Recent Advances in Biotechnology of Rumen Bacteria*
- Review -

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ABSTRACT : Recent advances in the biotechnology of ruminal bacteria have been made in the characterization of enzymes involved in plant cell wall digestion, the exploration of mechanisms of gene transfer in ruminal bacteria, and the development of vectors. These studies have culminated in the introduction and expression of heterologous glucanase and xylanase genes and a fluoroacetate dehalogenase gene in ruminal bacteria. These recent studies show the strategy of gene and vector construction necessary for the production of genetically engineered bacteria for introduction into ruminants. Molecular research on proteolytic turnover of protein in the rumen is in its infancy, but a novel protein high in essential amino acids designed for intracellular expression in ruminal organisms provides an interesting approach for improving the amino acid profile of ruminal organisms.
(**Key Words**: Rumen Bacteria, Gene Technology, Enzymes, Phylogenetic Diversity, Protein Quality, Review)

INTRODUCTION

Ruminants have the ability to convert low quality feeds into high quality protein. This is made possible by the complex population of ruminal microorganisms consisting primarily of bacteria, protozoa and fungi which produce cellulase and hemicellulase enzymes that bring about the hydrolysis of the plant cell wall. They utilize the hydrolysis products as a source of energy for growth. During growth they have the capability to use ammonium salts for the synthesis of microbial protein. However, only 10-35% of energy intake by the animal is captured as net energy because 20 to 70 % of cellulose may not be digested by the microflora (Varga and Kolver, 1997). There is a similar story for nitrogen metabolism in that as much as 60% of nitrogen entering the rumen does not reach the duodenum as microbial protein (Firkins, 1996). Because energy is the driving force for metabolic reactions much of the research focus has been on enzymes involved in plant cell wall degradation. Molecular genetic research, the under pinning of biotechnology, has assumed this same bias. Therefore this review will focus mainly on molecular aspects relating to the bacterial biodegradation of plant cell walls and related polymers, although other current aspects will be highlighted.

Bacteria are the primary countributors to the ruminal fementation; the fungi and protozoa have important, but less central roles. The fungi account for approximately 8% of the microbial biomass in the rumen (Orpin and Joblin, 1988) and this low density may be due to their slow generation time in comparison with the bacteria (6-9 vs. 0.5-3.5 h). Their elimination may only have a major effect if the animal is on a very poor quality forage ration (Gordon and Phillips, 1993). The protozoa have a greater role and their activities, for example, cellulase activity may account for 19-28% of total activity (Gijzen et al., 1988), and they may degrade only 5 to 21% of the cellulosic materials depending upon the diet (Dijkstra and Tamminga, 1995). Elimination of the protozoa may have a slightly deleterious effect on plant cell wall biodegradation, but with a general improvement in the overall efficiency of the ruminal fermentation (Jouany, 1996). Thus the focus is on novel features of the ruminal bacteria and the improvement of their metabolic characteristics by harnessing unique genes from other microorganisms.

RUMEN BACTERIA AS A SOURCE OF HYDROLASE GENES

Rumen bacteria contain a wide array of endoglucanases, cellobiosidases and β-glucosidases. They have

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* This paper has been presented at Pre-Conference Symposium 3 entitled "Rumen Micororganisms and Application to the Improvement of Ruminant Production" at the 8th World Conference on Animal Production on June 28, 1998 at Seoul National University, Seoul, Korea. The paper has been reviewed and edited by Prof. K. Ushida (Japan) and Prof. J. K. Ha (Korea).

been reviewed in detail by several authors (Chesson and Forsberg, 1997; Selinger et al., 1996). To provide perspective, a brief review of the mechanism of cellulose digestion is useful (figure. 1).

