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Assessing the survival of transgenic plant DNA in the human gastrointestinal tract

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The inclusion of genetically modified (GM) plants in the human diet has raised concerns about the possible transfer of transgenes from GM plants to intestinal microflora and enterocytes. The persistence in the human gut of DNA from dietary GM plants is unknown. Here we study the survival of the transgene *epsps* from GM soya in the small intestine of human ileostomists (i.e., individuals in which the terminal ileum is resected and digesta are diverted from the body via a stoma to a colostomy bag). The amount of transgene that survived passage through the small bowel varied among individuals, with a maximum of 3.7% recovered at the stoma of one individual. The transgene did not survive passage through the intact gastrointestinal tract of human subjects fed GM soya. Three of seven ileostomists showed evidence of low-frequency gene transfer from GM soya to the microflora of the small bowel before their involvement in these experiments. As this low level of *epsps* in the intestinal microflora did not increase after consumption of the meal containing GM soya, we conclude that gene transfer did not occur during the feeding experiment.

Human consumption of GM plants raises the question of whether transgenes can be taken up by the intestinal microflora. Of special concern is the possible transfer of genes conferring antibiotic resistance, leading to an increase in mammalian pathogens that are resistant to antimicrobial agents. This concern has been heightened by evidence that plant and gastrointestinal DNA can be transferred to mammalian and bacterial cells. Thus, gene transfer from plants to naturally competent bacteria has been observed[1], chloroplast DNA sequences were found in the tissues of chickens and lymphocytes of cows fed GM or non-GM maize[2], and bacteriophage DNA introduced into the mouse intestine has been detected in several body tissues[3–5]. Furthermore, plasmid DNA exposed to mammalian saliva retains the capacity to transform *Streptococcus gordonii*[6] and *Escherichia coli*[7]. In addition to gene transfer via transformation, prokaryotic DNA exchange can also be mediated by conjugation, which has recently been observed in the avian gastrointestinal tract[8].

Gene transfer from GM plants to the intestinal microflora requires survival of the plant DNA within the gastrointestinal tract. A transgene in the maize genome, *bla*, encoding β-lactamase, was completely degraded after the maize was exposed to rumen fluid for one minute[7], but was detectable for up to 1 h when the maize was incubated with ovine saliva. In chickens fed transgenic maize, *bla* was detected in the crop and stomach of the birds, but not in the small intestine or feces[9]. In contrast, phage DNA may be more stable in the gastrointestinal tract of mammals, appearing in the feces of mice[4]. An *in vitro* study that simulated the mammalian stomach and small bowel showed that 4% of the transgenes in GM soya and maize survived this treatment[10].

To the best of our knowledge, there are no published studies evaluating the survival of transgenic DNA in the human gastrointestinal tract. Here we assess transgene survival in the small intestine of human volunteers fed GM soya. Although a small proportion of transgenic DNA survived passage through the stomach and small intestine, all the transgenic DNA was degraded within the colon. There was some indication of low-frequency gene transfer from GM soya to the microflora of the small bowel before but not during the subjects' participation in these experiments.

RESULTS

DNA survival in ileostomists

To evaluate the survival of transgenic DNA in the stomach and small bowel, we fed seven ileostomists a meal containing GM soya and then quantified target DNA sequences in the digesta by quantitative competitive (QC) PCR. In ileostomists, the terminal ileum is resected, and digesta are diverted from the body to a colostomy bag. The transgene in our GM soya was *epsps*. The total *epsps* construct is 2,266 base pairs (bp) and encodes 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Examples of the PCR data and the subsequent quantification of *epsps* by QC-PCR are presented in **Figure 1**, and the full QC-PCR data set is displayed in **Table 1**. The meal fed to the ileostomists contained 3×10^{12} copies of the transgene and the indigestible marker PEG 4000 to determine the proportion of the food that had passed through the small bowel. Over 60% of the marker was recovered in the digesta of six subjects (**Fig. 2**). The transgene (**Table 1** and **Fig. 2**) was recovered in all seven subjects, although the rate of passage

