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Chesson A., Flint H.J.

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Genetically modified ingredients in animal nutrition: Their safety and future

A. Chesson and H.J. Flint
Rowett Research Institute, Bucksburn, Aberdeen AB21 9SB, UK

SUMMARY - The immense potential of genetic manipulation techniques is now being realized with a dramatic increase in the agricultural and industrial use of modified plants and microorganisms. Many animal feeds now include material from crop plants that have been modified for characteristics such as disease or pest resistance that are unlikely to affect their nutritional value. In addition crop plants are being modified to improve their nutritional characteristics as animal feed (e.g., amino acid composition, degradability) or to function as bioreactors for products, including bulk enzymes and hormones, that have applications in animal nutrition. Genetic modification will also be used to improve the properties of microorganisms used as silage inoculants and as probiotics, and to create new microbial products. The likelihood that very large quantities of GM material will soon be ingested by animals worldwide makes it imperative that safety issues are fully explored. These include possible direct toxicity or antinutritional effects of transgene products, indirect or unplanned effects on gut microbial ecology and metabolism, and possible onward gene transfer of transgenes to the resident microflora of the gut. While current assessments of risk can only be based on available scientific knowledge, there is a continuing need to increase our basic understanding of many aspects of biology relevant to the safe exploitation of gene technology.

Key words: Transgenics, biotechnology, risk assessment, animal nutrition, genetic modification, gene transfer.

RESUME - *"Ingrédients génétiquement modifiés en nutrition animale : leur sécurité et leur avenir". Le potentiel que représentent les techniques de manipulations génétiques est à l'heure actuelle utilisé de façon spectaculaire avec l'usage de plantes modifiées et de microorganismes, en agriculture et dans l'industrie. De nombreux aliments pour animaux incluent maintenant des matériaux issus de plantes modifiées afin d'augmenter leur résistance aux maladies ou aux parasites, sans provoquer de modifications notables de leur valeur nutritionnelle. Les plantes sont aussi modifiées pour améliorer leurs caractéristiques nutritionnelles dans l'alimentation animale (ex : la composition en acides aminés, la dégradabilité) ou pour être utilisées dans les bioréacteurs produisant notamment des enzymes enrobées et des hormones ayant une application en nutrition animale. Les modifications génétiques serviront aussi à améliorer les propriétés des microorganismes utilisés pour inoculer les ensilages, utilisés comme probiotiques ou pour créer de nouveaux produits d'origine microbienne. Bientôt une telle quantité de matériel génétiquement modifié sera ingérée par les animaux qu'il est nécessaire d'explorer largement les données de sécurité. Celles-ci incluent les risques de toxicité directe, les effets antinutritionnels des produits transgéniques, les effets indirects ou imprévus sur d'écologie microbienne et le métabolisme de l'intestin, et les possibles transferts de gènes du produit transgénique à la microflore intestinale naturelle. Bien que l'évaluation actuelle des risques ne peut être basée que sur la connaissance scientifique disponible à ce jour, il existe un besoin continuel d'augmenter notre compréhension des nombreux aspects biologiques concernant la sécurité dans l'utilisation des biotechnologies.*

Mots-clés : *Transgénique, biotechnologies, l'évaluation des risques, nutrition animale, modification génétique, transfert de gènes.*

Introduction

Some 15 million hectares of genetically modified crops were grown in 1997 in the USA alone. Maize, tomato and soybean predominated with lesser amounts of cotton and potato. Large areas devoted to transgenics exist in other countries, notably China, but information on which crops and the nature of the transformants is less readily obtained. As a result of these and earlier plantings feed manufacturers are already using, and will use in increasing amounts, GMO plants or their by-products in their formulations. In fact, since the products of most GMO plants currently are not separately handled after harvest but amalgamated with their non-GMO counterparts, manufacturers may have little opportunity to avoid their use.

