Genetically modified rumen bacteria protect sheep from fluoroacetate poisoning

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Summary

In two separate experiments, test sheep were inoculated with a mixture of four genetically modified strains of *Butyrivibrio fibrisolvens*, which attained a combined population level of 10^6–10^7 cells per ml in each animal. The bacteria had been modified to express the enzyme fluoroacetate dehalogenase. Uninoculated sheep were used as controls. Experiment I included two Merino/Border Leicester cross-bred sheep in each group, and Experiment II included three pure-bred Merinos in each group. Sheep were fed incremental doses of fluoroacetate, and their condition and behaviour was monitored continuously. In both experiments, the control sheep showed dramatic symptoms of fluoroacetate poisoning, judged by behaviour and heart-rate, while the inoculated sheep remained comparatively unaffected. At higher doses of poison, inoculated sheep showed symptoms of intoxication. In Experiment I, control sheep exhibited hyper–excitability as the first major symptom of poisoning, accompanied by compulsive eating. Vigorous movement of the control sheep broke loose the ECG monitoring cables, preventing comparison of heart-rate between groups. Both control sheep died from acute symptoms of fluoroacetate poisoning. The test sheep T2 with 10^6 modified bacteria/ml of rumen contents did not exhibit hyper-excitability or compulsive eating, but died of pulmonary oedema five hours after the control sheep. Sheep T1, with 10^7 bacteria/ml of rumen contents, survived without showing major symptoms of toxicity. In Experiment II, all three of the control sheep showed severe effects of poisoning that differed markedly from those seen in Experiment I. Activity was reduced to occasional periods of standing, and the animals ceased eating and drinking. ECG measurements showed frequent periods of extremely high heart rate (140 – 300 bpm) from 32 h to 72 h. The test sheep continued to eat and drink normally throughout the first 36 hours of the experiment, reducing intake by approximately 30% during the 36 – 72 hour period. Heart rates in the test sheep were occasionally raised to 80 – 100 bpm, during the later parts of the experiment. In Experiment II, none of the animals showed hyper-excitability at any stage of the experiment. The different responses of the animals to fluoroacetate poisoning appeared to be characteristic of the different breeds. The results showed clearly that rumen bacteria capable of detoxifying the poison fluoroacetate, significantly reduced the effect of this poison upon the host animals.

Introduction

The greatest single advantage of farmed ruminants is their ability to convert the relatively indigestible cellulosic plant tissues into animal tissues, and they owe this capability to the micro-organisms of the rumen. The microbes that ferment plant fibres within the rumen are diverse, but two major genera of bacteria are credited with performing a significant role in fibre digestion, *Ruminococcus* and ***Fibrobacter.*** Organisms such as Butyrivibrio also have a role in fibre digestion. It has been proposed that genetic modification of rumen bacteria might allow the process of fibre digestion to be performed more efficiently, thereby benefiting the animal (Smith and Hespell, 1983; Teather, 1985).

Despite wide discussion of the potential gains from genetic manipulation of rumen bacteria it is still uncertain whether bacteria can be grown in culture, genetically modified, and then reintroduced to the rumen to perform a useful new or enhanced function. Recent work has shown that useful genetic modifications can be made to rumen bacteria (Gregg *et* ***al.*** 1994) and that modified bacteria can indeed survive within the rumen (Gregg 1995; Gregg ***et al.*** 1995). But the question remains, whether modified organisms can make a genuine, beneficial difference to the host animal.

There are many ways in which the expression of a novel gene in rumen microbes might benefit the host animal, and therefore benefit the animal producer. Bacteria could be introduced to control parasites, to modify the faun1

composition of the rumen, or to reduce the effects of toxic or antinutritional chemicals. Of the uses visualised for genetically modified rumen bacteria, enhancement of fibre digestion may be one of the most complex to achieve, possibly requiring the modification of multiple bacterial strains and manipulation of multiple genes. It would be useful, therefore, to establish the practicality of this technology in less complex tasks, as an indicator of how effective this route to improved fibre digestion might be.

