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Review article

## Genetically modified organisms: consequences for ruminant health and nutrition

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**Abstract** — Many of the plants eaten by farmed ruminants are capable of being genetically modified, and may in the future be modified for nutritional, agronomic or industrial purposes. Techniques are also becoming available for genetic modification of silage and ruminal bacteria. Those working in agricultural biotechnology have a clear responsibility to detect and avoid any unintended or undesirable consequences of such modifications, whether direct or indirect, upon the animal, the consumer and the environment. One of the most general concerns that has been expressed is the possibility for onward transfer of modified gene sequences to gut microorganisms or host cells. Rare acquisition of diet-derived DNA fragments cannot be ruled out, but if this occurs, it must have also occurred throughout mammalian history. The possible impact of genes not normally present in ruminant diets must, however, be considered. Discussion of the use of antibiotic resistance markers in transgene constructs must take into account the wider debate on the likely impact of antibiotic use in animal agriculture on the spread of antibiotic resistant bacteria. There is increasing evidence that overuse of antibiotics has led to extensive transfer of antibiotic resistance genes between bacteria from the human and animal gut. In general this is likely to have a far greater impact than any rare transfer events involving resistance genes passing from transgenic plants to microbes. Our rapidly improving ability to use sophisticated molecular approaches to predict and track the consequences of genetic modification will help to ensure safe application of GM technology in agriculture in the future.

**GMO / rumen / ruminant nutrition / gene transfer**

**Résumé** — **Organismes génétiquement modifiés : conséquences pour la santé et la nutrition des ruminants.** La plupart des plantes actuellement consommées par les ruminants d'élevage peut être – ou est déjà – manipulée génétiquement, et il est probable que ces plantes seront encore modifiées dans le futur pour améliorer leurs propriétés nutritionnelles, agronomiques ou technologiques. Par ailleurs, il est maintenant techniquement possible de modifier génétiquement les ferments d'ensilage ou les bactéries du rumen. Les chercheurs impliqués dans ces biotechnologies doivent donc être capables

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de détecter et empêcher toute conséquence indésirable et préjudiciable, qu'elle soit directe ou indirecte, pour l'animal, le consommateur et l'environnement. Une des principales préoccupations concerne la possibilité de transfert des transgènes aux microorganismes du tube digestif ou à l'hôte. La possibilité d'intégration de fragments d'ADN présents dans l'alimentation ne peut pas être complètement exclue ; même si cet événement ne peut être qu'extrêmement rare, ce phénomène a pu se produire tout au long de l'évolution des mammifères. L'impact de gènes qui ne sont pas habituellement présents dans l'alimentation des ruminants doit être pris en compte. La discussion sur l'utilisation de gènes de résistance aux antibiotiques comme marqueurs des transgènes doit intégrer le débat plus large de l'impact de l'utilisation des antibiotiques en alimentation animale sur l'accroissement des bactéries résistantes aux antibiotiques. En effet, un nombre croissant de résultats suggère que l'utilisation exagérée d'antibiotiques a conduit à un transfert de gènes d'antibio-résistance entre les bactéries du tube digestif de l'homme et de l'animal. De façon générale, un tel transfert de gènes est susceptible d'avoir un impact beaucoup plus important que le peu probable transfert de gènes de résistance des plantes transgéniques aux microorganismes. Les rapides progrès dans l'utilisation d'approches moléculaires sophistiquées pour prédire et suivre les conséquences des modifications génétiques contribueront à l'avenir à une utilisation plus sûre du génie génétique en agriculture.

#### **OGM / rumen / nutrition des ruminants / transfert de gènes**

### **1. INTRODUCTION**

Ruminants rely on their resident rumen microorganisms for extensive degradation of feed materials. Considerable research effort has therefore been concentrated on improvement of feed quality and optimisation of ruminal microbial degradation and fermentation with the aim of maximising the efficiency of feed utilisation and increase ruminant productivity. Past and present methods include chemical pretreatments of the feed and use of dietary additives such as ionophores, antibiotics and probiotics acting on the rumen microflora. The development of genetic engineering techniques makes the use of genetically modified organisms (transgenic plants or modified microorganisms) now possible to improve ruminant productivity or quality of its products. Furthermore, an increasing number of currently grown plants have been genetically modified to improve their agronomic or technological properties, and ruminants may be fed with by-products of some of these GM-plants. The recent debate caused by the arrival of transgenic plants on the food market has revealed a resistance among European consumers towards GMO or GMO-derived products, which is likely to increase

concerns about quality and safety of animal products. We must consider several categories of possible risk from GMO feeding of ruminants, including possible deleterious effects upon animal health as a direct or indirect consequence of transgene expression or insertion, and the possibility that any such effects might carry through to the human consumer. In particular, it appears necessary to assess potential risks due to toxic or allergenic substances. The most general concern relates to the possible onward transfer of modified genes in the gut, body or wider environment. The increasing occurrence of multiple antibiotic resistance genes in medically important bacteria attributed to the use (or misuse) of antibiotics in humans and animals, has raised general concerns about the dissemination of resistance genes but it is the specific contribution, if any, of marker genes in GMOs that is relevant here.

