final report

Project code: TR.044 & TR.044B

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Date published: November 2010

ISBN: 9781741915327

PUBLISHED BY
Meat & Livestock Australia Limited
Locked Bag 991
NORTH SYDNEY NSW 2059

Construction of improved fluoroacetate degrading rumen bacteria

Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

Abstract

Genetically modified rumen bacteria were constructed to reduce loss of livestock from fluoroacetate poisoning by toxic native plants across northern and western Australia. Four strains of GM rumen bacteria were previously shown to protect sheep against fluoroacetate. In Projects TR.044 and TR.044B, five additional bacterial strains were modified, to double the total protective capability, and were tested for their ability to protect feral animals from poisoning by fluoroacetate (Compound 1080). All seven strains of *Butyrivibrio fibrisolvens* failed to colonise the digestive tracts of rabbits or cats after two oral inoculations of 10^8 bacteria. The two GM *Bacteroides* strains colonised cats and rabbits, but made no difference to their fluoroacetate sensitivity. Transfer of rumen bacteria between animals 30 metres apart was shown to occur in open fields, therefore preparations were made for a cattle toxicity trial to be performed in the AAHL PC2 facility. Administrative delays prevented commencement of the trial before regulatory changes created additional time requirements and the project was terminated. The improved bacterial consortium is expected to reduce significantly the effects of fluoroacetate on sheep and cattle. After proving the safety of the bacteria for release, they are expected to reduce financial losses from poisoning of livestock.

• Executive Summary

Background: The fluoroacetate detoxification project was initiated by a consortium of animal producers, who formed Applied Biotechnology Ltd (Qld) to find a solution to the poisoning of livestock by the Georgina Gidgee (*Acacia georginae*). Throughout a large area of Western Queensland and the Northern Territory, around 200 cattle per year are lost to poisoning on each property. In the Gidgee areas, animals browse the Gidgee trees continually. Most animals accumulate toxin in small doses and suffer major toxicity symptoms when mustered or after drinking large volumes of water. i.e. when the flow for rumen contents into the lower parts of the digestive tract is increased. In northern Queensland, the heartleaf bush (*Gastrolobium grandiflorum*) is more acutely toxic than the Gidgee tree and stock losses occur when sheep and cattle, which are normally kept away from the plant, come into contact with it accidentally. Similar poisoning of livestock occurs across the top of the Northern Territory, the north of Western Australia, and the south-west of WA, where the toxicity of fluoroacetate producing plants (*Gastrolobium spp.*) is extremely high. MLA (previously AMLRDC and MRC) agreed to fund the major portion of the research that was begun with funding from Applied Biotechnology Ltd.

Previous Research: Using molecular tools developed by this group at UNE (Beard *et al*., 1995), four strains of rumen bacteria had been genetically modified by inserting a gene from the soil bacterium *Moraxella* species strain B (recently reclassified as *Delftia acidovorans*). This enabled the rumen bacteria to produce the enzyme fluoroacetate dehalogenase, which can break down the Gidgee toxin (Gregg *et al*., 1994; attachment 1). The ability of the modified bacteria to reduce the effects of fluoroacetate on sheep was demonstrated in contained trials at the University of New England in 1996 – 1997 (Gregg *et al*., 1998; attachment 2).

Transfer of Project to Murdoch University: The Rumen Biotechnology team was transferred from UNE (Armidale NSW) to Murdoch University (Perth, WA) in late 1997. The objectives were:

1. to expand the number of modified bacterial strains, so that the efficiency and dependability of detoxification could be increased,
2. to conduct tests requested by GMAC on non-ruminant pest species, to establish the likelihood of colonisation by the bacteria and to determine whether pest species may be protected from 1080 poisoning (fluoroacetate is the active ingredient in 1080).
3. to conduct a field trial to test the effectiveness of the GM bacteria in protecting cattle from fluoroacetate.

Summary of Results:

1. Three new strains of *Butyrivibrio fibrisolvens* were isolated in WA and modified to express the protective enzyme, increasing the total to 7 GM strains. Together, the 7 strains possessed more than double the detoxifying ability of the original 4.
2. Shuttle plasmids capable of transforming ruminal *Bacteroides* species were constructed, allowing the fluoroacetate dehalogenase gene to be expressed in *Bacteroides* strains AR20 and AR29 (Wong *et al*., 2003; attachment 3).
3. The two strains of *Bacteroides* expressed the detoxifying enzyme in useful quantities, but their ability to detoxify the poison from their surrounding medium was very poor. This was concluded to be the result of *Bacteroides* lacking any transport system to bring fluoroacetate into the cell, and the retention of the detoxifying enzyme within the bacterial cell. These factors effectively prevented the detoxifying enzyme from making contact with the toxin. The modified *Bacteroides* strains were not included in plans for a cattle toxicity trial.
4. Tests on the ability of the GM bacteria to colonise and protect rabbits or cats showed that the *B. fibrisolvens* strains did not colonise either species, after two oral doses of 10^8 bacteria per animal. The two *Bacteroides* strains did colonise the large intestine of both species at level up to 10^7 cells per gram of faeces, but toxicity tests proved that there was no change in the animals' susceptibility to fluoroacetate.
5. Following our application to perform a field trial of toxin protection in cattle, GMAC requested that experiments be conducted to establish how readily rumen bacteria (unmodified) were transferred between animals in open fields. It was shown that bacteria could transfer over distances of 30 metres. A plot of distance *vs* the inverse of the time taken for transfer, produced a straight line relationship, suggesting that transfer over longer distances could occur, given enough time.

GMAC advised that a toxicity trial on cattle would need to be contained, e.g. in PC2 animal facilities at the Australian Animal Health Laboratories (Victoria).

6. Additional Project funding ($100,000) was committed by MLA to pay research staff for the 6 months needed to complete the cattle toxicity trial. This was insufficient to support the 4 existing staff on a full-time basis, over the 6-month period. The Technical Assistant was reappointed on a 10 hours per week basis and the Director accepted half-salary for this period, while continuing to work full-time. These economies allowed both Postdoctoral Researchers to be retained full-time.

7. Preparations for the trial were made in collaboration with Dr Chris Prideaux (CSIRO, Geelong) to conduct the cattle toxicity trial at the AAHL facility. An application for GMAC approval was unnecessary for conducting PC2 work in a PC2 faciltity, which required simple notification under the regulations existing at that time. All materials were prepared and stored and GMAC permission obtained for shipping them to Victoria and transfer between the laboratories at Geelong and the animal containment facility at Werribee. In order to complete the trial before the change-over from GMAC authority to OGTR authority (June 21, 2001) the trial needed to commence on or before May 14, 2001.

8. At May 1, 2001:

   - Technical preparations were completed and cattle were available at CSIRO.
   - A contract was completed between Murdoch University and CSIRO for the use of CSIRO facilitlies and staff. This agreement could not be executed until funding was secured through a contract between Murdoch and MLA.
   - By May 14, the Murdoch/MLA contract had not been executed and the project could not commence after that date. Overlapping the changeover from GMAC to OGTR authority would have contravened the new regulations, under which PC2 work within a PC2 facility required specific licencing as a “Dealing Not Involving Release” (DNIR) which involves a 90 working-day process of approval, following submission of the application. Funds to support the research staff expired at June 30, leaving no time for preparation of a DNIR application.

(*Note: Applied Biotechnology Ltd [Qld] employed K.Gregg to prepare a DNIR application later in 2001. That licence was granted in February 2002.*)

Benefits to Industry

If released for use, this technology could benefit animal producers in the affected areas by reducing cattle losses of around $120,000 p.a. on each affected property (200 animals lost p.a. at approximately $600 per head). The bacteria do not provide total immunity to the toxin, but reduce the risk of mortality in poisoned animals. Low-level continuous ingestion will be combatted through a continual reduction in toxin levels in the rumen, thereby reducing the amount that reaches the absorptive parts of the animals’ digestive tracts. In areas where *Gastrolobium* species occur, livestock are kept away from them. Accidental exposure occurs when animals break through fences or plants grow undetected within “safe” areas. When encountering a novel plant, sheep and cattle will take an exploratory taste of the new feed. When a novel plant is poisonous, animals that survive their first taste are known to develop an aversion to those plants, avoiding them in the future. Therefore it is expected that “protected” animals that survive initial exploratory doses of *Gastrolobium* plants will learn to avoid those plants in the future.

Main Research Report

Background to Project and the Industry Context

In Australia, plants of the genus *Gastrolobium* produce high levels of monofluoroacetate (commonly referred to as fluoroacetate). Most species are limited to the south-west of WA, with one species (*Gastrolobium grandiflorum*) spread across the north of WA, the Northern Territory, and Queensland where it is known alternatively as "wallflower poison" (WA) or "heartleaf poison" (Qld). In the Georgina river basin of central Australia, the Georgina Gidgee (*Acacia georginae*) also produces fluoroacetate, although at a lower level than in the *Gastrolobium* species.

Fluoroacetate is a highly toxic compound that enters aerobic metabolic pathways through the Citric Acid Cycle, where it is converted to fluorocitrate. Fluorocitrate binds to the enzyme Aconitase, and inhibits any further activity by that enzyme, blocking the Citric Acid Cycle and cellular energy production. This results in the death of cells and tissues, which, if the dose is sufficient, leads to the death of the victim. For most mammals, the dose required to cause death is very small: ranging from 0.04 mg/kg bodyweight for dogs to around 0.5 mg/kg for goats. The LD_{50} for most mammals is similar to that of Potassium Cyanide, which also attacks the energy producing pathways. Sodium fluoroacetate has been used as a pesticide, under then name of Compound 1080, where it has proven highly effective against feral pests such as rabbits and foxes. The use of 1080 has been particularly successful in the south-west of Western Australia, because native plants in the area naturally contain fluoroacetate. Therefore, native animals have evolved with the toxin and show tolerance approximately one hundred-fold higher than the introduced pests.

Throughout the Georgina Gidgee region, around 200 cattle per year are lost to poisoning on affected properties. In northern Queensland, the heartleaf bush is more acutely toxic than the Gidgee tree and cattle are kept away from it. However, losses occur through accidental contact of livestock with the toxic plant. Similar poisoning of livestock occurs across the top of the Northern Territory, the north of Western Australia, and the south-west of WA, where the toxicity of fluoroacetate producing plants is extremely high.

The fluoroacetate detoxification project was initiated by a consortium of animal producers, who formed Applied Biotechnology Ltd (Qld) and funded initial research to examine the feasibility of a biotechnological solution to Gidgee poisoning of livestock. Initial work demonstrated that a cloned gene from a soil bacterium produced an enzyme that could detoxify fluoroacetate when it was expressed in the laboratory bacterium *E.coli*. AMLRDC (later MRC) agreed to fund a major project to complete the work that was begun with funding from Applied Biotechnology Ltd.

Previous Research:

Molecular tools developed by this group at UNE (Beard *et al*., 1995), were used to genetically modify four strains of the rumen bacterium *Butyrivibrio fibrisolvens*. In that work, a gene encoding the enzyme fluoroacetate dehalogenase was cloned from the naturally occurring plasmid pUO1 from the soil bacterium *Moraxella* species strain B, which was characterised by Kawasaki *et al*. (1992). More recently, Strain B has been reclassified on the basis of 16S ribosomal RNA sequence as *Delftia acidovorans* (Sota *et al*. 2002). The dehalogenase gene was attached to shuttle plasmid pBH*erm* and transferred to the four *B. fibrisolvens* strains OB156, OB291, OR85, and 10/1. This enabled the rumen bacteria to produce the enzyme fluoroacetate dehalogenase, which can break down the Gidgee toxin (Gregg *et al*., 1994; Attachment 1).

In 1996 – 1997, toxicity trials on sheep housed in PC2 containment at UNE showed that the four modified bacterial strains were able to colonise the sheep rumen and to reduce the toxic effects of fluoroacetate on them (Gregg *et al*., 1998; Attachment 2).

Transfer of the Fluoroacetate Detoxification Project to Murdoch University:

The Rumen Biotechnology team from UNE was transferred to Murdoch University in Perth, Western Australia, in late 1997, for the purpose of testing the modified bacteria in cattle. Initial plans were for a field trial, under double-fence containment, and some improvements in the modified bacteria were believed essential for a clear demonstration of efficacy in protecting cattle from fluoroacetate poisoning.

The first approach to the regulatory authority GMAC raised questions on the safety of the GM bacteria: e.g. the likelihood of the bacteria colonising non-ruminants and whether pest species may be protected

from 1080 poisoning (fluoroacetate is the active ingredient in 1080). On this basis, additional objectives were defined.

Project Objectives:

1. to increase the number of modified bacterial strains, so that the efficiency and dependability of detoxification could be at least double that used in the sheep toxicity trials.
2. To test the ability of GM rumen bacteria to colonise feral species such as rabbits and cats.
3. Where colonisation occurs, to test whether these monogastric animals could be protected against 1080.
4. To examine the ability of rumen bacteria (unmodified) to transfer between ruminants in the field, without direct contact between animals.
5. To complete a trial for the protective capability of the GM bacteria in cattle.

Detailed Methodology:

The work in this project comprised six main experimental sub-projects, which require individual description to present them clearly.

1. Isolation and genetic modification of additional *B. fibrisolvens* strains

Novel, Western Australian strains of *B. fibrisolvens* were isolated from fresh rumen samples obtained from sheep and cattle at the Murdoch University Veterinary Farm. Samples were plated on rumen fluid agar plates, and screened microscopically for narrow, curved, motile organisms that could be classified as *B. fibrisolvens*. Strains identified phenotypically as *B. fibrisolvens* were tested for their ability to be transformed by the shuttle plasmid pBHf. Transformed strains were then classified conclusively by sequencing their 16S ribosomal RNA genes. Ribosomal RNA sequences were matched to those of species recorded in the GenBank database.

Results:

The novel *B. fibrisolvens* strains isolated in WA are listed in Table 1. Three new strains were modified, bringing the total number to 7, two from Canadian deer, three from cattle, and two from Australian sheep.

Table 1. Classification, source and taxonomic method for bacterial strains constructed at UNE (blue) and at Murdoch (red).

