PRACTICAL GENETIC ENGINEERING OF RUMEN BACTERIA.

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SUMMARY

Experiments have been conducted to test the survival of cultured rumen bacteria when returned to the rumen. A strain of *Butyrivibrio fibrisolvens* (AR10) isolated from a sheep in Armidale (NSW Australia) was shown to be capable of colonizing the rumens of pasture-fed Santa Getrudis cattle in north Queensland. The five animals inoculated with AR10 rapidly transferred the organism to other members of the herd and the introduced bacteria were detectable in a majority of the cattle over a period of three months, although detection frequencies declined during the dry season. A second strain of *B. fibrisolvens* (E14) from the rumen of a reindeer was tagged by genetic modification with a tetracycline resistance transposon (Tn916) and inserted into the rumens of four sheep in the UNE animal house. El4 was tracked successfully for 49 days, and the mean numbers across the four sheep stablilized at between 10^6 and 10^7 cells per ml of rumen fluid. In both AR10 and El4 tests, bacterial levels within any particular animal occasionally dropped below the levels of detection, but recovered subsequently. The sheep used in the El4 experiment were separated from one another, and the recovery of bacterial numbers was concluded to occur through reproduction of residual organisms rather than through reinoculation from other animals. *Prevotella ruminicola* AR20 was genetically modified by insertion of a recombinant plasmid (pBA) which conferred clindamycin resistance. In the presence of clindamycin, *in vitro* population growth of the plasmid-bearing organism was shown to be slightly faster than that of the unmodified organism in the absence of the antibiotic. This demonstrated clearly that the maintenance and expression of foreign genetic material did not impose a measurable burden upon AR20 *in vitro.*

INTRODUCTION

During the past ten years considerable interest has been expressed in the potential for genetic modification of rumen bacteria (Smith and Hespell 1983; Teather 1985; Armstrong and Gilbert 1985; Orpin et al. 1988;). It has been assumed that making alterations to the micro-organisms responsible for the ruminant's digestive processes will allow the efficiency or specificity of digestion to be modified in beneficial ways. As with many areas of biotechnology, progress within this field has been slower than initially predicted, because of the problems associated with manipulating DNA from exotic bacteria (Gregg 1992; White et al. 1990). In essence, development of the technology was expected to be simply a matter of applying laboratory bacterial methods to ruminal organisms, but the practice has proved far more complex.

Nevertheless, all the essential steps for genetic manipulation of rumen bacteria have been achieved. Many genes have been cloned from rumen bacteria, mostly those associated with digestion of carbohydrates (Teather et al. 1990). Some understanding of their expression has been gained (Vercoe and Gregg 1992; Flint et al. 1991; Huang and Forsberg 1990), and methods for returning DNA to several rumen strains have been

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reported (Thompson and Flint 1989; Ware et al. 1992; Whitehead and Hespell 1992; Cocconcelli et al. 1992; Klieve and Gregg, in preparation). The final step that is likely to be necessary for environmental release of genetically manipulated bacteria is the integration of novel genes into the bacterial chromosome. This process has been effectively demonstrated using homologous recombination (Whitehead et al. 1991), and is being attempted using the integrative mechanisms of temperate bacteriophage (Gregg, et al., in preparation).

The groundwork is now laid for practical application of the technology, to generate bacteria that have modified metabolic capabilities. Following the demonstration of useful genetic alteration *in vitro*, the major task will be to return the modified organism to the rumen and monitor its survival and function *in vivo*. The extremely sensitive method of PCR has recently been developed for quantitative detection of individual genetic strains within complex mixtures (Allen and Gregg, in preparation).

Since the first serious discussions of genetically manipulating rumen bacteria (Smith and Hespell 1983), opinion has been divided on the practicality of making useful changes to these little known organisms. More importantly, there has been dispute on whether a genetically altered bacterium would be capable of survival and competition within the rumen, which is regarded as a highly complex and competitive environment (Armstrong and Gilbert 1985).

The acknowledged complexity of the rumen supports the view that introduction of modified strains is unlikely to disrupt the overall function of the system. Reports on genetic diversity among rumen micro-organisms indicate that the rumen is more complex than is immediately apparent from physiological and ecological studies. On closer examination, some of the 14 dominant "species" (Hespell 1987) can each be shown to comprise several (or many) genetically distinct groups which correctly belong in a variety of species and even different genera (Manarelli 1988; Hudman and Gregg 1989). Their inclusion in a single species classification has arisen because of the quite remarkable convergence of phenotypes to suit the rumen environment.

