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

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Abstract

To assess the likelihood that the *bla* gene present in a transgenic maize line may transfer from plant material to the microflora associated with animal feeds, we have examined the survival of free DNA in maize silage effluent, ovine rumen fluid and ovine saliva. Plasmid DNA that had previously been exposed to freshly sampled ovine saliva was capable of transforming competent *Escherichia coli* cells to ampicillin resistance even after 24 h, implying that DNA released from the diet could provide a source of transforming DNA in the oral cavity of sheep. Although target DNA sequences could be amplified by polymerase chain reaction from plasmid DNA after a 30-min incubation in silage effluent and rumen contents, only short term biological activity, lasting less than 1 min, was observed in these environments, as shown by transformation to antibiotic resistance. These experiments were performed under in vitro conditions; therefore further studies are needed to elucidate the biological significance of free DNA in the rumen and oral cavities of sheep and in silage effluent. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Horizontal gene transfer; DNA survival; Transgenic plant; Antibiotic resistance; Transformation efficiency

1. Introduction

One of the main concerns regarding the use of bacterial antibiotic resistance genes in transgenic crops is the potential for horizontal gene transfer from plants to other organisms in the environment, most notably to human or animal pathogens. The most likely route by which bacteria may acquire plant DNA is transformation [1–2]. Until recently, attempts to transform naturally competent bacteria with transgenic plant DNA have been generally unsuccessful [3–4]. Gebhard and Smalla [5], however, showed that a kanamycin resistance gene integrated in the DNA of transgenic sugar beet was capable of transforming *Acinetobacter* sp. to kanamycin resistance, demonstrating that gene transfer can occur between different phylogenetic kingdoms.

Insect-resistant maize line CG00526-176 contains three bacterial genes: the lepidopteran-specific *cry1A*(*b*) gene, the *bar* gene conferring tolerance to glufosinate and a *bla* gene encoding TEM-1 β-lactamase [6]. The *bla* gene originates from the cloning vector pUC18 and is not expressed in maize, but has bacterial regulatory sequences that would allow it to become functional were it to be transferred back into bacteria. Southern transfer and hybridisation suggests that there are at least two copies of the *cry1A*(*b*) and *bla* genes integrated into the DNA of maize line CG00526-176 (unpublished data).

Maize is used in both animal and human food products. Heat and other processing of the crop can disrupt and even destroy the DNA present, effectively eliminating the risk of genetic transfer from the processed grain. The processes involved in producing different maize products and by-products used in animal feed vary considerably in their intensity. These result in different levels and fragment sizes of DNA in feed materials. In animals fed on unprocessed maize, DNA will be released during the digestive process. The oral cavity is the first site of contact between DNA released from food, the resident microflora and transient colonisers. Indeed, DNA that has been exposed to degradation by human saliva is able to transform naturally competent *Streptococcus gordonii* [7]. There is also poten-

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tial for gene transfer in the rumen [8] which, unlike the hindgut, is not preceded by an acidic barrier.

Much maize is ensiled for animal consumption and DNA released during the mechanical chopping of the crop and subsequent processing is also potentially available to the microflora found on plants and in the fermentation that produces silage [9]. The aims of this study are to examine the fate of free DNA in ovine saliva and rumen fluid, and maize silage effluent.

2. Materials and methods

2.1. Bacterial strains, maize and sheep

Escherichia coli strain DH5α [10] was grown in Luria–Bertani (LB) medium [11]. Where appropriate, the medium was supplemented with 50 mg l^{-1} ampicillin. *Zea mays* line CG00526-176 was grown under glasshouse conditions with minimum and maximum temperatures of 18 and 30°C, respectively, and a relative humidity of 88–90%. Rumen fluid, obtained via a cannula, and saliva were removed from two healthy adult sheep and clarified by centrifugation at $1000 \times g$ for 5 min.