Genetic engineering of crop plants is now an established fact and there are no major practical barriers to the creation of new varieties of cereals and legumes whose recognized deficiencies as feedstuffs have been corrected. However, "designer crops" modified to meet the needs of specific production systems, while an attractive proposition, are a long way from realization. For a variety of practical, economic and social reasons, the use of recombinant technology to improve the nutritional characteristics of plants used as feed for livestock production is not high on the agenda of most seed and biotechnology companies. Two other areas dominate at present, notably the introduction of disease or herbicide resistance to improve crop agronomy and the production of high value end-products, often with pharmaceutical applications.

The first generation of GMO crop plants can be considered primarily as those with improved agronomic characteristics of benefit to the grower. Crops expressing herbicide tolerance or pest resistance genes, together with any marker genes which were used to aid the selection of constructs, are the most commonly encountered. Plants of this nature are considered to have a substantial equivalence to the normal plant and thus confer no nutritional advantage for the livestock industry. Questions of safety for animals and consumers arise simply in relation to the potential toxicity of the gene products, the transfer of any antibiotic resistance genes remaining in constructs to the natural gut flora, and the broader environmental concerns about the consequences of the flow of pest or herbicide resistance to other plant species (Harding and Harris, 1997).

Aside from questions of profitability, an industrial end use for GMO plants avoids the immediate concerns of the public and regulatory bodies associated with the introduction of transgenes into the human food chain. Ironically, as a consequence of this, and as shown by the proliferation of new rapeseed (canola) varieties being developed for non-feed use around the world (Murphy, 1996), the number of different by-products available for incorporation into compound feeds could increase dramatically. The impact of this plethora of by-products on the feed industry is likely to be far greater in the medium term than any transgenic crops "designed" for feed use. Feed producers may well find that the parameters for by-products currently used in least cost formulation are no longer applicable and, certainly, a greater awareness of, and control over, the sourcing of ingredients will become essential. Plants also are being actively considered as bioreactors for the production of recombinant biopharmaceaticals including cytokines, hormones, monoclonal antibodies, bulk enzymes and vaccines (Miele, 1997). It is inevitable that the by-products after extraction of these products will find their way into feeding systems raising some concerns about residues and safety.

Engineering feed resources

Although few crops engineered specifically for improved feed qualities have reached the stage of field trials, considerable development work has been done in the laboratory and transgenic plants with modified seed proteins, starch and oil content and improved fibre degradability exist and are being evaluated. Seed producers favour hybrid crops at present because of the guaranteed annual returns on investment and for this reason rather less attention has been paid to cereals other than maize, where seed can be saved on-farm. However, probably the greatest practical problem limiting the introduction of transgenics designed for feed use is recovery of the added value. Where added value directly aids crop production, a premium price can be demanded of the grower who is able to offset this against the reduced use of pesticides or greater yield following better weed management. Crops with improved feed characteristics have added value only for the end user (the animal production industry) and the grower has to recover the premium paid to the seed company directly or indirectly from the livestock producer. This is only possible where the transgenic crop is separately harvested, stored and marketed and few have the facilities necessary.

Another factor mitigating against the development of some genetically modified crops are the measures already taken by the feed industry to correct the recognized inadequacies of existing feed resources. These range from the sophisticated mixing of ingredients in proportions which best meet the needs of livestock at least cost to the producer to the incorporation of specific additives to obtain greater nutritional benefit from diets. In theory at least, formulation could be greatly simplified and the use of additives avoided by the genetic modification of common feed resources. In practice, this is unlikely to happen because, firstly, additives offer flexible and cost-effective solutions not readily provided by genetic engineering and, secondly, investment in breeding new varieties, whether by

conventional or recombinant means, could not be justified by the small and variable returns offered by many additives.

Changing the amino acid profile of seed proteins

It is well recognized that amino acid profiles of seed proteins do not match livestock requirements. Typically lysine and threonine are present in limiting amounts in cereal-based rations while methionine is often present in legume seed proteins in amounts inadequate for some feed purposes. Traditional breeding methods to improve the complement of amino acids have met with only limited success, any gains being offset by undesirable seed traits. Not surprisingly recombinant technology has been identified as the means to introduce specific changes while avoiding the problems which have accompanied conventional breeding and mutagenesis.

