

Assessment of the risks of transferring antibiotic resistance determinants from transgenic plants to micro-organisms G01010

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Background

In the production of genetically modified (GM) plants, genes conferring ‘desirable’ traits are identified and are then assembled in bacteria before delivery into the target plants. The assembly of genes in bacteria is made simple by including antibiotic resistance marker genes. The presence of a gene that codes for resistance to an antibiotic in a single bacterial cell means that, by adding the appropriate antibiotic to a culture, the single cell can be isolated from millions or even billions of bacteria lacking the resistance gene present in the same culture. By linking a gene that confers antibiotic resistance on its host bacterium to the genes that are to be inserted into plants, sufficient quantities of DNA can be harvested from a bacterial culture to allow the novel DNA to be delivered easily to the plant. One marker gene commonly used in the early days of GM crop development, called *bla*_{TEM}, confers resistance to antibiotics such as ampicillin and amoxycillin. This gene causes the production of an enzyme, known as a β -lactamase, which breaks down the structure of these antibiotics, rendering them ineffective against bacteria. Although the bacteria that live in animal and human guts frequently carry *bla*_{TEM}, which occurs naturally at a high frequency, this project is designed to examine the risk that *bla*_{TEM} from GM maize can pass from the modified plant tissues into microbes in animals that are fed GM maize.

Rationale and Objectives

APPROACH

In essence, this project sought evidence for the occurrence of a very rare event. In order to tackle this problem, a critical step analysis was undertaken. This project was designed to address six main areas of concern. These are summarised below:

- 1) What is the risk that the *bla* gene will "loop out" from transgenic plant cells?** The DNA cassette inserted into the maize plant should be stably incorporated. If, however, it can "loop-out" from the plant DNA, its chances of spreading will be enhanced.
- 2) Do animals fed on transgenic maize release the *bla* gene from plants?** In order to transfer from GM plant tissues into microbes, it is necessary that the digestion processes release DNA in a form that can be taken up and also be expressed in bacteria. Sheep were used as the model ruminant animal; chickens were used as the non-ruminant. Both chickens and sheep may be fed significant quantities of maize or maize products.
- 3) With what efficiency can gut organisms take up and express the *bla* gene?** The demonstration that the antibiotic resistance marker gene is digested in the same way as other plant DNA and that it is thus unavailable for uptake and expression by bacteria in the lower intestine rendered this question of lesser importance than at the outset of the project.
- 4) Can the *bla* gene be mobilised from bacteria within an animal gut?** The transfer of *bla*_{TEM} from GM plant material to microbes in the animal gut, were it to happen, is the first step in spreading this gene to new hosts. In theory, once one bacterium has acquired *bla*_{TEM}, it may be transferable to other bacteria in the gut environment.
- 5) Does ensilage of transgenic maize result in the release of the *bla* gene?** Much of the maize feed in the United Kingdom is presented in the form of silage. To make silage, fresh plant material is fermented in the absence of air. Microbes associated with the fresh crop cause the production of organic acids, which preserve the plant material in a form that animals find desirable. The bacteria responsible for the later stages of silage production are fed to animals as part of the silage product. These have the potential to take up and express resistance genes, such as *bla*_{TEM} found in the maize that is the subject of this study.
- 6) To what extent does the *bla* gene undergo mutation to extended spectrum activity in the gut environment?** Over the past fifteen years, hospitals have seen the emergence of bacteria that are resistant to important first-line antibiotics. This resistance may arise from the mutation of *bla*_{TEM}, causing substitution of different amino acids in the enzyme that confers resistance to antibiotics. About ninety such mutations have now been described in *bla*_{TEM}. Many of these allow the enzyme to confer resistance on a much broader spectrum of antibiotics than the original enzyme. Were *bla*_{TEM} in GM plants to mutate, this could pose a risk, were it subsequently to transfer and be expressed in bacteria, particularly if those bacteria were capable of causing infections in humans or in animals.