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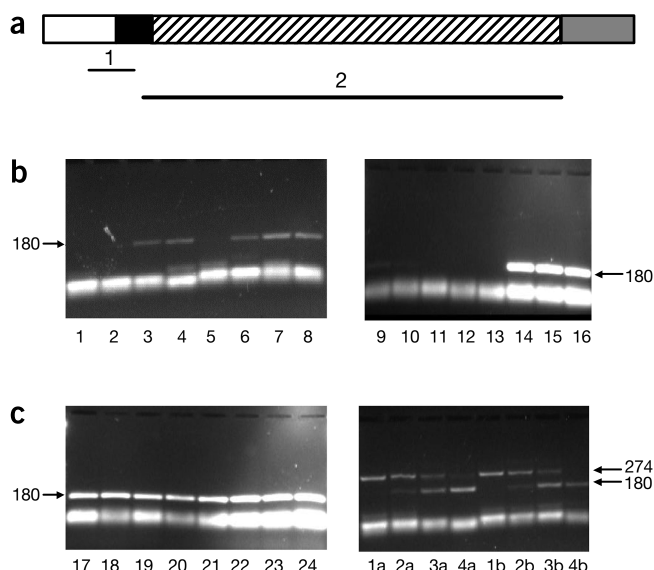

Figure 1 PCR amplification of *epsps* derived from the intestinal tract of ileostomists. (**a**) The *epsps* transgene, which consists of the cauliflower mosaic virus 35S promoter (white rectangle), the gene encoding EPSPS from *Agrobacterium sp.* strain CP4 (striped rectangle), fused to the region of the gene encoding the *P. hybrida* EPSPS chloroplast transit import signal (black rectangle), and the polyadenylation signal from the nopaline synthase gene (gray rectangle). The region of DNA that was amplified to quantify *epsps* (1) and detect the complete *epsps* gene (2) are indicated. (**b**) Examples of the initial PCR conducted on the various samples derived from the seven ileostomists using primers RR04 and RR05. The lanes contain the following samples: 1, subject 1 (0 min); 2, subject 4 (0 min); 3, microflora cultured in LB from subject 1 (0 min); 4, microflora cultured in LB from subject 4 (0 min); 5, subject 6 (0 min); 6, microflora cultured in LB from subject 6(0 min); 7, subject 1 (240 min); 8, subject 1 (270 min); 9, subject 1 (60 min); 10, subject 2 (60 min); 11, subject 3 (60 min); 12, subject 4 (60 min); 13, subject 5 (60 min); 14, subject 6 (240 min); 15, subject 6 (330 min); 16, subject 6 (360 min); 17, subject 7 (180 min); 18, subject 4 (180 min); 19, subject 4 (210 min); 20, subject 7 (210 min); 21, subject 7 (150 min); 22, subject 6 (270 min); 23, subject 6 (300 min); 24, subject 4 (240 min). (**c**) An example of QC-PCR. The PCRs were carried out as in **a**, except that pBNS2.2 was included at the following number of copies; lane 1, 1×10^4; lane 2, 2×10^3; lane 3, 4×10^2; lane 4, 8×10^1. Lanes 1a–4a contain the sample from subject 5 (180 min), whereas lanes 1b–4b contain the sample from subject 5 (210 min). The sizes (bp) of the major PCR products are shown.

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of digesta and total recovery of this nucleic acid was highly variable among individuals, ranging from 10^{11} copies of the transgene (or 3.7% of the 180 bp *epsps* region in the test meal) in subject 4 to only 10^5 copies in subject 3.

To determine whether we could detect the complete *epsps* gene, we subjected digesta to PCR using a primer pair that amplified the entire open reading frame. The full-length gene was detectable in samples that contained at least 10^4 copies of the 180 bp fragment per mg dry weight of digesta (**Table 1**). However, because such DNA molecules could have been produced by PCR-mediated recombination of *epsps* fragments, the conclusion that the full-length gene survived intact must be treated with caution.

