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Fibrobacter succinogenes, a Dominant Fibrolytic Ruminal Bacterium: Transition to the Post Genomic Era*

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ABSTRACT : *Fibrobacter succinogenes*, a Gram-negative, anaerobic ruminal bacterium is a major fibre digesting species in the rumen. It intensively degrades plant cell walls by an erosion type of mechanism, burrowing its way through the complex matrix of cellulose and hemicellulose with the release of digestible and undigested cell wall fragments. The enzymes involved in this process include a combination of glucanases, xylanases, arabinofuranosidase(s) and esterases. The genome of the bacterium has been sequenced and this has revealed in excess of 100 putative glycosyl hydrolase, pectate lyase and carbohydrate esterase genes, which is greater than the numbers reported present in other major cellulolytic organisms for which genomes have been sequenced. Modelling of the amino acid sequences of two glycanases, CedA and EGB, by reference to crystallized homologs has enabled prediction of the major features of their tertiary structures. Two dimensional gel electrophoresis in conjunction with mass spectroscopy has permitted the documentation of proteins over expressed in *F. succinogenes* grown on cellulose, and analysis of the cell surfaces of mutant strains unable to bind to cellulose has enabled the identification of candidate proteins with roles in adhesion to the plant cell wall substrate, the precursor to cellulose biodegradation. (**Key Words :** Cellulose, Cell Walls, *Fibrobacter succinogenes*, Cellulase, Xylanase, *Fibrobacter intestinalis*)

INTRODUCTION

Grasslands and savannas, covering about 20% of the earth's landscape are a major source of nutrients for wild and domestic herbivores. In addition, annual forage crops are often the primary source of nutrients for domestic herbivores. To maximize the value of these resources there is a continuing search for methods to improve the digestibility of both grasses and forage crops and the focus of these studies is the plant cell wall (Barrière et al., 2003). The cell wall is composed of an intertwined mesh of mainly cellulose, hemicellulose, and pectin (Cosgrove, 2005), but as the plant ages there usually is an increased content of lignin, likely the only component of the cell wall that is highly resistant to microbial degradation (Grabber et al., 2004). Satter et al. (1999) presented data demonstrating no relationship between lignin content and *in vitro* neutral detergent fibre degradability although other studies have shown an association between lignin content and indigestible neutral detergent fibre (Huhtanen et al., 2006), and with careful selection for strain fitness, genetic reduction in lignin concentration of forages can increase the availability of energy from cell-wall polysaccharides, improving the efficiency of livestock production (Casler et al., 2002; Grabber et al., 2004). Digestibility improvements may result from either manipulation of the genome of either the plant (Barrière et al., 2003) or the fibre digestive capabilities of the ruminal bacteria (Krause et al., 2003), or in the case of harvested forage by downstream processing (Huhtanen et al., 2006). With all of these approaches more knowledge of the mechanism of plant cell wall digestion by predominant ruminal organisms will contribute to the success of programs to improve forage utilization by ruminant and monogastric animals. The objective of this review is to survey recent advances in our understanding of the diversity, function and structure of microbial cellulases and hemicellulases with a focus on the primary cellulolytic

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ruminal bacterium *Fibrobacter succinogenes*.

MAJOR CELLULOLYTIC BACTERIA AND THEIR ASSOCIATION WITH PLANT CELL WALLS

The three species of ruminal bacteria considered to be primarily responsible for plant cell wall biodegradation are *F. succinogenes*, *Ruminococcus albus*, and *R. flavefaciens* (Forsberg et al., 1997; Krause et al., 2003). They are primarily associated with the solid phase of rumen contents and *F. succinogenes* often is the dominant population, and the most firmly attached (Michalet-Doreau et al., 2001). Koike et al. (2003) in a study of the kinetics of binding of bacteria to plant materials reported that *F. succinogenes*, *R. albus* and *R. flavefaciens in vivo* begin binding to hay stems within 5 minutes and increase to a maximum at 24 h of 10^9 cells per gram of dry matter for *F. succinogenes* and 10^7 per gram of dry matter for *R. flavefaciens* although for *R. albus* maximum binding of 10^6 per gram of dry matter did not occur until 48 h. *F. succinogenes* was the most dominant of the three in whole rumen digesta and on hay stems suspended in the rumen. Ozutsumi et al. (2006) reported that the numbers of *F. succinogenes* cells in a faunated rumen was 2.4-fold higher than in an unfaunated rumen while the numbers of *R. albus* and *R. flavefaciens* were decreased by 3.5 and 2-fold, respectively. Whether the apparent avoidance of predation by protozoa is related to the firmness, or location of binding of *F. succinogenes* to plant cell walls, or to some other characteristic of the bacterium, will prove particularly interesting to determine, and could have applied applications.

