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Unsuccessful search for DNA transfer from transgenic plants to bacteria in the intestine of the tobacco horn worm, *Manduca sexta*

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Abstract

DNA transfer from transgenic plants to native intestinal bacteria and introduced *Acinetobacter* BD413 was assessed in the gut of the tobacco horn worm (*Manduca sexta*). The marker was kanamycin resistance gene (*nptII*), and tobacco carrying the *nptII* gene in the chloroplasts served as the donor. We detected neither whole gene transfer to native bacteria, nor transfer of fragments of *nptII* to *Acinetobacter*, using a marker exchange assay. This negative result was attributed to a heat-labile activity that degraded DNA in the feces, probably DNAase. Nevertheless, a few intact leaf cells survived transit through the gut, and DNA extracted from feces did transform *Acinetobacter*, albeit at lower frequencies than DNA extracted from leaves.

Introduction

The inclusion of genetically modified plants in animal feed and human food has caused multiple concerns, including the possibility that plant transgenes might be transferred to intestinal microflora by natural transformation. Natural transformation occurs widely in bacteria. It can be defined as a DNAase-sensitive process that allows bacteria to assimilate naked DNA and biologically exploit its information. More than 40 bacterial species from different environments are naturally transformable (Lorenz & Wackernagel, 1994; Nielsen et al., 1998). Most studies describing natural transformation were conducted *in vitro* (Lorenz & Wackernagel, 1994; Paget & Simonet, 1994; Bertolla et al., 1997; Nielsen et al., 1997a, b; Bertolla & Simonet, 1999; Dubnau, 1999), under laboratory conditions well removed from natural environments, like the digestive tract examined here.

DNA transfer to gut bacteria would require that DNA be released from plant cells and remain undegraded long enough for transformation of receptive bacteria to occur. Plasmid DNA resisted exposure to human saliva and remained available for transformation in the oral cavity (Mercer et al., 2001). Plasmid DNA exposed to ovine saliva transformed competent bacteria, but incubation in silage effluent and rumen contents destroyed biological activity in less than one minute (Duggan et al., 2000). An antibiotic resistance marker in maize survived in the stomach of chickens, but not in their lower intestine (Chambers et al., 2002). On the other hand, DNA from transgenic leaves fed to mice was recovered in the gut, and it passed into various organs (Hohlweg & Doerfler, 2001). But what about insects?

Ideally, DNA transfer to bacteria would be assessed directly in the gut. We used the larvae of *Manduca sexta* (tobacco horn worm), attempting

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to detect DNA transfer both to resident bacteria and to introduced *Acinetobacter* BD413(pMR7), which takes up foreign DNA and uses it to repair a deletion in the *nptII* (carried by pMR7), thus restoring resistance to kanamycin (de Vries & Wackernagel, 1998). This marker exchange assay for DNA transfer does not rely on intact genes, allowing detection of partially degraded sequences. *Manduca sexta* was chosen because the larvae are large and voracious, producing copious feces. We examined microbial populations in these feces for indicators of gene transfer events in the insect gut. The evolution of these populations could thus be followed without sacrificing the animals. Feces were of particular interest, because they have the potential of transferring transformed gut bacteria to populations of soil microbes.

The donor plant, *Nicotiana tabacum* (tobacco), is a common host for *M. sexta*, and the line used here carries a functional *nptII* in its chloroplasts, providing copy numbers as high as 5,000 per leaf cell (Staub & Maliga, 1993; Daniell et al., 1998). Furthermore, chloroplasts and contemporary, free-living bacteria use similar gene expression mechanisms; thus, a chloroplast marker, such as kanamycin resistance, has a high probability of being expressed in bacteria.