2.2. Ensilage of maize

The maize crop was harvested at 11 weeks, when the plants were beginning to develop cobs. Plants were chopped mechanically and approximately 2 kg of this material was distributed between sterile plastic bags. Bag silos were evacuated before sealing and stored at ambient temperature. Effluent was recovered by squeezing the liquid from a bag into sterile universal bottles.

2.3. DNA purification and manipulation

Large-scale plasmid preparation was performed using alkaline lysis and CsCl density gradients [11]. Restriction endonuclease digestion with *Bgl*I was carried out according to the manufacturer's instructions (Gibco BRL). Maize chromosomal DNA was isolated from frozen maize leaves using the method previously described [12] and purified using CsCl density gradient centrifugation [11].

2.4. Degradation of plasmid DNA

The survival of plasmid pUC18 added to ovine saliva, rumen fluid and to silage effluent was estimated as described by Mercer et al. [7], with some modifications. Briefly, 200 ng of pUC18 DNA in 10 μl sterile H_2O was added to 30 μl silage effluent, rumen fluid or saliva, and mixed thoroughly. Silage effluent was incubated at ambient temperature, while saliva and rumen fluid were incubated at 39°C. At set times, the DNA was extracted, using Qiagen spin columns (Qiagen, Crawley, West Sussex, UK). The recovered DNA was resuspended in 50 μl sterile H_2O and 20-μl samples were run on 0.8% (w/v) agarose gels; 2-μl samples were used for the transformation of competent *E. coli* DH5α and polymerase chain reaction (PCR) amplification.

2.5. Degradation of chromosomal DNA

Chromosomal DNA (0.4 μg) in 4 μl sterile H_2O was added to 16 μl saliva, rumen fluid or silage effluent. The samples were mixed thoroughly and incubated for set times as described above. DNA was recovered using Qiagen DNeasy Tissue Kits, resuspended in 50 μl sterile water and 2.5-μl samples were used for PCR amplification.

2.6. Heat treatment of rumen fluid and silage effluent

One-ml samples were heated at 100°C for 10 min and were allowed to cool to ambient temperature before plasmid DNA was added.

2.7. PCR

PCR amplification of *bla* gene fragments was carried out using the primer pairs C and E, and C and B, described previously [13]. PCR amplifiers of a 1914-bp target within the *cry1A*(*b*) gene were generated using the primers of Hupfer et al. [14]. A target of 850 bp within the *nad*5 gene was amplified using primers described by Mannerlöf and Tenning [15].

2.8. Transformation of E. coli DH5α with pUC18

The transformation of calcium chloride-treated competent cells with pUC18 was performed as described by Sambrook et al. [11]. Transformation was confirmed by growth on LB ampicillin agar and the recovery of a plasmid with an appropriate restriction endonuclease profile.

2.9. Transformation of E. coli DH5α with pUC18 in the presence of filter-sterilised rumen fluid or silage effluent

The method of Mercer et al. [7] was used to transform calcium chloride-treated competent *E. coli* cells with pUC18 DNA in the presence of filter-sterilised rumen fluid or silage effluent. Competent cells (180 μl) were mixed with an equal volume of filter-sterilised silage effluent or filter-sterilised rumen fluid and pUC18 DNA (in a minimal volume of H_2O) was added to the mixture to give a final concentration in the transformation mixture of 2.5 mg l^{-1}. The transformation mixture was incubated at 37°C, with shaking for 0–300 min. At 30-min intervals, samples of the transformation mixture were removed, diluted appropriately and 100 μl was plated onto LB agar containing 50 mg l^{-1} ampicillin. Transformation was confirmed as described in Section 2.8.