We evaluated the differential survival of the transgene and a native gene in GM soya, the lectin gene *Le1*[11,12]. The persistence of the *Le1* and *epsps* genes was similar (**Fig. 3**), indicating that the transgene and the bulk soya DNA were degraded at similar rates.

To determine whether the indigestible marker PEG 4000 influenced DNA survival in the small bowel of the ileostomists, we assessed the capacity of this polymer to inhibit DNAase activity. At 4% (wt/vol), which is equivalent to the concentrations of PEG 4000 in the meal containing GM soya, the transit marker seemed to slightly increase the rate of DNA degradation (**Fig. 4**).

DNA survival in humans with an intact gastrointestinal tract

We fed the test meal containing GM soya to 12 human volunteers (with intact gastrointestinal tracts) and quantified the presence of the transgene in feces by PCR. For all volunteers, 90–98% of the indigestible marker was recovered in the feces but the transgene was not detected. This was not the result of PCR inhibition because a 180 bp product was amplified by PCR when the fecal material was spiked with 400 copies of the transgene in the aliquot used in the PCRs (data not shown). Thus, although the *epsps* transgene can survive passage through the small bowel of ileostomists, it is completely degraded in the large intestine.

Preexisting *epsps* transgene in the intestinal microflora

To assess possible gene transfer from GM soya to the intestinal microflora before the start of our experiments, we cultured microbes in the T 0 ileal digesta samples from the ileostomists in Luria broth (LB). Bacteria grew to a density of 10^8/ml (determined by plating onto LB agar) on each subculturing, and DNA from these microbes was subjected to PCR using the RRO4/RRO5 primer pair[13]. The PCR product was not detected in the original stoma digesta effluent (which contained ~10^6 bacteria/g wet weight, quantified by plating onto LB agar) taken from any volunteer before consumption of the meal containing GM soya (T 0). However, when the microbial populations in T 0 samples from subjects 1, 4 and 6 were cultured, the PCR product was evident in all the LB liquid subcultures at a very low concentration (150–650 copies/µg DNA; **Table 2**), equivalent to 1–3 copies of the transgene per 10^6 bacteria. Sequencing of the amplified DNA from subjects 1, 4 and 6 showed that the sequences were identical to that of the *epsps* transgene in our GM soya, suggesting that these subjects had consumed the transgene before enrolling in our study. In contrast, the lectin gene *Le1* was not detected in these bacterial samples from any subject.

Despite exhaustive attempts, we were unable to isolate bacteria harboring the transgene by colony blot hybridization or using a PCR pooling strategy. To investigate further the microorganism(s) that contain the transgene, we grew the *epsps*-containing cultures anaerobically, aerobically in M9 minimal medium containing glucose, with 32 µg/ml aztreonam (which inhibits Gram-negative bacteria) or with 4 µg/ml vancomycin (which inhibits Gram-positive bacteria, except *Lactobacillus*). The PCR product was evident in the anaerobic cultures and in cultures containing aztreonan, but was not detected in bacteria grown in minimal medium or in the presence of vancomycin (data not shown). These results indicate that either the bacteria containing the transgene or essential symbionts are likely to be auxotrophic, Gram-positive prokaryotes that are facultative anaerobes. When microbes from the feces of humans with intact gastrointestinal tracts fed the GM soya were cultured either aerobically or anaerobically, the *epsps* transgene was not detected by PCR.