A simple conclusion from such observations is that the rumen possesses a limited number of ecological niches, and that competition for occupancy depends upon the possession of major phenotypic characteristics. Possession of those major characteristics could be considered to define an "ecotype" of organism. Among organisms that have developed similar general features that allow them to occupy a particular niche, there are often numerous more finely variant features retained from their separate origins or accompanying their divergence from a common ancestor. In the presence of large numbers of phenotypically similar but subtly different strains, changes to the environmental conditions within a niche may be accompanied by changes in the "ecotype" subgroup that dominates the niche.

At present, this hypothesis is supported by preliminary observations that show dramatic fluctuations in population numbers of specific genetic types, while the overall population of the ecotype remains stable. Tests of individual strains of *B. fibrisolvens* have shown precisely this pattern. i.e. the individual genotype may fluctuate between $>10^7$ and $<10^4$ while the total numbers of *Butryrivibrio*-like organisms may remain constant.

Experiments to test the *in vivo* survival of laboratory-grown bacteria have reported the loss of introduced strains within a few days (Flint 1992) or even within a few hours (Attwood et al. 1988), leading to a generally held view that laboratory grown bacteria lose characteristics that are important for survival in the rumen. It has been argued, however, that the nature and source of the bacterial strain tested must seriously influence the outcome of *in vivo* tests (Gregg et al. 1987). Consideration of genetic plasticity in microbial populations, and the artificially selective nature of laboratory culture conditions, led us to

base our research on freshly isolated strains of rumen bacteria which were from Australian livestock and were stored frozen as soon as possible after isolation (Gregg et al. 1987).

EXPERIMENTAL RESULTS

Survival of Laboratory Grown Bacteria *In Vivo*

Strain AR10 of *Butyrivibrio fibrisolvens* was isolated from the rumen of a sheep from the University of New England animal house in 1985 (Hudman and Gregg 1989). Analysis by polymerase chain reaction (PCR) showed that AR10 was absent from 71 animals tested across 14 grazing properties in North and Central Queensland, including cattle, sheep and goats. To test the ability of AR10 to return to the rumen, we introduced cultures into the rumens of five Santa Getrudis cattle, within a herd of 40 animals at Headingly Station (Central Queensland). Rumen samples were removed at intervals of 1 week to 37 weeks, and the presence of AR10 was monitored by the same qualitative PCR technique. Animals within the herd that were not directly inoculated showed the presence of AR10 after four weeks (19 out of 25 animals tested), demonstrating that transfer of AR10 between animals occurred rapidly. A similar spread of novel organisms was demonstrated by Jones (1985) with an organism introduced to Australian ruminants from Hawaiian goats. It can be concluded that the inoculation of a herd, with a competitive rumen organism can be simply achieved by directly inoculating only a small proportion of animals.

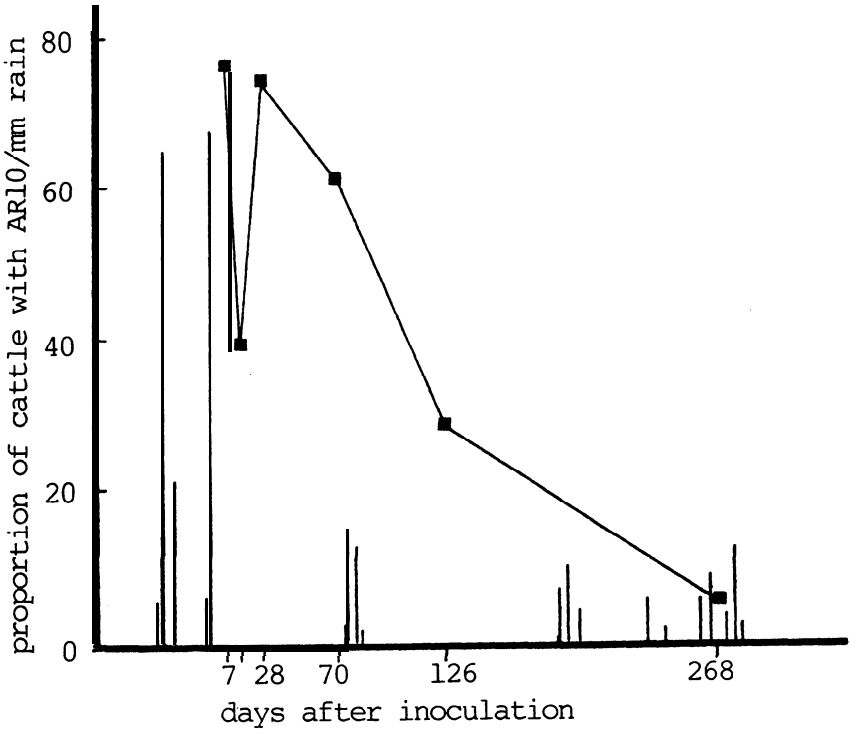

Figure 1. Retention of *Butyrivibrio fibrisolvens* strain AR10 in the rumen of Santa Getrudis cattle in the field. The proportion of animals with AR10 present was expressed as a percentage of those tested at each stage. This varied between 5 and 40 animals. Vertical lines depict rainfall.