SPECIES & STRAIN	SOURCE	TAXONOMY
Butyrivibrio fibrisolvens OB156	White-Tail deer: Canada	16S rRNA
Butyrivibrio fibrisolvens OB291	White-Tail deer: Canada	16S rRNA
Butyrivibrio fibrisolvens OR85	Holstein steer: Canada	16S rRNA
Butyrivibrio fibrisolvens 10/1	sheep: Australia	phenotype/biochem
Butyrivibrio fibrisolvens 0/10	cattle: Australia	16S rRNA
Butyrivibrio fibrisolvens 149/33	cattle: Australia	16S rRNA
Butyrivibrio fibrisolvens S2/10	sheep: Australia	16S rRNA

Modified strains were tested for their production of fluoroacetate dehalogenase by lysing cultures in a reaction buffer and measuring the release of fluoride ions from fluoroacetate, using an ion-selective electrode. To ensure that the detoxifying ability would be effective in practical situations, tests were also performed to ensure that fluoroacetate present in the surrounding medium could be detoxified by growing cultures of the modified bacteria. Fluoroacetate was added to culture medium at experimental time 0, aliquots of culture were withdrawn at a series of time intervals, and the levels of fluoride present in the medium were measured by ion-selective electrode. The detoxifying ability of *B. fibrisolvens* strains in growing cultures is shown in Table 2. Levels of activity are expressed as a function of the soluble bacterial protein present within the culture. Experiments showed that the modified *B.fibrisolvens* strains detoxified fluoroacetate with the same efficiency, whether tested as growing cells or as cell extracts.

Table 2. Fluoroacetate dehalogenase activity measured in growing cultures of each recombinant *B. fibrisolvens* strain from work at UNE (blue) and from Murdoch (red).

Species	Strain	Activity nmol/min/mg prot.
B. fibrisolvens (UNE project)	OB156	12
	OB291	10
	OR85	8
	10/1	5
Sub-Total Activity (UNE *B.fib* strains)		**35**
B. fibrisolvens (Murdoch Uni project)	S2/10	23
	0/10	8
	149/33	7
TOTAL ACTIVITY		**73**

The stability of plasmids in the modified bacteria was measured by the frequency with which untransformed revertants were generated when selective pressure was removed. The results from this study are shown in Table 3.

Table 3. The percentage of the bacterial population that lost the recombinant plasmid at each population doubling.

SPECIES	STRAIN	% PLASMID LOSS/GENERATION
B. fibrisolvens	OB156	0%
	149/33	0.19%
	10/1	0.435%
	S2/10	>3.0%
	0/10	0.57%
	OR85	2.08%
	OB291	0.28%

Conclusions:

The detoxifying capability of the seven *B. fibrisolvens* strains was more than double that of the original four strains used in sheep protection trials. The stability of plasmids varied, in culture, ranging from 0% to >3% loss of plasmid per population doubling. It is not certain how these figures relate to the retention of plasmid in the rumen. Plasmid loss did not appear to be related to a metabolic burden on the bacteria, since the cultures were not rapidly overgrown by the untransformed revertants.

The combination of modified strains was concluded to be sufficient for a toxicity trial on cattle.

2. Construction of shuttle vectors for genetic modification of *Bacteroides*

Background:

Methods for genetic transformation of *Bacteroides* have been developed in several laboratories, using both conjugation (Privitera *et al*., 1979; Hecht *et al*., 1991) and DNA transformation (Smith, 1985; Thomson and Flint, 1989; Smith, 1995). However, most molecular genetic studies on *Bacteroides* have involved colonic strains from non-ruminants, although some work has extended these techniques to related organisms from the rumen (Thomson and Flint, 1989; Shoemaker *et al*., 1991; Daniel *et al*., 1995; Aminov *et al*., 1998).

Ruminal *Bacteroides* strains have been reported to be more tolerant than many other species to environmental stresses such as lowered pH (Russell and Wilson, 1988) making them an excellent candidate for complementing the activity of modified *Butyrivibrio fibrisolvens* strains (Gregg *et al*., 1998). Despite advances made in understanding gene expression in ruminal bacteria, many questions remain unresolved regarding the molecular biology of these organisms and the development of agriculturally useful strains is far from routine. We have therefore developed systems for transforming ruminal isolates of *Bacteroides* with a long-term view to the development of chromosomal integration systems and for identification and characterisation of gene promoters. Of more immediate importance though, is to use them for the expression of the fluoroacetate dehalogenase gene. This report describes the construction of these plasmids and the expression of the heterologous dehalogenase gene in *Bacteroides* strains AR20 and AR29.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Bacteriophages, and Plasmids. *Bacteroides* strains AR20 and AR29 were isolated from the rumen of a sheep at the University of New England (Armidale, NSW, Australia; Hudman and Gregg, 1989). Sequences for their 16S ribosomal DNA were obtained by amplifying rRNA using the universal primers 27f and 1492r and sequencing them from both strands. Sequences are available through GenBank acquisition numbers: AF139524 (AR20) and AF139525 (AR29). *Bacteroides* strains were grown at 39°C in rumen fluid (RF) medium as described previously (Klieve *et al*., 1989). All manipulations of AR20 and AR29 were under anaerobic conditions, either in an atmosphere of CO_2:H_2 (95:5), in an anaerobic chamber, or N_2:H_2 (95:5) in a portable anaerobic bag. Plasmid pBI191 was propagated in *B. fragilis* 638 (Smith, 1985). Cultures of *E. coli* were grown in Luria-Bertani medium at 37°C. Construction of the recombinant plasmid pIF, containing the integration genes of bacteriophage ϕAR29 (Klieve *et al*., 1989; Klieve *et al*., 1991) in pTZ19U was completed by this group at UNE and has been described in Gregg *et al*. (1994b). Shuttle vectors pBA, pPPR and pBAC were propagated in *E. coli* strains K803, PMC112, GM2929, or SCS110 (Raleigh *et al*., 1988), and in *Bacteroides* AR20 and AR29. Population growth of *Bacteroides* was monitored by automatic sampling of a stirred culture and periodic measurement of absorbance at 550 nm in a flow-through cuvette. Transformed cultures were monitored in the same way, except that clindamycin (5 μg/ml) was added to the medium to ensure that plasmid-borne genes must be expressed.

Construction of Plasmids. Plasmid DNA was isolated using alkaline lysis procedures described by Maniatis *et al*. (1982). Methods for DNA electrophoresis and restriction endonuclease digestion have been described previously (Woods *et al*., 1989). Primers used in PCR for plasmid construction and copy-number analysis are shown in Table 1.

Bacterial Transformation. Transformation of *E. coli* was by electroporation as described by Dower *et al*. (1988). Electroporation of *Bacteroides* strains was by a modification of that method as follows: Washing and electroporation of *Bacteroides* was performed under N_2:H_2 (95:5). Mid-log-phase cultures were chilled rapidly by swirling in an ice slurry, transferred to 40-ml centrifuge tubes (Beckman), and the cells pelleted by centrifugation at 5000 × g for 5 min at 4°C (Beckman JA20.1 rotor). The bacterial pellet was washed once by resuspension in 40 ml of ice-cold, anaerobic, 10% (vol/vol) glycerol, 1 mM dithiothreitol, 0.1 g/l resazurin, and centrifuged as above. The bacterial pellet was resuspended in 100 – 300 μl of the same solution, and placed on ice.

Table 1. Primers used in the construction of recombinant plasmids.

Primer	Sequence 5' → 3'
EC	AGAAGTGAATTCACCTGTAAGAAGTTACTAATG
CT	GGTATATGGATTGTAAAAGCTT
CPRfor	ACAGAATTCTGATTAATAATTTGT
CPRrev	TAGTAACTTCTTACAGGTGAATTCACTTCT
MGRfor	TATCGATTGTCACAAAAATGTTAACTTATGGACTTTCCAGGATTCAA
MGRrev	ATCGCAAGAACTAGTGTTTCA

Bases underlined indicate sequence changes to introduce new restriction sites.

Primers used to track AR20 genomic DNA

GA3I - 5'- TGGCAGAAGTAGTGGAATATGCTG -3'

G2D2- 5'- TTGCCTCCGTCGGTGGTATT - 3' (PCR product size = 295bp):

Primers used to track pBA

SRep for- 5'- CTTCTCCAGTTTCAGTGTCGGCTAA -3'

SRep rev- 5'- GGCTGGTCCTCGGGCTATGAT -3' (PCR product size = 294bp)

Transforming DNA (1 – 5 μl containing up to 5 μg DNA) was mixed with a 40-μl aliquot of cells, transferred to a 1-mm path-length electroporation cuvette (Bio-Rad), and pulsed at a field strength between 10 and 25 kV/cm from a 25 μF capacitor, with a by-pass resistance of 200Ω. The cells were immediately transferred, using a syringe and needle, to Hungate tubes containing 1 – 2 ml of RF medium. The tubes were incubated at 39ºC for 1 – 4 h to allow recovery and expression of antibiotic marker genes. One ml of RF medium containing molten agar (20 g/l at 45ºC – 50ºC) was added to each tube, mixed rapidly, and poured as an overlay onto agar plates containing RF medium with 5 – 10 μg/ml clindamycin. Transformation plates were incubated at 39°C for 3 – 10 days, depending on the rate of colony development.

Determination of Plasmid Copy Number. The total DNA content of pBA-transformed AR20 cells was extracted from stationary-phase cells (Woods *et al*., 1989) and serially diluted in two-fold steps. Polymerase chain reaction was performed on dilutions of the DNA to determine the dilution at which the signal for chromosomal DNA was eliminated and the dilution at which signal for pBA was eliminated (primers shown in Table 1). The chromosomal marker was a segment of a single-copy endoglucanase gene isolated from AR20 (Woods *et al*., 1989; Vercoe and Gregg, 1992).

Measurement of Dehalogenase Activity. Dehalogenase activity was measured by release of fluoride ions, using an ion-specific electrode as described by Gregg *et al*. (1994a). Briefly this involved harvesting the cells by centrifugation at 5000 × g for 10 min, washing them in 1.0 volume of ice-cold 50 mM hepes buffer (pH 7.5) and resuspending in 0.2 volumes of the same buffer. The suspension was sonicated on ice-slurry for 4 × 15 s, with 15 s intervals, cell debris removed by centrifugation at 10 000 × g for 5 min, and the supernatant assayed for dehalogenase activity and protein content as described previously (Gregg *et al*., 1994a).

Plasmid Retention Tests. Transformed AR20 and AR29 were grown in antibiotic-free medium for a minimum of 15 subcultures (1:100 inoculum; total ≈100 doublings of population). Cells were plated at various dilutions on antibiotic-free plates, then colonies transferred by toothpick to both non-selective plates and to clindamycin plates. The percentage that failed to grow on clindamycin plates, while growing on non-selective plates was used to calculate the percentage of cells that lost their plasmids in each doubling of the population.

RESULTS

Shuttle Vector Construction. The construction of shuttle plasmids began with the assembly of pBA as a potential integrative vector (Fig. 1). It comprises replicons and antibiotic resistance markers for *E. coli* (ampicillin) and *Bacteroides* (clindamycin), plus integration-related sequences from bacteriophage ϕAR29 (Gregg *et al*., 1994b). The complete DNA sequence of pBA is available at GenBank Accession No AF203972. *********

Plasmid pBA was modified to construct the promoter rescue plasmid pPPR as shown in Fig. 2.

The bacteriophage integration determinants and part of the *mob*A gene, downstream of position +162 bp from the translational start-site, were deleted and the clindamycin resistance gene promoter was made removable by flanking it with *Eco*RI restriction sites. These modifications allow DNA fragments to be tested for their ability to function as gene promoters in *Bacteroides* by ligating them to the *Eco*RI termini of the linearised plasmid. Multiple attempts to transform AR20 and AR29 with the promoterless pPPR yielded no transformants, confirming that there was no significant transcriptional read-through of the clindamycin resistance gene from other promoter-like sequences in the plasmid. Reinsertion of the clindamycin-gene promoter into the *Eco*RI site of pPPR (pPPRcp) restored the ability of the plasmid to transform *Bacteroides* to the same level of clindamycin resistance. This demonstrated that the alteration of the DNA sequence 6 bases upstream of the putative clindamycin gene ribosome binding site, to create the new *Eco*RI site, did not significantly impair gene expression. As a further test, insertion of a PCR-generated copy of the pBA *rep*A gene promoter in place of the Cc^r promoter (pPPRrep) also restored the ability of the promoter rescue plasmid to transform AR20 and AR29 to clindamycin resistance. These results confirmed that pPPR can be used to identify DNA fragments with promoter activity in AR20 and AR29.

The coding sequence of fluoroacetate dehalogenase gene H1 was isolated by PCR amplification using plasmid pHS2 as template (Gregg and Sharpe, 1991; Gregg *et al*., 1994a) and inserted into pPPRcp to replace precisely the remaining 162 bp of the *mob* gene, creating the plasmid pBAC (Fig. 2a). Complete removal of the *mob* gene ensured that plasmids carrying a novel gene cannot initiate their own transfer to other bacteria, although transfer assisted by other epigenetic elements remains possible.

Bacterial Transformation. Optimum conditions for electroporation of AR20 were 15 – 20 kV/cm, with 25 μF capacitance, and 200 Ω by-pass resistance. The optimum recovery time before selective plating was 3 h at 39ºC. Results from these tests are summarized in Table 2.

Figure 1. Construction of plasmid pBA. The 2.6 kb *Hin*dIII/*Eco*RV fragment of lysogenic bacteriophage ϕAR29 was cloned into pTZ19U cleaved with *Hin*dIII and *Sma*I, to form pIF. pBI191 and pIF were linearised by cleavage with *Eco*RI and ligated to form the shuttle plasmid pBA. The ϕAR29 integrase and excisionase genes are shown as ORF2 and ORF3 respectively. MCS restriction sites: A, *Ava*I; K, *Kpn*I; E, *Eco*RI; S, *Sac*I; M, *Sma*I; B, *Bam*HI; X, *Xba*I; L, *Sal*I; P, *Pst*I; Sp, *Sph*I; H, *Hin*dIII. Amp^r, ampicillin resistance gene; Cc^r, clindamycin resistance gene; *Rep*A, replication control function for *Bacteroides*.