Materials and methods

Bacterial strain, plasmids, plants and larvae

Acinetobacter sp. strain BD413(pMR7) (de Vries & Wackernagel, 1998) is sensitive to kanamycin, but easily transformable to kanamycin resistance by *nptII*. It is naturally competent for transformation and carries a pRS1010 derivative, pMR7, which includes a *nptII* with a 10 bp deletion at the *Nco*I site, rendering the bacterium sensitive to kanamycin. Recombination with an intact *nptII* causes repair and activation of the gene. Plasmid pKT210 (Lorenz et al., 1992) is the pRS1010 derivative that served as a precursor to pMR7. Tobacco line pTNH32-70-2 (variety Petit Havana) (Carrer et al., 1993) carries a functional *nptII* in its chloroplasts. *Manduca sexta* eggs were provided by Jonathan Bohbot at the University of South Carolina.

Feeding experiments

Plants were grown in pots in the greenhouse at 25 °C under 18 h of light and 6 h of darkness. Five eggs were placed on each plant. Experiments were performed in triplicate. Containment was assured by growing plants in perforated plastic bags. Feces were collected every day from the beginning of the third through the end of the fifth instar. They were analyzed for bacterial content or stored at 4 °C.

Extraction and cultivation of feces bacteria

Fresh feces (5 g) were suspended in 45 ml sterile saline (0.85% w/v NaCl). After dispersion with a sterile spatula, the suspension was filtered (Miracloth, Calbioch) and centrifuged to obtain a crude preparation of bacteria. The pellets were washed three times with 45 ml 0.85% (w/v) NaCl and resuspended in 500 µl LB.

The suspension of feces bacteria was plated through a 10-fold dilution series onto LB plates containing 100 µg ml^{-1} cycloheximide (to inhibit fungal growth), sometimes with kanamycin (25 µg ml^{-1}), chloramphenicol (50 µg ml^{-1}) or streptomycin (750 µg ml^{-1}). The plates were incubated for 2 days at 28 °C before determining the number of colony-forming units (cfu).

Transformation of bacteria extracted from feces

The bacterial pellet was suspended in 5 ml LB containing $CaCl_2$ (0.25 mM) and $MgCl_2$ (0.25 mM) and 5 µg of plasmid pKT210 (Scholz et al., 1989; Lorenz et al., 1992) was added. Incubation followed at 30 °C for 1.5 h with agitation. Bacteria were concentrated by centrifugation; diluted aliquots were plated on selective medium (50 µg ml^{-1} chloramphenicol and 750 µg ml^{-1} streptomycin) and incubated for 48 h at 30 °C. For this experiment, only feces of third instar larvae were used.

DNA extraction

The bacterial pellet obtained from feces was incubated with 5 ml lysozyme (10 mg ml^{-1}) for 1 h at 37 °C, followed by the addition of 2 ml SDS (10% w/v) and incubated for 30 min at 65 °C. For DNA extraction, ammonium acetate (2.5 M

final concentration) was added, followed by centrifugation at 12,000 × *g* for 30 min at 4 °C. DNA in the supernatant was precipitated with isopropanol and resuspended in 100 µl TE buffer. DNA extraction from feces or from transgenic tobacco leaves was carried out using a standard method (Dellaporta et al., 1983).

Bacterial characterization by PCR

Colonies with various morphologies were resuspended in 100 µl of sterile NaCl (50 mM) and treated for 10 min at 95 °C. The PCR reaction mixture consisted of 34 µl of sterile water, 5 µl of 10 × PCR buffer (Invitrogen), 2.5 µl of 50 mM $MgCl_2$, 2 µl of 10 mM dNTP mix and 2 µl of 10 µM primers. To this was added 0.25 µl of *Taq* DNA polymerase (Invitrogen) and 2 µl of the heat-treated cell suspension. Amplification was carried out for 35 cycles, consisting of a denaturation step at 94 °C for 1 min, an annealing step at 55 °C for 1 min and an elongation step at 72 °C for 2 min.

To study the diversity of feces bacteria, PCR was conducted with the following primers, complimentary to part of the 16S ribosomal DNA gene: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-AAGGAGGTGATCCAGCGCCA-3′. The 1600 bp fragment obtained was then digested with *Hae*III (New England Biolabs), and the resulting patterns were compared. To determine whether the kanamycin resistance phenotype was encoded by *nptII*, PCR was conducted with the following primers: 5′-GAACAAGATGGATTGC ACGC-3′ (forward) and 5′-AGAAGGCGATAG AAGGCGATGC-3′ (reverse), amplifying a 773 bp fragment from the *nptII* gene.

