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# Fate of genetically modified maize DNA in the oral cavity and rumen of sheep

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The polymerase chain reaction (PCR) technique was used to investigate the fate of a transgene in the rumen of sheep fed silage and maize grains from an insect-resistant maize line. A 1914-bp DNA fragment containing the entire coding region of the synthetic *cryIA*(*b*) gene was still amplifiable from rumen fluid sampled 5 h after feeding maize grains. The same target sequence, however, could not be amplified from rumen fluid sampled from sheep fed silage prepared from the genetically modified maize line. PCR amplification of a shorter (211-bp), yet still highly specific, target sequence was possible with rumen fluid sampled up to 3 and 24 h after feeding silage and maize grains, respectively. These findings indicate that intact transgenes from silage are unlikely to survive significantly in the rumen since a DNA sequence 211-bp long is very unlikely to transmit genetic information. 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 incubation for 8 min 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.

**Horizontal gene transfer: DNA survival: Insect-resistant maize: Antibiotic resistance: Transformation efficiency**

Antibiotic resistance genes are frequently used as markers in the construction of genetically modified (GM) plants. The increasing use worldwide of GM plants in the production of animal feed has raised concerns that these genes may be spread horizontally to bacteria in the gastrointestinal tract of animals that are being fed the GM material. The consequences of such transfers would increase the load of resistance genes in bacteria that may act as potential human or animal pathogens.

Maize is preserved by ensilage to provide an important winter fodder for ruminants. The low pH and highly active bacterial and plant nucleases present in silos create a harsh environment for DNA survival outside the cellular environment. Nevertheless, evidence indicating that maize silage contains fragments of DNA large enough to contain potentially functional genes has been presented (Chiter *et al.* 2000). Moreover, temperatures of 95°C or above for more than a few minutes were necessary to prevent the survival of large DNA fragments in maize and wheat grains, the source of much animal feed (Chiter *et al.* 2000).

Natural genetic transformation is regarded as the most likely conduit for horizontal gene flow from GM plants to bacteria. Many bacteria, including oral and gut microbes, are known to be naturally transformable (Lorenz & Wackernagel, 1994). GM DNA integrated in the chromosomes of plants or bacteria has been shown to transform bacteria in soils by homologous recombination, albeit under optimised laboratory conditions (Gebhard & Smalla, 1988; Nielsen *et al.* 1988; De Vries & Wackernagel, 1998; Davison, 1999; De Vries *et al.* 2001). To address the possibility that bacteria in the gastrointestinal tract may take up and integrate diet-derived transgenes, consideration must be given to the stability of DNA in all regions of the digestive system. Previous studies have shown that a proportion of orally ingested bacteriophage M13 DNA can survive passage through the mouse gastrointestinal tract and can even traverse the intestinal epithelium into the systemic circulation (Schubbert *et al.* 1994, 1997). It has been shown that plasmid DNA previously exposed to human saliva *in vitro* or taken into

**Abbreviations:** $bla_{TEM}$, TEM-1 β-lactamase gene; GM, genetically modified; PCR, polymerase chain reaction.

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the human mouth was still capable of transforming competent bacteria *in vitro* (Mercer *et al.* 1999*a*, 2001). Additionally, competent *Streptococcus gordonii* cells were shown to take up and integrate foreign DNA sequences *in vitro*, in the presence of human saliva, based on homologous recombination (Mercer *et al.* 2001), implying that GM DNA released from the diet may provide a source of transforming DNA in the oral cavity.

Recently we showed that plasmid DNA exposed to degradation in ovine saliva *in vitro* was still capable of transforming competent *Escherichia coli*, even after 24 h, whereas only short-term biological activity, lasting less than 1 min was observed in ovine rumen fluid *in vitro* (Duggan *et al.* 2000). Here we examine the survival of free DNA *in vivo* in the ovine oral cavity and investigate the survival of a transgene in orally fed GM maize grain and silage *in vivo*, in the sheep rumen.

The TEM-1 β-lactamase gene ($bla_{TEM}$), conferring resistance to ampicillin and related β-lactam antibiotics, used as selectable marker during the construction of insect-resistant maize line CG00526-176 (Koziel *et al.* 1993) originates from the cloning vector pUC18. Since variants of this gene are widely distributed in the microflora of the gastrointestinal tract of animals and man, we investigated the fate of the insect resistance determinant, *cryIA*(*b*). The largest target sequence used in the present study is 1914-bp in length, which is larger than the 1600-bp fragment that would be necessary to contain the pUC18 $bla_{TEM}$ gene and its origin of replication. There is no reason to suppose that the survival of $bla_{TEM}$-containing DNA fragments should behave differently from that of any other DNA target sequence.

