TITLE

G01011 Dissemination of GM DNA and antibiotic resistance genes via rumen microorganisms.

Rowett Research Institute. Prof Harry Flint , Dr Karen Scott

Non-technical (Laypersons) Executive Summary

Background

The potential widespread use of genetically modified (GM) crops has led to concerns over the possible spread of transgenes and antibiotic resistance marker genes into gut microorganisms. One concern is the presence of a bacterial ampicillin resistance gene in transgenic maize and its potential for transfer. There is currently very little information on the potential for gene exchange between, or gene acquisition by, rumen microorganisms, and the possible consequences of such events. Ruminants consume vast quantities of unprocessed plant material which is only minimally digested before entering the rumen and encountering the large and complex rumen microbial community. The absence of passage through an acidic stomach before material reaches the main site of microbial activity, which is the case with hindgut animals such as pigs and humans, increases the possibility of gene transfer events.

Rationale and Objectives

In this proposal we addressed this problem from three angles.

1. **What is the potential for uptake of free DNA by rumen bacteria?** This depends on :-

a) how long free DNA can survive undegraded in the rumen.

b) whether any rumen bacteria are naturally transformable, i.e. able to take up and express foreign DNA.

2. **Are rumen bacteria resistant to ampicillin?** The potential impact of acquisition of the ampicillin resistance gene from GM maize by rumen bacteria is less important if rumen bacteria :-

a) Are already widely ampicillin resistant. Antibiotics have been used extensively as therapeutic agents and growth promoters in animal husbandry since their discovery more than 50 years ago. This has resulted in strong selective pressure on bacteria to develop or acquire resistance genes to survive, and has been a driving force in the development and spread of bacterial antibiotic resistance.

b) contain the same type of ampicillin resistance gene as the transgenic maize.

3. **How extensive is gene transfer between bacteria from different gut habitats?** The potential impact of the release and acquisition of novel genes, including antibiotic resistance genes, depends on the possibility for widespread gene transfer between rumen bacteria and bacteria from additional gut habitats e.g. pig/human hindgut. The closer the relationship between genes from diverse bacterial species from diverse habitats, the greater the probability of recent, widespread gene transfer events.

Key Results

1) The acquisition of transgenes by the bacterial uptake of free DNA is only possible if free DNA is not degraded immediately when it enters the rumen. A quantitative PCR approach, which had previously been validated in our laboratory, was used to investigate the survival of specific plasmid DNA sequences in rumen fluid. Although

50% of the DNA was degraded within a minute in sterile rumen fluid, DNA was still detectable after 5 minutes. Even in whole rumen fluid, containing the live microbial components (bacteria, protozoa and fungi), 50% of the DNA was detectable after one minute, although in this case no DNA remained after 5 minutes.

Although the survival time of the DNA was short, we had shown in previous work that an oral bacterium was able to take up and express free (exogenous) DNA within 1 minute. Thus even limited survival is potentially significant if rumen bacteria can take up exogenous DNA.

In laboratory experiments the rumen bacterium, *Streptococcus bovis*, proved to be "naturally transformable" (able to stably acquire foreign DNA without any special treatment) at high frequencies. Both acquisition of self-replicating plasmids and integration of free DNA into the bacterial chromosome were demonstrated, the latter depending entirely on the presence of similar sequences in the incoming DNA and the bacterial chromosome. However when these experiments were attempted in the presence of ovine saliva or rumen fluid, transformation was inhibited unless the rumen fluid was first diluted 10 fold with water.

Rumen *Escherichia coli* isolates were also assessed for their ability to incorporate and express foreign DNA. Strains of *E. coli* can be responsible for human and animal diseases. Again transformation efficiencies were high under optimised laboratory conditions, and were even possible in water supplemented with low concentrations of calcium ions that would typically be found in the environment. Again the presence of rumen fluid inhibited transformation.

These results indicated that plant DNA could survive briefly in rumen fluid, and that some rumen bacteria were capable of incorporating and expressing foreign DNA. However, for the two bacterial species studied, a component present in rumen fluid inhibited genetic transformation, implying that transformation was unlikely to happen frequently, if at all, *in vivo*. This finding might not apply to all rumen species and, without further investigation, the possibility of rare, *in vivo,* transformation events cannot be excluded.

2) A high proportion of rumen and pig *E. coli* isolated over a 10 year period proved to be ampicillin resistant (Amp^R). DNA sequencing of the genes indicated that the most common Amp^R gene was TEM-1, the same gene found in transgenic maize. Two isolates (one from the rumen and one from the pig) contained a slightly different gene, with a single amino acid difference that is predicted to change the enzyme activity considerably. The Amp^R genes were encoded both on plasmids and chromosomally. Plasmids were transferable between some of the rumen *E. coli* strains at high frequencies under simulated *in vivo* rumen conditions. The TEM-1 gene was also present in some rumen *Pseudomonas* isolates.

3) Tracking the incidence of similar genes in diverse bacteria from different hosts is an important way to elucidate pathways of gene flow. We have shown that identical *tet*(W) tetracycline resistance genes are present in oxygen sensitive anaerobic gut bacteria of different genera from Britain (rumen and human hindgut), Australia (rumen) and Japan (pig hindgut). This illustrates that there is little to prevent gene flow between the microbial gut communities of different mammalian hosts. The strong sequence conservation between these genes identified in diverse bacteria implies that gene transfer events have been both recent, since the gene sequences have not yet

been changed to suit the new bacterial hosts, and frequent since a large number of diverse bacteria contain the genes. The *tet*(W) gene is located on unrelated mobile elements in three of the bacterial species. Further investigation of the transfer mechanisms involved is the subject of a recent, successful BBSRC/SEERAD grant application.

A second novel Tc^R gene, *tet*(32), only 76% identical to its closest described relative, *tet*(O), was also discovered on a human anaerobic bacterium during this work. This gene was also found on a mobile chromosomal element. *tet*(32) can be transferred between rumen and human anaerobes in laboratory transfer experiments, directly illustrating gene transfer between bacteria originating from different habitats. *tet*(32) is also present in the microflora of the pig hindgut and rumen, although the bacterial hosts have not been identified.

What the research results mean and why they are important

DNA survives briefly in rumen fluid, and at least one species of rumen bacterium is capable of incorporating foreign DNA. This means that the possibility of rare acquisition of transgenes from ingested GM plant material by rumen bacteria cannot be entirely ruled out. However, the observation that rumen fluid inhibits the transformation process in the rumen bacteria studied here means that they at least are unlikely to acquire DNA under *in vivo* rumen conditions.

Since the same ampicillin resistance genes found in transgenic maize also occur naturally in rumen and pig hindgut *E. coli*, even successful acquisition of the maize gene by rumen *E. coli* would not equate to the introduction of a new gene into the rumen bacterial gene pool. Indeed the mobility of some of the *E. coli* Amp^R plasmids indicates that widespread dissemination of the "naturally occurring" ampicillin resistance gene occurs. Novel tetracycline resistance genes are readily identified among commensal bacteria, and such genes may be responsible, at least in part, for the unknown tetracycline resistance genes present among bacterial pathogens. The sequence similarity between native resistance genes from different environments emphasizes the extent of gene transfer between bacteria in nature, and illustrates that no bacterial environment can be considered in isolation. Indeed, bacteria appear to have access to a general gene pool, from which they can extend their genetic capabilities and enhance their ability to survive under adverse environmental conditions.

In general, genes of bacterial origin in GM plants may theoretically be capable of transfer back to bacteria in the gut or wider environment. However, the chance of acquisition of the same genes from another bacterial species in the environment is much greater. For genes of nonbacterial origin, the probability of expression in gut bacteria following transfer is very low, and there has always been a low risk of genes ingested in food being acquired by gut bacteria.

Finally, it should be noted that acquisition of foreign DNA by bacteria is most likely when there are similar sequences in the foreign DNA and the bacterium. The use of marker genes and other sequences of bacterial origin in GM crops may therefore increase the likelihood of gene transfer from GM plant DNA to gut bacteria, and should be avoided in future GM crops.

TECHNICAL REPORT

G0101: Dissemination of GM DNA and antibiotic resistance genes via rumen microorganisms

Objective 1: Assess the survival of free DNA in rumen contents

The advent of genetic modification of crop plants for human and animal consumption has raised questions about what happens to these transgenes upon entry into the gastrointestinal tract. The survival of transgenes in genetically modified (GM) crops is not expected to differ from that of non-GM DNA from the same source and with the same composition. In the case of processed or cooked food, the degraded state of the DNA reduces any potential impact. Ruminant animals, however, consume raw plant material that passes directly into the rumen, which is inhabited by large numbers of microbes. Therefore the fate of transgenes in ruminants may differ from that in non-ruminants.

