

Ralf Einspanier · Bodo Lutz · Stefanie Rief ·  
Oksana Berezina · Vladimir Zverlov ·  
Wolfgang Schwarz · Johann Mayer

## Tracing residual recombinant feed molecules during digestion and rumen bacterial diversity in cattle fed transgene maize

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**Abstract** The aim of this study was to trace selected nucleic acid and protein components of isogene versus Bt transgene maize within the bovine gastrointestinal tract (GIT). After feeding 22 cattle for 4 weeks with Bt176 maize, different plant genes and the recombinant protein CryIAb were quantified during digestion. Furthermore, a first initial characterization of rumen bacteria was approached, using 16rDNA gene sequencing comparing isogene- against transgene-fed animals. Ingesta samples of different GIT sections (rumen, abomasum, jejunum, colon) were analysed for chloroplast, maize invertase, zein and Bt toxin (CryIAb) gene fragments using quantitative real-time PCR. First, the initial gene dose of these maize genes was detected in maize silage. During digestion, a significant reduction of high-to-medium abundant plant gene fragments was shown depending on the dwell-time and the initial gene copy number. Immunoreactive CryIAb protein was quantified by ELISA in intestinal samples indicating a significant loss of that protein. Remarkable amounts of Bt toxin were found in all contents of the GIT and the protein was still present in faeces. For the first time, the influence of CryIAb transgene maize on rumen bacterial microflora was investigated compared to isogene material through analysis of 497 individual bacterial 16S rDNA sequences. In

principle, specific bacterial leader-species could be identified in all bovine rumen extracts, but no significant influence of Bt176 maize feed was found on the composition of the microbial population. This investigation provides supplementing data to further evaluate the fate of novel recombinant material originating from transgene feed or food within the mammalian GIT.

**Keywords** Bt maize · Cattle · Intestinal tract · CryIAb protein · Plant DNA

### Introduction

Genetically modified organisms (GMOs) such as insect-resistant Bt maize have been introduced in the food and feed market. Meanwhile the necessity of elucidating food and feed composition, origin and potential genetic modifications of such novel components has led to numerous research activities to prevent any risk to consumers. Based on the European novel food and feed regulation, a threshold of 0.9% transgene material within food and feed has been defined to require labelling of the concerned product. Most scientific effort has therefore focused on supplying technologies for quantifying recombinant material within food material [1]. However, the biological or physiological effects of transgene feed on target animals are difficult to study and have rarely been reported. As long as only sparse information about the fate of GMO during gastrointestinal passage of the farm animal is available, such experiments have to deal with highly complex circumstances. As proven in recent studies, the conventional opinion assuming the complete degradation of food or feed DNA into mononucleotides during digestion needs to be revised. Feed DNA fragments of about 200 bp have been shown to attain the bovine blood cells [2, 3], and even larger fractions of foreign DNA were found in rodent cells after feeding [4, 5, 6, 7]. For a well-founded risk-estimation, detailed information about the fate and influence of recombinant molecules (DNA and protein) during their passage

R. Einspanier (✉)  
Institute of Veterinary Biochemistry,  
Free University of Berlin,  
Oertzenweg 19b, 14163 Berlin, Germany  
e-mail: einspani@zedat.fu-berlin.de  
Tel.: +49-30-83-86-2575