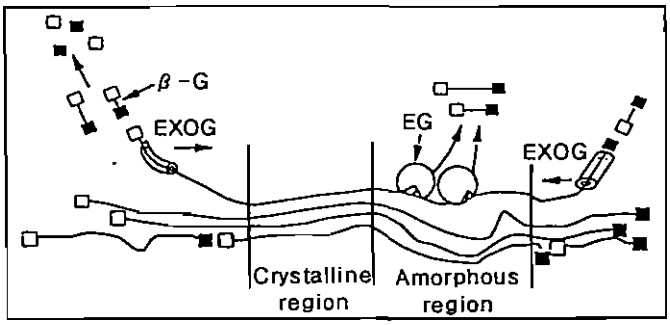

Figure 1. Schematic representation of the organization of crystalline cellulose and its hydrolysis by a cellulase complex. Cellulose is composed of crystalline and amorphous regions. Endoglucanases (EG) with open catalytic sites cleave internal amorphous regions while exoglucanases (cellobiohydrolases; EXOG) with tunnel shaped catalytic sites cleave exposed chain ends releasing cellobiose. Cellobiose is cleaved by β-glucosidase (β-G). Filled squares indicate reducing ends and open squares denote non-reducing ends. After Teeri (1997).

Although not mentioned, a large proportion of the information serving as the basis for the proposed mechanisms of cellulose hydrolysis stems from cloning and characterization of the genes. Cellulose hydrolysis is carried out either by free enzymes or cellulosomal structures composed of multiple enzymes non-covalently bonded to form an organized complex (Teeri, 1997). The aerobic fungi and some bacteria digest cellulose by the former mechanism while *Clostridium* species and probably the anaerobic ruminal fungi (Fanutti et al., 1995) hydrolyze cellulose by the latter mechanism involving cellulosomal structures. In systems involving free enzymes such as in aerobic fungi, for example, *Trichoderma reesei*, most cellulase enzymes are composed of a catalytic domain linked by an extended linker region to a cellulose-binding domain (CBD). CBDs are required for efficient cellulose hydrolysis. Cleavage occurs by a general acid catalyzed hydrolysis, leading to either inversion or retention of the configuration of the anomeric carbon. The key enzymes are endo- and exoglucanases. A typical endoglucanase cleaves bonds along the length of amorphous cellulose chains resulting in a rapid decrease in the degree of polymerization. Exoglucanases are processive enzymes initiating their actions from both the reducing and non-reducing ends producing primarily cellobiose. The complementary action of exo- and endoglucanases leads to synergy. The action of β-glucosidases prevents inhibition of the enzymes caused by accumulated cellobiose.

The cellulosomal mechanism of cellulose hydrolysis typified by that of *C. thermocellum* involves a multienzyme complex, the cellulosome, that is composed of a large non-catalytic glycoprotein CipA that acts as both a scaffolding component and a cellulose-binding factor. Catalytic subunits (endoglucanases, cellobiohydrolases, xylanases) of the cellulosome bear conserved, non-catalytic subdomains, termed dockerin domains, these bind to receptor domains of CipA termed cohesin domains. CipA contains nine cohesin domains, a cellulose-binding domain and a specialized dockerin domain. CipA molecules are bound via the dockerin to specific cohesins of cell surface proteins. Although there is a difference in structural organization between the free and cellulosomal cellulase systems, the comparable enzymes belong to the same families of enzymes. X-Ray crystallography has shown that in general the endoglucanases tend to have open active sites while exoglucanases have tunnel shaped active sites which limit their action to glucan chain ends. Despite considerable progress with other cellulolytic microorganisms (Beguin and Lemaire, 1996; Ohmiya et al., 1997, Teeri, 1997), the mechanisms by which ruminal bacteria degrade cellulosic materials have remained unclear. Cloned genes coding for enzymes involved in plant cell wall biodegradation have been primarily glucanases, cellodextrinases and β-glucosidases and most lack affinity for cellulose (table 1).