AR10 was detected in a proportion of the cattle, over the full period tested so far (268 days; Fig. 1). Interestingly, the proportion of animals retaining detectable levels of AR10 remained high for the first ten weeks of the experiment, but declined markedly during the dry season. The persistence or recovery of AR10 following the subsequent wet season will be known from the samples scheduled to be tested in the next few weeks Individual animals within the test group showed fluctuating populations of AR10, occasionally dropping to numbers too low to be detected and subsequently recovering. It was not possible in this field test to ascertain whether the restoration of population numbers was caused by multiplication of residual populations or by reinoculation through contact with other

This experiment demonstrated the ability of a laboratory grown organism to colonize the rumen under field conditions. It also served as a preliminary demonstration of how population numbers may vary with external influences. The success of AR10 in recolonizing the rumen supports the hypothesis outlined above, that wild-type characteristics can be retained if a minimum culture time is allowed, to isolate and characterize the bacterium before placing a culture in long-term storage at -70° C.

In *vivo* tests of modified bacteria

Proof that cultured bacteria can survive return to the rumen is encouraging for future prospects of placing modified organism in the field, but it has been argued that the process of altering a bacterium genetically may reduce its fitness for survival *in vivo* (Armstrong and Gilbert 1985). This argument has intuitive appeal, on the basis of fitness, energy efficiency, and competition, especially in those cases where the novel gene provides no metabolic advantage to the altered cell (Gregg et al. 1987). However, the hypothesis had not previously been tested empirically.

The *in vivo* survival of an artificially modified bacterium was tested using a cultured strain of *B. fibrisolvens* (E14) which was isolated from the rumen of a reindeer by Dr Colin Orpin (CSIRO Division of Tropical Crops and Pastures, Brisbane). El4 was genetically modified by Dr John Brooker (Waite Inst. S.A.) through conjugation with *Enterococcus faecalis* to introduce the transposon Tn916. This transposon is capable of integration into the chromosome, and imparts resistance to the antibiotic tetracycline. After inoculation into the rumens of four sheep within the UNE animal house, rumen-fluid samples were removed at intervals, diluted and plated onto culture plates containing rumen-fluid medium agar under conditions designed to favour the growth of *Butyrivibrio*-like organisms that were tetracycline resistant. After two days' growth, colonies were transferred to nylon membranes and processed for hybridization with radioactively labelled chromosomal DNA from E14. It was established before inoculation than no organisms naturally present in the rumen were capable of efficient cross-hybridization with El4 DNA, and tetracycline-resistant, hybridization-positive colonies were concluded to be the modified E14.

The survival of modified El4 is shown in Figure 2. Each point represents the mean number of bacteria per ml of rumen fluid, for the four sheep, and it should be emphasized that numbers within individual sheep fluctuated between 2 x 10^7 per ml and levels below the limit of detection by our method. Sheep were not in contact with each other and the recovery of El4 populations was concluded to be through reproduction of residual organisms, and not through reinoculation between animals.

It was clear that El4 carrying the added genetic material (under conditions where it conveyed no survival advantage) was able to compete with existing bacteria and maintain

a population at around 1% of rumen bacteria. Our observations suggest that this is a normal level for a single genetic strain.

