Technical report on the FSA project "Evaluating the risks associated with using GMOs in human foods

Introduction

The recent development of technologies that enable arable crops to be genetically modified (GM) has resulted in the inclusion of GM foods in the human diet. Many of the modifications introduced into plants by this technology are designed to confer resistance to herbicides and pesticides. Typical examples are the glyphosate resistant variants of soya [1], and pesticide tolerant maize strains that express *Bacillus thurinogenesis* δ-toxin (Bt maize; [2]). One of the major concerns in respect of GM foods is their possible threat to human health. Some of the current GM foods contain antibiotic resistance genes, which could contribute to the spread of pathogenic microorganisms that are immune to the antimicrobial agents currently available. In addition, there is a general concern that the transfer of plant-derived transgenes to the resident intestinal microflora could have safety implications. Intuitively one would predict that the chance of vertical gene transfer from plants to microorganisms in the gastrointestinal tract is extremely slight, due to the high levels of the pancreas-derived DNAase in the small intestine. This view is supported by Maturin and Curtiss who showed that bacterial DNA was rapidly degraded in the rat intestinal tract. In contrast, mammalian DNA infused into the duodenum of calves was detected in the distal

passage through the gastrointestinal tract is provided by two recent studies [3, 4], which showed that bacteriophage M13 DNA, fed to mice, was detected in the faeces, indicating that a proportion of the DNA survives in the GI tract. In addition, this group detected the phage DNA in the liver demonstrating that the nucleic acid was able to pass through the intestinal epithelium into the systemic circulation. These

studies cast doubt on the assumption that plant-derived transgenes will not transfer to the intestinal microflora because the nucleic acid will be rapidly and completely degraded by the digestive enzymes.

In addition to the transfer of antibiotic resistance another mechanism of gene transfer which can adversely effect human health is from microorganisms to intestinal epithelial cells. It is known that prokaryotes can adhere to the gastrointestinal epithelia, which some bacteria can invade intestinal enterocytes, and these interactions could result in gene transfer from the microorganisms into the mammalian cells. This view is supported by data demonstrating that bacteria can be used in gene therapy strategies designed to deliver DNA into mammalian cells.

The final risk to human health involving gene transfer is horizontal gene transfer between prokaryotes, which in the context of genetically modified foods would involve studying gene transfer within the intestinal microflora, a highly complex ecosystem. There have been numerous studies on the frequency of gene transfer between model microorganisms in complex ecosystems, however, these studies only explored gene transfer events between selected prokaryotes as the recipient need to be genetically marked.

The overall objective of this project is to develop methodology that is designed to evaluate whether there is a significant risk that genetically modified plants and bacteria can transfer their transgenes to other organisms in the intestinal tract of humans. To address this question the project has focussed on three issues:

1. Rare gene transfer events in complex ecosystems: To address this issue we have developed a donor microorganism that is designed to facilitate the measurement of rare gene transfer events in complex ecosystems by developing a specific kill system that enables survival of only the recipients of gene transfer events.
2. Vertical transfer between prokaryotes and the mammalian epithelium: To address this question we have examined the frequency of gene transfer between a *Lactobacillus* strain that is known to adhere to the epithelium of intestinal enterocytes and a *Salmonella typhimurium* strain that invades intestinal epithelial cells but does not cause cell death
3. We have evaluated the capacity of transgene DNA derived from genetically modified soya and maize to persist in the human intestinal tract *in vitro* and, in the case of GM soya, *in vivo*.

The data emanating from Objectives 2 and 3 are essentially described in the two manuscripts which accompany this report. Experimental methodology, the data and discussion of the results relating to Objective 1 are described below.

Report on Objective 1

Experimental Methodology

Bacterial strains, plasmids and culture conditions: The bacterial strains used in this study were *Escherichia coli* strains JM83 and *Pseudomonas fluorescens* subsp. *cellulosa*, *Pseudomonas putida*, the microbial ecosystem derived from the mouth of Dr Netherwood and the soil microbial community from Rivinton (sandy) and Hallsworth (clay). The two pseudomonad strains were resistant to 50 μg/ml rifampicin. The plasmids used in this study were pGT65mcs, pNG1 and p519*ngfp*. The genes present in pGT65mcs are displayed in Figure 1. All microbes were cultured

on Luria broth under aerobic conditions at either 37°C (*E. coli* strains) or 28°C (pseudomonad, soil and oral microbes). The antibiotics kanamycin (50 μg/ml) rifampicin (50 μg/ml), zeomycin (50 μg/ml) and chloramphenicol (20 μg/ml) were added as appropriate. AZT was routinely added at 5 μg/ml.

Filter mating: Filter mating experiments using the pseudomonads as the recipient were carried out essentially as described previously [5], except that transconjugants were selected on media supplemented with AZT and kanamycin. To verify that the colonies which appeared on media containing AZT and kanamycin were transconjugants, the colonies were patched onto rifampicin and, in the case of *P. fluorescens* subsp. *cellulosa*, onto LB supplemented with Blue xylan (*P. fluorescens* subsp. *cellulosa* is xylanolytic and thus colonies of these bacteria have a clear halo on a blue background when plated on Blue xylan). Rifampicin resistant colonies were also subjected to the catalase test to confirm that they were pseudomonads. Selected bacteria that display the appropriate phenotype for a transconjugant were subjected to Southern hybridisation using an appropriate probe to confirm that gene transfer had occurred. To carry out filter mating experiments using oral or soil bacteria as the recipients the following procedure was carried out: To 5 g of soil 10 ml of phosphate buffered saline was added and the soils were mixed vigorously. The suspension was allowed to settle for 5-10 min at room temperature and 500 μl of the soil-containing supernatant was used in the filter mating experiment. The soils were Rivinton (sandy) and Hallsworth (clay), and were both obtained from Cockle Park, a University of Newcastle farm.

Phylogenetic analysis of oral tranconjugants: The 16S ribosomal RNA gene (rDNA) from the transconjugants recovered from the filter mating experiment, using oral bacteria as the recipient, were amplified using the polymerase chain reaction. The

amplified DNA was ligated into pCR-blunt and the resultant plasmids were transformed into *E. coli* JM83. Recombinants of pCR-blunt containing rDNA was sequenced using M13 –20 universal forward and M13 universal reverse primers. The sequence was subjected to phylogenetic analysis using the ClustalW computer program to determine the taxonomy of the bacteria.

Assay of Green Fluorescent Protein (GFP): GFP was detected qualitatively by exposing bacterial colonies, grown on plates to longwave ultraviolet light, conditions in which GFP will fluoresce. The fluorescence of bacteria, due to GFP, was also qualitatively assessed using a fluorescent microscope. Quantitation of GFP was as follows: Cell-free extracts of bacteria derived from stationary phase cultures were prepared as described previously [6], and 100 μl of the material were subjected to spectrofluorimetry using excitation and emission wavelengths of 481and 507 nm, respectively.