Gene transfer into the intestinal epithelium

It is possible that transgenes derived from GM plants are transferred,

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via commensal or other bacteria, to the intestinal epithelium. Likely vectors for such genetic movement are *Lactobacillus plantarum*[14], which colonize the surface of intestinal enterocytes, or the intracellular pathogen *Salmonella typhimurium*[15]. To assess this possibility, we transformed *L. plantarum* GG and *S. typhimurium* SL3261 (refs. 14,15) with pBK-CMV and pLN1, respectively. Both plasmids contain a neomycin resistance gene that is active in all mammalian cells. The bacteria were incubated with Caco-2 cells, a model intestinal columnar epithelial cell line[16], and gene transfer from the prokaryotes into the mammalian cells was assessed by selecting for Caco-2 cells resistant to G418 (a neomycin analog). Transfection of Caco-2 cells with either pBK-CMV or pLPN generated G418-resistant cells at a frequency of 1 in 3,000. Incubation of 10^7 Caco-2 cells, at various stages of differentiation, with a 1,000-fold excess of either the recombinant *S. typhimurium* or *L. plantarum* generated no G418-resistant mammalian cells, indicating that no gene transfer from the bacteria to the mammalian cells had occurred.

DISCUSSION

We found that a small proportion of the *epsps* transgene in GM soya survives passage through the stomach and small bowel of all the ileostomists. The relatively low recovery of the transgene from subject 3 was accompanied by low recovery of PEG 4000, suggesting that digesta transit was slow in this individual. In the other six subjects, the quantity of the *epsps* fragment detected did not correlate with recovery of PEG 4000 at the stoma (**Fig. 2**), suggesting that the variation in transgene recovery was caused by factors other than small bowel transit time, such as differences in stomach acid or the quantity of DNAase I secreted from the pancreas .

It is surprising that even a fraction of GM soya DNA survives passage through the small bowel. The physiology of the small bowel in ileostomists may differ from that of individuals with an intact gastrointestinal tract, leading to an overestimate of DNA survival when extrapolating to normal individuals. However, the ileostomy model has been extensively validated as a means of determining digestion in the human small bowel[17–19] and is likely to be equally reliable for DNA. Our observation that the transit marker PEG 4000 does not inhibit DNA degradation suggests that we have not overestimated the stability of dietary DNA in the stomach and small bowel. The relative stability of the transgene in the stomach indicates that the food matrix had a pH buffering effect such that the concentration of H^+ in the microenvironment of the food was lower than in the bulk of the stomach lumen. It is possible that the GM soya retained some of its cellular structure, protecting the DNA from exogenous non-plant DNAases. But this is unlikely, as naked soya DNA and the DNA in processed food grade soya protein (the same material as used in this study)[10] are hydrolyzed *in vitro* at similar rates.

Our results in human ileostomists contrast with those of an earlier study in chickens, which found that transgenes in GM maize con-

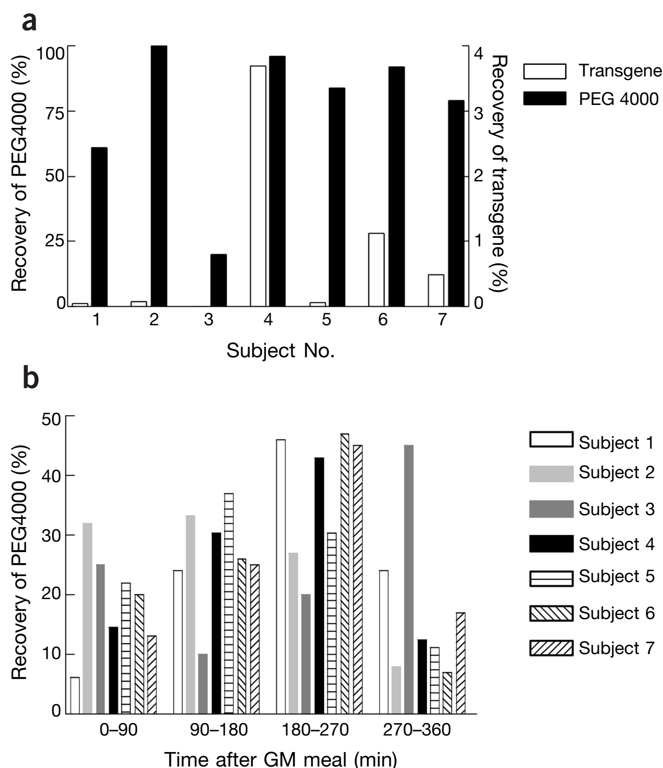

Figure 2 Recovery of the *epsps* transgene and the indigestible marker PEG 4000. (**a**) The total quantities of transgene and marker recovered in 6 h after consumption of the test meal. (**b**) The output of PEG 4000 during each 90 min period in the seven ileostomist volunteers.