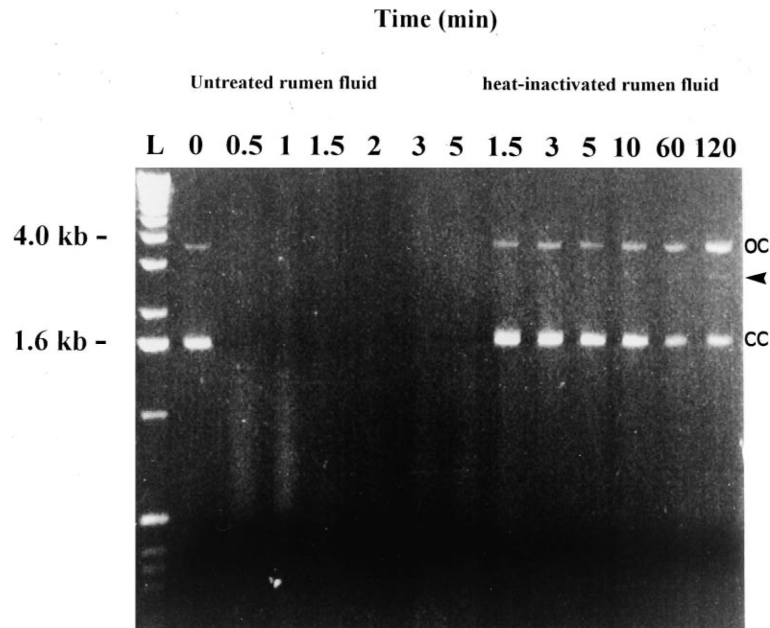

Fig. 1. Survival of pUC18 DNA in ovine rumen fluid and in heat-inactivated ovine rumen fluid for 5 and 120 min, respectively. After incubation for the times indicated, plasmid DNA was extracted immediately and samples were analysed on a 0.8% (w/v) agarose gel. OC, open circular DNA; CC, closed circular DNA. A faint band of linear DNA is visible in samples exposed to heat-treated rumen fluid (arrow head). The 1-kb DNA ladder (Gibco BRL), 0.075–12.2 kb, also included on the gel is indicated (L).

3. Results

3.1. Survival of DNA in ovine saliva, rumen fluid or silage effluent

The survival of pUC18 DNA after incubation in ovine rumen fluid is shown in Fig. 1. Degradation of pUC18 is evident from the rapid disappearance of the bands corresponding to the open circular and covalently closed forms of the plasmid, which are visible at time zero (Fig. 1). Similar experiments were performed with ovine saliva and maize silage effluent (data not shown). Results indicate that DNA, both plasmid and maize chromosomal, is incompletely degraded after at least 1 h after addition to ovine saliva. By contrast, plasmid DNA incubated in silage effluent was undetectable by this approach after 30 s (data not shown). Similarly, the physical integrity of maize chromosomal DNA was rapidly destroyed within 1 min of incubation in rumen fluid or silage effluent (data not shown). In contrast, plasmid DNA added to heat-inactivated rumen fluid (Fig. 1) or heat-inactivated silage effluent (data not shown) was still visible in an incompletely degraded state even after 2 h.

PCR experiments designed to estimate the persistence of target DNA sequences from pUC18 and maize chromosomal DNA following exposure to ovine saliva, rumen fluid or silage effluent indicated that a 350-bp *bla* gene fragment from pUC18 was still amplifiable after a 30-min incubation in rumen fluid (Fig. 2). The same target sequence from maize chromosomal DNA was, however, only amplifiable up to 1 min after addition to rumen fluid. Amplifiable *bla* sequences (350- and 684-bp fragments) were still detectable from pUC18 and maize chromosomal DNA after 30 min exposure to silage effluent. The same sequences from maize chromosomal DNA and pUC18 were still amplifiable after 24 and 2 h incubation in fresh ovine saliva, respectively.