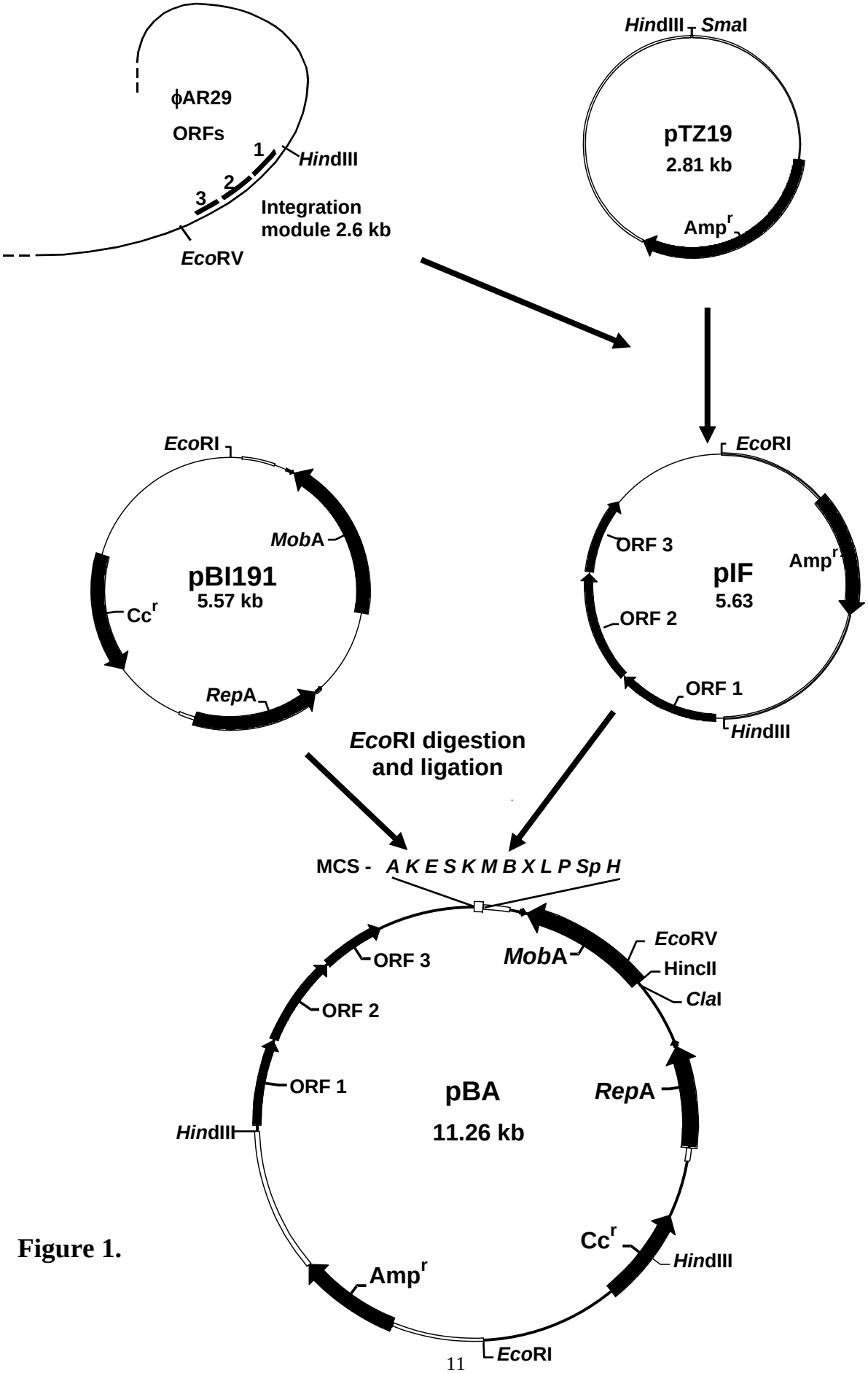

ϕAR29
ORFs
1
2
3
HindIII
Integration
module 2.6 kb
EcoRV
HindIII SmaI
pTZ19
2.81 kb
Ampr
EcoRI
MobA
pBI191
5.57 kb
Ccr
RepA
EcoRI
ORF 3
pIF
5.63
Ampr
ORF 2
ORF 1
HindIII
EcoRI digestion
and ligation
MCS - A K E S K M B X L P Sp H
ORF 3
MobA
EcoRV
HincII
ClaI
ORF 2
ORF 1
pBA
11.26 kb
RepA
HindIII
Ccr
HindIII
Ampr
EcoRI

Figure 1.

Table 2. Efficiency of transformation of AR20 with plasmid pBA.

Electroporation Field Strength kV/cm	Transformants/μg DNA $\times 10^{-4}$	Recovery Time (h)	Transformants/μg DNA $\times 10^{-4}$
10	5.1	1	0.03
15	12.0	2	4.0
20	10.3	3	20
25	9.2	4	24

Attempts to transform AR20 with the cryptic plasmid pBI191 extracted from *B. fragilis* 638 were unsuccessful. Similarly, *Bacteroides* strains were electro-transformed at extremely low efficiency with shuttle plasmids propagated in *E. coli* K803 or PMC112, giving less than 1 transformant/μg DNA. Total DNA was extracted from the first single putative transformant colony and restriction digests of the DNA showed a banding pattern typical of AR20 genomic DNA, overlaid with the characteristic restriction pattern of pBA (Fig. 3), demonstrating that pBA recovered from AR20 had not been integrated or significantly rearranged.

Figure 2.
(a) Plasmid pBA was digested with *Sph*I and *Eco*RV to remove *mob* gene sequences, downstream from position +162 of the *mob* transcriptional code, and a 1.1 kb fragment containing the dehalogenase gene and part of the pUK21 multiple cloning site (Vieira and Messing, 1991) was inserted. This construct was digested with *Hin*dIII and the 4.57 kb fragment containing pTZ19U and the upstream section of the *Cc*^{r} gene was ligated to the 2.6 kb fragment containing the downstream section of *Cc*^{r}, the *Rep*A gene, and the 162 bp remnant of the *mob* gene. The *Hin*dIII site* adjacent to the *mob* gene was inactivated by partial digestion, end-filling and religation to recircularise the whole plasmid, followed by selection of the construct in which the correct site was inactivated. (b) **Step A:** In an intermediary plasmid construct, the *Cc*^{r} promoter and the 5' half of the *Cc*^{r} gene ORF was removed by cleavage with *Eco*RI and *Hin*dIII, and replaced with a PCR product from primers EC and CT, which contained the region of the *Cc*^{r} ORF upstream from the *Hin*dIII site. This reformed the *Eco*RI site 23 bases upstream of the translational initiation codon of *Cc*^{r}. This process removed the *Cc*^{r} promoter to create plasmid pPPR. **Step B:** The complete *Cc*^{r} promoter region was PCR amplified using primers CPRfor and CPRrev, which conserved the *Eco*RI site at the distal end from the *Cc*^{r} gene and created a new *Eco*RI site at the proximal end. This fragment was inserted upstream of the promoterless *Cc*^{r} gene at the unique *Eco*RI site (pPPRcp). **Step C:** The dehalogenase gene H1 was amplified using primers MGRfor and MGRrev which introduced *Cla*I and *Spe*I sites at the 5' and 3' ends respectively. This was inserted into pPPRcp, which had been cleaved with *Spe*I and *Cla*I to remove the 162 bp fragment of the *mob* gene. The final construct (pBAC) was confirmed by DNA sequencing. MCS restriction sites: Bg, *Bgl*I; M3, *Xma*III; N, *Not*I; X, *Xba*I; Se, *Spe*I.

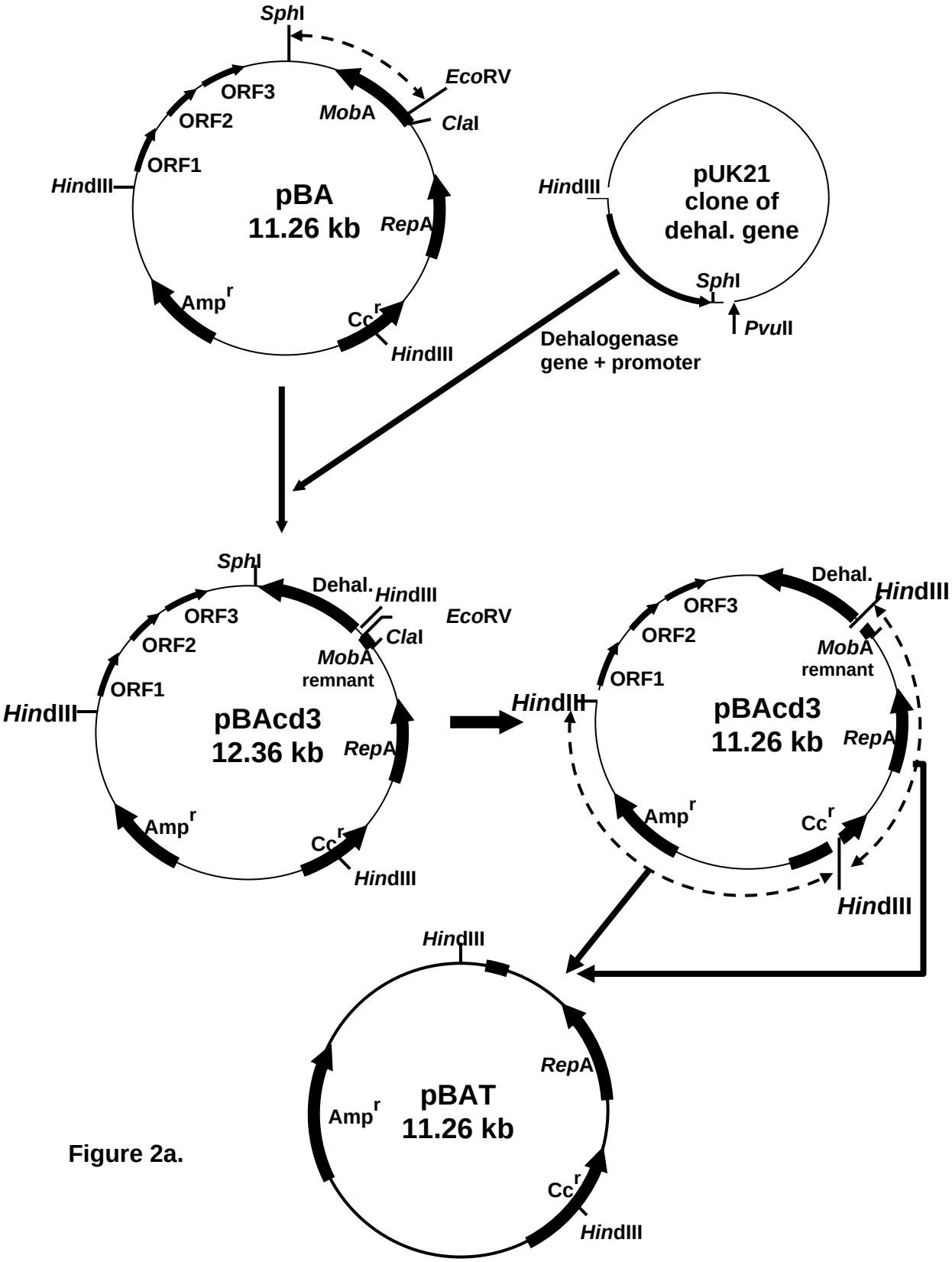

SphI
EcoRV
ClaI
ORF3
ORF2
ORF1
MobA
HindIII
pBA
11.26 kb
RepA
Ampr
Ccr
HindIII
pUK21
clone of
dehal. gene
HindIII
SphI
PvuII
Dehalogenase
gene + promoter
SphI
Dehal.
HindIII
EcoRV
ClaI
ORF3
ORF2
ORF1
MobA
remnant
HindIII
pBAcd3
12.36 kb
RepA
Ampr
Ccr
HindIII
Dehal.
HindIII
ORF3
ORF2
ORF1
MobA
remnant
HindIII
pBAcd3
11.26 kb
RepA
Ampr
Ccr
HindIII
HindIII
RepA
Ampr
pBAT
11.26 kb
Ccr
HindIII

Figure 2a.

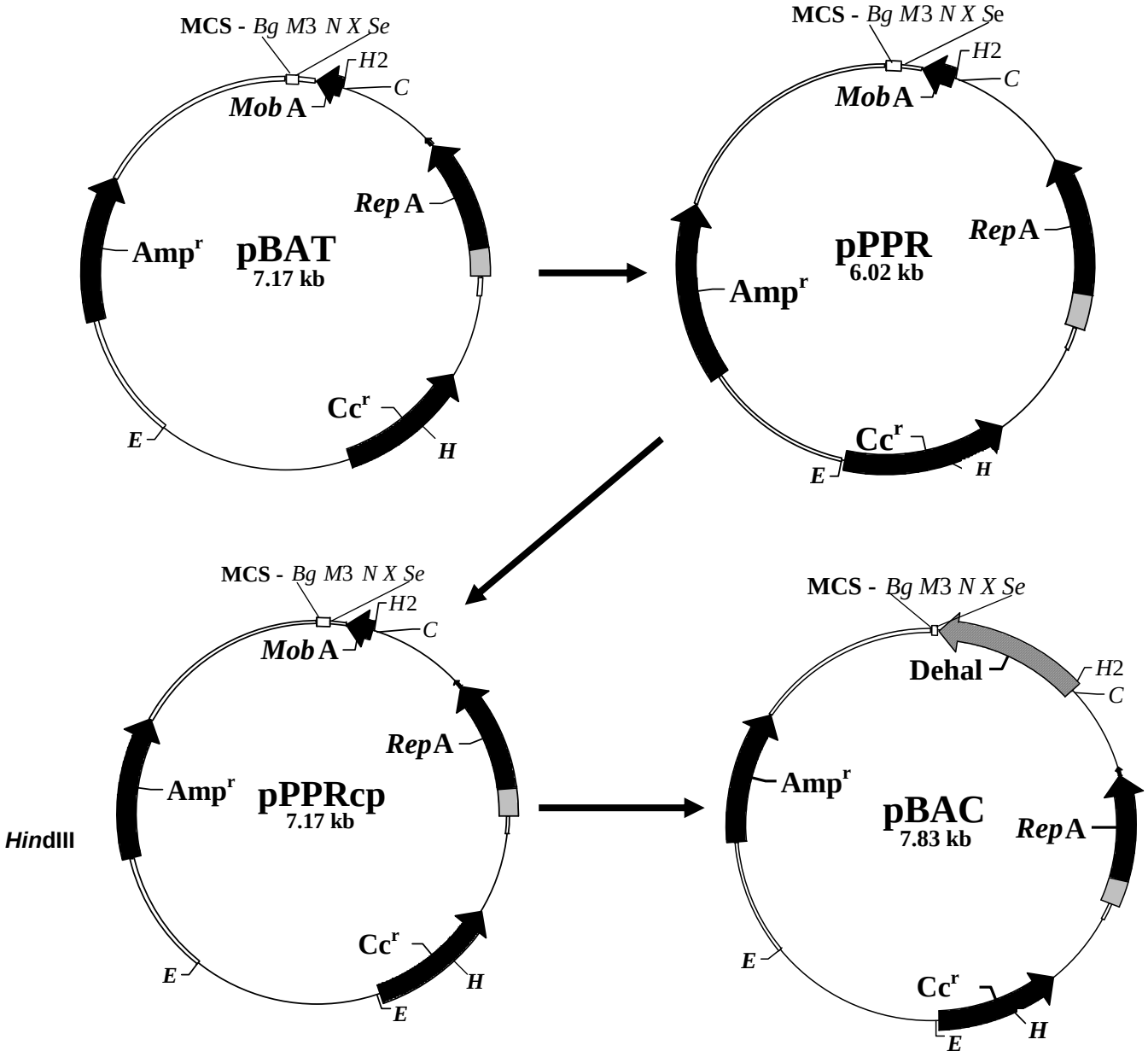

MCS - Bg M3 N X Se
H2
C
Mob A
Rep A
Amp^r
pBAT
7.17 kb
Cc^r
E
H
pPPR
6.02 kb
pPPRcp
7.17 kb
HindIII
Dehal
pBAC
7.83 kb

Figure 2b.