Results

Rationale for the donor kill system: The objective of this work was to develop a system for measuring rare gene transfer events to prokaryotes in complex ecosystems. To address this issue we have developed a specific kill system for donor microorganisms enabling the survival of only the recipients of gene transfer events. The basis of this kill system was to insert the herpes simplex virus thymidine kinase (hsvTK) gene into the donor bacterium, *E. coli*. hsvTK phosphorylates AZT to the monophosphate nucleotide. Endogenous kinases then further phosphorylate AZT to generate the nucleotide triphosphate which is then incorporated into DNA. Once incorporated AZT terminates DNA synthesis and causes cell death. In bacteria which do not contain hsvTK the endogenous kinases are unable to catalyse the initial

phosphorylation thus AZT is not converted into the triphosphate nucleotide, is not incorporated into DNA and thus does not cause cell death. To select for gene transfer events the donor bacterium, containing the hsvTK gene on a non-mobilisable plasmid and a mobile genetic element that confers antibiotic resistance, is mixed with the bacteria in the target ecosystem. The bacteria are then plated out on media containing AZT and the antibiotic encoded by the mobile genetic element, and thus any colonies that appear are likely to be the result of gene transfer events. The advantages of this system is that recepients do not need to be marked (e.g. rifampicin resistance), and thus rare gene transfer events into any bacterium in the ecosystem can be detected.

Verification of the strategy: To confirm that AZT did cause cell death in bacteria that expressed hsvTK, *E. coli* containing pGT65mcs (encodes hsvTK) was grown in liquid culture (LB containing zeomycin) overnight and then plated onto LB and LB containing various concentrations of AZT. The data, presented in Table 1, showed that *E. coli* expressing hsvTK did not grow in the presence of AZT at concentrations >1 μg/ml, while *E. coli* which lacked the viral kinase grew in the presence of the antiviral drug. Similarly, a variety of other bacteria including two pseudomonads and and the microbial community derived from soil and the human oral cavity were also able to grow in the presence of AZT (Table 1).

To assess whether continued growth of *E. coli* in rich media generated variants that were esistant to AZT, the bacterium was subcultured (10 μl from stationary phase cultures into 10 ml of fresh liquid medium) five times in liquid media containing zeomycin, and the number of AZT resistant bacteria was determined by plating the culture onto LB containing 5 μg/ml AZT. The data, presented in Figure 2, show that

continuous sub-culturing of *E. coli* in liquid media containing zeomycin generates AZT resistant variants. Thus, when using the hsvTK gene in gene transfer experiments it is critical that a fresh colony is taken and grown up only once, continuous sub-culturing of the strain generates too many AZT resistant variants for the donor kill system to be effective.

Gene transfer to P. fluorescens subsp. cellulosa and P. putida: To validate the donor kill system in studying gene transfer, *E. coli* containing p p519ngfp and the Tn5 delivery plasmid pTN1 was filter mated with *P. fluorescens* subsp. *cellulosa* and *P. putida*, respectively. Gene transfer was determined by the acquisition of kanamycin resistance, encoded by Tn5, in the recipient population, such organisms are known as transconjugants. The data, presented in Table 2 revealed that gene transfer into *P. fluorescens* was rare with only 10^{-5} bacteria displaying the Kan^r AZT^r expected on transconjugants. Gene transfer into *P. putida* was more frequent with 10^{-1} recipients displaying the Kan^r AZT^r phenotype . To evaluate whether these bacteria were transconjugants the donor and recipients were also plated out on AZT and kanamycin. No recipients were kanamycin resistant while AZT^r donor bacteria were present at 10^{-7}. To confirm the identity of the transconjugants, 100 of the Kan^r AZT^r colonies from each filter mating were plated out on Rif, as both recipient pseudomonads were Rif^r. The bacteria were all Rif^r and catalase positive confirming their identity as recepient bacteria. Furthermore, all 100 AZT^r Kan^r *P. fluorescens* subsp. *cellulosa* colonies were both cellulolytic and xylanolytic, again confirming their identity as pseudomonads. To ensure that the Kan^r phenotype in the putative transconjugants was the result of gene transfer, DNA extracted from 10 of the Kan^r AZT^r bacteria were subjected to Southern hybridization using Tn5 as the probe. All 10 colonies contained

the Tn5 sequence, which was absent in the original recipient strains (data not shown). To conclude, these data clearly show that the donor kill strategy is an effective method of killing Kan^r donor bacteria, enabling rare transfer events of the antibiotic resistance gene into two recipient pseudomonads to be easily detected.

Gene transfer into natural populations: To assess whether the donor kill strategy can be used to detect rare gene transfer events into natural populations, the microbes in the human oral cavity were filter mated with *E. coli* containing pTN1 and p519ngfp and gene transfer events were detected by selecting for Kan^r AZT^r bacteria. The data, presented in Table 3, revealed Kan^r AZT^r bacteria at a frequency of 10^{-3}/recepient and 10^{-5}/recepient for donor bacteria containing p519ngfp and pTN1, respectively. No Kan^r AZT^r were recovered from the oral microbial population and Kan^r AZT^r bacteria were present at 10^{-7} in the donor population. To assess the range of oral microbes that have acquired Tn5 and p519ngfp, DNA was extracted from 20 of the transconjugants and the rDNA genes were amplified by PCR, digested with *Rsa*I and electrophoresed on 1 % agarose gels. The data (Figure 3) revealed four different patterns indicating Tn5 had transferred into three different oral bacteria. The rDNA of the four different Kan^r AZT^r bacteria were sequenced and subjected to phylogenetic analysis. The data revealed gene transfer had occurred from *E. coli* into the gram-negative bacteria *Neisseria elongata*, and *Corynebacterium durum,* and the gram-negative bacterium *Streptococcus cristatus* (Figure 4). These data demonstrate, therefore, that the donor kill system can be used to detect gene transfer events from defined bacteria into natural ecosystems.

Gene transfer from E. coli into soil ecosystems: Initial attempts to measure gene transfer from *E. coli* into soil bacteria were unsuccessful due to the very high level of Kan^r (10^{-2}) in the ecosystem. The antibiotic resistance spectrum of soil ecosystems was therefore assessed. The data revealed chloramphenicol resistance (Cm^r) was very low ($<10^{-7}$) in the soil microbial population. To measure gene transfer a conjugative broad range gram negative plasmid was constructed that conferred Cm^r. The plasmid, which also encodes GFP was transformed into *E. coli* JM83 and the enteric bacterium was filter mated with the microbial ecosystem from a sandy and clay soil. The data, presented in Table 4, revealed a very high level of Cm^r AZT^r bacteria in the filter mated bacteria, which was not reflected in the donor or recipient organisms. Approximately 20 % of the transconjugants were GFP positive as judged by immunofluorescense, while quantitative analysis revealed large variation of GFP in those bacteria that fluoresced.

