"STATE-OF-THE-ART" IN RUMEN BACTERIAL GENETIC MANIPULATION

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SUMMARY

Genetic characterization of rumen bacteria is progressing rapidly, with the cloning of many genes, principally those encoding enzymes related to fibre digestion. Material is now available to allow gene regulatory sequences to be studied in detail. Direct transformation with recombinant plasmids has been reported for two species, and is being developed for others. A number of different techniques are now available to allow altered strains to be monitored when returned to the rumen, and the most sensitive are capable of detecting extremely low numbers. There are, so far, no reports of practical changes being made to rumen bacteria to enhance the capabilities of the rumen, but the present rate of progress suggests that such achievements are within reach.

INTRODUCTION

Using methods of dietary or ruminal manipulation it is already possible to make dramatic increases in animal production by making ruminants grow, reproduce, and lactate more efficiently (Leng 1990). It could therefore be argued that a biotechnological approach to the same problems may be unnecessary, since the technology is not practically applicable at present (Leng 1991). However, a sound reason for placing importance on the development of this technology is that it is likely to provide a variety of additional mechanisms for the enhancement of nutritional efficiency. Perhaps more importantly, the technology should provide possible solutions to other, non-nutritional problems in animal production.

Processes by which genetic alteration of rumen bacteria may improve nutritional efficiency include: enhanced fibre digestion (Smith and Hespell 1985); increased rumen by-pass of proteins containing essential amino-acids (Teather1985), or production of manipulative compounds which may increase the efficiency of rumen fermentation, such as anti-protozoal agents (Bird *et al.* 1990). Other uses for the technology include the possibility of constructing bacteria that are capable of detoxifying plant metabolites, or pesticides (Gregg and Sharpe 1991), or are engineered for the production of anti-parasitic agents

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Whatever the practical goal, attempts to manipulate the genetics of rumen bacteria involve certain fundamental steps that are common to most applications:

1. Selection of suitable bacterial species for alteration.

2. Cloning and characterization of rumen bacterial genes and their control mechanisms.

3. Methods for insertion of DNA and ensuring its retention.

4. Techniques for monitoring survival of altered organisms.

At present, the technology involved in each of these steps is still being developed, and in outlining the positive advances in the field it will be necessary to comment upon some of the difficulties that have been encountered. During early work, the extension of laboratory methods developed with ***Escherichia coli,*** to the anaerobes of the rumen, was approached with an expectation of rapid progress in many laboratories. In some respects this has proven justified, but in most respects it has not. Progress within this area of molecular biology has been slower than could be predicted five years ago, and it has become clear that extensive studies of the molecular genetics of rumen bacteria will be essential if genetic manipulation is to become routine. However, there have been considerable advances in this field since the first serious discussions of its application were published (Smith and Hespell 1983).

RUMEN BACTERIAL CULTURE AND CHARACTERIZATION

Classical taxonomy of rumen bacteria has defined approximately 15 phenotypic species which compose the major bacterial biomass of the rumen (Table 1, Hespell 1987). The role played by the major groups in rumen ecology is quite well understood (Russell 1988) and methods have been developed for the isolation and culture of the major species, which generally require strictly anaerobic conditions (Hungate 1969).

A complication that has arisen in this area of study, is that the phenotypes described in conventional taxonomy may not accurately reflect the genetic relationships of many of the major species. For example, it has been shown that independently isolated Australian strains of the species ***Butyrivibrio fibrisulvens*** are in many cases genetically distinct from one another, to the extent of comprising different species or even different genera (Hudman and Gregg 1989). In the same report, diversity was also noted among strains of ***Bacteroides ruminicola.*** Similar genetic divergence has been recorded among strains of ***Butyrivibrio*** from North America (Manarelli and Hespell 1987; Sewell et ***al.*** 1988) and among other rumen species (Hazlewood and Teather 1988; Ware et ***al.*** 1989).

