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ORIGINAL PAPER

Stimulation of bacterial DNA transformation by cattle saliva: implications for using genetically modified plants in animal feed

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Abstract To investigate the likelihood of DNA transfer from genetically modified plants (GMP) to bacteria, a rescue plasmid system for *Streptococcus gordonii* was modified. It was applied to monitor the DNA transformation into oral and intestinal bacteria in cattle. Transformation and recombination frequency of *S. gordonii* was dependent on the length of the transformed DNA. Beside horse serum, cow saliva also rendered the cells competent for DNA uptake. Competence induction was completely abolished by the addition of liquid from maize silage. Competence was partially suppressed by the addition of rumen liquid. In order to study native bacteria, 724 colonies sensitive to the antibiotics were isolated from either silage or the saliva and rumen of cows. Using horse serum, silage liquid, cow saliva or rumen liquid for competence induction, the isolates failed to integrate linearized pMK110 DNA and restore antibiotic resistance. Only 6 of the colonies obtained from the teeth of a silage-fed cow were sensitive to the antibiotics. Two isolates were related to *Staphylococcus warneri*. They could be transformed with the model plasmid pMK110 after induction by horse serum. DNA transformation, however, was not stimulated by incubation with cattle saliva, silage or rumen liquid. The response to competence-stimulating factors seems to vary between different bacterial species. These results suggest that the probability of DNA uptake from silage of GMPs is very low.

Keywords *Staphylococcus warneri* · Silage · Rumen liquid · Marker rescue plasmid · Genetically modified organisms

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Introduction

Since their commercial introduction, genetically modified plants (GMPs) play an important role as components of human as well as animal food. The transfer of recombinant DNA to bacteria associated with humans and animals, the horizontal (heterologous) gene transfer, is therefore a crucial factor in the risk assessment of GMPs. The fate of GMP-DNA in soil or in the intestine of humans and animals has been investigated (Einspanier et al. 2001, 2004; Philipps et al. 2003; Andow and Hilbeck 2004; Netherwood et al. 2004), as have the potential effects of transgenic feed on the microbial population in the rumen of cows (Einspanier et al. 2004) or in soil- and plant-associated bacterial communities (Bruinsma et al. 2003; Kowalchuk et al. 2003; Blackwood and Buyer 2004; Dunfield and Germida 2004).

One of the major problems in this area of research is that most of the microorganisms in the rumen or other body liquids of food-producing animals are not identified and/or cannot be cultivated successfully. Thus to date predominantly "representative" (i.e. well known) microorganisms have been used for comparative purposes (Tajima et al.

2001; Wiedemann et al. 2007). Under these circumstances horizontal gene transfer under field conditions is difficult to assay, especially if the recombinant DNA is present in all preparations and in ample quantity (Nielsen and Townsend 2004). Few studies have tackled this problem, although gene transfer from GMPs to soil bacteria have been found to be possible (Kay et al. 2002; Gebhard and Smalla 1998; De Vries et al. 2004; Badosa et al. 2004).

Considering the probability of foreign DNA to induce potentially harmful effects in other organisms, a series of cellular processes have to be taken into account: uptake of DNA into the cell, integration into the genome by recombination, and finally expression of an intact gene through a suitable promoter sequence, either attached to the transferred DNA fragment (and expressed in the respective host) or provided at the site of insertion in the correct orientation and distance. At least the transfer across the cell wall and membrane of the recipient bacterium (often through a competence system for the uptake of foreign DNA) together with the establishment of a DNA fragment within the cell (through a recombination event) are necessary for the expression of a gene in a given bacterium (Melville et al. 2001; De Vries and Wackernagel 2002; Kay et al. 2002; Nielsen et al. 2000). Once DNA (and hence an antibiotic resistance gene) is functionally combined with an appropriate bacterial promoter, the gene is activated and could be readily transferred into bacteria hazardous to human consumers and animals.

The transfer of short DNA fragments as usually assayed by PCR technology is only a risk under very limited circumstances, such as with the insertion of promoter or enhancer sequences. In the case of shortened and therefore defective genes, marker rescue (repair of homologous DNA) can occur (Kharazmi et al. 2003a). However, DNA is rapidly degraded in the rumen into small fragments (Lutz et al. 2006; Wiedemann et al. 2006) which are unlikely to retain the length necessary for biological activity.