Effect of rumen fluid on the survival of plasmid DNA

Initial experiments to investigate the degradative effect of rumen fluid on free DNA were carried out using plasmid pVACMC1. This plasmid is capable of replication in both Gram-positive and Gram-negative bacteria, and carries an inserted cellulase gene (Whitehead and Flint, 1995). It was chosen because we had already developed a competitive PCR protocol that enabled quantification of the amount of DNA remaining after various time points. In the case of DNA exposed to human saliva between 40% and 60% of the target sequences were detectable after 10 min at 37°C (Mercer et al 1999a).

The DNA purification method was modified to remove substances present in rumen fluid inhibitory to the action of *Taq* polymerase. DNA was found to be degraded rapidly following incubation at 39°C in sterile rumen fluid, with more than 90% of the DNA degraded within the first 5 minutes of incubation, although small amounts of DNA could still be detected after this time. Similar results were observed for clarified rumen fluid, where particulate matter had been removed by gentle centrifugation. Subsequent experiments investigated the degradation of DNA in whole rumen fluid containing the live microbial fraction and thus the associated degradative enzymes active against DNA. Again DNA degradation was rapid, and in this case no detectable sequences remained after 5 minutes. However, importantly, only 50% of the DNA was degraded in the first minute (Fig. 1). During bacterial transformation by free DNA the first step involves rapid DNA binding to the cell surface, whereupon the DNA is protected from further nucleolytic attack (Méjean and Claverys 1993). Thus the survival of DNA for even a few seconds in whole rumen fluid may be long enough to effect transformation.

Does the presence of feed affect the survival of DNA?

Any DNA entering the rumen environment will be ingested with feed, the components of which may have an effect on the rate of degradation. For example DNA bound to clay is protected from nuclease activity (Romanowski et al 1993), and thus would be expected to persist for longer. The protective effect of straw was investigated by preincubating the DNA with a suspension of ball-milled, ground straw for 5 minutes on ice (to prevent DNA degradation during this time) prior to exposure to sterile, clarified or fresh rumen fluid preparations for 0, 1, and 5 minute periods. Subsequent competitive PCR

amplification indicated that the survival time of DNA in the presence or absence of straw was very similar (Fig. 1). Again little difference was observed between sterile and clarified rumen fluid preparations, where target DNA sequences were still detectable after 5min. DNA degradation in fresh rumen fluid was more rapid and target DNA was not detected after 5min (Fig 1).

Objective 2: Assess the fate of free DNA in plant material after exposure to rumen contents

In the experiments described in Objective 1, the plasmid pVACMC1 was used to assess the degradative effects of rumen fluid on DNA, primarily because a quantitative competitive PCR approach for this plasmid had already been developed and perfected in our laboratory. This PCR approach targets survival of a 500bp sequence of DNA of a total 11.5kb plasmid, and thus would not necessarily pick up the first stages of plasmid degradation which would be sufficient to prevent the plasmid replicating in a new host bacterium. Assessment of the rate of degradation of actual plant DNA in rumen fluid would give more realistic information on the fate of GM material in the rumen.

Initial experiments to investigate the degradation of plant DNA under rumen conditions involved using DNA extracted from transgenic maize kernels, obtained from our collaborators in Newcastle. DNA was purified from the maize using a published CTAB extraction method (Hupfer et al 1997). Purified DNA was amplified using primers designed either to amplify a 520bp region of the inserted TEM-1 β-lactamase gene or a 135bp conserved eukaryotic genomic DNA region (control amplification). Amplification

was successful for the shorter eukaryotic product (Fig. 2a), illustrating that the purified maize DNA was of a suitable quality for PCR and contained no inhibitors. Unfortunately, it proved necessary to carry out two rounds of PCR amplification to obtain detectable PCR products for the TEM-1 gene (Fig. 2b). This may reflect the relative copy numbers of the TEM gene and the conserved eukaryotic sequence in the transgenic maize. The result meant that attempts to quantify the survival of maize DNA under different conditions at different time points would be heavily influenced by PCR bias. Other FSA-funded groups working under the Novel Foods Programme were attempting similar survival experiments and following discussions with our project manager it was decided that we would not continue this work, but would instead expand the work on Objective 4, which was producing important new results.

Objective 3: Investigate the potential for natural transformation of bacteria in the rumen

This part of the project became more important since the demonstration that plasmid DNA sequences were detectable following incubation in whole rumen fluid for at least 1 minute. If intact DNA coexists with competent bacteria it is likely that some DNA will be acquired by these bacteria, thus making transformation in the rumen a real possibility. A number of bacteria are known to become naturally competent at certain stages of their lifecycle eg during conditions of stress (Lorenz and Wackernagel 1994). Although natural competence has not previously been demonstrated for any rumen bacterium, many

members of the genus Streptococcus have competent phases in their lifecycles, and the rumen facultative anaerobe *Streptococcus bovis* is a member of this genus.

The natural competence of the rumen bacterium Streptococcus bovis

We were able to induce *Streptococcus bovis* JB1 to develop competence using the method perfected for the related oral bacterium *Streptococcus gordonii* DL1 (Mercer et al 1999a). Transformants were obtained at efficiencies of 5.5×10^{-6} per parent cell under optimised laboratory conditions. This was the first report of a rumen bacterium being capable of natural transformation and has been published (Mercer et al 2000).

In order for the uptake of free DNA to be detectable and meaningful the DNA must be expressed in the recipient, either by uptake of a suitable, replicating plasmid or by integration into the host genome by homologous recombination. The first transformation experiments were done using plasmid DNA (pAM120 and pVACMC1) and transformants selected based on the expression of antibiotic resistance. We were also able to transform *S. bovis* with an integrative vector that cannot replicate in *S. bovis* and is only maintained following incorporation into the host genome (Scott et al 2000). The chromosomal integration depends on homologous recombination between the *tet*(M) gene in the recipient (present on transposon Tn*916*) and a 458bp region of *tet*(M) incorporated into the vector (Fig. 3). The integrating plasmid construct also encoded the green fluorescent protein (GFP), which meant that transformants could be selected based on both antibiotic resistance and colony fluorescence, which was confirmed by epifluorescence microscopy. The transformation efficiencies of competent *S. bovis* cells using different plasmid constructs are shown in Table (a) below :-

Table (a)

	Strain	Transforming DNA	Transformation efficiency
1.	*S. bovis* JB1	pVACMC1	5.5×10^{-6}
2.	*S. bovis* JB1	pAM120	1.0×10^{-5}
3.	*S. bovis* JB1/Tn*916*	pKPSPgfp-int	7.2×10^{-4}
4.	*S. bovis* JB1	pKPSPgfp-int	0
5.	*S. bovis* JB1/Tn*916*	pKPSPgfp-int/*Bam*HI	0

Interestingly, higher transformation efficiencies (calculated as the number of transformants per parent cell) were obtained using the chromosomal integrative construct (pKPSPgfp-int) than the replicative plasmids (pVACMC1 and pAM120). However when there was no region of homology to permit chromosomal integration (Case 4) transformation did not occur. Additionally the plasmid must apparently be present in a circular form initially. In Case 5 where the plasmid had been linearised using *Bam*HI (adjacent to the *gfp* gene) no transformants were obtained. This may reflect either the greater uptake of supercoiled circular plasmid molecules by the bacterium, or a requirement to reconstitute circular plasmid within the cell prior to integration via a single crossover event. Thus any bacteria capable of taking in free plasmid DNA could potentially incorporate it into the genome providing there is homology between the incoming and host DNA. Antibiotic resistance genes, which were used as markers in producing the first generation GM plants, may provide such a region of homology. The potential for incorporation of linear DNA fragments was investigated in the concurrent

project (G01007). We found that transformation frequencies were 10-fold lower using linear DNA and depended on the presence of at least two regions of homology, flanking the gene to be integrated, to permit chromosomal recombination (Mercer et al 2001).

Transformation under "real" conditions

Although the initial transformation experiments used heat inactivated horse serum (HI-HS) to induce competence of the recipient bacteria, we were able to replace the HI-HS with sterile water with only a small reduction in transformation efficiency (Fig. 4), indicating that no competence stimulator was required for *S. bovis* (Mercer et al 1999). The experiment was further modified to simulate *in vivo* conditions by substituting the heat inactivated horse serum with either filter-sterilised ovine saliva or ovine rumen fluid. Both these substances markedly inhibited transformation, even though saliva stimulated growth of the parent cells (Fig. 4). Only when the rumen fluid was diluted 10 or 20 fold with water were any transformants obtained, and even then at considerably lower efficiencies than when heat inactivated horse serum, or even water, was used.