The importance of this extensive variability depends upon the purpose for which the bacteria are being studied (Gregg and Ware 1990). Research which attempts to transfer genetic

Table 1 Dominant bacterial species within the rumen

SPECIES
Anaerovibrio lipolytica
Bacteroides amylophilus
*Bacteroides ruminicola**
*Butyrivibrio fibrisolvens**
*Fibrobacter succinogenes*Θ
Lachnospira multiparus
Methanobrevibacter ruminantium
Methanosarcina barkeri
*Ruminococcus albus**Θ
*Ruminococcus flavefaciens*Θ
*Selenomonas ruminantium**
Streptococcus bovis
Succinivibrio dextrinosolvens
Wolinella succinogenes

* species being developed as DNA recipients
Θ highly cellulolytic species selected as gene donors

material between two organisms, may be profoundly affected by differences in DNA regulatory sequences (for example), which may cause genes to function differently within the two organisms.

Various methods have been developed for the identification of bacteria by their genetic content, rather than their outward appearance (Stackebrandt 1988), and these are being applied to rumen bacteria with increasing frequency.

GENE CLONING AND SEQUENCING

The genes that have been cloned from rumen bacteria can now be numbered in dozens (Table 2), with a majority being those which encode enzymes for digestion of complex carbohydrates, such as endoglucanase, endoxylanase, exoglucanase, ß-glucosidase, and ß-xylosidase genes. Complete DNA sequences have been established for more than 14 genes (Table 2), and genetic variability between strains provides a greater resource of distinct genetic material than was previously realized. For example, the endoglucanase genes cloned from *R. albus* AR67 differ markedly from those cloned from strain F-40 (Ware et *al.* 1989).

Descriptions of genes that have been cloned and sequenced have not generally given an indication of the difficulties that have been encountered in some cases. In many laboratories, evidence of cloning difficulties has been clear from the frequency with which cloned genes were subsequently lost

because of plasmid instability (Huang *et al.* 1989; White *et al.* 1990). In other cases, cloning inefficiency has been shown by the very large number of transformant colonies that had to be screened to isolate a small number of specific gene-clones. In some cases, screening an estimated 60 fold excess of transformant colonies was required, to isolate certain genes (Gong et *al.* 1989).

We have experienced particular difficulty in sub-cloning rumen bacterial DNA into the single-stranded DNA bacteriophage, M13. Reasons for this have not been established conclusively, but in many cases it has been necessary to sequence genes by using methods that circumvent the problem. In our laboratory,

Table 2 Genes cloned from rumen bacteria

SPECIES	GENE PRODUCT	SEQUENCED
Bacteroides ruminicola	endoglucanase[ab]	yes[BC]
	xylanase[c]	no
Butyrivibrio fibrisolvens	endoglucanase[def]	yes[df]
	ß-glucosidase[h]	yes[h]
	cellodextrinase[i]	yes[i]
	Xylanase[j]	yes[j]
	Xylosidase[k]	no
Fibrobacter succinogenes	endoglucanase[l]	yes[D]
	cellodextrinase[m]	no
	mixed-link-glucanase[n]	yes[E]
	Xylanase[o]	no
Ruminococcus albus	endoglucanase[epqrst]	yes[FGH]
	ß-glucosidase[uv]	yes[v]
	Xylanase[q]	no
Ruminococcus flavefaciens	endoglucanase[wxy]	no
	mixed-link glucanase[z]	no
	ß-glucosidase[y]	no
	xylanase[xz]	no
	cellodextrinase[A]	yes[A]