B. Lutz · S. Rief  
Institute of Physiology, TUM, Freising, Germany

O. Berezina  
Institute of Molecular Genetics RAS, Moscow, Russia, Russia

V. Zverlov · W. Schwarz  
Institute of Microbiology, TUM, Freising, Germany

J. Mayer  
Bavarian State Research Center for Agriculture, Poing, Germany

through the gastrointestinal tract (GIT) should be made available. Furthermore, knowledge of the endogenous degradation of foreign genetic material is the prerequisite to estimating a potential horizontal gene flow from feed towards e.g. microorganisms in the intestinal tract. The main focus of this study was on the fate of feed material during passage through different sections of the bovine GIT. Real-time PCR serves as the main analytical method for detection and quantification of GMOs in food as recommended earlier [8, 9] and was introduced here to quantify absolutely plant and transgene DNA within the cattle GIT. Plant genes with varying genomic copy numbers have been used as candidates to trace the concentration-dependent survival during ruminant digestion. In parallel, the recombinant CryIAb protein was quantified during the experiments to determine the degradation of a recombinant protein during digestion. In the last part of this study a first comparison and characterization of the bacterial microflora from the rumen of cows fed with silage of isogene or Bt176 transgene maize was performed, introducing a random genetic typing using 16S rDNA PCR, construction of gene libraries, sequencing and a phylogenetic evaluation. The aim was to monitor the possibility of either a foreign gene transfer from transgenic maize to rumen bacteria, or a change in the bacterial population. Therefore, this study elucidates the presence and concentration of plant DNA and transgene molecules from ingestion to excretion in cattle, as well as investigating for the first time the rumen bacterial population, screening for probable GM-feed-induced events.

## Material and methods

**Animals and diets.** Two different breeds of corn (isogene: Antares, transgene: Navares, Syngenta) were planted, grown and processed to silage. Authentic samples were taken at harvest (hackled corn) as well as from silage. The isogene material was always processed first to avoid any cross-contamination. During a 4-week feeding experiment 22 cows were treated as follows: 11 animals were fed

with the isogene Antares and the remainder (11) with silage of the transgene counterpart Navares. The daily diet contained 88.5% corn-silage, 2% barley-straw, 4.5% wheat, 4.5% rape-bruised grain extract and 0.5% mineral feed. Each cow was fed 19 kg of corn-silage in a total feed ration of 21.5 kg per day. After the feeding period, the cows were slaughtered and samples taken from different parts of the GIT: juices of rumen, abomasum, jejunum and colon, as well as intestinal epithelium were quick-frozen in liquid nitrogen and stored at  $-20^{\circ}\text{C}$  until further analysis. Blood and faeces samples were taken frequently during the whole experiment.

**CryIAb protein measurement.** The Bt-CryIAb/1Ac-ELISA (Agdia, Elkhart, Ind., USA) was carried out according to the manufacturer's instructions. Frozen samples (0.2 g) were ground using the FastPrep-System (Bio101, Carlsbad, Calif., USA) with 1.0 g of Green Bulk Matrix (6 m/s, 40 s) and finally dissolved in 1 ml MEB extraction buffer. For standards, a commercial control Bt-protein was introduced as follows: 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0 and 1.6 ng/ml. All samples were measured in duplicate showing an intra assay variation of 6.3% and an inter assay variation of 10.4% ( $n=8$ ). The total protein content of each sample was determined using the bicinchoninic acid assay as described in [10].

**DNA isolation.** DNA was isolated from the different sample materials either for detection of feed gene fragments or for construction of the bacterial 16S gene library as follows.

Samples of feed (100 mg), ingesta or bovine tissue were extracted using a combined method introducing the FastPrep (FP120) shaker (Q-Biogene, Heidelberg, Germany) with lysing matrix (Q-Biogene) and 600  $\mu\text{L}$  of nucleospin lysis buffer for 40 s (level 5.5) followed by the Nucleospin plant extraction kit protocol (Macherey & Nagel, Düren, Germany). The 16S rDNA libraries were constructed using DNA templates isolated from rumen contents with the Nucleospin Plant Kit, which was tested and found most suitable in preceding experiments yielding unfragmented DNA. The quantity and quality of the resulting DNA was measured by photometric determination at OD 260/280.

**PCR analysis.** Amplification of specific gene fragments using conventional PCR (Amp-R, 16S rDNA) was carried out as described previously [2].