## Materials and methods

### *Bacterial strains, plasmids, maize and sheep*

*E. coli* strain DH5α (Hanahan, 1983) was grown in Luria–Bertani medium (Sambrook *et al.* 1989). Plasmid pCRY6, constructed using pCR®Blunt (Invitrogen, Paisley, Strathclyde, UK), contains the entire coding region of the synthetic *cry1A*(*b*) gene, generated by polymerase chain reaction (PCR) amplification of GM maize line CG00526-176 chromosomal DNA using the previously described primer pair Cry01/02 (Hupfer *et al.* 1997). Where appropriate, the growth medium was supplemented with 50 mg kanamycin/l. The maize line G00526-176 was grown and ensiled as described previously (Duggan *et al.* 2000). Three mature Suffolk sheep were fitted with permanent ruminal fistulas and, unless stated otherwise, received diets of 1000 g dried grass pellets/d and 300 g hay/d, which was fed as single meals.

### *Sheep feeding study*

The first diet consisted of 500 g maize silage, 500 g pelleted dried grass and 100 g molasses, fed on three consecutive days. The second feeding study, performed approximately 2 months after the silage feeding study, consisted of 500 g fresh coarsely ground maize grain and 500 g pelleted dried grass, fed for three consecutive days. In both studies, the experimental meal was fed in the morning and rumen fluid was obtained via a cannula immediately before feeding (time zero) and at set times after the start of feeding. Fresh faeces were collected before feeding and 24 h after each experimental feed, i.e. for up to 72 h after the initial feeding of the test material. In addition to the experimental feed, each sheep received 300 g hay/d, which was fed in the afternoon. Each feed trial was performed twice on each of three sheep.

### *Extraction of maize chromosomal DNA from rumen and faecal samples*

Rumen and faecal samples (2–3 g) were ground to a coarse powder using a pestle and mortar, under liquid $N_2$. A 500 mg portion of the frozen powder was transferred to a 2 ml Eppendorf tube, mixed thoroughly with 1 ml lysis buffer supplied with the DNeasy™ Plant Mini Kit (Qiagen, Crawley, West Sussex, UK) and incubated at 65°C for 30 min. After centrifugation, the supernatant liquid was extracted once with phenol–chloroform (Phenol:chloroform:isoamyl alcohol (25:24:1, v/v)) and extracted further according to the DNA isolation protocol supplied with the DNeasy™ Plant Mini Kit (Qiagen). The DNA was finally eluted in 100 μl sterile water and 2 μl samples were used for PCR amplification. This method was also used to isolate genomic DNA from maize silage and crushed maize grain.

### *Experiments to examine the survival of DNA in the ovine oral cavity*

Plasmid pCRY6 (2·5 μg or 5 μg in 1 ml water) was added to the ovine oral cavity using a plastic pipette. At 1 min intervals over a 10 min incubation, saliva was removed using a swab, which was then submerged in 0·4 ml 10 mM Tris.HCl pH 8·0, 1 mM EDTA buffer. An additional sample was collected after 30 min and processed as described earlier. After thorough mixing, the plasmid was recovered using QIAprep® Spin Miniprep columns (Qiagen), resuspended in 50 μl sterile water and 2 μl samples were used for transformation of competent *E. coli* DH5α and PCR amplification. A portion of the DNA (30 μl) was visualised following electrophoresis on 1 % (w/v) agarose. As a modification to the procedure described earlier, plasmid (5 μg) was added to a slice of apple before feeding and saliva samples recovered at set times as described earlier.

Experiments with maize chromosomal DNA were performed as described earlier except that 10 μg chromosomal DNA was mixed with 1 ml water before addition to the oral cavity and was recovered from saliva samples using Qiagen DNeasy Tissue Kits. Recovered DNA was resuspended in 50 μl water, 2 μl samples were used for PCR amplification and 30 μl of each sample was subject to electrophoresis on 1 % (w/v) agarose gels.