Kharazmi et al. (2003a) showed by marker rescue transformation that plasmid and chromosomal DNA of bacteria as well as the DNA of transgenic potatoes was transferred to bacteria under laboratory conditions. In situ transfer has not been detected under experimental conditions but cannot be excluded since the hypothetical transformation rate of transgenic DNA to *B. subtilis* was calculated to be in the range of 8.5×10^{-19} to 1.2×10^{-27} per cell for homologous and non-homologous recombination, respectively (Kharazmi et al. 2003b). However, in this situation it must be considered that a region of homology between donor and recipient DNA is a prerequisite for stable integration (Kharazmi et al. 2003a) or, as demonstrated for *S. pneumoniae*, foreign DNA fragments have to be rescued by homology-directed illegitimate recombination during transformation (Prudhomme et al. 2002). Non-homologous integration of foreign DNA occurs at detectable frequencies only with the aid of highly specialized integrases, such as the *int* gene product of transposons (Malanowska et al. 2006). A potential transfer of recombinant DNA to intestinal and food-associated bacteria was assumed (Hertel et al. 1998), but has not been detected to date. However, under laboratory conditions it has been known for some time that DNA from GMPs can be transferred to bacteria if homologous sequences in the DNA of recipient cells are present (De Vries and Wackernagel 1998; De Vries et al. 2001).

Many Gram-positive bacteria are capable of natural transformation. This phenomenon is called competence, and has only been studied with a limited number of species (for a review see Redfield et al. (2006)). Kharazmi et al. (2003a) have developed a marker rescue system with an oral bacterium where DNA transfer events can be analysed in a laboratory setting resembling realistic conditions in the environment. It is based on the kanamycin resistance gene *nptII* and is suitable for studying transformation events of an intact gene in a food-related oral bacterium, *Streptococcus gordonii*. This system facilitates the quantification of DNA uptake and homologous recombination events. DNA transformation in *S. gordonii* was shown to be stimulated by human and rat saliva to an increased frequency (Kharazmi et al. 2003a; Mercer et al. 1999; Mercer et al. 2001), as its competence is controlled by a peptide-dependent quorum-sensing system (Cvitkovitch 2001; Havarstein 1998). *Streptococcus gordonii* may serve as a model organism for other bacteria associated with the mouth and intestinal bacterial flora in humans and other mammals such as cattle, which are likely to transfer the once established DNA horizontally to other bacteria.

The marker system of Kharazmi et al. (2003a) has been modified and exploited in this study to measure the transformation efficiency of linear DNA of different sizes into *S. gordonii* and other antibiotic-sensitive bacteria, which had been isolated from cattle tooth plaque. It was established that cattle saliva is a potent stimulator of cell competence, at least in *S. gordonii* but not in new isolates. This effect of saliva is completely neutralized by liquid derived from maize silage and partially by rumen liquid. The implications of these findings for the uptake of recombinant DNA during cow feeding are briefly discussed.

Materials and methods

Strains and media

Streptococcus gordonii NCTC7868 (strain Challis) derivatives containing plasmid pMK110 (ΔNcoI:nptII; Ery^R, Kan^S), with a deletion in the *nptII* gene (LTH5597), and

pMK111 with the intact *nptII* gene (LTH5743) were described by Kharazmi et al. (2002). Erythromycin and kanamycin were added to the media as indicated. Plasmid pUC19 was introduced into *E. coli* DH5α and grown in an LB medium at 37°C.

Streptococcus gordonii was grown anaerobically at 37°C in BHI (brain-heart infusion) broth (Merck). The medium was solidified with 1.5% (w/v) agar. *Streptococcus gordonii* colonies were grown for 36 h at 37°C for quantitative estimation. Selective media contained 10 μg/ml erythromycin and/or 1,000 μg ml^{-1} kanamycin (Sigma-Aldrich). *E. coli* DH5α was shaken at 37°C in LB broth with or without ampicillin (100 μg ml^{-1}).

Recombinant DNA techniques

To isolate plasmid DNA from *E. coli*, the QIAprep Spin Miniprep kit (Qiagen) was used in accordance with manufacturer's instructions. For plasmid DNA isolation from *S. gordonii*, the same kit was used with the following modifications: 10 ml overnight cultures were harvested by centrifugation (4,000×*g*, 8 min, 4°C) and washed with 1 ml Tris–HCl (10 mM, pH 8.0). The cell pellet was resuspended in 250 μl STE buffer (20% sucrose, 10 mM Tris–HCl [pH 8.0], 10 mM EDTA, 50 mM NaCl), with lysozyme (5 mg/ml) and mutanolysin (500 U/ml), and incubated at 37°C for 15 min. The DNA concentration was measured by absorption at 260 nm.