Moving to the species level, an interesting question is how the substrate may influence the proportions of different strains of a fibrolytic species to become the dominant population. Koike et al. (2004) studied the enrichment of strains *F. succinogenes* on different forages. *F. succinogenes* is divided into 4 different phylogenetic groups based on comparative sequence analysis of 16S rDNA (Amann et al., 1992). Group 1 is differentiated from other groups by its pleomorphic coccoid morphology and poor ability to digest cellulose in agar medium and includes strains S85, A3C, BL2, and B1; groups 2 and 4 are phenotypically indistinguishable and include strains REH9-1 and GC5, and MC1, respectively, while group 3 produces a yellow pigment and requires vitamin B12 and consists of strains HM2 and MB4. Of the four groups, group 1 dominated on ruminally incubated hay stems and when wethers were fed fresh orchard grass, while group 3 predominated in the rumen of wethers and steers received a hay diet (Koike et al., 2004). This information in conjunction with previously published research led to the conclusion that group 1 of *F. succinogenes* which includes strain S85 may contribute more to rumen fibre digestion than the other groups.

DIGESTION OF PLANT CELL WALLS BY *F. SUCCINOGENES*

Accessibility to cellulose digestion sites in the cell wall matrix has been suggested as the rate-limiting factor in cellulose digestion (Dehority and Tirabasso, 1998). It has been proposed that access to cell wall polymers is limited by the small pore size between polymers, which is in the order of 2 to 4 nanometers, and is not sufficient to allow free diffusion into the wall matrix of simple globular enzymes with masses greater than 20 kilodaltons (kDa) (Gardner et al., 1999). Furthermore, the authors reported that the porosity was not modified during digestion with a crude cellulase enzyme. They concluded that exclusion of enzymes from the wall matrix by low porosity limits bacterial attack in the rumen to a process of surface erosion. Because the plant cell wall is a matrix of different polymers the process of attack by surface digestion infers that for rapid digestion a combination of cellulase and hemicellulase enzymes acting simultaneously is essential. Matulova et al. (2005) indirectly and elegantly tested this possibility by growth of *F. succinogenes* strain S85 on ^{13}C enriched wheat straw and assessing the products of hydrolysis by a combination of nuclear magnetic resonance spectroscopy, and sugar linkage and compositional analyses. An important observation was the absence of acetylated xylooligosaccharides among the hydrolysis products thereby documenting extensive enzymatic deacetylation, since wheat straw cell wall materials are highly acetylated (Bourquin and Fahey, Jr., 1994). Deacetylation is an important step in cell wall digestion because it can enhance the rate of degradation of cell walls (Wood and McCrae, 1986). The identified free sugars accumulating in the culture fluid were xylose, arabinose and arabinoglucuronoxylan oligosaccharides indicating extensive hemicellulase action. There appeared to be simultaneous degradation of hemicellulose and cellulose, and furthermore, amorphous and crystalline regions of cellulose were degraded at the same rate, which supports the concept of concerted action in the surface degradation of the cell walls. Glucose did not accumulate in the medium indicating rapid utilization of cellooligosaccharides with minimal cellodextrin export and recycling as previously suggested (Wells et al., 1995), while the accumulation of xylose and arabinose was expected since they are not used as a carbon source (Matte and Forsberg, 1992a). However, 4-OMe-α-glucuronic acid was not detected despite the presence of α-glucuronidase (Smith and Forsberg, 1991), which may be explained by low activity of the enzyme. In conclusion, this research clearly documents that digestion of the cell wall matrix involves

Table 1. Glycosyl hydrolases encoded by the S85 genome as compared to those present in the genomes of other cellulolytic bacteria[a]

