Genetic modification of fibre digestion

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Plant biomass is an extensive renewable resource which already plays a central role in herbivore nutrition, but has the further potential to serve as a raw material for industrial processes leading to the production of bulk organics, fine chemicals and biofuel. Microbial enzymes which reside in the gut of both ruminant and non-ruminant herbivores and degrade plant structural polysaccharides, represent the means by which most of the energy and carbon contained in plant biomass may be released. Such enzymes have been the subject of much biochemical research, but since the advent of recombinant DNA (rDNA) technology during the late 1970s, various research groups have, in addition, focused on molecular aspects of these enzymes, and in particular on the introduction of cellulose-degrading capacity into non-cellulolytic organisms by techniques known broadly as genetic engineering.

The major components of plant cell walls are hemicellulose, cellulose and lignin (Demeyer, 1981). Cellulose, the linear $\beta1 \rightarrow 4$ homopolymer of glucose forms tightly packed parallel molecules linked together by hydrogen bonds. The resulting fibrils are embedded in a ligno–hemicellulose complex. The most abundant hemicelluloses are branched chain $\beta1 \rightarrow 4$ xylans and glucomannan which are covalently bound to lignin, an heterogeneous phenolic polymer (Whistler & Richards, 1970). Pectin, which is an $\alpha1 \rightarrow 4$ polymer of galacturonic acid, is a minor component of grass cell walls.

To realize the full potential of plant biomass in animal nutrition requires an efficient system for the complete enzymic hydrolysis of plant structural polysaccharides into their constituent monomers. The weighty problem of improving the effectiveness of naturally-occurring cellulolytic micro-organisms or introducing plant-cell-wall hydrolysing capacity into non-cellulolytic organisms, has two main aspects. First, the analysis of enzymes which hydrolyse plant cell walls and the genes which encode them. This will facilitate the future construction of novel genes, encoding multi-functional hybrid enzymes with enhanced activity against cellulose and hemicellulose. Second, the development of host–vector systems which will enable the genes developed in the first phase to be efficiently expressed in organisms that would then be used to elicit improved fibre utilization by both ruminants and non-ruminants.

ANALYSIS OF PLANT-FIBRE-DEGRADING ENZYMES

Fungal cellulases. Cellulose is hydrolysed by the synergistic action of a number of different enzymes. In fungi these enzymes are defined as exo-1,4-β-glucanases (cello-biohydrolases), endo-1,4-β-glucanases and β-glucosidases (Wood, 1989). Cellulases synthesized by *Trichoderma reesei* have been extensively studied. At the molecular level,

the enzymes consist of two distinct functional domains; a cellulose-binding domain (CBD) and a catalytic domain (CD). These domains are linked by short sequences rich in hydroxy O-glycosylated amino acids (Teeri *et al.* 1987; Tomme *et al.* 1988). Typical of fungal cellulolytic systems, *T. reesei* expresses multiple (Van Tilbeurgh *et al.* 1986; Tomme *et al.* 1988) endoglucanases (EG) and cellobiohydrolases (CBH). The role of the CBD in these enzymes has received considerable attention. Tomme *et al.* (1988) showed that removal of the CBD from CBHI of *T. reesei* greatly reduced the enzyme's capacity to bind and hydrolyse crystalline cellulose, although it still digested amorphous and soluble cellulose efficiently. Knowles *et al.* (1988) argued that these findings showed that the CBD of CBH disrupts the H bonding between cellulose fibrils and consequently allows the CD to hydrolyse the β-1,4-bonds. Many fungal cellulases retain catalytic activity after proteolysis. Indeed, Eriksson & Pettersson (1982) report that *Sporotrichum pulverulentum* proteases activate the organism's EG. Knowles *et al.* (1988) suggested that cleavage of the CBD of EG may play an important role in altering substrate specificity dependent on the nature of the cellulosic molecules present. Thus, the fungus can increase the range of substrate specificities of existing enzymes without increasing the number of cellulase genes.