[a]Woods *et al.* 1989; [b]Russell and Wilson 1989; [c] Whitehead and Hespell 1989; [d]Berger *et al.* 1989; [e]Mann 1988; [f]Hazlewood *et al.* 1991; [h]Lin *et al.* 1990; [i]Berger *et al.* 1990; [j]Manarelli *et al.* 1990; [k]Sewell *et al.* 1989; [l]Crosby *et al.* 1984; [m]Gong *et al.* 1989; [n]Irvine and Teather 1988; [o]Sipat *et al.* 1987; [p]Kawai *et al.* 1987; [q]Romaniec *et al.* 1989; [r]Ohmiya *et al.* 1988; [s]Howard and White 1988; [t]Ware *et al.* 1989; [u]Ware *et al.* 1990; [v]Ohmiya *et al.* 1990; [w]Barros and Thomson 1987; [x]Howard and White 1990; [y]Huang *et al.* 1989; [z]Flint *et al.* 1989; [A]Wang and Thomson 1990; [B]Matsushita *et al.* 1990; [C]Vercoe *et al.* 1991a; [D]McGavin *et al.* 1989; [E]Teather and Erfle 1990; [F]Poole *et al.* 1990; [G]Vercoe *et al.* 1991b; [H]Ohmiya *et al.* 1989.

complications caused by plasmid instability and inability to obtain single-stranded DNA clones have been overcome by sequencing double-stranded DNA using synthetic oligonucleotide primers to "walk" through the sequence. This has eliminated. the need for sub-cloning. Difficulty of cloning has not been observed with all rumen bacterial DNA; for example, rapid progress has been made with genes cloned from *F. succinogenes* strain S85, using the conventional sub-cloning and single-strand sequencing techniques generally applied to eukaryotic DNA (McGavin *et al.* 1989; Teather and Erfle 1990).

Sequencing rumen bacterial genes is essential to reveal the mechanisms by which gene expression is controlled. Structure alone cannot define which sequences have regulatory function, but provides the information essential for more definitive experiments.

In many cases, rumen bacterial genes have been shown to contain regions with strong similarity to the regulatory sequences of *E. coli,* and these have been cautiously assumed to control expression of the genes in their native bacterium (Teather 1990). One study has demonstrated that the mRNA encoding an *R. albus* endoglucanase is synthesized from a point close to sequences which strongly resemble an *E. coli* consensus promoter (Ohmiya *et al.* 1989). However, for most of the genes sequenced so far, there has been no definitive proof that transcription does indeed commence adjacent to the observed promoter-like sequences. In one case, primer extension studies have shown that transcription of a *B. ruminicola* endoglucanase gene does not originate from any of the *E.* coli-like sequences that are present upstream from the protein-coding region, but from a region in which there is no obvious consensus sequence (Vercoe and Gregg 1991a). In some cases, therefore, gene regulatory requirements are likely to differ from familiar bacterial systems and will require detailed study.

It is clear that the rapid accumulation of sequence data that is occurring at present will allow the general situation in each rumen species to be established, but it is not yet possible to predict whether a gene will be functional if introduced into any particular species. At present, essentially nothing is known about molecular mechanisms of gene induction within rumen bacteria.

DNA TRANSFER VECTORS FOR RUMEN BACTERIA

Mechanisms for the insertion of novel DNA into bacteria usually involve epigenetic elements such as plasmids, bacteriophages, or transposons. All three systems are being tested for their application to rumen bacteria.

Transfer of plasmids and transposons from non-rumen species into rumen bacteria, by the process of conjugation, has been demonstrated to occur (Hazlewood and Teather 1985; Russell and Wilson 1988), and transfer between rumen species is also possible (Flint et *al.* 1988). However, the broad host-range

plasmids used in some experiments have proven unstable and unsuitable as vectors for foreign DNA (Teather 1985). Plasmids derived from the intended recipient species appear more promising as transfer vectors (Thomson and Flint 1989; Hazlewood and Gilbert 1989). Bacteriophage, and plasmids that have been used for direct DNA transformations are listed in Table 3.

Successful attempts have been made to insert naked DNA directly into rumen bacteria using polyethylene glycol shock (Lockington *et al.* 1989). However, more widespread success has resulted from the use of electroporation, a process by which a pulsed electrical field induces bacteria (and other cells) to take up DNA from the surrounding medium (Dower *et al.* 1988; Thomson and Flint 1989; Hazlewood and Gilbert 1989; Lockington *et al.* 1989; Gregg and Ware 1990).

