Use of Engineered Rumen Bacteria to Degrade Fluoroacetate

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Plants containing fluoroacetate represent a serious problem for the livestock industry in several regions of Australia, notably North Queensland, the Northern Territory and Western Australia.

A strain of the rumen bacterium *Butyrivbrio fibrisolvens* has beenmodified by inserting a gene isolated from a soil bacterium which allows it to degrade fluoroacetate. By allowing bacterial detoxification in the rumen, these modified bacteria have the potential to save large numbers of animals from lethal poisoning. However a number of questions have been raised about the potential environmental effects of releasing such genetically modified organisms. The fluoroacetate detoxificating bacterium provides a unique model for the consideration of these questions and as a measure of the benefits this type of research may provide by modifying ruminant nutrition.

The Project Outline

Fluoroacetate toxicity is a significant problem in Africa and Central America as well as in Australia, where about 40 species of trees and shrubs in the genera *Acacia, Gastrolobium* and *Oxylobium are* considered poisonous. Fluoroacetate is lethal to domestic rurninants at doses of 0.25 - 0.5 mg/kg live weight (Jensen *et al.,* 1948) It is incorporated into the TCA cycle and, on conversion to fluorocitrate, inhibits the enzyme aconitase (Elliot and Kalnitsky 1950). Native animals in poison-affected areas commonly exhihit high levels of resistance to fluoroacetate which appears to be conferred by the evolution of enhanced systemic tolerance (King *et al.,* 1978).

The impetus for this project came in 1988 from investigations made on behalf of a group of Northern Territory cattle producers with herds affected by Gidyea tree *(Acacia georginae*) toxicity. Although the leaves and pods of these plants are highly toxic, they are somewhat palatable to livestock and losses of up to 20% of affected herds are not uncommon. Detoxification of fluoroacetate by modification of the rumen ecosystem was one of the remedies examined. In the best current example of this approach it has been shown that the transfer of rumen flora from Indonesian and Hawaiian goats resistant to the poisonous legume *Leucaena leucocephala* is able to confer toxin resistance to Australian livestock (Jones and Lowry, 1984, Jones and Megarrity, 1986). The toxin, the non-protein amino acid mimosine, is degraded in the rumen before its absorption by the animal. The bacterium responsible for the detoxification colonises the rumen efficiently and is maintained at sufficient levels to confer resistance to previously sensitive animals (Jones, 1985).

For fluoroacetate the situation is somewhat different because, unlike mimosine, this compound is of little nutritional value for bacteria under anaerobic conditions. Under these circumstances rumen microbes capable of metabolising this compound would be unlikely to evolve naturally (Gregg and Sharpe, 1991), and there was no experimental evidence of natural fluoroacetate degradation by rumen fluid. The possibility of introducing defluorination by gene-transfer was investigated. A search for defluorinating organisms from which a gene could be isolated was made. This resulted in the selection of the soil bacterium *Moraxella sp.* (Kawasaki *et al.,* 1991a,b), which was found to contain a gene encoding an enzyme that degraded fluoroacetate with high specificity and activity. This gene was isolated and cloned in *E. coli* (Kawasaki *et al.,* 1984). The cloned enzyme also exhibited high activity indicating that degradation of fluoroacetate does not require *Moraxella* cell machinery. Importantly, activity was retained under anaerobic conditions and in the presence of conditions and compounds found in the rumen.

The cloned gene was sequenced (Kawasaki *et al.*,1992) and the operon structure and regulatory sequences determined (Gregg *et al.,* 1994). In *Moraxella*, the dehalogenase gene is part of an operon. The dehalogenase gene and another gene with an undetermined function, (but not apparently

involved in defluorination) share common regulatory sequences. As there is no guarantee that regulatory sequences which work in one organism will function in another, the *Moraxella* sequence was replaced with sequences known to function in a rumen bacterium. To accomplish this, the gene promoter from the Erythromycin resistance gene from the shuttle vector pBHerm (Beard et *al* 1995), which functions in *B. fibrisolvens*, and dehalogenase gene from *Moraxella*, were amplified in separate PCR reactions. These fragments were subsequently fused using Synthesis by Overlapping Extension (SOEing) to produce a new chimeric gene. (Gregg et *al.*, 1994) (Fig. 1) This gene was cloned into an *E. coli* plasmid vector and assayed for activity.