In late 1994, a putatively useful genetic modification was made to a rumen bacterium, *B. fibrisolvens* OB 156 (Gregg et *al.* 1994) using plasmid pBHf (Beard et *al.* 1995). In this work, *B. fibrisolvens* was modified to allow it to inactivate the naturally occurring plant toxin monofluoroacetate.

This was followed by experiments that showed it was possible to return the same genetically modified bacterium to the rumen, and to monitor its continued survival over a five-month period (Gregg, 1995). By extending the work on fluoroacetate detoxification, we have now shown that genetically modified bacteria can perform a useful task in vivo.

Methods

Sheep and housing

For Experiment I, 4 Merino/Border Leicester crossbred sheep were used, two inoculated test sheep and two uninoculated control sheep. The two sheep that were to receive recombinant bacteria were housed in a PC2 classified room, with negative pressure and a HPA filtered outlet fan. Individual animals were placed in metabolism crates that allowed collection of all urine and faeces. Sheep had been surgically cannulated to allow direct access to the rumen, removal of fluid samples and addition of bacterial cultures. For Experiment II, six pure-bred Merino sheep were housed in the same way as for experiment I, except that they were harnassed to prevent escape from the metabolism crates. Three sheep were used in each group.

Feeding

Each sheep received four meals daily, of 150 g oaten chaff and 50 g lucerne (alfalfa) chaff, at approximately 10 am, 12.00 noon, 2.00 pm and 4.00 pm. Water was provided *ad libitum.*

Intra-ruminal inoculation with modified bacteria

Cultures of four distinct strains of rumen bacteria containing the dehalogenase gene (Gregg *et al.* 1994) were grown in 20 – 50 ml flasks of rumen fluid medium (Klieve et *al.* 1989) in which the peptone component had been replaced by yeast extract (Oxoid batch L21) approved by AQIS for *in vivo* use. Sixty ml of an equal mixture of the four cultures was inoculated by syringe, via the rumen fistula, into the fluid phase of the rumen contents. The four strains used were: Butyrivibrio fibrisolvens OB 156 and OB29 1 (from Canadian White–Tail deer), and OR85 (from a Holstein steer). All three were isolated at the CFAR, Agriculture and Agrifood Canada, Ottawa, and kindly supplied by Dr R. Forster and Dr R.M. Teather. Strain 1 O/l was isolated in this laboratory from the sheep rumen. Plasmid pBHf, carrying the fluoroacetate dehalogenase gene, was inserted into these strains as described by Gregg et *al.* (1994).

Tracking the dehalogenase gene *in* vivo

Rumen fluid samples were removed, via the fistulae, using disposable plastic pipettes with an aperture that allowed collection of feed particles only if below approximately 1 mm. Samples were heated at 95°C for 1 O-l 5 minutes to inactivate nucleases and were prepared for PCR analysis as follows.

A 100 ml sample was centrifuged (12,000 *x* g) for 90 seconds, and the fluid removed. The pelleted material was resuspended in 1 ml of sterile distilled water and re–centrifuged as before. This procedure was repeated once and the final pellet was suspended in 1 ml of water. This represented a ten-fold dilution of particles from the original sample. Further dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} were prepared, and all dilutions were tested by PCR for the presence of dehalogenase gene sequences. PCR reactions were prepared using the procedure described by the supplier of the thermostable *Taq* DNA polymerase (Bresatec, SA). Reaction volumes of either 10 μλ or 20 μλ volume were incubated in either a Bartelt thermal cycler, or an MJ Research PTC-100 thermal cycler. Products were analysed by electrophoresis on 1.5%–2% agarose gels in TBE buffer. Positive-control samples contained washed cells from a culture of *B. fibrisolvens* OB 156, containing the plasmid pBHf. PCR amplification used primers CC5 and CC6.2i, which amplified a 300 bp fragment from the coding region of the dehalogenase gene.