Several strategies for the modification of seed protein have been identified but only two to date have resulted in the production of transgenic plants (Altenbach and Townsend, 1995). The first and most successful is the introduction of novel seed proteins which have more desirable amino acid profiles. Seed proteins from a wide range of plants have been identified which contain a high proportion of methionine residues (12-22%). Amongst these, constructs involving the methionine-rich 2S albumin from Brazil nut with a 19% methionine content has been widely and successfully used in gene transfer experiments (Saalbach *et al.*, 1994). Expression of this gene in rapeseed (canola) accounted for 4% of total seed protein and increased the overall methionione content of the seed by 33% (Altenbach *et al.*, 1992). Additional tryptophan codons have now been introduced into the Brazil nut gene further improving the nutritional balance of the protein (Marcellino *et al.,* 1996). However the extent of expression of the introduced gene cannot be predicted with any certainty. In a similar exercise with the Brazil nut gene expressed in soybean, the overall increase in methionine was only one-quarter of that calculated. In this case synthesis of the Brazil nut protein occurred at the expense of a minor soybean protein also rich in methionine when competition for sulphur amino acids simply led to a redistribution of supply rather than stimulating additional synthesis (Saalbach *et al.*, 1994). Thus, consideration also may need to be given to the flux of amino acids and rate limitations in their synthesis adjusted.

While the successful production of transgenic plants with improved methionine concentrations demonstrated that manipulation of seed proteins can be achieved, work with the Brazil nut gene also provides a useful object lesson in the need for safety consideration. Unfortunately, expression of the 2S protein in soybean also transferred the potent Brazil nut allergen precluding its practical use (Frick, 1995).

Existing seed proteins with a naturally high lysine content have proved more difficult to identify. Alternatives, such as expressing non-seed protein in seeds or engineering existing cereal protein genes by substituting codons for non-essential amino acids with those for lysine, have yet to be proved feasible. Down regulation of one storage protein to stimulate compensatory production of others with benefit to the overall amino acid profile has proved an option in a limited number of cases, notably rapeseed (Kohnormurase *et al.*, 1995). A more general approach of circumventing the normal feedback regulation of key enzymes in the lysine biosynthetic pathway resulted in 100-fold increase in free lysine in rapeseed and soybean with evidence of additional incorporation into storage proteins (Falco *et al.,* 1995).

Modifying the energy-yielding components of crops

Lipid metabolism

After cereals, oil crops are the most important source of calories for human societies and are the source of many industrial products and have the capacity to provide feedstock for many more. The four major oil crops in order of importance are soybean, oil palm, rapeseed and sunflower seed and, together, they account for over 70% of world-wide vegetable oil production (approximately 70 Mt). Not surprisingly a major objective of plant biotechnology is to manipulate the amount and composition of seed storage lipids for medium to high-value industrial applications. The first transgenic crop with modified seed composition, a lauric-oil rapeseed intended for the detergent market, was approved for

commercial use in the USA in 1995. Other constructs undergoing field trials will provide oils for use in polymer synthesis, cosmetics, lubricants and the pharmaceutical industry. There are applications in foods, notably a transgenic rapeseed variety in which the stearate desaturase gene has been down-regulated by incorporation of an antisense copy of a bacterial gene, resulting in seeds containing 40% stearic acid (Knutzon *et al.*, 1992). Saturated acids such as stearate find a market in margarine production and as a cocoa butter substitute. It is both easier and more desirable from an industrial viewpoint to suppress desaturase activity and increase the stearic and oleic acid content of seed oils at the expense of di- and trienoic polyunsaturates. Raising levels of linolenic acid in seed oils is a potential target, but not primarily for any nutritional reason. If linolenic acid could be produced in economic quantities it could compete with petrochemicals for use in paints, coatings and drying oils.

The low value of oilseeds in the feed market ensures that nutritional targets are far less attractive than those with an industrial application. Oil crops commonly find their way into animal feeds as co-products after oil extraction rather than as whole seed. Feed use is seen more as a means of disposal rather than a prime market. However, large numbers of transgenic oilseeds will mean that by-products cannot automatically be assumed to come from an unmodified plant with known nutritional characteristics. Seedmeals will invariably reflect the plants from which they derive and many may well have come from GMO's expressing high levels of oils including euricic acids. The nutritional properties and safety of such products cannot be assumed.

