize lines, isogenic and transgenic for the *Bacillus thuringiensis* toxin gene; control for possibility of the Bt-gene transfer from transgenic maize to rumen bacteria.

1. Isolation of total DNA from rumen contents of cows, fed with transgenic and isogenic maize; amplification of 16S rDNA using general oligonucleotide primers for bacteria; construction of 16S rRNA gene libraries and sequencing of 16S rRNA genes, computer aided analysis of the sequence data; revelation of possible alterations in rumen-microflora in cows fed with transgenic and isogenic maize on the basis of differences between artificial phylogenetic groups of 16S rRNA genes.

2. PCR-monitoring for possibility of *Bacillus thuringiensis* toxin gene transfer from transgenic maize to rumen bacteria using primers for Bt-gene and HotStarTaq DNA Polymerase.

DNA isolation from cows fed transgenic and isogenic maize

In cooperation with Mayer (BLT-Grub, Germany) adult cows were fed isogenic and transgenic maize silage (*Antares* / *Novares* resp.) under controlled conditions (n=3, each). **Well mixed rumen samples** were taken from different sections and quickly deep frozen in aliquots. Different methods for total DNA preparation were tested. The „NucleoSpin Plant kit“ (Macherey-Nagel, Germany) was shown to yield the highest amount of unfragmented DNA and the best 16S rDNA amplification results.

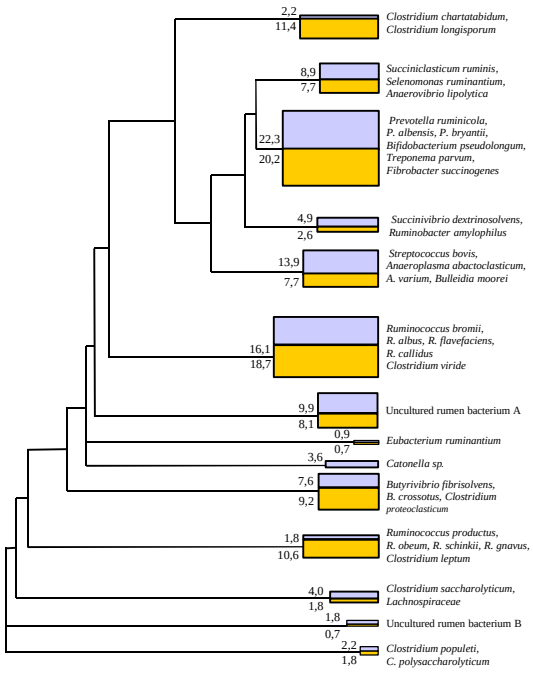
16S rDNA amplification and clone libraries

Bacterial 16S rDNA was amplified from mixed rumen samples of 2 cows of each set. The oligo-cucleotide primers p616V (5'- AGA GTT TGA TYM TGG CTC - 3') and p630R (5'- CAK AAA GGA GGT GAT CC - 3') were used with the "puReTaqTMReady-To-GoTM PCR Beads" (Amersham). The obtained **fragments of about 1490 bp** were taken for the construction of gene libraries in *E. coli*. A total of 576 clones was isolated from the four libraries and sequenced.

The **ARB program package** (Inst. for Microbiology, Bioinformatics group, TUM) was used to edit the data manually for removing insecure sequences and to perform **multiple alignment**. Questionable bases were corrected by viewing the original sequence gel data. Each single sequence was then screened for the most related known 16S rRNA members in the two databases, Genbank and ARB. Although there was no exact 16S rDNA sequence correspondence with a defined species, near relatives of a defined species could generally be identified in the phylogenetic tree of ARB. The majority of the homologous sequences was from unidentified, **uncultured rumen bacteria**, giving an impression of the unlimited variability.

In general, species definition requires sequence similarities greater than 98%, but most of the new sequences had a value below this with a most related defined species. Therefore a classification of "**Artificial Taxonomic Groups**" was constructed and is shown in figure1.

All bacteria usually prevalent in rumen were also found to be prevalent in our data sets. No statistically significant differences between transgenic and isogenic rumen samples were observed.



