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Opportunities to improve fiber degradation in the rumen: microbiology, ecology, and genomics

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Abstract

The degradation of plant cell walls by ruminants is of major economic importance in the developed as well as developing world. Rumen fermentation is unique in that efficient plant cell wall degradation relies on the cooperation between microorganisms that produce fibrolytic enzymes and the host animal that provides an anaerobic fermentation chamber. Increasing the efficiency with which the rumen microbiota degrades fiber has been the subject of extensive research for at least the last 100 years. Fiber digestion in the rumen is not optimal, as is supported by the fact that fiber recovered from feces is fermentable. This view is confirmed by the knowledge that mechanical and chemical pretreatments improve fiber degradation, as well as more recent research, which has demonstrated increased fiber digestion by rumen microorganisms when plant lignin composition is modified by genetic manipulation. Rumen microbiologists have sought to improve fiber digestion by genetic and ecological manipulation of rumen fermentation. This has been difficult and a number of constraints have limited progress, including: (a) a lack of reliable transformation systems for major fibrolytic rumen bacteria, (b) a poor understanding of ecological factors that govern persistence of fibrolytic bacteria and fungi in the rumen, (c) a poor understanding of which glycolyl hydrolases need to be manipulated, and (d) a lack of knowledge of the functional genomic framework within which fiber degradation operates. In this review the major fibrolytic organisms are briefly discussed. A more extensive discussion of the enzymes involved in fiber degradation is included. We also discuss the use of plant genetic manipulation, application of free-living lignolytic fungi and the use of exogenous enzymes. Lastly, we will discuss how newer technologies such as genomic and metagenomic approaches can be used to improve our knowledge of the functional genomic framework of plant cell wall degradation in the rumen.

Keywords: Rumen; Plant cell wall; Metagenome; Diversity; Functional genome

Contents

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1. Introduction

Ruminants make up a significant proportion of the domesticated animal species worldwide, and among farmed livestock are the best adapted to utilization of plant cell walls [1]. Almost half of the global carbon fixed annually by photosynthesis is incorporated into plant cell walls making it the most renewable carbon source on earth [2]. Lignocellulose will therefore always be important in ruminant diets, and even in intensive finishing systems is incorporated into the ration because it is both economical and necessary for normal healthy rumen function. Improvement in the ability of the rumen microbiota to degrade plant cell wall is generally highly desirable and usually leads to improved animal performance [3].

In the developing world ruminants have an important role in the sustainability of village communities, and in many cases form the major source of income. Smallholders rely on subsistence farming, with few or no inputs, but forages are usually available and generally provide the sole source of nutrition to the animal [4,5]. Even in the developed world, forage is the major source of nutrition in many situations. For example, in the major beef-producing regions of northern Australia, forage is virtually the only source of nutrition, and supplementation is impractical and expensive. This is also true in North and South America where grazing animals are common, and lignocellulose makes up the majority of the diet.

The symbiosis between animal and microbe in the rumen allows for a cooperative system in which both the host and animal derive a benefit [6]. The rumen is a capacious pre-gastric fermentation chamber that sustains a rich community of microorganisms that rapidly colonize and digest feed particles. Carbohydrate polymers in plants are indigestible to most animals but can be hydrolyzed and fermented by a range of microorganisms in the rumen. The end products of this fermentation are fatty acids, which form a major metabolic fuel for the ruminant, and microbial cells that are a major source of protein and amino acids when absorbed in the lower digestive tract of the animal. One cost of this relationship is the breakdown, or sacrifice, of dietary protein by rumen microorganisms before digestion by the host enzymes [6].

Over the last 50 years, significant improvements in our understanding of the digestion of fiber in the rumen have occurred and much of this information has been translated into practical nutritional management strategies. For example, an understanding of the importance of nitrogen to the degradation of fiber by fibrolytic microorganisms has led to the inclusion of urea supplements in ruminant diets [1], and mechanical and chemical treatments of forages has improved their digestibility [7]. Probably one of the most effective means of exploiting information on rumen microbiology and digestion has been the construction of computer models that predict animal performance from the characteristics of feed ingredients [3,8,9]. These models have the ability to make significant improvements in dietary formulation, and although they do not directly manipulate fiber digestion in the rumen, they do optimize the utilization of scarce nutrients by rumen microbiota. Models are ideal examples of how incremental advances in our knowledge of rumen microbial processes have been utilized to make significant advances in rumen function.

The supposed inability of rumen microbiota to express the appropriate suit of enzymes to maximize fiber digestion is often given as a reason for research into improving the function of rumen microbiota. For example, this has led to the inoculation of bacteria into the rumen that ap-

parently have 'superior' abilities. Hungate [1] discussed early examples in which fibrolytic bacteria dosed into the rumen had little effect on fiber digestion. In fact the notion that the rumen microbiota lacks appropriate fibrolytic activities has persisted, and in recent years genetically modified rumen bacteria have been the focus of intensive research [10,11]. Unfortunately, much of the effort in producing ruminal inoculants has been unsuccessful because no improvements in rumen fiber digestion have resulted in vivo.

What has become obvious from these efforts is that our understanding of the rumen microbial ecosystem is still superficial in comparison with the complexity it encompasses. If one reflects on the fact that a substantial proportion of the rumen microbiota has not been cultured then these results are not surprising. Far less effort has been expended on the rumen fungi and protozoa [12], and even less work has been done on bacteriophage inhabiting the rumen [13,14]. The importance of this complexity has become apparent when inoculation studies revealed that dosed organisms usually disappear below detectable levels in the rumen and that rumen protozoa may play an important role in this decline [15,16].

The purpose of this review is to examine research that has attempted to improve fiber digestion in the rumen. We will not discuss some of the well known aspects of rumen

Table 1
GH identified in cultured rumen microorganisms and the family to which they belong

Enzyme[a]	GH families[b]	Rumen microorganism (GH families)
Endocellulase 3.2.1.4	5, 6, 7, 8, 9, 10, 12, 26, 44, 45, 48, 51, 61, 74	*Butyrivibrio fibrisolvens* (5, 9)
		Fibrobacter succinogenes (5, 51, 9)
		Neocallimastix frontalis (5)
		Neocallimastix patriciarum (5, 6)
		Orpinomyces joyonii (5)
		Orpinomyces sp. (5, 6)
		Piromyces equi (45, 5, 48)
		Piromyces sp. E2 (9, 48)
		Piromyces rhizinflata (5)
		Prevotella ruminicola (26, 5, 9, Nc)
		Ruminococcus albus (5, 9)
		Ruminococcus flavefaciens (44, 5, 9, Nc)
Exocellulase 3.2.1.91	5, 6, 7, 9, 10, 48	*Neocallimastix patriciarum* (6)
		Orpinomyces sp. (6)
		Piromyces rhizinflata (6)
		Piromyces sp. E2 (6)
β-Glucosidase 3.2.1.21	1, 3	*Butyrivibrio fibrisolvens* (3)
		Orpinomyces sp. (1)
		Piromyces sp. E2 (3)
		Prevotella albensis (3)
		Prevotella ruminicola (3)
		Ruminococcus albus (3)
Endoxylanase 3.2.1.8	5, 8,10,11, 16, 26, 43, 52, 62	*Butyrivibrio fibrisolvens* (10)
		Eubacterium ruminantium (10)
		Fibrobacter succinogenes (10, 11)
		Neocallimastix frontalis (11)
		Neocallimastix patriciarum (10, 11)
		Orpinomyces sp. (11)
		Piromyces communis (11)
		Piromyces sp. (11)
		Prevotella bryantii B14 (10)
		Prevotella ruminicola (10, 26, 5)
		Pseudobutyrivibrio xylanivorans type strain: Mz5 (11)
		Ruminococcus albus (11)
		Ruminococcus flavefaciens (10, 11, 16)
β-Xylosidase 3.2.1.37	3, 10, 39, 43, 52, 54	*Butyrivibrio fibrisolvens* (43)
		Prevotella ruminicola (43)
α-Amylase 3.2.1.1	13, 14, 57	*Butyrivibrio fibrisolvens* H17c (13)
Licheninase 3.2.1.73	16	*Fibrobacter succinogenes* (16)
		Orpinomyces sp. PC-2 (16)
Mannanase 3.2.1.78	26	*Piromyces* sp. (26)
		Prevotella ruminicola (26)

[a]Enzymes are identified by their IUPAC designations.
[b]GH enzymes are identified by their designation in the Carbohydrate Active Enzyme server (http://afmb.cnrs-mrs.fr/CAZY). This provides a hierarchical based web database that can easily be queried for organisms or enzymes of interest.

Table 2
Carbohydrate esterases (CE) identified in cultured rumen microorganisms and the family to which they belong

Enzyme[a]	CE[b]	Rumen microorganism (GH families)
Acetyl xylan esterase 3.1.1.72	1, 2, 3, 4, 5, 6, 7	*Fibrobacter succinogenes* (6)
		Neocallimastix patriciarum (3, 2, 6)
		Orpinomyces sp. (6)
		Ruminococcus albus (1, 4)
		Ruminococcus flavefaciens (1, 3)
		Ruminococcus sp. (1)
		Butyrivibrio fibrisolvens (Nc)
		Piromyces equi (1)
Feruloyl esterase 3.1.1.73	1	*Orpinomyces* sp. (1)

[a]Enzymes are identified by their IUPAC designations.
[b]CE enzymes are identified by their designation in the Carbohydrate Active Enzyme server (http://afmb.cnrs-mrs.fr/CAZY). This provides a hierarchical based web database that can easily be queried for organisms or enzymes of interest.

microbiology such as taxonomy involved in fiber digestion, but rather will focus on attempts that have been made to improve fiber digestion with inoculants, fungal treatments, and exogenous enzymes, but will preface this with an update of the glycosyl hydrolases (GH) (Table 1), and other enzymes (Table 2) produced by rumen microorganisms. In addition, we will discuss the applications of genomics and metagenomics to rumen microbiology and how this new technology will affect our knowledge of rumen cell wall degradation. This review will provide a roadmap of where we have been, and where this research is likely to go in the future.

2. Fibrolytic ruminal microorganisms and enzymes

A complex community of fibrolytic microorganisms catalyzes the degradation of fiber in the rumen. The taxonomy of these organisms has been extensively reviewed in recent years and the reader should refer to these publications for further information [12,17–19]. In brief, the major fibrolytic bacteria are the Gram-negative *Fibrobacter succinogenes*, and two species of Gram-positive bacteria, *Ruminococcus albus* and *Ruminococcus flavefaciens*. *Butyrivibrio fibrisolvens* are a group of highly xylanolytic Gram-positive bacteria inhabiting the rumen, which have a central role in fiber digestion. *Prevotella* are not regarded as highly cellulolytic bacteria but do produce a range of xylanases. A number of less well characterized cellulolytic bacteria occur, such as *Eubacterium cellulosolvens*. In addition, the anaerobic rumen fungi are considered important in fiber digestion and one of the best-studied fungi is *Neocallimastix* sp. There is also increasing evidence that the rumen protozoa may have the capacity to digest fiber, although this is not particularly well understood [20].

2.1. Glycosyl hydrolases

Most of the enzymes involved in cellulose and hemicellulose degradation are GH (Table 1) that hydrolyze the glycosidic bond between carbohydrates, or between a carbohydrate and a non-carbohydrate molecule [21]. Hydrolysis of the glucoside results in the formation of a sugar and another compound, and the 'hydrolase' signifies that C–O, C–N, or C–C bonds can be broken during hydrolysis. We have included the carbohydrate esterases (CE) in the discussion of GH, as they hydrolyze C–O bonds, but there may be some definitional issues (Table 2). The hydrolysis step is via general acid catalysis, and requires a proton donor and a nucleophile, or base [21]. The reaction results in either a retention, or an inversion of the anomeric carbon. Retaining GH retain the anomeric carbon configuration via a double displacement mechanism. Inverting GH invert the anomeric configuration via a single nucleophilic displacement.

GH and related enzymes can be classified based on their amino acid sequence similarity using hydrophobic cluster analysis, rather than simple substrate specificity [22] (Table 1). This method of classification results in all members of a family possessing a conserved catalytic mechanism, even though they may act on different substrates [23]. The CAZy (Carbohydrate Active enZyme) database (http://afmb.cnrs-mrs.fr/CAZY) maintains and updates information on GH and their classifications via amino acid sequences. Currently there are 91 structurally defined GH families, 65 glycosyltransferase families, 13 polysaccharide lyase families, 13 carbohydrate esterase families, and 32 carbohydrate-binding module families.