### *Oligonucleotide primers and polymerase chain reaction*

Detection of GM maize-specific target sequences was performed using the previously described primer pairs,

Cry01/02, which flank the synthetic *cryIA*(*b*) gene, yielding an amplimer of 1914-bp (Hupfer *et al.* 1997) and Cry03/04, which yields a 211-bp amplimer. Expression of the *cryIA*(*b*) gene in maize line CG00526-176 is controlled by a combination of the phosphoenolpyruvate carboxylase promoter, and the pollen-specific, Ca-dependent protein kinase promoter (Koziel *et al.* 1993; Hupfer *et al.* 1998). The 211-bp amplimer generated using primer pair Cry03/04 consists of the last 73-bp of the Ca-dependent protein kinase promoter and the first 138-bp of the 5′ region of the *cryIA*(*b*) gene (Hupfer *et al.* 1998). The positions of primer pairs Cry01/02 and Cry03/04 are shown in Fig. 1. PCR amplification of a 226-bp maize-specific region of the invertase gene ivr1 was performed using primers *ivr1*-F and ivr-R, described by Ehlers *et al.* (Hupfer *et al.* 1999). Amplification of a 214-bp fragment of pCRY6 was carried out using T7 universal primer and the previously described Cry04 primer (Hupfer *et al.* 1998). Amplification of a 370-bp fragment of the 16S rRNA gene was performed using the previously described primer pair, DG74 and RW01 (Greisen *et al.* 1994). Amplification of *bla* gene fragments was carried out using the previously described primer pairs C and E and C and B (Mabilat *et al.* 1990). Amplification of the 211-bp *cryIA*(*b*), the 226-bp *ivr1*, the 214-bp pCRY6-specific amplimer, the 370-bp 16S rRNA and the *bla* gene (350-bp and 600-bp) target sequences was carried out with the following cycle times: 1 min at 94°C, followed by thirty cycles of 94°C for 30 s, annealing for 30 s at temperatures ranging from 52°C to 63°C (depending on the melting temperatures of the primers used) and 72°C for 45 s, with a final elongation step of 5 min at 72°C. For amplification of the longer *cry1A*(*b*) gene target the programme described earlier was modified by increasing the number of cycles to thirty-two and lengthening the elongation step to 2·5 min.

### *Transformation of* Escherichia coli *DH5α with plasmid pCRY6*

Transformation of calcium chloride-treated competent *E. coli* cells with pCRY6 was performed as described by Sambrook *et al.* (1989). Transformation was confirmed by growth on Luria–Bertani kanamycin agar and recovery of a plasmid with an appropriate restriction endonuclease profile.

## Results and discussion

### *Survival of DNA in silage and maize grains fed to sheep*

A PCR strategy previously used for the detection of a chromosomally integrated transgene, *cry1A*(*b*), in foods derived from GM maize and in GM maize silage (Hupfer *et al.* 1999) was employed to investigate the stability of the transgene in GM maize silage and grain fed to sheep. Before starting the feeding trials, tests were performed to determine whether PCR-amplifiable DNA could be isolated from maize tissues in the presence of rumen fluid. Samples (0·2 g) of silage or crushed maize grains were mixed with 1 ml samples of fresh rumen fluid and DNA isolation performed as described earlier (p. 162). Amplification of a 1914-bp *cryIA*(*b*) target sequences was possible with DNA isolated from both crushed maize grains and silage (data not shown).

With regard to the survival of DNA in maize tissues fed to sheep, amplification of the 1914-bp *cryIA*(*b*) coding region was possible with extracts prepared from rumen fluid sampled 5 h after feeding maize grain (Fig. 2). By contrast, all attempts to amplify the same target sequence from silage-fed sheep were unsuccessful, suggesting that DNA is rapidly degraded in the rumen following its release from plant cells, disrupted, presumably, through mastication and/or during transfer to the rumen (data not shown). By decreasing the length of the target sequence chosen for PCR amplification, from 1914-bp to 211-bp we were able to achieve more sensitive detection of surviving transgene sequences within maize grains in the rumen. In these experiments a 211-bp transgene target sequence flanked by primers Cry03/04 was still amplifiable from DNA extracted from rumen fluid sampled 24 h after feeding maize grain (data not shown) and 3 h after feeding silage (Fig. 3 (A)). Likewise, detection via amplification of a 226-bp fragment of the *ivr1* gene, included as a control to confirm the suitability of the isolated DNA as a target for PCR amplification, was still possible up to 3 h after feeding silage (Fig. 3 (B)) and 24 h after feeding maize grain (data not shown).