Starch synthesis

Starch is a primary and obvious target of interest both to the food/feed sectors and to the many industries which make use of starch, in original or modified form, as feedstock. Cereal starch dominates the European food/feed market and there is considerable interest in manipulating grain characteristics useful to processors and manufacturers. Research, however, has focused on potato not least because of the relative ease of its transformation using *Agrobacterium*.

The amylose: amylopectin ratio, degree of branching and chain length determine the physicochemical properties of starch and the genes responsible for the enzymes controlling these properties have been cloned from a variety of sources. Manipulation of these biosynthetic enzymes, largely by use of antisense constructs, has shown that it is possible to markedly change the properties of the starch granule and its component polymers. Thus suppression of the granule-bound starch synthase, responsible for amylose synthesis, results in the production of starch consisting only of amylopectin. (Kuipers *et al.*, 1994). The degree of amylopectin branching has been altered by manipulation of starch branching enzyme activity (Kortstee *et al.*, 1996). Suppression of soluble starch synthases, also involved in amylopectin synthesis, does not greatly effect the amylose: amylopectin ratio or the total amount of starch present, but has profound effects on granule morphology, indicative of significant changes in the amylopectin polymer (Abel *et al.*, 1996; Marshall *et al.*, 1996). At present no clear functions can be assigned to the various isoforms of the soluble starch synthases and a better understanding of granule formation is needed before tailor-made starch becomes a reality. Although not an immediate prospect, feed producers should be aware that modification of starches to better suit industrial processes may affect the nutritional characteristics of the many by-products which derive from starch-based industries.

Lignin, tannin and the availability of structural polysaccharide

There exists the potential to manipulate the production of any one of the large number of secondary metabolites produced by plants. Amongst these compounds, products of the phenylpropanoid pathway which include lignin, tannins and the flavonoids have attracted particular attention. Several of the enzymes involved in the section of the phenylpropanoid pathway leading to the production of the lignin precursors have been the target of sense and antisense technology. The selection of activities for regulation have been heavily influenced by studies of the brown midrib mutants of maize, sorghum and pearl millet. These mutants have sometimes shown a reduction in lignin content and improved degradability (Cherney *et al.*, 1991) associated with reduced O-methyl transferase (OMT) and/or cinnamyl alcohol dehydrogenase (CAD) activities (Grand *et al.*, 1985; Pillonel *et al.*, 1991). However, it is questionable whether lignin is a major barrier to cell wall digestion in forages and thus an appropriate target for manipulation. Other factors, such as cell wall thickness,

may prove to be more important and better traits for selection for improved degradability (Chesson and Travis, 1997). This view is supported by the poor response shown to down-regulation of OMT or CAD in tobacco where little effect on either lignin content or cell wall degradability could be demonstrated (Bernard-Vailhé *et al.*, 1996).

The related polymer, condensed tannin, built from flavanol units, has both anti-nutritional protein-binding effects and, perversely, the potential to improve protein utilization in ruminants. Condensed tannins are closely related to the anthocyanins responsible for many flower colours and their synthesis share a number of biosynthetic enzymes. Some attempts have been made to manipulate tannin biogenesis, often with flower colour as a marker (Carron *et al.*, 1994). Selection of various forage legumes for high tannin has shown greater amounts of protein nitrogen surviving breakdown in the rumen and passing to the small intestine of grazing ruminants (Lowry *et al.*, 1996). However, in the longer term a more important application of tannin manipulation may be to reduce levels in the many tree legumes whose leaf protein content could add substantially to ruminant production in many parts of the world where protein-rich supplements are not readily available (Roothaert and Paterson, 1997).

Other targets for genetic manipulation

There are many changes being considered which directly or indirectly might influence nutritional value. These range from altering the production of natural antioxidants in order to reduce the need for food additives, to manipulating phytase and β-glucanase expression in the endosperm of cereals. In addition to the crop plants that form the basis of animal nutrition, recombinant technology also will affect the various feed additives derived from microrganisms or their genes products. All such manipulations raise questions of safety regardless of whether such concerns have a basis in science. Rigorous testing of all transgenics entering the food chain for toxicological and anti-nutritional effects is essential.