Efficient breakdown of cellulose in the rumen usually requires a number of GH including endoglucanases (endo-1,4-β-D-glucan hydrolase, EC 3.2.1.4), exoglucanases (exo-1,4-β-D-glucan cellobiohydrolase, EC 3.2.1.91), and β-glucosidases (β-D-glucosidase, EC 3.2.1.21), which work synergistically to hydrolyze cellulose [24]. The model (Fig. 1) for synergism between the three types of enzymes has been proposed to be that of endoglucanase attack on amorphous regions of cellulose fibers, creating sites for cellobiohydrolases to proceed into the crystalline region of cellulose [25]. The cleaved cellobiose and short-chain cellodextrins are then converted to glucose by β-glucosidases to stop end product inhibition. All three types of GH have been isolated from different rumen cellulolytic

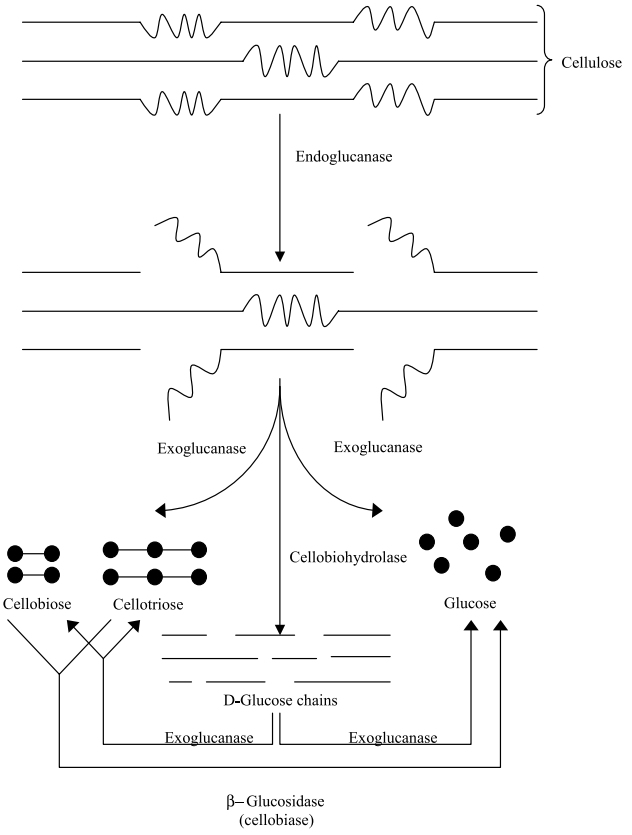

Fig. 1. The cellulase enzyme system consists of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). The mode of action of each of these is: (1) endo-*p*-glucanase, 1,4-β-D-glucan glucanohydrolase, carboxymethyl cellulase: 'random' scission of cellulose chains yielding glucose and cello-oligosaccharides. (2) Exo-*p*-glucanase, 1,4-β-D-glucan cellobiohydrolase: exo-attack on the non-reducing end of cellulase with cellobiose as the primary structure. (3) β-Glucosidase, cellobiase: hydrolysis of cellobiose to glucose [31].

microorganisms. There are 14 GH families containing endoglucanases, and the rumen microorganisms predominantly fall within families 5 and 9. This is hardly surprising, as these families are comprised of the most members, suggesting that many different types of organisms have found this to be the best form for an endo-acting cellulase.

Of the exoglucanases isolated from the rumen, family 6 exo-acting enzymes have only been found in anaerobic fungi (Table 1). Members of this family have an inverting mechanism that proceeds from the non-reducing end of the cellulose chain. Family 7 enzymes act with a retention mechanism and proceed from the reducing end of the cellulose chain. Currently no family 7 exo-acting enzyme has been isolated from rumen microorganisms. The absence is notable because in aerobic fungi such as *Trichoderma reesei* and *Humicola insolens* these enzymes can act in synergy with the family 6 exo-acting enzymes to attack the cellulose fibers from both ends and thus increase the degree of digestion [26].

Xylanases act on xylan, converting it to its constitutive sugars, with endo-β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase, EC 3.2.1.8), which hydrolyze the 1,4-β-xylopyranosyl linkages of xylan, and β-xylosidases (1,4-β-xylan xylohydrolase EC3.2.1.37), which hydrolyzes the xylo-oligosaccharides produced by the endoxylanases (Table 1). These enzymes have been found in the rumen, mostly from GH families 10 and 11. A series of enzymes, which cleave side chain sugars or remove acetyl groups from the xylan backbone [27], are also involved in xylan degradation and can be found in the rumen (Table 2). Acetylxylan esterases are responsible for deacetylation of xylans and xylo-oligosaccharides. With 22–50% of xylose residues being acetylated at the *O*-2 and/or *O*-3 positions, acetylation is an important factor influencing the digestibility of plant cell wall material in ruminants [28,29]. In addition arabinoxylan is one of the main hemicelluloses comprising the backbone structure of β-1,4-linked xylose to which are attached the arabinose side chains. The arabinose also has ester-linked *p*-coumaric and ferulic acid, and the hemicellulose is linked to lignin via ferulic acid links (Fig. 2) [30–32].

In this era of whole genome sequencing, tabulation of GH is in some ways redundant, because the sequence information of each organism contains the whole complement of enzymes. Traditionally, genes have been cloned and screened on various substrates that indicate a particular type of GH activity, and the inability to recover a gene of a particular class may just mean that the cloning

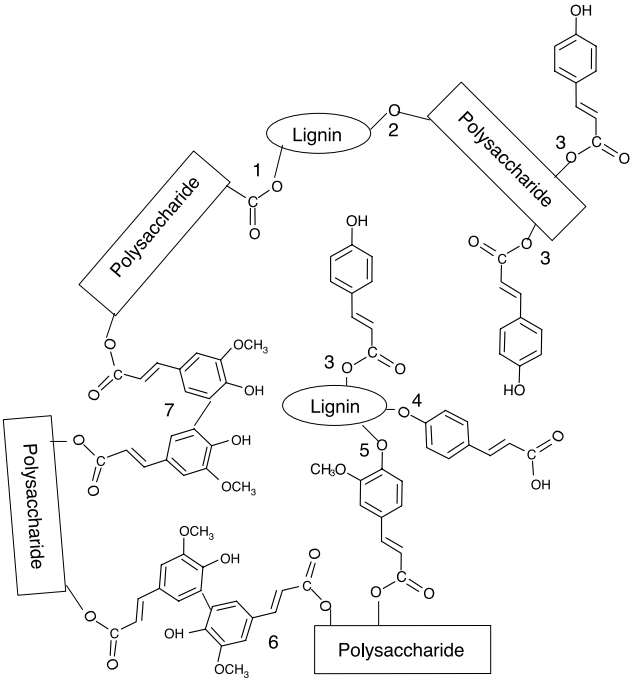

Fig. 2. A representation of the different kinds of lignin interactions in plant cell walls. A summary of the kinds of aromatic ester and ether cross-links between carbohydrate and lignin. Linkages are: 1 = direct ester linkage, 2 = hydroxycinnamic acid ester, 3 = hydroxycinnamic acid ether, 4 = ferulic acid bridge, 5 = direct ether linkage, 6 = dehydrodiferulic acid diester bridge, 7 = dehydrodiferulic acid diester–ether bridge. Redrawn from [30].

and screening strategy did not work. A simplistic resolution to this problem is to mine the genome and identify the genes in question. The only problem with this is that so much of the genome has no sequence homology with known genes, and as many as 30–40% of the open reading frames (ORFs) have no discernible homology/function. There is an increasing number of ORFs without known function, or ORFans, and in the case of the rumen bacteria could represent GH that have not yet been cloned [33].

The following discussion describes GH from *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Prevotella*, and anaerobic rumen fungi, and is not based on discovery via genome mining techniques. Genome data for rumen bacteria are available in special purpose databases such as the 'The comprehensive microbial resource' [34]. Research on the GH of rumen organisms should always be done within the context of the genome sequence. The discussion below is based largely on functional analysis which genome sequence annotation relies upon, and these two approaches need to be pursued in parallel.

2.2. Fibrobacter succinogenes

The fibrolytic enzymes of *F. succinogenes* are amongst the best studied within the rumen bacteria (Tables 1 and 2). Initial enzyme purification work characterized the endoglucanases EG1 and EG2 [35], a chloride-stimulated cellobiosidase [36], and a cellodextrinase [37]. Overall, the cloning of endoglucanases is most common [24]. An endoglucanase gene, *cel-3*, was one of the first to be sequenced [38] and at least seven distinct glucanase activities in *F. succinogenes* strain S85 have been reported [24,25]. Genomic libraries have subsequently revealed the endoglucanase genes *endB*, *celD*, *celE*, *celF*, and *celG* [39–42]. A gene for a mixed linkage β-glucanase (*mlg*) has also been cloned and characterized [43]. More recently, a new GH family 5 endoglucanase gene, *endA*, from strain S85 has been reported [44]. The cellobiosidase gene (*cedA*), which encodes a previously purified cellodextrinase, has also been cloned and characterized [45]. Strain S85 also contains two β-glucosidase activities [46] and a cellobiase activity [47]. Where gene and derived protein sequence information is known, these genes fall into either GH family 5 or family 9, with the exception of *celF* that is in family 51.

Xylanases have also been purified from strain S85. The xylan debranching enzyme endoxylanase 1, and a dual function endoxylanase 2 (xylanase and endoglucanase activities) have been characterized [48]. Four xylanase genes have been cloned and fully characterized. The xylanase gene *xynC* encodes two catalytic domains, both with GH family 11 xylanase activity [49]. The *xynB* gene encodes a dual function GH family 10 domain, with both xylanase and endoglucanase activities [50]. Genomic fragments of *F. succinogenes* strain S85 containing GH family 10 xylanase genes have also been deposited in GenBank by two groups. A 6688-bp fragment of *F. succinogenes* S85 DNA encoding four open reading frames showing homology to GH (*celJ*, *cel5K*, *xyn10L*, and *xyn10M*) and showing xylanase activity on RBB xylan plates (GenBank AY007248 [51]). Another overlapping fragment (7223 bp) was sequenced and three genes were identified. One had homology to a xylanase (*xynD*) that is identical to *xyn10L*, and *xynE* that is identical to *xyn10M*, previously reported as *xynB* (GenBank AF180368 [52]).

Glucanase genes have also been isolated from *F. succinogenes* strains AR1, SD35, 135 and BL2. Three glucanases were cloned from AR1, and one, *endAFS*, was characterized in detail and found to be a GH family 9 endoglucanase [53–55]. Strain SD35 contained a GH family 51 endoglucanase (*end1*) [56] while strain BL2 had a GH family 9 endoglucanase (*egC*) [57]. An endoglucanase (*end3*) [58] and a xylanase [247] have been described in *F. succinogenes* strain 135. The glucanase genes from these strains seem to be distinct from strain S85 glucanases [24,59].

2.3. Ruminococcus albus

A large range of GH have been isolated from *R. albus* strains (Tables 1 and 2). *R. albus* strain F-40 has attracted substantial attention and seven distinct endoglucanases, a cellobiosidase, and two β-glucosidases have been reported [24,60]. Enzyme purification and characterization studies have in addition identified two endoglucanases [61], a cellobiosidase [62], and a β-glucosidase [63]. The β-glucosidase was later cloned and sequenced [64]. Recently, a family 5 GH was cloned and sequenced (*egV*), and contained a dockerin domain thought to be involved in cellulosome assembly [65]. This was followed by the isolation of the strain F-40 cellulosome complex [66] and the demonstration of cellulase and/or xylanase activity in 11 of at least 15 proteins that comprised the complex.

The fiber-degrading enzymes and associated genes from an *R. albus* strain isolated in Australia, AR67, have also been studied [67,68]. This particular strain is extremely interesting as it is one of the most active strains yet cultured [69]. A gene encoding exo-1,4-β-D-glucosidase from *R. albus* AR67 was expressed in *Escherichia coli*. The cloned enzyme was located in the cytoplasm (40%) and attached to insoluble cell components (48%). After purification to homogeneity, the enzyme was found to be specific for cellulose with a β-D-glucopyranosyl configuration and was inactive against α-glucosides, lactosides and xylosides.

Strain SY3 of *R. albus* is another highly active degrader of plant cell walls [69]. To determine which subcellular proteins might be involved in adhesion and a possible cellulosome complex, SY3 wild-type was compared with an SY3 adhesion-deficient mutant. The adhesion-defective mutant produced significantly less (5–10-fold) overall gly-

canase activity, and the 'true cellulase activity' appeared to be entirely confined to the cell membrane fractions. Two family 4 GH, endoglucanases EGA (*celA*) and EGB (*celB*), have also been cloned from *R. albus* SY3 and sequenced [61,70].