It is generally agreed that synergism between endoglucanases and exoglucanases during cellulose hydrolysis is apparent when crystalline cellulose is the substrate, but is less significant when the enzymes are cleaving amorphous or soluble forms of the polysaccharide (Wood, 1989). This synergism is not restricted to enzymes from the same organism, but occurs between EG and CBH from different cellulolytic fungi (Wood *et al.* 1988).

Recently, the molecular biology of fungal cellulases has received considerable attention. Much of the work has focused on the *T. reesei* system. To date, four cellulase genes from this organism have been characterized: two CBH, CBHI (Shoemaker *et al.* 1983) and CBHII (Chen *et al.* 1987; Teeri *et al.* 1987), and two EG, EGI (Penttila *et al.* 1986) and EGIII (Saloheimo *et al.* 1988). The genes coding for CBHI and EGI are derived from a common gene and show respectively 50% and 45% nucleotide and amino acid homology. In contrast CBHII and EGIII show very little similarity either with each other or with other *T. reesei* cellulases. The thirty-five amino acid sequence at the N-(CBHII, EGIII), and C-(CBHI, EGI) termini of these enzymes are 70% conserved and constitute the CBD. The four genes analysed contain short introns typical of filamentous fungal genes (Knowles *et al.* 1988). In two genes (CBHII, EGI) the introns are located near the hydroxy amino acid linker sequence, supporting the idea that fungal cellulases have been assembled by exon-shuffling.

Recent studies have shown that *T. reesei* EG and CBH (Penttila *et al.* 1987, 1989) can be expressed in heterologous lower eukaryotic hosts. Thus, the feasibility of transferring the cellulolytic system of *T. reesei* to other non-cellulolytic organisms is demonstrated.

Cellulolysis is effected not only by aerobic fungi, but also by the rumen anaerobic fungi *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis* (Bauchop, 1981; Orpin, 1984). Each of these organisms produces an extracellular cellulase system that degrades crystalline cellulose. Some strains of *N. frontalis* express cellulases which are even more active than the comparable enzymes from *T. reesei* (Wood, 1989). Although the organism contains EG and β-glucosidases, evidence for the presence of a CBH is not definitive. However, when the crude cellulase is heated to 45° for a prolonged period, the enzyme complex no longer degrades crystalline cellulose, even though EG activity is still apparent. When *Trichoderma koningii* CBH is added to the heat-treated

cellulase the capacity to degrade cotton fibres is restored (Wood *et al.* 1988). Thus, on balance the evidence suggests that *N. frontalis* does synthesize all three of the enzymes typically found in fungal cellulases and probably secretes them as a high-molecular-weight multi-enzyme complex (Lowe *et al.* 1985).

Bacterial cellulases. The enzymology of bacterial cellulases is less well defined than for corresponding fungal systems. All cellulolytic prokaryotes express multiple cellulases which individually exhibit little activity against crystalline substrates (Béguin, 1990). Exoglucanases have been described in very few cellulolytic bacteria, and only one has been shown to act synergistically with EG to degrade insoluble cellulose (Creuzet *et al.* 1983). The structural organization of bacterial cellulases is variable. In aerobic soil organisms such as *Pseudomonas fluorescens* ssp. *cellulosa* and *Cellulomonas fimi*, cellulases do not associate into large multi-enzyme complexes. In contrast, many anaerobic bacteria, such as *Clostridium thermocellum* and those which reside in the rumen, synthesize a cellulase system consisting of a large multi-enzyme complex whose structural organization appears to play a pivotal role in the cellulolysis of crystalline substrate (Lamed & Bayer, 1988). For example, the cellulase complex of *C. thermocellum*, termed the cellulosome, contains fourteen to eighteen different polypeptides (Lamed *et al.* 1983). The high affinity of the cellulosome for cellulose is thought to be mediated by an apparently non-catalytic binding factor of M_r 210 000–250 000 (Lamed & Bayer, 1988; Wu *et al.* 1988). The enzyme system, although active against crystalline cellulose, does not appear to contain a CBH. The molecular architecture of cellulases from aerobic bacteria shows a number of constant features. The enzymes contain two distinct regions, a CD and CBD (Ghangas & Wilson, 1988; Gilkes *et al.* 1988; Hall & Gilbert, 1988; Gilbert *et al.* 1990), analogous to aerobic fungal cellulases. These functional domains are linked by sequences rich in hydroxy amino acids. Deletion of the CBD does not result in a dramatic decrease in the enzyme's activity against amorphous or crystalline cellulose (Gilkes *et al.* 1988). Thus, there is some debate as to whether bacterial CBDs play a crucial role in the hydrolysis of crystalline substrates.