Conclusions: The data presented in this study demonstrated that the AZT-based donor kill system is a highly effective in measuring gene transfer into natural microbial populations. The primary advantage that this system over standard gene transfer methodology is that there is no need to include a marked recipient bacterium and thus gene transfer into the complete microbial population can be assessed. This system can be exploited in studying gene transfer from food grade microorganisms into natural populations, such as the intestinal microflora, into which these prokaryotes are introduced. This methodology is this of considerable value when developing risk assessment protocols for GMOs that are introduced into the human food chain. It was hoped that the strategy could also have been exploited in detecting gene transfer into viable but non-culturable populations, which comprise >90 % of natural microbial

ecosystems. This proved not to be feasible as the endogenous fluorescense in natural populations created a high background, the donor cells, although killed did not lyse, and GFP was expressed at very variable levels when *gfp* was transferred into the recipient bacteria. In the original project we had intended exploiting the AZT kill system to measure gene transfer from the food grade microorganism *Lactococcus lactis*, which is a target GMO, into the microbial ecosystem of the large bowel. In a parallel study, however, H. J. Flint's group showed that *L. lactis* was very rapidly lost from *in vitro* large colon simulations with no evidence of gene transfer (erythromycin resistance) occurring. For these reasons we did not further explore this system in the large bowel but focussed more on Objectives 2 and 3 as agreed with FSA.

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Table 1 **Survival of bacterial populations in the presence of AZT**

Bacterial strain	No AZT	AZT 1 μg/ml	AZT 5 μg/ml	AZT 10 μg/ml	AZT 20 μg/ml
	Colony forming units/ml of culture				
E. coli JM83	2.9×10^8	1.5×10^8	4.5×10^7	7.8×10^7	2.3×10^8
E. coli JM83: pGT65mcs	3.1×10^8	5×10^3	11	15	5
Oral Bacteria	3.9×10^7	2.7×10^7	5.1×10^7	3.1×10^6	1.6×10^7
Soil bacteria	4.5×10^6	1.5×10^6	2.8×10^6	1.1×10^6	2.4×10^6
P. fluorescens subsp. cellulosa	7.8×10^7	9.2×10^7	4.1×10^7	2.1×10^7	3.9×10^7
Pseudomonas putida	1.1×10^8	7.5×10^7	3.2×10^7	7.8×10^7	9.1×10^8

Table 2 **Filter mating between *E. coli* containing a mobilsable kanamycin resistance gene and two pseudomonads**

Bacterial strain	Frequency of Kan^r AZT^r bacteria
E. coli JM83: pGT65mcs:pTN1	1.2×10^{-7}
E. coli JM83: pGT65mcs:p519ngfp	1.6×10^{-7}
E. coli JM83: pGT65mcs:pTN1 and *P. fluorescens* subsp. *cellulosa*	3.7×10^{-5}
P. fluorescens subsp. *cellulosa*	0
E. coli JM83: pGT65mcs:p519ngfpi and *Psudomonas putida*	4.2×10^{-1}
Psudomonas putida	0

E. coli containing the non-mobilisable plasmid pGT65mcs encoding HSVTK and either p519ngfp (mobilisable plasmid that replicates in a broad range of gram negative bacteria and encodes kanamycin resistance and GFP) or pTN1 (plasmid that is mobilisable but only replicates in *E. coli.* It contains the transposon Tn5 that confers kanamycin resistance). These strains were filter mated with two pseudomonads and gene transfer was detected by the appearance of Kan^r AZT^r transconjugants. The data is the average of three independent filter mating experiments.

Table 3 **Filter mating between *E. coli* containing a mobilsable kanamycin resistance gene and an oral microbial ecosystem**

Bacterial strain	Frequency of Kan^r AZT^r bacteria
E. coli JM83: pGT65mcs:pTN1	2.7×10^{-7}
E. coli JM83: pGT65mcs:p519ngfp	1.1×10^{-7}
E. coli JM83: pGT65mcs:pTN1 and oral microflora	$2.6 \times 10^{-5} \pm 1.2 \times 10^{-5}$
Oral microflora	0
E. coli JM83: pGT65mcs:p519ngfpi and oral microflora	$4.3 \times 10^{-3} \pm 2.4 \times 10^{-3}$

The methodology and genotype and phenotype of the strains used were as described in Table 2 and the data are derived from three independent experiments.

Table 4 **Filter mating between *E. coli* containing a mobilsable chloramphenicol resistant plasmid and soil microbial ecosystems**

Bacterial strain	**Frequency of Cm^r AZT^r bacteria**
E. coli JM83: pGT65mcs:pCMgfp	1.1×10^{-7}
E. coli JM83: pGT65mcs:pCMgfp and Rivinton soil bacteria	$1.6 \times 10^{-1} \pm 2.5 \times 10^{-2}$
Rivinton soil bacteria	0
Hallsworth soil bacteria	3×10^{-7}
E. coli JM83: pGT65mcs:pCMgfp and Hallsworth soil bacteria	$6.8 \times 10^{-2} \pm 9.4 \times 10^{-3}$

The methodology and genotype and phenotype of the strains used were as described in Table 2 and the data are derived from three independent experiments.

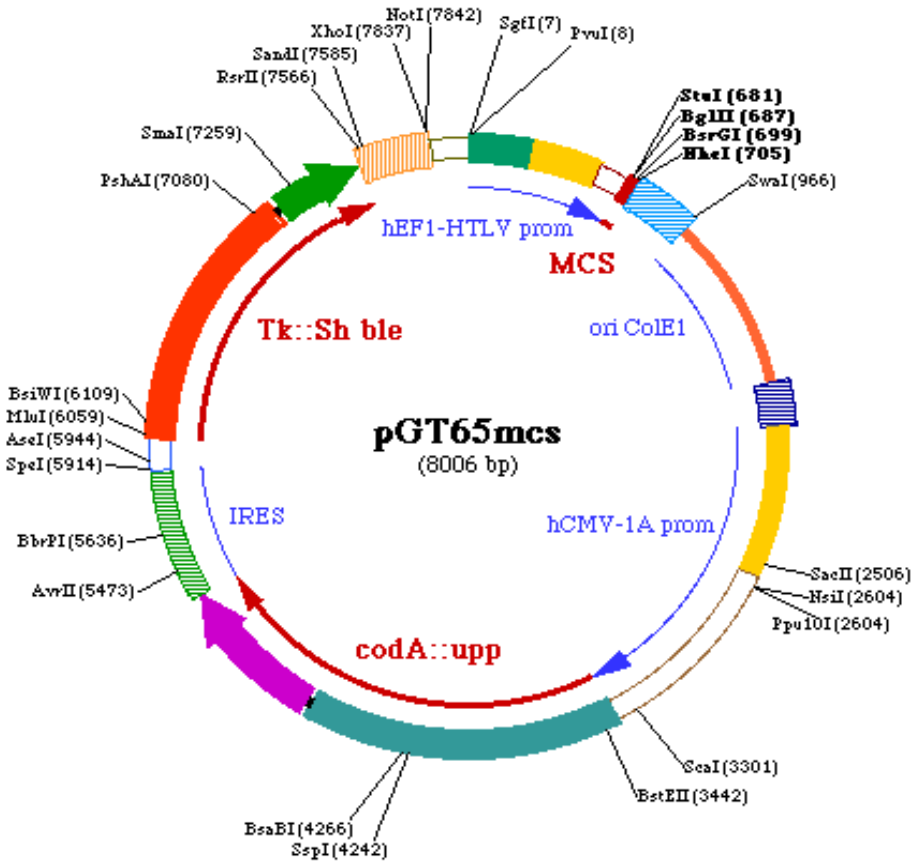

Figure 1 **Physical map of pGT65mcs**

The map of pGT65mcs displays the HSVTK and zeomycin resistance genes (TK::Sh ble) organised into an operon.