Xylanase activity has been reported in *R. albus* strain 8 [71]. Xylanase 1 and 2 have been purified and are thought to interact synergistically with an arabinofuranosidase during degradation of alfalfa cell walls [71]. *R. albus* strain 7 has been found to harbor a β-glucosidase, an endoglucanase, and three xylanases [72–74]. The *xynA* gene from strain 7 encodes a protein with three domains: a GH family 11 catalytic domain, a xylanase-stabilizing domain similar to those from *R. flavefaciens*, and a domain with sequence similarity to deacetylases [74]. The *xynB* gene encodes a protein of 680 amino acid residues, including a family 11 GH catalytic domain, a repeated asparagine-tyrosine region and a C-terminal domain showing homology to the *Clostridium stercorarium xynY* domain E [73]. The *xynC* gene also show discrete domains: an N-terminal domain with homology to xylanases of *Bacillus subtilis* and *Erwinia chrysanthemi*, a region with homology to the *C. stercorarium xynY* thermostabilizing domain and a linker sequence containing a 20-residue repeat of asparagines [73]. An unidentified *Ruminococcus* species has also been found to contain a xylanase gene (*xyn1*) belonging to the family 11 GH, which shows homology to the *R. flavefaciens* xylanase gene, *xynE* [75] and *R. albus* 7 xylanase (*xynB*) [74].

2.4. Ruminococcus flavefaciens

GH from *R. flavefaciens* strains are common (Tables 1 and 2). *R. flavefaciens* FD-1 has an exoglucanase activity [76], a cellodextrinase [77], and at least four glucanases [78–82]. Three of these glucanase genes (*celB*, *celD* and *celE*) are inducible, while one glucanase (*celC*) and the FD-1 cellodextrinase (*celA*) are expressed constitutively [76,82]. Strain 17 has an endoglucanase in GH family 5 (*endA* [83]), and a recently characterized GH family 44 endoglucanase gene, *endB* [84]. *R. flavefaciens* is able to degrade xylan, and four xylanase genes have been identified. The genes *xynA* and *xynD* encode xylanases with dual catalytic domains [85,86]. Although the XynA domains exhibit similar substrate specificities, they fall into different GH families; domain A is in GH family 11 while domain C belongs to GH family 10 [86]. Similarly, the two *xynD* domains fall in different GH families: domain A is a family 11 xylanase while domain B is a family 16 glucanase [85]. On the other hand the *R. flavefaciens* 17 xylanase, *xynB*, encodes an enzyme with a single GH family 11 catalytic domain [86].

Three enzymes carrying esterase domains have recently been identified in the rumen cellulolytic anaerobe *R. flavefaciens* 17 [75]. The *cesA* gene product includes domains for an N-terminal acetylesterase and an unidentified C-terminal domain, while the previously characterized XynB enzyme (781 amino acids) includes an internal acetylesterase domain in addition to its N-terminal xylanase catalytic domain. A third gene, *xynE*, is predicted to encode a multidomain enzyme of 792 amino acids including a family 11 xylanase domain and a C-terminal esterase domain. A family 3 GH gene, *xylA*, has been identified in an 11-kb genomic DNA fragment from *R. flavefaciens* 17 [75] which also carries two putative *AraC*-type regulators, *xynD*, and a putative xylose isomerase and ABC-type sugar transporter genes.

A phage library constructed from strain 186 genomic DNA produced a carboxymethylcellulose (CMC)-degrading clone containing four ORFs corresponding to an endoglucanase (*renA*), an exoglucanase (*rex*), a β-glucosidase, and a xylanase (*rxy*) [87]. The predicted protein encoded by *renA* had an N-terminal proline-threonine-serine-rich region, a central catalytic domain and a C-terminal binding domain. The *rex*-encoded exoglucanase had its catalytic site located at the N-terminus followed by a proline-threonine-serine-rich region and a putative binding domain at the C-terminus.

2.5. Butyrivibrio/Pseudobutyrivibrio

Ruminal strains of *Butyrivibrio* are genetically diverse and have recently been reclassified with the creation of a new genus, *Pseudobutyrivibrio* [88]. The phylogenetic diversity of the *Butyrivibrio*/*Pseudobutyrivibrio* assemblage is mirrored by the large number of strains in which GH have been detected (Tables 1 and 2) [89]. Although cellulolytic strains of *Butyrivibrio* have been isolated in the past [90], this activity is often lost upon subculture in the laboratory. The *Butyrivibrio* are, however, efficient utilizers of xylans [91,92].

Correspondingly, an abundance of xylanase genes have been isolated and only a few endoglucanases have been described. *B. fibrisolvens* strain H17c(SA) contains a GH family 5 endoglucanase (*endI* [93]), a GH family 9 cellodextrinase (*cedI* [94]), a GH family 3 β-glucosidase (*bglA* [95]) and three xylanase genes (*xynB* [96], *xynE* and *xynF* [89]. *B. fibrisolvens* strain A46 produces a GH family 5 glucanase (*celA* [97]) while GS113 produces an interesting GH family 43 enzyme which has both xylosidase and arabinofuranosidase activity [98,99]. Rumbak et al. [100] cloned and sequenced an α-amylase gene (*amyA*) from strain H17c and found that it shared homology with prokaryotic and eukaryotic amylases of GH family 13. Mannarelli et al. [101] cloned and sequenced a xylanase gene (*xynA*) from strain 49 and showed via hybridization studies that a similar gene was present in strains H17c and CF3. Subsequent to this, Dalrymple et al. [89] used polymerase chain reaction (PCR) primers designed to different xylanase gene families to survey the xylanase genotypes in 28 *Butyrivibrio* strains. They found 11 new xylanase genotypes, all of which fall into GH family 10. More recently

multiple xylanases have been reported from *Pseudobutyrivibrio xylanovorans* strain Mz5 [102]. Strain Mz5 was found to have high xylanolytic activity and the cell-associated fraction showed 14 xylanase activities on sodium dodecyl sulfate–polyacrylamide gel electrophoresis zymograms ranging in molecular mass from 26.7 kDa up to 145 kDa. Two of these xylanases (99.8 and 77.4 kDa) showed weak CMCase activity indicating some endoglucanase activity. Xylanase expression was generally inducible by growth on oat spelt xylan, but two xylanases showed low-level expression when glucose, cellobiose, maltose and soluble starch were used as the carbon source. Subsequently, a partial xylanase sequence (*xynT*) was deposited in GenBank [103] and is placed within family 11 GH.

2.6. Prevotella

Prevotella are able to utilize a wide variety of polysaccharides, and are thought to be important contributors to xylan degradation in the rumen [104] and as such a range of GH have been identified (Table 1 and 2). *Prevotella bryantii* has been found to contain a GH family 26 endoglucanase, which was notable due to a frame shift in its coding region [105]. Matsushita et al. [106] sequenced an endoglucanase gene (*celA*) encoding an enzyme with substantial homology to members of the GH family 26 cellulases. A membrane-associated glucosidase with cellodextrinase and cyanoglycosidase activities has also been characterized [107]. The enzyme appears to have an exo-1,4-β-glucosidase activity as it attacks cellodextrins from the non-reducing end. It was able to cleave the cyanogenic glycosides, amygdalin and prunasin, and may play a part in cyanide toxicity in ruminants. Another study by Verco and White [108] identified a phage clone from a $B_1$4 genomic library expressing endoglucanase and mannanase activities. Subclones containing *Eco*RI fragments were sequenced and six ORFs were identified: ORFs 1 and 2 had no significant homology with any database sequences, ORF 3 had regions of homology with a cellulose-binding protein (CBP) from *Clostridium cellulovorans*, ORFs 4 and 5 encoded two related β-1,4-endoglucanases, while ORF 6 encoded a mannanase. The gene sequences up- and downstream of the ORFs indicated that these genes were transcribed as a single unit.

A xylanase gene from *Prevotella ruminicola* 23 [109] with endoglucanase activity has a catalytic domain that shares homology with *B. fibrisolvens*, *R. flavefaciens*, and *Clostridium thermocellum* xylanases. A phage library of *P. ruminicola* (*bryantii*) $B_1$4 screened for xylanase activity revealed four chromosomal regions associated with activity [110]. One clone encoded an endoxylanase, with *p*-nitrophenyl (pNP)-xylosidase and pNP-arabinofuranosidase activities. The DNA sequence of this clone [111] consisted of two genes, *xynA*, which was responsible for endoxylanase activity, and *xynB*, which encoded xylosidase activity (using an exoxylanase mechanism), and a weak arabinofuranosidase activity. Two other xylanases, *xynC*, from strain $B_1$4, and a strain D31d xylanase, have been characterized [112]. They were found to possess highly unusual structures in which their catalytic domains were interrupted by possibly non-coding sequences. The sequence of a cellulase gene from *P. ruminicola* strain 23 showing homology with GH family 5 enzymes has also been lodged (GenBank AB022865). Recently, further characterization of the *P. bryantii* $B_1$4 gene cluster containing *xynA* and *xynB* identified additional genes [113]: *xynD* which appears to be involved as a solute transporter, *xynE* which has homology to genes encoding acylhydrolases and arylesterases, *xynF* which shares homology with β-glucuronidases, and *xynR*, a multidomain regulatory protein which may serve to activate the *xynABD* gene cluster and therefore xylanase expression.

2.7. Anaerobic rumen fungi

The actual role of anaerobic fungi in the rumen is still an issue that has not been completely resolved [104] but our knowledge of their GH is increasing as evidenced by the range of enzymes identified (Tables 1 and 2). It is, however, known that anaerobic fungi colonize plant tissue and appear to degrade lignified tissue that is not degraded by other microorganisms [114]. Having said this, it is also true that the rates of growth and degradation of fungi are much slower than those of the bacteria, and their ability to persist is limited because their growth rates are much lower than the rumen dilution rate. Fungi are able to degrade up to 34% of the lignin in plant tissue, can penetrate the plant tissue as a result of their filamentous growth [115], have a broad range of highly active enzymes, and are the only known rumen organisms with exo-acting cellulase activity [24]. The importance of fungi is further supported by the synergy that occurs with rumen bacteria [116]. Methanogenic co-cultures of non-autoclaved stem fragments were degraded more extensively by *Neocallimastix frontalis* and *Piromyces* isolates than by *Caecomyces* isolates and *N. frontalis* and *Piromyces* isolates showed the greatest rates of stem degradation. When interactions between *F. succinogenes* and methanogenic co-cultures of fungi growing on ryegrass stems were investigated, *N. frontalis* inhibited *F. succinogenes*. In contrast, a *Caecomyces* species grown with *F. succinogenes* increased stem degradation, indicating that *F. succinogenes* and *Caecomyces* spp. may have complementary fibrolytic activities [117].

Neocallimastix spp. are the best studied of the rumen fungi and are highly active against crystalline cellulose. Earlier work [118] reported that the activity on crystalline cellulose by RK21 was even higher than that of *T. reesei* C30. To unravel the cellulases a cDNA library prepared from *Neocallimastix patriciarum* CX was constructed [119]. Four cDNAs of note were identified: *celA*, *celB*, *celC*, and *celD*. The *celA* was a cellobiohydrolase that

hydrolyzed crystalline cellulose, while *celB* and *celD* had endoglucanase activities. The most interesting cDNA was *celD* that had multiple activities including endoglucanase, licheninase, cellobiosidase, and xylanase. Several researchers have also identified cellobiohydrolase (*celA*) [120], glucanase (*celB*) [121], glucosidase [122], β-glucosidase [123], and cellobiase [124] from rumen fungi.

Cellulase activity is also associated with anaerobic *Orpinomyces joyonii* SG4, which has significant cellulase activity [125]. Two cellulases, *celB29* and *celB2*, were isolated from a cDNA library. The cloned enzymes had high activities towards barley β-glucan, lichenin and CMC, but not Avicel, laminarin, pachyman, xylan and pullulan. In addition, CelB29 and CelB2 showed activity against pNP-β-D-cellobioside and pNP-β-D-cellopentaoside but not pNP-β-D-glucopyranoside with preferential activity against pNP-β-D-cellotrioside [125]. Recently identified from a cDNA library of *Orpinomyces* PC2 [126] was a cDNA designated *celF* encoding a cellulase with a signal peptide, a carbohydrate-binding module (CBM), a linker, and a catalytic domain similar to that of *celA* from *N. patriciarum*. The catalytic domain was also homologous to CelA and CelC from the same fungus which contain N-terminal docking domains for a cellulase–hemicellulase complex.

Xylanases have also been cloned (*xynA* and *xynB*) from *N. patriciarum* [127]. The *xynA* encodes an enzyme with two catalytic domains and is highly active [127]. Interestingly, the truncated form of this enzyme has at least a five-fold higher activity than the native. The *xynB* encoded both xylanase and cellobiosidase activity [127]. Similar data were obtained with *Piromyces* sp. [128].

Forages are rich in xylan containing hemicellulose and as many as 22 ± 50% of the xylose residues are acetylated at the *O*-2 and/or *O*-3 positions and acetylation is an important factor influencing the digestibility of plant cell wall material in ruminants [129,130]. It can be demonstrated that chemical deacetylation of xylan is easily achieved using dilute alkali solutions and significantly increases the digestibility of cellulose by enzymes [131]. Several workers [132,133] have suggested that enzymic deacetylation may be a prerequisite for the breakdown of acetylxylan or may enhance the rate of its hydrolysis by other enzymes.