Genetically modified microorganisms and their products AS feed additives

Recombinant feed enzymes and other microbial products

Many current enzyme additives used in pig and poultry feed derive from filamentous fungi such as *Aspergillus* or *Trichoderma* spp. Since gene cloning and strain modification by genetic manipulation are now technically straightforward in these organisms it can be anticipated that the market will become dominated by products derived from genetically modified microbial strains or from transgenic plants. Heterologous genes can be introduced that enhance a desired activity, or resident enzymes can be modified to improve their characteristics (e.g., stability, pH tolerance) through site-directed mutagenesis or the construction of hybrid genes. This type of application is generally felt to raise few new safety considerations, unless the producing organism is, intentionally or unintentionally, part of the final product. It is worth stressing however that any new hybrid or modified protein has an outside chance of producing unexpected allergenicity problems in humans, while any change in the stability or activity of enzymes may have effects on gut metabolism that go beyond those originally envisaged. Therefore thorough risk assessments will always be important to detect possible adverse effects of the novel proteins or activities present. Similar comments apply to enzymes and other recombinant gene products derived from genetically modified bacteria.

Genetic modification of microbial inoculants

Live microbial inoculants and probiotics (also referred to as direct fed microbials) are used widely in animal nutrition. Again it is clear that genetic modification will be used increasingly in attempts to improve the characteristics and efficacy of inoculants, and for some applications the necessary research is already well advanced. The extensive, deliberate release of genetically modified microorganisms for agricultural purposes clearly raises more fundamental safety concerns than their contained use.

Ruminants

Because of the absence of a preceding acidic barrier, the rumen is quite unusual among gut habitats in being open to inoculation with foreign microbes. Grazing ruminants can potentially ingest a variety of modified microorganisms and viruses that may be applied to crop plants to suppress pests and diseases, and this possibility has to be considered in the relevant risk assessments. Lactic acid bacteria used as silage inoculants have been modified by insertion of polysaccharidase genes that are intended to allow them better access to energy sources for growth, or to increase the subsequent digestibility of the silage by the animal (Scheirlinck *et al.*, 1989). Clearly large numbers of viable modified bacteria are likely to enter the rumen of animals ingesting such silage. Deliberate introduction of other genetically-modified microorganisms into the rumen is also likely in the future. Existing probiotics, including those based on yeast, filamentous fungi and lactic acid bacteria, will surely be modified to enhance their efficacy as soon as their mechanism of action is well enough understood to allow this to be done successfully. Furthermore, the emerging ability to genetically modify ruminal bacteria themselves creates some entirely new 'probiotic' possibilities. Techniques for genetic manipulation of some oxygen tolerant species, notably *Streptococcus bovis* (Whitehead and Flint, 1995), and the more numerous oxygen intolerant species, such as *Butyrivibrio fibrisolvens* (Gregg *et al.*, 1994), are now available. In the latter case it has been proposed to use a strain carrying a plasmid-encoded fluoroacetate dehalogenase gene to help detoxify this forage plant toxin in Australia (Gregg *et al.*, 1994) and numerous other proposals for using modified rumen organisms have been made previously (Smith and Hespell, 1983).

Monogastric animals

Probiotics are widely used as dietary additives for monogastric animals and are also part of the human diet, since live yoghurts fall within this definition. A number of benefits are claimed but, in animals, their most important potential lies in suppressing gut infections. It is easy to envisage strain modification having a major role in improving this aspect of probiotic use in the future, e.g., through expression of selective antimicrobial activities, enhanced survival in the host, or expression of particular recombinant antibodies or receptor-binding specificities. Most existing probiotic preparations are based on lactic acid bacteria that have GRAS (generally regarded as safe) status, although this definition is being questioned in the case of *Enterococcus* spp. in particular which include pathogenic relatives. Recent progress in the genetics of lactic acid bacteria, including *Lactococcus* and *Lactobacillus* spp. (Gasson, 1993) means that the necessary techniques for strain manipulation are already well advanced. Strains have been manipulated to overexpress foreign proteins for various applications including use as oral vaccines (Wells *et al.*, 1993). In *Lactococcus,* vector systems are available that are based entirely on lactococcal DNA, therefore classifying as self-cloning systems (MacCormick *et al.,* 1995). The availability of methods for genetic modification creates entirely new possibilities for using recombinant microorganisms to deliver specific enzymes (such as xylanases, glucanases or phytases) and other gene products to the lower gut to overcome nutritional problems.