Name	S85	*T. fusca* YX	*C. thermocellum*	*C. acetobutylicum*	*C. hutchinsonii*
Cellulase	33	8	32	16	9
Xylanase	24	4	4	13	11
Carbohydrate esterase [b]	14	-	-	2	-
Arabinofuranosidase	2	1	1	1	-
Mannanase	8	1	1	4	-
Lichenase	3	-	-	-	-
α-Galactosidase	1	-	-	-	-
β-Galactosidase	3	1	9	3	-
β-Glucosidase	2	3	9	5	8
Arabinase	3	-	-	1	-
Pectate lyase	7	1	2	6	-
Amylase	4	3	1	1	2
Total	104	22	59	52	30

[a] Accession number: *T. fusca* YX, CP000088; *C. thermocellum*, AABG00000000; *C. acetobutylicum*, AE001437; *C. hutchinsonii*, AABD00000000.
[b] Carbohydrate esterases include acetyl xylan esterases, coumaric acid esterases, and rhamnogalacturonan acetyl esterases.

the interaction of a complex array of enzymes.

ENZYMOLOGY OF PLANT CELL WALLS DEGRADATION BY *F. SUCCINOGENES*

The paper by Matulova et al. (2005) clearly showed that *F. succinogenes* S85 possesses the essential enzymes for the digestion of wheat straw cell walls. This fact was originally assumed because of the capacity of individual *Fibrobacter* cells to burrow into the plant cell wall matrix (Cheng et al., 1983). Enzymology and cloning studies prior to sequencing the genome of *F. succinogenes* had documented the presence of seven endoglucanases, a cellodextrinase, a chloride-stimulated cellobiosidase, a lichenase, and an α-glucuronidase (Forsberg et al., 2000). Added to this array of enzymes were at least 3 xylanases that produce xylooligosaccharides as products (Jun et al., 2003), an arabinose debranching xylanase (Matte and Forsberg, 1992b), at least three acetyl xylan esterases (McDermid et al., 1990a; Kam et al., 2005), an arabinofuranosidase and a ferulic acid esterase (McDermid et al., 1990b). Many of the genes coding for these enzymes were cloned and sequenced. The debranching nature of both the acetylxylan esterase and the one xylanase clearly points to the very important roles of this group of enzymes in plant cell wall biodegradation because they improve access of other enzymes to previously inaccessible substrates.

Béra-Maillet et al. (2004) conducted experiments to determine the presence of 10 glycosyl hydrolase genes, previously cloned from *F. succinogenes*, in other strains of *F. succinogenes* and in *F. intestinalis* strain NR9. Almost all of the glycosyl hydrolase genes were detected in strains of *F. succinogenes* closely related to strain S85, and a few were present in *F. intestinalis* NR9. Only the 118 kDa family fifty one glycosyl hydrolase, *celF*, with family 11 and family 30 carbohydrate binding modules was detected in all strains of *F. succinogenes* and in *F. intestinalis* NR9. They noted that cell and extracellular culture fluid samples from all strains studied exhibited low hydrolytic activity on crystalline cellulose, which confirmed earlier observations by others, both published and unpublished. A concluding remark from this study was that strain S85 of *F. succinogenes* is a good model for studying the fibrolytic properties of the species as the strain has conserved the enzymatic characteristics representative of the species.

To elucidate the role of adhesion of *Fibrobacter* cells in the of digestion of cellulose, adhesion defective mutants were isolated from both *F. succinogenes* S85 (Gong and Forsberg, 1989) and *F. intestinalis* DR7 (Miron and Forsberg, 1998). In both studies it was observed that the mutants either grew more slowly on cellulose as a carbon source, or not at all, but their growth on glucose was unaffected, which suggested that the adhesion process was a key factor in cellulose digestion. Antibodies prepared against the wild type strains and adsorbed with the mutant strains efficiently blocked adhesion of wild-type cells to cellulose, which showed that key adhesins were not synthesized by the non-adherent mutants (Miron and Forsberg, 1999; unpublished data). Furthermore, the adsorbed antibodies reacted with numerous cellulose binding proteins in the outer membrane of both *F. succinogenes* and *F. intestinalis*. When the cellulose-binding proteins were treated by periodate oxidation, which blocks sugar residues, the reaction with the antibodies were reduced. These data suggest that adhesion is important in cellulose digestion and that glycosylated cellulose binding proteins ranging from approximately 36 to 225 kDa may have roles in the binding process.