Acetylesterases able to remove *O*-acetyl groups from xylose residues in xylan and xylo-oligomers are classified as acetylxylan esterases [132,133]. Although not all esterases that hydrolyze such substrates are acetylxylan esterases, it is likely that under conditions of high levels of synthesis of fiber-degrading enzymes, a significant proportion of esterases able to hydrolyze naphthyl acetate (a-NA), or similar compounds, may be acetylxylan esterases. The limited number of cloned acetylxylan esterases isolated is partly due to the difficulties encountered in finding suitable substrates for screening of libraries for acetylxylan esterase clones. However, a number of acetylxylan esterases have been reported as having activity against a-NA and other artificial substrates [134].

More recently acetylesterase and cinnamoyl ester hydrolase activities were described in *N. patriciarum* [135,136]. None of the enzymes had true cinnamoyl ester hydrolase activity, but two of the enzymes had acetylxylan esterase activity, and *bnaA*, *bnaB* and *bnaC* encode proteins with several distinct domains. Carboxy-terminal repeats in BnaA and BnaC were homologous to protein-docking domains in other enzymes from *Neocallimastix* species and another anaerobic fungus, a *Piromyces* sp. The catalytic domains of BnaB and BnaC are members of Ser/His active site hydrolases [137]. Feruloyl and *p*-coumaroyl esterases have also been purified from *Neocallimastix* strain MC2 [138,139], and significant extracellular acetylesterase activity can be detected in the MC2 strain [140].

3. Adherence to cellulose and evidence for the cellulosome

3.1. General nature of adherence to cellulose

Fiber-degrading bacteria, and fungi, usually adhere to the surface of plant cell walls (Fig. 3) and a lack of understanding in exploiting this process may be one of the reasons for the difficulty in establishing inoculant microorganisms in the rumen [141–143]. The intimate association

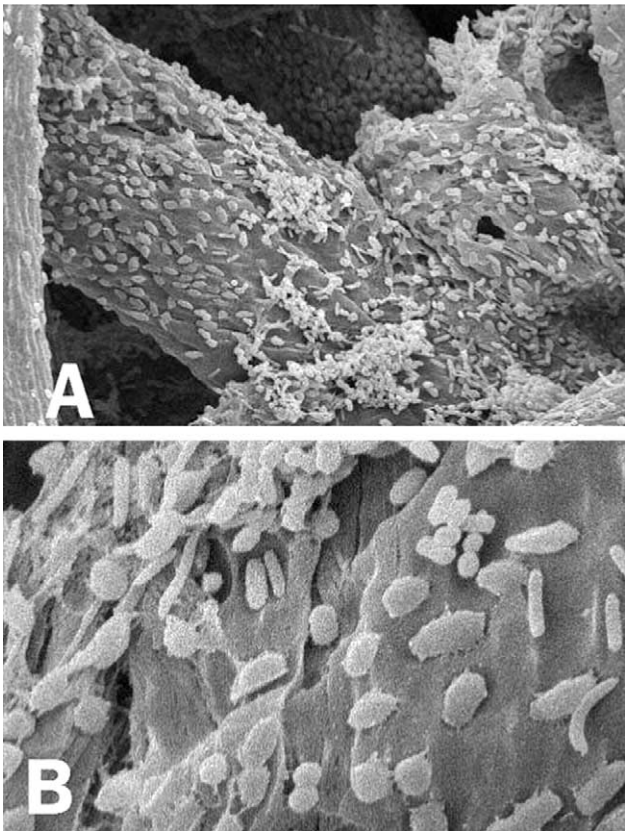

Fig. 3. Adherence of mixed rumen bacteria to plant material. A: Scanning electron micrograph of adherence to plant cell wall. B: Close examination of bacterial cells reveals protuberances that are likely adherence factors that bind the cells to the plant surface [147].

with plant cell walls has evolved in conjunction with a sophisticated molecular structure, the cellulosome, which facilitates the adherence process (Fig. 4). The cellulosome is an extracellular structure, and appears in many cases to be essential for degradation of crystalline cellulose and associated plant cell wall polysaccharides [144]. The cellulosome arrangement also promotes adherence to the plant cell walls, and provides individual microbial cells with a direct competitive advantage in the utilization of the soluble hydrolysis products [144]. It also appears that the anaerobic fungi have cellulosome-like machinery that is involved in adherence to cellulose [145,146].

Presently the adherence of bacteria to cellulose in rumen ecosystems can be divided into four phases in *F. succinogenes*, *R. flavefaciens*, and *R. albus* [147], but it is likely that many of the steps are similar in fungi. (1) Transport of the nonmotile bacterium to the plant substrate. (2) Nonspecific adhesion of bacteria to available sites on the plant cell wall [147]. (3) Specific adhesion via adhesions or ligand formation with the substrate, that may be facilitated by structures such as cellulosome complexes, fimbrial connections, glycosylated epitopes of CBP or the glycocalyx, and CBM [147]. (4) Proliferation of the attached bacteria on potentially digestible plant tissues [147]. Bacterial adhesion is not straightforward and can be affected by a range of factors including: (1) bacterial age, glycocalyx condition, and competition with other microorganisms [147], (2) the nature of the substrate, including cuticular covering, surface area, hydration, and ionic charge [147], and (3) environmental factors such as pH, temperature, and presence of cations and soluble carbohydrate [147].

3.2. F. succinogenes *adherence to cellulose*

F. succinogenes binds tightly to the surface of plant materials via adhesions leading to extensive plant cell wall degradation [148–151]. Three *F. succinogenes* enzymes, the endoglucanases EG2 and EGF and the chloride-stimulated cellobiosidase, are likely to contain a CBM, and may be involved in bacterial adhesion to cellulose [152,153]. There is strong evidence which suggests that seven CBPs, with masses of 40, 45, 50, 120, 180, 220, and 240 kDa located in the outer membrane of *F. succinogenes*, may be involved in adhesion. Immunogold labelling of the 180-kDa CBP demonstrated its importance in adhesion to cellulose via a common glycosidic epitope [154]. The importance of glycoproteins to the adhesion process can be demonstrated more directly by removing the carbohydrate structures on the cell surface with periodate and surface proteins with proteases as has been described for *Streptococcus bovis* and *E. coli* [155,156]. In a recent study with *Fibrobacter intestinalis* DR7 carbohydrate components of a glycosylated CBP isolated from the outer membrane and periplasm of *F. intestinalis* DR7 were shown to

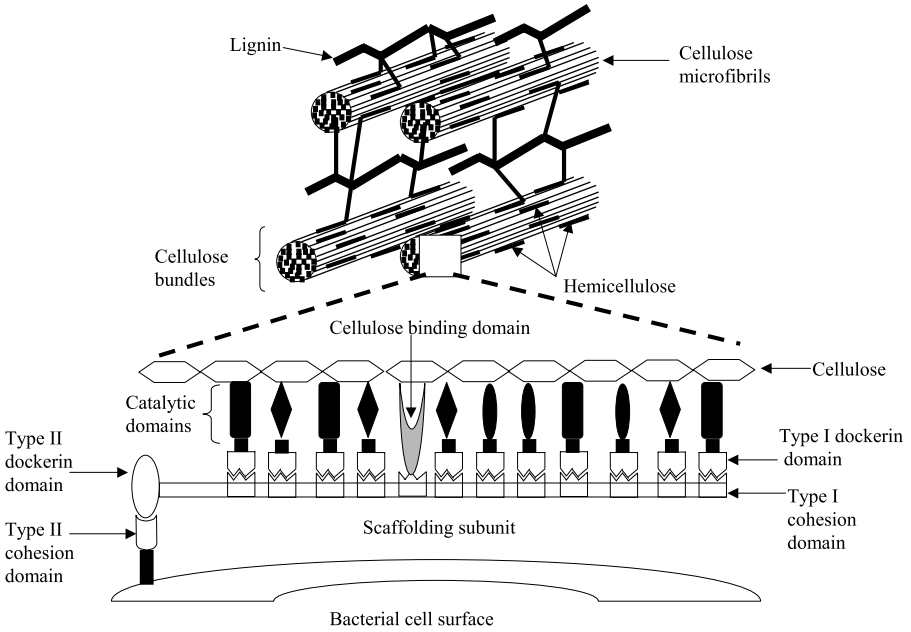

Fig. 4. Idealized representation of fiber and its component cellulose, microfibrils, hemicellulose, and lignin that are degraded via the cellulosome complex. The 'cellulosome' is a multienzyme complex produced by many cellulolytic rumen organisms. The cellulosomes are associated with the microbial cell surface, mediate cell attachment to the insoluble substrate, and degrade it to soluble products that are then absorbed. The multiple subunits of the cellulosome are composed of numerous functional domains that interact with each other and with the cellulosic substrate. One of these subunits, a large glycoprotein, which is called the 'scaffoldin', comprises a distinctive class of non-catalytic scaffolding polypeptide. The scaffoldin subunit selectively integrates the various cellulases and xylanase subunits into the cohesive complex, by combining its 'cohesin' domains with a 'dockerin' domain present on each of the subunit enzymes. The cellulose-binding domain attaches the cellulosome to the cellulose surface. The 'catalytic domains' constitute the various GH. Redrawn from various authors [30,147,174].

play a significant role in adhesion to cellulose. The isolated CBP included residues of glucosamine, galactosamine, glucuronic acid, and galacturonic acid that blocked adhesion to cellulose [148,157]. However, additional biochemical and genetic evidence is needed to explore the role of glycocalyx carbohydrates in the adhesion process for *F. succinogenes*. Thus, both the glycosidic residues of the outer membrane CBP and especially of the 180-kDa CBP, and the CBM of EG2, EGF, and Cl-stimulated cellobiosidase may play a role in the adhesion of *F. succinogenes*.

Even though a number of proteins appear to be involved in adherence, this is still indirect evidence. The only direct evidence to support the existence of either a cellulosome complex or fimbria structures involved in the adhesion mechanism of this bacterium is scanning electron microscopy observations [148,150,151,158,159]. However, in bacterial cells prestained with cationized ferritin, the presence of ultrastructural protuberances is sometimes connected to growth rate rather than to induction of cellulolytic systems [160].

3.3. R. flavefaciens *adherence to cellulose*

R. flavefaciens adheres immediately and strongly to fiber particles and degrades plant cell walls at a comparatively rapid rate [161–163]. For example, *R. flavefaciens* FD-1 has a maximum dilution rate on crystalline cellulose (0.1 h^{-1}), which is higher than that of other ruminal bacteria on the same substrate [164]. When *R. flavefaciens* is allowed to grow in the presence of plant cell walls, the cells will attach, and close examination of the areas of attachment reveals the existence of protuberances extending from the bacterium to the plant surface [148,161,165]. It has also been reported [166] that the endoglucanases of *R. flavefaciens* FD-1 exist in two forms: a large enzyme complex with a molecular mass of 3000 kDa called complex A, and a smaller fraction (89 kDa) designated complex B. It appears that complex A contains at least 13 different endoglucanases, whereas complex B has only five unique endoglucanases and some of the polypeptides in these complexes are glycosylated. Gene sequence analysis of three endoglucanases, and one cellodextrinase in *R. flavefaciens* FD-1, and three xylanases (*xynB*, *C*, and *D*), one endoglucanase (*endA*), and an esterase (*estA*) in *R. flavefaciens* 17, demonstrates that these enzymes lack any distinct CBM ([82,166,167]. However, some *R. flavefaciens* 17 enzymes including XynB, XynD, EndA, and EstA contain a dockerin-like domain, suggesting that they may in some way interact to form a cellulosome-like complex that could be involved in the adhesion mechanism [167,168].

Recently, a scaffoldin protein, a member of the cellulosome complex of *R. flavefaciens* 17, has been identified, and several cohesins connecting the scaffoldin with a type 1 dockerin associated with several catalytic enzymes have been identified [147]. A 30-kDa protein attached to the CBP in *R. flavefaciens* has been identified but its role in adhesion is not clear [169,170]. Early work [161] suggested the possible role of glycocalyx glycoproteins in mediating adhesion of *R. flavefaciens* cells to cellulose, which was supported by a study of lectins which inhibited adhesion probably by blocking specific epitopes [171]. The finding that polypeptides of the two complexes identified in *R. flavefaciens* FD-1 are glycosylated [166] supports the possible importance of carbohydrate epitopes in adhesion of the bacterium. The adhesion of *R. flavefaciens* to cellulose was inhibited when methylcellulose or CMC was added to medium (0.1%) but not by the addition of cellobiose (1%), suggesting that the recognition site of cellulose-binding factors of this bacterium is larger than a repeating cellobiose moiety [169,170,172]. Thus, at least two mechanisms, cellulosome-like complexes and carbohydrate epitopes of the glycocalyx layer, are involved in the adhesion of *R. flavefaciens* to cellulose.