The aims of the present article are thus (1) to summarise the proposed utilisation of GMO to improve the ruminant performance and health, which includes feeding of transgenic plants, improving silage quality, and genetic engineering of the rumen flora (2) to review the present knowledge about

mechanisms of genetic exchanges between bacteria, from plants to bacteria, and from bacteria to mammalian cells, particularly considering the case of antibiotic-resistance genes (3) finally to provide a basis for general discussion about risks and possible consequences of the deliberate release of GMO to improve ruminant nutrition and health.

## 2. POSSIBLE USES OF GMOs TO IMPROVE ANIMAL NUTRITION

### 2.1. Transgenic plants

The number of plant species that are amenable to genetic modification is increasing rapidly and includes many of those used in animal feed. Several of these GM-crops have already received regulatory approval for feed use (Tab. I). So far, manipulations aimed at improving nutritional characteristics are generally not as well advanced as those aimed at agronomic traits, but the potential is clearly enormous. These manipulations have been the subject of recent

reviews [8, 21] and will be discussed here only in outline. One obvious target that has been explored is improving the rumen degradability of fibrous plant material and the possibility of modifying lignin content, for example through antisense inhibition of lignin synthesis enzymes [33]. An alternative is to increase the degradability of non-GM plant material through pretreatment of feed, but even here the development of enzyme cocktails that include recombinant enzymes is being actively pursued [11, 57, 73]. Indeed, transgenic crop plants may be used for the large scale production of recombinant enzymes intended for use in animal feed or elsewhere [39, 48] or might be designed to switch on controlled expression of degradative activities before feeding to ruminants.

Another obvious target is to increase the availability of essential amino acids derived from plant proteins. Manipulation of the amino acid composition of plant seed proteins through the expression of foreign gene products, and altered regulation of lysine

**Table I.** Examples of plants that can be used in animal feed and that have been approved as plants with novel traits. These GM-plants have received regulatory approval (environmental and/or food and/or feed approval) in one or more countries. A summarisation of the nature and types of approved plant with novel traits can be found at <http://www.agbios.com>.

Plant	Phenotypic Trait	Source of the transgene
Canola (oilseed rape)	Herbicide tolerance Modified seed fatty acid content Pollination control system	Bacteria Plant Bacteria
Corn	Herbicide tolerance Male sterility Resistance to insects	Bacteria Bacteria Bacteria (Bt)
Potato	Resistance to viruses or insects	Virus or bacteria (Bt)
Rice	Herbicide tolerance	Bacteria
Soybean	Herbicide tolerance Modified seed fatty acid content	Bacteria Plant
Sugar beet	Herbicide tolerance	Bacteria

biosynthesis, have both proved effective in transgenic plants [5, 20, 55]. The relative abundance of different storage proteins that vary in nutritive value is also subject to manipulation [46]. A particular problem in the ruminant is to decrease losses due to rumen microbial activity, and the stability of plant proteins to rumen degradation might also be tackled through plant manipulation. Manipulation of the free carbohydrate content in forages has also been investigated and provides an important approach for increasing the supply of readily available energy which can help to improve nitrogen retention [38].

To these possibilities can be added a host of others, including removal of antinutritional factors, selective antimicrobial action and delivery of bioactive compounds (Tab. II). Soybeans expressing fungal phytase (an enzyme that catalyses the release of phosphate from plants), for example, increase phosphate availability in chickens [13].

## 2.2. Genetically modified silage bacteria

Another indirect approach to the enhancement of fibre digestion in ruminants is through modification of silage inoculants. Silage preservation involves the growth of

lactobacilli (indigenous or added) on soluble sugars present in the plants with the production of lactic acid. In silages containing low carbohydrate contents, inclusion of amylase, cellulase or hemicellulase enzymes has been shown to increase lactic acid production by releasing sugars for growth of lactobacilli [37]. Thus, inoculation of silage bacteria genetically modified to produce such enzymes has been proposed to obtain better ensiling and/or pre-digest the plant material in order to lead to better digestibility in the rumen. Recombinant *Lactobacillus plantarum*, a species used as silage starter, were constructed to express alpha-amylase, and cellulase or xylanase genes [65, 66]. The competitive growth and survival of such modified lactobacilli has been shown in silage [74], although the impact on silage digestibility has not been assessed. Considering the genetic tools already available for lactic acid bacteria, there is now a great potential to modify *L. plantarum* strains in order to increase their efficacy.