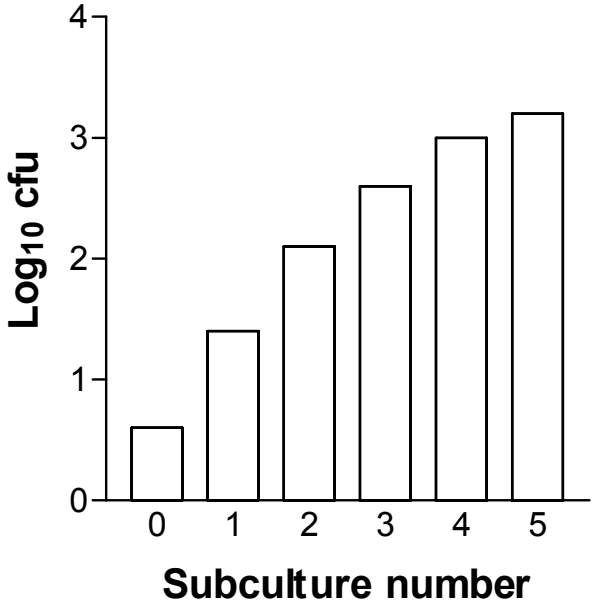

Figure 2 The influence of subculturing on the acquisition of AZT resistance

E. coli JM83: pGT65mcs was cultured in LB in the presence of zeomycin through five subcultres, in which the bacteria were diluted 1000-fold when inoculated into fresh medium. The bacteria were plated out onto LB-agar containing 5 μg/ml AZT and incubated for 16 hours at 37°C.

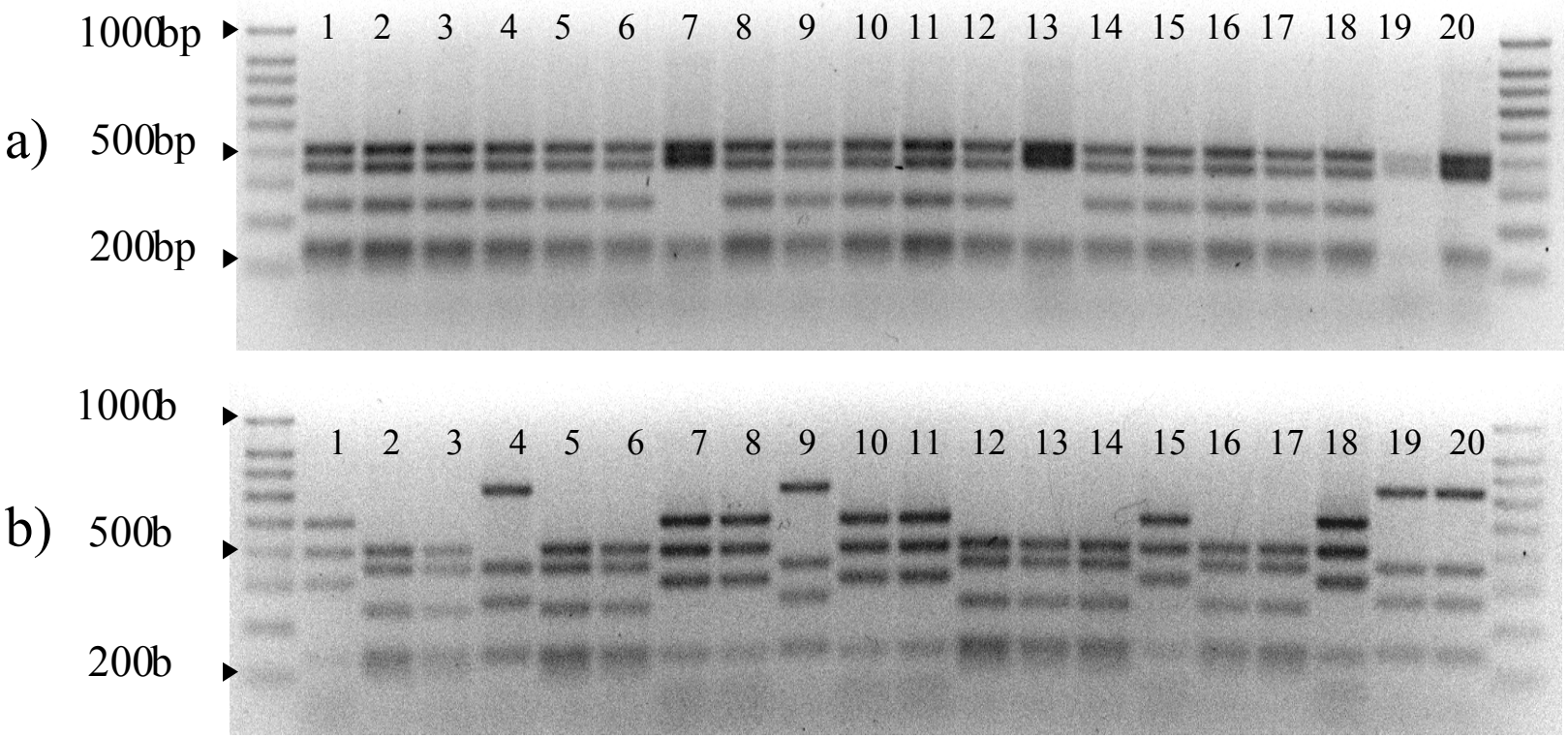

Figure 3: 16SrDNA ARDRA of 20 oral microflora transconjugants from gene transfer by a) p519*ngfp* and b) Tn5

The 16S bands were digested with restriction enzyme *Rsa*I, and the unique profiles sequenced (I.e. 2a, 7a, 1b and 4b)