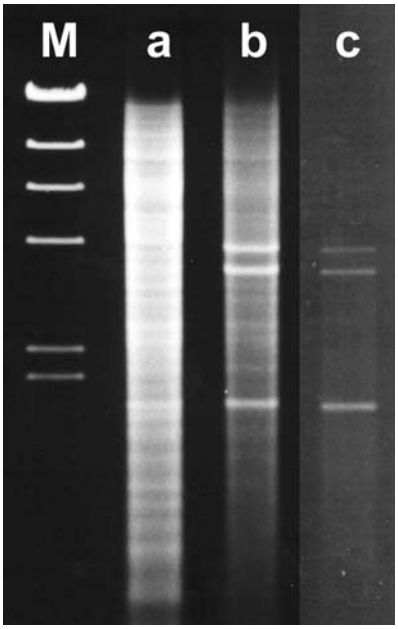


Figure 3. Agarose gel showing: (M) λ bacteriophage DNA digested with *Hin*dIII; (a) *Ava*I digest of genomic DNA from untransformed AR20; (b) *Ava*I digest of a total DNA preparation from AR20 transformed with pBA, showing plasmid bands against the genomic DNA background; (c) Purified plasmid pBA digested with *Ava*I;

Plasmid pBA propagated in AR20 gave maximum transformation efficiencies of $1 – 2 \times 10^5$ transformants per μg DNA, compared with fewer than one transformant per μg for plasmid pBA propagated in *E. coli* K803 or PMC112. Restriction-protected pBI191 DNA was recovered by isolating pBA from AR20, cleaving with *Eco*RI and recircularising the pBI191 component. This restriction protected form was able to transform AR20 at efficiencies comparable with pBA. It was concluded that AR20 possesses a formidable restriction barrier to DNA from *B. fragilis* 638 and from *E. coli* K803 and PMC112. In contrast, plasmids pBA, pBAC, pPPRcp, and pPPRrep, isolated from *E. coli* strains SCS110 or GM2929, which lack DNA methylation mechanisms, was able to transform AR20 and AR29 at $10^2 – 10^3$ transformants/μg DNA. These results suggest that part of the *Bacteroides* restriction systems may involve the recognition of "foreign" methylation patterns.

Electrocompetent AR20 cells snap frozen and stored at –70ºC were found to transform with 10-fold lower efficiency than freshly prepared cells. Washing cells with distilled water during preparation reduced transformation efficiency 1000-fold.

Stability of pBAC in Bacteroides AR20. When grown under non-selective conditions pBAC-transformed AR20 and AR29 exhibited an apparent plasmid loss of 0.16% and 0.11% per generation respectively. Loss of the native *Bacteroides* plasmid pBI191 from AR20 was significantly higher at 0.53% per generation. These observed frequencies could be influenced by differences in growth efficiency between transformed and untransformed cells. However, plasmid-bearing organisms showed no measurable reduction in population growth rate despite the energy requirements of replicating and transcribing the plasmid (Fig. 4). This was supported by the observation that plasmid loss from bacterial populations under non-selective conditions was a slow, progressive phenomenon.

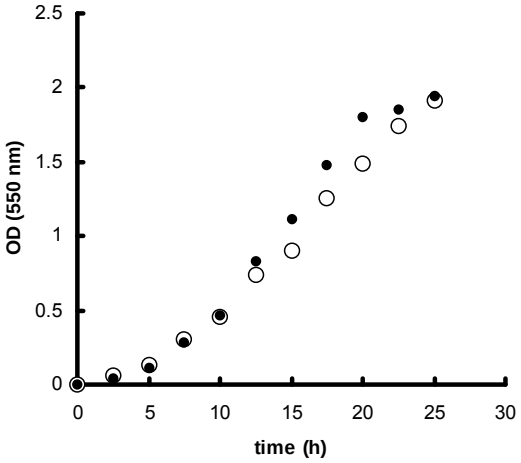


Figure 4. Growth curve of *Bacteroides* AR20 with (•) and without (o) pBA, measured as increase in OD_{550}. Curves are the average of two experiments each.

Mixed populations of transformed and untransformed bacteria were not rapidly dominated by untransformed cells. Serial two-fold dilution of total DNA preparations from AR20 showed that PCR signal from pBA was present in the 3 dilutions following loss of chromosomal signal. This indicated that AR20 contained around 8 copies of the plasmid per cellular genome.

Expression of Fluoroacetate Dehalogenase. from pBAC in AR20 and AR29. The fluoroacetate dehalogenase gene in pBAC was expressed in *Bacteroides* AR20 and AR29, with activities in unfractionated cell lysates of 7 nmol fluoroacetate/min/mg soluble cellular protein in AR20 and 4 nmol/min/mg in AR29. Fluoroacetate added to bacterial cultures was defluorinated significantly less rapidly at 0.45 ± 0.04 and 0.50 ± 0.04 nmol/min/mg soluble cellular protein in late log-phase cultures of

both strains. This was equivalent to 0.30 ± 0.03 and 0.29 ± 0.02 nmol/min/ml growing culture for AR20 and AR29 respectively. Parallel experiments measuring dehalogenase activity in cell-free supernatant medium showed 0.27 ± 0.01 and 0.33 ± 0.01 nmol/min/ml medium respectively. It was concluded that defluorination of fluoroacetate in the culture medium could be fully accounted for by traces of enzyme that were released from the cells, or from a small proportion of cells lysing in culture.

Discussion

The low efficiency of transformation of *Bacteroides* AR20 and AR29 with plasmid propagated in *E. coli* suggests the presence of highly effective restriction systems in these two strains. Enzymic studies on strain AR20 have demonstrated the presence of multiple nucleases (Hamdorf, 1998). Propagation of plasmid in non-methylating *E. coli* strains GM2929 and SCS110 reduced cleavage by AR20 endonucleases *in vitro* (Hamdorf, 1998), suggesting that at least one nuclease in AR20 recognises foreign methylation patterns on DNA. Transformation efficiencies of 10^2 – 10^3 transformants/μg unmethylated DNA, compared with <1 transformant/μg with DNA from methylation-competent strains of *E. coli*, indicated that a methylation-specific nuclease could reduce transformation efficiency by at least 100-fold and up to 1000-fold. The improvement created by the use of unmethylated exogenous DNA in transformation experiments has made this shuttle plasmid system a practical tool for genetic modification of these strains. If observations of methylation-specific nuclease are confirmed in future work, this enzyme could prove to be a valuable tool for the study of DNA methylation.

Plasmid pBAC expressed the *Moraxella* dehalogenase gene in *Bacteroides* strains AR20 and AR29 and was moderately stable, imposing no measurable genetic or metabolic burden to these bacteria. Although there was some loss of plasmid after repeated subculture without antibiotic selection, the plasmid-bearing bacteria were not rapidly outgrown by those without plasmid: a consequence that would be predicted if the expression of plasmid genes placed a significant burden on the host. The observation that the growth-rate of AR20 *in vitro* was not measurably impaired, when the expression of plasmid-borne genes was essential for growth in clindamycin, suggests that the extra genetic load caused no significant disruption to normal cellular function or impediment to replicative efficiency *in vitro*. Further research is required to ascertain whether this useful property is maintained *in vivo*.

In previous work it was shown that genetically modified *B. fibrisolvens* exhibited fluoroacetate detoxification levels that were very similar from both cell lysates and from whole, viable bacteria, even though dehalogenase activity was overwhelmingly intracellular (Gregg *et al*., 1994a). In contrast, the dehalogenase activity of whole, viable AR20 and AR29 cultures was only a small fraction of the activity of lysed cells, presumably because *Bacteroides* lack an active transport for the uptake of fluoroacetate. In contrast, *Butyrivibrio* have active mechanisms for the uptake of acetate, which can be regulated according to their environmental conditions (Latham and Legakis, 1976). Fluoroacetate appeared to be actively transported into *B. fibrisolvens*, judging by the comparable levels of defluorination observed from intact cells and equivalent amounts of unfractionated cell lysate (Gregg *et al*., 1994a). Tests on the cell-free culture medium of AR20 and AR29 confirmed that the defluorinating activity seen when adding fluoroacetate to growing cultures could be entirely accounted for by activity associated with the medium alone. It is not clear at this stage whether activity in the medium resulted from a small loss of enzyme from viable cells, or from the lysis of a minority of cells within the culture.

From these results, AR20 and AR29 appear unlikely to be useful for the protection of ruminants against fluoroacetate poisoning, because toxin present in the surrounding medium was defluorinated at low efficiency.. However, the demonstration of stable heterologous gene expression is encouraging for the use of this plasmid system in other applications, such as the synthesis and storage of proteins rich in essential amino-acids. One interesting possibility is the expression of specific *Bacillus thuringiensis* toxins in *Bacteroides*, which could offer ways to control the breeding of harmful insects and parasitic nematodes in animal faeces.

Analysis of DNA sequence upstream of the dehalogenase gene in pBAC suggested that transcriptional initiation did not occur within that region. The nearest identifiable promoter sequences are those that regulate the *rep*A gene. If so, transcription would have to proceed through *rep*A gene, and continue through intergenic sequences to the dehalogenase gene. It is expected that the moderate level of heterologous gene expression observed in AR20 and AR29 might be increased significantly by adding promoter sequences immediately upstream of the novel gene. The promoter rescue plasmid pPPR was developed to identify promoter sequences applicable to this task.

3. Aerobic Transmission of Ruminal Bacteria

Background:

Ruminal bacteria are an essential part of the ruminant digestive system, processing many of the dietary components that mammalian enzymes cannot digest (Hungate, 1966). The ruminal microflora is acquired at an early age, but the precise mechanism by which this occurs has not been reported. We have observed horizontal transmission of a Butyrivibrio fibrisolvens strain within a herd of cattle in open pasture (Gregg et al, 1993), and we have observed transmission of B. fibrisolvens between sheep that were separated by 2 metres, within an enclosed room (G. Allen unpublished). In the former case it was assumed that direct contact between the animals was largely responsible for passing the bacterium from one animal to another. However, the second observation suggested that anaerobic ruminal bacteria might survive long enough in aerosol droplets to be passed between animals.

The process of rumination ensures that the ruminant mouth contains bacteria freshly regurgitated from the rumen. Eructation of ruminal gases can be expected to create aerosol droplets of saliva containing bacteria. Obligately anaerobic ruminal bacteria cannot grow with prolonged exposure to oxygen, but it is not precisely known how long they can remain sufficiently viable to pass from one animal to another. Experiments in which sheep were treated with surfactants to remove the ruminal protozoa showed that the animals remained free from protozoa while they were separated from untreated sheep by a distance of 3 metres (S. Bird, University of New England, personal communication). The obligately anaerobic bacteria of the rumen might be expected to have similar limits on their horizontal transmission.

For reasons of containment, before conducting open-air trials with the GMOs, it was necessary to establish the distance over which these rumen bacteria can be passed from one animal to another. A test enclosure for toxicity testing could then be constructed with an exclusion zone that provides a satisfactorily high probability of containment. In open enclosures, bacteria may be transmitted from one animal to another by insects, rodents, wind-borne dust or aerosol droplets. In establishing the distance of transfer for safety purposes, it is important to include these variable factors. The precise environmental conditions would also be expected to govern the spread of these often delicate microbes.

This section reports tests on the transmission of ruminal species *B. fibrisolvens*, *Bacteroides uniformis*, and *B. thetaiotaomicron* between animals in open-air pens.

Material and Methods

Bacterial strains. *B. fibrisolvens* strains OB156, OB291, and OR85, were obtained from Drs R. Forster and R. Teather (Agriculture and Agrifood Canada). *Bacteroides* strains AR20 and AR29 were isolated by Dr J.F. Hudman at the University of New England and identified by 16S ribosomal gene sequence (GenBank acquisition numbers: AF139524: AR20; AF139525: AR29).

Animals and animal enclosures. Sheep used in this experiment were cross-bred Merino/Dorset ewes and wethers between 1 and 2 years of age. Goats were Angora does and bucks of similar age. Animals were housed in outdoor pens, provided with water *ad libitum*, and fed 1 kg each, once a day, of oaten chaff:alafalfa chaff (lucerne) in the ratio 3:1. Shelter from rain and sun was provided by metal roofing over approximately 20% of the pen. Raised metal grids were provided, under shelter, to allow the choice of dry ground during rain.

The arrangement of pens within the trial area is shown in figure 1. Five sheep in pen 1 were inoculated with the target bacteria. Pens 2 - 6 each contained five sheep and two goats and were respectively located: 0.5 m, 5 m, 10 m, 20 m, and 30 m from pen 1. Access for feeding and sampling was by a spiral path, ensuring that workers were never closer to the inoculated animal pen, than the next pen with which they had contact. After leaving the inoculated animal pen, boots and gloves were washed and sterilised and workers left by the route that took them furthest from all pens. No worker was allowed to return to any pen, within two hours of contacting a closer pen.