In ruminants, the physical breakdown of fibrous feed results primarily from mastication during eating and re-mastication during rumination. Maize grains have a tough exterior and it is not surprising, therefore, that a proportion of the plant cells remain intact, surviving mastication, rumination and the carbohydrate-degrading enzyme activity of

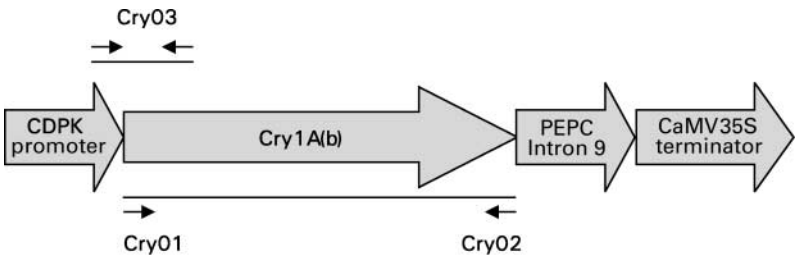


**Fig. 1.** Schematic diagram showing part of the genomic region of transgenic maize line CG00526-17b and positions of the primers (Cry01–Cry04) used for amplification of transgene sequences. CDPK, Ca-dependent protein kinase; PEPC, phosphoenolpyruvate carboxylase; CaMV35S, terminator from the cauliflower mosaic virus 35S gene.

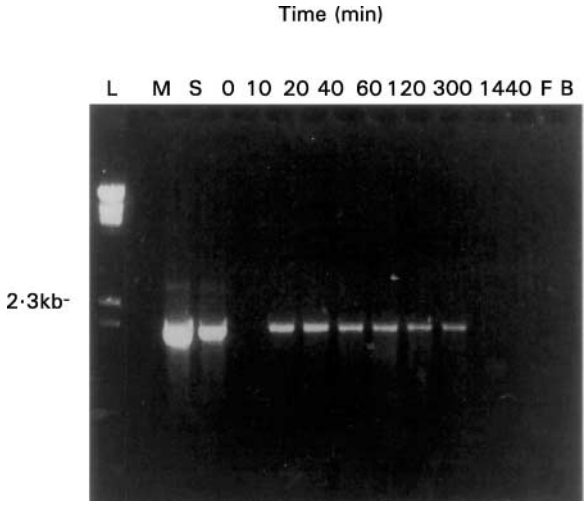


**Fig. 2.** Survival of a 1914-bp fragment containing the entire synthetic *cry1A*(*b*) gene in genetically modified (GM) maize grains fed to sheep. Feeding experiments were performed as described on p. 162 and DNA was isolated from rumen fluid collected at the times indicated. Polymerase chain reaction (PCR) analysis of purified DNA was performed using primers Cry01 and Cry02 and the products were analysed on a 1% (w/v) agarose gel. The blank (B) contained all the components of the PCR reaction except template DNA. PCR was also performed using DNA isolated from faecal material collected 24 h after feeding maize grain (F). Positive controls (M) and (S) were performed using 0·2 μg chromosomal DNA isolated from GM maize leaves and GM silage, respectively. The molecular size marker, also included on the gel (L), was generated by digesting bacteriophage λ DNA with *Hin*dIII.