Two reports have been published describing plasmid transformation of ***Bacteroides ruminicola*** by electroporation. One involved the naturally occurring plasmid pRRI4 (Thomson and Flint 1984) and the other involved a recombinant vector constructed from a cryptic native plasmid (Hazlewood and Gilbert 1989). We have demonstrated the use of electroporation for the transformation of ***Butyrivibrio fibrisolvens*** strain AR10 (Gregg and Ware 1990), using a recombinant plasmid which was constructed from a cryptic plasmid native to AR10.

Table 3 Bacteriophages and plasmids used for transformation

SPECIES	EPISOME	TRANSFORMATION METHOD
B. ruminicola	plasmid pRRI4[a]	electroporation[b]
	*plasmid pE5-2[c]	electroporation[c]
		conjugation[d]
B. fibrisolvens	plasmid RP4[e]	conjugation[e]
	plasmid pRK248[f]	PEG/spheroplasts[f]
	*plasmid pCW2[g]	electroporation[g]
S. ruminantium	bacteriophage[h]	electroporation[h]
		PEG/lysozyme[h]
	plasmid[i]	culture with DNA[i]

* recombinant plasmids; PEG = polyethylene glycol treatment.

[a]Flint *et al.* 1988; [b]Thomson and Flint 1989; [c]Hazlewood and Gilbert 1989; [d]Russell and Wilson 1988; [e]Teather 1985; [f]Hazlewood and Teather 1988; [g]Gregg and Ware 1990; [h]Lockington, *et al.* 1988; [i]Orpin *et al.* 1986.

The process of introducing DNA into a bacterium requires that the organisms containing the new material be separable from those that do not. This is achieved in most cases by using what is known as a selectable marker gene. This may be a gene that allows the bacterium to use an unusual nutrient, but more often it is a gene that provides protection for its host, against some toxic compound. The majority of selectable marker genes provide resistance to an antibiotic.

A tetracycline resistance gene carried on a native rumen plasmid (pRRI4) has been used successfully for the selection of transformed *B. ruminicola* (Thomson and Flint *1989).* Similarly, several species of bacteria have been shown to be capable of expressing the Tet(M) gene from transposon Tn916 (J. Brooker, pers. comm. *1990)* which was obtained originally from *Streptococcus faecalis* (Senghas et *al. 1988).*

Interestingly, antibiotic resistance genes from non-rumen sources are frequently ineffective in rumen species. In our work (Ware *et al.* unpublished) it has been demonstrated that the ampicillinase gene from pUC plasmids (Yanisch-Perron *et al.* *1985)* works only poorly as a selectable marker in *B. fi bri solvens*, and the CAT gene from pKK232-8 (Brosius *1984)* and the clindamycin resistance gene from *Bacteroides fragilis* (Smith *1985)* appear non-functional in this species. It remains to be shown whether this is a consequence of differences in gene regulatory factors, translation and/or protein folding under anaerobic conditions, or inappropriate partitioning of the resistance factor within the bacterium. In contrast, the clindamycin resistance gene from *B. fragilis* has been shown to function in *B. ruminicola* (Hazlewood and Gilbert *1989).*

Development of transformation vectors and. methods-for rumen bacteria is in its infancy, but it is clear that recombinant plasmids based upon naturally occuring replicons will be useful for the transfer of foreign DNA into rumen bacteria. The availability of suitable vectors is expected to lead to a rapid expansion in genetic knowledge.

Most genetic manipulation projects which entail the release of altered bacteria require the genomic integration of the added genes. Work with transposons has shown that DNA can be transferred to rumen bacteria and incorporated into the chromosome. The inherent mobility of transposons makes them unsuitable for practical genetic alteration, unless their mobility can be regulated, but their usefulness as genetic research tools may be considerable (Hespell *1987).*

In our own laboratory we are examining the possibility that the integrative function of bacteriophages may be useful in attaching newly introduced genes to the bacterial chromosome. A *2.8* kilobase DNA fragment containing an integration attachment site from the temperate bacteriophage ϕAR29 (Klieve *et al.* 1989) has been cloned into an *E. coli* plasmid and is now being tested for its ability to direct chromosomal integration (A. Klieve and K. Gregg, unpublished).