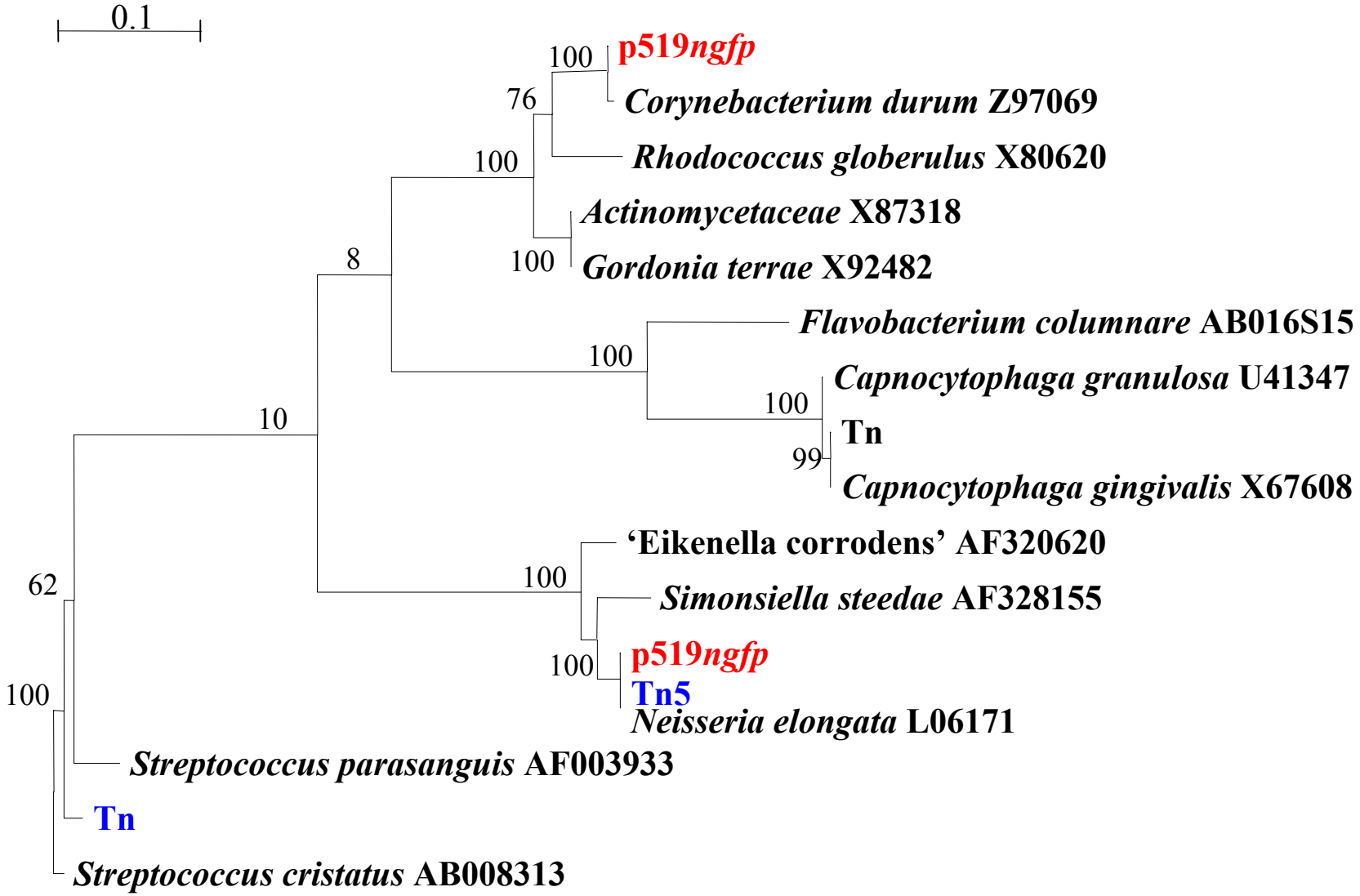

Figure 4 Phylogenetic analysis of the transconjugants

The transconjugants that inherited the transposon Tn5 and p519*ngfp* are depicted as blueand red, respectively

Transgenes in genetically modified Soya survive passage through the human small bowel but are completely degraded in the colon

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The introduction of genetically modified plants (GMPs) into the human food chain has caused considerable debate with regard to the associated risks to human health. One of the major issues following the inclusion of GMPs in the human diet is the possible transfer of transgenes to the commensurate intestinal microflora and/or the epithelial cells lining the intestinal lumen, both of which could have health implications. Recent data indicate that a significant proportion of the transgenes in GMPs does survive *in vitro* simulations of the small bowel[7], and bacteriophage M13 DNA gavaged into the mouse intestines is detected in the faeces, blood and liver[3, 4, 8]. The persistence of dietary GMP-derived DNA in humans, however, is unknown. In this study we have evaluated the survival of transgenes in GMPs during passage through the gastrointestinal trat of humans. To track DNA survival through the small intestine seven ileostomists were given a single meal containing genetically modified Soya (GMS), and the appearance of transgene DNA on the digesta collected from the stoma was monitored. Whilst the amount of transgene that survived passage from the small bowel was highly variable between subjects, the nucleic acid was detected in all seven subjects. In one individual as much as 3.7 % of the transgene DNA was recovered at the stoma. In a second trial, human volunteers with an intact gastrointestinal tract were fed a single meal containing GMS. No transgene DNA was detected in the faeces indicating that the nucleic acid did not survive passage through the complete intestine.

Seven ileostomists were recruited for a trial in which the volunteers were fed a meal containing GMS purchased from a standard food retailer. The transgene (*epsps*) present in the plant material comprised the CP4 5-enolpyruvylshikimate-3-phosphatesynthase (EPSPS) gene from *Agrobacterium sp*. strain CP4 fused to the region of the EPSPS *Petunia* CTP gene encoding the mitochondrial import signal[9]. The transgene was detected and quantified by competitive PCR using the RR04/RR05 primer pair described by Studer *et al.*[10]. The meal, consisting of 454 g wet weight, contained 3 x 10^{12} copies of the transgene. The diet contained the indigestible marker PEG 400 to determine the proportion of the meal that had passed through the small bowel during the course of the experiment. Greater than 90 % of the marker was recovered in the digesta collected from the stoma in six out of the seven subjects. The transgene, Table 1 and Figure 1, was recovered in each subject although the rate of passage of digesta and total recovery of this nucleic acid was highly variable between individuals. The low level of transgene recovery from Subject 3 may reflect the poor recovery of the indigestible marker indicating very slow passage of the digesta. It is interesting to note, however, that as much as 1.1 x 10^{11} copies of the transgene, equating to 3.7 % of the 180 bp *epsps* cassette in the original meal was detected in samples retrieved from subject seven. The survival of the *epsps* fragment did not correlate with the transit time of the digesta (Figure 2), suggesting that other factors were responsible for variation in transgene recovery. It is possible that individual differences in the amount of DNAase I, secreted from the exocrine pancreas and the quantity of acid secreted from the gastric cells could explain the variation in transgene survival. To investigate whether the complete *epsps* gene survived passage through the small bowel, samples were subjected to PCR using the primer pair GMS01 and 35S1 that amplifies the entire open reading frame[10]. A reasonable correlation was observed with the competitive PCR results; the full length gene was detectable in samples which contained at least 10^4 copies of the 180 bp fragment per mg dry weight of digesta. It is possible that the survival of the transgene in GMS did not parallel that of Soya DNA. This was evaluated by amplifying the endogenous Soya lectin gene, *Le1*[11, 12]. The data showed that the persistence of the *Le1* lectin gene was similar to that of *epsps* (Figure 3) indicating that the transgene was degraded at a rate similar to the bulk Soya DNA. It is surprising that a relatively large

proportion of GMS DNA survives passage through the small bowel in view of the depurination that occurs in the stomach, and the high level of pancreatic DNAase in the intestinal lumen. The relative stability of the transgene in the stomach may indicate that the food matrix, in some way, had a buffering effect such that the proton concentration in the microenvironment of the DNA was not as high as in the bulk of the stomach lumen. The protection of the transgene to DNAase attack could reflect its very tight association with histones, particularly within the highly structured heterochromatin regions of the genome. It is also possible that GMS retains some cellular structure, which would protect the intracellular DNA from exogenous DNAases. This explanation, however, is unlikely as naked soya DNA was hydrolyzed *in vitro* at a similar rate to the DNA in processed food grade Soya protein (the same material used in this study), indicating that the cellular structure of the plant material had been significantly disrupted[7].