In recent years, the molecular biology of bacterial cellulases has also received considerable attention. In excess of forty different prokaryotic cellulase genes have been sequenced and the primary structures of the encoded enzymes deduced (Béguin, 1990). The resultant findings have revealed that multiple cellulases observed in prokaryotes are not the result of post-translational processing of single gene products, but are encoded by multiple genes. The *C. thermocellum* proteins contain a highly conserved twenty-three amino acid repeated sequence which is generally at the C-terminus (Béguin *et al.* 1989). This reiterated region is not essential for catalytic activity or cellulose binding (Grépinet *et al.* 1988; Hall *et al.* 1988; Chauvaux *et al.* 1989) and, thus, probably plays a key role in the structural organization of the cellulosome. Interestingly, apart from the twenty-three residue sequence, there is little homology between the *C. thermocellum* cellulases indicating that they did not evolve from a single gene. Indeed, apart from *Bacillus* and *Ruminococcus albus* enzymes, (Fukumori *et al.* 1989; Poole *et al.* 1990) the catalytic domains of multiple cellulases produced by a single organism do not show a high degree of sequence conservation. This argues in favour of isoenzymic cellulases arising from gene tranfer between different bacteria. In contrast, the CBDs of cellulases appear to be highly conserved, suggesting that they might be encoded by a mobile genetic element. Alternatively, because of the relatively short sequences which constitute a CBD (100 amino acids), the scope for divergence of structure is limited.

It is generally recognized that fungal cellulase systems contain fewer enzymes, although they are more active against insoluble substrates when compared with the bacterial enzymes. This probably reflects the presence of CBH in the lower eukaryotes, while in prokaryotes exoglucanases are less common and exhibit a low specific activity against crystalline substrates. Why highly active CBH should be confined to fungi remains an important unresolved question.

Xylanases. Xylan, the most abundant hemicellulose, constitutes up to 35% of the total dry weight of higher plants. Because of the heterogeneity of xylans, several types of endo- and exo-acting xylanases are required to effect hydrolysis. These include 1,4-β-D-xylanases, β-xylosidases, α-L-arabinofuranosidases, α-glucuronidases, acetyl-xylan esterases and ferulic acid esterases (Wong *et al.* 1988). Multiple xylanases have been reported in numerous micro-organisms. Five different xylanases have been purified from *Aspergillus niger* (Takenishi & Tsujisaka, 1975) and at least three from *Clostridium stercorarium* (Bérenger *et al.* 1985), *Streptomyces exfoliatus* (Sreenath & Joseph, 1982), *Trichoderma* sp. (Dekker, 1985) and *Penicillium janthinellum* (Takenishi & Tsujisaka, 1973). The underlying reasons for the multiplicity of xylanases remain largely unresolved. For example, xylanase heterogeneity may arise from post-translational modifications such as differential glycosylation and proteolysis, or both. Support for this view is provided by the fact that many xylanases are glycosylated, and can be proteolytically cleaved to produce smaller catalytically active products (Gorbacheva & Rodionova, 1977). However, recent studies by Gilbert *et al.* (1988) and Hazlewood *et al.* (1988) have shown that isoenzymic forms of these enzymes are encoded by multiple genes at least in *C. thermocellum* and *P. fluorescens* ssp. *cellulosa.* It is likely that the large number of xylanases in *A. niger* results from a combination of both multiple genes and post-translational modification. The rationale for expressing multiple xylanases probably resides in the fact that xylosidic linkages in ligno-cellulose are not all equivalent and equally accessible to xylanolytic enzymes (Wong *et al.* 1988). The presence of true exoxylanases has been demonstrated by Reilly (1981), but these enzymes are not thought to be prevalent among xylanolytic micro-organisms.