To examine the survival of a second transformed bac-

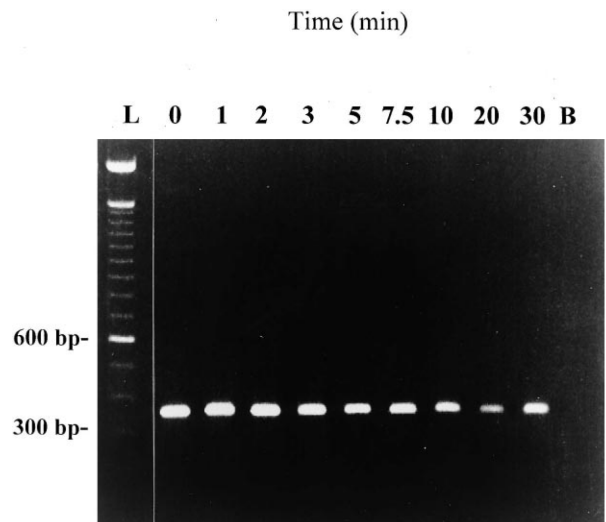

Fig. 2. Survival of a 350-bp *bla* gene fragment of pUC18 DNA after incubation with fresh ovine rumen fluid for 30 min. PCR amplification of degraded DNA was performed as described in Section 2, and products were analysed on a 1.4% (w/v) agarose gel. The 100-bp DNA ladder (Gibco BRL), 100 to 2072 bp, also included on the gel is indicated (L). The blank (B) contained all the components of the PCR reaction except template DNA.