For quantifying specific plant genes, a real-time cycler (Light-Cycler, Roche, Mannheim, Germany) was used: the SybrGreen technique as described elsewhere [11]. In brief, the Fast Start SybrGreen Master Mix (Roche; 10  $\mu\text{L}$  total volume) was used to amplify 90 ng or 60 ng of sample DNA as indicated: initial denaturation at  $95^{\circ}\text{C}$  for 10 min, 45 cycles of amplification at  $95^{\circ}\text{C}$  for 15 s, annealing 10 s (for plant 2,  $60^{\circ}\text{C}$ ; invertase (ivr)1,  $65^{\circ}\text{C}$ ; zein,  $60^{\circ}\text{C}$ ; CryIAb,  $58^{\circ}\text{C}$ ), and elongation at  $72^{\circ}\text{C}$  for

**Table 1** Primers used for PCR amplification and detection of specific genes. SMT Specific melting temperature during real-time PCR

Gene	Primer	Sequence (5'-3')	Product/ modification	Quotation EMBL Acc. Nr.	SMT ( $^{\circ}\text{C}$ )
Plant 2	plant 2 for plant 2 rev	GGA AGC TGT TCT AAC GAA TCG CTC GAA AAC AAT GAA TTG AAG G	199 bp	[2]	74
Invertase	ivr1 for ivr1 rev	CCG CTG TAT CAC AAG GGC TGG TAC C GGA GCC CGT GTA GAG CAT GAC GAT C	226 bp	[17] U161231	93
Zein	zein for zein rev	AGT GCG ACC CAT ATT CCA G GAC ATT GTG GCA TCA TVA TTT	275 bp	[18] M235371	88
CryIAb	cry 03 cry 04 176 FL 176 LC	CTC TCG CCG TTC ATG TCC GT GGT CAG GCT CAG GCT GAT GT GGG GTG TAG CCG GTC TCG ATG C-X X-TCG CCG CCC AGC ACC TCC AC	211 bp	[19] Unpublished	
16S rDNA	616V 630R	AGA GTT TGA TYM TGG CTC CAK AAA GGA GGT GAT CC	1,490 bp	[20]	
AmpR	Ap1 Ap2	TAT TCA ACA TTT CCG TGT CGC TTT CGT TCA TCC ATA GTT GCC	810 bp	AF427133	

25 s. Finally, a melting curve analysis was performed (95 °C/5 sec, 60 °C/10 s with continuous heating until 99 °C) verifying the specificity of PCR products. Dilutions of known concentrations of corresponding purified PCR fragments served as external standard curves. The resulting PCR products were sequenced to confirm authenticity (Table 1).

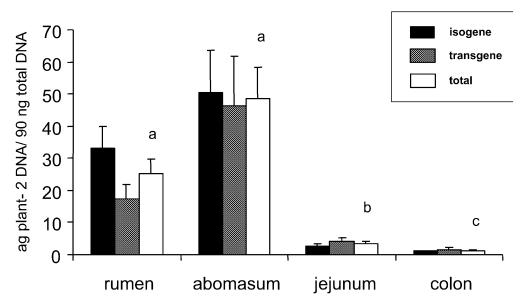
**16S rDNA clone libraries.** Primer pair 616V and 630R was used with the “puReTaq™ Ready-To-Go PCR Beads” (Amersham) under the following conditions: initial denaturation at 94 °C for 4 min, followed by 20 cycles of denaturation at 94 °C for 20 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 2.5 min (Table 1). The obtained fragments of about 1,490 bp were ligated to TA-cloning vectors (Invitrogen, USA) to construct gene libraries. A total of 576 clones were isolated from four libraries and sequenced commercially (MediGenomics, Munich, Germany).

**Statistical analysis.** The Mann-Whitney U-test was performed to compare the quantity of specific gene detection (plant 2, zein, ivr, CryIAb) in any material comparing iso- and transgene with each other as well as quantities in different sections of the GIT. Values are presented as means±standard error, SEM at different levels of significance (*P*). All reference sequences obtained from the GenBank database were aligned and the artificial phylogenetic tree was created using the ARB program package (<http://www.arb-home.de/>) developed at the Institute for Microbiology, TUM.