Safety implications

Products of transgenes

The most obvious and immediate safety issue with any genetically modified plant or microbe is whether the product of the novel transgene can have deleterious effects in the environment or upon ingestion by humans or animals. Clearly this has to be assessed on a case by case basis.

Direct toxicity

The most economically important group of transgenic crops are currently those expressing insecticide or pesticide genes. Where possible genes are chosen whose products are known to be highly specific in their action. The Bt toxin, for example, is believed to be toxic only for lepidopteran and dipteran larvae and there is no evidence that it can be toxic for mammals. Its specificity depends on recognition by toxin receptors said to be found only in the insect gut. Other toxins, however, show less specificity and wheat germ agglutinin has antinutritional effects when fed to rats (Peferoen and

Rudelsheim, 1993). It is extremely important therefore to establish the range of target species that might be affected by any new toxin, and it is also important to determine how toxins are modified, resulting in activation or inactivation, during passage through the gut. Possible consequences of transfer of the gene to gut bacteria must also be considered, as discussed later. Finally, even for toxins that appear highly specific, it is still important to consider possible ecological consequences of inhibition of harmless or beneficial species that happen to be closely related to the target species. There are recent suggestions that bees can be affected by pollen from transgenic oilseed rape expressing a protease inhibitor (Crabb, 1997) and that ladybirds are affected by consuming aphids that have been feeding on transgenic potatoes expressing snowdrop lectin (Gledhill and McGrath, 1997).

Indirect effects

It is always possible that modifications made for one purpose will have unpredicted ecological consequences. For example, it is conceivable that equipping a silage organism with novel polysaccharidases might radically alter microbial competition through the release of readily usable carbohydrate, perhaps encouraging the growth of spoilage organisms or even pathogens such as *Listeria*. Creating plants that are more degradable by ruminants through manipulation of their lignin content and composition might have the consequence of releasing more phenolic compounds into the rumen which might in turn radically affect the balance of the microflora. It will be important to develop more convenient methods for monitoring the effects of modified organisms and feed material upon gut microbial ecosystems.

Gene transfer in the gut and the dissemination of transgenes

A more general concern has been the possibility of onward transfer of transgenes from modified organisms. In the case of modified plants most attention has been given to the potential spread of transgenes through outbreeding with weedy relatives. Both for modified plants and microorganisms, however, the potential for onward gene transfer in the digestive tract needs to be established. Recent experimental evidence challenges several comfortable assumptions that have been made. Although it would be reasonable to predict that DNA is rapidly destroyed by nuclease activity in the gut, a recent report shows that M13 viral DNA not only survived passage through the digestive tract of mice but was detected in host tissues (Schubbert *et al.*, 1994). Meanwhile it has also been shown that sequences present in the bacterial chromosome can become incorporated into the chromosome of mammalian cells (Courvalin *et al.*, 1995). It is therefore not out of the question that modified genes acquired by elements of the gut flora might be come incorporated into cells lining the gut wall.

Gene transfer mechanisms

Most is known about mechanisms of transfer that involve cell-cell contact (conjugation) by which self replicating plasmids and mobile chromosomal elements can pass between bacteria (Fig. 1). Conjugation occurs in both Gram-positive and Gram-negative bacteria, and can involve distantly related partners, the main limitation being the range of hosts in which the element can express its genes and replicate. It might be thought that onward transfer of transgenes from GMM could be avoided entirely by locating modified DNA in the bacterial chromosome and by choosing strains that lack known transmissible elements, but unfortunately this is not so. Released strains can readily acquire new, possibly unknown genetic elements from their environment that can effect transfer of any chromosomal gene at low frequency. Another mechanism of gene transfer that may have been underestimated is bacteriophage (virus)-mediated generalized transduction, where random chromosomal fragments can become packaged into viral heads and thus transported to a new host. Conjugation requires live, metabolically active donor and recipient cells, while transduction depends on the survival of transducing particles, but not of the donor bacterium.