Transformation of rumen E. coli

Rumen isolates of *E. coli* were also tested for their ability to uptake and express foreign DNA. Although *E. coli* form only a minor component of the rumen microflora, numbers can increase significantly under certain dietary regimes e.g. starvation prior to slaughter. Transformation of laboratory *E. coli* is a routine procedure where the bacteria are grown in the presence of 10mM $CaCl_2$ to weaken the cell wall, prior to limited heat shock at 42°C in the presence of free DNA to stimulate DNA uptake.

A selected rumen *E. coli* strain, F318, was transformed with the *E. coli* plasmid vector pUC18 at high efficiencies using the standard laboratory protocol to promote competence. Transformation also occurred when the 42°C heatshock step was omitted and replaced with incubation at 39°C, representative of the temperature in the rumen. Transformants were also obtained at lower concentrations of Ca^{2+} ions, which could represent environmental conditions. The concentration of Ca^{2+} ions normally present in the rumen is 2mM. The results of the transformations at 39°C in the presence of various "inducers" are shown Table (b).

Table (b)

Substance added to facilitate transformation	No. transformants
0mM $CaCl_2$	0
2mM $CaCl_2$	0
5mM $CaCl_2$	21
10mM $CaCl_2$	1.3×10^3
20mM $CaCl_2$	66
30mM $CaCl_2$	0
Sterile rumen fluid	0
Sterile rumen fluid + 10mM $CaCl_2$	polysaccharide layer

Clearly the optimal $CaCl_2$ concentration is 10mM, that used routinely in the laboratory. However transformation is possible, albeit at lower frequencies, at both 5mM and 20mM $CaCl_2$ concentrations. $CaCl_2$ may be present in the rumen at 5mM concentrations under certain dietary regimes, although the average is 2mM. Although we initially thought that

transformation had been successful in the presence of rumen fluid, supplemented with 10mM $CaCl_2$, transformants could not be isolated from the slimy polysaccharide layer, formed by the combination of rumen fluid and $CaCl_2$. Thus the presence of rumen fluid inhibited transformation completely, making it unlikely that rumen bacteria could be transformed by free DNA *in vivo*.

To summarise the results of **Objectives 1, 2 and 3**, although at least one resident rumen bacterium is capable of natural transformation, the inhibitory effects of rumen fluid and, to a lesser extent, saliva make it unlikely that this will occur frequently under natural conditions. However the impact of rumination, whereby rumen bacteria are removed from the rumen into the oral environment and the rumen fluid is effectively diluted out with saliva and water has not been investigated. Since *S. bovis* transformants can be obtained when only water is present, and low numbers of transformants are obtained in the presence of saliva or diluted rumen fluid, rumination may create a potential route for transformation to occur.

Objective 4: Investigate the incidence of *tet*(W) in bacteria from other gut habitats

The aim of this part of the project was to investigate gene transfer between diverse bacterial populations, including both commensal and pathogenic bacteria, by focussing on the spread of the tetracycline resistance (Tc^R) gene *tet*(W), recently identified in this laboratory (Barbosa et al 1999), in rumen anaerobes. Tetracycline has been widely used

in animal husbandry since the 1960s and there has been a concomitant rise in the number of tetracycline resistant bacteria. Thus monitoring the spread of Tc^R genes illustrates the extent to which gene transfer can occur under ideal conditions where a selective pressure is exerted over an extended time period.

Distribution of tet(W) in anaerobic bacteria

Identical *tet*(W) genes were present in a range of important commensal rumen anaerobes isolated over a 25 year period, including isolates from the UK, Japan and Australia (Barbosa et al, 1999). Screening of tetracycline resistant colonies from faecal samples from three human volunteers by hybridisation identified the same *tet*(W) gene in two of the individuals. One positive individual was on long-term tetracycline therapy but the other had not been exposed to tetracycline. The two different bacterial species identified were predominant members of the colonic microflora, a *Clostridium* relative and *Bifidobacterium longum* (Scott et al, 2000). Sequencing of the genes from these diverse bacteria indicated minimal sequence variation along the 1.9kb gene sequence (Table 1). The high degree of sequence identity between the *tet*(W) genes found in different genera of bacteria isolated from different hosts strongly implies that recent gene transfer events must have occurred, although the direction of transfer cannot easily be deduced. Interestingly, with the exception of *Butyrivibrio fibrisolvens* and the *Clostridium* spp., *tet*(W) always seems to be associated with higher G+C content bacterial species (Table 1). *tet*(W) itself has a much higher G+C content than most ribosome protection-type tetracycline resistance genes, which may be important in elucidating the direction of gene flow, as it implies origin from a high G+C content genome.

Analysis of the conjugative transposon carrying tet(W)

In the *B. fibrisolvens* host bacterium *tet*(W) is located on a mobile conjugative transposon, Tn*B1230*. Sequence analysis of this transposon has identified several potential open reading frames flanking the *tet*(W) gene (Fig. 5). Comparing the %G+C content of the ORFs clearly shows that the *tet*(W) gene itself is the only one with a G+C ratio significantly higher than that of the *B. fibrisolvens* genome, implying that the *tet*(W) gene may have been integrated into an existing transposon, as a result of gene acquisition from a higher G+C content bacterium. Several of the other ORFs in *TnB1230* have identity to some found on other conjugative transposons. The degree of identity is low and indicates that Tn*B1230* is a novel conjugative transposon, the first to be identified from a rumen anaerobe (Scott et al 1997). Both PCR amplification and Southern blotting show that Tn*B1230* is not present in any of the other bacteria carrying *tet*(W).

Transfer of other tetracycline resistance genes between anaerobic bacteria

The high homology between the *tet*(W) genes on the diverse bacterial hosts implies that there may be a common transfer mechanism, yet to be identified. Onward transfer of tetracycline resistance from the human faecal isolates containing *tet*(W) was therefore investigated. Both the human *Clostridium*-like K10 and *Bi. longum* donors were able to transfer tetracycline resistance to the rumen *B. fibrisolvens* 2221^{R} recipient, which equates to transfer across the host barrier. Transfer efficiencies (K10 – 1×10^{-4}; *Bi. longum* –1×10^{-3}) were almost as high as those observed for Tn*B1230* transfer between

rumen *B. fibrisolvens* strains (Scott et al 1997). In the case of *Bi. longum* the *tet*(W) gene seems to be encoded on a mobile, transferable plasmid rather than chromosomally. Analysis of the transconjugants arising from the *Clostridium* K10 donor showed that they, unexpectedly, did not contain *tet*(W) but rather another new ribosome protection-type of tetracycline resistance gene. This gene, which we have designated *tet*(32), was cloned and sequenced and found to be 76% identical to the *tet*(O) gene, its closest relative (Fig. 6, Melville et al 2001). Southern blot analysis of transconjugants (Fig. 7) indicated that transfer of *tet*(32) appeared to be associated with the movement of a mobile chromosomal element (Tn*K10*). This element showed no hybridization to either Tn*B1230* or to Tn*916*, again implying the presence of a novel mobile element.

Incidence of tet(O) among commensal bacteria

We also showed that the *tet*(O) gene itself, initially described in the human pathogen *Streptococcus pneumoniae* was also present in several low %G+C Cluster XIVa isolates obtained from human faeces. This bacterial group is the most important numerically in the human gut. *tet*(O) was also found in a rumen *B. fibrisolvens* isolate (Barbosa et al 1999). Sequence analysis of the *tet*(O) genes indicated considerable sequence conservation extending upstream of the coding sequence to include the regulatory regions (Fig. 8). This sequence was highly conserved between the human, rumen and pathogenic isolates containing *tet*(O), indicating that expression of *tet*(O) is regulated in the same way in diverse host bacteria. *tet*(O) is known to be a mobile gene when present on plasmids but these genes appeared to be chromosomally encoded. At the limits of the available sequence of the *Butyrivibrio* and *Eubacterium* genes, a short open reading

frame was identified with identity to a 123 amino acid protein encoded on the *Clostridium perfringens* transposon Tn*4451*. This sequence may be involved in dissemination of these *tet*(O) genes.