THE POSTGENOMIC ERA

In 2001, the North American Consortium for the

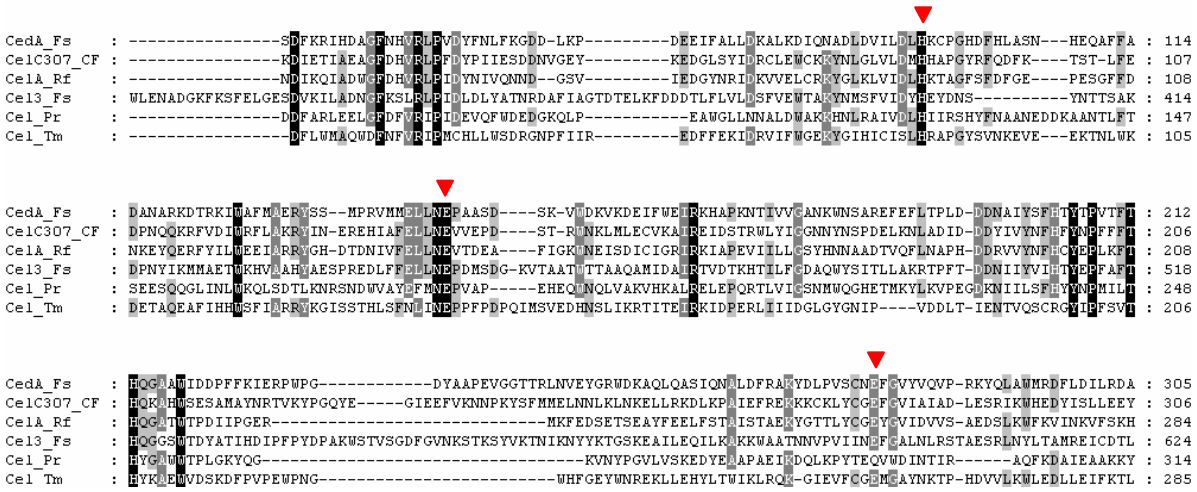

Figure 1. Alignments of cellulases belonging to GH family 5 showing similarity to CedA (FSU2070). CelC307_CF1 (P23340) from *Clostridium sp.* strain F1; CelA_Rf (P16169) from *Ruminococcus flavefaciens*; Cel3_Fs (P14250) from *F. succinogenes* S85; Cel_Pr (Q9ZN63) from *Prevotella ruminicola*; Cel_Tm (Q9X274) from *Thermotoga maritima*. The catalytic sites are indicated by red arrow heads.

Genomics of Fibrolytic Ruminal Bacterial in conjunction with the Institute for Genomics Research (http://www.tigr.org/) initiated projects to sequence the genomes of the three principal fibrolytic bacteria, *F. succinogenes* S85, *Ruminococcus albus* 8 and *R. flavefaciens*. Of these, the *F. succinogenes* genome is the only one that has been completed and closed (M. Morrison, Personal Communication). The genome of *F. succinogenes* S85 is 3.8 mbp and contains 3252 open reading frames putatively coding for proteins. Of these open reading frames, 104 code for putative enzymes involved in plant cell wall biodegradation including 83 glycosyl hydrolases, 7 pectate lyases and 14 carbohydrate esterases (Morrison et al., 2003). Among the glycosyl hydrolases 33 cellulases are present in families 5, 8, 9, 10, 45, 51 and 74, and the 24 xylanases are in families 10, 11, 30, 39 and 43. Only five of the cellulase proteins have carbohydrate binding modules (CBMs) while 14 xylanases have CBMs. A comparison of the numbers of putative plant cell wall hydrolases in *F. succinogenes* as compared with those in other fibrolytic bacteria is shown in Table 1. As is evident from the table, *F. succinogenes* has a far greater number of cellulases, xylanases, carbohydrate esterases and pectate lyases than the other bacteria, which in part may help to explain its efficient digestion of plant cell walls. However, no proteins were found with similarity to known scaffoldin, cohesion or dockerin proteins characteristic of cellulosomal cellulase complexes present in the ruminal bacteria *Ruminococcus albus* and *R. flavefaciens* and the cellulolytic ruminal fungi (Lynd et al., 2002; Doi and Kosugi, 2004). Furthermore, there were no genes coding for cellulases from families 6 and 48, which typically contain exoglucanases found in both cellulosomal and non-cellulosomal cellulase systems that degrade crystalline cellulose, for example, that of *Thermobifida fusca*. This information superimposed upon the fact that once growth of *F. succinogenes* is interfered with, cellulose biodegradation immediately ceases (Maglione et al., 1997), a characteristic very different from other cellulolytic organisms, leads to the conclusion that the mechanism of cellulose digestion by *F. succinogenes* is very different from that of other organisms in nature.