A third mechanism, transformation, requires only DNA and live recipient cells in a state competent to take up DNA and can potentially occur with DNA from any source. An unknown proportion of

bacteria are naturally competent, taking up DNA indiscriminately, or in a sequence specific manner (Lorentz and Wackernagel, 1994). Genetic transformation in bacteria is assumed to be limited to those situations where the incoming DNA can reconstitute to form a self replicating entity (e.g., plasmid) or where there is sufficient sequence homology with the recipient chromosome to allow insertion by recombination.

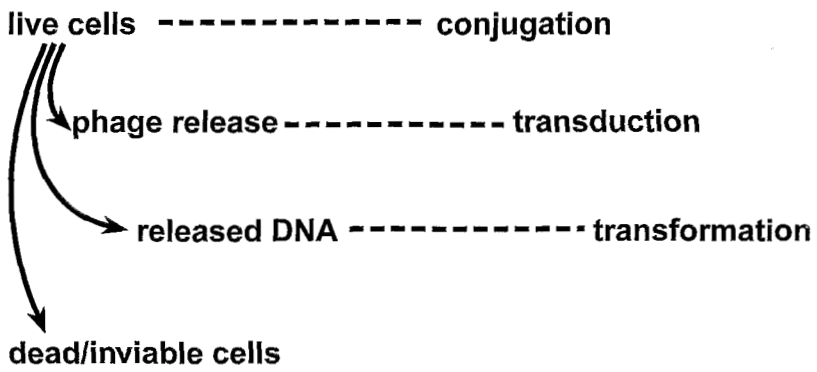

Fig. 1. Mechanisms of gene transfer in gut bacteria.

Gut conditions

With regard to transformation by DNA released from feed or ingested GMMs, it seems likely that those regions preceding the acidic stomach (the rumen, avian crop, oesophagous and mouth) might see the highest concentrations of intact DNA entering with the diet. In fact DNA has been shown to survive for significant times in human saliva and to be capable of transforming a human oral bacterium (Mercer *et al.*, 1998). DNA turnover in most other gut environments, including the rumen (McAllan and Smith, 1973) is certainly rapid, but it is still conceivable that microenvironments exist where it is not degraded, or that certain dietary components afford protection against degradation. Under *in vitro* conditions conjugation is often found to be favoured in populations associated with surfaces, but it is not known whether this applies in the gut.

Potential impact of gene transfer

It is sometimes argued that gene transfer between bacteria is already so extensive in natural ecosystems that any transfer of transgenes will have negligible impact. As a general argument this does not hold up. While some natural bacterial populations show rapid assortment of genes (Maynard Smith, 1995) others, including *E. coli,* exhibit a largely clonal structure which suggests that for most genes horizontal transfer is a rare event (Whittam, 1995). Rare transfer events can have enormous significance however, and can be amplified very rapidly under favourable selective conditions, as evidenced by the spread of antibiotic resistances and by the emergence of new pathogens. Verocytotoxic *E. coli* 0157, for example, are thought to have acquired their shiga-like toxin through a bacteriophage mediated transfer event (Whittam, 1995). With regard to transgenes the two most pertinent questions are whether release of a modified organism is likely to create a new route for acquisition of novel genes by organisms that are unlikely to have been able to acquire them naturally, and whether such acquisition could have any deleterious consequences. As an illustration it is useful to consider the use of antibiotic resistance markers.