MONITORING BACTERIAL LEVELS IN THE RUMEN

When altered bacteria are returned to the rumen, the first measure of their success will be their ability to persist in sufficient numbers to exert a significant biological effect. It is therefore essential to have a reliable mechanism for measuring the predominance of the altered strain within the rumen population.

DNA hybridization has been used to estimate bacterial numbers in a variety of locations including rumen fluid. Attwood et al. (1989) demonstrated the detection of a target species in rumen fluid, with an estimated sensitivity of around $10^6 - 10^7$ cells per ml. However, in a population of 10^9-10^{10} bacteria per ml this represents a sensitivity of only 1% – 0.1%. The genetic diversity that has been shown to occur within single phenotypes (see above) indicates that any one genotype is likely to compose only a small proportion of the apparent representation by that phenotypic species: i.e. where a phenotypic species is 5% of the rumen population, a single genotype may be less than one tenth of this. As a consequence, the level of sensitivity provided by standard hybridization techniques may be inadequate to follow fluctuations of individual genotypes within the rumen.

An alternative method has been described which uses monoclonal antibodies to detect specific strains of bacteria in a mixed population with considerably greater sensitivity (Brooker and Stokes 1990). However, in some cases this method may be too specific, since the cell-surface antigens of bacteria can change with a changing environment. For example, the loss of a particular nutrient receptor, with a change in diet, could result in false negative results from monoclonal antibody detection processes.

A more promising mechanism for detecting and enumerating individual species lies in the recently developed technique of polymerase chain reaction (PCR; Mullis and Faloona 1987). This method uses a DNA synthesis reaction to duplicate a particular DNA sequence. Repeated denaturation, annealing and polymerization steps, using a thermostable enzyme, allows the duplication to be repeated many-fold in an automated system (Saiki et al. 1988). In principle, this technique makes possible the detection of a single copy of a DNA sequence in a mixed population.

PCR has been applied in the detection and quantitation of bacterial species in non-ruminal ecosystems as well as being developed in our laboratory for application to the rumen. Early work has shown considerable success in detecting DNA equivalent to 1^3 cells among non-target DNA equivalent to approximately 10^8 cells (i.e. detecting an organism that is 0.0001% of the total population) while remaining well within the sensitivity limits of the technique. For quantitation of individual species within the rumen, the most challenging part of this process is the development of methods which ensure that DNA extracted from rumen samples, is representative of the entire bacterial population. It is expected that, for

most purposes, quantitation down to 0.0001% of the bacterial population will be adequate to detect those bacteria which play a significant role in the rumen.

APPLICATIONS FOR MODIFICATION OF RUMEN BACTERIA

Many reviews have been published, describing the potential benefits to be obtained by manipulating the genetics of rumen micro-organisms (Teather 1985; Smith and Hespell 1985; Armstrong and Gilbert 1985). However, it is still too early to predict which alterations are most likely to provide real benefits. Although DNA has been inserted into rumen bacteria, demonstrating the feasibility of genetic alteration, there are still no reports of useful changes being made to rumen bacteria, and some of the strains used in genetic studies have an unpredictable likelihood of surviving if returned to the rumen (Attwood et *al.* 1988).

It is expected that some genetic changes to rumen bacteria will be effective when expressed as a small percentage of the rumen population (e.g. <0.1%) while others may require a major proportion to be altered (e.g. >5%) to have an adequate effect on the physiology of the animal. The following are examples of three different types of manipulation which are likely to require different levels of altered bacteria within the rumen population.