Despite the fact that availability of the genome has not led to an immediate solution for the mechanism of cellulose biodegradation by *F. succinogenes*, it has been valuable in exploring the genome of *F. intestinalis* DR7 by the application of the technique of suppressive subtractive hybridization (Qi et al., 2005). By the use of *F. succinogenes* strain S85 as the driver they identified 30 unique glycosyl hydrolases in strain DR7 that, on the basis of the subtractive method, were genetically different from those of *F. succinogenes*. However, once the cloned inserts were sequenced, blast searches revealed that they aligned with genes in the genome of *F. succinogenes*. Four belonged to glycosyl hydrolase (GH) family 5, two to family 8, four to family 9, one to family 16, one to family 18, one to family 45, one to family 51 (Cel F; Malburg et al., 1997) and one to family 74. There were two family 43 and one family 10 xylanases, one family 26 mannanase, three family 57 amylases, 1 family 77 α-glucanotransferases and one carbohydrate phosphorylases. There were two pectate lyase families represented, two family 1 and one family 11. There was one representative of the carbohydrate esterases, a family 12 acetyl esterase. Of the 23 glycosyl hydrolase genes tested for expression in *F. intestinalis* at least 16 were expressed and 8 of these were expressed at a higher level along with a pectate lyase, but none of the three xylanases

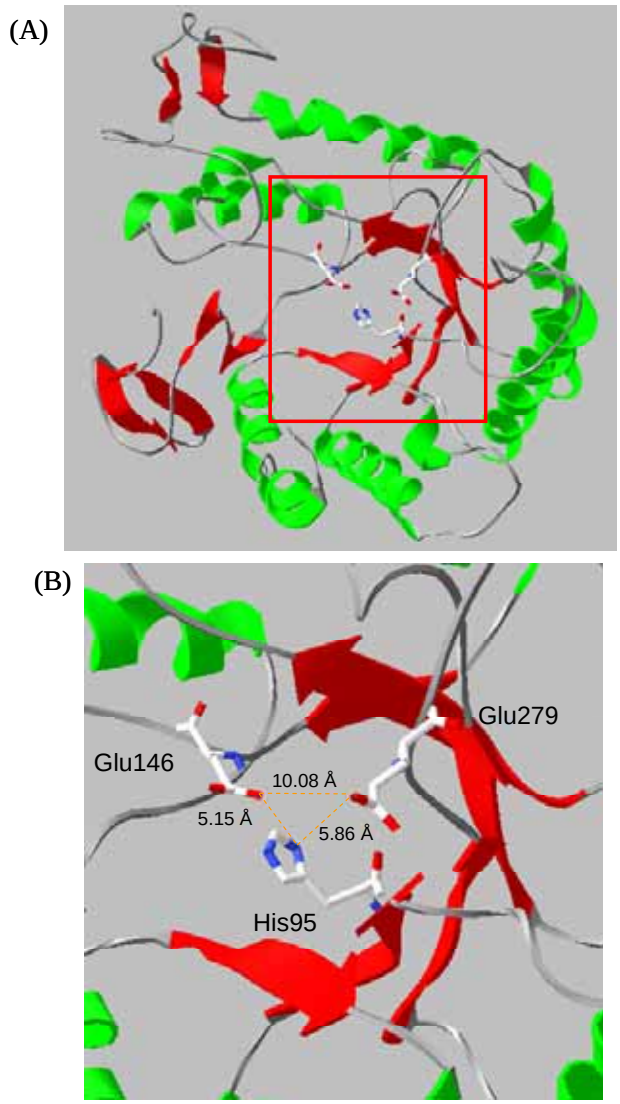

Figure 2. (A) Predicted tertiary structure of cellodextrinase CedA (FSU2070) and (B) enlargement of active-site cleft (red box in A). α-helices (green) and β-strands (red) are shown. The catalytic residues are indicated as sticks (white, carbon chain; blue, nitrogen; red, oxygen) and the distances (Å) between molecules are shown as dotted line.