Feeding experiments using bacteria

Naturally competent *Acinetobacter* sp. BD413 (pMR7) was prepared as described (De Vries & Wackernagel, 1998). Five milliliter of an overnight preculture (30 °C) in LB medium containing chloramphenicol (50 µg ml^{-1}) were used to inoculate a 200 ml culture in the same medium. After approximately 6 h of growth (OD_{650} 0.6), bacteria were concentrated by centrifugation and resuspended in 20 ml of LB containing $CaCl_2$ (0.25 mM) and $MgCl_2$ (0.25 mM).

Tobacco plants (6 leaf stage) were sprayed with 20 ml of a stationary phase culture of *Acinetobacter* BD413(pMR7) and air dried for 1 h, coating the adaxial leaf surfaces with bacteria. Plants were then inoculated with four larvae (second instar) that had been raised only on wild type tobacco plants. Fresh feces (third instar) were collected every day following day 1. Bacteria were extracted from 5 g of feces, resuspended in 1 ml LB and plated in a 10-fold dilution series onto LB plates, containing cycloheximide (100 µg ml^{-1}) and chloramphenicol (50 µg ml^{-1}) with or without kanamycin (25 µg ml^{-1}). Plates were incubated for 2 days at 30 °C. BD413(pMR7) was identified on chloramphenicol selective plates by RFLP analysis of 16S ribosomal DNA, and *nptII* was detected by PCR (see above).

Transformation of BD413(pMR7)

A culture of *Acinetobacter* BD413(pMR7) (100 ml OD_{650} 0.6) was concentrated by centrifugation and resuspended in 10 ml of LB. Aliquots of 500 µl, containing approximately 10^9 bacteria, were added to 25 ml LB containing $CaCl_2$ (0.25 mM) and $MgCl_2$ (0.25 mM). Incubation followed at 30 °C for 1.5 h with agitation (100 rpm) in the presence of transforming DNA. When feces or ground leaves were used (see below), the suspension was first filtered (Miracloth, Calbioch). Bacteria were concentrated by centrifugation and diluted aliquots were plated on selective medium (25 µg ml^{-1} kanamycin, 25 µgl ml^{-1} chloramphenicol) and incubated for 48 h at 30 °C. Repair of *nptII* was confirmed by PCR and *Nco*I digestion. Sequencing of the PCR product (using the reverse *nptII* primer) was used to corroborate the restoration of the *Nco*I site deleted in pMR7.

In experiments to test for DNA transfer from feces to BD413(pMR7), 2 g of feces were dispersed by grinding with a mortar and pestle in liquid nitrogen, allowed to warm to ambient temperature and added to the bacterial suspension, then the above transformation protocol was followed. The effect of feces on the transformation of BD413(pMR7) was examined by mixing 5 µg DNA extracted from transgenic tobacco and 2 g of feces that had been dispersed in 25 ml LB containing $CaCl_2$ (0.25 mM) and $MgCl_2$ (0.25 mM) with a sterile spatula.

Stability of plant DNA incubated with the feces filtrates

Fecal pellets (1 g) were suspended in 2 ml of LB medium. After centrifugation the suspension was filtered (0.2 μm), and 100 ng of transgenic tobacco DNA was mixed with 15 μl of filtrate and incubated for 1, 2 or 5 min at room temperature. The incubation was stopped by heating the samples in a boiling water bath for 10 min. Aliquots were analyzed on agarose gels or diluted 1:10, from which 1 μl served as the template in a PCR amplification of *nptII*. Controls consisted of heating fecal filtrates or exposing DNA samples to ground tobacco leaves (1 g) instead of filtrate.

Heat treatment of fecal suspensions

The samples were heated in a boiling water bath for 10 min and allowed to cool to ambient temperature before adding DNA with or without *Acinetobacter*.