Distribution of tetracycline resistance genes in gut environments

Further analysis of the distribution of these three Tc^R genes, *tet*(O), *tet*(W) and *tet*32), was done by amplifying total DNA extracted from rumen fluid or pig faeces with primers specific for each of the individual genes. All the genes were found in all the samples tested (Table 2).

The results obtained from **Objective 4** confirm that the commensal anaerobic bacteria constitute an important reservoir of Tc^R genes, potentially transferable to incoming pathogenic bacteria. Although only *tet*(O) has so far been identified in pathogens it is likely that tet*(W)* and *tet*(32) account for, at least some, of the 50% or so of Tc^R genes that remain unidentified in pathogenic bacteria. The fact that all the genes were present in all the environments tested shows that widespread gene transfer can, and does, occur between diverse bacterial isolates from diverse environments, and that there are no environmental barriers to gene transfer. Thus any perturbation of one environment, perhaps by the introduction of a novel gene, could have a significant effect on an unlinked ecosystem. This point should be borne in mind when considering the ultimate potential impact of novel genes.

Objective 5: Investigate the occurrence of *bla*TEM-related ampicillin resistance genes among predominant rumen anaerobes, and determine the type of ampicillin resistance in existing, and new, *E. coli* isolates of ruminal origin

There has been considerable interest recently as to whether the presence of β-lactamase genes, conferring ampicillin resistance, in transgenic maize engineered by Ciba-Geigy to be herbicide resistant and produce the Bt toxin, could spread into bacteria present in the environment. This was perceived to be particularly relevant in the feeding of forage maize to cattle, where there is no heat treatment, and the ingested maize mixes directly with the large rumen microbial population.

Ampicillin resistance genes in rumen bacteria

The incidence of ampicillin resistance among enteric and rumen bacterial populations is well established but the types of resistance genes present have not been documented. A number of *Escherichia coli* isolated from both the ovine rumen (1987) and the pig hindgut (1997) had been found to be resistant to ampicillin (100μg/ml). Only two of these isolates were not also tetracycline resistant (20μg/ml). A further set of ampicillin resistant *E. coli* isolated between 1992 and 1997 were received from the Scottish Agricultural College (SAC). These isolates were all faecal isolates from ovine or bovine sources. Nine out of ten *Pseudomonas* isolates cultured from ovine rumen fluid samples obtained from three out of six adult sheep were also found to be ampicillin resistant (Table (c) below).

Table (c)

Bacterial Source	no. Amp^R Isolates	Year	Tc^R	PCR product	PCR products sequenced?
Ovine rumen	3	1987	2/3	3/3	3/3
Pig hindgut	15	1997	14/15	15/15	15/15
SAC	7	1992-1997	nd	3/7	3/3
Pseudomonas	9	1997	nd	4/9	4/4

Sequence comparisons of ampicillin resistance genes

The ampicillin resistance genes were amplified using PCR primers designed to amplify a 520bp fragment internal to the TEM-1 β-lactamase gene (Sutcliffe 1978). PCR products were obtained for the isolates detailed above and were sequenced. The genes were found to be highly homologous, all related to TEM-1, and there were only five variable nucleotide positions (Table 3). Of these variable positions only three resulted in changes in the amino acid sequence (positions 69, 84 and 184) and only one of these (Met_{69} – Val_{69}) was a non-conservative change. The original Class A (TEM) β-lactamases, TEM-1 and TEM-2, are broad spectrum B-lactamases resulting in bacterial resistance to amino/carboxy-penicillins and 1^{st} or 2^{nd} generation cephalosporins. Minimal sequence changes near the active site (Ser_{70}) result in the formation of extended spectrum β-lactamases (ESBLs), which are additionally resistant to oxyimino β-lactams and aztreonam. The amino acid change in two of the three SAC isolates tested (Met_{69}-Val_{69}) is adjacent to this residue and is also found in the inhibitor resistant B-lactamase TEM-78. However this enzyme has two additional aminoacid changes compared to the translated sequences of SAC-4 and SAC-5. Extension of the sequences to compare full-length TEM sequences did not identify any additional changes.

Included in the sequence analysis was the PCR product amplified from the TEM-1 gene in the transgenic maize (Objective 2). Significantly this sequence was identical to one of the *Pseudomonas* isolates and differed from the other *Pseudomonas* sequences at only one nucleotide position. This includes conservation of the T nucleotide at 752 which is important as this position corresponds to the *Pst*I site CTGC*AG. Thus the recognition sequence for this enzyme is not present in maize or *Pseudomonas* spp. but is in the other isolates. The presence of this site was confirmed by restriction digestion of a number of PCR products with *Pst*I, and the products of all except *Pseudomonas* and maize were cut with the enzyme. Thus the absence of a *Pst*I site cannot be used to confirm the presence of an ampicillin resistance gene of transgenic origin.

Genomic location and transfer of ampicillin resistance genes

The genomic location of the ampicillin resistance genes on each of the pig hindgut and the 1987 rumen isolates was confirmed by Southern blotting. Plasmid DNA was purified from each sample and digested with *Eco*RI to linearise the plasmids. The DNA was then separated by electrophoresis, Southern blotted and hybridised to the PCR amplified TEM gene probe (Fig. 9). There were four distinct hybridisation patterns. Three differently sized plasmids hybridised, and in 2 samples the chromosomal DNA hybridised. The same hybridising plasmid was present in the two amp^R, Tc^R rumen strains (lanes 1 and 2), the remaining rumen strain (amp^R, Tc^S, lane 3) contained a chromosomal copy of the amp^R gene. A single pig isolate contained a smaller plasmid (lane 4), the remainder contained a plasmid of intermediate size (e.g. lanes 5 and 6). Once again the isolate that was amp^R,

Tc^S contained a chromosomal copy of the gene (not shown). These results suggest that amp^R and Tc^R are encoded by the same plasmid when both genes are present.
In vitro transfer studies had been carried out previously using one of the two amp^R, Tc^R rumen strains (Scott and Flint, 1995) and had shown that in this strain the two resistances were indeed linked on a single mobile plasmid estimated to be 62kb in size. These transfer experiments had included investigating transfer under simulated rumen conditions, and although frequencies were lower than those found under optimal laboratory conditions, plasmid transfer was still detectable.

In **Objective 5** we have shown that ampicillin resistance genes are readily detectable in the enteric flora of farm animals, the sequences are highly conserved and in some cases are identical to those of transgenic maize. [These results are currently being prepared for publication.] Although it is possible that the TEM-1 ampicillin resistance gene could be acquired from transgenic maize by ampicillin sensitive gut bacteria, they are much more likely to acquire resistance through bacterial – bacterial gene transfer. The greatest risk from the acquisition of the TEM-1 gene from either route is not simply the development of ampicillin resistance but rather the possibility that homologous recombination between TEM-1 and other TEM determinants may extend the resistance spectrum of the bacteria. This is not however a risk unique to genetically modified material. The simple conclusion is that since ampicillin resistance genes identical to those used in transgenic maize are already present and easily detected in the rumen microbial population, feeding transgenic maize containing ampicillin resistance genes to ruminants is unlikely to have a significant impact on resistant populations of bacteria.

General Conclusions

- Free DNA can survive for at least 1 minute in raw rumen fluid (approximately 50% survival of a 500bp target sequence)
- Some rumen bacteria (*Streptococcus bovis* and *Escherichia coli*) are "naturally" transformable under certain conditions
- The presence of homologous sequences in foreign and genomic DNA can result in chromosomal integration of exogenous DNA

However the presence of rumen fluid inhibits transformation in the two species studied, indicating that foreign DNA is unlikely to be incorporated into rumen bacterial genomes in vivo.

- Many rumen enterobacteraciae isolates are ampicillin resistant
- The ampicillin resistance genes are predominantly TEM-1, the same gene contained on transgenic maize
- The ampicillin resistance genes are located both on plasmids and chromosomally
- At least some of the plasmid encoded ampicillin resistance is transferable

The presence of the same ampicillin resistance genes in transgenic maize and rumen bacteria means that the potential impact of feeding transgenic maize to ruminants is negligible, with respect to the ampicillin resistance

- Identical *tet*(W) genes are present on different bacterial species from diverse environments
- Two novel tetracycline resistance genes have been identified among commensal bacteria
- Both these genes are located on mobile chromosomal elements transferable between rumen and human bacterial isolates

- *tet*(W), *tet*(32) and *tet*(O) genes have been identified in gut samples from human, pig and ruminant sources

Recent widespread gene transfer appears to have occurred among commensal bacteria from diverse habitats. The commensal flora may contribute to antibiotic resistance among bacterial pathogens

REFERENCES

Barbosa, T.M., Scott, K.P. and Flint, H.J. (1999). Evidence for recent intergeneric transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butyrivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environmental Microbiology **1:**53-65.