or the acetyl esterase were expressed. All of the glycanases showed the highest similarity to their counterparts in *F. succinogenes.* However, when a BLASTX search was conducted against the GenBank non-redundant sequence database, not including the *F. succinogenes* genome, half of the genes showed highest similarity to those of *Cytophaga*, indicating a close relationship between the cellulase systems of these species. In contrast to the similarity of the glycanases between the *Fibrobacter* species, there were major differences between the two species in the numbers of transposases and restriction modification enzymes, both of which were more numerous in *F. intestinalis.*

STRUCTURAL ANALYSIS OF SEVERAL *F. SUCCINOGENES* CELLULASES

Molecular level structural analysis of the *F. succinogenes* S85 1,3-1,4-D-glucanase has been reported for the enzyme in both the absence and presence of β-1,3-1,4-cellooligosaccharides (Tsai et al., 2005). This has provided detailed knowledge of the subsites for binding of glycosyl residues during catalysis and the organization of the catalytic site during cleavage of the glycosidic bond. Although providing a less complete description, the availability of amino acid sequences of the glycosyl hydrolases offers the opportunity to model enzymes by reference to crystal structures of closely related cellulases. This is accomplished by submission of the query sequence to SWISS-MODEL server (http://swissmodel.expasy.org) (Schwede et al., 2003). The predicted protein models are viewed and analyzed using Swiss PDB-viewer (version 3.7) and RasTop (version 2.1). Two cellulases have been modelled, the glycosyl hydrolase family 5 cellodextrinase, CedA (Iyo and Forsberg, 1994) and the GH family 9 endoglucanase EGB (Broussolle et al., 1994).

CedA, now Cel5C (FSU2070), has a mass of 41.9 kDa. It exhibits low activity on amorphous cellulose, limited activity on microcrystalline cellulose, but cleaves cellooligosaccharides releasing cellobiose as the primary product (Huang and Forsberg, 1987). CedA exhibits homology with the CelC endoglucanase from *Clostridium thermocellum* that was previously crystallized (Figure 1, Dominguez et al., 1996) and has enabled modelling of the three dimensional structure. The model shown for CedA in Figure 2 illustrates the general organization of α-helices and β-sheets, forming an $(\alpha/\beta)_8$-barrel, a common protein fold of cellulases belonging to GH 5 family. The putative catalytic residues Glu 146, Glu 279 and His 95 are illustrated.

The second enzyme, endoglucanase EGB, now Cel9G (FSU0451), has a mass of 62.5 kDa and exhibits high catalytic activity on barley β-glucan, lichenan and carboxymethyl cellulose producing the hydrolysis products cellobiose and cellotriose from carboxymethyl cellulose (Broussolle et al., 1994; Forano et al., 1994). It has a requirement for divalent cations satisfied by magnesium and calcium. This enzyme exhibits high similarity to the family 9 cellobiohydrolase, CbhA from *C. thermocellum* (Figure 3, Schubot et al., 2004). CbhA is a multimodular enzyme comprising an N-terminal CBM4, an Ig-like module, a catalytic module, several unknown modules, a CBM3, a dockerin module, and the catalytic region contains two atoms of calcium that are essential for activity. The structure on which the EGB enzyme was modelled was that of the CbhA Ig-like module and the catalytic module. It is fascinating to note from the alignment that EGB is missing