An exception to these is the *celF* gene product, EGF from *F. succinogenes* (Malburg et al., 1997) formerly known as endoglucanase 2 (McGavin and Forsberg, 1989). It contains separate catalytic and cellulose binding domains, neither of which exhibit substantive similarity to other known families of catalytic and binding domiains. It has been suggested that the presence of protuberant structures on the cell surface of some ruminal bacteria resemble cellulosomes of *Clostridium thermocellum* (Miron et al., 1989), but biochemical evidence is lacking. Kirby et al. (1997) recently demonstrated that the *R. flavefaciens* 17 glucanase gene *endA* encodes a product larger than was originally estimated whose non-catalytic region includes sequences similar to the 80 amino acid conserved region found in xylanase XYNB and XYND (Zhang et al., 1994). These regions showed distant but significant similarities to dockerin sequences from *C. thermocellum* (Schimming et al., 1992), suggesting that this enzyme and the two xylanases may form part of a cellulosome-like multienzyme complex.

Glycanases with dockerins have not been detected in *F. succinogenes*, however, a highly basic C-terminal

Table 1. Cellulase and hemicellulase genes from rumen bacteria and substrates hydrolyzed by their gene products

Bacterium	Gene[a]	Enzyme	Reference
B. fibrisolvens A46	*celA* [A2]	Glucanase	(Hazlewood et al., 1990)
B. fibrisolvens H17c	*endI* [A4]	Glucanase	(Berger et al., 1989)
B. fibrisolvens H17c	*cedI* [E9]	Cellodextrinase	(Berger et al., 1990)
B. fibrisolvens H17c	*bglA* [A3]	β-Glucosidase	(Lin et al., 1990)
B. fibrisolvens 49	*xynA* [F10]	Xylanase	(Mannarelli et al., 1990)
B. fibrisolvens H17	*xynB* [F10]	Xylanase	(Lin and Thomson, 1991)
B. fibrisolvens GS113	*xylB* [43]	Xylosidase	(Sewell et al., 1989; Utt et al., 1991)
B. fibrisolvens E14	*cinA*	Cinnamoyl ester hydrolase	(Dalrymple et al., 1996)
B. fibrisolvens E14	*cinB*	Cinnamoyl ester hydrolase	(Dalrymple and Swadling, 1997)
F. succinogenes S85	*celF*	Glucanase	(Malburg et al., 1997)
F. succinogenes S85	*cel-3* [A3]	Glucanase	(McGavin et al., 1989)
F. succinogenes S85	*celD* [9E]	Glucanase	(Malburg et al., 1996)
F. succinogenes S85	*celE* [9E]	Glucanase	(Malburg et al., 1996)
F. succinogenes S85	*celG* [5A]	Glucanase	(Iyo and Forsberg, 1996)
F. succinogenes S85	*cedA* [A5]	Cdsase	(Iyo and Forsberg, 1994)
F. succinogenes S85	*xynC* [G11]	Xylanase	(Paradis et al., 1993; Zhu et al., 1994)
	xynC-A [G11]	XynC-A	
	xynC-B [G11]	XynC-B	
F. succinogenes S85	PJ110 [A5]	Lichenase	(Erfle et al., 1988; Teather and Erfle, 1990)
F. succinogenes S85	*endB* [E9]	Glucanase	(Broussolle et al., 1994)
F. succinogenes S85	*endC* [9E]	Glucanase	(Bera et al., 1996)
F. succinogenes AR1	$endA_{FS}$ [E9]	Glucanase	(Cavicchioli and Watson, 1991)
R. albus F-40	*egI* [E9]	Glucanase	(Deguchi et al., 1991)
R. albus F-40	*egIV* [A2]	Glucanase	(Karita et al., 1993)
R. albus F-40	pRA201 [A3]	β-Glucosidase	(Takano et al., 1992)
R. albus 8	*celA* [A5]	Glucanase	(Attwood et al., 1996)
R. albus AR67	*celA* [A5]	Glucanase	(Vercoe and Gregg, 1993)
R. albus SY3	*celA* [A4]	Glucanase	(Poole et al., 1990)
	celB [A4]	Glucanase	
R. flavefaciens 17	*endA* [A5]	Glucanase	(Kirby et al., 1977)
R. flavefaciens 17	*xynA*	Xylanase	(Zhang and Flint, 1992)
	xynA-A [G11]	XynA-A	
	xynA-C [F10]	XynA-C	
R. flavefaciens 17	*xynB* [G11]	Xylanase	(Zhang et al., 1994)
R. flavefaciens 17	*xynD*	Xylanase/glucanase	(Flint et al., 1997)
	xynD-A [G11]	A-Xylanase	
	xynD-C [16]	C-β-1,3-1,4-glucanase	
R. flavefaciens FD-1	*celA* [A5]	Cdsase	(Wang and Thomson, 1992)
R. flavefaciens FD-1	*celB*	Glucanase	(Vercoe et al., 1995)
R. flavefaciens FD-1	*celD* [E9]	Glucanase	(Vercoe et al., 1995)
R. flavefaciens FD-1	*celE* [−]	Glucanase	(Wang et al., 1993)
P. ruminicola B_14	[26]	Glucanase	(Matsushita et al., 1991)
P. ruminicola B_14	*cdxA* [A3]	Exo-glucosidase	(Wulff-Strobel and Wilson, 1995)
P. ruminicola AR20	*celA* [A4]	Glucanase	(Vercoe and Gregg, 1992)
P. ruminicola 23	[A5]	Glucanase	S27500
P. ruminicola 23	[A5]	Xylanase	(Whitehead, 1993)
P. ruminicola B_14	*xynA* [10F]	Xylanase	(Gasparic et al., 1995)
P. ruminicola B_14	*xynB*	Exoxylanase	(Gasparic et al., 1995)
P. ruminicola B_14	*xynC*	Xylanase	(Flint et al., 1997)
P. ruminicola D31	*xyn*	Xylanase	(Flint et al., 1997)
S. bovis JB1		β-1,3-1,4-Glucanase	(Ekinci et al., 1997)
S. bovis		α-Amylase	(Whitehead and Cotta, 1995)