OUTCOME/KEY RESULTS OBTAINED

There is no evidence that the antibiotic resistance gene, *bla*_{TEM}, can "loop-out" of the maize plant DNA. The survival of *bla*_{TEM} DNA was then studied in a series of experiments that modelled various environments. Sheep saliva, sheep rumen fluid and the fluid that runs off from silage production, effluent, were collected and survival of DNA was monitored under laboratory conditions. It was discovered that in rumen fluid and in silage effluent, *bla*_{TEM} very rapidly lost its biological activity. In contrast, *bla*_{TEM} survived in sheep saliva and in this environment it retained its ability to cause susceptible bacteria to become resistant to ampicillin. Feeding experiments were then carried out with chickens. Although the plant-associated *bla*_{TEM} could be found in the crops of all birds fed on GM maize, it could only be detected in the stomach contents of five birds and was undetectable in any samples taken from below the stomach. This experiment further showed that the plant-associated *bla*_{TEM} was digested in the same way as other plant DNA. Parallel experiments feeding GM plant material to sheep indicated that *bla*_{TEM} DNA survives very poorly in the rumen of animals that are fed transgenic maize or silage made from transgenic maize. In contrast, free DNA in the sheep oral cavity retains the ability to transform susceptible bacteria to resistance for up to eight minutes, implying that DNA released within the mouth from GM plants in the diet may retain sufficient biological activity for genes to transfer from plant material to susceptible oral bacteria. Culture experiments, selecting for ampicillin resistant bacteria, showed no evidence that the bacteria involved in silage formation could take up and express GM plant-associated *bla*_{TEM}. Data from molecular studies suggest, however, that bacteria in silage may take up the *bla*_{TEM} sequence but once taken up, it remains silent.

WHAT IT MEANS AND WHY IT'S IMPORTANT

It can be concluded that the antibiotic resistance marker is stably incorporated into the GM maize that was the subject of this study. Thus, the chances that it will escape easily and transfer to microbes associated with the GM plant or animals feeding on GM material are slight. DNA is, however, released from plants during digestion, and may be available to transfer to microbes. The oral cavity receives a large number of incoming bacteria with the diet and these, along with bacteria that are normally resident at this site may include microbes that can cause infection in humans or animals. This project indicates that biologically active DNA from GM plants can survive in the oral cavity for long enough to transfer to microbes in this site. Similarly, the bacteria involved in silage production may acquire *bla*_{TEM} from GM maize but nevertheless silage bacteria do not express the gene and are not resistant to antibiotics such as ampicillin.

Publications arising from this work:

Duggan PS, Chambers PA, Heritage J, Forbes JM (2000)

Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent

***FEMS Microbiology Letters* 191, 71-77.**

Chambers PA, Duggan PS, Forbes JM, Heritage J (2001)

A rapid, reliable method for the extraction from avian faeces of total bacterial DNA to be used as a template for the detection of antibiotic resistance genes

***Journal of Antimicrobial Chemotherapy* 47, 241-243.**

Chambers PA, Duggan PS, Forbes JM, Heritage J (2001)

The fate of antibiotic marker genes in transgenic plant feed material fed to chickens ***Journal of Antimicrobial Chemotherapy* 49, 161-164.**

Duggan PS, Chambers PA, Heritage J, Forbes JM (In print)

Fate of genetically modified maize DNA in the oral cavity and rumen of sheep
(Submitted for publication: *British Journal of Nutrition*)

Parts of this work have also been presented by John Heritage as invited papers at the April 2000 meeting of the British Society for Animal Science, Scarborough, and at the Second CSL / JIFSAN Symposium, University of Maryland, July 2001.

John Heritage has also presented part of this work as a research seminar at the University of Liverpool, Department of Medical Microbiology in November 2000.

Progress on this project was reported at the MAFF workshop in Stafford, in July 1998 and at the FSA Oxford workshop in July 2000.

Parts of this work have contributed to presentations made by John Heritage at sixth-form workshops at the Universities of Leeds and York. John Heritage also referred to this work in a presentation to the Society for General Microbiology Education group in September 2000, in a debate on GM crops at the University of Lancaster in October 2000 and in a University of Leeds seminar in March 2001. It was referred to in a presentation on GM crops to the Whitby Rural Deanery in April 2000.