To assess whether there was any evidence of gene transfer from GMS to the intestinal microflora, the microbes in the ileal digesta samples were cultured through 6 passages in Luria Broth containing glyphosate. Bacteria grew to a density of 10^8/ml on each sub-culturing. In each sub-culture of digesta derived from the samples taken from Subjects 1, 4 and 7 at 180, 240 and 300 min post GMS consumption, a 180 bp PCR product was generated using the RRO4/RRO5 primer pair[10]. Significantly, the nucleotide sequence of the amplified DNA confirmed that it originated from the GMS transgene. Although this PCR product was not detectable in samples taken from these three subjects prior to GMS consumption (time 0 min), when the microbes in this material were cultured in LB containing glyphosate the PCR product was evident from passage 1, although at very low concentration. Importantly, despite exhaustive attempts, we were unable to isolate the bacteria harbouring the transgene by colony blot hybridisation or a PCR pooling strategy, confirming that, although present, the bacterium containing the GMS transgene represented a very small proportion of the indigenous intestinal microflora of these subjects.

To determine whether GMS survived passage through the complete gastrointestinal tract, a further 12 human volunteers were fed the GMS-containing meal and the presence of the transgene in the faeces was investigated by PCR. Greater than 90 % of the undigested marker was recovered in the faeces, showing that the meal had passed

through the large and small bowel, but the transgene was not detectable in any of the subjects. Studies confirmed that this was not the result of PCR inhibition since a 180 bp product was amplified by PCR when the faecal material was spiked with the plasmid containing *epsps*. When microbes from the faecal material were cultured either aerobically or anaerobically, PCR again demonstrated the absence of the GMS transgene. These data indicate that GMS, although surviving passage through the small bowel, is completely degraded in the large intestines. This is consistent with studies[13, 14] (H.J. Flint personal communication) which demonstrated that naked DNA is extremely rapidly degraded by the very high levels of DNAase activity associated with the rumen microflora, a microbial ecosystem with similarities to the human large bowel. It is worth noting that in subjects with intact gastrointestinal tracts, no GMS-derived transgenes were detected in bacteria cultured from faeces. This suggests that the transgene-containing bacterium in the small bowel of the ileostomists did not survive passage through the human colon. This conclusion, however, should be viewed with some caution, as it is possible that the physiology of the small bowel in ileostomists differs from that in subjects with an intact gastrointestinal tract, in ways which could influence gene transfer from plant material to the intestinal microfolora. For example, ileostomists might secrete lower levels of DNAase from the exocrine pancreas resulting in a reduced rate of DNA degradation, which would increase the probability of gene transfer from the GMS into the intestinal microflora. Alternatively the rate of passage of the digesta and/or the structure of the microbial ecosystem in the small bowel may be quite different between ileostomists and normal subjects.

Another area of concern with the inclusion of GMPs in the human diet is that transgenes might be transferred, *via* bacteria, to the intestinal epithelium. The most likely vectors for such genetic movement are lactabacilli, which colonise the surface of intestinal enterocytes[15], or the intracellular pathogen *Salmonella typhimurium* [16]. Indeed, the intracellular location of *S. typhimurium* has resulted in its exploitation as a vector in gene therapy strategies targeted at monocyte/macrophages [17]. To assess the possibility of such gene transfer events *Lactabacillus* GG and *S. typhimurium* SL3261[16] were transformed with pBK-CMV and pLN1, respectively. Both plasmids contain a neomycin resistance gene under control of the SV40 early promoter that is active in all mammalian cells. The bacteria were incubated with

Caco-2 cells, a model intestinal cell line[18], and gene transfer from the engineered prokaryotes into the genome of the mammalian cells assessed by selecting for G418 (neomycin analogue) resistant Caco-2 cells. Transfection of Caco-2 cells with either pBKCMV or pLPN generated G418 resistant cells at a frequency of 1 in 3000. In contrast, incubation of 10^7 Caco-2 cells, at various stages of differentiation, with a 1000-fold excess of either *S. typhimurium* SL3261 or *Lactabacillus* GG containing the respective recombinant plasmids generated no G418 resistant mammalian cells. These data indicate that gene transfer from GMPs to the intestinal epithelium, either directly or *via* the intestinal microflora, is unlikely to occur. Further support for this view is provided by the lack of the GMS transgene in the faeces of subjects fed the GMS-containing diet; material which is known to contain significant numbers of exfoliated colonocytes. This is in contrast to studies with mice demonstrating that naked bacteriophage DNA is taken up into mammalian intestinal cells, and even appears in systemic tissues Schubbert, 1997 #6}. It is possible that the gastrointestinal tract of mice is more leaky than humans, or the amount of DNA gavaged into the murine intestinal lumen was far in excess of the nucleic acid present in the human diet. Thus, the mouse data does not accurately reflect the fate of dietary DNA in humans.

This report demonstrates that transgenes in GMS although surviving passage through the small intestine appear to be completely degraded in the human colon. Although there was some evidence of gene transfer from GMS to intestinal microflora of the small bowel, the transfer event reflected previous exposure of the subjects to GMPs, and the microbial population containing the plant-derived DNA sequence was an extremely minor component of the intestinal microflora. These data support the view that GMS does not represent a significant risk to human health through gene transfer to either the intestinal epithelium or the microflora within the intestinal lumen.

Methods

Plasmids

The plasmid pBK-CMV (Stratagene) was from Invitrogen and pLPN was constructed by cloning the *SV40-neo*^{r} cassette from pBK-CMV into the *Bam*HI site of the gram-positive vector pLPM11[19].

Culturing of Caco-2 cells with *Lactobacillus* and *Salmonella*

Caco-2 cells which had been passaged through seven generations were seeded at a density of 3 x 10^5 per cm^2 and grown in standard conditions until confluent[20]. At various times after the initial seeding a 1000 fold excess of bacteria were added. After 28 days the bacteria were killed with appropriate antibiotics and the mammalian cells were exposed to G418. The transfection of Caco-2 cells by lipofection and selection of transfectants was as described previously[20].

Culturing of intestinal and faecal microorganisms

To 10 ml of Luria broth (LB) was added 500 mg of either digesta or faeces, and the mixture incubated for 16 hours. A 50 μl aliquot of the culture was inoculated into 10 ml of fresh LB and the bacteria were again incubated for 16 hours. The bacteria were sub-cultured for a further six passages.