[a] [ ]glycosyl hydrolase family.

domain of 43 to 76 residues with isoelectric points between 10.8 to 11.7 has been observed in the glucanases EndC (Bera et al., 1996), CelD and E (Malburg et al., 1996), CelG (Iyo and Forsberg, 1996), the mixed linkage glucanase (Teather and Erfle, 1990) and the XynC xylanase (Paradis et al., 1993). Alignment of the BTDs revealed no significant similarity. The role of the basic terminal domain is not fully resolved, but it does not appear to be involved in binding of the enzyme to the cell surface polymers; instead it may modulate catalytic activity of the enzyme (unpublished data).

An unexpected observation was the report on the isolation of a gene coding for an extracellular β-(1,3-1,4)-glucanase from *Streptococcus bovis* (Ekinci et al., 1997). The enzyme had a pH optimum of 6.5 although it was tolerant to low pH. It was speculated that the enzyme improves the access of *S. bovis* to plant starch by degrading structural polysaccharides present in the endosperm cell walls.

The rumen bacteria posses a rich array of xylanase enzymes. These vary from single domain enzymes, for example, the XynA from *B. fibrisolvens* (Mannarelli et al., 1990), to multicatalytic domain enzymes such as XynC from *F. succinogenes* (Zhu et al., 1994) with two xylanase catalytic domains and XynD from *R. flavefaciens* which contains both a xylanase catalytic domain and a glucanase catalytic domain (Flint et al., 1993). In addition to the range of enzymes degrading hemicellulose in plant cell walls, Dalrymple et al. (1996) reported the cloning of a gene encoding a 30 kDa cinnamoyl ester hydrolase which cleaves the ester linkage between an arabinose side chain of xylan and cinnamic acid, and which in the plant may be linked to lignin.