Co-operativity between xylanases is a common phenomenon. For example, the maximal rate and degree of hydrolysis of two different xylans, required the action of three distinct xylanases from *Trichoderma harzianum* (Wong *et al.* 1988). Similar results were observed for xylanases from *Neurospora crassa* (Deshpande *et al.* 1986) and *S. exfoliatus* (Sreenath & Joseph, 1982). Furthermore, the concerted action of debranching enzymes and endoxylanases increases the rate and the extent of xylan hydrolysis (Lee & Forsberg, 1987).

Recently, the molecular biology of xylanases has been much studied. Xylanases from *C. thermocellum* (Grépinet *et al.* 1988), *Bacillus* sp. (Paice *et al.* 1986; Hamamoto *et al.* 1987), *Caldocellum saccharolyticum* (Lüthi *et al.* 1990) and *P. fluorescens* ssp. *cellulosa* (Hall *et al.* 1989) have been cloned and sequenced. The expression of these genes in the non-xylanolytic bacterium *Escherichia coli*, has enabled the determination of the substrate specificities of these enzymes. The resultant findings confirmed the previously held view that xylanases can either show narrow substrate specificities (Gilbert *et al.* 1988) or are capable of hydrolysing cellulosic substrates in addition to xylan. Recent studies by Ferreira *et al.* (1990) have shown that xylanase A from *P. fluorescens* ssp. *cellulosa* contains two distinct functional domains, a CBD, which shows little avidity for xylan, and a CD. Furthermore, Kellett *et al.* (1990) have demonstrated that a second

xylanase and an arabinofuranosidase, synthesized by the pseudomonad, exhibit the same molecular architecture as xylanase A. The chromosomal location of xylanase and cellulase genes has also been investigated. In *P. fluorescens* ssp. *cellulosa xynA* and *celA* (Hall & Gilbert, 1988; Hall *et al.* 1989) are adjacent on the chromosome as are the *xynB* and *xynC* genes. The CBD and serine-rich linker sequences of the pseudomonad cellulases and xylanases show a high degree of sequence conservation (Hall & Gilbert, 1988; Hall *et al.* 1989; Gilbert *et al.* 1990) which is particularly pronounced in XYLB and XYLC, where the first 300 residues of the mature proteins show complete identity (Kellett *et al.* 1990). Other examples of close linkage of cellulases and xylanases include two homologous EG genes from *Bacillus* sp. strain N-4 (Fukumori *et al.* 1986), *celX* and *celE* of *C. thermocellum* (Hall *et al.* 1988), *xynA* (xylanase) and *xynC* (xylan esterase) of *C. saccharolyticum* (Lüthi *et al.* 1990). However, apart from these examples, cellulase and xylanase genes are not clustered on bacterial or fungal genomes.

IMPROVING THE ACTIVITY OF CELLULASES AND XYLANASES

The major limitation in cellulolysis is the very low specific activity of cellulases and xylanases when acting on native substrates such as plant cell walls. The understanding of the molecular architecture, and the mechanisms by which functional domains of the proteins contribute to enzyme action, is required before the primary structure of these enzymes can be modified to elicit an improvement in catalytic activity. Particularly important questions which must be resolved are: (1) What, if any, is the contribution of fungal and bacterial CBD to the hydrolysis of cellulose? (2) What is the role of the CBD in xylanase action? (3) What is the minimum number of enzymes required for complete plant-cell-wall hydrolysis? (4) Can the activity of cellulases and xylanases be improved by altering residues at the active site or within substrate-binding domains? (5) Can hybrid genes be designed which encode enzymes exhibiting increased activity against cellulose and hemicellulose? To investigate the last question Warren *et al.* (1987) constructed a gene encoding both EG and exoglucanase activities. The rationale of this approach is based on the synergism observed between endo- and exo-acting cellulases. If these complementary activities are expressed by a single polypeptide chain an increase in the synergism between the enzymes could be anticipated. However, hybrid enzyme did not exhibit improved cellulase activity. In contrast, a gene encoding only the CD of EG E from *C. thermocellum* exhibited a 400% increase in specific activity compared with the native enzyme; although, interestingly, the truncated enzyme had a decreased affinity for cellulosic substrates (Durrant *et al.* 1991). There are numerous other possibilities for constructing novel plant-cell-wall-degrading enzymes, and only future research will determine whether this approach will prove fruitful.