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

Time (min)	Transgene copies × 10^6 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	0
60	0	0	0	0	0	0	0
90	0	0	25 ± 4	340 ± 43[a]	0	0	0
120	0	1,720 ± 34[a]	0	380 ± 31[a]	51 ± 4	0	0
150	0	210 ± 15[a]	0	460 ± 56[a]	23 ± 3	0	4,700 ± 346[a]
180	250 ± 35[a]	0	0	3,790 ± 321[a]	330 ± 41[a]	0	2,300 ± 268[a]
210	430 ± 57[a]	0	0	8,200 ± 920[a]	630 ± 70[a]	523 ± 85[a]	7,000 ± 850[a]
240	140 ± 23[a]	0	0	93,000 ± 10,500[a]	490 ± 55[a]	3,200 ± 367[a]	410 ± 5[a]
270	160 ± 21[a]	0	0	4,100 ± 650[a]	250 ± 33[a]	10,200 ± 1,200[a]	0
300	110 ± 15[a]	0	0	230 ± 45[a]	5 ± 1	12,340 ± 2,105[a]	0
330	180 ± 31[a]	0	0	470 ± 76[a]	0	4,600 ± 552[a]	0
360	14 ± 3	0	0	420 ± 54[a]	0	2,700 ± 324[a]	0

The values corresponding to the total number of copies of the transgene in the whole sample ± s.e.m.
[a]Samples in which full-length *epsps* was detected.

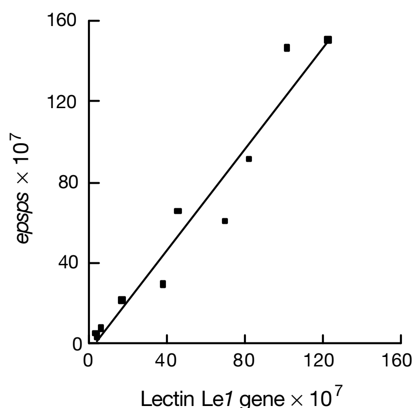

Figure 3 Relationship between recovery of the *epsps* transgene and the soya lectin gene *Le1*. The correlation coefficient (r^2) was 0.95 and $y = 1.25 \pm 0.09x - 3.76 \pm 5.77$.

sumed by the animals were completely degraded before entering the small intestine[9]. This may reflect differences in the physiology of the intestines of humans and the avian. Chickens have a crop where stored food may be subject to microbial DNAases. Grinding in the gizzard, by increasing the surface area of the food, may further expose DNA both to acid and to small intestinal DNAases. Our data show comparable degradation kinetics for *epsps* and the native plant lectin gene *Le1*. Similarly, the degradation of antibiotic resistance transgenes in GM maize fed to chickens mirrored that of *nad5*, a plant mitochondrial gene[9].

Our finding that *epsps* is completely degraded in the large intestine is consistent with studies[7,20] demonstrating that naked DNA is rapidly hydrolyzed by DNAases associated with the rumen microflora, a microbial system similar to that of the human large bowel. In contrast, DNA fed to mice was detected in the small intestine, cecum and feces in amounts that suggest that nucleic acid that survived the stomach was not subject to further degradation[4]. This may reflect the shorter transit time of digesta through the mouse intestines (compared with humans), allowing less time for hydrolysis by intestinal microbial and pancreas-derived DNAases. The mouse may therefore not be an ideal model for studying transgene survival in humans.