The organization of the hydrolase genes on the bacterial chromosome has received comparatively little attention. Gardner et al. (1997) cloned a glucanase and a mannanase from *P. ruminicola* which are present in an operon of 6 open reading frames and are under the control of a single regulator. Closely linked genes have been isolated from several rumen bacteria. These include *xyn*A and *xyn*B from *P. ruminocola* (Gasparic et al., 1995) which encode endo- and exoxylanases, respectively, and *cel*D and *cel*E from *F. succinogenes* which code for a catalytically active endoglucanase and a silent gene product, respectively, but that share 73% similarity (Malburg et al., 1996).

A genome map of 3.6 Mb has been prepared for *F. succinogenes* (Ogata et al., 1997) (figure 2). The authors determined the locations of 7 hydrolase genes and three ribosomal operons on the map, and reported that the genes were randomly scattered in the chromosome. Although details are still very sparse, this provides a beginning to our understanding of the *F. succinogenes* chromosome.

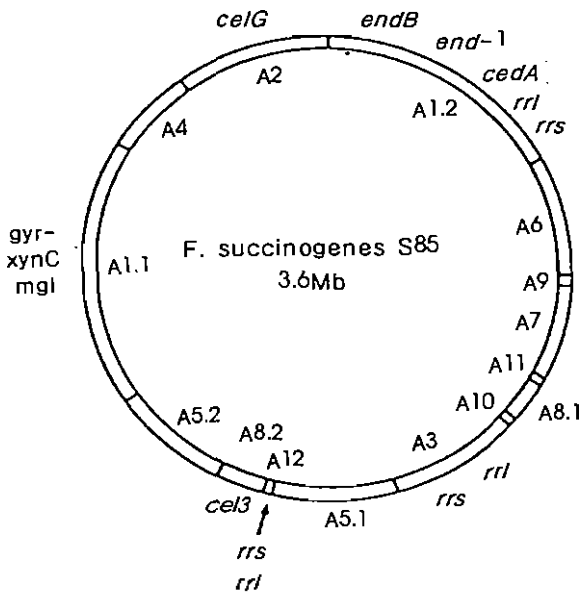

Figure 2. *Asc*I restriction map of the *F. succinogenes* chromosome. Genetic markers illustrated for glycanase genes are from table 1. Abbreviations: *rrs*, rRNA small subunit; *rrl*, rRNA large subunit. After Ogata et al. (1997).

GENE TRANSFER IN RUMEN BACTERIA

Gene transfer systems are powerful tools in genetic analysis, and are essential for the construction of recombinant rumen bacteria. Gene transfer systems have now been developed and refined for prominent species of ruminal bacteria including *B. fibrisolvens*, *P. rumincola*, *R. albus*, *S. bovis* and *S. ruminantium* (Forsberg et al., 1997; Selinger et al., 1996). Bacteriophage have also been studied, but none have been used for genetic transfer (Selinger et al., 1996). In the search for suitable cloning vectors, plasmids have been isolated from practically all species of ruminal bacteria studied (Selinger et al., 1996) except *F. succinogenes*. Most plasmids are between 1.4 and 8 kb and lack an antibiotic resistance marker. One of the most novel plasmids reported is a cellulose dependent replication plasmid from *R. albus* (Ohmiya et al., 1993). Due to the lack of antibiotic resistant markers ruminal plasmids are often used to construct chimeric shuttle vectors with antibiotic marker genes from other sources. For example, a recently reported shuttle vector for *B. fibrisolvens* (pSMerml) was constructed from the small 2.8 kb *B. fibrisolvens* plasmid pOM1 with the *ermAM* gene coding for erythromycin resistance from the pAMβ1

plasmid and as ampicillin resistance marker from pUC19 (Hefford et al., 1998).

Steady progress is being made to develop more sophisticated vectors, such as the suicide vector system for chromosomal integration in *S. bovis* (Brooker et al., 1995). Brooker and McCarthy (1997) used the suicide vector to assess the importance of the intracellular α-amylase in *S. bovis*. A transformant lacking intracellular amylase activity exhibited a slower growth rate, thereby demonstrating the importance of the amylase in metabolism. It was speculated that intracellular amylase may have a role in either maltose or glucose transport. Thus the technique has raised an important physiological question. As initially intimated, this class of vectors provides a mechanism for stable integration of heterologous recombinant genes into the host chromosome, thereby avoiding problems such as loss of the plasmid in the absence of a strong selective agent.