Since the genetic manipulation of rumen bacteria was first suggested (Smith and Hespell 1983, Hespell 1985, Teather 1985) progress has been impeded by the lack of practical techniques for adding new genes. Several systems have recently been reported for inserting plasmids into rumen bacteria (Thomson *et al.*, 1992; Cocconelli *et al.*,1992; Beard et *al.*, 1995). Most conveniently, a system should allow gene manipulation to be performed in *E. coli* laboratory strains and the resulting DNA mass-produced for further manipulation or for insertion into the rumen bacteria. To perform this function of "shuttle vector', a plasmid must possess replication control mechanisms that allow it to operate in both *E. coli* and *B. fibrisolvens*. It must also possess antibiotic resistance genes that allow newly modified (transformed) bacteria to be separated from unmodified organisms. For greatest convenience a shuttle plasmid should also contain a number of places at which it can be cut with restriction endonucleases, for the addition of foreign genes (Fig 2). An *E. coli/B. fibrisolvens* shuttle vector gene transfer system was developed in this laboratory (Beard *et al* 1995) and 'was used to transfer the engineered dehalogenase gene to *B. fibrisolvens strain* OB156 (Gregg *et al.*, 1994).

The genetically modified *B. fibrisolvens strain* expressed dehalogenase sufficient to detoxify fluoroacetate from the surrounding medium at a rate of 10nmol/min/mg bacterial protein in *in vitro* testing . The plasmid carrying the dehalogenase gene has proved to be very stable and was retained by 100% of the transformed bacteria after 500 generations of growth in non-selective media (Gregg *et al.*, 1994). The modified bacteria were able to colonise the rumens of two sheep and were shown to persist for an experimental period of 5 months. Experimental evidence indicates that colonisation by modified bacteria was comparable to that by non-engineered organisms. Populations in both cases were observed to fluctuate independently with changes in the sheeps' diet. (Fig. 3).

With the observed level of activity, and if the bacteria represented 0.5% of the rumen flora, the likelihood of death in animals eating toxic plants should, in theory, be reduced. However many factors remain unknown about the ingestion of poisons by

Fig. 1 Construction of recombinant dehalogenase gene and insertion into shuttle plasmid.

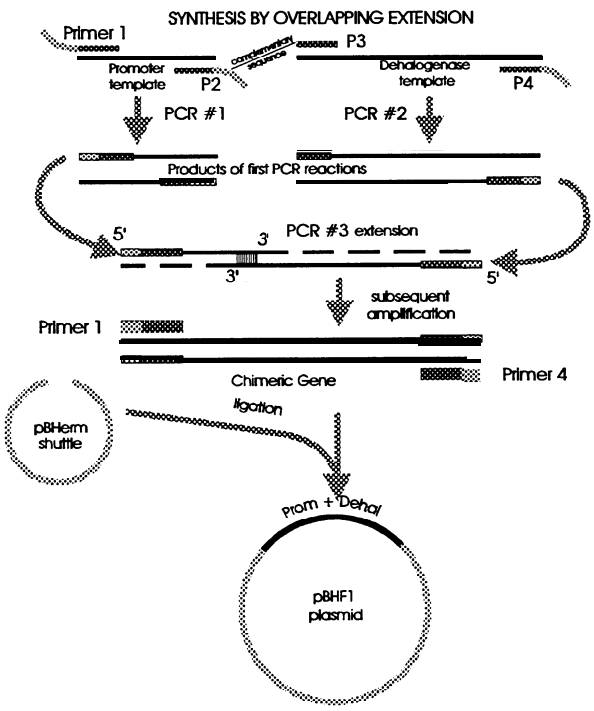
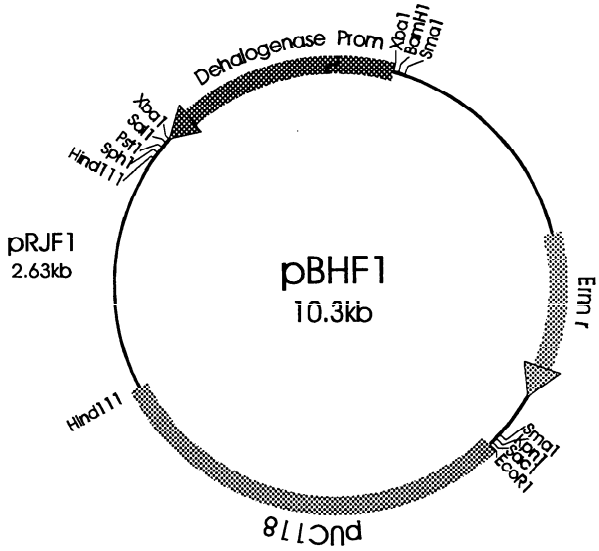