Hupfer, C., Hotzel, H., Sachse, K. and Engel, K-H. (1997). Detection of genetically modified insect-resistant Bt maize by means of polymerase chain reaction. Z Lebensm Unters Forsch A **205;** 442-445.

Lorenz, M. G., and W. Wackernagel. (1994). Bacterial gene transfer by natural genetic transformation in the environment. Microbiology Reviews, **58:** 563-602.

Méjean, V. and Claverys, J.-P. (1993). DNA processing during entry in transformation of *Streptococcus pneumoniae*. Journal of Biological Chemistry, **268:** 5594-5599.

Melville, C.M., Scott, K.P., Mercer, D.K. and Flint, H.J. (2001). Novel tetracycline resistance gene, *tet*(32), in the *Clostridium*-related human colonic anaerobe K10 and its transmission in vitro to the rumen anaerobe *Butyrivibrio fibrisolvens.* Antimicrobial Agents and Chemotherapy **45:**3246-3249.

Mercer, D.K., Scott, K.P., Bruce-Johnson, W.A., Glover, L.A. and H.J. Flint (1999a). Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Applied and Environmental Microbiology **65;**6-10.

Mercer, D.K., C.M. Melville, K.P. Scott, and H.J. Flint (1999b). Natural genetic transformation in the rumen bacterium *Streptococcus bovis* JB1. FEMS Microbiology Letters **179:** 485-490.

Mercer, D.K., Scott, K.P., C.M. Melville, L.A. Glover and H.J. Flint (2001). Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. FEMS Microbiology Letters **200:**163-167.

Romanowski, G., M.G. Lorenz and W. Wackernagel (1993). Plasmid DNA in a groundwater aquifer microcosm: adsorption, DNAase resistance and natural genetic transformation of *Bacillus subtilis.* Molecular Ecology **1:**171-181.

Scott, K.P. and Flint, H.J. (1995). Transfer of plasmids between strains of *Escherichia coli* under rumen conditions. Journal of Applied Bacteriology **78:** 189-193.

Scott, K. P., Barbosa, T. M., Forbes, K. J. and Flint, H. J. (1997). High-frequency transfer of a naturally occurring chromosomal tetracycline resistance element in the rumeinal anaerobe *Butyrivibrio fibrisolvens.* Applied and Environmental Microbiology **63;** 3405-3411.

Scott, K. P., D.K. Mercer, A.J. Richardson, C.M. Melville, L.A. Glover and H.J. Flint. (2000). Chromosomal integration of the green fluorescent protein gene in lactic acid bacteria and the survival of marked strains in human gut simulations. FEMS Microbiology Letters **182:**23-27.

Scott, K. P., C.M. Melville, T.M. Barbosa, and Flint, H. J. (2000). Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrobial Agents and Chemotherapy **44:** 775-777.

Sutcliffe, J. G. (1978). Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proceedings of the National Academy of Science USA **75:**3737-3741.

Whitehead, T.R. and Flint, H.J. (1995). Heterologous expression of an endoglucanase gene from the ruminal anaerobe *Ruminicoccus flavefaciens* 17 in *Streptococcus bovis* and *Streptococcus sanguis.* FEMS Microbiology Letters **126:**165-169.

Figure Legends

Fig. 1 Competitive PCR analysis to assess the survival of DNA in whole rumen fluid after 0, 1, 5 minutes incubation at 37°C, with or without preincubation with a 100mg/ml straw suspension. Results are expressed as a percentage of the starting DNA concentration.

Fig. 2 PCR amplification of purified maize DNA using primers designed to amplify 137nt of eukaryotic genomic DNA (a) or 540nt of the ampicillin resistance gene (b). The appropriately sized PCR products are indicated by the arrows.
(a):lanes 1-10: transgenic maize DNA; lane 11: *E. coli* TEM sample (negative control); lane 12: no DNA (negative control). (b):lanes 1, 2, 3, 5: transgenic maize DNA (2^{nd} amplification; lane 4: no DNA (negative control); lanes 6, 7: TEM genes (positive control). M – 1kb DNA ladder.

Fig. 3 Diagrammatic representation of the chromosomal integration of vector pKPSP-gfp-int into the chromosome of bacteria containing Tn*916,* by homologous recombination. The region of homology is indicated by a hatched box, and the location of unique restriction sites is shown.

Fig. 4 Transformation efficiency of *Streptococcus bovis* JB1 with the integrative vector pKPSP-gfp-int. Transformation efficiencies are expressed as the number of transformants per parent cell, and efficiencies using different competence inducers are compared.

Fig. 5 Genetic organization of the 13kb sequence of Tn*B1230,* showing the positions of the identified open reading frames. The arrow indicates the direction of transcription, and the %G+C content of each ORF is indicated.

Fig. 6 Phylogenetic tree showing the evolutionary relationships between ribosome protection type tetracycline resistance genes. The % amino acid identity to Tet(32), and the DNA %G+C content are stated.

Fig. 7 Southern blot of genomic DNA from *B. fibrisolvens* 2221^{R} transconjugants Tcm1 (lanes 1-4) and Tcm8 (lanes 5-8) digested with *Bam*HI, *Eco*RI, *Hin*dIII and *Sma*I, in that order, and hybridised to a probe specific for *tet*(32). Controls of the parent strain K10 digested with *Bam*HI (lane 9) and *Eco*RI (lane 10) and a transconjugant containing only *tet*(W) (lane 11) are included.

Fig. 8 Diagrammatic illustration of the sequence conservation between *tet*(O) genes and upstream regions from two human pathogens and two commensal bacteria. The percentage of amino acid identity between different regions is stated.

Fig. 9 Southern blot of purified DNA probed with the ^{32}P-labelled TEM-1 β-lactamase PCR product to show the genomic location of the ampicillin resistance genes. Lanes 1, 2, 3 are rumen *E. coli* isolates and lanes 4, 5 and 6 pig hind gut *E. coli* isolates.

Fig. 1 COMPETITIVE PCR TO MEASURE DNA SURVIVAL IN RUMEN FLUID .