3.4. R. albus *adherence to cellulose*

Electron microscopic and antibody studies provided some evidence for the existence of cellulosome-like structures in *R. albus* [148,165,173,174]. It has been reported [175] that the cellulase activity of cellobiose-grown *R. albus* SY3 was cell-associated, had a high molecular mass, but formed an unstable complex (1.5 MDa) that could be easily disassociated into proteins of low molecular mass. Later it was found that phenylpropanoic acid (PPA) or phenylacetic acid (PAA) stabilized this complex [176]. Miron et al. [148] reported the isolation and separation of the glycocalyx capsule, inner membranes, and peptidoglycan cell walls of cellobiose-grown *R. albus* SY3, from the extracellular fluid and the cytoplasm [147]. They found that most of the cellulases, xylanases, and cellulose-degrading activities of *R. albus* SY3 were associated with the capsule and cell walls. These findings are consistent with electron microscopic observations that adherent *R. albus* cells are surrounded by a glycocalyx capsule and may not actually touch the cellulose surface [177,178]. Genetic evidence is provided by sequence analysis of endoglucanases *celA* and *celB* from *R. albus* SY3 and endoglucanases I, II, III, and IV from *R. albus* F-40, as well as several xylanases. These analyses indicate that these enzymes lack an epitope for either a CBM or a dockerin-like domain, suggesting that they are not integrated as part of a cellulosome complex [61,66,166].

Even though most of the *R. albus* SY3 endoglucanases are not integrated into cellulosome-like organelles, high-molecular-mass complexes have been isolated that contain mainly xylanases and some endoglucanase activity. Most of this activity is associated with cellulose [165]. Thus it is suggested that the cellulosome complex of *R. albus* may contain a CBM or enzymes employing CBM [147]. In parallel, a cellulosome complex was isolated from the culture supernatant of *R. albus* F-40 grown on cellulose, and its components were identified as three previously se-

quenced endoglucanases (*egV*, *egVI*, and *egVII*). In addition five endoglucanases, three xylanases, and four non-enzymatic proteins were described that had not previously been identified [65,66].

3.5. Fungal adherence to cellulose

Molecular evidence is accumulating that enzymes are associated with a fungal cellulosomes in the genera *Neocallimastix*, *Orpinomyces*, and *Piromyces*, and are modular [145,179]. In addition to a catalytic domain, they contain several copies of a conserved 40-amino acid cysteine-rich, non-catalytic docking domain (NCDD), which do not show sequence homology to bacterial dockerins [125,127,180,181]. NCDDs are interspaced by short linkers and are separated from the catalytic domain(s) by a serine-threonine-rich linker(s). Given these molecular signatures the NCDD subunits can be regarded as members of fungal cellulosomes.

Recently, it was shown that a glutathione *S*-transferase (GST) reporter protein fused to one, two, or three NCDDs from *Piromyces equi* was able to specifically recognize a 97-kDa protein present in a cellulosome preparation purified by a cellulose affinity procedure [182]. These results strongly indicate the presence of a scaffoldin analog in the fungal cellulosome and show that, in contrast to bacterial dockerins, a single NCDD may serve as the interacting unit. Thus far, *estA* from *P. equi* [182] and *celB2* from *O. joyonii* [125] are the only examples of genes encoding a cellulosome component containing only one NCDD. The majority of the genes encoding components contain two NCDDs.

Significant progress has been made in unravelling the nature of the cellulosome in *Piromyces* spp. The cellulosome produced by *Piromyces* sp. strain E2 during growth on filter paper was purified [183]. Three dominant proteins were identified in the cellulosome preparation, with molecular masses of 55, 80 and 90 kDa. To investigate the major 90-kDa cellulosome protein further, the corresponding gene, *cel9A*, was found to be an endoglucanase. Cel9A includes a 445-residue GH family 9 catalytic domain, and is the first fungal representative of this large family. The catalytic domain was succeeded by a putative β-sheet module of 160 amino acids with unknown function, followed by a threonine-rich linker and three fungal docking domains. Homology modelling of the Cel9A dockerins suggested that the cysteine residues present are all involved in disulfide bridges. Further investigations of cDNAs in *P. equi* and *Piromyces* sp. strain E2 revealed that they both encoded a GH 48 cellulase, containing two C-terminal fungal dockerin domains [184]. Immunoscreening with anti-cellulosome antibodies was used to isolate β-glucanase activity in the strain E2 cellulosome. The C-terminal end of the encoded Cel3A (GH family 3) protein consisted of an auxiliary domain and three fungal dockerins, typical for cellulosome components. Cel3A catalytic domain was specific for β-glucosidic bonds and functioned as an exo-glucohydrolase on soluble substrates as well as cellulose [179].

There has also been significant progress elucidating the molecular determinants of substrate recognition in *Piromyces*. Two CBM were described [146], which were highly discriminatory for gluco- and manno-configured ligands and accompanying data suggested that these sugars were likely to play a pivotal role in the efficient degradation of the plant cell wall by the *Piromyces* cellulosome. This unusual ligand specificity presents an excellent model system for studying protein–carbohydrate recognition. A model for CBM ligand recognition in *Piromyces* based on a crystal structure has recently been elucidated [185]. The major molecular determinant in recognition is the orientation of the aromatic residues in the binding site which complement the conformation of gluco- an manno-configured ligands.

4. Improvement of fiber digestion by rumen inoculation

4.1. Background

The idea that the microbiota of the digestive tract does not always comprise an optimum balance was noted in the early 1800s. Brugnone (referenced by Hungate [1]) stated that "...as one encounters it [the bolus] in ruminants, a bolus which is removed very easily from the mouth, and which some veterinarians give as a sure remedy to induce rumination in animals, in which this function is suspended due to illness". Metchnikoff [186] discussed a similar concept in relation to human gut health [187]. He believed that the human colon was a reservoir of potentially pathogenic bacteria and the toxic substances produced by these bacteria were harmful to the host. The observation that milk fermented by lactic acid bacteria did not support the growth of potentially pathogenic bacteria allowed him to arrive at this theory.

Inoculation clearly is of benefit to rumen function. Hungate [1] describes a number of studies in which 'supposedly' fibrolytic ruminal inoculants were added to the rumen, but there was no evidence suggesting that ruminal fiber degradation was enhanced. He did, however, cite studies that showed quite convincingly that inoculation modifies rumen function, other than fiber digestion. For example, rumen inoculation improves performance during transition from a forage ration to a high-grain diet. If very large amounts of rumen contents are transferred from grain-adapted animals to unadapted animals the transition to the high-grain diet can be made quite quickly without the ensuing problems of ruminal acidosis [188]. Similarly, adaptation of calves to a roughage ration can be accelerated if normal adult microbiota is dosed into calves [189]. More recent studies have confirmed that dosing of newborn dairy calves with rumen fluid from adult cattle causes

them to gain more weight and have less diarrhea than untreated controls [190].

Forage utilization can also clearly be improved with inoculation. The forage legume *Leucaena leucocephala* produces a goitrogenic compound (3-hydroxy-4[1*H*]pyridone) that causes toxicity in ruminants. *Synergistes jonesii*, is able to break down this compound, and was originally found in Hawaiian goats but not in cattle in Australia. This organism when inoculated into susceptible ruminants grazing *L. leucocephala* conferred resistance to the toxin [191–193]. Similarly, monofluoroacetate is found in various pasture plants at levels of up to 5 g kg^{-1} [194] and has an LD_{50} in ruminants of approximately 0.3 mg kg^{-1} of body weight. Gregg et al. [143] inoculated four strains of recombinant *B. fibrisolvens*, transformed with a gene encoding fluoroacetate dehalogenase into sheep. The inoculated sheep showed a significant reduction in toxicological symptoms after fluoroacetate poisoning when behavioral, physiological, and histological data were compared with those of five uninoculated sheep. It also appeared as if the inoculated strains persisted at approximately 10^6–10^7 ml^{-1}.

4.2. Genetically modified fiber-degrading bacteria

With regard to fiber degradation in the rumen, much effort has been expended in developing genetically modified bacteria that would have superior fiber-degrading abilities. The construction of genetically modified bacteria has proceeded under the assumption that the rumen microbiota does not produce the correct mixture of enzymes to maximize plant cell wall degradation. For example, *Ruminococcus* and *Fibrobacter* do not produce exocellulases that are active against crystalline cellulose, so that adding this activity would make them more potent. Transformation of *Ruminococcus* and *Fibrobacter* (the most fibrolytic rumen bacteria) [11] has not been successful, but it has been possible with *B. fibrisolvens* [195–197], *S. bovis* [198], and *Prevotella* spp. [199]. *Bu. fibrisolvens* is primarily hemicellulolytic [200,201], and is considered ecologically robust making it a good choice as a host for recombinant plant cell wall-degrading enzymes. Published studies [202–204] have demonstrated that modification of *B. fibrisolvens* with GH has been successful, and in vitro digestibility of fiber can be improved, but unfortunately this still does not allow them to compete with the far more fibrolytic *Fibrobacter* and *Ruminococcus* species.

Initially *B. fibrisolvens* was modified with a xylanase (family 10 GH) from *N. patriciarum*, a GH family different from the family 11 typically present in *B. fibrisolvens* [195,205]. GH 10 and GH 11 xylanases differ in their catalytic properties [206] and it was reasoned that the introduction of a GH 11 xylanase would increase its ability to digest fiber [195]. Fiber digestibility could be improved in vitro by more than 28% in comparison with the native untransformed strain [195]. Subsequent studies demonstrated that these recombinants were unable to compete with highly cellulolytic *Ruminococcus* strains in vitro and did not persist in the rumen beyond 10–15 days [207].

Cotta et al. [204] transformed the human colonic bacterium *Bacteroides thetaiotaomicron* strain BTX with a xylanase. In a continuous fed-batch culture system with mixed rumen bacteria this strain only persisted when chondroitin sulfate (a mucopolysaccharide used by *B. thetaiotaomicron*) was added to the medium. In a subsequent study [208], *B. thetaiotaomicron* was inoculated into dual flow continuous culture fermenters and persisted at approximately 1% of the total population for at least 144 h. Some increase in fiber digestion could be observed. It should be noted that the fermenters used did not support a full complement of rumen microorganisms, and in particular, no protozoa were present [209].

An alternative approach is to create recombinant bacteria that can degrade fiber at low pHs. It is well known that fiber digestion declines in animals on high-grain diets because the pH of the rumen drops below 6.5 [210] and *Ruminococcus* and *F. succinogenes* are sensitive to even mildly acidic pH [211]. Russell and Wilson [212] proposed that the addition of fibrolytic activity to an acid-resistant species such as *Prevotella* would create an organism which would be far more competitive because it would be filling an 'acidic niche' which autochthonous cellulolytic bacteria are unable to fill. Subsequent studies have constructed the appropriate organisms but no in vivo studies have been conducted [213,214].

4.3. Non-genetically modified fiber-degrading bacteria

The establishment of non-cellulolytic bacteria in the gnotobiotic rumen is possible [215,216], but persistence of fibrolytic strains is more difficult and there are no cases of *Ruminococcus* becoming established [217]. *F. succinogenes* S85 has been successfully established in gnotobiotic lambs but introduction as a member of a mixed microbial community is a prerequisite [216,218]. Fonty et al. [216] tried to define which combination of 182 rumen cultures was essential to colonization but could only conclude that increased complexity increased colonizing ability. Alternative approaches to establishing cellulolytic strains have included repeated dosing of lambs [15] and adult sheep [219], and completely replacing the contents of the rumen with single strains of cellulolytic bacteria [220] (D.O. Krause and C.S. McSweeney, unpublished). All these attempts have been unsuccessful.

Given that measurement of fiber digestion in vivo would require the inoculant to be at reasonably high levels for at least 2 weeks, and that dosed strains disappear quickly, attempts have been made to measure digestibility by artificially elevating cellulolytic bacteria in the rumen. Dehority and Tirabasso [221] increased the numbers of fibrolytic bacteria in the rumen by feeding a high-cellulose diet composed of purified wood cellulose. There was a 10-fold

increase in the number of cellulolytic bacteria but they could not demonstrate a significant increase in the digestion of alfalfa (lucerne) cellulose using in situ nylon bag digestibility. Similar results were obtained if the number of *Ruminococcus* species was elevated by continuous inoculation [222]. Collectively, these results would indicate that microbial enzyme activity is not the limiting factor in ruminal fiber digestion.