Gene transfer from GM soya to the intestinal microflora appears to have occurred before the feeding experiment in three of the ileostomists, as a single meal containing very high levels of GM soya did not enhance plant to bacterial gene transfer; the levels of transgene in the microbes (measured in subculture 6) derived from the digesta of the T 0 min (before GM meal ingestion) and T 360 min (after consumption of the GM meal) samples from Subjects 1, 4 and 6 were similar (T 0 min results in **Table 2**; T 360 min data not shown). This gene flow is therefore likely to reflect long term consumption of GM foods. The transgene-derived PCR product was evident only when the microbes in the T 0 digesta had been expanded by culturing, indicating that the *epsps*-containing bacteria was a very small component of the small intestinal microflora. It should be noted that these bacteria contained only a fragment of *epsps*; the full-length gene was not detected in these microbes. The observation that the bacteria containing the GM soya transgene could not be identified from $>2 \times 10^6$ colonies screened on agar plates again indicates that the microbes containing the transgene represent a very minor component of the small intestinal microflora.

The inability to isolate this organism(s) on agar media is not unusual. Indeed, molecular evidence indicates that 90% of microorganisms in the intestinal microflora remain uncultured[21]. That the target prokaryote(s) grew in liquid culture but not as colonies on agar media suggests that they can grow only in mixed culture, a phenomenon seen with other microorganisms[22,23]. The observation that the transgene fragment was detected only when the microbial population had been amplified indicates that the DNA was stably maintained in the bacteria and thus had integrated either into the microbial genome or into a stable extrachromosomal element. Although the plant lectin gene was not detected in the microbial population (data not shown), it is premature to conclude that the *epsps* transgene is more likely than endogenous plant genes to transfer into the microbial population.

No microbes containing the transgene could be cultured from the feces of humans with an intact gastrointestinal tract. This may indicate that the bacteria containing the GM soya transgene are viable only in the small bowel and do not survive in the large bowel where the environment is more anaerobic and there is a much higher density of competing organisms. Alternatively, it is possible that gene transfer from plant DNA to the human intestinal microflora is less likely to occur in people with intact gastrointestinal tracts than in ileostomists because of differences in gastrointestinal physiology between these two groups of people.

Our results from coculture experiments with transformed bacteria and Caco-2 cells suggest that gene transfer from GM plants to the intestinal epithelium is unlikely to occur. This is consistent with the absence of the transgene in the feces of subjects fed the diet containing GM soya, as feces contain substantial numbers of exfoliated colonocytes[24]. The interpretation that gene transfer from bacterial cells to enterocytes is unlikely must be viewed with caution, however, because the complex environment of the intestinal mucosa and lumen cannot be simulated perfectly *in vitro*.

In the mouse, by contrast, bacteriophage DNA was shown to be taken up by the columnar cells in the cecum[4]. However, the DNA may have entered the M cells and leukocytes in the Peyer's patches, rather than the enterocytes, for which Caco-2 cells provide an excellent model[16]. Stable incorporation of bacteriophage DNA into the mouse genome was evident only after feeding the mice with 50 μg of bacteriophage DNA daily for one week, indicating that gene dosage may be a key factor in gene flow from GM plants to mammalian cells. In a recent study, chloroplast DNA, which is present in multiple copies in plant cells, was detected in the tissues of

Table 2 Detection of the epsps transgene in the intestinal microflora

Subculture	Copies of the 180 bp epsps fragment/μg of bacterial DNA[a]					
	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
0[b]	ND[c]	ND	ND	ND	ND	ND	ND
1	475	ND	ND	320	ND	185	ND
2	404	ND	ND	120	ND	190	ND
3	150	ND	ND	250	ND	210	ND
4	265	ND	ND	643	ND	156	ND
5	423	ND	ND	312	ND	312	ND
6	197	ND	ND	500	ND	256	ND

[a]One microgram of extracted DNA was used in each QCPCR reaction. [b]Subculture 0 comprises the stoma effluent which was taken before consumption of the meal containing GM soya (time 0 sample; see **Table 1**). ND, no DNA product was detected. An amplified DNA product can be detected when samples containing 80 copies or more of the transgene are subjected to PCR[10].