Results

Antibiotic resistance in fecal bacteria from larvae grown on control and transgenic tobacco plants

To determine whether ingestion by *M. sexta* of leaves containing *nptII* could lead to the transfer of *nptII* from chloroplasts to aerobic gut bacteria, eggs were placed on the surface of leaves of wild type and transgenic tobacco plants. Fresh fecal samples from the third through the fifth instar were tested for kanamycin resistant aerobic bacteria (Table 1).

Culturable bacteria numbered about 10^8 cfu g^{-1} feces during the third instar. No kanamycin, chloramphenicol or streptomycin resistant bacteria were detected until the end of the fourth instar, when kanamycin resistance appeared progressively and reached a maximum in the fifth instar. The timing of appearance and numbers of resistant bacteria were similar in feces from larvae that had consumed control and transgenic tobacco plants. About a 100 colonies resistant to kanamycin were restreaked, and isolates were tested for *nptII* by PCR. In no case was the expected *nptII* fragment at 773 bp obtained. We concluded that DNA transfer experiments using *nptII* are only meaningful before the fourth instar, and that ingestion of leaves containing high copy numbers of *nptII* under the control of a chloroplast promoter during the third instar does not confer kanamycin resistance on gut bacteria.

Qualitative characterization of the bacterial population in fecal pellets

To assess the diversity of fecal bacteria, purified colonies from third instar feces were classified according to their 16S rRNA genes by PCR-RFLP. We distinguished only two 16S rRNA patterns, corresponding to two colony morphologies (Figure 1(a)). However, kanamycin resistant bacteria appeared during the fourth and fifth instars, and they produced three PCR-RFLP patterns (Figure 1(b)). When kanamycin resistant bacteria reached the maximum level (approximately 10^4 cfu g^{-1}) in the fifth instar, only one pattern remained (Figure 1(b), lane 5), and it had the same 16S RNA profile as one of the kanamycin sensitive isolates from feces from the third instar (Figure 1(a), lanes 3–6). These fifth instar

Table 1. Microbial colony-forming-units (cfu) in feces (means of three replicates ± standard deviation)

	cfu g^{-1}[a]		cfu g^{-1} (Kan^R)[b]		cfu g^{-1} (Sm^R, Cm^R)[c]	
	Normal	Transgenic	Normal	Transgenic	Normal	Transgenic
Instar 3	$2.0 (\pm 1.25) \times 10^8$	$1.44 (\pm 1.05) \times 10^8$	0	0	0	0
Instar 4–5	$5.5 (\pm 1.7) \times 10^{11}$	$5.6 (\pm 0.8) \times 10^{11}$	10^2–10^4[d]	10^2–10^4[d]	ND[e]	ND[e]

[a]Determined with LB agar.
[b]Determined with LB agar with kanamycin.
[c]Determined with LB agar with chloramphenicol and streptomycin.
[d]Variable number. The values show the minimum and the maximum.
[e]Small colonies observed but not counted.