Preparation of chromosomal and plasmid DNA, endonuclease digestion, and ligation were carried out using standard procedures or in accordance with supplier protocols. Restriction digests of DNA were performed as recommended by the manufacturer (MBI Fermentas). *E. coli* cells were transformed with plasmid DNA by electroporation (GenePulser, BioRad). Plasmids were sequenced from double-stranded DNAs of selected colonies. Sequence data was analysed, edited and then compared with the DNASIS/PROSIS for Windows package (Hitachi Software Engineering).

Construction of rescue plasmid pUC18/nptII

The intact *nptII* DNA fragment (792 bp) was amplified from pMK111 template DNA (Kharazmi et al. 2003b) by PCR using the oligonucleotide primers pNpt-*Eco*RI (AGGGAATTCGGCTATGACTGGGCAC) and pNpt-*Hin*dIII (GAGGAAGCTTTCAGCCCATTCGCCGCCA) and the Expand-High-Fidelity PCR-System (Roche Diagnostics). The amplification product was cloned in opposite *lac*-promoter orientation into the pUC18 vector using *Eco*RI-*Hin*dIII sites. The orientation was verified by DNA sequencing. DNA for transformation experiments was digested with restriction enzymes: pUC18-*nptII*(*Sca*I)—cut with *Sca*I (3,492 bp), and pUC18/*nptII*(*Eco*RI-*Hin*dIII)—cut with *Eco*RI and *Hin*dIII (*nptII* DNA fragment 792 bp).

Transformation of *S. gordonii*

Overnight cultures of *S. gordonii* in BHI broth were diluted 1:1000 with BHI containing 10% (v/v) thermally treated horse serum (56°C, 30 min). After 4 h at 37°C donor DNA was added and the mixture was incubated for 1 h at 37°C before plating on BHI agar (Kharazmi et al. 2003b). A second experiment was carried out, with 10 μl of overnight grown *S. gordonii* LTH 5597 (pMK110 (Δ*NcoI nptII*)) culture, diluted 1:10 with BHI broth (to 1.5–2.3 × 10^8 CFU/ml) and added to 990 μl of thermally treated cow saliva, cleared rumen or silage liquid (clarified by centrifugation). The rumen liquid was obtained from fistulated non-lactating cows fed a conventional maize silage diet, as described by Wiedemann et al. (2007). The mixture was incubated at 37°C for 4 h. DNA was added to 1 ml of this culture and incubated again for 1 h before plating on selective media.

Cow saliva was collected from non-lactating cows fed with maize silage and was frozen in aliquots. Dilutions of bacterial cultures were carefully pipetted at constant speed and with the same batch of pipette tips to ensure a uniform shearing of cell-chains. Preliminary experiments have shown that those experimental parameters influence the quantification of cell counts.

Isolation of bacteria from tooth plaque of cows

Tooth plaque was scraped from the neck of several teeth from a cow fed with maize silage. The plaque material was fully immersed in a buffer. Aliquots were plated on agar plates and incubated anaerobically at 37°C under nitrogen atmosphere (COY anaerobic chamber). Bacteria sensitive for antibiotics were selected by replica plating on selective plates with 10 μg erythromycin ml^{-1} and 1,000 μg kanamycin ml^{-1}.

Nucleotide sequence Accession Number

The nucleotide sequence of the 16S rRNA gene has been deposited in GenBank, acc. no. AM396434.

Results and discussion

Marker rescue experiments with *S. gordonii*

Streptococcus gordonii of the *S. oralis*-group B of the streptococci was selected for marker rescue experiments because it is an abundant, commensal bacterium which