Fig. 2 Structure of plasmid pBHF1



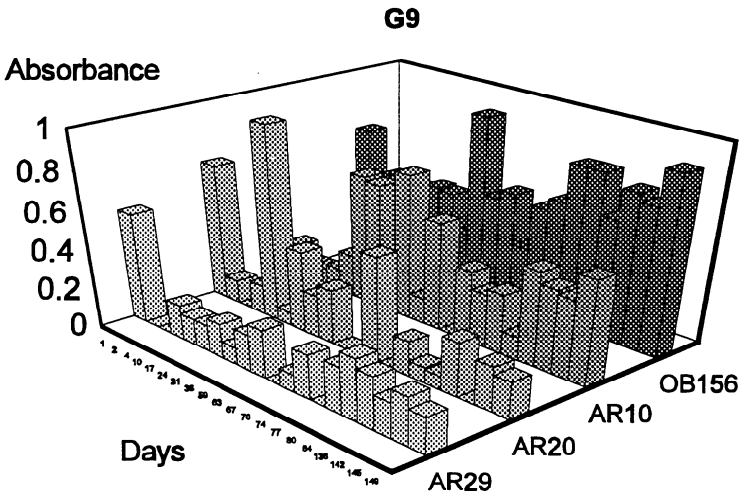


Fig. 3 Relative variation in population densities of three non-recombinant bacterial strains (AR29, AR20, AR10) and one recombinant strain (OB156/pBHF1) in the rumen of an experimental sheep over a five month period.

ruminants. For example, in the case of fluoroacetate, the rate of release of toxin from plant material is unknown, as is the distribution of the toxin within the rumen and its subsequent absorption by the host. It has yet to be demonstrated empirically whether the detoxification observed *in vitro* will be adequate *in vivo.* Trials are currently being conducted, under containment, to determine the ability of the modified bacteria to protect host animals from poisoning.

Field Release of Genetically Modified Rumen Bacteria

The release of Genetically Modified Organisms (GMO's) is a subject which has generated much public anxiety, often without justification. In Australia the Genetic Manipulations Advisory Committee (GMAC) has the task of advising government on whether releases should proceed. GMAC is in turn advised by representatives from a range of scientific, environmental, legal and ethical professions. GMAC sees its main role to be risk assessment and risk management. In applying for a general release a number of criteria need to be addressed in order to minimise the risk of creating new environmental or agricultural problems.

Firstly the GMO must perform its task sufficiently well to justify release. In the case of fluoroacetate detoxification the altered bacteria must be tested empirically under contained trial conditions and be shown to work. Additionally a comprehensive report on the attributes of the GMO is required by GMAC. Antibiotic sensitivities, which will allow the engineered bacteria to be removed from host animals, have been determined. The survival time of the GMO in air, water and on surfaces has been measured and the presence of recombinant genetic material in animal faeces and rate of plasmid loss tested.

Secondly the potential of the released organism to change the environmental impact of its host must be assessed. One major concern is the possible overgrazing and associated land-degradation caused when previously ungrazed land becomes safe for livestock. Should this problem arise, it would require adjustments to be made to pastoral practices. As the extent to which individual graziers would make these adjustments can only be guessed at, assessing this risk will be difficult.

Thirdly the potential of the released organism to colonise hosts other than the intended ones, and what effects it might have in that event, must be evaluated. There is a concern that fluoroacetate degradation could be acquired by feral animals such as rabbits, foxes, cats and dogs. Fluoroacetate, under the name Compound 1080, is widely used in this country to control these animals by baiting. It will therefore be necessary to test empirically whether the GMO is capable of colonising a range of non-target hosts, and if it is, whether it confers resistance. While this may not be a problem with monogastric animals when dealing with an easily absorbed poison such as fluoroacetate, feral ruminants such as goat and camels may become resistant. It thus becomes necessary to determine whether feral goats are currently limited in their range by the presence of poisonous plants and what environmental effects could be anticipated by the development of resistance. To further complicate matters, in Western Australia 1080 is used to control a serious feral goat problem in rangelands (Norbury, G. 1993). It would therefore be necessary to test the efficacy of poison dosage rates on "protected" animals.

A fourth concern lies in the ability of many bacteria to exchange genetic information, sometimes between unrelated species. It is therefore necessary to ensure that the risk of manipulated genes spreading promiscuously is minimised. This may be achieved by integrating the gene into the host's genome or by demonstrating that plasmids, such as the one carrying the dehalogenase gene, are not mobilisable and passed to other bacteria.

After consideration of the responses made to its requests and after seeking input from government departments, community, industry and special interest groups, GMAC will advise the Federal Government

whether or not to release the GMO, based on its assessment of the probable risks and benefits entailed. Alternatively it can request that further trials be conducted to its satisfaction or that the proposed release be referred to the Federal or State environmental protection agencies for full impact assessment.

The general release of a GMO is therefore not a trivial matter. The process can take a substantial period of time and may cost a great deal of money. While most applications to GMAC produce little public discussion, the dehalogenase project has generated a great deal of comment because of the many potential environmental concerns which need to be addressed before a release can be allowed. By providing technical guidance on release procedures and a forum for communication between interested parties, GMAC plays an essential role in the safe implementation of biotechnology.

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