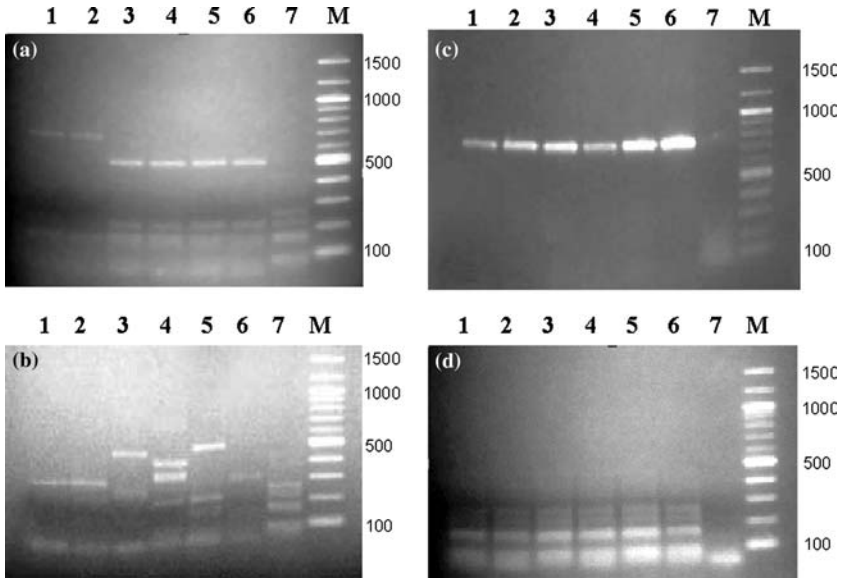

Figure 1. Characterization of gut bacteria using RFLP of 16S RNA sequences and PCR. (a) RFLP study of 16S RNA sequences from third instar gut bacteria. Lanes 1–6, gut bacteria. Lane 7, *Acinetobacter* BD413(pMR7). Lane M, 100 bp DNA ladder. (b) RFLP study of 16S RNA sequences from bacteria fourth and fifth instar bacteria, isolated on kanamycin. Lanes 1–6, gut bacteria. Lane 7, *Acinetobacter* strain BD413(pMR7). Lane M, 100 bp DNA ladder. (c) PCR detection of *nptII*. Lanes 1–5, bacteria isolated on chloramphenicol and streptomycin. Lane 6, *Acinetobacter* BD413(pMR7). Lane 7, negative control. Lane M, 100 bp DNA ladder. (d) RFLP study of 16S RNA sequences from bacteria isolated on kanamycin. Lanes 1–5, bacteria isolated on chloramphenicol and streptomycin. Lane 6, *Acinetobacter* BD413(pMR7). Lane 7, negative control. Lane M, 100 bp DNA ladder.

bacteria probably acquired resistance to kanamycin by conjugation with one of those that appeared during the fourth instar. Conjugation has been described in the intestines of various organisms (Hoffman et al., 1998; Thomas et al., 2000, 2001).

In an effort to detect natural transformation in bacteria from fecal pellets of third instar larvae (before the appearance of spontaneous antibiotic resistances), the protocol for transformation of *Acinetobacter* BD413 was used with a broad host range plasmid, pKT210 (Scholz et al., 1989; Lorenz et al., 1992). No transformants were obtained (data not shown).

In order to test for *nptII* in gut bacteria, independently from their culturability, we used PCR to search for *nptII* in bacteria isolated from feces from larvae (of varying stages) that had consumed transgenic tobacco. No *nptII* sequences were detected (data not shown).

Attempts to detect DNA transfer to Acinetobacter in the larval gut

Acinetobacter BD413 assimilates and integrates transgenic DNA from disrupted leaves (Gebhard & Smalla, 1998; Tepfer et al., 2003), and it is transformable in groundwater, river water, and in soil (Chamier et al., 1993; Williams et al., 1996; Nielsen et al., 1997, 2000; Tepfer et al., 2003). To determine whether DNA transfer to *Acinetobacter* BD413 is possible in the gut of *M. sexta*, third instar larvae (before the appearance of chloramphenicol and streptomycin resistance) were placed on either control or transformed tobacco plants onto which *Acinetobacter* BD413(pMR7) had been sprayed.

Survival of *Acinetobacter* in the gut was first assessed by counting the number of chloramphenicol and streptomycin resistant bacteria in the feces. *Acinetobacter* BD413(pMR7) was identified by PCR detection of *nptII* in pMR7 (Figure 1(c)) and by RFLP-PCR (Figure 1(d)). After one day of feeding on sprayed leaves, larvae produced fecal pellets containing BD413(pMR7) at about 10^4 cfu g^{-1}. This number remained stable for the time that the larvae ate tobacco leaves. However, BD413(pMR7) was not detected in fecal pellets 3 days after feeding on sprayed leaves was stopped. Kanamycin resistance was not observed in BD413(pMR7), in spite of survival of the

bacteria in the gut; thus DNA transfer was not detected.