adheres, for example, to the epithelial cells of the oral mucosa (Kilian et al. 1989). For marker rescue experiments *S. gordonii* strain LTH5597 containing plasmid pMK110 was the recipient (Kharazmi et al. 2003a). The plasmid contains a promoter for expression in Gram-positive bacteria upstream of an inactivated neomycin phosphotransferase II (deletion of 10 bases in the reading frame, Fig. 1) and an active erythromycin resistance gene for selection in the bacterium host. The marker rescue plasmid pMK111 (Kharazmi et al. 2003a) was modified, and the intact but promoter-less *nptII* gene was cloned from pMK111 into plasmid pUC19, in the opposite direction to the *lac* promoter. The resulting plasmid pUC-*nptII* does not replicate in Gram-positive bacteria and does not provide a promoter for the expression of *nptII*. Therefore, both plasmids cannot express kanamycin resistance in the recipient *S. gordonii*, unless the deletion in the resident *nptII* gene in pMK110 is repaired by recombination with the homologous DNA fragment in pUC-*nptII* (marker rescue, Fig. 1). This rescue system allows for the measure of the combination of uptake and establishment of DNA through DNA transfer and homologous recombination in native Gram-positive bacteria, not only from cattle.

To test the marker rescue plasmids, cells of strain LTH5597 were incubated with linearized pUC-*nptII* DNA and plated on kanamycin plates. Only through prior incubation of the cells with heat-treated horse serum were kanamycin resistant colonies obtained (Table 1). Isolated plasmids from resistant colonies showed a restored restriction site for NcoI endonuclease, which is deleted in plasmid pMK110, indicating restoration of the intact *nptII* gene. The single NcoI site within the *nptII* gene was verified by digesting the PCR amplicon of the *nptII* gene from plasmid DNA of *S. gordonii* as well as through restriction fragment analysis using gel electrophoresis.

This experiment shows that transformation of, and DNA recombination in, *S. gordonii* cells took place in the expected way with the new plasmid combination. Hence, the experimental design was suitable for the intended marker rescue tests. A transformation frequency of up to 1.6 transformants/recombinant per 100 bacterial cells was achieved in vitro after induction of the DNA uptake competence by horse serum (Table 1).

Table 1 Marker rescue of *Streptococcus gordonii* LTH5597 with linearized pUC19-*nptII* plasmid DNA and dependence on fragment length with standard transformation protocol (heat-treated horse serum)

Transforming DNA	*nptII* (*Eco-Hin*d)	*nptII* (*Sca*I)
Fragment size (bp)	792	3,472
DNA conc (μg ml^{-1})	6.0	2.3
No. of *nptII* genes (ml^{-1})	6.9×10^{12}	6.1×10^{11}
No. of recipient cells (c.f.u. ml^{-1})	$1.7 \pm 0.2 \times 10^{8}$	$2.4 \pm 0.1 \times 10^{8}$
No. of Kan^R transformants (c.f.u. ml^{-1})	$1.4 \pm 0.6 \times 10^{5}$	$3.8 \pm 0.7 \times 10^{6}$
Transformation frequency	$8.2 \pm 0.4 \times 10^{-4}$	$1.6 \pm 0.7 \times 10^{-2}$
No. of transformants per *nptII* copy	$2.02 \pm 0.4 \times 10^{-8}$	$6.2 \pm 0.3 \times 10^{-6}$

Plasmids: restriction digest *Eco*RI-*Hin*dIII (792 bp *nptII* fragment) cuts upstream as well as downstream of the *nptII*-fragment, *Sca*I (3,472 bp fragment) cuts the vector opposite to the *nptII*-insert

Influence of DNA fragment size

The influence of the DNA fragment size on transformation frequency was investigated by removing the *nptII* insert from the pUC19-*nptII* plasmid with restriction enzymes *Eco*RI and *Hin*dIII instead of *Sca*I. The latter enzyme digests the plasmid at a single site opposite to the insertion site. The loss of "protecting" DNA sequences on both ends of the insert (fragment size 792 bp instead of 3,472 bp) resulted in a reduction of the transformation frequency (which includes the recombination step) by a factor of approximately 20 (Table 1). The number of transformants per *nptII* copy was reduced by a greater extent. This protecting effect of non-homologous DNA on transformation efficiency has also been observed in *Bacillus subtilis* (Kharazmi et al. 2003b), and underlines that any potentially harmful effect from the uptake of foreign DNA is strongly dependent on the size of the DNA fragments.