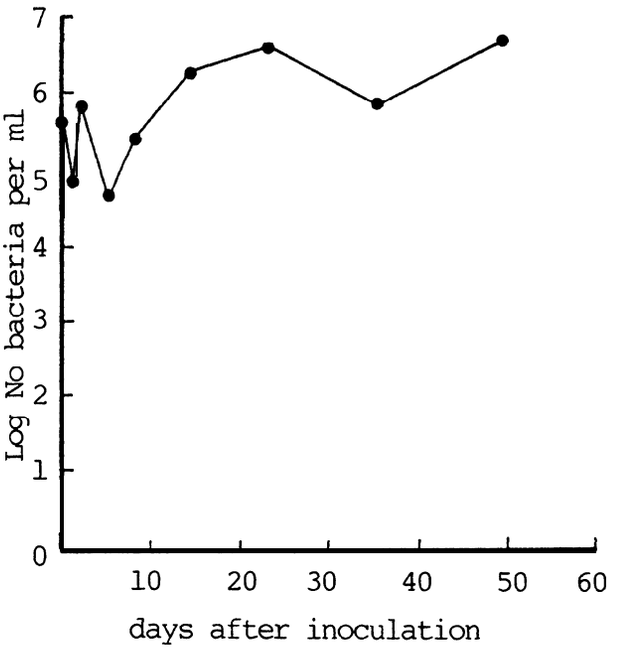

Figure 2. Survival of transposon modifed *B. fibrisolvens* El4 in the rumen of sheep. Each point represents the mean of four measurements from separate sheep. Measurements from individual sheep varied widely between different sampling times.

Despite the mechanism of modification for E14, which has an extremely low probability of occurring in nature, it may be argued that the insertion of Tn916 by conjugation represents a "natural" event, rather than an artificial genetic alteration. Less natural alterations may be seen as more likely to interfere with growth and the maintenance of additional genetic material might slow the reproductive rate of a bacterium. We have tested this proposition in vitro, using the recombinant shuttle-plasmid pBA, developed within this laboratory for transformation of *P. ruminicola* AR20 (Klieve, et al. in preparation). Plasmid pBA is an 11 kb plasmid constructed by combining the *E.* coli plasmid pUC18, the *B. fragilis* plasmid pBI191 (Smith 1985) and a 2.7 kb segment of bacteriophage ϕAR29 (Gregg et al., in preparation). pBA conveys resistance to the antibiotic clindamycin and has been introduced by electroporation into *P. ruminicola* AR20. To determine whether the metabolic load of replicating the plasmid and expressing the clindamycin resistance gene caused any reduction in population growth, the rate of growth of AR20 was tested both in the absence of pBA, and was compared with the rate of growth in the presence of pBA and medium containing 5 μg/ml clindamycin.

Growth curves for the two strains are shown in Fig. 3. Clearly the maintenance and expression of plasmid pBA did not reduce the rate of reproduction of AR20 to any detectable degree. This demonstration supports the notion that reproductive efficiency is determined by a complex series of factors, and the presence and expression of a single novel gene does not seriously unbalance these factors at least in *P. ruminicola* AR20. When the complexity of bacterial genomes is considered, it appears likely that most bacteria carry a battery of genes that confer metabolic advantage only under specific environmental conditions. In many cases such genes may be regulated to be expressed

only when it is advantageous to do so (Flint et al. 1991), but the emerging picture of gene expression in rumen bacteria suggests that this is not necessarily so for all genes (Huang and Forsberg 1990). Our observations with AR20 are almost the opposite of those described for other rumen bacteria (Russell and Wilson 1988) and the cause of the difference is not clear.

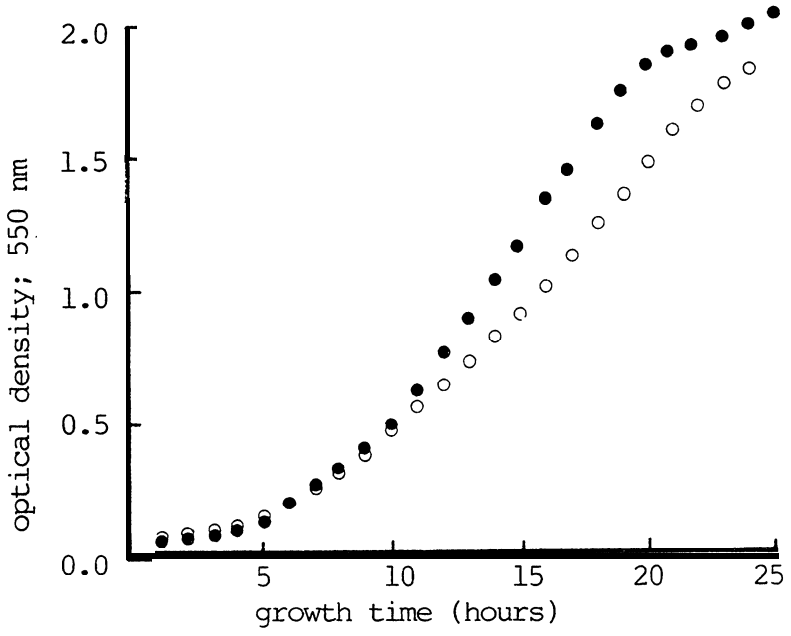

Figure 3. Growth of *P. ruminicola* AR20 in rumen fluid medium. Open circles represent growth of the unmodified strain. closed circles represent growth of AR20 containing plasmid pBA, in the presence of 5 µg/ml clindamycin. Each curve is the average of two measurements.