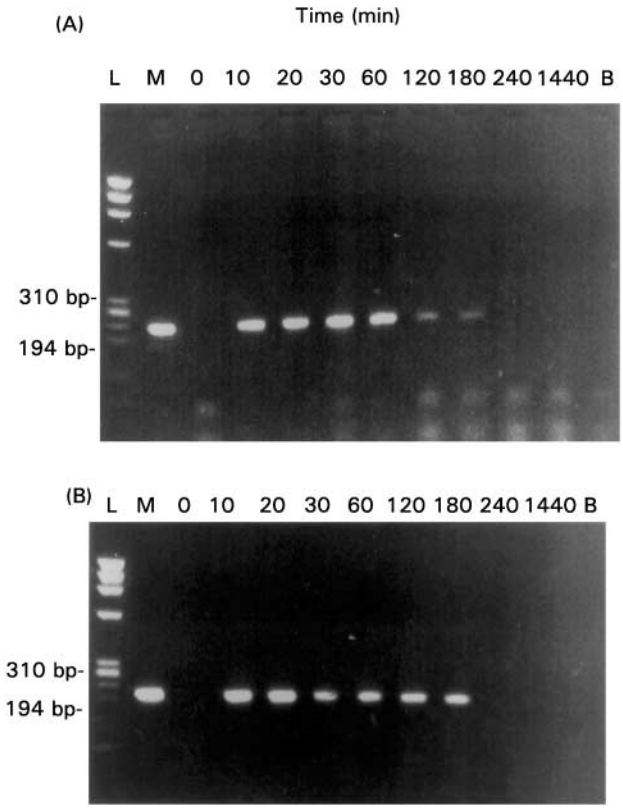


**Fig. 3.** Survival of DNA target sequences in maize silage tissues after feeding to sheep. DNA was isolated from rumen fluid collected at the times indicated and DNA survival estimated by polymerase chain reaction (PCR) amplification. (A), Persistence of a 211-bp DNA fragment containing parts of the 5′-end of the coding region of the synthetic *cry1A*(*b*) gene and the adjacent promoter. (B), Persistence of a 226-bp *invr1* gene fragment. The blank, B, contained all the components of the PCR reaction except template DNA. The positive control PCR reaction (M) was performed using 0·2 μg chromosomal DNA isolated from transgenic maize leaves. The molecular size marker comprising ϕX174 RF DNA digested to completion with *Hae*III (Gibco BRL), also included on each gel, is indicated (L).

protozoa and bacteria within the rumen. Both maize grains and material resembling silage in its macroscopic appearance were observed in rumen fluid sampled just 10 min after feeding and were still present in rumen fluid samples collected after 24 h. Attempts to amplify maize-specific sequences from DNA prepared from portions of rumen fluid lacking grains or silage tissue were unsuccessful (data not shown). In these experiments the material was removed by hand before DNA extraction. This suggests that the maize sequences amplified in the present study most probably originated from DNA released from plant cells during the DNA isolation process. This finding supports previous suggestions that naked DNA is unlikely to survive the high level of nuclease activity in the rumen (Flint, 1994). As predicted, amplification of *bla* gene fragments was possible with DNA extracted from faecal material and from all rumen fluid samples, including those removed before feeding GM material (data not shown), suggesting that the template DNA in these reactions was of bacterial origin. Indeed, amplification of the 370-bp 16S ribosomal gene fragment using previously described universal eubacterial primers was possible with DNA prepared from faecal and rumen extracts (data not shown). The survival times for DNA in silage and maize grain in the rumen are summarised in Table 1.

Neither maize grain nor silage was detected in faecal material and DNA prepared from faeces did not contain amplifiable plant DNA target sequences. Rate of passage of digesta is slow in ruminants, but the last sample of faeces was collected 72 h after initial feeding of the test material and, with a daily intake of about 1000 g DM/d it is clear that plenty of opportunity was given for test food to have passed completely through the digestive tract (Faichney, 1993). Considering that only the smaller transgene sequence was detected in rumen samples collected after 24 h, the possibility of biologically significant DNA sequences in material passing through the sheep intestine, however, seems extremely low. Nevertheless, orally

**Table 1.** Persistence of DNA target sequences from insect-resistant maize tissues fed to sheep

| | Survival time (min)* | |
|---|---|---|
| Target sequence | Silage-fed | Maize grain-fed |
| 1914-bp *cryIA*(*b*) | 0 | 300 |
| 211-bp *cryIA*(*b*) | 240 | 1440 |
| 226-bp *ivr1* | 240 | 1440 |

* Survival of DNA sequences was estimated by polymerase chain reaction following extraction of maize chromosomal DNA from rumen fluid samples removed from sheep fed silage or dry maize grain.

ingested DNA has been detected in the faeces of mice (Schubbert *et al.* 1994) and there is some recent evidence to suggest that GM DNA fragments in foodstuff may survive passage through the human intestine (Martín-Orúe *et al.* 2002).

One of the prerequisites for successful transformation is the availability of free DNA. Here we show that DNA fragments large enough to be of biological significance survive within maize grains for a considerable time in the rumen. Since the rumen is characterised by a high level of DNA-degrading activity (Russell & Wilson, 1988; Morrison, 1996; Ruiz *et al.* 2000), it is questionable whether free DNA can survive for significant time in this environment. DNA released into soils is protected from nuclease attack by binding to solid material, such as plant polysaccharides and minerals (Lorenz & Wackernagel, 1994; Davison, 1999). Recent evidence suggests that certain food components can reduce DNA degradation in gut simulations, *in vitro* (Martín-Orúe *et al.* 2002). It remains to be established whether DNA can be protected from rumen nucleases by binding to components of the diet. A second prerequisite for transformation is the availability of naturally transformable bacteria. The resident bacterial population of the rumen is high in terms of both numbers ($>10^{10}$ microbes/ml) and diversity. Additionally, it receives a large range of transient microbes that are ingested with the feed. At least one rumen isolate is known to develop natural genetic competence *in vitro* (Mercer *et al.* 1999*b*). Additionally, nutrients are abundant and there are numerous surfaces, such as plant particles, to which bacteria can adhere. It could be argued therefore that the rumen could provide opportunities for bacteria to come into close contact with DNA released from the diet.