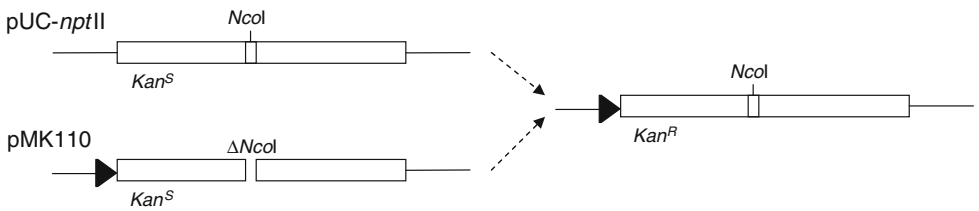

Fig. 1 Scheme of the marker rescue model: the *nptII* gene is shown as a box together with the plasmid designations and the promoter region (*black triangle*). The sensitivity, respectively resistance for the antibiotic kanamycin contributed by the plasmid (Kan^S resp. Kan^R) and the restriction site for endonuclease *NcoI* are indicated

Influence of rumen and silage liquid

Heat-treated horse serum triggers the cell competence and thus enables *S. gordonii* DNA transformation. However, the exact mechanism for the stimulation has not yet been identified. Other protein-containing and well buffered solutions such as saliva of rats were also found to stimulate cell competence (Kharazmi et al. 2003a). Cattle saliva was therefore also tested to identify if it contained a stimulating factor for cell competence. Growing cells of *S. gordonii* were incubated with saliva collected from cows which had been fed a corn silage diet. Cells with a linearized (*ScaI*) 3,472 bp DNA fragment of pUC18*npt*II were rescued with a transformation frequency of 1.3×10^{-2} (Table 2). This rate was in the same range as the transformation frequency after horse serum treatment (Table 1). Compared to the complete failure of transformant formation without the addition of competence stimulants, this is an enhancement of DNA rescue by a factor of more than 10^7, if the maximum number of screened cells is taken into account.

These results have significant implications for the potentially hazardous transfer of DNA from GMPs to mucosal bacteria in the natural environment (such as the mouth of cattle fed with genetically modified maize). Further experiments were designed to determine DNA transfer under experimentally imitated natural conditions. Genetically modified maize is usually fermented and fed to cattle as silage. It was tested to see whether the acidic silage liquid also has a stimulatory effect on DNA transformation. In contrast to horse serum and cattle saliva, no transformants were obtained by addition of silage liquid to a growing culture of *S. gordonii* prior to DNA transformation with the marker rescue system (Table 2). On the other hand, the addition of silage liquid to saliva had a severely negative effect on the stimulatory mechanism of cattle saliva (Table 2), and no transformants were obtained. Rumen liquid also had no stimulatory effect on DNA transformation. However, the addition of rumen liquid to saliva diminished DNA transformation by a factor of approximately 10^{-3} (Table 2).

Isolation of bacteria from cows and DNA transformation

To verify the model marker rescue experiment with bacteria derived from the native microflora of cows, samples from rumen liquid, maize silage liquid, saliva and tooth plaque of non-lactating cows fed with maize silage were grown anaerobically on agar plates at 37°C. The majority of the bacterial colonies from these sources were resistant to the concentrations of erythromycin and kanamycin used in the marker rescue experiment. Following this observation, colonies grown on non-selective BHI plates were replica plated on erythromycin and kanamycin plates and colonies sensitive to both antibiotics were obtained: 243 from saliva, 264 from rumen liquid and 217 from silage liquid. The colonies from each source were pooled and transformed with plasmid pMK110, with erythromycin selection according to the standard *S. gordonii* protocol with heat-treated horse serum as well as with cattle saliva, silage liquid and rumen liquid. No erythromycin resistant colony was observed upon incubation with plasmid DNA under the given conditions. Thus none of the 724 bacterial isolates was susceptible to DNA transformation or resistance expression under these conditions. Although this could be due to intracellular fragmentation of the *S. gordonii* modified plasmid DNA by restriction endonucleases, or to the type of selection (antibiotic concentration etc.), it is very likely that this is caused by the lack of competence (see *S. gordonii* experiment above).