Bacterial numbers within the rumen were calculated from the dilution at which a PCR signal was no longer obtained. Detection of a single bacterium was possible, but inclusion of approximately 1 ml of sample in the PCR mixture dictated that minimum detection level was 10^3 cells/ml. Therefore, detection of plasmid sequences at a dilution of 10^{-3}, indicated a ruminal population ≥ 10^6 cells/nil.

Fluoroacetate dosage

Fluoroacetate was administered with feed, either injected as a 5 mg/ml solution into snow-peas, or absorbed as a 10 mg/ml solution into feed-pellets or specially made lucerne biscuits. In this form the toxin was eaten voluntarily. The lucerne biscuits were made from lucerne, blended with a 10% sucrose solution, pressed into disc shapes to remove most of the fluid, and dried overnight at 60°C. On the two occasions that sheep C2 (Experiment II) refused to eat the biscuits, they were placed into the

rumen via the fistula, and sheep T2 was treated similarly, to ensure balance between groups.

Experiment I

Day 1: The first dose of fluoroacetate was 0.045 mg/kg live weight, at time zero, and subsequent doses of 0.03 mg/kg were administered at two-hourly intervals, to a total of 0.195 mg/kg.

Day 2: Two more doses were administered, at 22 h and 24 h, bringing the total dose to 0.255 mg/kg.

Behaviour and health of the animals was monitored for a further 24 h.

Day 3: Doses were administered at 0.0 15 mg/kg (45 h), 0.12 mg/kg (51.25 h) and 0.015 mg/kg (56.45 h) bringing the dose to a total of 0.297.

Day 7: A repeat series of 0.015 mg/kg doses was adminstered at hourly intervals fiom 140 h to 152 h, to bring the total dose over a 7-day period to 0.492 mg/kg.

Experiment II

In this experiment, six sheep (two groups of three) were dosed with fluoroacetate in the same schedule as for experiment I, up to a total dose of 0.255 mg/kg. However, further doses of 0.0 15 mg/kg were administered at 3 8 h, 40 h and 42 h after commencement of the experiment. A further 0.03 mg/kg was administered at 46 hours. The total dose was 0.33 mg/kg.

Heart-rate data was recorded throughout the experiment, together with video monitoring of behaviour, which was observed remotely to avoid disturbance to the animals.

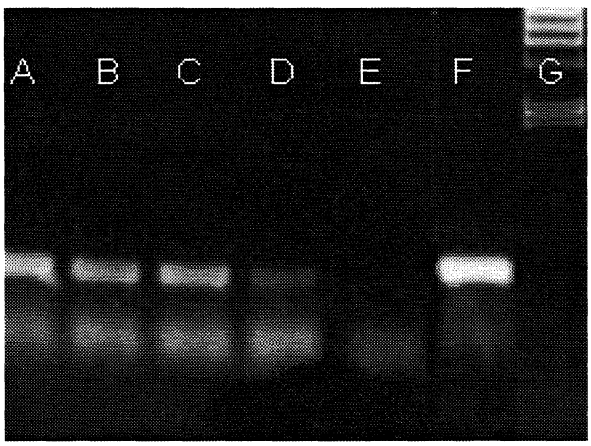

Fig. 1 Agarose gel showing PCR products from amplification of the dehalogenase gene fragment in a rumen sample from test-sheep 1 in trial I. Serial dilutions of washed rumen particles were used as template for PCR: A, 10^{-1}; B,10^{-2}; C, 10^{-3}; D, 10^{-4}; E, 10^{-5}; F, positive control; G, molecular weight standards, HindIII digest of l bacteriophage DNA

Results

Colonization of the rumen by modified bacteria

Experiment I was the fourth experiment in which we had inoculated laboratory-grown bacteria into the sheep rumen. As with previous experiments, the inoculated bacteria had established populations within the rumen of the inoculated sheep within 10 days. Test sheep T1 showed a population of $>10^7$ bacteria per ml, while T2 showed $>10^6$/ml. Tests on the control sheep showed that recombinant bacteria were undetectable. An example of PCR detection results is shown in Figure 1. In Experiment II, recombinant bacteria were slower to colonize the rumen, and some difficulties were encountered with rumen contents inhibiting the PCR detection process. It was approximately 5 weeks after the initial inoculation and several, low-volume, reinoculations that we were able to demonstrate stable levels of $>10^6$ recombinant bacteria per ml of rumen contents.