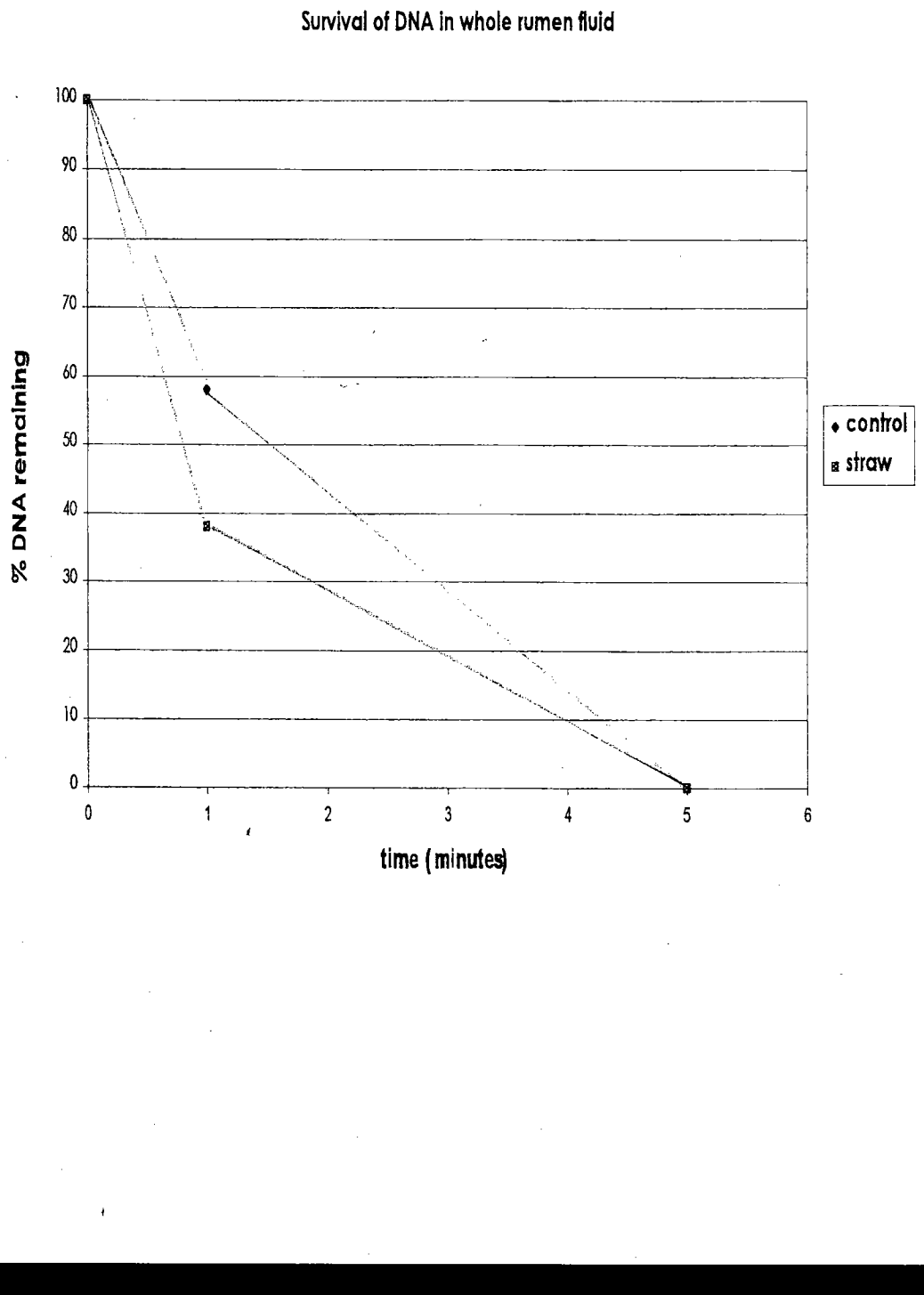
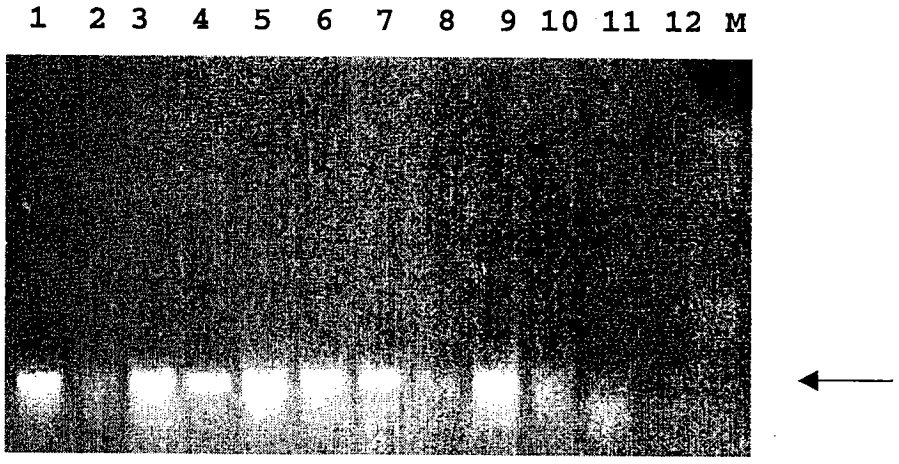


Fig. 2 Maize DNA PCR amplification

(a)



(b)

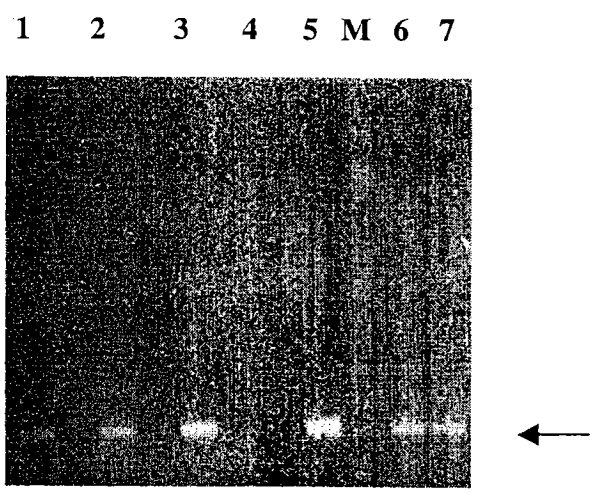


Fig 3: Diagram illustrating chromosomal intergration

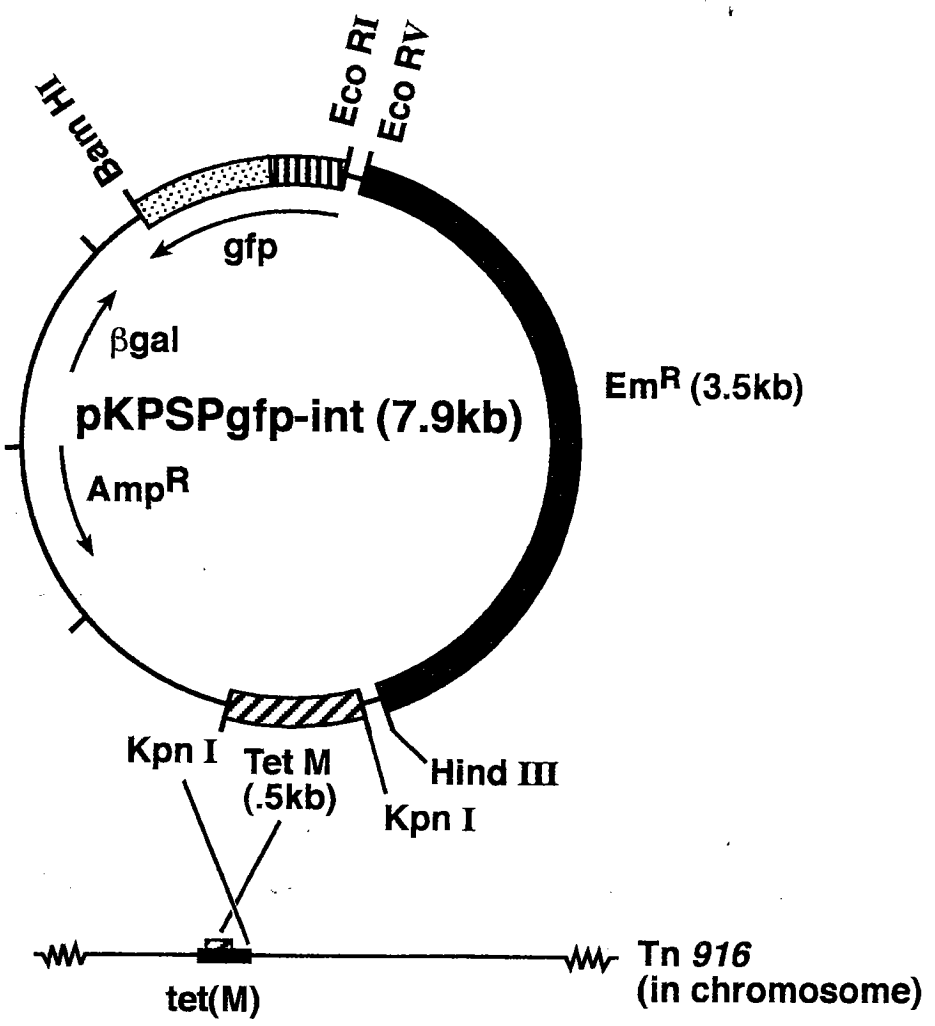
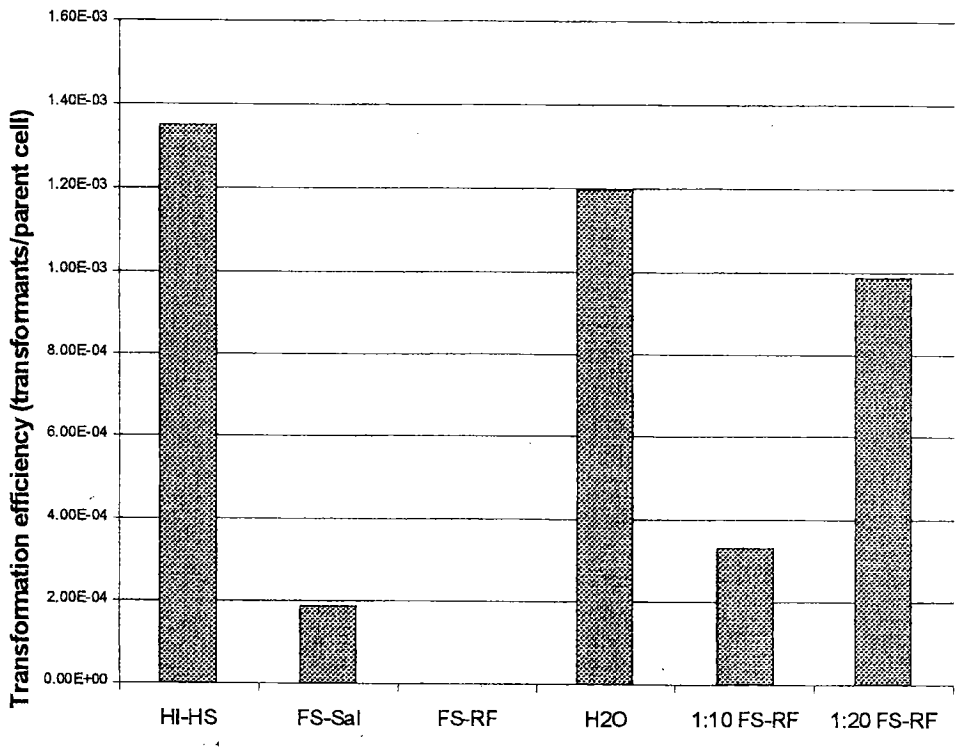
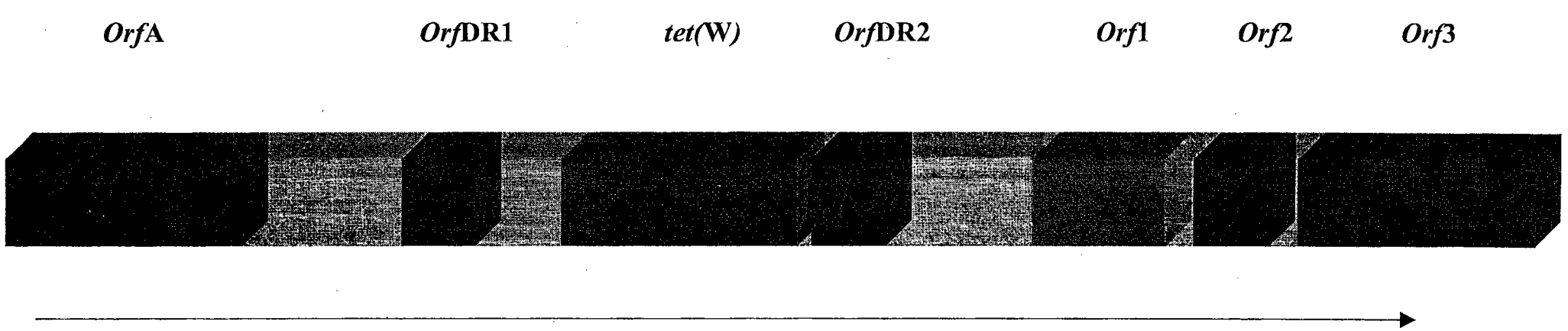