The experiment was repeated with 274 colonies from tooth plaque scraped from the teeth of a cow. Among them 6 strains were sensitive to erythromycin and kanamycin. Only two of these colonies could be transformed with plasmid pMK110 with erythromycin selection according to the standard *S. gordonii* protocol with heat-treated horse serum. The transformation frequency was 6.9×10^{-3} to 1.5×10^{-2}, depending on the DNA concentration in repeated experiments (data not shown). The two unidentified new isolates with plasmid pMK110 were then transformed with the marker rescue plasmid pUC-*nptII* and

Table 2 Elicitor dependence of marker rescue efficiency in *S. gordonii*

	Colony counts of recipients (c.f.u. ml^{-1})	Colony counts of Kan^R transformants (c.f.u. ml^{-1})	Transformation frequency	Colony counts of transformants per *nptII* copy
Saliva	$2.3 \pm 0.11 \times 10^8$	$2.90 \pm 0.72 \times 10^6$	$1.3 \pm 0.4 \times 10^{-2}$	$4.7 \pm 1.2 \times 10^{-6}$
Saliva + rumen liquid	$1.45 \pm 0.23 \times 10^8$	$0.95 \pm 0.20 \times 10^3$	$6.5 \pm 0.06 \times 10^{-5}$	$6.5 \pm 0.50 \times 10^{-10}$
Rumen liquid	$1.74 \pm 0.49 \times 10^8$	<1	$<5.7 \pm 0.21 \times 10^{-9}$	$<5.3 \pm 0.28 \times 10^{-13}$
Silage liquid	$1.93 \pm 0.15 \times 10^8$	<1	$<5.2 \pm 0.14 \times 10^{-9}$	$<5.3 \pm 0.28 \times 10^{-13}$
Saliva + silage liquid	$1.16 \pm 0.23 \times 10^8$	<1	$<0.8 \pm 0.06 \times 10^{-8}$	$<5.3 \pm 0.28 \times 10^{-13}$

DNA pUC18-*nptII* (*Sca*I) with fragment size 3,492 bp was used for transformation. Final DNA concentration for the saliva only and the other experiments was 2.3 and 7.24 μg ml^{-1}, respectively

horse serum with a transformation frequency of 5.1×10^{-13} and 7.6×10^{-13}, respectively. For these experiments the linearized and longer "end-protected" DNA fragment pUC-*nptII*(*Sca*I) was used.

A repetition of the transformation experiments with cattle saliva instead of horse serum did not result in a single transformation event. The stimulatory effect of cattle saliva seems to be restricted to a few bacterial species, such as *S. gordonii*.

Phylogeny of new isolates

The 16S rDNA of both transformed new isolates was amplified with universal bacterial primers 616 V and 630R as described in Einspanier et al. (2004), cloned in *E. coli* and then sequenced. The 1400 bp sequences of both strains were found to be identical. They were subjected to phylogenetic analysis with the ARB software package (Ludwig et al. 2004). The most closely related species is *Staphylococcus warneri*, a coagulase-negative bacterium and a common commensal organism on the skin and mucous membranes of mammalia, which may cause, for example, nosocomial infections in immuno-compromised patients (Kamath et al. 1992).

Conclusions

Cattle saliva had a stimulatory effect on at least one Gram-positive model bacterium, *S. gordonii*, but not on another Gram-positive bacterium, a new isolate from cattle related to *S. warneri*. The standard competence-stimulating agent, heat-treated horse serum, was not effective in any of the 724 bacteria isolated at random from cattle saliva or liquid from maize silage and rumen. This does not necessarily imply that those bacteria cannot be transformed by foreign DNA at all, but suggests that they were not stimulated for DNA uptake by horse serum. The possibility of DNA uptake by at least one genuine mouth or rumen bacterium in cattle was shown with an isolate of *S. warneri*. The low incidence rate of DNA transformation among bacteria associated with cattle does not, however, exclude a potentially higher transformation rate when the longer exposure of cells to DNA in natural biofilms is considered.

The stimulatory effect of cattle saliva on *S. gordonii* was completely removed by silage and drastically diminished by rumen liquid. Therefore, at least for silage made from GMPs and used as feed for cattle, the probability of DNA uptake can be assumed to be very low. It cannot be quantified, because the response to competence-stimulating factors was observed to vary among different bacterial species. However, in a natural setting the cattle ruminates recombinant plant material, whereby the GMP material is continuously mixed with rumen liquid. Moreover, GMPs used as feed for cattle are often given in ensiled form. As both rumen liquid and ensiling seem to effectively prevent DNA transformation, the risk of recombinant DNA transfer to bacteria associated with cattle seems to be low. In addition, the marker rescue effect of ingested DNA is directly correlated to the size of the DNA fragments, which are rapidly degraded during the passage through the rumen and the digestive tract. Although a degradation process of DNA also takes place in the human digestive tract, these results cannot simply be extended to the situation in humans, and should instead be separately tested with experimental parameters adapted to human physiology.

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