Poison symptoms

In Experiment I the first obvious symptoms of fluoroacetate poisoning in the cross-bred sheep were heightened wariness and tendency to panic at minor sounds. These phenomena were observed in the control sheep but not in the inoculated test sheep. Constant attendance was required with the control sheep to prevent them from jumping out of the metabolism crates in which they were housed. The test-sheep remained calm throughout this period, eating and drinking normally.

During the later phases of poisoning, hyper-excitability symptoms recurred in the control sheep, accompanied by compulsive eating. During the same time, the test-sheep reduced their food and water intake slightly. At day 7, the control sheep were startled by extraneous noises during the early morning and died. Approximately four h later, test-sheep T2 died without any signs of excitability. Autopsy showed the T2 had died of pulmonary oedema which is typical of chronic fluoroacetate poisoning . T1 survived the tests without overt symptoms of toxicity.

In Experiment II following the initial 24 h poisoning period, control sheep became visibly unwell. All three refused to eat or drink. During the same period, the test-sheep remained apparently normal, eating and drinking as usual. Heart-rate measurements showed considerable stress in the control-sheep, with heart-rates frequently above 140 and sometimes exceeding 200 bpm. Some periods of raised cardiac activity in the test-sheep gave rates between 80 – 100 bpm (Fig 2).

At this stage, sheep C2 refused to eat the lucerne biscuits in which the toxin was incorporated, and further doses were administered by direct placement into the rumen, via the rumen fistula. The closest matched test-sheep T2 was treated similarly, to ensure balanced treatment.

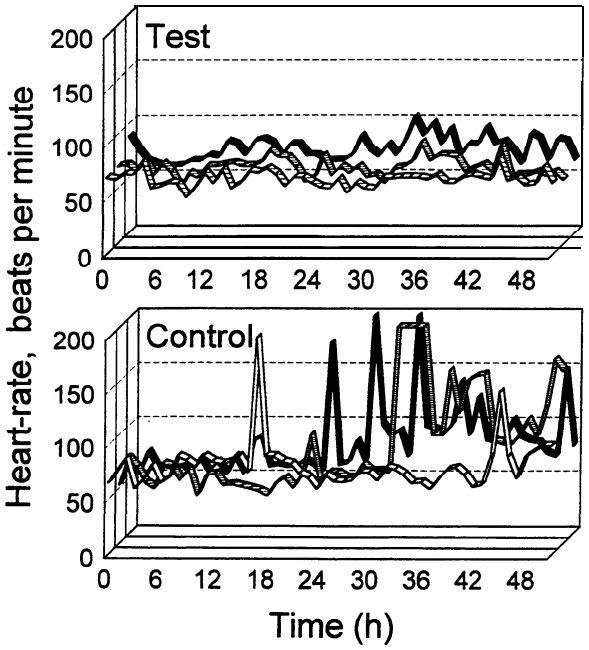

Figure 2 Heart-rate measurements on sheep in trial II, comparing the heart rates of inoculated test-sheep T1 (white), T2 (grey), T3 (black) with the uninoculated controls, C1–C3. Normal hear&-rates for all sheep, measured prior to toxicity testing, were in the range 60–80 beats/min.

During the later stages of this experiment, T2 showed minor signs of intoxication, with feed intake reduced approximately 30%. The control-sheep appeared seriously ill, with high, erratic heart rates, refusal to eat or drink, and unsteadiness of posture.

During the recovery stage, two of the three test–sheep returned to normal eating habits within 24 h and the third T3 continued to eat approximately 30% less than usual for 3 days. The control sheep displayed reduced appetite for 3 days, continued unsteadiness on standing for 2 days, and involuntary muscular activity (head twitching) for 24 h. The results of these two experiments strongly support the hypothesis that rumen bacteria, modified to detoxify fluoroacetate, could significantly reduce the symptoms suffered from fluoroacetate poisoning in the host animal.