GENETIC MANIPULATION OF ORGANISMS

It can be visualized that the introduction of fibre-degrading enzymes into suitable gut micro-organisms will result in an improvement in the efficiency of plant-cell-wall utilization by ruminant and non-ruminant herbivores. The present review will discuss the possibility of improving plant-fibre degradation by genetic manipulation of rumen and silage bacteria, and also by the novel strategy of introducing cellulase genes into the germline of simple-stomached livestock.

Rumen. The two major limitations in rumen fibre digestion are the rate and extent of plant-cell-wall hydrolysis. The total amount of cellulose and hemicellulose degraded in the rumen depends on the degree of lignification which in turn is related to the age of the crop (Demeyer, 1981). Is it reasonable to expect an increase in plant-fibre digestion in the rumen in response to increasing the activity or expression of cellulases and xylanases in rumen micro-organisms? Pretreatment of forage-based silages with cellulases resulted in an increase in solubility, and although the enzyme had no effect on the total degradability of plant cell walls, there was an increase in the rate of plant-fibre hydrolysis in the rumen (van der Meer & van Es, 1987; Van Vuuren & Spoelstra, 1987; Gomez Cabrera & van der Meer, 1988). Thus, the introduction of genes encoding very active cellulases into key rumen bacteria may improve the rate, if not the extent, of cellulolysis. Such a genetic modification could reduce N loss which occurs when feeding ruminants silage-based diets, due to the imbalance of ammonia release and energy generated by metabolism of plant structural polysaccharides (Johnson, 1976). However, this presupposes that the rate of plant-cell-wall hydrolysis is limited by the activity of rumen cellulases and xylanases, rather than by the structure of plant fibre. Such an assumption is not proven. Russell & Wilson (1988) have suggested that the introduction of cellulase and xylanase genes into rumen micro-organisms which predominate when ruminants are fed on cereal-based diets, could improve fibre digestion. The rationale for this proposal is that in cereal diets the predominant polysaccharide, starch, is rapidly fermented resulting in a decrease in rumen pH to below 6. Under these more acidic conditions the rumen cellulolytic bacteria, *R. albus*, *Ruminococcus flavefaciens*, *Fibrobacter succinogenes* and *Butyrivibrio fibrisolvens* will not persist (Stewart, 1977; Russell & Dombrowski, 1980) and, thus, fibre digestion will diminish (Ørskov & Fraser, 1975). In view of the fact that cereal diets contain a considerable fibre component, and may be supplemented with 100–150 g forage/kg, the decrease in rumen pH will diminish the maximum utilization of structural carbohydrates contained in cereals. Russell & Wilson (1988) argue that the introduction of a complete cellulolytic system into a rumen micro-organism, which grew in acid conditions, could improve fibre digestion when animals were fed on grain. A potential candidate for such a genetic alteration is *Bacteroides ruminicola*. The bacterium grows in acidic conditions, and will attach to food particles and express EG activity. The introduction of CBH and hemicellulase activities could potentially enable the organism to efficiently degrade plant cell walls.