## Results and discussion

### Quantification of maize DNA in fresh and ensilaged material

To assess the fate of plant DNA during feed processing and digestion, various genes were quantified in material provided from isogene or transgene hackled maize directly after harvest, 4-week-old silage and ingesta of cattle. Marker genes with different copy numbers were selected to elucidate the effect of various initial gene doses: a chloroplast-specific amplicon (plant 2) with high copy number (>1,000 copies/cell [12]), ivr and zein with medium-abundant copy numbers and CryIAb representing a proposed single copy gene. Absolute quantities of such genes were initially measured in fresh compared with ensilaged maize through real-time PCR and statistically evaluated. The concentrations of specific plant DNAs decreased remarkably during the ensiling process to 1.3–3% of the starting material, supporting previous findings [13]. The final DNA concentration decreased more rapidly when correlated with the starting copy number of the investigated gene (plant 2, 30.98 fg:0.98 fg per 60 ng total DNA; ivr, 1.76 fg:0.04 fg per 60 ng total DNA; zein: 0.6 fg:0.008 fg per 60 ng total DNA). No quantitative difference of these maize genes was observed between the isogene and transgene group. The single copy gene CryIAb, representing the genetic modification of Bt176 maize, was also present in silage (CryIAb 1.97 fg in fresh:0.06 fg in silage per 60 ng total DNA). However, AmpR DNA could not always be reliably quantified in transgene silage material because of unspecificity of the amplicon (inherent melting curves). Due to varying numbers of plastids within each plant cell, the absolute amount of chloroplast-specific fragments appeared some-



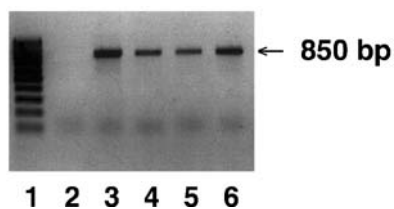
**Fig. 1** Degradation of ubiquitous plant chloroplast DNA in intestinal juice of cattle. Different letters (*a*, *b*, *c*) indicate significant differences between the columns ( $P < 0.05$ )

what lower than expected indicating a varying predictability of final foreign gene doses within animal products.

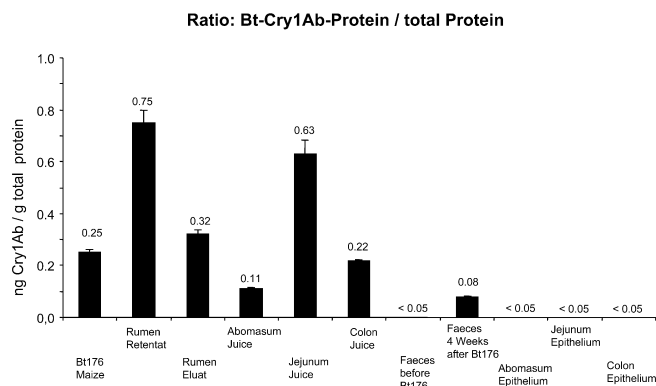
### Quantification of plant genes in the GIT of cows

The amount of plant 2 DNA fragments in rumen ingesta and abomasum ingesta was significantly higher than that in chyme of jejunum or colon (Fig. 1,  $P < 0.001$ ). Thus, in the last section of the GIT (colon), significantly lower plant 2 DNA was detectable. No statistically significant differences between the isogene- and transgene-maize-fed group was observed. Reliable quantification of any chloroplast DNA fragments in faeces was not possible due to reaching the detection limit and the generation somehow of unspecific amplicons. Similar problems arose while attempting the quantification of low copy (ivr, zein) or single copy (CryIAb, AmpR) genes and did not lead to utilizable results. These findings correspond well with earlier published data [3, 14, 15], showing that only chloroplast DNA, but not recombinant low copy genes were detectable in the GIT of pigs. Summarized, a detection of high-copy genes in ingesta of different sections of herbivore GIT is possible, whereas we were not able to reliably quantify low-abundant genes in the GIT of cows using real-time PCR.