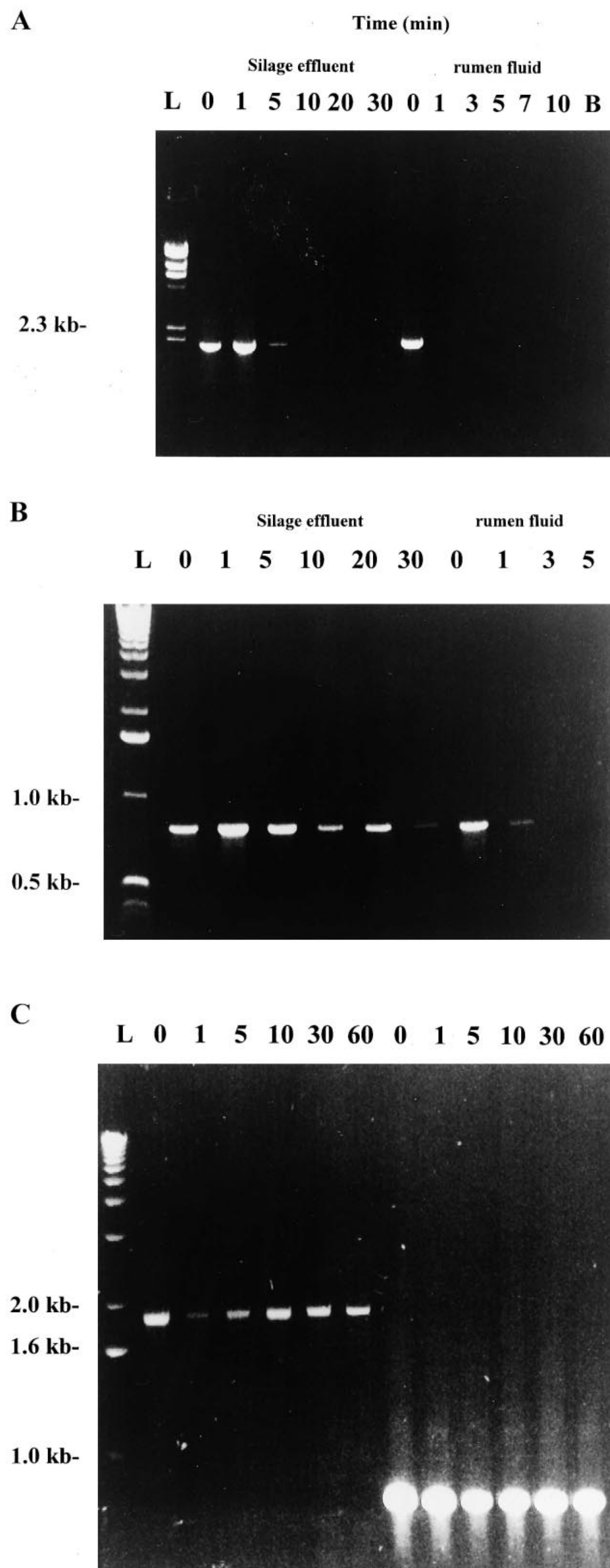

Fig. 3. Survival of target DNA sequences from maize chromosomal DNA after incubation with ovine rumen fluid, maize silage effluent and ovine saliva. (A) Persistence of a 1914-bp *cry1A*(*b*) gene fragment after incubation in silage effluent and rumen fluid for 30 and 10 min, respectively. (B) Persistence of a 850-bp *nad*5 gene fragment after incubation in silage effluent and rumen fluid for 30 and 5 min, respectively. (C) Persistence of a 1914-bp *cry1A*(*b*) gene fragment and a 850-bp *nad*5 gene fragment after incubation in ovine saliva for 60 min. A 1-kb DNA ladder (Gibco BRL), 0.075 to 12.2 kb, was used in (B) and (C). The molecular size marker used in (A) was generated by digesting bacteriophage λ DNA with *Hin*dIII.
←

terial gene in maize, a 1914-bp *cry1A*(*b*) gene fragment could be amplified from maize DNA after a 5-min incubation with silage effluent, but was not amplifiable after a 1-min incubation in rumen fluid (Fig. 3A). As a control, an 850-bp *nad*5 mitochondrial gene target was still amplifiable from maize DNA after a 30-min incubation in silage effluent and after a 1-min incubation in rumen fluid (Fig. 3B). The *nad5* and *cry1A*(*b*) targets were still amplifiable after a 60-min incubation in saliva (Fig. 3C). The survival times for plasmid and chromosomal DNA target sequences in ovine saliva, ovine rumen fluid and maize silage effluent are summarised in Table 1.

3.2. *Effect of saliva, rumen fluid and silage effluent on transformation by plasmid DNA*

The biological stability of plasmid DNA was investigated by incubating plasmid pUC18 for increasing amounts of time in ovine saliva, rumen fluid and silage effluent. After incubation for the desired length of time plasmid DNA was immediately extracted to inhibit further degradation and used to transform competent *E. coli* DH5α. Plasmid pUC18 that had previously been exposed to clarified ovine saliva for 24 h was still capable of transforming competent *E. coli* DH5α to ampicillin resistance, albeit at low efficiency (20 cfu ml^{-1}). This compares with 1.6×10^3 cfu ml^{-1} transformants observed after incubation of pUC18 DNA in sterile water for 24 h. Pre-exposure of plasmid DNA to rumen fluid markedly inhibited transformation of *E. coli*, since the number of transformants obtained after just 30 s of incubation was some five-fold lower than for unexposed DNA. No transformants were obtained after exposure longer than 1 min. Similarly, no transformants were observed after incubations longer than 1 min in silage effluent (Table 2).

Table 1
Persistence of DNA target sequences of plasmid and maize chromosomal DNA after incubation with ovine saliva, ovine rumen fluid or maize silage effluent

Target sequence	Survival time (min)[a]		
	saliva	rumen fluid	silage effluent
bla[b]	120	30	30
bla[c]	1440	1	30
cry1A(*b*)	60	0	5
*nad*5	60	1	30

[a]Survival of DNA sequences was estimated by PCR following incubation of plasmid pUC18 and maize chromosomal DNA with ovine saliva, ovine rumen fluid or maize silage effluent.
[b]Plasmid-borne *bla* gene fragments (350- and 684-bp target sequences).
[c]Maize-encoded *bla* (350- and 684-bp target sequences).

Table 2
Transformation of competent *E. coli* with pUC18 DNA previously exposed to ovine rumen fluid and maize silage effluent

Time (min)	Mean number of transformants[a]	
	rumen fluid	silage effluent
0	$2.87 \pm 1.07 \times 10^4$	$3.8 \pm 1.75 \times 10^4$
0.5	$3.06 \pm 1.35 \times 10^3$	157 ± 18
1	53 ± 7	95 ± 12
2	NG[b]	NG
3	NG	NG
4	NG	NG

[a]Plasmid pUC18 DNA, at an initial concentration of 5 mg l^{-1}, was exposed to silage effluent or rumen fluid for the times indicated and then immediately extracted to inhibit further degradation. Samples (2 μl) of extracted plasmid were used to transform competent *E. coli*. These results were obtained from three independent experiments using silage effluent recovered 24 h after the initial chopping of the crop and from rumen fluid removed from a single sheep.
[b]No growth.