Human trial

Seven ileostomists were recruited from within the Newcastle upon Tyne. These volunteers fasted overnight and DNA was extracted from the contents of their stoma prior to the start of the trial. The GMS-containing meal consisted of burgers and a milk shake. The meal was prepared by deep frying in vegetable oil a burger mix comprising three eggs, 150 g Soya meal (El Corte Ingles), 300ml water and 24 g PEG400. The GMS-containing milk shake consisted of 100 g Soya Protein Supplement (Holland and Barrett), 20 g PEG400, 600 ml Soya milk (Holland and Barrett) and 20ml milk shake flavouring. Each subject was fed 454 g of the meal after which their stoma bag was emptied every 30 min for six hours and the digesta free-dried. DNA was extracted and the PEG400 quantified following standard procedures. In the study in which the subjects had an intact gastrointestinal tract, the volunteers were fed the same GMS-containing meal, except that the indigestible marker consisted of radio-opaque markers. After consumption of the test meal the subjects ate a normal diet and collected their stools over 72 hours. The faeces were freeze-dried and the DNA was extracted and analysed.

DNA analysis

The concentration of a 180 bp region of *epsps* and the Soya lectin gene in the digesta were determined by quantitative competitive PCR as described previously[7]. The presence or absence of full-length *epsps* was determined by performing PCRs using primers that bind to the 3' end of the transgene and the cauliflower mosaic virus 35S promoter.

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Table 1 Survival of the *epsps* transgene in the small bowel of ileostomists

Time (min)	Transgene copies x 10^5						
	Subject 1	**Subject 2**	**Subject 3**	**Subject 4**	**Subject 5**	**Subject 6**	**Subject 7**
0	0	0	0	0	0	0	0
30	0	0	1	0	0	0	0
60	0	0	0	0	0	0	0
90	0	0	25	340*	0	0	0
120	0	1720*	0	380*	51	0	0
150	0	210*	0	460*	23	0	4700*
180	250*	0	0	3790*	330*	0	2300*
210	430*	0	0	8200*	630*	523*	7000*
240	140*	0	0	93000*	490*	3200*	410*
270	160*	0	0	4100*	250*	10200*	0
300	110*	0	0	230*	5	12340*	0
330	180*	0	0	470*	0	4600*	0
360	14	0	0	420*	0	2700*	0

*Samples in which full length *epsps* was detected

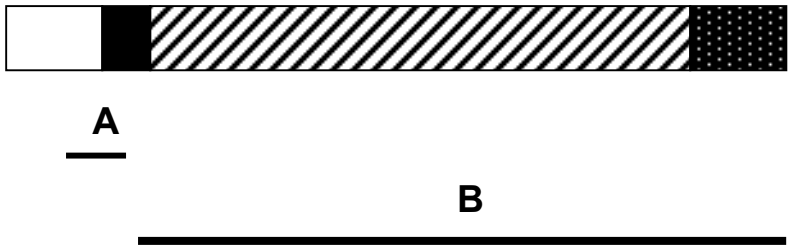
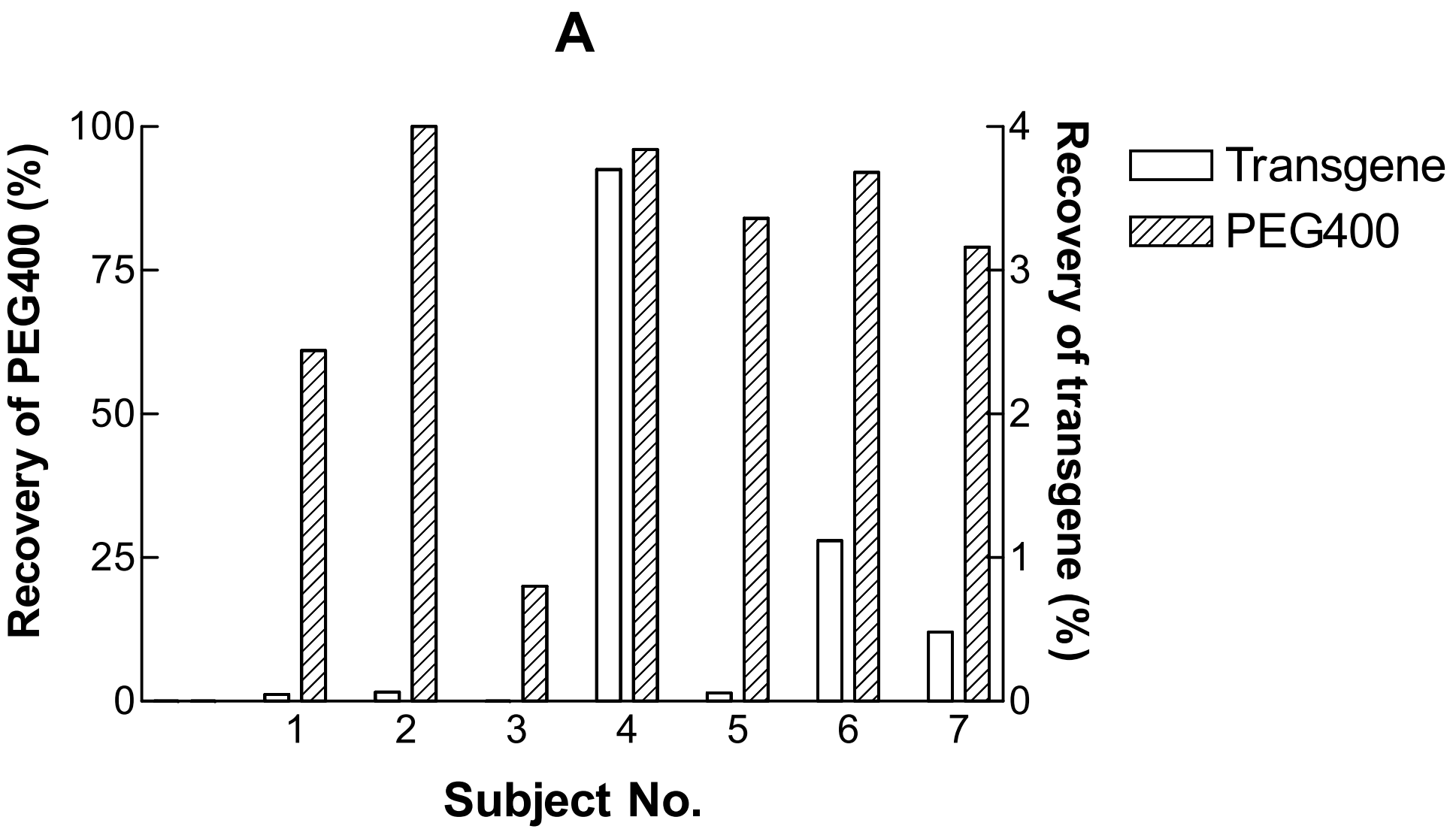


Figure 1 The *epsps* construct in GMS. The transgene consisted of the cauliflower mosaic virus 35S promoter (▭), the gene encoding the CP4 5-enolpyruvylshikimate-3-phosphatesynthase gene from *Agrobacterium sp*. strain CP4 (▨) , fused to the region of the EPSPS *Petunia* CTP gene encoding the mitochondrial import signal (■), and the polyadenylation signal from *Nos* (▩.). The region of DNA that was amplified in order to quantify *epsps* (A) and detect the complete EPSPS gene (B), respectively are indicated.