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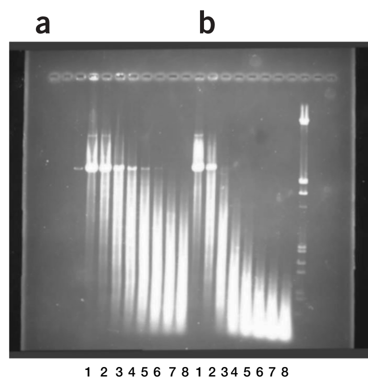

Figure 4 Influence of PEG4000 on DNAase activity. (**a**,**b**) Plasmid DNA containing the *epsps* transgene was incubated with ileal digesta in the absence (**a**) and presence (**b**) of 4% (wt/vol) PEG4000. At various intervals aliquots were removed, DNAase activity was inactivated by the addition of EDTA to 20 mM and the samples were subjected to agarose gel electrophoresis. Lanes depict the time the DNA was incubated with the DNAase: 1, 0 min; 2, 5 min; 3, 10 min; 4, 15 min, 5, 22 min; 6, 30 min; 7, 40 min; and 8, 60 min.

chickens and in the lymphocytes of cows fed GM-maize, whereas the plant transgene was not detected[2].

In conclusion, we have shown that a small proportion of the transgenes in GM soya, like the native soya DNA, survives passage through the human upper gastrointestinal tract but is completely degraded in the large intestine. Although we found some evidence of preexisting gene transfer between the GM soya and the human small intestinal microflora, the bacteria containing the transgene represented a very small proportion of the microbial population, and there was no indication that the complete transgene had been transferred to the prokaryotes. Thus, it is highly unlikely that the gene transfer events seen in this study would alter gastrointestinal function or pose a risk to human health. Nevertheless, the observed survival of transgenic DNA from a GM plant during passage through the small intestine should be considered in future safety assessments of GM foods.

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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[25].

Culturing of Caco-2 cells with *L. plantarum* and *S. typhimurium*. Caco-2 cells which had been passaged through seven generations were seeded at a density of 3×10^5 cm^{-2} and grown under standard conditions until confluent[26]. At various times after the initial seeding a 1,000-fold excess of bacteria was added. After 28 d 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[26].

Culturing of intestinal and fecal microorganisms. To 10 ml of Luria broth (LB) we added 500 mg of either digesta or feces, and the mixture was incubated for 16 h. A 50 µl aliquot of the culture was inoculated into 10 ml of fresh LB and the bacteria were again incubated for 16 h. The bacteria were subcultured for a further five passages. Microorganisms derived from feces were also cultured anaerobically as described previously[27].

Human studies. After approval from the Joint Ethics Committee of the University of Newcastle upon Tyne, seven ileostomists were recruited. These volunteers fasted overnight and DNA was extracted from their stoma effluent before the start of the trial. The test meal consisted of GM soya burgers and a GM soya milk shake, the ingredients for which were purchased from retail outlets. The meal was prepared by deep frying in vegetable oil a burger mix comprising three eggs, 150 g texturized soya protein, 300 ml water and 24 g PEG4000. The milk shake containing GM soya consisted of 100 g soya protein supplement, 20 g PEG4000, 600 ml soya milk and 20 ml milk shake flavoring. Each subject was fed 190 g of the GM soya burger and 264 g of the GM soya milk shake. The contents of their stoma bags were collected every 30 min for 6 h and the digesta freeze dried. DNA was extracted and the PEG4000 (a marker of digesta flow[28]) quantified following standard procedures[29]. Subjects with intact gastrointestinal tracts received the same meal containing GM soya except that the indigestible marker consisted of radio-opaque markers ('Colon Transit 20'). After consumption of the test meal the subjects consumed their usual diet and collected their stools over 72 h. Feces were freeze dried, radio-opaque markers were counted on X-ray plates and the DNA was extracted and analyzed.