Figure 1
Pen Arrangement

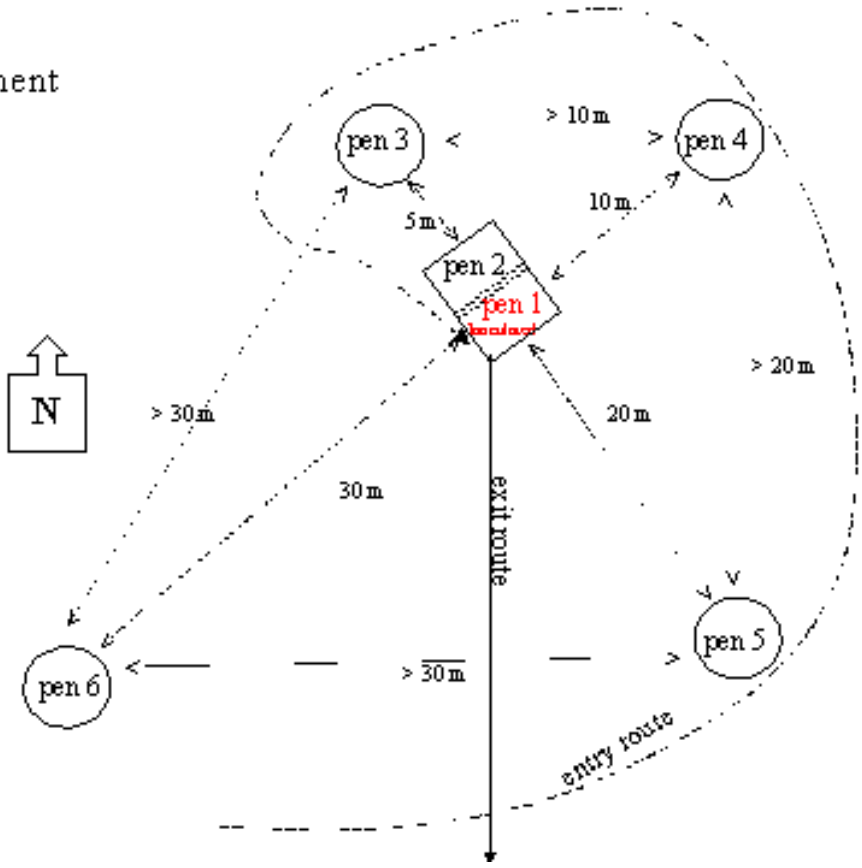
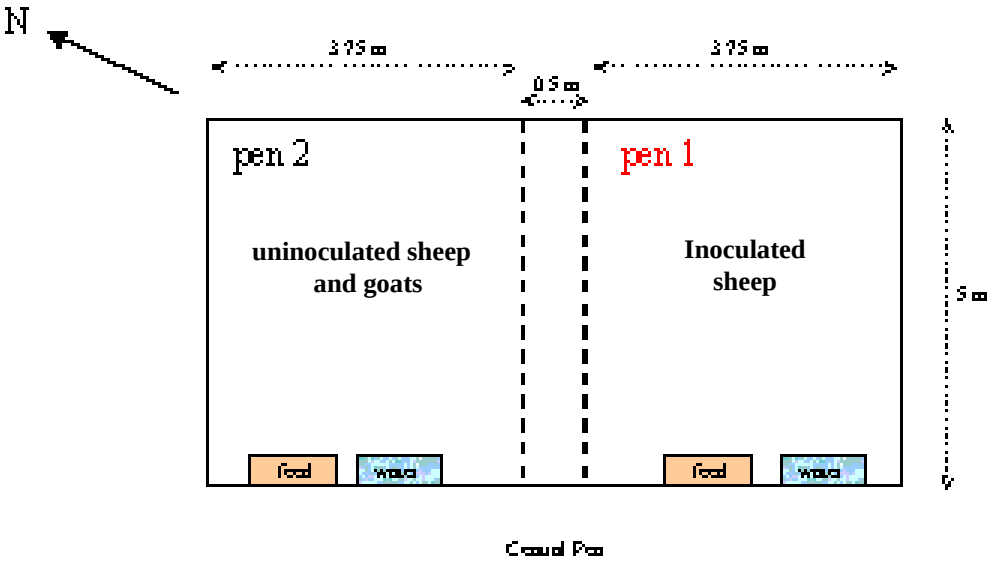


Figure 1(ii)
Structure of pens 1 & 2.



Casual Pen

Weather conditions. Details of wind speed and direction, rainfall, and temperatures, were provided by the Murdoch University weather station, which was located approximately 400 metres north-east of the test area.

Ruminal inoculation and sampling. Stationary phase cultures of *B. fibrisolvens* and *Bacteroides* strains were grown in rumen fluid medium (Klieve *et al*., 1989) in which the peptone component had been replaced by yeast extract (Oxoid). Cultures of the different organisms were pooled, harvested by centrifugation, and resuspended in sterile, anaerobic, distilled water equal to their original volume. Five sheep were each inoculated with 5 ml of the mixed five strains, by inserting a throat tube approximately 20 cm into the esophagus and delivering the cell suspension with a syringe.

Rumen samples were obtained by inserting a tube via the esophagus into the rumen and applying negative pressure with a 50-ml syringe.

Sample preparation and detection of bacterial strains. Rumen fluid containing particulate feed material was centrifuged at 14,000 × g, for 5 minutes, resuspended in an equal volume of sterile water, and recentrifuged. The pellet was washed once more in one volume of water and finally resuspended in one volume of water.

Polymerase chain reaction (PCR) was used to detect the presence of the target bacteria in 1 ml of washed rumen particles, using primers that amplified a characteristic region of the chromosome. All animals were tested before commencing the experiment, to ensure that no organisms naturally present in the rumen gave a positive signal with the PCR detection process.

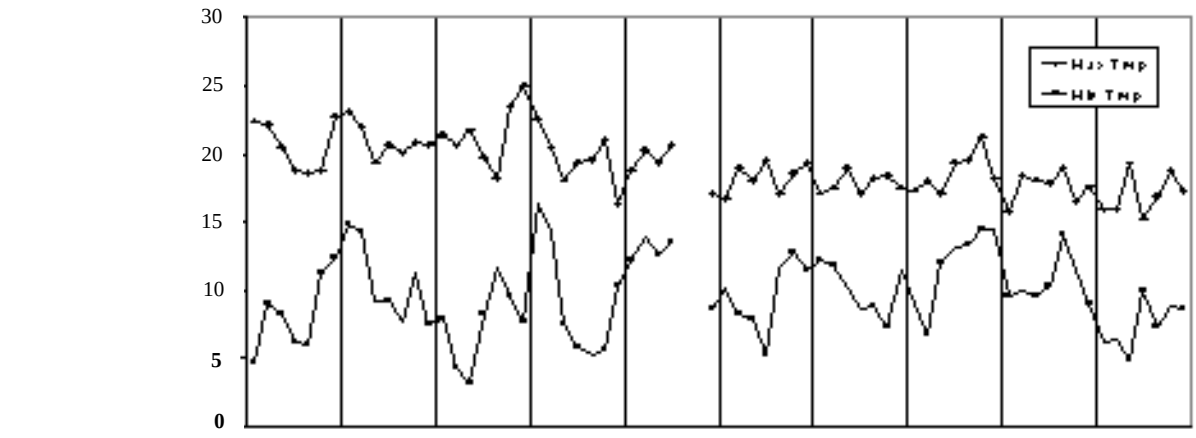
PCR was performed over 35 cycles, with 30 s at 94°C, 30 sec at 60°C, and 35 sec at 72°C for each cycle. Products were analysed by electrophoresis on 1.3% agarose gels in TBE buffer. Positive controls were preinoculation rumen samples to which washed target bacteria had been added. Negative controls included both a reaction blank, with water in place of rumen sample, and preinoculation rumen samples.

Results

Weather Conditions. The rainfall, wind-speed, wind direction, barometric pressure, and minimum and maximum temperatures are shown in figure 2.

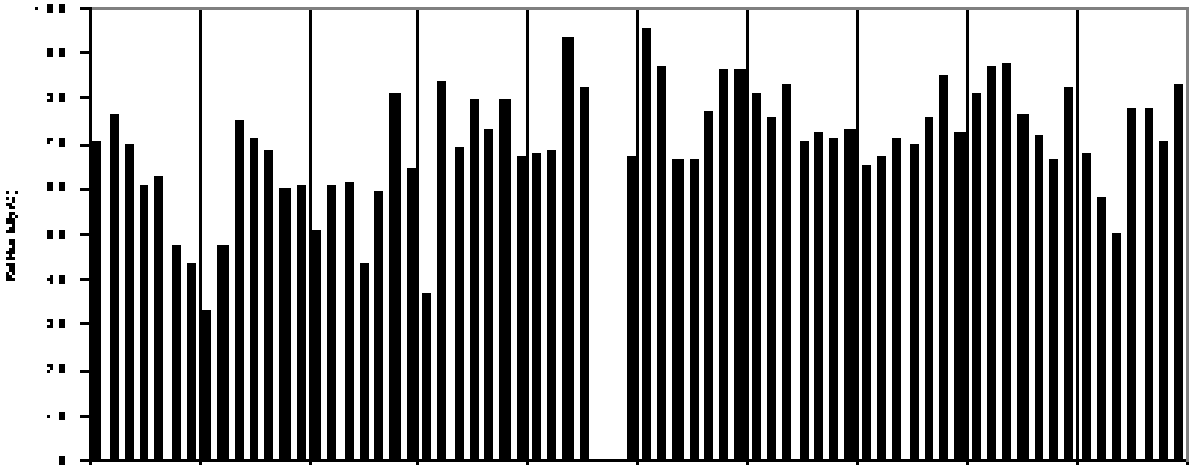
Figure 2. **Weather conditions over the 10-week period of the experiment. The weather station is located approximately 400 m north-east of the test site.**

Max. and min. temp.



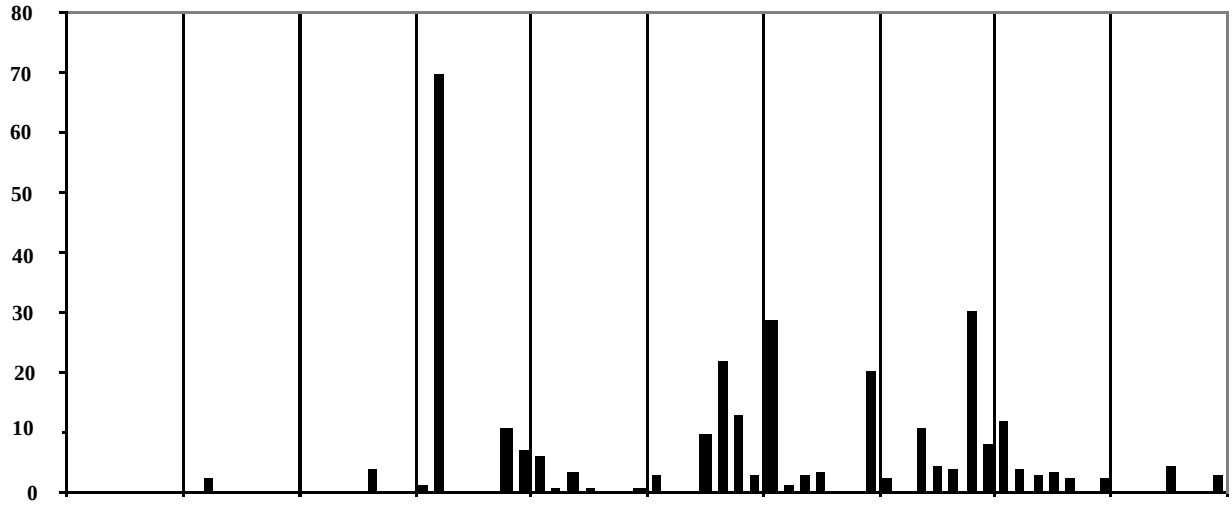
Rel. Humidity (%)

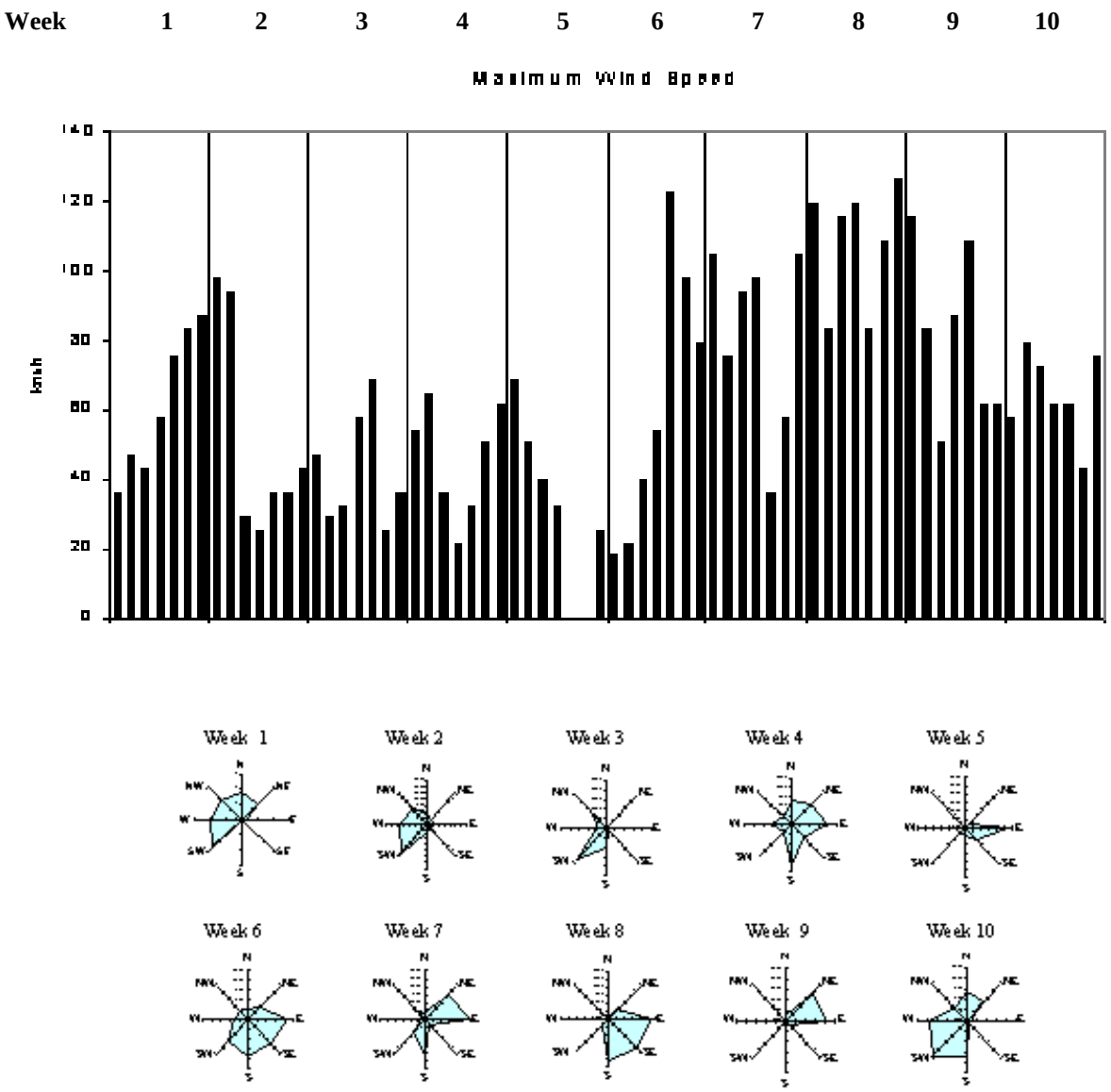
Weeks: 1 2 3 4 5 6 7 8 9 10



Rainfall (mm)