How feasible is the genetic manipulation of rumen bacteria? Evidently, the first stage in such a process is the insertion of DNA into the selected organism. Teather (1985) has described the transfer of the broad host-range conjugative plasmid RP4 from *E. coli* to *B. fibrisolvens*. Furthermore, transformation and transfection of cells with phage DNA have been reported for *Selenomonas ruminantium* (Orpin *et al.* 1986*a*; Lockington *et al.* 1988). To achieve stable introduction of heterologous DNA into rumen bacteria requires the development of a stable vector system. It should be noted that cryptic plasmids have been described in *R. albus*, *R. flavefaciens* (Asmundson & Kelly, 1987), *B. fibrisolvens* (Teather, 1982; Mann *et al.* 1986) and *S. ruminantium* (Orpin *et al.* 1986*b*; Martin & Dean, 1989). Plasmids from the latter organism have been cloned into pUC-based vectors, and their mode of replication is being examined to assess their utility as cloning vectors (J. Murray and G. P. Hazlewood, unpublished results). The range of bacteria which can now be successfully transformed has been greatly extended by the use of

electroporation (Luchansky *et al.* 1988), and it is likely that in the near future transformation systems for rumen bacteria, based on electroporation, will be developed.

The second stage in the genetic manipulation of rumen bacteria is to obtain efficient expression of heterologous genes. Several rumen bacterial genes encoding cellulases and other catabolic enzymes have now been cloned and in some cases sequenced (for example, see Gilbert & Hall, 1987; McGavin *et al.* 1989; Ohmiya *et al.* 1989; Hazlewood *et al.* 1990; Poole *et al.* 1990). S1-mapping will delineate the regulatory sequences which could then be utilized in the efficient transcription and translation of foreign genes in rumen bacteria.

Having constructed the required genetically engineered rumen bacterium, the organism must be re-introduced into the rumen. This is likely to be the most problematic step in manipulating rumen function. Recent studies (Flint *et al.* 1989) have shown that *S. ruminantium*, but not *Bacteroides multiacidus*, could be re-established in the original ecosystem. In addition, an organism harbouring extra DNA (plasmid) may not be as competitive as the unmodified bacterium. This view is supported by Russell & Wilson (1988) who showed a dramatic decrease in growth rate of *B. ruminicola* containing a heterologous erythromycin resistance (Em)^r plasmid. The introduced genetic trait was rapidly lost if selection pressure was not applied. Thus, the introduction of exogenous DNA must confer a clear selective advantage to the organism if the exogenous nucleic acid, and the genetically altered bacterium, are to persist in the rumen. It could be argued that functional cellulase genes will fufil this criterion. It should, of course, be pointed out that the use of antibiotic resistance genes as selectable markers, although widespread in laboratory experiments, would be totally inappropriate in the genetic manipulation of rumen micro-organisms.

Silage bacteria. The introduction of cellulolytic activity into *Lactobacillus plantarum* could also improve fibre digestion in ruminants. This organism, which is commonly inoculated onto freshly cut herbage during ensiling, ferments water-soluble carbohydrates (WSC) to predominantly lactic acid. The resultant low pH (3·6–4·2) of the silage prevents the proliferation of deleterious anaerobes such as *Clostridium* sp., so that the preserved crop may be used primarily for winter feeding to ruminants. Although silage inoculants, consisting of *L. plantarum*, are widely used their effectiveness when WSC levels are low (wet summer) is limited, as insufficient substrate is available to generate the required lactic acid. The addition of plant-fibre-degrading enzymes could overcome the substrate limitation by releasing di- and monosaccharides from plant cell walls, which could then be fermented by *L. plantarum* to lactic acid.

Recent studies by several groups have demonstrated that the addition of polysaccharidases to ensiled herbage improves the rate of acid production, thus ensuring better-quality silage (Huhtanen *et al.* 1985; Jorgensen & Cowan, 1989). Furthermore, the use of these enzymes also improves the rate of plant-fibre degradation and, thus, plant-cell-wall utilization in ruminants, probably by catalysing initial depolymerization of plant cell walls during ensiling. From the foregoing discussion it is apparent that the addition of cellulases and xylanases to ensiled herbage improves the efficiency of ruminant production. However, the cost of these enzymes prevents their extensive use. The introduction of cellulolytic–hemicellulolytic capacities into *L. plantarum* could provide a more cost effective method for applying these enzymes during ensiling.