To monitor the presence of Bt176 maize-derived recombinant ampicillin resistance (AmpR) DNA in rumen samples referring to a possible horizontal gene transfer, an AmpR-specific 810 bp fragment was used in a conventional PCR setup. However, trace amounts of that antibiotic resistant gene present in the transgenic Bt construct were amplified from all rumen samples, including samples from the isogene-fed cows and from total DNA of isogenic maize silage (Fig. 2). This surprising result was repeatedly observed with a number of variations in experimental conditions and controls. It could be shown that all amplified AmpR sequences were identical within the sequencing error rate with the ubiquitous AmpR gene, which is present in the majority of the cloning vectors. A contamination during the extraction or PCR set-up can be excluded due to negative controls. Further experiments must take into account such



**Fig. 2** Detection of AmpR gene fragments in different samples by PCR. 1 100 bp DNA ladder, 2 control without DNA, 3 rumen juice DNA from animals fed Bt176, 4 rumen juice DNA from animals fed isogene maize, 5 isogene maize silage DNA, 6 transgene maize silage DNA. Inverted image, 42 PCR cycles



**Fig. 3** Quantification of immunoreactive CryIAb protein during the intestinal passage in Bt176 maize-fed cattle. Numbers over the bars indicate the absolute CryIAb protein concentration

a probable general distribution of AmpR genes through e.g. using PCR primers specific for regions flanking the AmpR construct in the transgene plant genome to verify the forage plant origin of the resulting amplicon. However, the unreliable detection of even high-copy genes (plant 2) in bovine faeces may indicate a very low risk of spreading probably functional recombinant genes into the environment.

#### CryIAb protein in the GIT of Bt maize fed cattle

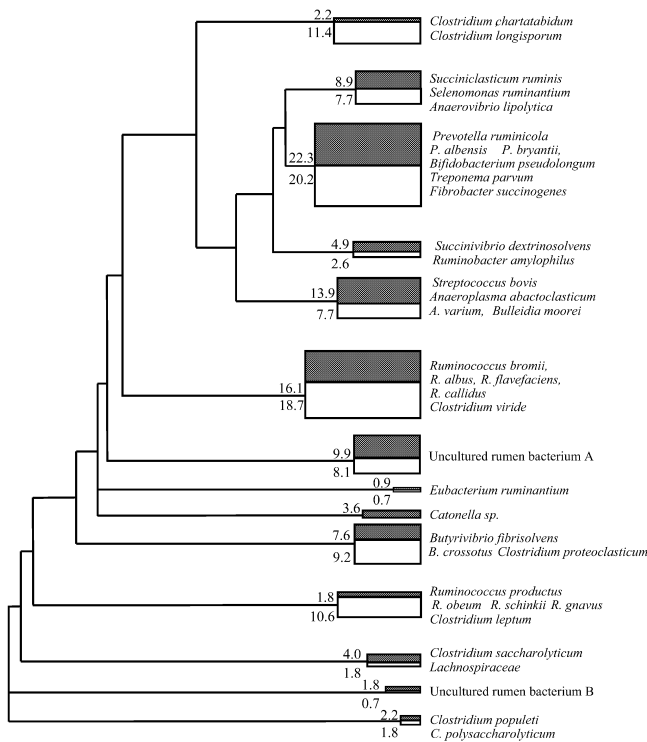
Immunoreactive CryIAb protein could be detected in different concentrations only in the intestinal juice of the transgene-fed cattle (Fig. 3). However, in the extensively washed intestinal epithelia the CryIAb protein was not detectable, in agreement with a recently published observation [16]. All faeces samples investigated were found somewhat positive for immunoreactive Bt toxin, but near the detection limit of the assay. Corresponding samples from isogene-fed cattle were always found to be negative; therefore any antibody cross-reactivity toward animal, microbial or plant components can be excluded. Approximately 4.3 ng CryIAb protein/g rumen sample (=0.75 ng/g total protein) was found. Calculating the total amount of the daily maize intake, an absolute amount of about 21–148 mg of immunopositive CryIAb protein can