Weeks: 1 2 3 4 5 6 7 8 9 10



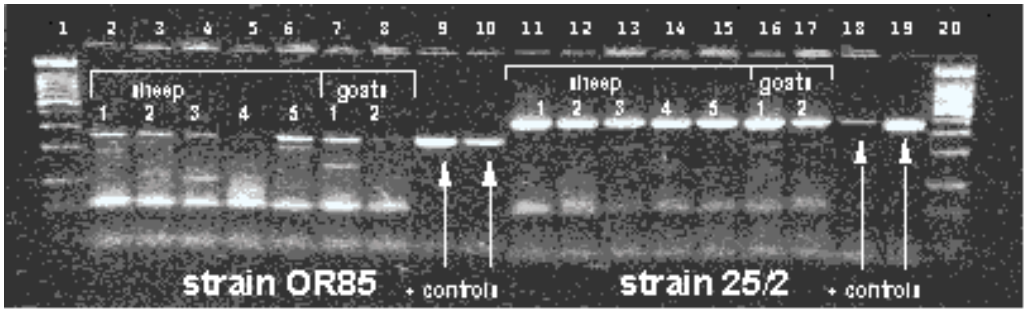


Radar Graphs (above) showing the wind direction during the field trial period. The shaded area represnts direction and proportion of time in that direction. Note: Figures indicate the direction towards which the wind was blowing.

Tracking GM bacteria in rumen contents

Typical tracking results, with positive signals are shown in figure 3.

Figure 3. Group 6 (30 metres from pen 1) Week 10. Lanes 1 and 20: 100 base-pair ladder mixed with lambda/HindIII markers. Lanes 2 - 8: rumen samples from sheep and goats, 10 weeks after inoculation of animals in pen 1, tested with primers specific to B. fibrisolvens OR85. Size of PCR product is 250 base pairs. Lanes 9 and 10: positive controls comprising whole washed cells or genomic DNA (respectively) of OR85 added to washed rumen particles from pre-inoculation samples. Lanes 11 - 17: rumen samples from the same sheep and goats tested with primers able to detect both B. fibrisolvens 25/2 and OR85. Size of PCR product is 372 base pairs. Lanes 18 and 19: positive controls comprising whole washed cells or genomic DNA (respectively) of strain 25/2 added to washed rumen particles.



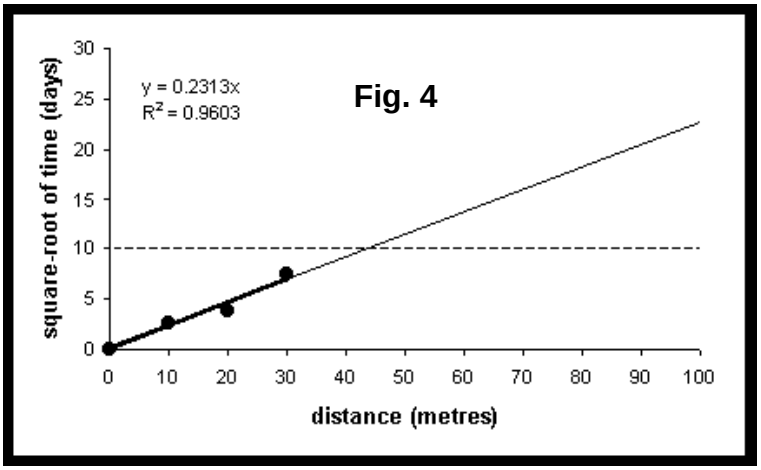
A summary of bacterial detection results across the 6 pens is shown in table 3.

Table 3. Detection of tracker strains in animals at increasing distance. nd = not determined

TIME AFTER INOCULATION (days)	DISTANCE FROM INOCULATED ANIMALS					
	0	0.5 m	5 m	10 m	20 m	30 m
3	-	-	-	-	-	-
10	+	-	-	-	-	-
17	+	+	+	+	-	-
24	+	+	+	+	+	-
38	+	+	+	+	+	-
45	-	+	-	+	+	-
52	nd	nd	+	+	+	-
59	nd	nd	nd	+	+	+
65	nd	nd	nd	nd	+	+

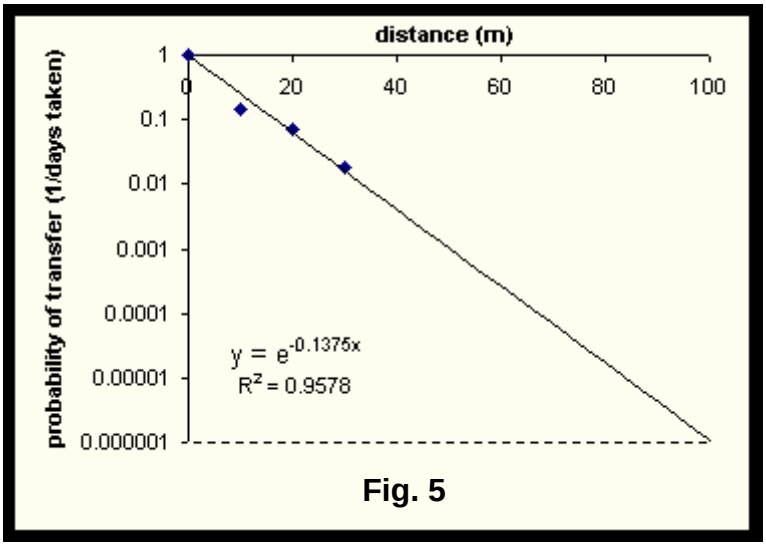
When plotted as distance of transfer (x-coordinate) *vs* square-root of the time taken for transfer to be detected (days; y-coordinate) an apparently linear relationship was obtained.

Figure 4. Time taken for bacteria to transfer to animals at various distances. Day 0 on the graph represents the first day at which tracker strains were detected in the inoculated animals (day 10 of the trial). The point for the 5 metre pen has been omitted because the first measurement after day 0 was day 7. Transfer to the 5 metre pen appears likely to have occurred earlier than day 7 since transfer to the 10 metre pen was observed in that time.



It is highly probable, that transfer to the 5 metre pen occurred significantly before transfer to the 10 metre pen, but samples were not collected in the period between days 3 and 10. Because the detection of bacteria in the 5 metre pen could be misleading, almost certainly occurring at an earlier time, this point has been omitted when extrapolating to establish probability of transfer beyond the test distances. Figure 5 shows extrapolation of the data to 100 metres, vs probability of transfer.

Figure 5. Probability of bacterial transfer each day. Probability was defined simply as the reciprocal of the time taken for transfer (in days).



Discussion.

Transfer of bacteria between ruminants that have direct contact with one another is probably the mechanism by which young ruminants acquire their ruminal flora. The ability of these obligately anaerobic organisms to survive for a few minutes when exposed to oxygen makes transfer feasible, without direct contact between animals. Transfer through direct contact appears to occur rapidly, since the presence of a particular bacterium within a pen, was detected in more than one animal, on all occasions. This was particularly notable with the 30 metre pen, where transfer was delayed for 7 weeks after the first detection of the tracker bacteria in the inoculated sheep. There was, nevertheless, widespread occurrence of bacteria among the seven animals when transfer was first detected in that pen (Figure 3).

Movement outward from a single source is often subject to the inverse-square rule, in which increasing the distance by a factor of 2 decreases the observed effect by a factor of 4. Consistent with this, a plot of distance vs the square-root of the time taken for transfer to occur (in days) produced a reasonable fit to a straight line The mechanism of transfer in this case is not known, although carriage of aerosols or insects by wind is likely. The presence of wild birds and rodents in the area might account for transmission of organisms, but the 30 metre distance might be expected to be breached more quickly if large vectors were involved.

The inverse square relationship would imply that the time of transfer would be linearly related to the square of the distance between inoculated and uninoculated pens. However, plotting the data in this way did not produce a line from which extrapolations could be made. In contrast, plotting the square-root of the time taken for transfer against linear distance provided an apparently linear relationship. In addition, plotting the distance of transfer *vs* the inverse of the time taken also produced an apparently straight line. The regression line produced from these data provided a method for extrapolating to longer time-periods to estimate the probability that transfer would occur.

The effects of weather conditions on transfer of obligately anaerobic bacteria may be significant. Throughout this experiment, wind direction and speed varied as shown in Figure 2, with no particular bias to wind direction over the ten-week period.

In summary, this experiment shows that transfer of ruminal bacteria between animals without direct contact can occur over at least 30 metres. The sensitivity of the bacteria to oxygen and to desiccation did not prevent transfer, probably because the time taken for wind-born particles to travel 30 metres can be very short. During the test period, maximum wind speeds for each day varied from 20 km/h to 120 km/h. At those speeds, aerosol droplets could cover the 30 metres in 0.9 – 5.4 seconds. The potential for obligately anaerobic bacteria to remain viable over such short times is extremely high.

As the distance increases, the major factor in the probability of transfer is likely to be the probability of the bacteria being taken quickly into an anaerobic environment. Animals with prefermentative digestive systems offer a suitably rapid return to anaerobic conditions if the bacteria are swallowed. Non-ruminant animals do not provide hospitable conditions to ruminal bacteria that may be swallowed, because of the proteolytic, acid-hydrolysis processes that occur within the monogastric stomach. Therefore, the transmission of these bacteria to monogastric animals is predicted to follow an entirely different pattern and to occur with considerably lower probability, governed principally by the ability of the bacteria to survive within a gastric environment.

4. Survival and Effects of Genetically Modified Rumen Bacteria in Rabbits

It has been proposed by ideological opponents of this GM technology, that rumen bacteria modified to detoxify fluoroacetate might confer resistance to the pesticide 1080 on feral species such as rabbits, cats, foxes, etc. The proposal is biologically untenable because the physiology of monogastric (non-ruminant) animals allows absorption of fluoroacetate before it reaches the caecum or colon: the only sites of significant bacterial fermentation. On this physiological basis, modified rumen bacteria cannot be expected to protect a monogastric animal from poisoning with 1080. Nevertheless, the importance of 1080 control of monogastric pests was perceived by GMAC as being sufficiently great that the inability of the modified rumen bacteria to protect these animals should be demonstrated empirically.

Methods

Housing and care of rabbits. New Zealand White Rabbits were housed singly in cages of 0.25 m^2, fed a diet of commercial pelleted rabbit food. Water was available at all times.

Oral inoculation of rabbits with GMOs. An anaerobic suspension containing equal quantities of nine strains of GMO (two *Bacteroides* + seven *Butyrivibrio*) was delivered orally, to each of ten rabbits. The 1-ml inoculum, containing equal quantities of each strain totalling approximately 10^8 cells, was delivered on day 1 and again on day 8 of the experimental period. Delivery was via a flexible silicone tube attached to a 5-ml syringe.

Screening for presence of GMOs. Faecal pellets were collected from the rabbits, macerated in sterile water, and the particulate material was washed twice by centrifugation and resuspension in sterile water. A series of dilutions was prepared from the suspension and analysed by PCR for the presence of the dehalogenase gene. Samples that gave positive results for the presence of the dehalogenase gene were also tested by PCR using primers specific for the *Butyrivibrio* plasmid pBHf and the *Bacteroides* plasmid pBAC.

Toxicity testing. Inoculated and uninoculated rabbits were dosed, orally, with 0.5 mg fluoroacetate for each kg of body weight (125% of the published LD_{50}). Clinical condition and behaviour were monitored for 24 hours.

Results

Colonisation by GMOs. From four weeks after the first inoculation with modified rumen bacteria (day 28), rabbit faeces showed positive PCR signal when analysed for the presence of the dehalogenase gene. It was calculated that GMOs were present at between 10^4 and 10^7 cells/g faeces, varying among rabbits and among sampling times.
Specific tests for the two different recombinant plasmids showed that the dehalogenase gene was present only in the form of the *Bacteroides* plasmid. The *Butyrivibrio* plasmid was not detectable in the rabbit faeces.

Response to Fluoroacetate. Both inoculated rabbits and uninoculated controls showed no obvious signs of toxic effects until acute symptoms occurred. At various times, individual animals showed an acute response, characterised by muscular spasms for 5 - 10 seconds, loss of conciousness, and death. The response from the two groups is shown in Figure 6.Histological analysis of tissues from the two groups showed no difference between inoculated or uninoculated animals.

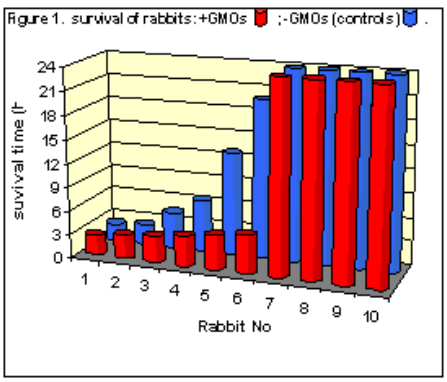


Figure 6. Survival of rabbits with GMOs (red) compared with rabbits without GMOs (blue). The Y axis shows the time for which rabbits survived, up to the end of the experiment at 24 h.

Conclusions

All seven strains of *B. fibrisolvens* failed to colonise the gastrointestinal tract of rabbits, despite inoculation with large numbers of viable cells on two occasions.

Bacteroides strains inoculated in similar numbers were able to colonise rabbits and were detected in faeces at levels of 10^4 – 10^7 cells/g.