## 2.3. Genetic manipulation of the rumen flora

The possibility of genetic modification of rumen microorganisms in ways that would benefit the host animal has been discussed since the early 1980s and has been extensively reviewed [26, 28, 62, 76, 79]. The main targets are the same as those that motivated the manipulation of plants (Tab. II). One of the first objectives, as with transgenic plants, has been to increase the extent of degradation of fibre components, and one of the possible strategies is the establishment of recombinant organisms expressing specific polysaccharidase activities at a high level. Recent work showing that enzyme (cellulase and xylanase) supplementation of diet can increase the performance of ruminants [88], will probably renew interest in such manipulation since genetically modified microorganisms would be more suitable, especially in extensive

**Table II.** Targets for nutritional manipulation.

- Increase degradation of plant cell wall material.
- Improve amino acid supply/ reduce ammonia loss.
- Improve availability of other nutrients (e.g. phosphate)
- Decrease methane loss/ improve VFA proportions.
- Prevention of digestive disorders (e.g. acidosis, bloat).
- Detoxification of plant constituents – extend variety of feed plants.
- Suppression of human and animal pathogens.

systems, because they theoretically only require one inoculation rather than daily feeding as with enzymes.

A major goal has been the manipulation of predominant rumen bacterial species such as *Prevotella ruminicola*, *Butyrivibrio fibrisolvens* or *Streptococcus bovis* with most of the initial work aimed at the expression of heterologous polysaccharidase genes (Tab. III). One aim of the expression of fibrolytic enzymes in *P. ruminicola* or *S. bovis* is to increase fibrolytic activities in animals fed with concentrate-rich diets. Such diets can decrease rumen pH and consequently inhibit the native cellulolytic species that are very sensitive to pH below 6.0 [62]. Recent developments include the expression of an endoglucanase gene from *Ruminococcus flavefaciens* in *S. bovis* [84], expression of an endoglucanase/xylanase gene from *P. ruminicola* in another strain of the same species [12], and expression in *B. fibrisolvens* of a xylanase gene originating from the rumen fungus *Neocallimastix patriciarum* [87] or from *Eubacterium ruminantium* [45]. Effort is now being directed towards constructs with more efficient

promoters and regulatory and secretion signals [87]. Work is still in the developmental stage, however, and in no case has the optimal combination of gene product, target species and expression system for enhanced ruminal fibre breakdown been identified. Since the stability of the newly acquired gene(s) in the host would probably be better assured by insertion of the gene onto the host's chromosome, the use of phage sequences or a suicide vector were investigated to develop an integration system for *P. ruminicola* and *S. bovis*, respectively [6, 30].

Another area that has been investigated is detoxification. Many plants produce compounds that may be toxic, or reduce productivity in ruminants. These include oxalic acid, fluoroacetate and a range of different tannin and related phenolic structures. The possibility to enhance the capacity of rumen bacteria for detoxification of these plant poisons may increase the range of vegetation that can be used for grazing livestock. There are several examples of successful detoxification by the establishment of new organisms, not genetically modified, in the rumen.

**Table III.** Predominant rumen bacteria and gene transfer.

Species	Presumed major function in the rumen ecosystem	In vitro gene transfer techniques	Evidence of natural genetic exchange
<i>B. fibrisolvens</i>	hemicellulolysis sugar fermentation	conjugative plasmids and transposons, phages	yes
<i>Prevotella</i> sp.	hemicellulose, starch and protein degradation	conjugative plasmids, phages	yes
<i>S. ruminantium</i>	sugar fermentation	artificial transformation, phages	yes
<i>S. bovis</i>	amylolysis, sugar fermentation	natural transformation, phages	no
<i>Ruminococcus</i> sp.	cellulolysis	artificial transformation, conjugation	no
<i>F. succinogenes</i>	cellulolysis	no	no

Bacteria that detoxify DHP (3-hydroxy-4(1H)-pyridone), the ruminal metabolite of mimosine, a toxic amino acid present in the leguminous shrub *Leucaena leucocephala*, were introduced and established in the rumen of Australian goats and steers which were then able to eat the plant [42]. Furthermore, the mimosine tolerance spread naturally through sheep flocks. Similarly, inoculation in sheep of a novel bacterium isolated from the rumen of feral goats reduced the toxic effect of tannins of woody plants such as *Acacia aneura* [7]. These successes have led to the investigation of the possibility of detoxification by introduction of genetically modified bacteria [7, 28], and the following example represents one of the first successful utilisations of GMOs in ruminants under contained conditions. Recombinant strains of *B. fibrisolvans* were produced to detoxify monofluoroacetate, a compound present in the gidgee bush *Acacia georgina*, and in other Australian, African and Central American plants. A fluoroacetate dehalogenase-encoding gene from *Moraxella* sp., a soil bacterium, was cloned on a shuttle vector and expressed in *B. fibrisolvans* [29]. The modified bacteria were then introduced into the rumen of sheep, and on trials, the host animals showed reduced toxicological symptoms, although the protection was not total [31]. However, the release of the modified *B. fibrisolvans* strains has not been achieved because it raises several questions such as their potential spread to other animals. For example, fluoroacetate is used as a pesticide in Australia for controlling feral animals such as rabbits, and transfer of the transgenic resistant bacteria to these animals may confer resistance [28].