Several groups of workers have dosed fibrolytic bacteria into the rumen but have found that the inoculant usually disappears from the rumen [223–226]. One of the reasons given for this is that we do not yet understand how fibrolytic bacteria exist and reproduce at the fiber surface. What we do know is that organisms do not exist in ecosystems on their own, but reproduce and persist as members of complex microbial communities. Ultimately bacteria persist, or survive, because they reproduce within the physiological and ecological limits of the ecosystem (community-level reproductive strategies) [227]. These strategies derive from the evolution of cooperative networks among microorganisms in which some members cleave specific bonds, others utilize particular substrates, and still others produce inhibitors [227]. A ruminal example is the production of cellodextrins by cellulolytic bacteria [228], which are utilized by non-structural carbohydrate-fermenting bacteria (NSC). The NSC in turn produce ammonia and branched-chain volatile fatty acids that are consumed by cellulolytic bacteria [229,230]. In the case of fibrolytic organisms, it is likely that our understanding of nutrient requirements and other interactions between organisms at the fiber surface is inadequate.

In the continuous dosing studies of both lambs [15] and adult sheep [219] 16S rRNA-based observations indicated that there was a significant increase in the eukaryotic population and this appeared to be primarily the result of an increase in protozoa [15]. Protozoa have a considerable capacity to ingest rumen bacteria [231–233] and in vitro studies with sheep rumen fluid have shown that lysis of *Methanobrevibacter*, and *Selenomonas ruminantium* decreased significantly when protozoa were absent from the rumen fluid [234]. Sharp et al. [235] have also demonstrated that an inoculated *Lactobacillus plantarum* strain disappeared from the rumen largely because of protozoal predation. There were also suggestions of protozoal predation of *F. succinogenes* S85 in the gnotobiotic study of Fonty et al. [216]. In contrast, there did not appear to be a suppression of *F. succinogenes* S85 when dosed to protozoa-free gnotobiotic lambs in the presence of a complex mixture of axenic rumen bacteria [236].

Bacteriocin production by certain cellulolytic bacteria has only recently become a subject of research and may be an essential component in the formation of cooperative microbial networks. *R. albus* strains can produce bacteriocin-like substances that inhibit the growth of *R. flavefaciens* but not *F. succinogenes* [237,238]. There also appears to be an unusually high incidence of bacteriocin-like activity among *Butyrivibrio* isolates and butyrivibriocin has been isolated from *B. fibrisolvens* AR10 [239]. How the ability to produce bacteriocins, or resistance to bacteriocins, is involved in the establishment and persistence of dosed ruminal bacteria is difficult to assess with current knowledge, but these compounds may have important ecological consequences.

When cellulolytic bacteria are grown together in diculture, cellulose degradation is often below that of the pure culture [201,240], which is probably the result of competitive and non-competitive interactions between cellulolytic bacteria. Shi [241] demonstrated that cell numbers of individual species were approximately equal in cellulose excess dicultures of *R. albus* plus *R. flavefaciens*, *R. albus* plus *F. succinogenes*, and *R. flavefaciens* plus *F. succinogenes*. However, when cellulose was limiting *R. flavefaciens* > *R. albus*, *R. flavefaciens* > *F. succinogenes*, and *F. succinogenes* > *R. albus*. These competitive outcomes were likely the result of the superior ability of *R. flavefaciens* to adhere to cellulose [242]. It is interesting to note that *R. albus* survived under cellulose-limited conditions. This was probably a combination of its ability to utilize glucose (*R. flavefaciens* does not) [243], grow at low concentrations of cellobiose [244], and its capacity to produce bacteriocins [238].

5. Modification of fiber by exogenous means

5.1. Chemical and mechanical treatments

There are a number of well-established technologies that can reliably be used to increase the digestibility of fiber in the rumen based on mechanical and chemical treatment of plant material before it is consumed. In this review we will not discuss this technology, which has been previously considered [7,245–247]. We will only concentrate on developments of some newer technologies.

5.2. Plants genetically modified for plant cell wall composition

5.2.1. Lignin synthesis

The relationship between lignin and carbohydrates in the plant cell wall is complex, constituting a range of compounds (Fig. 5), various lignin iterations with plant cell walls (Fig. 2), within the context of a multifaceted lignin biosynthetic pathway (Fig. 6). Changes to the components of lignin result in improvements in digestibility. Natural or chemically induced '*brown midrib*' (*bm*) mutations in several forage species result in altered lignin contents and consequently higher digestibilities [248]. Two of the *bm* mutations in maize occur in genes that encode enzymes in the lignin biosynthetic pathway (Fig. 6). The *bm1* mutation affects the gene encoding cinnamyl alcohol dehydrogenase [249] and the *bm3* mutation results in changes to

the gene encoding caffeic acid *O*-methyltransferase (COMT) [250]. These mutants clearly indicate the potential for manipulation of the lignin biosynthetic pathway to improve digestibility of forages.

Recently, molecular strategies for manipulating the enzymes of lignin biosynthesis have contributed greatly to our understanding of the process of lignin biosynthesis and the effects of altered lignin content and composition on digestibility [251]. The enzymes that catalyze lignin formation are encoded by multi-gene families and may carry out a number of related reactions with variable specificity. These factors generate an enormous plasticity in lignin structure that allows adaptation during development and environmental stress [251]. Consequently, the effects on lignin structure of suppressing a single enzymic reaction are not entirely predictable.

Modification of lignin biosynthesis has been achieved by sense and antisense suppression of plant gene expression. These experiments have produced somewhat conflicting results showing that changes both to lignin content and to composition appear to alter cell wall digestibility. Transgenic tobacco plants with reduced expression of cinnamaldehyde dehydrogenase (CAD) produced lignin with a decreased S/G ratio and increased degradability even though there was no change to the total amount of lignin [252]. Similarly, down-regulation of CAD activity in alfalfa altered the composition of the lignin and led to improved digestibility in the rumen [253]. The activity of COMT has also been reduced by sense or antisense suppression in a number of species. The digestibility of cell walls from tobacco increased following suppression of COMT activity, which caused a reduction in the S/G ratio without altering total lignin content [254]. Similar improvements to digestibility were seen in *Stylosanthes humilis*, a tropical forage legume, when the composition of

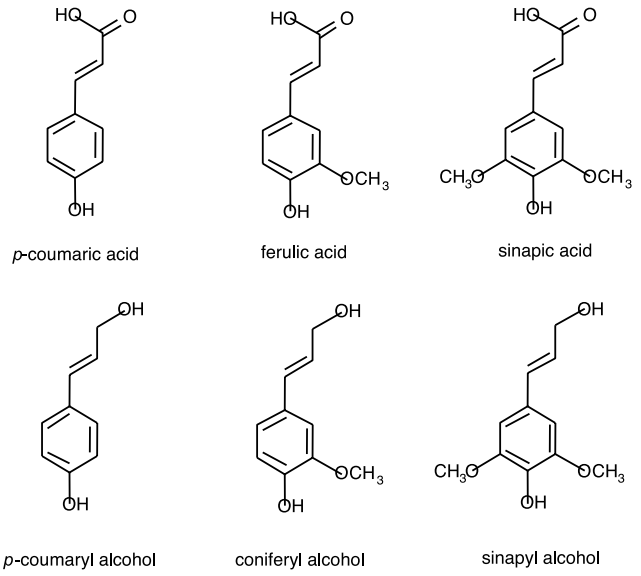

Fig. 5. Major monolignol constituents of lignin in their hydrocinnamic acid. Redrawn from [30].

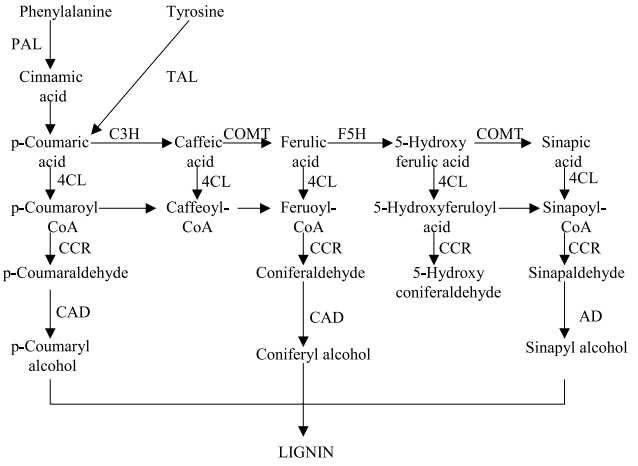

Fig. 6. The lignin biosynthetic pathway. The lignin biosynthetic enzymes are: PAL = phenylalanine ammonia-lyase, TAL = tyrosine ammonia-lyase, C4H = cinnamate 4-hydroxylase, C3H = 4-hydroxycinnamate 3-hydroxylase, COMT = caffeic acid 3-*O*-methyltransferase, F5H = ferulate 5-hydroxylase, 4CL = 4-coumarate:CoA ligase, CCoA-3H = coumaroyl-coenzyme A 3-hydroxylase, CCoA-OMT = caffeoyl-coenzyme A *O*-methyltransferase, CCR = cinnamoyl-CoA reductase, and CAD = cinnamyl alcohol dehydrogenase [352].

lignin was altered by antisense suppression of the COMT gene [255]. Although these results suggest that lignin composition is an important factor in digestibility, contradictory results were obtained from a study of induced lignin polymerization in a model system. The digestibility of cell walls from maize suspension-cultured cells was investigated following induced polymerization of monolignols added in various proportions.

While lignification substantially reduced the degradability of the cell walls compared to controls, varying the ratios of monolignols (Fig. 5) had no effect [256]. Some experiments with transgenic plants concur with these results. Sewalt et al. [257] showed that the increased digestibility of tobacco stems with suppressed COMT activity was associated with reduced lignin content, not with changes in composition. An increase in digestibility was also achieved in transgenic tobacco plants by down-regulation of caffeoyl coenzyme A 3-*O*-methyltransferase, which caused a small change in lignin content but no change in S/G ratios [258]. Furthermore, major changes to lignin composition have been shown to have no effect on digestibility. The *fah1* mutants of *Arabidopsis thaliana* are deficient in ferulate-5-hydroxylase and produce lignin with no syringyl units. There was no difference in cell wall degradability of these mutants compared to wild-type plants [259].

It seems clear that a reduction in lignin content can improve digestibility. In all cases where a reduction in lignin content was achieved in transgenic plants, digestibility increased. Presumably, this increase is due to improved access of rumen microorganisms and secreted enzymes to plant cell wall polysaccharides. Lignin appears to play a major role in cross-linking and anchoring cell wall

polysaccharides. In an *A. thaliana* mutant in which lignin was reduced by 50% by a defect in the gene encoding cinnamoyl-CoA reductase, the cell wall architecture was altered to the extent that xylem vessels collapsed [260]. Detrimental changes to plant growth and metabolism have been described in other transgenic plants with reduced lignin contents [261].

Notwithstanding the effects of reduced lignin content, there are many documented examples of improvement to digestibility achieved through altered lignin composition. These improvements may be due to associated changes in cell wall composition and structure. Alterations of single enzymes can affect other products of the phenylpropanoid pathway, which are known to have multiple roles in metabolism. Furthermore, altering one enzyme may interrupt the very fine control mechanisms that maintain the heterogeneity of lignin across different cell types and developmental stages [251]. A recent study confirmed that different alterations to lignin biosynthetic enzymes could cause different changes to spatial organization of lignin and cell wall polysaccharides at the cellular and subcellular levels [262]. The interactions between lignin and cell wall polysaccharides are likely to be a very important determinant of digestibility.

An improved understanding of the types and quantity of lignin required by plant cells for normal development will be important in genetic manipulation of plant cell walls for improved digestibility. Large decreases in lignin concentration appear to cause defects in plant growth and metabolism [259,260]. More subtle changes to lignin concentration and cell wall ultrastructure may be as effective as reducing the overall lignin content. Molecular tools for manipulating plant cell wall architecture are becoming available, and offer new approaches for understanding and improving forage digestibility.

5.2.2. Cellulose synthesis

Some progress has also been made recently in our understanding of the regulation of cell wall polysaccharide synthesis and structure. The model plant species *A. thaliana* has proved particularly valuable in these studies because of the ease of screening large numbers of plants and the availability of a complete genome sequence. Mutations have been found in genes encoding cellulose synthesis that act in primary cell wall synthesis [263] and in secondary cell wall synthesis [264]. The mutant plants had no changes in concentrations of lignin or non-cellulose polysaccharides, but major changes to cell wall ultrastructure. There is some evidence for coordinated regulation of lignin and cellulose deposition in tree species. In transgenic *Populus* trees with reduced activity of 4-coumarate:CoA ligase, the reduction in the amount of lignin was counteracted by an increase in the amount of cellulose, so that the total lignin–cellulose mass was unchanged [265]. Genes encoding other enzymes involved in cell wall synthesis have also been isolated from *Arabidopsis* recently, including a family of genes related to xyloglucan fucosyltransferases [266]. Analysis of cell walls from plants altered in expression of these genes will help assemble models of cell wall structure.