Tetracycline resistance strains of *B. fibrisolvens* were recently demonstrated to be capable of transferring a resistance gene distantly related to Tet M between strains at a high rate by a chromosomally mediated mechanism (Scott et al., 1997). These studies illustrate a parallel to Tet Q transfer between rumen strains of *P. ruminicola* and hind gut *Bacteroides* except that in this case the element was transferred on a plasmid (Nikolich et al., 1994). The chromosomally mediated transfer agents are not well known, but as their structure is unraveled they may become very important in the development of gene transfer systems for the genetic modification of ruminal bacteria.

Most bacteria are equipped with restriction enzymes, which are sequence-specific endonucleases that recognize foreign DNA moleeules (such as bacteriophage DNA) that may invade the bacterial cytoplasm. In specific cases a cognate modification enzyme is responsible for methylating self-DNA with the same recognition sequence, rendering it protected from endonucleolytic cleavage. Restriction endonucleases have been charaeterized for *B. fibrisolvens* (Mohn and Teather, 1995), *F. succinogenes* (Lee et al., 1992), *R. albus* (Morrison et al., 1992b; Morrison et al., 1993), *R. flavefaciens* (Morrison et al., 1992a), *S. ruminantium* (Pristas et al., 1994; Pristas et al., 1995; Vanat et al., 1993b), and *S. bovis* (Vanat et al., 1993a). Of these, *F. succinogenes* contains a highly active non-specific endonuclease (Lee et al., 1992). These systems obviously have a deleterious influence on the efficiency of gene transfer by transformation/electroporation and may partially explain the difficulty indevelop of a gene transfer system for *F. succinogenes*.

EXPRESSION OF HETEROLOGOUS GENES IN RUMINAL BACTERIA

In the quest to develop genetically modified organisms (GMOs) containing genes to enhance ruminal digestion, novel heterologous genes have been introduced into ruminal bacteria (table 2).

Table 2. Expression of heterologous genes in rumen bacteria

Host	Heterologous gene	Promoter/Vector	Reference
P. ruminicola 2202	*P. ruminicola* 23 xylanase/endoglucanase gene (Whitehead and Hespell, 1990)	pRH3-pRRI2 replicon & *tetQ* marker	(Daniel et al., 1995)
S. bovis JB1	*R. flavefaciens* 17 *endA*	pVA838 shuttle vector	(Whitehead and Flint, 1995)
S. ruminantium	Undefined lactate utilization	Unknown promoter, no plasmid detected	(Gilmour et al., 1996)
B. ruminicola B_14	*P. ruminicola* B_14 glucanase gene (pGF7) ligated in frame with the cellulose binding domain segment on the *Thermomonospora fusca* endocellulase E2 gene (Maglione et al., 1992)	Xylanase promoter (*R. ruminicola* vector pTC-XRCM)	(Gardner et al., 1996)
Bacteroides thetaiotaomicron 5482	*Ruminicola* 23 xylanase (Whitehead and Hespell, 1990)	Indigenous promoter	(Cotta et al., 1997)
B. fibrisolvens OB 156	*Moraxella sp.* fluoro- acetate dehalogenase	*erm* promoter, plasmid pBH *erm*	(Gregg et al., 1994)
B. fibrisolvens OB 156	*N. patriciarum xynA*	*B. fibrisolvens* 49 *xyn* promoter, amylase signal peptidase	(Xue et al., 1997)