### *Survival of free DNA in the ovine oral cavity*

In addition to examining the fate of a transgene in maize fed to sheep, the present study also examined the survival of free DNA in the oral cavity. The survival of plasmid pCRY6 *in vivo* in the ovine oral cavity is shown in Fig. 4. Extensive degradation of the plasmid is evident after incubation for just 1 min in the oral cavity and DNA was no longer visible after 30 min (Fig. 4). Densitometry and spectrophotometric determinations showed that at least 70 % of the plasmid DNA added to the mouth was lost within the first minute, and samples recovered after 10 min contained less than 10 % of the original plasmid DNA concentration. The physical integrity of maize chromosomal DNA was similarly destroyed within 1 min of addition to the oral cavity (data not shown). PCR experiments designed to estimate the persistence of target sequences from pCRY6 and maize chromosomal DNA following incubation in the oral cavity indicated that both the 1914-bp *cryIA*(*b*) and the plasmid-specific 214-bp fragment could be amplified from saliva-degraded pCRY6, even after 30 min of incubation (Figs. 5 (A) and (B), respectively). PCR amplification of the 1914-bp *cryIA*(*b*) target from maize chromosomal DNA was, however, only possible up to 5 min of exposure to the oral cavity, whereas a shorter 211-bp *cryIA*(*b*) target sequence was still available for amplification after 30 min (data not shown). The results shown in Fig. 5 were obtained from sheep receiving 5 μg plasmid pCRY6, although identical DNA survival times were observed with DNA recovered from sheep receiving 2·5 μg of the plasmid (data not shown).

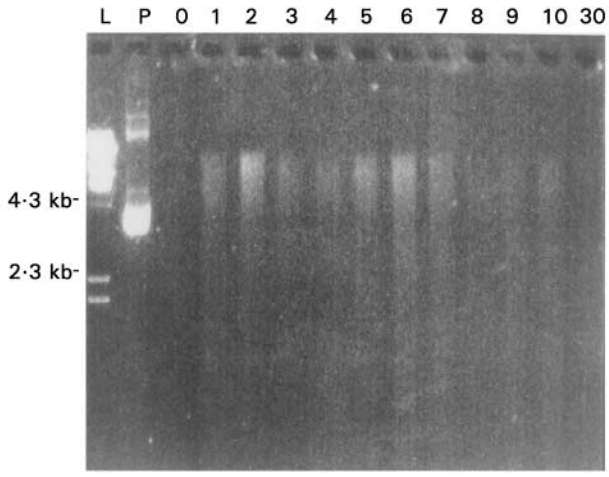


**Fig. 4.** Survival of pCRY6 DNA in the ovine oral cavity for 30 min. Plasmid DNA was added to the ovine oral cavity and samples of saliva removed at the times indicated. Plasmid DNA was recovered from saliva as described on p. 162 and samples were analysed on a 1·0 % (w/v) agarose gel. Plasmid DNA not exposed to degradation in the oral cavity, also included on the gel, is indicated (P). The molecular size marker, also included on the gel (L), was generated by digesting bacteriophage λ DNA with *Hin*dIII.

The biological stability of plasmid DNA in the ovine oral cavity was examined using *in vitro* transformation assays. Plasmid pCRY6 (2·5–5·0 μg) was added to the ovine oral cavity and then purified from saliva samples removed at set time intervals. Kanamycin-resistant transformants, albeit at very low frequencies, were observed up to 8 min of incubation in the oral cavity (Table 2). Indeed, the half-life for transforming activity was considerably shorter *in vivo* (30–114 s) than was previously observed under *in vitro* conditions (approximately 20 min; PS Duggan and PA Chambers, unpublished observations). Indeed, under *in vitro* conditions, plasmid DNA exposed to ovine saliva was capable of transforming *E. coli* cells to antibiotic resistance, even after 24 h, albeit at an extremely low frequency (Duggan *et al.* 2000). The long-term biological activity observed in ovine saliva *in vitro* may be due to a decrease in salivary nuclease activity over time. Although salivary nucleases may contribute to the short-term biological activities observed *in vivo*, most of the DNA that is added to the oral cavity will be lost through copious salivation and rapid swallowing. Thus, a major limiting factor in these experiments is the inefficient recovery of saliva-degraded DNA, suggesting that the true survival time of free DNA in the oral environment may be much higher than that reported here. Nevertheless, the low initial concentrations of plasmid DNA (2·5–5·0 μg) that had been added to the oral cavity was still able to transform competent *E. coli in vitro*, suggesting that DNA released from feed material within the mouth has potential to transform naturally competent oral bacteria.