Fig 2a


A
Recovery of PEG400 (%)
100
75
50
25
0
Recovery of transgene (%)
4
3
2
1
0
1
2
3
4
5
6
7
Subject No.
Transgene
PEG400

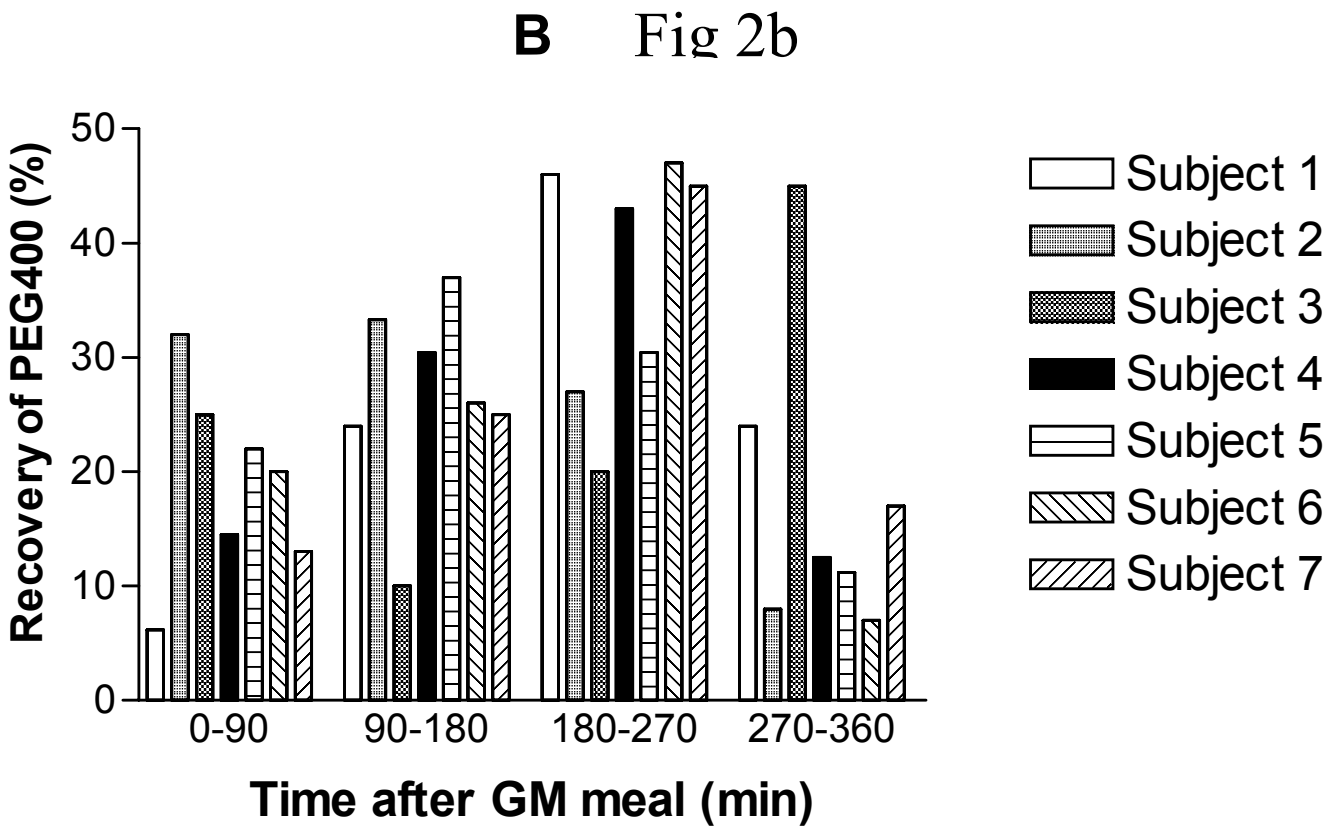


Figure 2 Recovery of *epsps* transgene and the indigestible marker PEG400. Panel A displays the total quantity of transgene and marker recovered, while Panel B displays the transit time of PEG400 in the different subjects.

Fig. 3

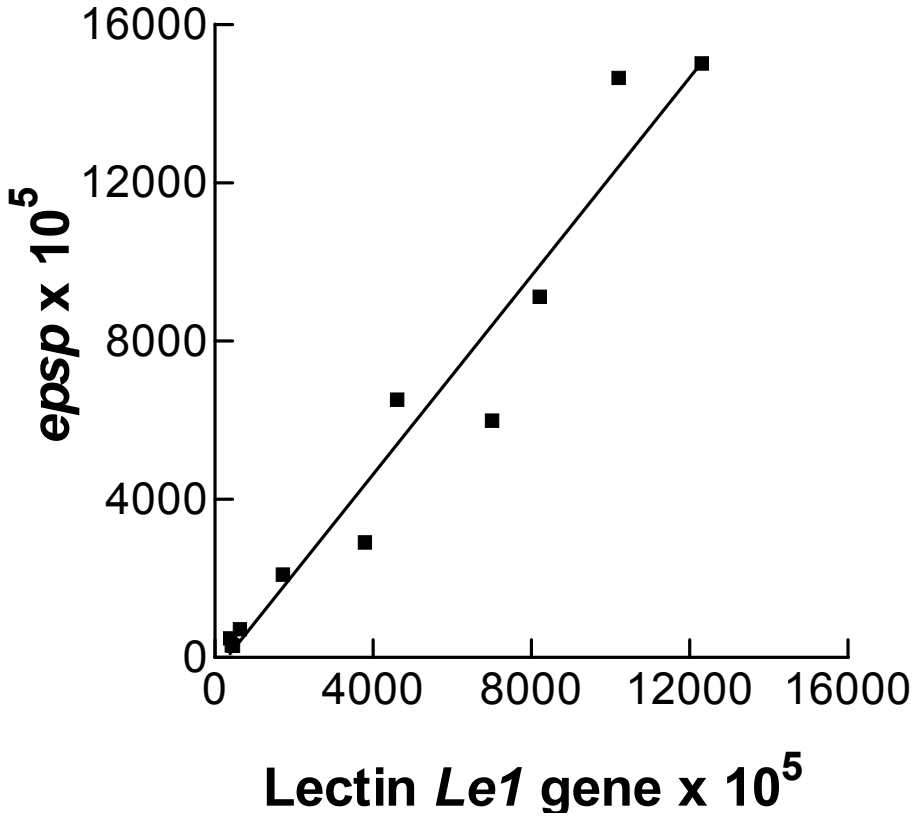


Figure 3 Relationship between recovery of the *epsps* transgene and the Soya lectin gene *Le1*. The regression coefficient was 0.9507