5.3. Lignolytic fungi

In nature, lignin is degraded by several groups of organisms, among which white-rot fungi (WRF) belonging to the basidiomycetes are one of the most active groups [267,268]. Two families of ligninolytic enzymes are widely considered to play a key role in this process: phenol oxidase (laccase) and peroxidases (lignin peroxidase (LiP), manganese peroxidase (MnP)) (see reviews [32,269]). A number of genera produce these enzymes, which include *Pleurotus*, *Phanerochaete*, *Coprinus*, and *Pycnoporus* spp. [270].

Initially fungal cells colonize plant material and utilize soluble carbohydrates (although this is not true for all strains) before degrading lignin. Initial work by Agosin et al. [271] indicated that in vitro digestibility of wheat straw increased from 38 to 68% when treated with strains of *Dichomitus squalens* and *Cyathus stercoreus*. Agosin et al. [272] did detailed analyses of structural components of wheat straw to assess the effects of WRF on forage chemistry. There was a rapid degradation of ^{14}C-labelled lignin until a plateau was reached, but further weight loss was a result of carbohydrate degradation.

Gamble et al. [273] used nuclear magnetic resonance to investigate the structural changes that occurred when *Ceriporiopsis subvermispora* and *Cyathus stercoreus* delignified Bermuda grass (*Cynodon dactylon*) stems. The in vitro digestibility of Bermuda grass after 6 weeks of incubation was improved from 29 to 32% by *C. stercoreus*, and from 63 to 77% by *C. subvermispora*, while dry matter losses were about 20%. *C. subvermispora* removed 23% and *C. stercoreus* 41% of total aromatics. Removal of aromatic compounds, including ester-linked *p*-coumaric and ferulic acid, occurred before that of carbohydrates. However, even highly specialized lignolytic fungi that preferentially degrade lignin will start consuming the structural carbohydrates once the tissue has been delignified [274–276], making it important to stop the process if animal feeding is contemplated.

One of the problems with this process at an agricultural scale is that putrefying bacteria on the forage will overgrow causing the forage to rot. To solve this problem the material can be pasteurized during solid-state fermentation. This is the most successful delignification process available because temperature, oxygen, aeration etc., are tightly controlled. The process is technically viable at an industrial scale but not economically feasible for farmers because of the energy inputs. For example, Zadrazil et al. [277] incubated 1500 kg of wheat straw with *Pleurotis* spp. and demonstrated improved in vitro digestibility (40% to 53.7% dry matter disappearance).

Hüttermann et al. [278] described a novel process for the recycling of agricultural wastes by *Pleurotus* spp. of fungi. This included pasteurization of wet straw by solar heat, treatment with detergents and amendment of straw with wastes from the food industry, such as potato pulp and tomato pomace. Careful analysis found this methodology to be suitable for on-farm application where low energy requirements are a prerequisite. In vitro and in vivo experiments have clearly shown that biological treatment with *Pleurotus* spp. improves the availability of poor roughage for animals, owing to its effect on cellulose and lignin. In feeding experiments, rams fed fungus-treated straw exhibited increased body weight [278].

Alternative means of pasteurizing the material are the use of chemicals such as NaOH, H_2SO_4, and NH_3 that inhibit putrefying bacteria before treatment with WRF [104,113,118]. However, WRF are typically sensitive to high-pH environments and this can restrict the use of chemicals. *Coprinus* spp. of fungi are alkaliphilic and can grow at pHs as high as 10.0 [279]. This particular property of *Coprinus* fungi suggests a solution to two of the practical requirements of forage conservation, prevention of putrefactive microorganisms and increasing the protein content of the feed. Urea on forage rapidly converts to ammonia due to the activity of autochthonous microorganism on the forage; the pH consequently increases resulting in inhibition of many putrefying organisms. If *Coprinus* fungi are used delignification can proceed in the absence of putrefying organisms while at the same time increasing the NPN content of the forage [279–282].

Another mechanism by which putrefying bacteria are inhibited and the degradation of cellulose retarded is by ensiling plant material after initial treatment with WRF. The anaerobic conditions will prevent activity by fungi due to the lack of oxygen, and the low pH produced by the acidic fermentation in the silage will retard the growth of putrefactive bacteria. Yang et al. [283] used a two-stage process that combined solid-state fermentation and ensiling with corn straw. The solid-state fermentation increased the level of protein in the feed from 6.7% to 14.7% but decreased the cellulose by 38.0% and hemicellulose by 21.2%. A high-protein roughage with increased digestibility resulted after ensilage of the material.

5.4. Exogenous enzymes

5.4.1. Historical perspective

During the 1960s evidence that the direct application of enzymes to forage before consumption can improve cattle performance became available [284]. In comparison to controls, cattle gained 6.8–24.0% more weight and converted feed 6.0–21.2% more efficiently when ground maize, oat silage, maize silage, or lucerne hay was treated with an enzyme cocktail containing amylolytic, proteolytic, and cellulolytic enzymes. Four different enzyme preparations given in combination with the growth stimulant diethylstilbestrol allowed cattle fed a maize–lucerne hay diet to gain 14% more weight than controls [285]. Further studies confirmed that enzyme supplements could improve the performance of cattle on silage diets [286], but other studies have not necessarily supported this work and have even demonstrated negative results. Leatherwood [287] added a fungal enzyme extract to a grain-supplemented lucerne diet fed to young calves and found no improvement in gain or feed utilization. Unfortunately enzyme cocktails containing enzymes have also resulted in quite significant decreases in animal performance when applied to forage-based diets fed to ruminants [288–290].

These earlier studies have provided valuable information on the potential benefits of enzymes for beef cattle production. More recent studies have been designed to address issues related to the inconsistency of results by investigating the effects of feed type [291], application levels [292], enzyme products [293,294], and enzyme applications [292,295]. Other factors, such as whether dry forage or silage is used [291,293], the enzyme is infused directly into the rumen or not [295,296], have been investigated. Given this variation in responses it is obvious that a mechanistic understanding of how enzymes interact with forages and how synergies between exogenous and autochthonous microbiotas in the rumen occur are still not well understood.

5.4.2. Mode of action

An early criticism of enzyme inoculation into the rumen was that the enzymes were likely to undergo inactivation by the proteolytic activity of rumen fluid. It has subsequently been demonstrated that CMCase and xylanase activities contributed from endogenous sources can remain active in the rumen [297] and all exogenous enzyme activity is not necessarily inactivated once introduced into the rumen [297]. The survival of exogenous enzymes in the rumen raises the prospect that enzymes may improve digestion through the direct hydrolysis of ingested feed. Several researchers have demonstrated that exogenous enzymes may enhance fiber digestion by ruminal microorganisms in vitro [298,299] as well as in situ but these observations do not confirm work by other researchers [298,300].

Release of reducing sugars by exogenous enzymes is probably an important mechanism by which exogenous enzymes operate [292]. The degree of sugar release is dependent on the feed type as well as the type of enzyme. For example, only two of 11 enzymes tested released significant amounts of reducing sugars from barley silage [299]. In addition, the enzymes most effective at releasing reducing sugars from lucerne hay were not those that released the most reducing sugars from barley silage. The release of sugars from feeds is at least partially the result of solubilization of NDF (neutral detergent fiber) and ADF (acid detergent fiber) [301,302] and is consistent with the observed increases in the soluble fraction and

rate of in situ digestion [295,301]. However, most studies have not found that exogenous enzymes improve the extent of in situ or in vitro dry matter digestion [301,303,304]. These studies would suggest that exogenous enzymes only digest substrates that would normally be digested by enzymes produced by the autochthonous microbiota in the rumen. Additionally, although exogenous enzymes affect the release of soluble carbohydrates, the amount liberated represents only a minute portion of the total carbohydrate present in the diet. It would be difficult to attribute any production responses solely to the increased availability of reducing sugars given that comparable increases in yield were not seen when up to 9% of total dietary dry matter was supplied as molasses [305].

Although adding exogenous enzymes may increase the activity of xylanases and cellulases in ruminal fluid, enzyme activity in the rumen fluid usually represents less than 30% of the total enzyme activity in the rumen, the remainder being associated with the feed particles [306–308]. For example, application of fibrolytic enzymes to a grass hay diet fed to sheep prior to consumption increased endoglucanase activity and xylanase activity in ruminal fluid, but this activity accounted for only 0.5% of the total endoglucanase activity in the rumen [298]. Given that exogenous enzymes represent only a small fraction of the ruminal enzyme activity, and that the ruminal microbiota is inherently capable of digesting fiber it is difficult to envision how exogenous enzymes would enhance ruminal fiber digestion through direct hydrolysis [298].

A more likely explanation for the mode of action of exogenous enzymes is that they work synergistically with the rumen microbiota. This would only be true if the exogenous enzyme inoculant contained enzymes not produced by rumen microbes and was therefore contributing unique activity. Presently, it does not seem likely that the addition of exogenous enzymes has added unique activity to the rumen as only the rate, and not the extent, of cell wall digestion has been improved [306,309]. Of course, as discussed previously, fibrolytic enzymes normally act as members of a cellulosome complex, presumably because there are a range of glycosidic bonds that need to be hydrolyzed synergistically. A logical extension of this reasoning would therefore be to add enzymes that are not produced by rumen microorganisms and attack structures in forage that result in an increased extent of digestion. Lignolytic enzymes clearly fit within these requirements.

5.4.3. Lignolytic enzymes

As discussed, lignin is clearly one of the major constraints to plant cell wall degradation (Figs. 2 and 5). Most of our understanding of the enzymology of lignin biodegradation comes from studies of a single WRF species, *Phanerochaete chrysosporium*. The principal enzymes implicated in this process are LiP and MnP [310–312]. Despite the extensive literature on LiP and Mn-dependent peroxidases, there is relatively little information on the depolymerization of synthetic lignin in vitro [310,313–315], and it has not been possible to demonstrate extensive lignin depolymerization using isolated LiP or MnP to date. Furthermore, many WRF, including a number of aggressive lignin degraders, seem to operate without expression of LiP activity [316]. Conversely, the WRF *Lentinula edodes* achieves an almost imperceptible rate of lignin biodegradation despite producing a greater specific LiP activity than optimally expressed *P. chrysosporium* [251]. Similarly, a LiP-overproducing mutant of this fungus did not show an increased rate of lignin mineralization [317].

Laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are produced by WRF in combination with LiP and MnP [318,319]. The role played by laccases in lignin degradation has remained obscure since the low redox potential of this enzyme appeared to make it incapable of oxidizing non-phenolic lignin constituents. However, the identification of efficient lignin-degrading WRF that lack LiP and MnP has stimulated research on the role played by laccases in this process. The redox potential of laccases is lower than that of LiP and horseradish peroxidase [320] and it was thought that this low redox potential precluded laccases from playing a significant role in the oxidation of non-phenolic polymeric lignin. However, recent studies show that, in the presence of appropriate low-molecular-mass 'mediators' (such as 2,2′-azino-bis-(3-ethylthiazoline-6-sulfonate (ABTS) or 3-hydroxyanthranilic acid (3-HAA)) laccase is able to oxidize a wide range of other aromatic lignin compounds [321–323]. These recent studies have indicated that laccases may play a far more important role in lignin biodegradation than was previously thought.

The WRF *Pycnoporus cinnabarinus* was recently demonstrated to degrade lignin in the absence of both LiP and MnP [323–325]. Under ligninolytic conditions, laccase was the predominant phenol oxidase secreted and neither LiP nor MnP was produced [322]. Despite the absence of these two LiPs, lignin appeared to be degraded just as rapidly, and to the same extent, as *P. chrysosporium*. The mechanism by which laccases can assume some of the function of the LiP was described by [251,326–328]. The identification of 3-HAA as a naturally occurring redox mediator for laccase is the first evidence to support this ligninolytic system as having equivalent potential to ligninolytic systems based on LiP or MnP [251,316,322–324].

Synthetic compounds that have been used as redox mediators in combination with fungal laccases include 3-HAA, ABTS and 1-hydroxybenzotriazole which has been used in a process-scale pulp bio-bleaching application [329]. The laccase/3-HAA system was significantly more effective in degrading ^{14}C-labelled lignin (polymerized guaiacyl dehydroperoxidase) to fragmentation products (with molecular masses ranging from 4000 to monomeric units) than was the ABTS/laccase couple [322]. The compound 3-HAA is produced as an intermediate in the bio-

synthesis of a major fungal orange pigment (cinnabarinic acid) by the WRF *P. cinnabarinus*.