Another aim of manipulation of the rumen ecosystem has been to limit protein degradation or increase amino acid production by rumen microorganisms. The use of recombinant DNA technology was thus proposed to produce proteins from synthetic genes that match the particular amino acid requirements of the ruminant under certain

production conditions. Attempts were made 15 years ago to construct a synthetic gene for a polypeptide composed of the most limiting amino acids (lysine, methionine and threonine) in the objective of expressing this gene in a rumen bacterium [79]. Recently, such a *de-novo* protein designed to incorporate the nutritionally important amino acids was successfully produced in *Escherichia coli* [4, 36], and experiments are underway to express this protein in rumen bacteria such as *B. fibrisolvans*.

Progress on the genetic modification of obligately anaerobic rumen species has been relatively slow due to limited input, technical difficulties and the previous lack of genetic work. Nevertheless there are now systems to transfer genes into several rumen species. The detoxification of fluoroacetate by recombinant *B. fibrisolvans* demonstrates the efficiency of the technology, and the potential of using GMO to optimise animal health and/or production. Other possible targets for genetic manipulation of rumen bacteria may include, for example, control of biohydrogenation to improve nutritional qualities of animal food products, overexpression of phytase, etc. (Tab. II). An alternative is to introduce into the rumen modified strains of non-ruminal species, which can potentially include a range of aerotolerant bacteria and fungi including yeast. The yeast *Saccharomyces cerevisiae* and filamentous fungi such as *Aspergillus oryzae* are widely fed to ruminants as probiotics, and their mode of action is being determined [18]. Since the genetics of these organisms are very well known, it seems logical to modify them in order to increase their efficiency, or to use them as the vehicle of new activities by expressing added genes. In addition, more tractable anaerobes such as the human colonic species *Bacteroides thetaiotaomicron* have been engineered to express a recombinant xylanase gene originating from the rumen species *P. ruminicola* [85]. Although derived from an ecosystem similar to the rumen, the modified *B. thetaiotaomicron* strain was found not to



be able to compete against the resident rumen microflora [10].

Now that the testing and development of GM-microorganisms to manipulate rumen functions is a practical possibility, several questions must be addressed before using such modified strains. These include: how might the genetically engineered microorganism spread and persist in a ruminant population under field conditions? Would the presence of the GMMs influence the environmental impact of its host? Could the GMMs spread to the environment (feral animals) and to humans? Could the engineered gene(s) be transferred to other microorganisms, to the host, or to the consumer and if so could this have any deleterious effects? Finally, can the GMMs have any unexpected, deleterious consequences for the animal or human consumer?

These questions, which are posed by existing regulations in most countries, demand solutions through the design of GMMs that minimise potential risks and can become acceptable for release. The first desirable step will be to maximise the genetic stability and minimise the transfer of modified traits by achieving targeted chromosomal insertion of modified genes. It is also important to know whether the strain harbours native genetic elements that are likely to enhance chromosomal transfer. This requires much improved knowledge of natural agents of gene transfer in gut anaerobes, and this knowledge would undoubtedly be greatly accelerated by genome sequencing of representative species. This said, there is every likelihood that a modified strain will acquire such genetic elements from the native flora following introduction into the gut. Thus in principle the transfer of modified sequences from GMMs to other gut bacteria cannot be excluded, making it essential still to include potential consequences of transfer into any risk assessment. Special suicide mechanisms to limit transfer, or conditional expression systems to limit gene expression other than in the modified

host, offer potential solutions that should be developed in the future. Survival of the modified strain itself will often be limited by its oxygen intolerance and history as a laboratory strain, but the cautious view dictates that non-reversible mutations (e.g. leading to a nutritional requirement) may have to be built, in order to limit survival to defined conditions. Finally, while a selectable marker is necessary for the initial insertion, it is possible to arrange that the marker can subsequently be eliminated, thus avoiding the release of further resistance genes in any manipulated strain. Ultimately the most acceptable GMMs may well be products of self-cloning, where no heterologous DNA is present.

#### **2.4. Transgenic ruminants**

Despite the development of techniques for the introduction of genes into domestic animals [41], there is no known example of a transgenic ruminant with improved performance, whereas there are several reports of gene transfer to monogastric animals, enhancing their digestive capabilities [25]. This may be due to difficulties in genetic modification of large animals and in identification of the best targets.

### **3. CONSUMPTION OF GMOs DESIGNED FOR AGRONOMIC/ INDUSTRIAL PURPOSES**

In general, modifications aimed at nutritional improvement are likely to be accompanied by extensive feeding trials to assess efficacy, which should help to reveal possible unintended consequences of manipulation, although specific safety evaluation is of course also necessary. Despite the potential of GM technology for nutritional upgrading of plant material, however, it is important to recognise that most GM plants currently grown and fed to ruminants have been modified for quite different reasons.



In particular, agronomic traits such as resistance to pests and diseases and herbicides are the most widespread targets of manipulation (Tab. I). Furthermore ruminants are very commonly fed on residues from crops that are primarily grown for other purposes, which may range from grain production for human food through the production of oils, chemical feedstocks or biopharmaceuticals that are of industrial, not nutritional, use [54]. Modification of such crops through GM technology is likely to increase greatly in the future.