Detoxification

An adaptation of rumen bacteria that could be applicable to a large number of specific problems, is modification to allow detoxification of plant poisons, pesticides, or other pollutants which contaminate ruminants through their presence as residues in soil and pastures (Gregg and Sharpe 1991). A precedent has been set by the work of Jones and Megarrity (1986), in which a bacterial culture was transferred from the rumen of Hawaiian goats to Australian goats and sheep, transferring resistance to the goitrogenic amino-acid mimosine and its degradation product dihydropyridone. Populations of the new bacteria were detected at a level around 0.01% of the rumen flora ($\approx 10^5$/ml), but were able to protect the animals against toxin levels taken in by eating *Leucaena leucocephala* (R Jones, personal communication). Bacteria that are engineered to produce anti-parasitic compounds or anti-protozoa1 agents may also function adequately at quite low population numbers.

Protein quality supplementation

An attempt may be made to supplement dietary protein by causing "storage" or "by-pass" proteins to be generated within rumen bacteria (Teather 1985; Brooker et *al.* 1989). Altering a small proportion of bacteria to carry high levels of proteins that are rich in essential amino acids might be of considerable nutritional benefit to the host animal. However,

the ecological competitiveness of such organisms may be attenuated if they store large quantities of proteins that are of no advantage to them. It may be necessary to provide external support mechanisms to enable the altered bacteria to survive in useful numbers. As an alternative, it may be more beneficial to alter a larger proportion of the rumen population to produce low levels of an enriched protein,, for example by adding essential amino acids to existing structural proteins. While this would almost certainly require the alteration of many organisms, it appears more likely to be successful, on evolutionary principles.

Enhanced fibre digestion

At present, the process of cellulose digestion remains poorly understood and might therefore appear impractical as a genetic modification. Nevertheless, it has been demonstrated that cellulase genes from a bacterium can be added to a normally non-cellulolytic organism (in this case a yeast), to make it capable of digesting filter paper and treated wood-chips (Wong *et al.* 1988). The process of fibre digestion involves a major proportion of the rumen microflora, and manipulations to enhance fibre digestion might require large changes in the bacterial population in order to show significant increases in digestive efficiency. The remarkable genetic diversity among rumen bacteria increases the complexity of this task.

Expression of a foreign cellulase gene in a modified rumen bacterium has been reported, although this apparently did not confer true cellulolytic capability (Hazlewood and Gilbert 1989). The ease with which cellulolytic capabilities may be transferred to non-cellulolytic organisms may depend upon the particular system chosen for transfer. For example, cellulases isolated from *F. succinogenes* appear to retain little of their ability to digest native cellulose as a cell-free extract (Groleau and Forsberg 1981). In contrast, we have demonstrated that *R. albus* strain AR67 produces extracellular cellulases capable of digesting filter-paper and Avicel extensively in cell-free preparations (A. Lachke et *al.* in preparation). Such enzymes may be ideal for transfer to species that are normally only weakly cellulolytic, such as *B. fibrisolvens* (Bryant 1974). With five cellulolytic genes cloned from AR67 (Ware et *al.* 1989; Ware *et al.* 1990), it remains to be shown which of these, or which additional ones, are required to confer cellulolysis on a non-cellulolytic species.

CONCLUSIONS

It is widely accepted that a greater understanding of rumen microbial molecular genetics is necessary if manipulation of rumen bacteria is to become routine, and the knowledge required to make it so is expanding rapidly.

Bacterial species suitable for modification have been selected from the large number present in the rumen. Genes from those

species are being cloned and sequenced at an accelerating rate and much structural information is already available. Experiments to study the operation of these genes ***in vivo*** are in progress in several laboratories.

Methods for the insertion of new DNA have been developed for at least two species, and possible ways to stabilize genes by chromosomal integration are being investigated. The most recent developments in PCR technology lend themselves to the process of monitoring fluctuations in bacterial populations in the rumen. As a consequence, it will be relatively simple to assess the viability of modified bacteria when reintroduced to the rumen.

At present, practical genetic alteration of rumen bacteria remains a thing of the future. However, the rate of progress during the past five years, and the rate of acceleration of progress, has made it clear that the capability to make useful modifications to these important organisms is no longer in question.

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