Given that the enzymes involved in lignin oxidation are too large to penetrate the unaltered wood cell wall, the use of low-molecular-mass, diffusible compounds to oxidize the polymer is a logical solution. Although the exact reactive species that mediate the laccase-catalyzed oxidation remain unidentified, it is clear that diffusible and reactive low-molecular-mass compounds are responsible for the degradative attack on the lignin polymer [251]. It is likely that each laccase has a preferred low-molecular-mass 'mediator' substrate, which may represent a major secreted metabolite. It has been proposed that LiP uses the veratryl cation radical as mediator [329]. Finding the physiological laccase substrate and matching it to the laccase secreted by the fungus has recently been applied to an industrial process. It appears that a laccase and mediators with NO, NOH, or HRNOH groups can be combined in a laccase–mediator system (Lignozyme® process) and are effective in delignifying wood in a pilot pulp and paper process [329]. Given the success with this process it is likely that it could be extended to forage.

6. (Gen)omics and metagenomics

High-throughput DNA sequencing and the advent of the '-omics' disciplines now offer the potential to obtain a complete blueprint for the lifestyle of a specific microbe, and to assess its genetic potential in a comparative and functional manner. Although early initiatives in microbial genome sequencing focused on microbes with either small genomes (e.g. mycoplasmas and some archaebacteria) and (or) pathogens, similar approaches with agriculturally and environmentally relevant microbes have been conducted. The genomes of the rumen microbes with relevance to fiber degradation for which genome sequence is available are *F. succinogenes*, *R. albus*, and *P. ruminicola* strain 23.

The *F. succinogenes* genome has yielded significant insights, and some of those related to GH are detailed in this publication [330]. From the gene list at least 24 genes encoding endoglucanases and cellodextrinases were identified, far exceeding the six genes previously characterized by recombinant DNA strategies employing *E. coli* as a cloning host. A relatively large number of these genes (13/24) appeared to encode GH belonging to enzyme family 5, but family 7 and one family 45 GH were also identified. Three genes, not previously described, appeared to encode CelF homologs, which possess multiple catalytic domains. Interestingly, no family 6 or family 48 GH were identified, up until now thought to be the source of exo-acting cellulases and processive endoglucanases in eubacteria. An additional 23 genes were identified that were presumed to encode xylanases and other enzymes that hydrolyze non-cellulosic polysaccharides. The *F. succinogenes* genome encodes type I and type II secretion systems, in addition to PilT homologs that coordinate twitching motility in other Gram-negative bacteria. The genome sequence data have already revealed the limited scope of our knowledge of the genetic blueprint of polysaccharide hydrolysis in *F. succinogenes*, the most intensively studied aspect of this bacterium's physiology. The vast majority of this genome sequence carries sequence information that cannot confidently be assigned to any specific function.

The *R. albus* project has revealed a number of ORFs containing type I dockerins, supportive of recent biochemical and genetic evidence that *R. albus* produces a cellulosome-like complex. It has also long been recognized that efficient cellulose hydrolysis by *R. albus* is conditional on the provision of micromolar amounts of PAA/PPA, which elicit substantial changes to cell surface ultrastructure. Cellulase activity is also retained within cell-associated, high-molecular-mass protein complexes, thought to be cellulosomes. A combination of several '-omics' approaches was recently used to define this response [331,332]. Two-dimensional polyacrylamide gel electrophoresis revealed two proteins, 'PpaA' (108 kDa, p*I* 5.6) and 'PpaB' (94 kDa, p*I* 5.4), which increase dramatically in extracts prepared from cells cultured in the presence of PAA/PPA. These polypeptides were subjected to N-terminal sequence analysis by Edman degradation and a peptide mass fingerprint from tryptic digests of each protein was generated by matrix-assisted laser desorption/ionization time of flight analysis. These proteomic data were then used to query the *R. albus* genome sequence, and the two ORFs encoding these proteins were identified. Based on these findings, 'PpaA' is a family 9 GH (Cel9B) and 'PpaB' is a family 48 GH (Cel48A), and homology searches suggest these proteins provide processive endoglucanase (Cel9B) and exoglucanase (Cel48A) activities. These results show for the first time some of the enzymes rate-limiting to cellulose hydrolysis by *R. albus*. PAA/PPA apparently does not have the same effect when xylan is the substrate [333].

Despite the unequivocal value associated with whole genome sequencing, it is still cost-prohibitive for many investigators to obtain a similar degree of sequence data for multiple strains of the same microbe, or for related species. Subtractive hybridization (SH) can be used as a means to recover 'unique' genomic information from other strains of *Ruminococcus* spp. and *F. succinogenes*. Using these methods Antonopoulos and White [334] have generated a set of 288 clones that are unique to *R. flavefaciens* FD-1 with respect to *R. flavefaciens* JM1. This clone library includes sequences with homology to a non-ribosomal peptide synthetase from *Streptomyces avermitilis*, and the restriction endonuclease *Sal*I. These clones were also used to query the *R. albus* genome, and clones with significant sequence identity to genes present in *R. albus* include a putative acetyl-CoA synthetase/carbon monoxide dehydrogenase (78% identical) and a nitrogenase subunit (60% identical). These results substantiate how SH can broaden the scope of functional and comparative ge-

nomics in a cost-effective manner, and will facilitate the examination of gene diversity and genome plasticity among related ruminal microbes. These methods might also help elucidate which gene(s), as well as other ecological or physiological process(es), are rate-limiting to fiber degradation. By doing so, some of the major 'informational' constraints to improving ruminal fiber degradation may ultimately be alleviated, and hypothesis-driven rather than empirical experimental designs will be employed.

Microbial genome sequencing is a useful tool that will allow us to extend our understanding of individual fibrolytic species of rumen bacteria. However, we should keep these genome sequencing efforts in context. It is by no means the end game given that the majority of microbial species cannot be, or have not been, cultured. As discussed, microorganisms live in community and analyses of individual organisms do not comprise the complex ecological networks that optimize ecosystem function found in most microbial systems, including those for fiber digestion. These complex systems are not well understood and it is by studying fiber digestion at a community genomic level (the metagenome) that the new clues to manipulating fiber digestion will emerge.

The analysis of the rumen metagenome is complicated by the fact that the majority of microorganisms have not been cultured (estimates range from 85 to 95% [335–337]), and probably comprise upwards of 1000 individual species of bacteria, fungi, and protozoa [338]. This situation is not unique to the rumen and the vast majority of microbes in the biosphere, often thousands of species in a single environmental niche [339]), cannot be grown in the laboratory. It is estimated that, on average, less than 1% have ever been identified [340]. Traditional methods for culturing microorganisms fail to represent the scope of microbial diversity in nature and they limit analysis to those that grow under laboratory conditions [341,342].

The use of PCR in conjunction with phylogenetically stable molecular markers like 16S rDNA as species-specific identifiers has provided us with the ability to detect single cells in microbial ecosystems [337,343], irrespective of their culturability, and thereby widened our view of biodiversity [343]. The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel types of microorganisms in high numbers and provides an entirely different approach for tapping into the potentially limitless resource of uncultured bacteria. The rumen is no different, and clone libraries based on 16S rRNA clearly suggest that a vast reservoir of physiologies is present in the rumen [335–337]. Handelsman et al. [344] first coined the term 'metagenome' to describe genomic and associated functional analysis at the community level. One of the keys to metagenomic studies is the ability to clone large fragments of community DNA (>100 kb) into bacterial artificial chromosomes (BAC). BAC vectors are controlled for copy number and insert stability, allowing single genes and even operons to be cloned. An extremely useful phylogenetic spin-off is that because of the insert size many clones will contain 16S genes, which together with an analysis of associated functional genes provides a phylogenetically informed view of uncultured diversity [344,345].

This technology is still in its infancy and issues such as representative cloning, quantitative lysis, and expression still need to be addressed [346]. In particular, quantitative lysis is difficult to overcome. It is imperative that large fragments be cloned, and to do this techniques based on pulsed field gel electrophoresis must be employed [345]. To prevent shearing of DNA in aqueous solutions cells are embedded in agarose plugs and lysed in situ. This immediately presents the problem of quantitative lysis of the microbiota. We have developed technology in our laboratory that allows us to obtain quantitative lysis and at the same time obtain clones, on average, of above 150 kb (D.O. Krause et al., unpublished). The importance of clone size should not be underestimated. If insert size averages 100 kb, a library of 50 000 clones will have a low probability of covering the genome of an organism that is less than 0.5% of the population. If, on the other hand, insert size is 200 kb a library of 50 000 clones will contain organisms of as little as 0.2% of the population. Libraries of less than 100 kb require clone libraries of impossible dimensions. Handelsman et al. [344] estimated that in the order of 10^6 BAC clones with an average insert size of 100 kb will be necessary for complete representation of the soil metagenome. This seems an impossible task, and with the resources available to most institutions, it is. However, if one accepts that major microbial species are the target of these activities, and that the metagenomes include incredible molecular diversity, 50 000 clones may be adequate.

The choice of appropriate clone recipients will be important as cloning of metagenomic DNA will be in heterologous hosts, and primarily in *E. coli*, simply because high clone numbers are necessary to get anywhere near a representation of the microbial diversity present in the metagenome [346]. Similarly, downstream cloning in *Bacillus* species or *Streptomyces lividans* for the sake of optimizing gene expression will be the method of choice [346]. Although activity screening based on the functional expression of enzymes is attractive because of unique physiologies that will be discovered, problems associated with heterologous gene expression would naturally limit its success. However, it appears that diversity in metagenomic DNA is so great that even inherently flawed cloning strategies represent so much molecular novelty that at present optimization of these strategies seems irrelevant in comparison to the constraints in downstream processing of this 'mega-diversity'.

Already metagenome research has provided some unique insights into microbial ecosystems. Analysis of large clones derived from oceanic libraries has revealed the importance of proteorhodopsin obtained from BAC inserts of uncultured bacterioplankton [347]. It was dem-

onstrated that photoactive proteorhodopsin is present in oceanic surface waters and that there may be an extensive family of globally distributed proteorhodopsin variants. The protein pigments comprising this rhodopsin family were spectrally tuned to different habitats and absorb light at different wavelengths in accordance with light available in the environment. These authors suggest that proteorhodopsin-based phototrophy is a globally significant oceanic microbial process.

In another study [348] on oceanic microbial assemblages, large-scale genome sequence analysis was conducted on marine archaea to better describe the population genetics, genome content, and biological properties of naturally occurring, uncultivated pelagic crenarchaeotes. Sequencing and analysis of the entire DNA insert from one Antarctic marine archaeon revealed differences in genome structure and content between Antarctic surface water and temperate deep water archaea. Analyses of the predicted gene products revealed many typical archaeal proteins but also several proteins that so far have not been detected in archaea.

Metagenome analysis is not simply limited to discovery of more and novel diversity, but has practical use in finding fresh enzymes for industrial and agricultural use. This is a logical assumption when one realizes that because of the poor ability to culture environmental microorganisms, most industrial enzymes will necessarily have originated from the small percentage that have been cultured. Using BAC-based technologies, direct DNA extraction and cloning strategies from environmental DNA have already been demonstrated and revealed novel enzyme activities that are of industrial importance [344,349–351]. The list of reported enzyme activities discovered this way (lipase, esterase, amylase, nuclease, chitinase, xylanase) is still rather small, but will undoubtedly grow rapidly. This approach has significant scope for mining the rumen microbiota for novel enzyme activities, or lack of, so that more effective exogenous enzyme additives can be designed.

7. Conclusions

This review has discussed the major problems that face scientists trying to improve plant cell wall digestion in the rumen. A semi-historical survey covered the various attempts and strategies that have been employed to improve fiber digestion in the rumen. It seems clear from this review that several major challenges are presented and that improvement of fiber digestion is possible providing that it is based on a rational scientific basis, something that until now has not always been obvious.

In retrospect, many of the strategies that have been undertaken to improve rumen digestion have floundered because of our lack of understanding of a very complex system. First among these is the fact that we have based almost all our knowledge about rumen microbiology on just a few species of bacteria, and in fact just a few strains. For example, it is not clear that we are even working with the major cellulolytic rumen bacteria in pure culture. In addition, attempts to improve rumen function by addition of exogenous enzymes have been based largely on availability of enzymes and little attention has been paid to actual enzyme 'requirements' of the rumen. This of course has been because of the fact that we have essentially been ignorant of the functional genomic framework within which the rumen operates; the cellulosome is an example of this. This situation can be likened to a ship's captain who can only see the tip of an iceberg, the mass of which is vast in comparison to what sticks up above the water. To say the least it makes navigation difficult.

(Gen)omics technologies, including metagenomes, now provide rumen microbiologists with their best opportunity, to date, to see the iceberg in its entirety, rather than just its tip, in both a comparative and functional system. This is both an exciting and daunting prospect. Clearly, (gen)omics will provide a massive increase in the rate of information acquisition, and both novel and conventional methodologies and techniques must flourish for the potential of (gen)omics to be fully realized. For instance, there will be a need for novel in silico methods to mine and extract relevant information from seemingly disparate systems. Renewed interest in microbial physiology and in the isolation of 'unculturable' or 'not-yet-cultured' microbes is also necessary, if we are to fully exploit the opportunities provided by (gen)omics.

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