In view of this, it is particularly important for risk assessments to take account of possible unexpected consequences from ingestion of GM crop residues. In the case of insecticidal proteins, for example, a wide range of genes have been proposed for use including *Bacillus cry* (BT toxin) genes, genes coding for protease and amylase inhibitors of microbial, plant and animal origin and genes for several different plant lectins. Most of these proteins must be sufficiently stable and active in the insect gut to be effective as insecticides, and it is clearly essential to rule out antinutritional or toxic effects in mammalian systems at the relevant dosages [69].

In most forms of plant manipulation there is no single, controlled site of insertion of the GM construct in the chromosome. This leaves open the possibility of indirect and unknown consequences of such insertions upon the expression of flanking, and perhaps distant, genes. In practice it will often be very difficult to demonstrate such disturbances affecting nutritional value against the background of normal inter-batch variation. However rapid advances in genomics and proteomics offer the prospect of very precise monitoring both of insertion sites and of the physiological consequences of insertions. It should therefore become much easier in the future to rigorously exclude any unexpected and unfortunate 'freak' effects, e.g. on the expression of native plant toxins or antinutritional agents, that might affect the animal or human consumer [34, 86].

#### 4. GENE TRANSFER

Perhaps the most general concern felt over the consumption and release of GM feed material or establishment of GM microorganisms in the rumen is that the modified genes will spread via the intestinal tract or environment with unpredictable consequences. Thus genes that are deemed harmless in one host might behave quite differently when transferred to another. The most obvious examples are antibiotic resistance marker genes, which pose no risk in a plant, but might do so if transferred to certain pathogenic bacteria. The potential for gene transfer from plant to rumen bacteria, between rumen bacteria, or from rumen to non-rumen (and particularly human) bacterial species must thus be examined.

##### 4.1. Mechanisms of gene transfer between microorganisms

Gene transfer is a natural phenomenon that can provide microorganisms with the means to survive under unfavourable environmental conditions. The sequencing of entire microbial genomes in the last few years has shown that microbes are mosaics of acquired genes, suggesting that horizontal gene transfer has been a major driving force in microbial evolution. The three recognised mechanisms of prokaryotic gene transfer are transformation, conjugation, and transduction, but it is likely that the three mechanisms combine in natural environments such as the gut. Transformation involves the uptake and expression of genes encoded by exogenous, unprotected DNA from the environment. This could be chromosomal DNA fragments, plasmids or transposons. Transformation is a normal, physiological function of some bacteria that express an ability to uptake DNA at some time in their life cycle [49]. Conjugation is a plasmid or transposon encoded mechanism of gene transfer that requires contact between cells. In Gram-negative bacteria

the transfer is mediated by a specific pilus, produced by the donor strain, that binds to a receptor present on the recipient strain. Transduction is a process of gene transfer that involves bacteriophages. In generalised transduction, a phage mistakenly packages some host DNA (chromosomal DNA fragment or plasmid) in the proteic capsid and transfers it to another bacterium upon subsequent infection. These mechanisms have been well described in [56]. Although less studied, other mechanisms of gene transfer may also be involved. For example, several reports indicate that membrane vesicles released by some bacteria contain DNA and RNA, and may have a role in the exchange of genetic material [16, 47]. Furthermore, it is possible that other mechanisms, still unknown, exist in nature. It is clear that genetic exchanges have also occurred between prokaryotes and eukaryotes on an evolutionary timescale [78]. The predation of rumen bacteria and fungi by protozoa may have favoured inter-kingdom genetic transfer, since a major mechanism of acquisition of genes by eukaryotes during evolution may have been grazing on prokaryotes and/or eukaryotes (“you are what you eat”, [15]). Indeed the striking similarity between family 11 xylanases from certain ruminal bacteria and protozoa noted recently provides a very likely example of such genetic interchange [14].

The rumen environment has several qualities that are thought to favour inter and intraspecies gene transfer. First, it has a very high microbial population density ( $10^{11}$  cells·mL<sup>-1</sup>). Second, most of the bacteria live in biofilms covering feed particles or the rumen epithelium, and some bacteria are also found attached to fungi or protozoa, resulting in a close cell-to-cell contact. Third, extrachromosomal mobile elements (plasmids and bacteriophages) have been found in several rumen bacterial species (e.g. [23, 44]), and recently a transmissible chromosomal element was reported in *B. fibrisolvans* [71]. Finally, the rumen microbial population is likely to be in

permanent contact with exogenous free DNA, coming from ingested plants, microorganisms and others, but also from indigenous microorganisms and particularly bacteria, as a large proportion of the rumen bacteria undergoes spontaneous (or phage-induced) lysis. This bacterial lysis also leads to a release in the rumen fluid of extrachromosomal infectious particles or plasmids.

All the studies concerning the transfer of genetic material into or between ruminal microorganisms have involved bacteria (Tab. III), and have generally been aimed at introducing new genes into chosen species in order to manipulate rumen functions. Only few recent studies have focussed on the occurrence of gene transfer between rumen microorganisms in vivo. No information is available about rumen protozoa and there is only one report of artificial gene transfer to a rumen fungus [17].