Discussion

In Experiment I, and in three previous experiments, modified bacteria colonized the rumen rapidly, attaining population levels around 10^4–10^7 bacteria/ml rumen contents (Gregg, 1995). Populations of individual strains have been shown to fluctuate from day to day, independently of one another (Gregg, 1995). The combination of four strains produces a more even total population. It was estimated, from early work, that a total of ten strains would be ideal for field use, but time constraints made it necessary to attempt these protection experiments with the four strains available.

Experiment II was delayed slightly by difficulties with the PCR tracking process, and through rapid changes in rumen microbial populations. This may be attributable to the recent change of feed regimen, from pasture to chaff diet, and to the recent surgical modification of the animals used in Experiment II.

Interesting differences were observed between the toxic response of the Merino/Border-Leicester cross-bred sheep in Experiment I and the pure-bred Merino sheep used in Experiment II. The major response of control sheep in Experiment I was heightened nervousness, frequent attempts to break free of the metabolism crates, and compulsive eating. In Experiment II, the control sheep showed no increase in their normal wariness-indeed there was possibly a somewhat reduced response to sharp stimuli such as sudden noises. Equally notably, Experiment II resulted in complete cessation of eating and drinking in control sheep, which remained largely inactive.

In both experiments, the inoculated test sheep remained visibly normal throughout the greater part of the experiment, with slight increase in nervousness (Expt. I) or slightly decreased feed intake (Expt II) at the highest doses of fluoroacetate. The recorded heart–rates from Experiment II showed clear differences between the two groups. During Experiment I, agitation of the control sheep resulted in the monitoring equipment being accidentally disconnected and it was not possible to obtain reliable, comparative heart-rate measurements.

The experiments described here were performed with four bacterial strains, three of which expressed dehalogenase activity at approximately 10 nmol/min per mg bacterial protein. The fourth strain, OR85, gave approximately twice this activity depending upon the phase of bacterial population growth. In earlier work it was estimated that this level of expression would require approximately 10^7 cell/ml to offer significant protection to the host. Because of time constraints on this project, it was necessary for us to proceed with the strains available. In Experiment I, test sheep T1 showed dehalogenase-bearing cells to be present above 10^7 cells/ml and showed major resistance to a lethal dose of the toxin. T2 was shown to have between 10^6–10^7 cells/ml, resisted the acute symptoms of poisoning, but succumbed to the very high, final dose. In Experiment II, all three inoculated sheep gave levels of 10^6–10^7 cells/ml. Because bacterial levels in most sheep were only marginally satisfactory, the toxicity tests were performed with gradual build-up of fluoroacetate, in order to detect even marginal protection. However, there was a clearly protective effect of the modified bacteria on the host animals. The level of protection offered could, presumably, be increased by achieving higher levels of recombinant bacteria in the rumen, and would be more effective still, if each strain expressed dehalogenase more efficiently.

Over the past 10 years, in the field of rumen bacterial biotechnology research activity has been declining, because of difficulties experienced in applying recombinant DNA technology to bacteria from the

rumen. The present work demonstrates that returning modified bacteria to the rumen is feasible, and that the host animal can be advantaged by the presence of those organisms. It is possible that the research field may be rejuvenated by this demonstration, leading to some beneficial effects for animal production.

Acknowledgements

This work was supported by Applied Biotechnology Ltd (Qld) and the Australian Meat Research Corporation. Additional funds were provided for animal care by the Faculty of the Sciences, through the Dean, Professor Keith Entwistle. The authors wish to thank Dr Steve Atkinson for his valuable expertise, Mr Graham Chaffey, for his assistance with sheep handling and physiological monitoring, Mr Frank Ball, for his assistance in setting up the experiments, and the UNE Animal House staff for their continued help throughout these experiments. We also wish to thank Dr Barrie Entsch, for bearing the complex administrative burden necessary to keep the project running.

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