Antibiotic resistances as genetic markers

To date antibiotic resistance have been the most widely used markers in bacterial molecular genetics, and play important roles in plant manipulation. The reason for this is the requirement for a selectable marker that allows cells transformed with the desired DNA constructs to be rapidly recovered from a background of non-transformed cells, and the easiest way to achieve this is by

killing the non-transformed cells. Antibiotics do not provide the only means of doing this - alternatives are genes that encode antimicrobial compounds that have no little or no therapeutic value (e.g., some amino acid analogues, heavy metal resistance) or that allow utilization of new substrates for growth. It so happened that antibiotic resistance genes were present on many of the plasmids used for early work in microbial genetics and became the most obvious and convenient method for selection. It is now generally agreed that genes encoding resistance to clinically useful antibiotics should not be present in modified microorganisms intended for release into the environment, although it may be argued in specific cases that resistance is already so prevalent as to make any contribution from released microorganisms insignificant. In bacteria it is perfectly possible to find alternatives to antibiotic resistance, and it is also possible to design constructs in such a way that resistance genes used in the initial construction can subsequently be excised.

In transgenic plants the arguments are less clear. Some markers (e.g., ampicillin resistance) are only useful in selecting constructs in the bacterial host but have in some cases been incorporated into the transgenic plant even though they are not expressed in it. Other markers (e.g., kanamycin resistance) are important for selection in the plant itself if they are expressed from a suitable plant promoter, in which case they will not normally be found to express in a bacterial host. The following arguments have been advanced to justify inclusion of antibiotic resistance markers in plants:

(i) Transfer of resistance genes present in plant chromosomal DNA to gut bacteria is exceedingly unlikely.

(ii) Rare transfer events arising from ingestion of modified DNA would have little impact on the frequency of resistance genes in the environment, and their impact would be insignificant compared with the effects of indiscriminate use of antibiotics which is known to have dramatically increased the incidence of resistance.

(iii) In the case of the ampicillin resistance gene found in pUC and similar vectors, this gene is already widely distributed among gut bacteria and among clinical isolates of bacterial pathogens.

In relation to (i), it is necessary to know whether vector sequences present in plant DNA can somehow loop out and reform to give functional plasmids (which is not inconceivable particularly where multiple copies are present), or whether linear fragments of plant chromosome have any potential to transform gut bacteria. An obvious mediating factor would be the survival time for DNA in gut environments. While it is generally assumed that DNA turnover is rapid in environments such as the rumen, the possibility that special microhabitats exist in which DNA could survive or that dietary components may bind and protect DNA from degradation has already been mentioned. Another factor is the prevalence of natural transformation among gut bacteria. Little is known about this for most gut species, but it is worth noting that *E. coli*, always cited as a bacterium capable only of artificial Ca^{++} induced transformation, was recently shown to be naturally transformable in the presence of Ca^{++} concentrations found in ground water (Baur *et al.*, 1996). In the case of pUC plasmids whose host range is limited to coliform bacteria, only transformation of *E. coli* and its relatives appears relevant.

With regards to points (ii) and (iii), these arguments conceal some important considerations. In particular, do they apply to all pathogens and to all geographic locations? It only requires there to be a significant risk that the treatment of one disease might be compromised somewhere in the world as a result of resistance gene transfer to justify the cautious view. The real question is whether a new route of transfer might be created for a gene to enter a pathogen that does not yet possess it. Approval has been given for use of kanamycin resistance as a marker in plants, and for feeding of ampicillin resistant maize to animals, because on current evidence it is difficult to perceive a genuine risk. Further research into gene transfer possibilities is still very necessary, however, and it cannot be assumed that antibiotic resistance markers will be deemed acceptable in the modified plants of the future.

Conclusions

From a safety perspective the main lesson appears to be that the intensive effort in molecular biology and testing to establish the efficacy of transgenic products has to be coupled with equally

intensive and high quality research aimed at predicting and avoiding potential risks. It should be apparent that at this stage even relatively trivial negative consequences may adversely affect public opinion to an extent that is out of all proportion with the real risks. On the other hand complete certainty over risks is not possible. For example it is impossible to completely exclude or eliminate the chance that transgenes will be transferred, particularly from modified microorganisms. However, to block the development of genetically modified organisms on the basis of largely hypothetical risks will mean failing to exploit a technology that has immense potential benefit to mankind, and one able to help to eliminate the undesirable side effects of the widespread use of chemical insecticides and antibiotics. The pragmatic view is that we continue to make the most realistic risk assessments and regulatory decisions based on the often limited scientific evidence currently available, while at the same time striving to improve our basic understanding of aspects of biology relevant to risk assessment.

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