#### 4.2. Gene transfer to ruminal bacteria under laboratory conditions

Advances in genetics of rumen bacteria have been the subject of several reviews [24, 80, 82]. Native plasmids have been isolated from most of the predominant rumen bacteria (Tab. III). With a few exceptions, the role of these plasmids remains unknown, but some have been used to construct shuttle vectors for use in rumen strains. The introduction of plasmids or marker genes into ruminal bacteria was attained mainly by electroporation and conjugation (Tab. III). Electroporation-mediated artificial transformation has been reported in several species [81, 83] while many other species remain resistant to transformation by transposons or plasmids (unpublished results). The very efficient protection (restriction-modification) systems described in these rumen species may tend to limit the acquisition of foreign DNA [22, 61]. At least one member of the rumen microflora, *S. bovis* displays ‘natural’ competence for transformation. Competence development appears,

however, to be inhibited by rumen fluid [53]. Failure to demonstrate transformation of many rumen bacteria may result from inappropriate physiological and medium conditions; studies in aquatic ecosystems have indicated that environmental conditions may lead to transformation of strains that are apparently not transformable under normal laboratory culture conditions [60]. Even in *E. coli*, which was considered for many decades to be the classic example of a bacterium that required artificial methods such as calcium chloride treatment to induce competence, it has been found that relatively low levels of calcium such as those found in ground water are sufficient to induce competence [3]. Given that natural competence has been reported for an increasingly large number of bacterial species [49], it is reasonable to assume that many gut bacteria will prove to be capable of natural transformation.

The conjugative transfer of plasmids between species and within a species has been shown to occur *in vitro* in several species (Tab. III). For example, a plasmid carrying a tetracycline-resistance ( $Tc^r$ ) gene isolated from a *P. ruminicola* strain was transferred by conjugation to several other strains from the same species [23]. In addition, conjugal transfer was achieved from *P. ruminicola* to several human colonic *Bacteroides* species (*B. uniformis*, *B. thetaio-taomicron*, *B. distasonis*), as well as from *Bacteroides* to *P. ruminicola* [75]. Another mechanism for cell-cell transfer involves conjugative transposons which may prove more significant than plasmids in gut bacteria [64]. Conjugative transposons are self-transmissible elements that are normally integrated into the chromosome or a plasmid but can excise themselves and transfer by conjugation to a recipient. They have been shown to be able to transfer naturally between a variety of unrelated bacteria [64]. Transfer and chromosomal integration of the streptococcal transposon Tn916 has been achieved by conjugation from *Enterococcus faecalis* into several rumen species

(*S. bovis*, *B. fibrisolvans*) [40]. Another self-mobilising transposon (Tn1545) was transferred from a clostridial strain to the ruminal *Eubacterium cellulosolvans* [1]. Furthermore, a conjugative transposon recently identified in *B. fibrisolvans* shows rates of transfer under anaerobic conditions *in vitro* that are comparable with the highest transfer rates observed for such elements in aerotolerant bacteria [71].

The presence of high numbers of bacteriophages has been demonstrated in the rumen (up to  $10^{10}$  particles per mL), and a wide variety of morphotypes has been identified [43]. A large number of these phages are lysogenic [44]. However, natural gene transfer to rumen bacteria by transduction has not been investigated.

#### 4.3. Evidence and potential for gene transfer *in vivo*

There are two main approaches for obtaining information on gene transfer events *in vivo*. One is to monitor transfer events as they are happening, either in a simulation of the natural gut system or in the gut itself. The second is to infer transfer events from sequence relationships. For the rumen ecosystem, there is still very limited information available from either approach, but what information is available relates mainly to the transfer of antibiotic resistance genes.

##### 4.3.1. Conjugal transfer

It has been argued that anaerobic environments such as the rumen would not be conducive to transfer of genes by conjugation, partly because of limited energy availability for plasmid transfer. Nevertheless Scott and Flint [70] demonstrated conjugative transfer of an antibiotic resistance plasmid between two strains of *E. coli* under anaerobic conditions in the presence of whole rumen contents. Transfer occurred at substantially lower rates compared with

in vitro matings performed aerobically but was still detectable. Plasmid transfer between *E. coli* strains has also been demonstrated in the rumen [77]. Conjugative transposons have already been identified in *B. fibrisolvens* [71], and are likely to also be present in the abundant *Prevotella/Bacteroides* group [59]. Despite the paucity of direct experimental evidence under gut conditions, there is every reason to believe that conjugal transfer of plasmids and transposable elements will be a major factor in gene transfer between ruminal bacteria given the exceedingly high cell densities, and the availability of surfaces on feed particles, in the rumen system. Indeed, recent work has shown that conjugation in biofilms occurs far more frequently than previously thought [35]. In addition, numerous studies document the transfer of conjugative or mobilisable plasmids in the gut of rats or mice, both in gnotobiotic animals and in animals associated with human gut microflora [19, 32].