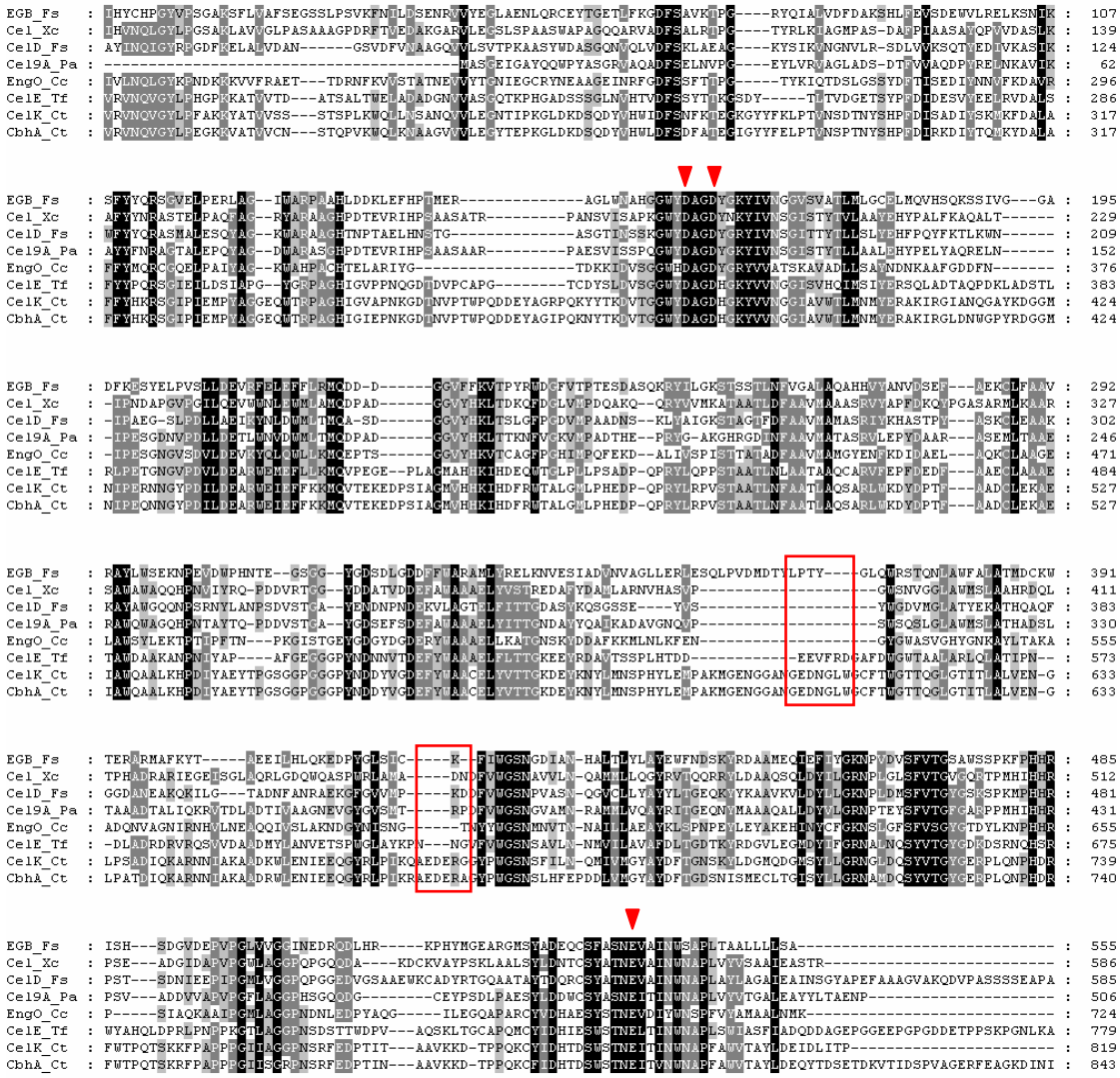

Figure 3. Alignments of cellulases belonging to GH family 9 showing similarity to Cel9G (formerly EGB; FSU0451). Cel_Xc (Q4VVY1) from *Xanthomonas campestris*; CelD_Fs (P77864) from *F. succinogenes* S85; Cel9A_Pa (Q9APG3) from *Pseudomonas aeruginosa*; EngO_Cc (Q6DTY2) from *Clostridium cellulovorans*; CelE_Tf (Q08166) from *Thermobifida fusca*; CelK_Ct (O68438) from *Clostridium thermocellum*; CbhA_Ct (Q6RSN8) from *C. thermocellum*. The catalytic sites are indicated by red arrow heads. The loop regions responsible for the exo activity of CbhA of *C. thermocellum* are highlighted by a red rectangular outline.

the loop regions responsible for the exo-activity of CbhA. The model for EGB in Figure 4 illustrates a topology of the $(\alpha/\alpha)_6$-barrel mainly found in GH9 cellulases, with the location of the putative catalytic residues, Asps 157 and 160 and Glu 537. Precise details of the structure and locations of the cation binding sites will require the crystal structure for the enzyme.