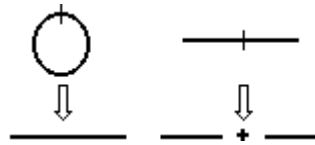
Paula Duggan has presented her work at two seminars for LIBA, one in March 1999 and one in January 2001.

Phil Chambers has successfully gained his PhD.

Scientific Objectives

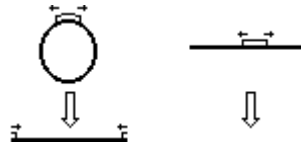
1) What is the risk that the *bla* gene will "loop out" from transgenic plant cells?

It was intended to use Southern transfer and hybridisation techniques linked to restriction fragment length polymorphism (RFLP) studies to establish whether loop out occurs in the transgenic maize that was the subject of this study. Circular DNA when cut with restriction endonucleases will yield a different fragment pattern from its linear counterpart:



Results from this study showed, however, that the insert was more complex with at least two *bla* cassettes inserted into the plant.

Assuming that "loop out" is a rare phenomenon, an alternative approach was used. PCR primers were designed that extend outwards from the *bla* gene. These were used in PCR reactions with DNA isolated from transgenic plants and with circular DNA of various sizes. If the *bla* gene is associated with "looped out" DNA it will be in a circular configuration. PCR will yield a product from such a reaction. Conversely, if the *bla* gene remains integrated in all cells it will be in a linear conformation and will thus not be detectable using PCR:



There was no evidence of circularisation of DNA isolated from transgenic plants carrying the *bla* gene.

2) Do animals fed on transgenic maize release the *bla* gene from plants?
& 5) Does ensilage of transgenic maize result in the release of the *bla* gene?

To assess the likelihood that the *bla* gene present in a transgenic maize line may transfer from plant material to the microflora associated with animal feeds, we examined the survival of free DNA in maize silage effluent, ovine rumen fluid and ovine saliva. Plasmid DNA that had previously been exposed to freshly sampled ovine saliva was capable of transforming competent *Escherichia coli* cells to ampicillin resistance even after 24 h, implying that DNA released from the diet could provide a source of transforming DNA in the oral cavity of sheep. Although target DNA sequences could be amplified by PCR from plasmid DNA after 30 min incubation in silage effluent and rumen contents, only short term biological activity, lasting less than one minute, was observed in these environments, as shown by transformation to antibiotic resistance. These experiments were performed under *in vitro* conditions; therefore further studies are needed to elucidate the biological significance of free DNA in the rumen and oral cavities of sheep and in silage effluent.

A PCR-RFLP protocol for the detection of the *bla*_{TEM} gene associated with plasmid pUC18 was shown to differentiate this gene from the wild-type *bla*_{TEM} in material derived from

throughout the gut of chickens. This technique was subsequently applied to an examination of the fate of pUC18-derived *bla*_{TEM} gene present in transgenic maize, which was fed to chickens. Amplifiable sequences derived from the pUC18 *bla*_{TEM} gene were detected in the crops of each of the five birds fed on transgenic maize and in the stomach contents of only two of these birds. In none of the birds was this gene detected lower down the intestinal tract or in faecal material, although the wild-type *bla*_{TEM} gene could be found in samples taken throughout the intestinal tract and in faecal material. The persistence of the pUC18-derived *bla*_{TEM} DNA mirrors the survival of a plant-derived sequence, showing that the *bla*_{TEM} gene in transgenic maize does not survive any better than any other DNA in the feed. These findings suggest that it is very unlikely that bacteria in the lower gut of chickens will be transformed to ampicillin resistance when the birds are fed on transgenic maize containing this gene.