Fig. 4 Transformation of *S. bovis* with pKPSPgfp-int



HI-HS: heat inactivated horse serum
FS-sal: filter sterile ovine saliva
FS-RF: filter sterile ovine rumen fluid

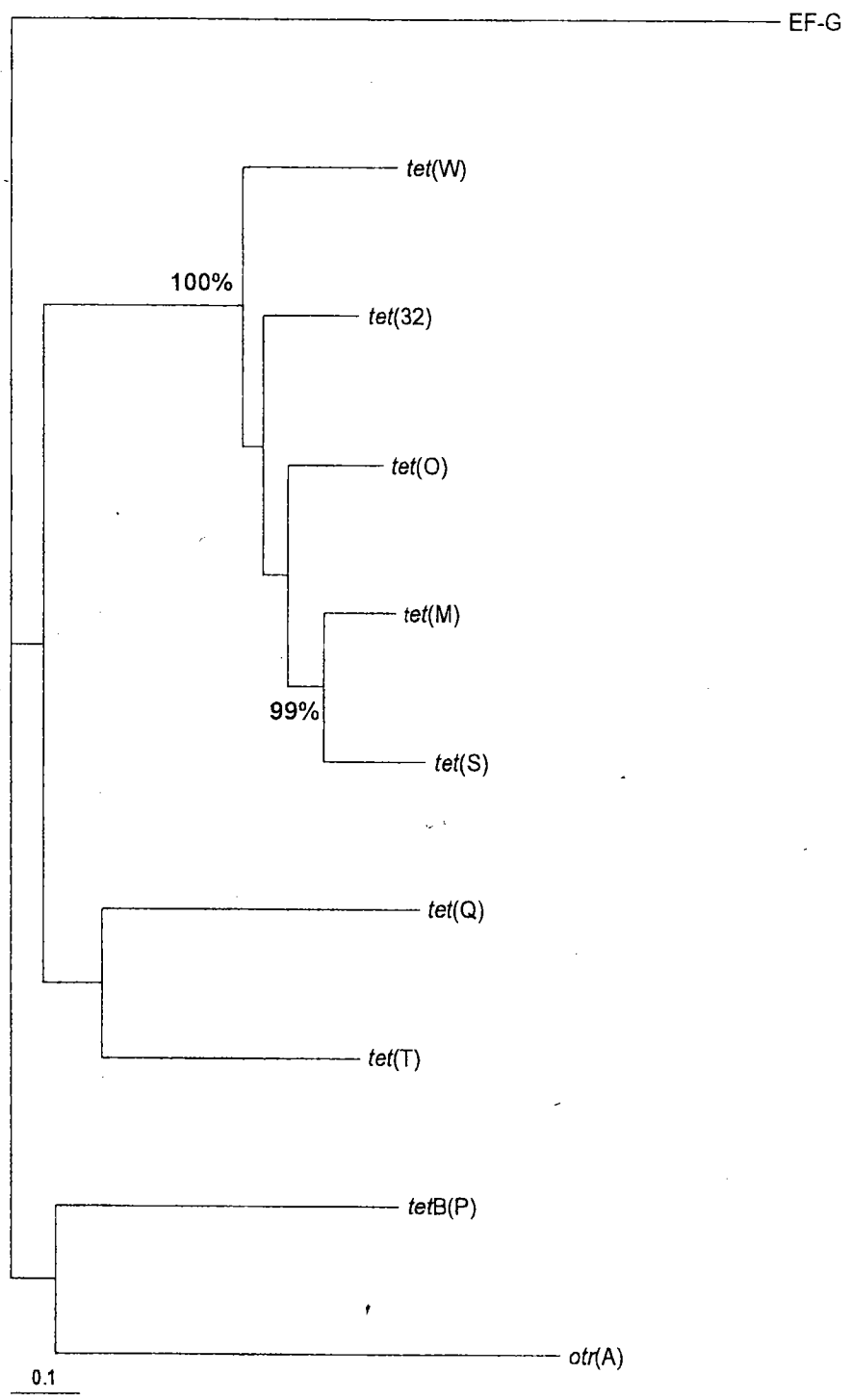
Fig. 5: Genetic organisation of Tn*B1230*



ORF %G+C content

41%	37%	53%	37%	43%	43%	42%

Fig. 6 Phylogenetic tree



	% Amino Acid identity to Tet(32)	% DNA G+C ratio
tet(W)	68%	53%
tet(32)		40%
tet(O)	76%	40%
tet(M)	71%	35%
tet(S)	68%	33%
tet(Q)	43%	39%
tet(T)	40%	32%
*tet*B(P)	42%	32%
otr(A)	35%	72%

Fig. 7: Southern blot of genomic DNA from *B. fibrisolvens* 2221^{R} transconjugants