The presence of *Bacteroides* carrying the dehalogenase gene in the rabbit digestive tract made no measurably difference to the rabbits' survival, when dosed with fluoroacetate.

It was concluded that the fluoroacetate degrading GMOs constructed in this project provide no measurable protection for rabbits against fluoroacetate (1080). This is consistent with the biological fact that fluoroacetate eaten by a rabbit is absorbed in the stomach or small intestine, before reaching the normal site of bacterial colonisation in the large intestine and caecum.

5. The Effects of Genetically Modified Rumen Bacteria in Cats.

The ability of the GM rumen bacteria to colonise the digestive tract of cats was also investigated by oral inoculation. The effect of inoculation on fluoroacetate toxicity was measured by the clinical, pathological, and histological comparison of test and control groups.

Experimental Methods

Inoculation of cats with live GMOs. Ten female cats, between 5 - 14 months old were separated into two groups that were matched as closely as possible on their age, and size. The five test animals were inoculated orally on day 1, with 1 ml of a bacterial suspension that contained equal amounts of the two GM strains of *Bacteroides* and the seven GM strains of *Butyrivibrio* (10^8 bacteria per ml, total). The inoculation process was repeated on day three.

Screening for the presence of GM bacteria in cat faeces. Faeces were collected twice a week, and samples extracted to perform PCR analysis on the DNA. PCR primers used in the analysis amplified the fluoroacetate dehalogenase gene. Subsequent tests used PCR primers that specifically amplified part of the plasmid carried by *Bacteroides*, or the plasmid carried by *Butyrivibrio*. Products of PCR were analysed by electrophoresis on agarose gels, visualised by ethidium bromide staining.

Testing the effects of fluoroacetate. Toxicity tests were performed by dosing the cats (orally) with 0.25 mg of fluoroacetate per kg body weight, delivered as an aqueous solution at 10 mg/ml. This dose is 25% above the published LD_{50} for cats, which is 0.2 mg/kg. The dosing of test-cats was performed at 11:00 am, and over the following 24 hours the cats were monitored for clinical symptoms. At 25 hours they were euthanased with barbiturate and post mortem examination was made to record pathological changes in each animal. Control groups were tested in the same way the following day as the inoculated test animals, except that the experiment was commenced at 8:00 am.

Results

Colonisation by the GMOs. Faecal samples from the five inoculated cats showed the presence of the dehalogenase gene, and plasmid specific PCR showed that it was present in the form of plasmid pBAC, which is carried by *Bacteroides uniformis* AR20 and *Bacteroides thetaiotaomicron* AR29 (16S rRNA sequence available at GenBank Nos BankIt261430 AF139524, AR20; and BankIt261431 AF139525, AR29). PCR results indicated that the plasmid-bearing organisms were present at 10^4 - 10^7 cells per gram of faeces. GM *Bacteroides* strains remained detectable in the faeces of the inoculated cats to the end of the 10-week test period.

Response to fluoroacetate. Clinical responses to the fluoroacetate dose of 0.25 mg/kg were recorded by the attending veterinarian. Some minor difference were observed between groups in the type of clinical response to fluoroacetate. However, the attending veterinarian interpreted these symptomatic differences as relating to the difference in feed-cycle, starting time of the experiments, and the corresponding difference in gut contents.

Both groups of animals showed pathological changes of similar nature and extent, although the gastrointestinal tract of the control animals contained less material than in the inoculated test animals. Cell damage in the cats caused extensive scouring of the large intestine. There was no significant difference between test and control groups.

Histologist's report on post-mortem tissue samples from experimental cats. Histological analysis of tissue samples from the cats is shown in table 4. Cats were listed in order from "most seriously affected" at the top of the list, to "least seriously affected" at the bottom of the list, as judged by clinical symptoms. These observational ratings did not appear to correlate clearly with the severity of histological symptoms. There was no significant difference between test and control groups in histological tissue damage.

PCR analysis of contents of gastrointestinal tract. Post-mortem analysis of contents from the proximal duodenum, distal duodenum, ileum, and colon, showed no recombinant organisms to be present when tested for the presence of the dehalogenase gene. The disappearance of GM *Bacteroides* strains from the colon was attributed to the extreme diarrhea caused by the toxin, which effectively scoured the lower gastrointestinal tract. It is not known whether recolonisation of the colon would occur if the animals were allowed to recover fully from a sub-lethal dose of fluoroacetate.

Table 4.	**HISTOPATHOLOGICAL FINDINGS**			
Rumen Biotech Fluoroacetate Toxicity Trials			**Test Animals** - inoculated	*Control animals* uninoculated
CAT I.D.	TISSUE			
	KIDNEY	LIVER	HEART	LUNG
Control 1	Histologically NAD	Mildly congested	Histologically NAD	Areas of moderate to severe congestion/haemorrhage & mild/moderate perivascular oedema
Control 2	Histologically NAD	Mild congestion and subtle peri-portal zonar pattern of mild hepatocyte swelling	Histologically NAD	Histologically NAD
Test 1	Mild congestion, mostly at cortico-medullary margin	Histologically NAD	Histologically NAD	Histologically NAD
Test 2	Mild congestion, mostly at cortico-medullary margin	Areas of mild congestion & peri-portal zonal pattern of mild hepatocyte swelling	Histologically NAD	Collapsed & mildly congested
Control 3	Histologically NAD	Areas of mild congestion & subtle periacinar hepatocyte swelling	Histologically NAD	Areas of moderate congestion
Test 3	Areas of mild congestion & moderate/marked infiltration of renal crest with mononuclear inflammatory cells forming lymphoid follicles*	Mildly diffusely congested	Histologically NAD	Collapsed & diffusely congested
Test 4	Mild congestion, mostly at cortico-medullary margin, & some renal tubular epithelial cells contained occasional darkish brown pigment granules	Mild increase in periportal fibrous connective tissue associated with mild lymphocytic infiltration in some areas	Histologically NAD	Collapsed, mildly congested with focal lymphoplasmacytic bronchiolitis
Test 5	Focal areas of mild lymphocytic infiltration of renal crest and ureter	Mild congestion and peri-portal zonar pattern of mild hepatocyte swelling, pale in some areas	Histologically NAD	Congested, mild chronic bronchiolitis in some areas
Control 4	Histologically NAD	Mildly congested	Histologically NAD	Severe diffuse congestion & mild perivascular oedema
Control 5	Histologically NAD	Mild periportal swelling	Histologically NAD	Histologically NAD

Note: NAD = No Abnormality Detected.

Cats are listed in the order: most severe clinical symptoms (top) to least severe clinical symptoms (bottom).

Discussion

The test group of cats showed clinical symptoms that differed slightly from the control animals. The major difference was the more obvious neurological effects of the toxin in the control group, during the observation period. Dr Allan Sheridan (the attending veterinarian) attributed this to a more rapid absorption of the toxin due to differences in gut contents. The animals were fed a dry-feed/wet-feed diet alternating daily. The day before the toxicity experiment, test cats received wet-feed (canned cat food). The toxicity test on control animals fell on a day following dry-feed (cat biscuits). This difference was reflected in the greater "gut-fill" of test animals during the toxicity test. Increased digestive tract contents cause toxin absorption to be more gradual (A. Sheriden, pers. comm.).

Despite those differences between groups, clinical observations ranked the animals to show no difference in severity of symptoms between the two groups (Table 4). The pathological and histological effects from

fluoroacetate poisoning were slightly more obvious in the test (inoculated) animals, but overall no significant difference could be drawn between the two groups. During the observation period, three animals from each group appeared likely to succumb to the toxin. However, despite the fluoroacetate dose exceeding the published LD_{50}, only one animal (control group, Cat 1) died from the effects of fluoroacetate poisoning within the 25 hour period.

Conclusions

The presence of two strains of genetically modified rumen bacteria in their lower digestive tract gave the inoculated cats no measurable protection from the effects of fluoroacetate. This is consistent with the result predicted from monogastric physiology which allows absorption of fluoroacetate before it reaches the fermentative regions of the gastrointestinal tract.

6. Preparations for the Cattle Toxicity Trial

Background:

Two applications were made to GMAC for approval to conduct a toxicity trial in cattle. Since containment facilities large enough to house ten cattle were not available in WA, it was proposed to conduct this trial in an open pen, surrounded by a vermin-proof safety zone of 100 metres. Following the demonstration that rumen bacteria could be transferred over at least 30 metres in the open field, GMAC did not approve an open pen trial, but suggested a contained trial at the facilities belonging to the Australian Animal Health Laboratory. This option simplified the preparations considerably, since the GMAC requirement for PC2-classified research in a PC2-classified facility did not require specific permission. It required only that GMAC be notified of the project. Communications with GMAC filled that requirement.

At 21 June 2001, the regulator of GM work in Australia (GMAC) was scheduled to be replaced by the newly formed Office of the Gene Technology Regulator (OGTR). This included changes to the approval and reporting requirements for any work involving recombinant organisms. Under OGTR regulations, the performance of PC2 work in a PC2 facility must be licenced. Licence application requires 90 working days for approval of the project. Therefore it was necessary for the cattle trial to be completed by June 21 to avoid breaching the new OGTR regulations.

Preparations:

In collaboration with Dr Chris Prideaux of AAHL, arrangements were made for the use of a cattle containment room at the CSIRO station at Werribee, Victoria. A group of cattle suitable for the trial was held at the facility. Electronic heart-rate monitors were purchased, to allow recording of physiological status during the trial. The seven strains of *B. fibrisolvens* were cultured for frozen storage and shipment, revived from these frozen stocks and tested to confirm their continued activity for detoxifying fluoroacetate. Materials for culturing bacteria for inoculation and for tracking GM bacteria in rumen samples were packed for shipment to the AAHL laboratories. Approval by the CSIRO Ethics committee was for work to be conducted at AAHL was completed by early May, 2001.

Administrative requirements:

Two contractual agreements were required for the toxicity trial to commence.

- An agreement was required between MLA and Murdoch University, formally acknowledging that MLA would provide to Murdoch University the funds necessary for experimental costs and to pay operating, rental and staff cost to CSIRO.
- An agreement was required between Murdoch University as a principal and CSIRO as a sub-contractor, for the use of the AAHL animal rooms and staff. This agreement was a formal agreement that operating costs, staff allocation, and rental costs would be paid from Murdoch university to CSIRO.

Murdoch University was unwilling for the latter agreement to be executed before the former agreement was finalised. The commitment of funds to CSIRO, without formal commitment that MLA would provide the necessary funds, was seen as inappropriate financial practise.

Outcomes:

In order to complete the toxicity trial before 21 June 2001, the 4 – 5 week project needed to commence by 14 May 2001. At 14 May 2001, the contract between MLA and Murdoch University not been executed, and the contract between Murdoch University and CSIRO could not be executed. The trial was therefore unable to proceed. The possibility of starting the trial after 14 May was ruled out, since this would overlap the OGTR commencement date, and would place the researchers and their organisations in breach of the new regulations.

Equipment and materials important to the project were stored, to be available for future use. Bacterial strains were stored at -80°C in the State Agricultural Biotechnology Centre at Murdoch University. Research was wound up and terminated on 30 June 2001.

Success in achieving objectives

The objectives of the Project changed somewhat during its progress, on the basis of advice and requests from GMAC. All of the objectives within this Project were met, with the exception of the final cattle toxicity trial.

- The detoxifying capability of the bacterial consortium was increased two-fold, with bacterial numbers increased from 4 to 7. The larger number of bacteria is important for maintaining the stability of the protective system, in an environment where fluctuations in bacterial numbers are routine. The increased dehalogenase activity is important for maximising the protective value of the bacteria.
- The inability of the GM bacteria to colonise and protect non-ruminant animals was shown clearly in rabbits and cats, both of which are feral pests with a high impact on the Australian environment. The possibility of feral pests being protected against Compound 1080 was raised as a major concern by commentators responding to a GMAC information release on the project. Demonstrating that this does not occur is a crucial step in obtaining permission to release these bacteria for use on-farm.
- The ability of obligately anaerobic rumen bacteria to be transferred between animals without direct contact was an important observation. This has important implications for the difficulty in containing the bacteria, should it be necessary. But also for the ease of application, when approval is received for field use of the organisms.
- The cattle toxicity trial was unable to proceed because of financial and regulatory limitations on the duration of research. Application for a DNIR licence from OGTR was successful, and the trial has since been completed successfully, at the AAHL facility at Werribee (Vic). A separate report deals with that trial.

Attachments:

1. Family of Shuttle Vectors for Ruminal *Bacteroides*. Wong *et al*., 2003.
2. Detoxification of the Plant Toxin Fluoroacetate by a Genetically Modified Rumen Bacterium. Gregg *et al*., 1994.
3. Genetically Modified Rumen Bacteria Protect Sheep from Fluoroacetate Poisoning. Gregg *et al*., 1998.

References

(*publications from the Rumen Biotech Group UNE & Murdoch)

*Allen, G.A. 1997. Polymerase chain reaction techniques for the study of rumen bacterial ecology and biotechnology. PhD thesis, University of New England. Armidale, NSW, Australia.

Aminov, R.I., K. Tajima, K. Ogata, T. Nagamine, M. Sugiura, and Y. Benno 1998. Transcriptional regulation of the Prevotella ruminicola recA gene. Curr Microbiol 36:259-65.

*Beard, C.E., Hefford, M.A., Forster, R.J., Sontakke, S.,Teather, R.M., Gregg, K. (1995) Stable and efficient transformation system for *Butyrivibrio fibrisolvens* OB156. Current Microbiol. 30:105-109.

Daniel, A.S., J. Martin, I. Vanat, T.R. Whitehead, and H.J. Flint 1995. Expression of a cloned cellulase/xylanase gene from Prevotella ruminicola in Bacteroides vulgatus, Bacteroides uniformis and Prevotella ruminicola. J Appl Bacteriol 79:417-24.

Dower, M.J., J.F. Miller, and C.W. Ragsdale 1988. High efficiency transformation of *E. coli* by high-voltage electroporation. Nucleic Acids Res. 16:6127-6145.

*Gregg, K., H. Sharpe 1991. Enhancement of rumen microbial detoxification by gene transfer. p. 719-736. in T. Tsuda, Y. Sasaki, R. Kawashima. (Ed.) Physiological Aspects of Digestion and Metabolism in Ruminants. Academic Press Inc. Tokyo.