The important follow-up to *in vitro* studies, with experiments to examine the survival of AR20 carrying the pBA plasmid in the rumen without antibiotic selection, are planned for the immediate future. Approval for in *vivo* experiments has been obtained, but commencement of such experiments is presently awaiting the completion of animal housing facilities that satisfy Cl containment criteria.

CONCLUSIONS

In developing methods for genetic manipulation of rumen bacteria, most effort has been concentrated on technical requirements such as direct DNA transformation of selected species. Throughout the past ten years there has been no serious doubt that the technology for modifying rumen bacteria would be developed. Methods for transformation now exist and a variety of genes are available for transfer into rumen species. However, serious doubt has been levelled at the ability of modified organisms to compete with their unmodified couterparts when returned to the rumen.The data presented here demonstrate that predictions based upon theories of fitness and competitiveness are not supported by practical observations, probably because factors affecting bacterial reproduction are more complex than is presently understood. Indeed, it appears likely that an understanding of ruminal ecology that is based upon observations of bacterial ecotypes, deals only with gross aspects of this complex

ecosystem. The ability to follow single strains of bacteria will undoubtedly add another dimension to ecological studies within the rumen, and results obtained so far provide optimistic indications for the possibility of adding usefully modified bacteria to the rumen.

METHODS

Bacterial culture. Rumen bacteria were grown routinely in rumen-fluid medium, at 39° C, and handled under strict anaerobic conditions. Growth of AR20 cultures was monitored continuously by automatic sampling of a stirred anaerobic culture into a flow-through spectrophotometer (Hitachi). Clindamycin was used at a final concentration of 5 μg/μl, and tetracycline at 10 μg/μl, by addition of a concentrated stock solution made with distilled water. Rumen samples containing strain El4 were diluted in rumen-fluid medium and spread onto selective plates of rumen fluid medium containing 1.5% agar and 0.2% oat-spelt xylan as the major carbohydrate source, containing 10 μg/ml tetracycline. Colonies were allowed to grow for 2 days at 39° C in sealed, anaerobic cannisters.

Sampling from the rumen. Sheep with rumen fistulae were used for reintroduction of bacteria by inserting 50 ml of a dense culture. Samples were removed by means of a tube and syringe, diluted and plated for anaerobic growth. Sheep were housed in the UNE animal house and fed on oaten chaff. Cattle used in field tests were range-fed, inoculated with 250 ml cultures using a throat tube, and rumen samples were extracted by means of a nose-tube while the animals were held in a crush. After removal, rumen samples were heated to 100° C for 10 minutes before sealing the lids and shipping on wet-ice to the laboratory.

Colony Hybridization. Colonies of *B. fibrisolvens* El4 were transferred to nylon hybridization membranes and processed by the procedure of Maniatis et al (1982). El4 genomic DNA was extracted, radiolabelled with ^{32}P-dATP by the process of nick translation, and used to probe the filters as described previously (Hudman and Gregg 1989). After washing twice at a stringency of 0.1 x SSC at 65° C for 30 minutes each, the membranes were sealed in plastic and autoradiographed overnight at -70° C with an intensification screen (DuPont HiPlus), using Fuji X-ray film.

Polymerase Chain Reaction. *B. fibrisolvens* strain AR10 was detected by PCR amplification of a 300 bp DNA fragment that was shown empirically to be specific for strain AR10 and very closely related strains from the same source. Reactions were performed with 25 cycles of 90s at 94° C; 60s at 60° C; 60s at 72° C. Rumen samples were centrifuged to sediment micro-organisms and small fragments of plant material. The pelleted material was washed by resuspension in distilled water and resuspended in the original volume of sterile distilled water. Suspended particles were added to the PCR reaction, and heating to 94° C in reaction buffer was sufficient to allow reagents access to genomic DNA. and permit amplification.

Products were visualised by electrophoresis on 1% agarose gels in TBE buffer (Maniatis et al. 1982), stained with ethidium bromide (1 μg/ml) and photographed over an ultraviolet transilluminator.

ACKNOWLEDGEMENTS

The authors wish to thank Jenny Druitt and Helen Watson for technical assistance.. Thanks also go to Mr Clayton Paige of Headingly Station and Dr Barry Robinson of Queensland's Department of Primary Industries for their practical assistance in performing field tests on cattle and, together with Mr Terry McCosker of Resource Consulting Services, for help in the organization of field trials. A large proportion of this work was funded by the Australian Meat Research Corporation and Applied Biotechnology (Qld).

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