PCR was used further to investigate the fate of a transgene in the rumen of sheep fed silage and maize grains from an insect-resistant maize line. Because of the potential for contamination with *bla* sequences, a 1914-bp DNA fragment containing the entire coding region of the synthetic *cry* IA(b) gene was targeted. This was still amplifiable from ovine rumen fluid sampled up to 5 h after feeding maize grains. The same target sequence was not detected, however, in rumen extracts prepared from sheep fed silage prepared using the transgenic maize line. PCR amplification of a shorter (211-bp) but nevertheless highly specific target sequence was possible with rumen fluid sampled up to 4 h and 24 h after feeding silage or maize grains, respectively. These findings indicate that intact transgenes from silage are unlikely to survive in the rumen. By contrast, DNA in maize grains persists for a significant time and may, therefore, provide a source of transforming DNA in the rumen. In addition, we have examined the biological activity of plasmid DNA that had previously been exposed to the ovine oral cavity. Plasmid extracted from saliva sampled after 8 min of incubation was still capable of transforming competent *Escherichia coli* to kanamycin resistance, implying that DNA released from the diet within the mouth may retain sufficient biological activity for the transformation of competent oral bacteria.

Duggan PS, Chambers PA, Heritage J, Forbes JM (2000) Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent *FEMS Microbiology Letters* **191**, 71-77.

Chambers PA, Duggan PS, Forbes JM, Heritage J (2001) A rapid, reliable method for the extraction from avian faeces of total bacterial DNA to be used as a template for the detection of antibiotic resistance genes *Journal of Antimicrobial Chemotherapy* **47**, 241-243.

Chambers PA, Duggan PS, Forbes JM, Heritage J (2002) The fate of antibiotic marker genes in transgenic plant feed material fed to chickens *Journal of Antimicrobial Chemotherapy* **49**, 161-164.

Duggan PS, Chambers PA, Heritage J, Forbes JM (*Submitted for publication: British Journal of Nutrition*) Fate of genetically modified maize DNA in the oral cavity and rumen of sheep.

3) With what efficiency can gut organisms take up and express the *bla* gene?

The PBP mutants that were to be used in this work were unavailable to the group, if ever they existed in reality. The feeding experiments described above, however, indicate that the *bla* gene is unlikely to survive transit to the lower intestine where bacteria that would take it up and to express it are likely to be found. See also Question 4.

4) Can the *bla* gene be mobilised from bacteria within an animal gut?

Since earlier experiments established that the *bla* gene was unlikely to survive to the lower intestine where bacteria that are likely to acquire and express this gene reside, this work was scaled down, in consultation with the Project Officer. Attempts were made, however, to establish the efficiency with which plasmid pUC18 could be mobilised by a conjugative plasmid, when supplied with the TnA transposase. This enzyme can cause the fusion of two replicons when one is carrying one copy of the TnA 38-bp terminal sequence. Plasmid pUC18 carries such a sequence but it proved impossible to detect replicon fusion when using this plasmid in artificial culture. No attempts were made, therefore, to replicate these experiments in animals, where the problems of recovery would have made the detection of very rare events extremely difficult, particularly in view of the high level of ampicillin resistance seen in the commensal bacteria from animals used in this work

[**Chambers PA, Duggan PS, Forbes JM, Heritage J** (2002) The fate of antibiotic marker genes in transgenic plant feed material fed to chickens *Journal of Antimicrobial Chemotherapy* **49**, 161-164.].

5) Does ensilage of transgenic maize result in the release of the *bla* gene?

The ensilage of crops is an anaerobic fermentation process, carried out by a range of bacteria associated with the crop at harvest. As the fermentation proceeds, different bacteria predominate. Culture and PCR experiments reveal that lactic acid bacteria involved in the later stages of silage fermentation may have acquired the *bla*_{TEM} sequence that codes for ampicillin resistance and that is present in the modified maize plant. These bacteria, however, remain sensitive to ampicillin.

6) To what extent does the *bla* gene undergo mutation to extended spectrum activity in the gut environment?

Nucleotide sequence determination of the *bla* genes recovered from transgenic plant material revealed no change in the nucleotide sequence from that of pUC18, the vector used to assemble the cassette used to construct this transgenic maize line.