*Gregg, K., G. Allen, T. Bauchop, A.V. Klieve, and M. Lincoln 1993. Practical genetic engineering of rumen bacteria. p. 13 – 21. in D.J. Farrell (Ed.) Recent Advances in Animal Nutrition in Australia. University of New England, Armidale, NSW, Australia.

*Gregg, K., C.L. Cooper, D.J. Schafer, H. Sharpe, C.E. Beard, G. Allen, and J. Xu 1994a. Detoxification of the plant toxin fluoroacetate by a genetically modified rumen bacterium. Bio/Technology 12:1361-1365.

*Gregg, K., B. G. Kennedy, and A.V. Klieve 1994b. Cloning and DNA sequence analysis of the region containing *attP* of the temperate phage ϕAR29 of Prevotella ruminicola AR29. Microbiol. 140:2109-2114.

*Gregg, K., B. Hamdorf, K.P. Henderson, J. Kopecny, and C. Wong 1998. Genetically modified ruminal bacteria protect sheep from fluoroacetate poisoning. Appl. Environ. Microbiol. 64:3496-3498.

*Hamdorf, B. J. 1998. Isolation and characterisation of endonucleases from Prevotella ruminicola AR20. PhD Thesis. University of New England, Armidale, NSW, Australia.

Hecht, D.W., T.J. Jagielo, and M.H. Malamy 1991. Conjugal transfer of antibiotic resistance factors in *Bacteroides fragilis*: the btgA and btgB genes of plasmid pBFTM10 are required for its transfer from *Bacteroides fragilis* and for its mobilization by IncPb plasmid R751 in *Escherichia coli.* J Bacteriol 173:7471-80.

*Hudman, J.F., and K. Gregg 1989. Genetic diversity among strains of bacteria from the rumen. Curr. Microbiol. 19:313-319.

Hungate RE. 1966. The Rumen and its Microbes. Academic Press, NY.

Kawasaki, H., K. Tsuda, I. Matsushita, and K. Tonomura. 1992. Lack of homology between two haloacetate dehalogenase genes encoded on a plasmid from *Moraxella* sp. strain B. J. Gen. Microbiol. 138:1317-23.

*Klieve, A.V., J.F. Hudman, and T. Bauchop 1989. Inducible bacteriophages from ruminal bacteria. Appl. Environ. Microbiol. 55:1630-1634.

*Klieve, A.V., K. Gregg, and T. Bauchop 1991. Isolation and characterization of lytic phages from *Bacteroides ruminicola* sub-sp. *brevis*. Curr. Microbiol. 23, 183-187.

Latham M.J., Legakis N.J. 1976. Cultural factors influencing the utilization or production of acetate by *Butyrivibrio fibrisolvens*. J Gen Microbiol 94(2):380-8.

Maniatis, T., E. F. Fritsch, and J. Sambrook, 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Privitera, G., A. Dublanchet, and M. Sebald 1979. Transfer of multiple antibiotic resistance between subspecies of *Bacteroides fragilis*. J. Infect. Dis. 139:97-101.

Raleigh, E. A., N. E. Murray, H. Revel, R. M. Blumenthal, A. D. Westaway, P. W. J. Rigby, J. Elhai, and D. Hanahan, 1988. McrA, McrB restriction phenotypes of some *E. coli* strains and implications for gene cloning. Nucleic Acids Res. 16:1563-1575.

Russell, J.B., and D.B. Wilson 1988. Potential opportunities and problems for genetically altered rumen microorganism. J. Nutr. 118:271-279.

Sota M, Endo M, Nitta K, Kawasaki H, and Tsuda M (2002) Characterization of a Class II Defective Transposon Carrying Two Haloacetate Dehalogenase Genes from *Delftia acidovorans* Plasmid pUO1. Appl. Env. Microbiol. 68:2307-2315.

Shoemaker, N.B., K.L. Anderson, S.L. Smithson, G.R. Wang, and A.A. Salyers 1991. Conjugal transfer of a shuttle vector from the human colonic anaerobe *Bacteroides uniformis* to the ruminal anaerobe *Prevotella* (*Bacteroides*) *ruminicola* B(1)4. Appl Environ Microbiol 57:2114-20.

Smith, C.J. 1985. Development and use of cloning systems for *Bacteroides fragilis*: cloning of a plasmid-encoded clindamycin resistance determinant. J. Bacteriol. 164:294-301.

Smith, C.J. 1995. Genetic transformation of Bacteroides spp. using electroporation. Methods Mol Biol. 47:161-9.

Thomson, A.M., and H.J. Flint 1989. Electroporation induced transformation of *Bacteroides ruminicola* and *Bacteroides uniformis* by plasmid DNA. FEMS Microbiol Lett. 52:101-104.

*Vercoe, P.E., Gregg, K. 1992. Sequence and transcription of an endoglucanase gene from *Prevotella* (*Bacteroides*) *ruminicola* strain AR20. Molec. Gen. Genetics. 233:284-292.

Wong, C.M., Klieve, A.V., Hamdorf, B.J., Schafer, D.J., Brau, L., Seet, S.G.M., Gregg, K. 2003. Family of shuttle vectors for ruminal Bacteroides. J. Mol. Microbiol. Biotech. 5: 57 – 66.

*Woods, J.R., J.F. Hudman, and K. Gregg 1989. Isolation of an endoglucanase gene from Bacteroides ruminicola sub-sp. brevis. J. Gen. Microbiol. 135:2543-2549.

Additional publications from the Rumen Biotech Group (UNE & Murdoch):

Journal Publications

Beard, C.E., Gregg, K., Kalmokoff, M. & Teather, R.M. (2000) Construction of a Promoter-rescue plasmid for *Butyrivibrio fibrisolvens*, and its use in characterization of a flagellin promoter. Current Microbiol. 40:164-168.

Cooper, C.L., Vercoe, P.E., Ford, M.D., Gregg, K. (1993) Single-step amplification-sequencing reactions (ASR) on genomic DNA. Asia-Pacific J. Mol. Biol. Biotech. 1:122-125.

Engelke, C.F. McSweeney, C.S. Vercoe, P.E. Gregg , K. (2002) MALDI-TOF mass spectrometry can be used to identify changes in the protein composition of rumen bacteria: the response of the novel ruminal bacterium LPLR3 7 when grown in the presence of diaminopropionic acid. Anim. Prod. Aust. Vol. 24: 61 64.

Gold JJ, Heath IB, Bauchop T. (1988) Ultrastructural description of a new chytrid genus of caecum anaerobe, Caecomyces equi gen. nov., sp. nov., assigned to the Neocallimasticaceae. Biosystems.;21:403-415

Gregg, K., Allen, G., Beard, C. (1996) Genetic manipulation of rumen bacteria: from potential to reality. Aust. J. Agric. Res. 47:247-256.

Gregg, K., Kennedy, B., Klieve, A.V. (1994) Cloning and DNA sequence analysis of integration determinanats of the temperate phage *phi*-AR29 of *Prevotella ruminicola* AR29. Microbiol. 140:2109-2114.

Hamdorf, B.J., Gregg, K. (1994) Rapid and precise gender determination from DNA samples using polymerase chain reaction. Asia Pacific J. Mol. Biol. Biotech. 2:136-142.

Ho YW, Bauchop T. (1991) Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. J Gen Microbiol. 137: 213-7.

Klieve AV. (1991) Bacteriophages from the forestomachs of Australian marsupials. Appl Environ Microbiol. 57: 3660-3663.

Klieve AV, Bauchop T. (1991) Phage resistance and altered growth habit in a strain of *Streptococcus bovis*. FEMS Microbiol Lett. 64: 155-159.

Klieve AV, Bauchop T. (1988) Morphological diversity of ruminal bacteriophages from sheep and cattle. Appl Environ Microbiol. 54: 1637-1641

Klieve AV, Swain RA. (1993) Estimation of ruminal bacteriophage numbers by pulsed-field gel electrophoresis and laser densitometry. Appl Environ Microbiol59: 2299-2303

Klieve AV, Bauchop T. (1988) Morphological diversity of ruminal bacteriophages from sheep and cattle. Appl. Environ. Microbiol. 54:1637-41.

Swain RA, Nolan JV, Klieve AV. (1996) Natural variability and diurnal fluctuations within the bacteriophage population of the rumen. Appl Environ Microbiol. 62: 994-7.

Vercoe, P.E., Gregg, K. (1995) Sequence and transcriptional analysis of an endoglucanase gene from Ruminococcus albus AR67. Anim. Biotech. 6:59-71.

Ware, C.E., Bauchop, T., Gregg, K. (1989) The isolation and comparison of cellulase genes from two strains of *Ruminococcus albus*. J. Gen. Microbiol. 135:921-930.

Ware, C.E., Lachke, A.H., Gregg, K. (1990) Mode of action and substrate specificity of a purified exo-1,4-Beta-glucosidase cloned from the cellulolytic bacterium *Ruminococcus albus* AR67. Biochem. Biophys. Res. Commun. 171: 777-786.

Ware, C.E., Bauchop, T., Hudman, J.F., Gregg, K. (1992) Cryptic Plasmid pBf1 from *Butyrivibrio fibrisolvens* AR10: its use as a replicon for recombinant plasmids. Current Microbiol. 24:193-197.

Xue, G-P., Johnson, J.S., Bransgrove, K.L., Gregg, K., Beard, C.E., Dalrymple, B.P., Gobius, K.S., Aylward, J.H. (1997) Improvement of expression and secretion of a fungal xylanase in the rumen bacterium *Butyrivibrio fibrisolvens* OB156 by manipulation of promoter and signal sequences. J. Biotechnol. 54:139-148.

Book Chapters

Teather, R.M., Forster, R.J., Gregg, K., Hefford, M., Kobayashi, Y., Ware, C.E. (1993) Structure and function of plasmids from *Butyrivibrio fibrisolvens*. in "Genetic, Biochemistry and Ecology of Lignocellulose degrdation". Eds. K. Shimada, S. Hoshino, K. Ohmiya, K. Sakka, Y. Kobayashi, S. Karita. UNI Publishers Co. Ltd. Tokyo. pp 216-225.

Gregg, K. (1989) Genetic Engineering of Rumen Bacteria. In "The Biology of Wool and Hair". Eds. G.E. Rogers, P.J. Reis, K.A. Ward, R.C. Marshall. Chapman and Hall, London/New York. 417-424.

Refereed Conference Papers

Gregg, K., Rowan, A.M., Ware, C.E. (1993) Digestion of filter paper by cellulases cloned from *Ruminococcus albus* AR67. in "Genetics, Biochemistry and Ecology of Lignocellulose degrdation". Eds. K. Shimada, S. Hoshino, K. Ohmiya, K. Sakka, Y. Kobayashi, S. Karita. UNI Publishers Co. Ltd. Tokyo. pp 166-172.

Gregg, K., Schafer, D., Cooper, C., Allen, G. (1995) Genetic manipulation of rumen bacteria: now a reality. in "Rumen Ecology Research Planning. Eds J. Wallace, A. Lahlou-Kassi. Intl. Livestock. Res. Inst. Nairobi, Kenya. pp. 227-240.

Gregg, K., Teather, R.M. (1992) The genetic manipulation of rumen bacteria. in "Manipulation of rumen microorganisms". Ed. K. El-Shazly. Alphagraph, Alexandria, Egypt. pp 1-12.

Gregg, K., Ware, C.E. (1990) Importance of genetic diversity in manipulation of rumen bacteria. In "Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants". Eds, D.E. Akin, L.G.Ljungdahl, J.R. Wilson, P.J. Harris. Elsevier, New York, pp357-366.

Reviews

Bauchop T. (1989) Biology of gut anaerobic fungi. Biosystems.;23(1):53-64

Gregg, K. (1992) Developments in rumen bacterial molecular biology. AgBiotech News and Information. 4:375N-380N.

Gregg, K. (1995) Engineering gut flora of ruminant livestock to reduce forage toxicity: progress and problems. TIBTECH 13:418-421.

Video Production:

Gregg, K., Hamdorf, B.J., Hendersons, K.P. (1997) The Fluoroacetate Research Project. 24 min. A documentary production, describing the origins, purpose, and progress of the Rumen Bacterial Genetic Manipulation Program, up to the proof-of-concept stage. Made primarily for the informaton of financial contributors to Applied Biotechnology Ltd (Qld)

Research Students Supervised in Rumen Biotech

(UNE: 1987 – 1995; Murdoch: 1999 – 2001; *writing thesis currently; **still in progress)

PhD	Masters	Honours	Honours
Vercoe, P.E. (UNE)	Everard, D.T. (UNE)	Woods. J. (UNE)	Clausen, P. (UNE)
Beard, C.E. (UNE)	Helen Sharpe (UNE)	Townsend, M. (UNE)	Xu, J-W. (UNE)
Hamdorf, B.J. (UNE)	Shi, J (Murdoch)	Rourke, I. (UNE)	Schoep, T.D. (Murdoch)
Allen, G. (UNE)		Kennedy, B. (UNE)	Seet, S.G.M. (Murdoch)
Schoep, T.D. (Murdoch)		Rowan, A. (UNE)	Kane, C. (Murdoch)
*Seet, S.G.M. (Murdoch)		Waters, D. (UNE)	Limadinata, V. (Murdoch)
		Hamdorf, B.J. (UNE)	

* Students in progress now.

Administrative Details Report

Funds budgeted and received 01/07/97 – 30/06/01.

DATE	OPERATING COSTS	OVERSEAS TRAVEL	CAPITAL	TOTAL $
TR.044				
01/07/97	79,004	2,500	-	81,504
01/10/97	64,004	-	-	64,004
01/01/98	70,254	5,000	-	75,254
01/04/98	70,254	-	-	70,254
01/07/98	75,254	-	9,000	84,254
01/10/98	70,254	-	-	70,254
01/01/99	47,754	2,500	-	50,254
01/04/99	70,254	-	-	70,254
03/05/99	5,200	Variation to add casual salary (T. Schoep) for tech. assistance		5,200
TOTAL TR.044				**571,232**
TR.044B				
01/01/00	100,000	-	-	100,000
01/04/00	100,000	-	-	100,000
01/07/00	100,000	-	-	100,000
TOTAL paid on TR.044B				**300,000**
TOTAL paid to date:				**871,232**
		Not yet invoiced:		
01/10/00	*105,000*	-	-	*105,000*
Committed for 2001	*100,000*	-	-	*100,000*
Remaining for invoice:				**205,000**
TOTAL Committed				**1,076,232**