Fate of DNA in ingested leaves

Microscopic observation of a suspension of feces revealed that fecal pellets were composed of leaf fragments, and that some of these contained intact cells. Although we did not attempt to quantify the total number of intact cells, the number of intact cells per fragment varied from zero to a maximum, roughly estimated to be 40% (Figure 2). The persistence of plant *nptII* in feces was examined using rescue of kanamycin resistance in BD413(pMR7) as a bioassay. Heat treatment was used to kill endogenous bacteria. Transformation of BD413(pMR7) was not observed using feces (Table 2), but successful transformation occurred using DNA extracted from feces. The fecal DNA transformation frequency represents only 2.5% of the leaf DNA transformation frequency (Table 2). This result was confirmed by amplifying *nptII* after serial dilution of DNA from leaves and feces. The lower detection limit for *nptII* was approximately 100 times higher in feces than in the leaves (Figure 3(a)). We can conclude that at best only a few percent of the ingested *nptII* sequences survive transit through the intestine.

Effect of feces on transformation

The suitability of feces as a medium for transformation was examined using rescue of kanamycin resistance in BD413(pMR7). The presence of feces totally inhibited transformation. Heat treatment of feces (100 °C for 10 min) resulted in the restoration of transformation at 24% (Table 3). To determine whether inhibition of transformation resulted from the degradation of *nptII* by DNAase, filtrates with or without heat treatment were mixed with DNA extracted from transgenic tobacco and incubated at room temperature. Gel electrophoresis showed that DNA was completely degraded 1 min after addition of fecal filtrates. Heat treatment inactivated this DNA degradation

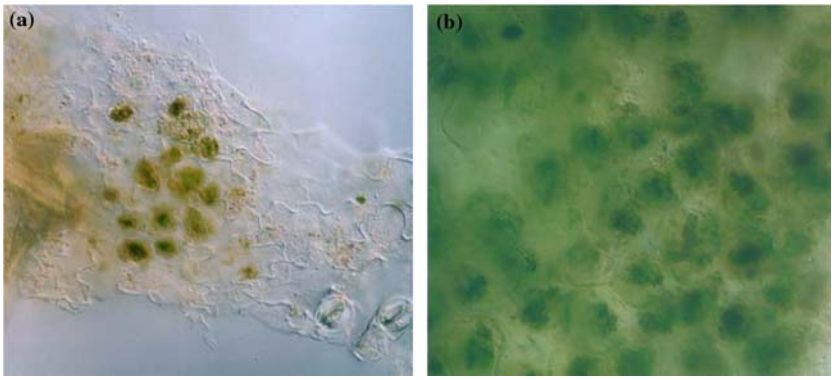

Figure 2. Photomicrograph of leaf fragments. (a) From feces and (b) From crushed leaves.

Table 2. Transformation of *Acinetobacter* BD413(pMR7) with feces and DNA extracted from leaves or feces. No transformation was observed with wild type tobacco

Crushed fecal pellets		DNA[a]	
No heat treatment	Heat treatment	Leaves	Feces
0	0	$5.3\ (\pm 1.04) \times 10^{-8}$[b,c]	$1.3\ (\pm 0.76) \times 10^{-9}$[c]

[a]DNA was extracted from 1 g of leaves and 1 g of feces. The amount of DNA used in the transformation was equal to that extracted from 20 mg of leaves or feces.
[b]Transformation frequency is given as the mean ($n = 3$) $\pm$ the standard deviation, and defined as the ratio between the kanamycin-resistant transformants and the number of *Acinetobacter* BD413(pMR7) colony-forming units.
[c]Significant difference ($P > 0.001$).