DECODING THE CELLULASE SYSTEM OF *FIBROBACTER* SPECIES

The easiest path to solving the mechanism of crystalline cellulose biodegradation by *F. succinogenes* would probably be through transposon mutagenesis (Reznikoff et al., 2004) of the bacterium and characterization of the mutated genes. Unfortunately, this technique is not available for genetic manipulation of *F. succinogenes* at this time. Therefore the focus will be on more detailed characterization of the cellulase system; which includes the identification of proteins overproduced when cells are grown with cellulose as a carbon source as compared when cells are grown on glucose and cellobiose, identification of cellulose binding proteins, and those missing from non-adherent mutants previously isolated (Gong and Forsberg, 1989). An interesting feature with the non-adherent mutants is that several exhibit differential binding to amorphous and crystalline cellulose. From this observation one presumably can conclude that there are different cellulose binding

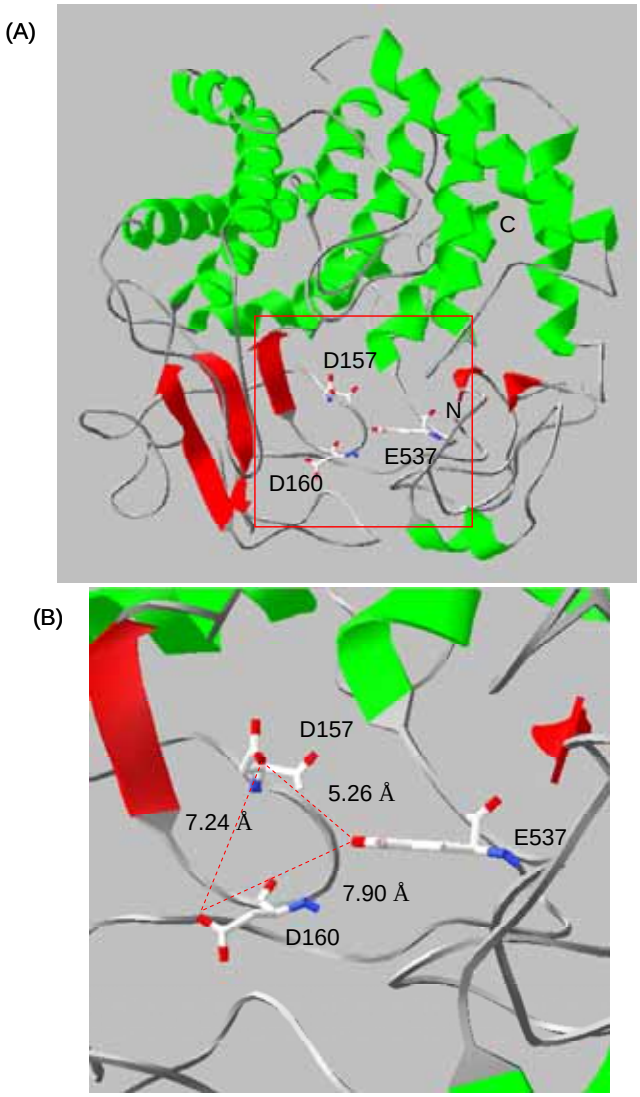

Figure 4. (A) Predicted tertiary structure of Cel9G (formerly EGB; FSU2070) and (B) enlargement of active-site cleft (red box in A). α-helices (green) and β-strands (red) are shown. The catalytic residues are indicated as sticks (white, carbon chain; blue, nitrogen; red, oxygen) and the distances (Å) between molecules are shown as dotted line.

proteins for adhesion to amorphous and to crystalline cellulose. We have discovered that these mutants are pleiotropic, exhibiting multiple defects, which perhaps suggests that they are mutated at different levels of a hierarchical control system. Consequently, there remain many novel aspects of the cellulase system of this group of bacteria that require resolution.

CONCLUSION

The information presented in this short review documents that *Fibrobacter succinogenes* has an important role in the ruminal environment. The mechanism by which it degrades the hemicellulose fraction of plant cell walls by the combination of xylanases and debranching enzymes is now obvious although more detailed information is needed to better understand the functionality of the enzymes involved. Similarly pectin metabolism will yield to enzymological analysis. However, despite the identification of numerous cellulase enzymes, there is still the challenge of solving the mechanism of cellulose digestion. Part of this puzzle may be contained in the open reading frames that are categorized as coding for hypothetical, conserved hypothetical and lipoproteins that account for over half of the open reading frames of the genome, and await assessment to determine whether they are expressed, and of those expressed to characterize their functionality.

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