Heat treatment of silage effluent and rumen fluid before the addition of pUC18 DNA did, however, result in the recovery of ampicillin-resistant colonies, even after a 2-h incubation. In these experiments, the maximum number of transformants observed after 2 h with heat-inactivated silage effluent or rumen fluid, was 3.2×10^4 and 2.8×10^4 cfu ml^{-1}, respectively. This compares with 6×10^4 cfu ml^{-1} transformants observed when pUC18 DNA was incubated in an identical volume of sterile, distilled water for 2 h.

3.3. *Transformation of E. coli by plasmid DNA in the presence of rumen fluid and silage effluent*

In these experiments transformation was observed when competent *E. coli* DH5α cells and pUC18 DNA, at a final concentration of 2.5 mg l^{-1}, were simultaneously added to filter-sterilised silage effluent or rumen fluid. There were 4.75×10^3 cfu ml^{-1} transformants recovered after a 4.5-h incubation in filter-sterilised rumen fluid and 11 cfu ml^{-1} transformants were recovered after 3 h in filter-sterilised silage effluent. The number of transformants recovered after incubating competent *E. coli* with pUC18 DNA for 4 h in sterile dH_2O was 9.6×10^3 cfu ml^{-1}. Whole silage effluent and rumen contents gave a background of ampicillin-resistant colonies of approximately 80 and 40 cfu ml^{-1}, respectively, from total bacterial populations of approximately 2×10^4 and 1.9×10^5 cfu ml^{-1}. In the present study, the total bacterial population represents the total number of organisms recovered following an overnight incubation at 37°C on LB agar under aerobic conditions. No ampicillin-resistant colonies were observed when samples of filter-sterilised rumen fluid or silage effluent were plated on LB agar containing 50 mg l^{-1} ampicillin, indicating that ampicillin-resistant colonies must have arisen by transformation of competent *E. coli* included in each experiment.

4. Discussion

The survival of free plasmid and maize chromosomal DNA in ovine saliva, rumen fluid and in silage effluent has been examined using agarose gel electrophoresis and PCR and the survival of biologically active plasmid DNA has been investigated in these environments by transformation assays. Amplifiable *bla* gene target sequences remained detectable for at least 30 min in silage effluent and rumen fluid, while the same *bla* gene regions were still amplifiable using PCR after a 24-h incubation in ovine saliva.

Plasmid DNA that had previously been exposed to degradation by ovine saliva was capable of transforming competent *E. coli* cells, even after 24 h, albeit at a very low frequency. The decreasing transformation frequencies over time most likely result from DNA fragmentation and degradation by nucleases. The sensitivity of DNA to nuclease attack, or other biological factors resulting in inactivation, was nullified following heat treatment of the matrix in which these experiments were performed. Plasmid DNA incubated in heat-inactivated silage effluent or rumen fluid was able to transform competent *E. coli*, even after 2 h of incubation. By contrast, there was a rapid loss of transformation, within 1 min, in unheated rumen fluid or silage effluent, indicating the discrepancy between the detected physical stability of DNA, measured by PCR, and its functional biological activity.

Although *E. coli* is not an inhabitant of the oral cavity, it was chosen as the recipient organism in this study because it is known to be transformable by plasmid pUC18 from whence the *bla* genes in maize line CG00526-176 originate. *E. coli* is a minor constituent of the resident microflora of the rumen, representing some 10^5 ml^{-1} cells in the rumens of healthy adult cattle and sheep [16,17]. The population levels of *E. coli* strains carrying transmissible multiple antibiotic resistances can reach populations of 10^4 ml^{-1} in the rumen of sheep maintained under common dietary conditions [18]. *E. coli* populations are known to increase rapidly under conditions of fasting [19]. Furthermore, *E. coli* can be induced to take up DNA and is known to develop natural competence under certain conditions [20].