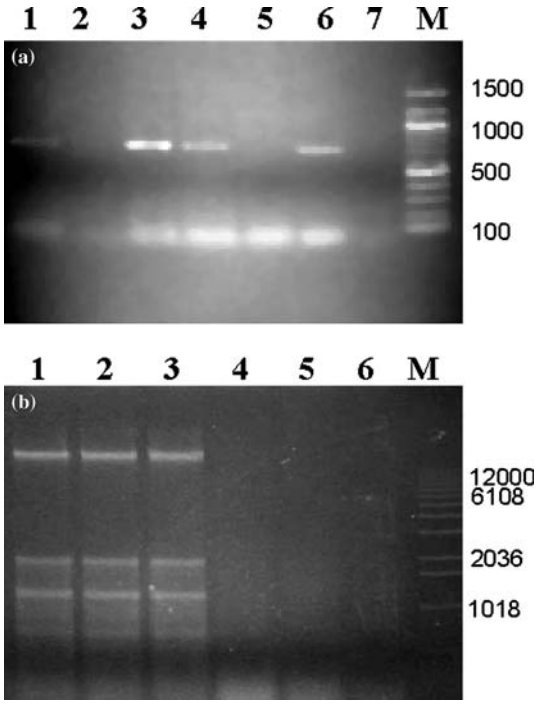

Figure 3. (a) PCR detection of *nptII*. Lane 1, undiluted DNA from feces. Lane 2, diluted 1:10. Lanes 3–5, undiluted DNA from leaves, diluted 1:10 and diluted 1:100. Lane 6, *Acinetobacter* BD413(pMR7). Lane 7, negative control. Lane M, 100 bp DNA ladder. (b) Survival of DNA extracted from transgenic tobacco in fecal filtrates. Lane 1, control. Lane 2, DNA exposed to heat-treated fecal filtrates for 5 min. Lane 3, DNA exposed to crushed leaf filtrates for 5 min. Lanes 4, 5 and 6, DNA exposed to fecal filtrates for 1, 2 and 5 min. Lane M, 1 kb DNA ladder.

(Figure 3(b)). The loss of *nptII* in the plant DNA after 1 min of exposure to the fecal filtrate was confirmed by PCR (data not shown).

Discussion

Some wild bacteria are naturally competent for transformation. The feces of *M. sexta* contain partially digested leaf fragments, suggesting that gut bacteria could assimilate plant DNA along with other nutrients liberated during transit. We were, nevertheless, unable to detect whole gene transfer to resident bacteria; nor could we demonstrate rescue of a deletion in *nptII* by homologous recombination in the naturally competent *Acinetobacter*, which had been introduced in high numbers into the gut flora with ingested leaves. These experiments were performed with larvae from the third instar, because kanamycin resistance spontaneously appeared during the fourth instar.

There are numerous ways to explain this negative result. (1) Transformation might occur in intestinal bacteria that did not grow under the culture conditions used. (2) Antibiotic stress was not applied in the gut, but only on synthetic media. (3) Conditions for gene or DNA transfer might be propitious at other stages in the development of the insect: e.g. later when leaf fragment sizes are larger. (4) Because *Acinetobacter* is naturally competent for transformation only when growing, the ingested bacteria might have only survived transit through the gut, without growing sufficiently to be competent for transformation and (5) Conditions in the gut and feces could inhibit DNA transfer.

Of these explanations, the fourth was easiest to test. When DNA extracted from transgenic plants was mixed with feces it was rapidly degraded. On the contrary, DNA extracted from feces did produce transformants, a result explained by the presence of intact plant cells in the leaf fragments that compose the bulk of the fecal material. Heat treatment of feces partially restored the ability of added DNA to transform *Acinetobacter*.

We conclude that the lack of DNA transfer is due to a heat-labile activity that degrades plant DNA as soon as it is liberated from the leaf frag-

Table 3. Effect of feces on transformation frequencies of *Acinetobacter* BD413(pMR7) with 5 μg of DNA extracted from transgenic tobacco

Control	1 g suspended feces[a]	1 g heat-treated feces[a]
$9.3\ (\pm 0.91) \times 10^{-8}$ [b,c]	0	$2.2\ (\pm 0.43) \times 10^{-8}$[b]

[a]Fecal pellets were suspended in LB medium, heat-treated (10 min at 100 °C) and allowed to cool to ambient temperature before bacteria and DNA were added.
[b]Significant difference ($P > 0.001$).
[c]Means ($n = 3$) ± standard deviation.

ments. DNAase activity is known in various intestines (Liven, 1976, 1977; Liven & Staveland, 1976; Maturin & Curtiss, 1977). The intestine of *M. sexta* is, thus, not a suitable environment for DNA transfer to *Acinetobacter*. High DNase activity would likely keep other bacteria from absorbing and using intact DNA sequences as sources of information. Although acquisition of DNA via the vertebrate gut was reported, it would seem unlikely in *M. sexta,* given the DNA degrading activity we have observed.

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