Important information on gene transfer involving gut anaerobes has recently come from the study of tetracycline resistance genes. Tetracyclines are still widely used in agriculture in many parts of the world, including their use in ruminants as growth promoters or prophylactics. The types of tetracycline resistance gene present in rumen anaerobes have only recently been identified but include tetQ in the Gram-negative *Prevotella* group [23, 59], together with tetO and a completely new determinant, tetW, in the Gram positive *Butyrivibrio* [2]. TetW genes found in isolates from three rumen genera, *Butyrivibrio*, *Selenomonas* and *Mitsuokella* share more than 99% base sequence identity, arguing for very rapid recent genetic exchange between them [2]. TetW genes have now been found in anaerobic bacteria from pigs and humans and again show remarkable sequence similarity to the original ruminal isolate [72]. The tetW product shows only 68% amino acid sequence homology with its closest relatives (tetM and tetO) among the products of ribosome protection type resistance genes [2]. Some

clue to the tetW origin may lie in its higher DNA % G +C content compared with other ribosome protection genes, but it is currently unclear whether tetW is a recent acquisition from soil microorganisms, or a long standing resident within the gut microbial community. In addition to tetW, a tetO gene found in *B. fibrisolvens* was 100% identical in its sequence with tetO from the human pathogen *Streptococcus pneumoniae* [2]. Thus it appears that resistance genes do not only exchange readily between different genera of rumen bacteria, but these genes can also exchange with bacteria that normally inhabit other gut ecosystems including the human digestive tract. The most likely candidates for such rapid transfer events in the case of tetW and tetQ are conjugative transposons.

#### 4.3.2. Bacterial transformation

The likelihood of conjugal transfer between rumen bacteria must clearly be taken into account when considering the design and potential use of any recombinant bacteria intended for silage or rumen inoculation. On the contrary, it is not directly relevant to the design of GM plants used for animal feed since it does not provide a potential route for plant genes to enter bacteria (although it would clearly increase the dissemination of any genes so acquired). In contrast, bacterial transformation by DNA released from GM plant or microorganisms might be considered as a route for acquisition of transgenes by the native microflora. To assess the likelihood of this, we have to consider the state of the transforming DNA, its degradation rate under gut conditions and the potential of the native bacteria to become naturally competent for transformation by free DNA.

As discussed above, it is very likely that many gut bacteria are capable of natural transformation. The rumen, and rumen microorganisms, are known to be rich in nucleases, and nucleic acids are rapidly degraded [22, 50]. Nevertheless DNA added

to rumen contents does not disappear instantaneously. In studies with human saliva *in vitro*, it has been shown that plasmid DNA survives in a state capable of transforming the naturally competent oral bacterium *S. gordonii* for several minutes [52]. Survival may be shorter *in vivo*, but uptake of transforming DNA by naturally competent bacteria into a DNase resistant state requires only a few seconds [51]. In addition, foreign DNA administered orally to mice was shown to persist in fragmented forms in the gastrointestinal tract [68]. Thus while rapid turnover of DNA may reduce the frequency of transformation events in the gut, it is unlikely to eliminate them altogether given the diversity of gut habitats and the possibility of protective effects of certain feed and soil components on DNA survival [49].

It is therefore important to consider how the nature of released GM DNA sequences of plant or microbial origin will affect their ability to be acquired by a bacterial host. Broad host range plasmids including some of those used in manipulating Gram positive bacteria by definition have the potential to replicate in a wide range of native gut species. In the case of narrow host range plasmids such as pUC (based on *colE1*) which are the most widely used in genetic modification, only certain coliform bacteria would be predicted to be potential hosts. Self-replicating plasmids of bacterial origin might therefore be acquired by certain gut bacteria via transformation [53]. However most potentially transforming DNA can be expected to be in the form of partially degraded linear fragments in the gut. Unless these fragments contain transposable elements or insertion sequences, they will generally only lead to stable bacterial transformation when they share close homology with the chromosome of the recipient bacterium. In general plant DNA fragments must be considered unlikely to transform bacteria because of the lack of close sequence homology. This probability must be significantly increased however when bacterial sequences, including antibiotic

resistance marker genes, have been incorporated into the chromosome of a transgenic plant. Indeed, recent studies have shown transfer of marker genes based on such homologous recombination from transgenic plant DNA to soil bacteria [27, 58]. The probability of acquisition of transgene sequences from plant DNA by gut bacteria by transformation will therefore depend on DNA survival in the gut, the likelihood that surviving DNA will encounter a naturally transformable bacterium, and the probability of recombination events, or reconstitution of a self replicating entity, within the host bacterium. The currently available data do not allow a precise quantitative estimate of this probability, but it has been theoretically calculated to be very low (e.g.  $10^{-19}$ , cited in [9]).

#### **4.4. Acquisition of GM DNA by mammalian host cells**

The work of Doerfler and coworkers [67, 68] indicates that in rats naked DNA molecules are incompletely degraded in the gut and can become incorporated into the host chromosome. If so, as the authors point out [68], mammalian cells have been subjected to challenge with foreign DNA from food throughout their evolutionary history. GMO DNA therefore presents a potential new risk only where the transgenes are not normally present at any significant concentration in the diet, or in the commensal flora. The risks from oncogenic DNA are of course well known to regulatory authorities, but there is little information on which to assess the possibility of deleterious effects resulting from rare insertion of other sequences.