In most cases a DNA fragment (with suitable restriction sites) containing the gene have been inserted into the best vector available and introduced into the host by transformation or mating and antibiotic selection. In experiments which investigated expression of the xylanase in *P. ruminicola* 2202 by Daniel et al. (1995) and the glucanase in *S. bovis* by Whitehead and Flint (1995) expression of the genes in the new host was dependent upon the functionality of the indigenous promoter or another sequence to substitute for a promoter. Expression of the heterologous gene was demonstrated, but activity was low. The transfer of lactate utilization in a lactate negative strain of *S. ruminantium* observed by Gilmour et al. (1996) is a novel observation and according to the authors could involve transduction or transformation. Expression of a modified *P. ruminicola* gene with a *T. fusca* CBD in *B. ruminicola* B_14 was designed to develop an acid tolerant rumen bacterium with cellulase activity at a lower pH than normally observed, since the presence of this modified bacterium in the rumens of feedlot cattle putatively would permit greater cellulose digestion and thereby improve the economics of beef production. However, the *P. ruminicola* strain 23 xylanase promoter was inactive in strain B_14 (Gardner et al., 1996). This, perhaps, is not surprising since they are sufficiently different to be reclassified as different species (Avgustin et al., 1997).

The experiment to determine whether *B. thetaiotaomicron* containing a *P. ruminicola* 23 xylanase integrated into the chromosome would compete efficiently against ruminal organisms (Cotta et al., 1997) raises several interesting points. The fact that it was not competitive may not be surprising since the bacterium is not a competitive ruminal organism. Furthermore, the bacterium does not utilize xylose and consequently is not advantaged by this hydrolytic activity (Whitehead et al., 1991). In the case of the fluoroacetate dehalogenase from *Moraxella* sp. (Gregg et al., 1994), and *xyn*A gene from the ruminal fungus *Neocallimastix patriciarum* (Xue et al., 1997) expressed in *B. fibrisolvens*, the promoter, signal sequence (as appropriate), and the plasmid were carefully designed to function within the host. In both cases the plasmids were stably expressed in the host without antibiotic selection. The observation by Gregg (1995) that *B. fibrisolvens* is able to persist in the sheep rumen for months lends credibility to the expectation that the modified organisms will persist in the rumen, although the cell numbers may be below the threshold to provide observable beneficial effects. This indicates the direction research must take to obtain stable GMOs for ruminal function. To sustain a new catalytic activity in the rumen the new gene would need to be introduced into more than a single strain, unless it was in a very promiscuous plasmid.

PHYLOGENETIC DIVERSITY OF RUMINAL BACTERIA

The application of a combination of ribosomal RNA analysis, DNA-DNA hybridization and analysis of phenotypic diversity is staking out the claims for new species of ruminal bacteria. Bacteria classified as *P. ruminicola* (formerly *Bacteroides ruminicola*) constitute one of the most numerous groups recovered from the rumen and hind gut fermentations. A recent 16S rDNA analyses separated it into 3 groupings (Avgustin et al., 1994). Further analysis of DNA G + C content and phenotypic analysis confirmed the groupings. Strains B_14, GA33 and strain 23 were recommended as type species *P. bryantii*, *P. brevis* and *P. ruminicola*, respectively (Avgustin et al., 1997). Their different physiological properties suggested that they occupied different niches within the rumen. The *Butyrivibrio* species represent another diverse group of ruminal bacteria (Willems et al., 1996). They form 3 distinct lineages and exhibit 12 distinct rRNA sequence types. *B. fibrisolvens* which represents the second lineage consisted of 12 strains which exhibited seven distinct rRNA sequence types. The type strain *B. fibrisolvens* NCDO 2221 was a member of this lineage, but was peripheral. Willems et al. (1996) recommended that only the lineage containing the type strain be designated the genus *Butyrivibrio*. The *Ruminococcus* sp. fall within two distinct unrelated clusters (Rainey and Janssen, 1995). The one group contains *R. flavefaciens*, *R. albus*, *R. bromii*, and *R. callidis* which with the exception of *R. callidis*, are commonly found within the rumen. *Streptococcus bovis* strains from the rumen form two homology groupings, one contains *S. bovis* and the other includes *S. equinus* (Nelms et al., 1995). The cluster of ruminal strains could be considered one species. The *F. succinogenes* species although reasonably well defined are still incomplete. The detection of new strains from the equine large intestine with genus specific probes for 16S rRNA sequences, but not with the species specific probes documents the expansion of the species (Lin and Stahl, 1995). These data clearly document the broad diversity and complexity of the populations of bacteria present within the ruminal and lower intestinal environments. This of course challenges our initiative in devising means to genetically modify the metabolic properties of ruminal organisms in such a way as to have an impact on the ruminal

fermentation since modifying one isolate would be insufficient.