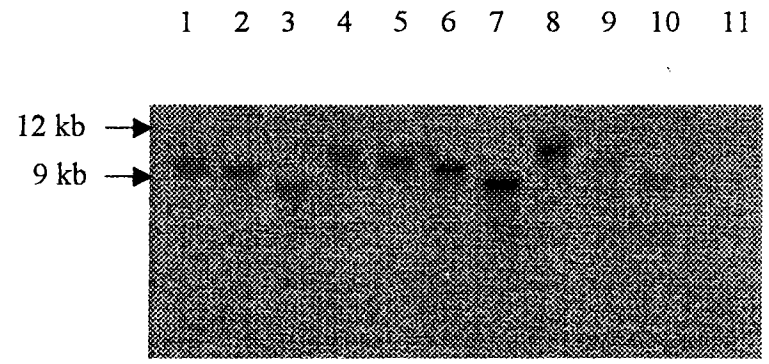


Fig. 8 Sequence conservation between *tet*(O) genes

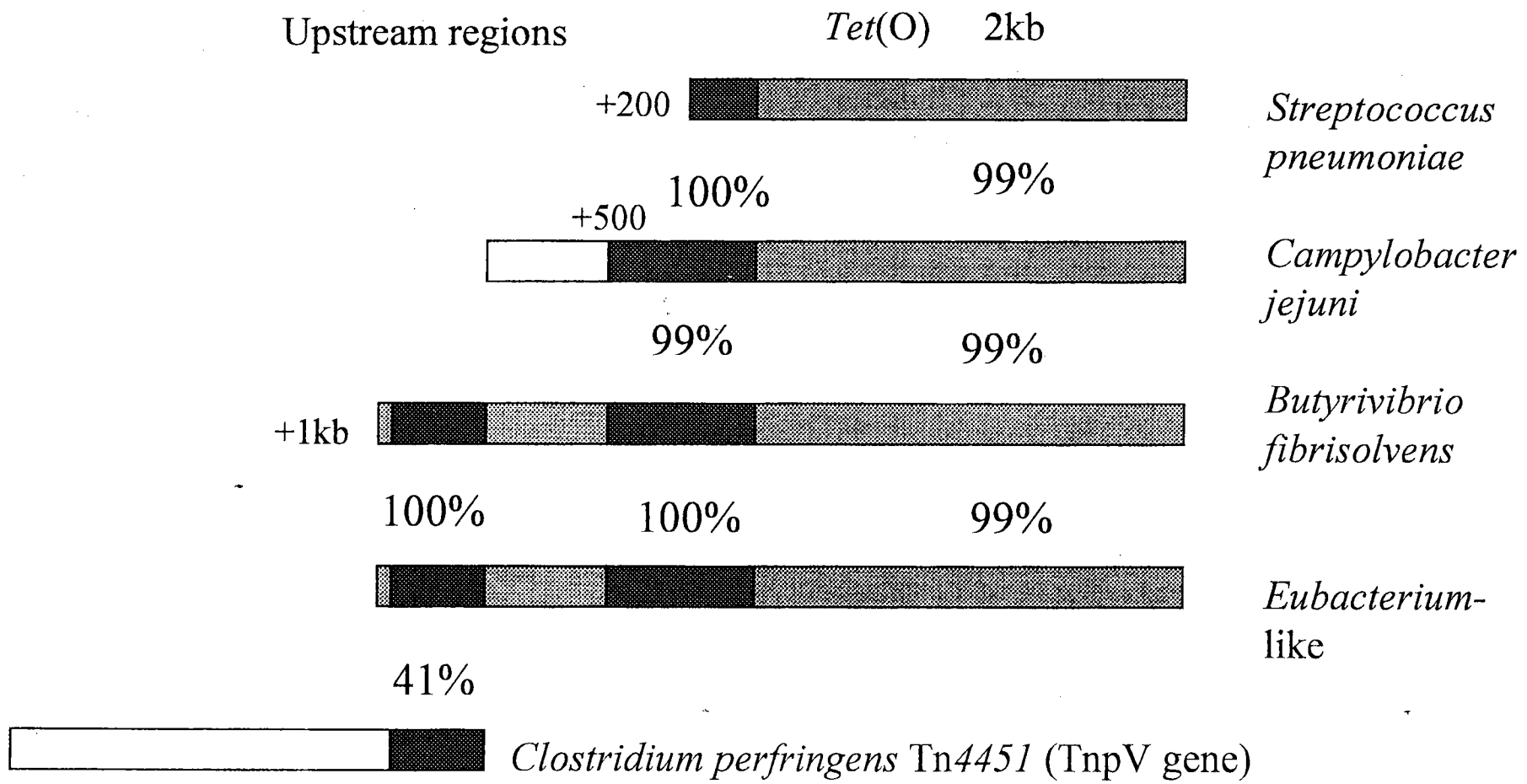


Fig. 9 Southern blot illustrating the location of ampicillin resistance genes

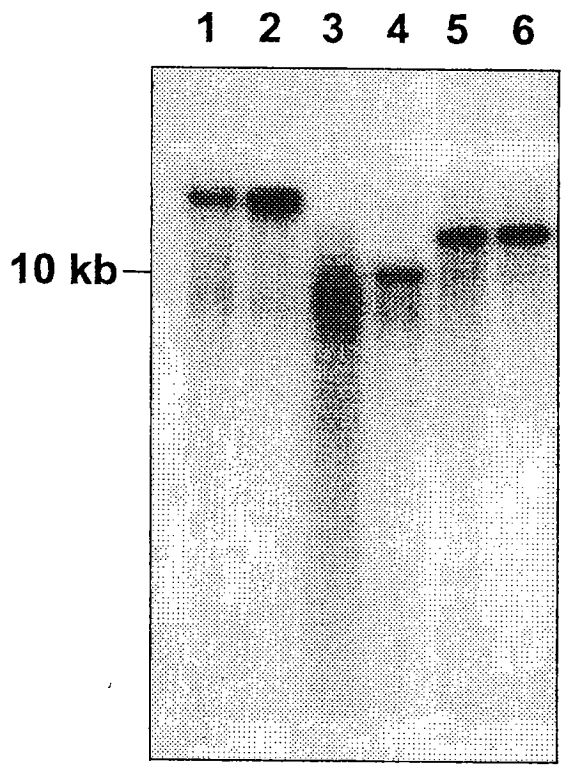


Table 1: Characteristics of strains harbouring *tet*(W) (%G+C=53%)

Strain	Source	Year	nt changes from 1.230	genome %G+C content
B. fibrisolvens				
1.230, 1.23	bovine (R)RRI	1993	0	36-41%
JK51, JK214	ovine (R) Australia	1996	0,0	
S. ruminantium				
FB32, FB34	bovine (R)RRI	1989	0,0 (plasmid)	54-61%
FB322	bovine (R)RRI	1989	1	
M. multiacidus				
46/5(2)	ovine (R)RRI	1987	0	56-58%
P208-58	pig (F) Japan	1974	7	56-58%
Clostridium spp.				
K10	human (F) UK	1999	0	~40%
Bi. longum	human (F) UK	1999	1 (plasmid)	58%
F5, F8, F10				

(R)-rumen, (F)-faecal

maximum nucleotide changes over 1919nt =7 = **99.6% identity**

Table 2: Distribution of tetracycline resistance genes

	tet(W) 53%	*tet*(32) 40%	*tet*(O) 39%
Rumen commensal	*B. fibrisolvens* 36 -41% *S. ruminantium* 54 - 61% *M. multiacidus* 56 -58%		*B. fibrisolvens* 36 -41%
Human commensal	*Clostridium* spp. ~40% *Bi. Longum* 58%	*Clostridium* spp. ~40%	*Eubacterium* spp. 30 - 55%
Pig hindgut	*M. multiacidus* 56 -58%	+	+
Human pathogen	**?**	**?**	*S. pneumoniae* 40% *C. jejuni* 35%

Within groups all genes are >99% identical at nucleotide level; % G+C content is indicated
? Not tested

Table 3: Differences in sequences of ampicillin resistance genes

Nucleotide position [1]	**406**	**435**	**451**	**603**	**752***	**No. strains**	**Origin of Amp^R strains**
Corresponding amino acid [2]	**69**	**78**	**84**	**134**	**184**		
Source of Amp^R gene (Year)							
E. coli (1994)	G	T	G	T	C	2/4	SAC bovine faeces (SAC-5, SAC-6)
E. coli (1996, 1992)	A	T	G	T	C	2/4	SAC bovine faeces (SAC-3, SAC-7)
E. coli (1987)	A	T	G	T	C	3/3	RRI rumen (2 ovine, 1 bovine)
E. coli (1997)	A	T	G	T	C	14/15	RRI porcine faeces
E. coli (1997)	A	C	G	G	C	1/15	RRI porcine faeces
E. coli TEM-1	A	C	G	G	C		*E. coli* plasmid pBR322 (Sutcliffe 1978)
Pseudomonas (1997)	A	C	G	G	T	3/4	RRI ovine rumen
Pseudomonas (1997)	A	C	A	G	T	1/4	RRI ovine rumen
Transgenic maize	A	C	A	G	T		
pUC18	A	C	A	G	T		*E. coli* cloning vector pUC18

*position of *Pst*I site

1- nucleotide position corresponds to the numbering system of Sutcliffe.[8]

2- Amino acid position corresponds to the numbering system of Ambler et al.[6] Thus the active site serine which is residue 68 [8] corresponds to residue 70 using the numbering system of [6].