DNA amplification of recombinant plant genes from rumen samples

The possibility of Bt- and *bla*-gene transfer from transgenic maize to rumen bacteria was investigated by PCR using oligonucleotide primer pair Bt1/Bt4 and total DNA from rumen samples. Plant DNA samples of *Antares* and *Navares* maize lines were used as controls. For PCR reactions the HotStarTaq DNA Polymerase was utilized. This method of amplification is highly sensitive and allows to get a specific product with a minimal amount of template DNA (Fig. 2).

A number of attempts with varying PCR conditions and amounts of template DNA failed to show amplification of the Bt gene from rumen samples (Fig. 2A). Only from the transgenic ***Novares*** DNA amplification was possible (positive control from down to 1 ng of template DNA). In contrast, the *bla* gene present in the transgenic Bt-construct was amplified from all samples, also from the isogenic fed cows and from total DNA of isogenic ***Antares*** maize line (Fig. 2B). This surprising result was repeatedly observed with a number of variations in experimental conditions (the negative control was void of amplificate). **Interpretation:** recombinant bacteria containing the *bla* gene are ubiquitously found in any experimental setting and override any signal from the recombinant plants.

As a control for the unexpectedly high DNA degradation in the rumen, the calcium-dependent protein kinase gene from *Z. mais* was amplified from rumen and plant samples (Fig. 2C). No amplification for the 870 bp fragment was observed, indicating heavy DNA degradation in the rumen, already early in the digestion process.

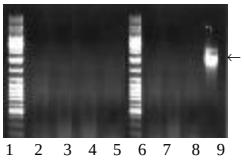


Fig. 2A: PCR amplification of Bt-gene.
1 - MassRuler^{TM} DNA Ladder Mix;
rumen DNA: 2 - 1t; 3 - 2t; 4 - 3t; 5 - 4i; 6 - 5i;
7 - 6i; plant DNA: 8 - *Antares* DNA; 9 - *Navares* DNA

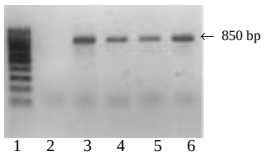


Fig. 2B: PCR amplification of the *bla*-gene:
1 - GeneRuler^{TM} 100 bp DNA Ladder;
2 – none DNA
3 - 1t rumen DNA
4 - 4i rumen DNA
5 - *Antares* DNA
6 - *Navares* DNA

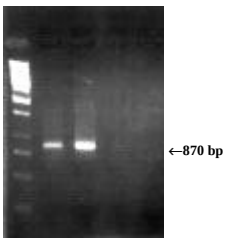


Fig. 2C: PCR amplification of *Z. mais* calcium-dependent protein kinase gene.
1 - 1 kb DNA Ladder; 2 - *Antares* DNA; 3 - *Navares* DNA;
4 - 1t rumen DNA; 5 - 4i rumen; DNA

Summary

The influence of feed containing *Bacillus thuringiensis*-toxin (Bt) transgenic maize on the rumen bacterial microflora was investigated. Cows were fed silage of either isogenic or transgenic lines of maize. Total DNA was isolated from samples of rumen content and 16S rDNA was amplified with bacteria-specific general oligonucleotide primers. We present the results of about 125 partial 16S sequences from each fed cow (n=2). The resulting sequences were aligned, assigned to artificial taxonomic groups and specifically compared using the ARB program package. The Bt-gene from forage material could not be amplified applying DNA extracted from rumen content.
In principle, specific leader bacterial species could be identified in these bovine rumen extracts. Such results will provide the basis for further studies on taxonomic investigations as well as analyzing the diversity of rumen bacteria concerning transgenic feed.

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Figure 1: Similarity tree of 16S rRNA sequences obtained from clones of PCR-amplified rumen DNA. The sequences were grouped by relatedness to known reference sequences (artificial taxonomic groups, ATG); the depth of the branching is indicated by the left border of the box. The width of the boxes indicates the relative number of sequences contained: blue - transgenic fed cows; yellow - isogenic fed cows. Reference strains contained in these ATGs is indicated in the right column.