TACKLING RUMINAL PROTEIN QUALITY

In the rumen there is rapid proteolytic degradation of freshly consumed protein rich forage and a large proportion of the nitrogen is excreted as ammonium salts (Siddons et al., 1985). In this complex environment bacteria provide the major source of dietary protein for the animal. However, the microbial protein is low in the essential amino acids lysine and methionine and to a lesser extent threonine (Schwab, 1995). Several approaches have been used to protect feed proteins from ruminal degradation including roasting, chemical treatment and encapsulation in order to provide supplemental essential amino acids (Ashes et al., 1995). Unfortunately, these techniques are not totally effective (Rulquin et al., 1995). Therefore, to provide a basis for improving the low efficiency of nitrogen utilization research is directed to characterize the proteases and peptidases of ruminal bacteria (Attwood and Reilly, 1995; Madeira et al., 1998). Measures are being sought to control and reduce protease, peptidase and deaminating activities by chemical and biological inhibitors (Wallace, 1996; Wallace and McKain, 1996).

In a totally different and novel approach, Beauregard et al. (1995) designed and synthesized a gene that encodes a protein enriched in the limiting amino acids lysine, methionine, threonine and leucine, such that they accounted for 60% of the protein on a molar basis. The idea is to express the gene in ruminal bacteria in situ and thereby increase the concentrations of limiting dietary amino acids. The protein was designed with a compact structure, adopting an α-helical bundle fold. It is 100 residues in length with a molecular weight of 11.34 kDa and a predicted isoelectric point of 6.1. Lysine accounts for 12% of the residues, methionine for 14%, threonine for 22% and leucine for 9%. The protein was expressed in *Escherichia coli* as a fusion protein. Studies with several proteases demonstrated that the protein is readily degradable (MacCallum et al., 1997). The authors have not reported on expression of the gene in ruminal bacteria, therefore considerable developmental research may be required before the principle can be tested. A concern is the metabolic load synthesis of this protein would add to a cell's metabolism, and whether it may be toxic to the bacterial host at a concentration sufficient to provide a benefit to the animal. As discussed by Gregg (1995) with regard to fluoracetate dehalogenase expression in *B. fibrisolvens*, because of the diversity of strains of any one bacterial species within the rumen, the new protein would need to be expressed in many ruminal bacteria to ensure its presence at a sufficiently high concentration to have an impact on the nutrition of the animal. Since achieving widespread expression of the recombinant gene in ruminal bacteria will be difficult, there may be a case for generating transgenic cattle which contain the bacterial genes coding for synthesis of the essential amino acids, as has already been done for sulfur amino acids in sheep (Ward et al., 1997; Ward and Nancarrow, 1995). Unfortunately, the metabolic pathways for lysine and methionine involve numerous enzymatic transformations, consequently extensive work will be needed before transgenic cattle can be produced. However, in the future this type of molecular manipulation undoubtedly will become practical.

FUTURE RESEARCH

Recent advances in the biotechnology of ruminal bacteria has been with the numerically and physiologically dominant bacteria, *B. fibrisolvens*, *F. succinogenes*, *R. albus*, *R. flavefaciens*, *P. ruminicola*, *S. ruminantium* and *S. bovis*. These advances have relied heavily on DNA sequence data. Therefore as we look to the future, with DNA sequencing costs decreasing, the initiation of a global project to sequence the genomes of selected ruminal bacteria in the next five to six years is feasible. This would substantially enhance our research productivity, far exceeding the cost of the project, by making the genomes of these bacteria fully available for dissection and modification.

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