A significant finding of this work is that plasmid DNA survives in a biologically functional state in ovine saliva for a considerable time, implying that DNA released from food is a potential source of transformation for naturally competent oral bacteria. In a similar study, Mercer et al. [7] demonstrated that a recombinant plasmid was able to transform a naturally competent oral bacterium after incubation in fresh human saliva for 9 min. There is evidence that human saliva may contain factors that promote competence development in bacteria of the oral flora [7]. Whether ovine saliva contains similar competence-promoting factors remains to be established. It may be worthy to note, however, that incubation with ovine saliva did not

induce natural competence in the rumen bacterium, *Streptococcus bovis* JB1 [22].

Various sized target sequences from maize chromosomal DNA, including a 1914-bp fragment of *cry1A*(*b*), remained detectable after a 30-min incubation in silage effluent and for at least 24 h in ovine saliva. By contrast, in rumen fluid the same target sequences were not amplifiable after just a 1-min incubation. This finding supports the suggestion that DNA is unlikely to survive the activity of nucleases in the rumen [21], suggesting that transformation in that site is likely to be a rare event. The short-term biological activity of plasmid DNA exposed to rumen fluid reported above supports this suggestion. Indeed, transformation of *S. bovis*, a rumen bacterium, is reported to be inhibited following pre-exposure to rumen fluid and saliva [22]. Similarly, plasmid DNA exposed to silage effluent rapidly lost biological activity, suggesting that transformation in this environment may be rare also.

DNA uptake in vitro is known to occur rapidly [23]. It could be argued, therefore, that the persistence of functional plasmid DNA for up to 1 min in rumen fluid or silage effluent may suffice to enable competent bacteria to be transformed. The recovery of ampicillin-resistant transformants after just 30 s of exposure to competent cells in the presence of filter-sterilised rumen or silage effluent raises this possibility. The rumen contains high concentrations of bacteria, some of which are naturally competent [8,22,24] and the resulting cell-to-cell contact enhances the possibility of gene transfer events. Similar arguments apply to the microflora responsible for silage fermentation.

DNA incubated in silage effluent was unable to transform competent *E. coli* cells after 1 min of incubation. The apparent efficiency of transformation, in which ampicillin-resistant transformants were recovered after 4.5 h, probably reflects the survival of transformed cells in that environment rather than survival of biologically active DNA. This assumption is supported by the observation that *E. coli* cells lost viability after incubation for 4 h in silage effluent (data not shown). Similarly, the short-term biological activity of plasmid DNA in rumen fluid contrasts with the transformation yield data, where ampicillin-resistant transformants were obtained after 4 h in the presence of filter-sterilised rumen fluid. Again, this discrepancy is probably attributable to the survival of transformed *E. coli* in that medium rather than the survival of biologically active DNA. Raw, clarified silage effluent or rumen fluid were used in the experiments represented by Table 2. There was, however, no difference between the fate of free DNA in raw clarified silage or rumen fluid compared with that in filter-sterilised rumen or silage effluent (data not shown).

Prerequisites for successful transformation include the availability of free DNA, the development of natural competence and the stable integration of the captured DNA into the genome of the recipient organism. Maize line CG00526-176 contains sequences derived from plasmid pUC18, including the complete *bla* gene. Should this transfer to a recipient bacterium, it has the potential to confer resistance to ampicillin.

Thus, we conclude that free DNA can survive in a functional state for a significant time in ovine saliva and survives for much shorter periods in rumen fluid and silage effluent. The persistence of DNA in these environments, particularly in saliva, suggests the possibility of natural transformation to antibiotic resistance of bacteria within the microflora associated with transgenic plants that contain antibiotic resistance marker genes. In view of the continued use of transgenic plants with such genes, this possibility warrants further investigation in vivo.

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