#### **4.5. Transgene and antibiotic marker gene transfer in perspective**

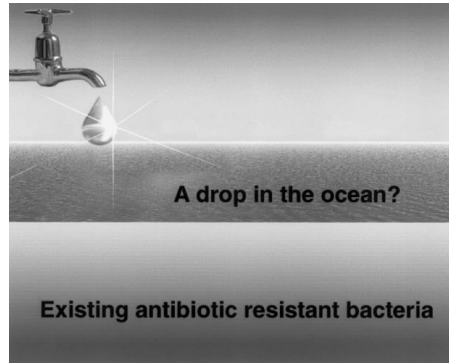
In conclusion, transgene transfer from plants to rumen bacteria is theoretically possible, but probably occurs at an extremely



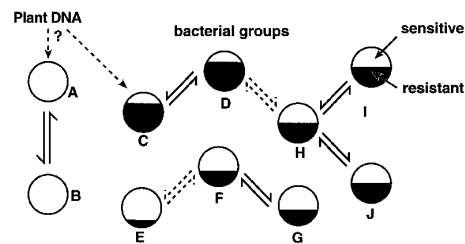
low frequency, if any. Gene transfer from rumen to human colonic bacteria is more likely to occur, given the relatedness of several rumen and colon bacterial species, and the recent data about conjugative transposons [2, 59]. However, the consequences of such transfer would clearly differ, for example, between a polysaccharidase gene that are already prevalent in the human gut flora, and a hypothetical antibiotic-resistance or toxin gene that is currently absent from the gut flora. The probability of further dissemination of the transgene to the wider environment beyond the gut must also be assessed. Transfer of a rumen transgenic bacterium from cattle to feral ruminants would be much more likely than transfer from a transgenic plant to an unrelated plant species. Again the risks resulting from dissemination will also depend on the nature and the function of the gene, and on the selective force acting on the outcome. Accurate prediction of possible consequences of introduction of novel genes in an open environment is thus a highly complex issue that requires substantial further research, and must currently proceed on a case by case basis.

With respect to antibiotic resistance marker genes, antibiotic resistances have become common and widespread since the corresponding antibiotics have become widely used in medicine and agriculture. In view of this, it has been argued that any rare transfer events from ingested plant DNA to gut bacteria could have no significant impact on the incidence of resistance and the risk to human health [63]. The *bla-TEM* ampicillin resistance gene used in some varieties of transgenic maize, for example, is already detectable in ruminal *E. coli* strains (Scott et al., in preparation), and 10 to 50% of the human gut strains are already ampicillin-resistant [9]. It is possible to imagine situations where this argument does not hold, however. For example if a certain group of bacterial pathogens has hypothetically failed to acquire the resistance gene through inter-bacterial transfer, it is conceivable that a

### Risk posed by antibiotic resistance marker gene transfer from transgenic crops?



### Or Opening up a new route for acquisition of resistance?



**Figure 1.** Two views of the threat posed by antibiotic resistance marker gene transfer from GM plants.

new route of acquisition might be opened up by the feeding of transgenic plant material (Fig. 1).

## 5. CONCLUSIONS

The use of GMOs to improve ruminant nutrition and health will, as for the other applications of GMOs, in all probability lead to controversy between proponents of this technology who emphasise its potential advantages, and opponents who highlight risks for human health and the environment. The acceptability of transgenic approaches should depend on a rational assessment of the balance between benefit and carefully assessed risks. Unfortunately,

the necessary careful scientific debate is all too easily obscured by simplistic and emotive arguments in the media.

On the positive side, GM technology has the potential to increase the digestibility and nutritive values of forage plants and crop residues and also to improve the health status of farm animals. The consequent increase in production efficiency should reduce dependence on dietary antimicrobial agents and hormones for altering gut function, and has the potential to deliver substantial environmental and health benefits to mankind. Particularly in the developing world, the need for appropriate application of this technology for food production is already urgent, although many of the applications so far envisaged are more relevant to intensive rather than extensive agricultural systems.

On the other side, there is an obvious need to exclude and avoid undesirable consequences of GMO use. As discussed earlier these include possible direct effects of the transgene product (allergenicity, toxicity) effects exerted through alterations in metabolic activity, physiology or microbial ecology, or possible consequences of gene transfer. It is clearly vital to arrive at a scientific consensus over what constitutes GM safety. We can all agree that risk assessments should ensure that any GM plants and microorganisms used in agriculture should pose no significant risks to human and animal health or to the environment. The problems come in deciding (1) how exhaustive and therefore how costly tests on individual products should be (2) what tests will satisfy both the scientific community and the public, and (3) what to do in cases where the basic scientific knowledge required for full risk assessment may be lacking. For example, we are still on a steep scientific learning curve with respect to gene transfer events in the GI tract. If we insist on awaiting complete scientific knowledge and certainty, however, this can only mean abandoning the potential benefits of GM technology altogether. A balance has to be

found based on a combination of meaningful experimentation and careful reasoning.

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