DNA analysis. DNA was extracted from digesta, feces and the cultured intestinal microflora[10] in duplicate. Briefly, the digesta and fecal samples were freeze-dried and 100 mg of ground material were resuspended in 400 µl of water, and 600 µl of 6 M guanidinium hydrochloride was added. For the bacterial cultures, cells from 10 ml of an overnight culture were also resuspended in 400 µl of water followed by the addition of 600 µl of 6 M guanidinium hydrochloride. The samples were incubated at 58 °C for 3 h, vortexing every 15 min. The mixture was centrifuged at 13,000*g* for 10 min and DNA was purified from 500 µl of the supernatant fraction using the Wizard DNA cleanup system Protocol (Promega). The DNA was eluted from the columns in 50 µl of sterile Milli-Q water and quantified by measuring the A_{260} and A_{280} of the DNA samples. The 25 µl PCR reactions comprised 2.5 µM of each primer, 1× Amplitaq Gold buffer (Perkin Elmer), 2 mM dNTPs, 10 mM $MgCl_2$, 0.5 units Amplitaq DNA polymerase and ~1 µg target DNA; the reaction was made up to volume with sterile PCR grade water (Sigma) and overlaid with 40 µl of mineral oil. The PCR was carried out in a PTC-100 Programmable Thermocontroller (M.J. Research) using the following conditions: initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation for 1 min at 95 °C, annealing at 65 °C for 1 min and extension at 72 °C for 1 min, with a final extension step of 15 min at 72 °C. The primers used in these reactions to detect the region of the *epsps* transgene encompassing the CaMV E35S promoter and DNA encoding the *P. hybrida* chloroplast N-terminal transit peptide were RR05 (5′-TGCGGGCCGGCTGCTTGCA-3′) and RR04 (5′-CCCCAAGTTCC TAAATCTTCAAGT-3′). To detect the soya lectin gene *Le1*, PCR was carried as described above using the primers GM03 5′-GCCCTCTACTCCACCCC CATCC-3′ and GM04 5′-GCCCCATCTGCAAGCCTTTTTGTG-3′ (ref. 12). To quantify *epsps* and *Le1*, quantitative competitive PCRs (QC-PCRs) were carried out using the reaction conditions described above. For *epsps* detection the reactions included the competitor DNA pBNS2.2 at 8×10^1 to 1×10^4 copies. The plasmid pBNS2.2 contains a 550 bp fragment of *epsps* into which 94 bp of DNA derived from pET16b had been inserted[10]. To quantify the lectin gene (*Le1*) the plasmid pJG1 was included also at 80 to 10,000 copies. The plasmid, pJG1 contains the *Le1* gene into which a 100 bp sequence of pCR-Blunt had been inserted in the region amplified by the primers GM03 and GM04. QC-PCR was carried out in duplicate on each sample. The presence or absence of full-length *epsps* was determined by PCRs using primers that amplify the complete *epsps* transgene encoding the *P. hybrida* chloroplast transit peptide fused to the *Agrobacterium* sp. CP4 EPSPS[30] (**Fig. 1**). To ensure that the PCR product detected in the microbial cultures was not derived from the culture medium, repeated PCRs on the uninoculated culture medium (LB-broth) were carried out and no product was detected. To minimize the risks of cross-contamination, PCR analysis was carried out in a separate location (different building) where we kept our stock plasmids containing the *epsps* transgene sequence, and laboratory clothing and equipment were not exchanged between the different sites. Furthermore, PCRs lacking test DNA but including all other components of the reactions were incorporated in every amplification series as negative controls to ensure that there was no transgene contamination of our reagents, water, mineral oil or the reaction vessels.

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COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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