still be expected in the rumen after a routine Bt176 maize feeding. It cannot be excluded that the conformation of the inactive recombinant CryIAb protein may resist a rapid decomposition of its relevant immunological recognition sites. During the passage of the ingesta through the different sections of the GIT the CryIAb protein concentration was astonishingly rising, probably due to water elimination. In faeces, the immunoreactive Bt toxin concentration was still about 0.08 ng/g total protein, calling into question the complete degradation of that protein during the bovine digestion process. Therefore, a potential distribution of Bt protein fragments on fields may be feasible considering the routine spreading of manure in e.g. dairy farms, and should be addressed in future studies. Any remaining biological effect of the digested Bt toxin can only be the subject of speculation.

Generation and identification of bacterial 16S rDNA libraries from cattle rumen

Bacterial 16S fragments of about 1,490 bp were used for the construction of specific gene libraries either from isogene- or transgene-fed cattle. A total of 497 sequences (273 isogene and 224 transgene), obtained from a PCR-amplified clone library of four individual animals (two isogene, two transgene), were analysed in detail. Each single sequence was screened for the most-related known 16S rRNA members in two databases (Genbank and ARB). Although an exact 16S rDNA sequence correspondence with a defined species was found for none of the obtained sequences, nearest relatives with defined species name were identified in the phylogenetic tree of ARB. 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” (ATG) was constructed. Only 28 sequences from our libraries could be assigned within the 98% rule to a defined “species”. This reflects the great diversity of the microbiota in the rumen of cows.

Since for a majority of the sequences the similarity was too low to allocate them with a reasonable degree of confidence to the corresponding taxa, a phylogenetic analysis was performed to clarify their taxonomic position. The artificial phylogenetic tree of microorganisms from both libraries, and differences in rumen microflora composition between rumen contents from cattle fed either isogene or transgene maize are shown in Fig. 4. The majority of the homologous sequences derived from unidentified, uncultured rumen bacteria, giving an impression of their unlimited variability. All bacteria usually prevalent in rumen were also found to be prevalent in our data sets. Finally, no statistically significant differences between transgenic and isogenic rumen samples were observed. This observation does not indicate for any remarkable effect of Bt transgene maize silage on the rumen bacterial population in cattle fed for a month. To elucidate the potential risk of a horizontal gene transfer from genetically modified plants into non-target organisms, e.g. rumen microbes, will not be easy even if highly sophisticated methods are introduced. Taking into ac-



**Fig. 4** Species comparison of the bacterial rumen population identified using 16S rDNA sequences. Similarity tree of 16S rDNA sequences obtained from bovine rumen DNA. The sequences were grouped by relatedness to known reference sequences [artificial taxonomic groups (ATGs)]; the relative depth of the branching is indicated at the left border of the box. The width of the boxes indicates the relative number of sequences contained: grey box transgene-fed cows, open box isogene-fed cows. Reference strains of these ATGs are named

count our results, the widespread ampicillin resistance (AmpR) gene will not serve as a suitable target gene for that purpose.

To conclude, depending on the initial copy number of foreign feed genes ingested, DNA will rapidly be degraded within the more caudal bovine intestinal organs. Gene fragments of high-abundant plant genes were not found in bovine faeces. However, the fate of the recombinant CryIAb protein is somewhat different: remarkable concentrations are not only present in all GIT samples, but traces are still found in faeces. Significant differences in the rumen microbiota population between isogene- and transgene-fed cows could not be distinguished after a 4-week feeding period. If searching for the AmpR gene, it must be recognized that natural or even recombinant bacteria containing such genes may be ubiquitous in many environments and will override the specific signal expected from the Bt176 maize. To date, no specific biological risks of feeding

transgene Bt176 maize to cattle have been discovered in short-term experiments, either for the animals or the consumers health when using such transgene-derived cattle products.

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