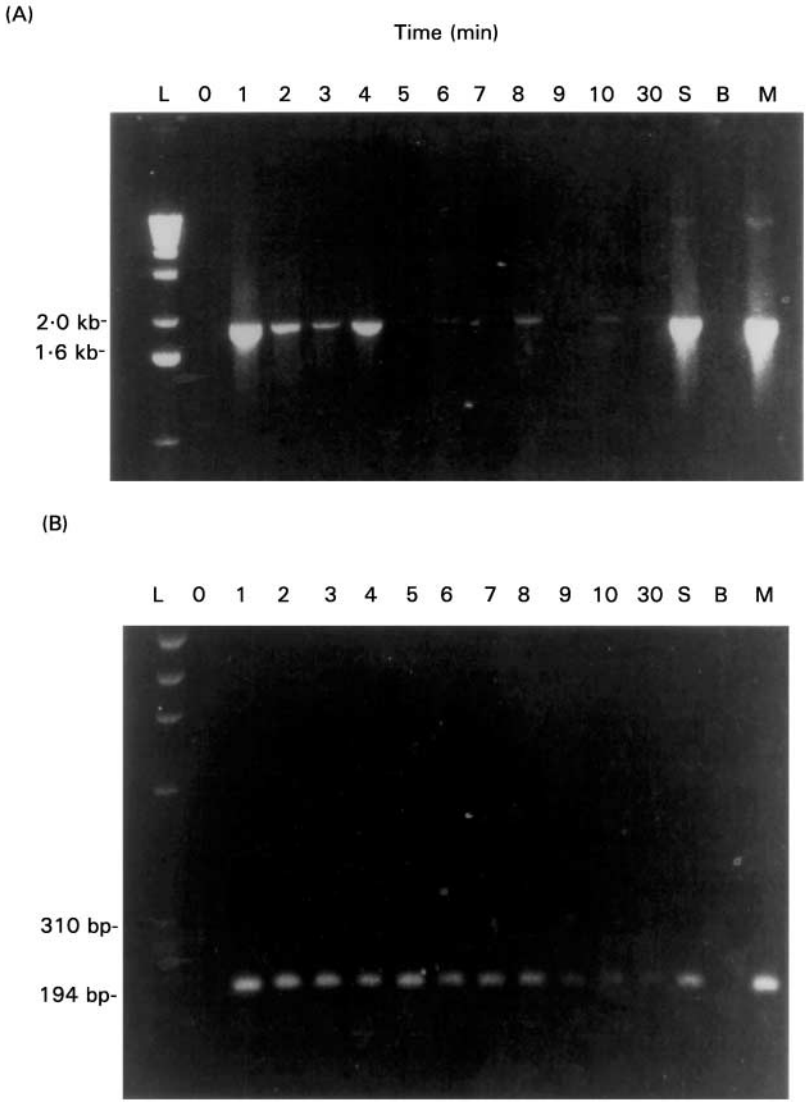


**Fig. 5.** Survival of target sequences from plasmid pCRY6 after incubation in the ovine oral cavity. Plasmid DNA was added to the oral cavity and then extracted from saliva samples removed at the times indicated. Samples (2 μl) of recovered DNA were used for polymerase chain reaction (PCR) analysis. (A), Persistence of a 1914-bp *cry1A*(b) gene fragment; (B), a 214-bp fragment flanked by primers Cry04 and T7 universal primer. The blank, B, contained all the components of the PCR reaction except template DNA. The positive control (M) was performed using 0·2 μg pCRY6 DNA. To test whether saliva extracts contained components inhibitory to PCR (S), PCR amplification of pCRY6 DNA (0·2 μg) was performed in the presence of 2 μl ovine saliva (time zero). The 1-kb DNA ladder (Gibco BRL), 0·075 to 12·2 kb used in (A) is indicated (L). The molecular size marker comprising ϕX174 RF DNA digested to completion with *Hae*III (Gibco BRL) used in (B) is indicated (L).

Acid conditions have been shown to accelerate fragmentation of GM maize DNA (Hupfer *et al.* 1998). Fruit and vegetables are characterised by acidic pH conditions. Addition of plasmid DNA to slices of apple (pH 3·6) before feeding did not significantly increase the rate of DNA degradation. The entire *cry1A*(*b*) gene target could still be amplified after 6 min and detection of the shorter 214-bp, but still plasmid-specific, target was still possible after 30 min (data not shown). Plasmid DNA added to apple before feeding was still capable of yielding kanamycin-resistant transformants after 6 min incubation in the oral cavity, indicating retention of both structural and functional integrity (Table 2). The oral cavity is inhabited by a large range of bacteria, many of which adhere to teeth, forming a complex biofilm community, known as a dental plaque. Biofilm-growth of the oral bacterium *S. mutans* resulted in transformation rates 10- to 600-fold higher than those of their free-living counterparts, suggesting that biofilm environments, such as dental plaque, are conducive to the development of natural genetic competence (Li *et al.* 2001). Little is known about natural transformation and competence development *in vivo* in the oral cavity, although many oral bacteria are known to be naturally transformable *in vitro* (Westergren & Emilson, 1983; Kuramitsu & Trapa, 1984; Lunsford, 1998). Human saliva appears to contain factors that may actually promote competence development in oral bacteria (Mercer *et al.* 1999*a*). Whether ovine saliva contains similar competence-promoting factors remains to be established. It may be worthy to note, however, that natural transformation

**Table 2.** Transformation of competent *Escherichia coli* DH5α with pCRY6 previously exposed to the ovine oral cavity* (Ranges of colony-forming units)

| | No. of transformants (cfu/ml culture) | | |
|---|---|---|---|
| Plasmid pCRY6 added... | 2·5 μg | 5 μg† | 5 μg |
| Time (min) | | | |
| 0 | NG | NG | NG |
| 1 | 60–4250 | 30–230 | 30–140 |
| 2 | 20–620 | 0–40 | 10–60 |
| 3 | 10–70 | 0–10 | 10–40 |
| 4 | 10–270 | 10–20 | 0–10 |
| 5 | 0–10 | 0–10 | NG |
| 6 | NG | 0–10 | NG |
| 7 | NG | NG | NG |
| 8 | 0–10 | NG | 0–10 |
| 9 | NG | NG | NG |
| 10 | NG | NG | NG |

Cfu, Colony-forming units; NG, no growth.

* Plasmid pCRY6 was added to the oral cavity and purified from saliva sampled at the times indicated. Samples (2 μl) of the recovered plasmid were used to transform *E. coli* DH5α *in vitro*. Saliva was also removed before plasmid addition (time zero). The results presented were obtained from three independent experiments, executed on three different days, using the same sheep for each experimental treatment.

† Results observed with plasmid DNA recovered from the saliva of a sheep fed an apple slice containing 5 μg plasmid DNA.

of the rumen bacterium *S. bovis* JB1 was inhibited in the presence of ovine saliva (Mercer *et al.* 1999*b*). The possibility that bacteria in the ovine oral cavity may develop competence, however, should not be ruled out. In summary, free DNA exhibited long-term biological activity *in vivo*, in the ovine oral cavity, lasting 8 min. DNA acquisition, by competent bacteria *in vitro*, is known to occur rapidly, at a rate of 100 nucleotides/s in the case of *Streptococcus pneumoniae* DP1601 (Méjean & Claverys, 1993). If DNA-competent oral bacteria can access DNA at similar rates *in vivo*, in the oral cavity, then the significant biological activity, reported here, would be more than sufficient to permit their transformation.

## Conclusions

Acquisition by bacteria of GM DNA, integrated in the chromosomes of plants, is most likely to occur by homologous recombination. This process depends on the survival of plant DNA fragments bearing significant regions of sequence homology with the DNA of gut bacteria. Although the results presented here are largely qualitative, they do indicate that DNA fragments, larger than the 1600 bp necessary to contain the pUC18 $bla_{TEM}$ gene and its origin of replication, persist for considerable time in maize grains in the rumen. Biologically significant DNA is, however, unlikely to persist in silage material in the rumen, indicating that there is little likelihood of DNA transfer from ensiled maize to bacteria in the ruminant digestive system. A significant finding of the present study is that free DNA survived in a functional state for significant time in the ovine oral cavity, suggesting that DNA released from the diet may transform competent oral bacteria. By contrast, the chances of microbial transformation in the rumen and lower regions of the ovine digestive system are likely to be low due to a high level of nuclease activity. Nevertheless, rare transformation events can be significant if the donor DNA is an antibiotic resistance gene and the recipient is a human or animal pathogen. The use of GM crops harbouring antibiotic resistance genes, in particular the use of unprocessed grains in animal feed, possibly deserves further evaluation.

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