The genetic manipulation of silage bacteria, such as *L. plantarum*, poses many of the problems encountered in the insertion of heterologous genes into rumen micro-

organisms. Again, the first requirement is for a transformation system for the lactic acid bacterium. Recent studies (Luchansky *et al.* 1988; Bates *et al.* 1989; Scheirlinck *et al.* 1989) have described the insertion of various plasmids into *L. plantarum* by electroporation. Scheirlinck *et al.* (1989) recognized the importance of obtaining stable maintenance of foreign DNA in the absence of selection pressure. Subsequently, this group, using a vector which does not replicate in *L. plantarum* but contains a cloned segment of the organism's genomic DNA, integrated heterologous DNA into the genome of *L. plantarum* through homologous recombination. Such an approach requires a high transformation frequency, which is not generally available for *L. plantarum* strains (Bates, 1990). Rixon *et al.* (1990) have utilized an unstable *L. plantarum* plasmid to direct foreign DNA into the organism's chromosome, by growing lactobacilli containing the vector under non-selective conditions. Such an approach has wide applicability in *L. plantarum* strains, as there is no requirement for a high transformation frequency. Both Scheirlinck *et al.* (1989) and Rixon *et al.* (1990) have demonstrated that *L. plantarum* strains containing chromosomally integrated heterologous DNA maintain these foreign sequences in the absence of selection pressure, although amplification of the foreign genes does occur when selection pressure is applied. Furthermore, these integrated sequences do not affect the growth rate of the recombinant strains in monoculture or in silage ecosystems. Both groups have demonstrated that *C. thermocellum* endoglucanases (Bates *et al.* 1989; Scheirlinck *et al.* 1989) function in *L. plantarum* and the encoded enzymes are efficiently secreted by the lactic acid bacterium. Obviously, to achieve efficient plant-cell-wall hydrolysis requires the introduction of the full complement of enzymes which cleave cellulose and hemicellulose. In addition, an improvement in gene expression over that observed for the *C. thermocellum* genes is required. A potential approach for achieving the latter objective is to insert foreign genes into endogenous high-copy-number plasmids (300/cell) which are stably maintained in the absence of selection pressure. Several groups have described numerous cryptic plasmids in *L. plantarum* strains (West & Warner, 1985) one of which has recently been sequenced (Bates & Gilbert, 1989). The resultant findings showed that the plasmids belonged to the group of Gram positive extrachromosomal elements which replicate by the rolling circle mechanism. It is widely believed that these plasmids may be unsuitable for cloning vectors, as the insertion of heterologous DNA causes the plasmids to undergo severe DNA rearrangements.

When genetically manipulating organisms which are designed to inhabit open ecosystems, the question of gene transfer to indigenous micro-organisms must be considered. Because the frequency of genetic exchange must be minimized, it is desirable to incorporate heterologous DNA into the organism's genome in order to reduce the risk of horizontal transfer.

What are the realistic expectations of genetically manipulating rumen and silage bacteria? We believe the rumen organisms present a particularly challenging problem. Can we reasonably expect an organism which harbours exogenous DNA to compete in an energy-limited symbiotic environment? Will any change in fibre digestion achieved by modifying one organism, be buffered by a shift in the microbial population of the rumen to accommodate the introduced bacterium? In comparison, the silage ecosystem appears to be a relatively simple system. Silage inoculants are added at 10^6 colony forming units/g forage. The organism will increase in mass 1000-fold which is equivalent to ten generations over the first 48 h of ensiling. In addition, once the pH of the ensiled herbage

starts to drop, due to proliferation of the *Lactobacillus*, the competing bacteria in the ecosystem will not multiply. Thus, the goal of using a genetically manipulated *L. plantarum* in the ensiling process appears to be more feasible than establishing an anaerobic organism, harbouring heterologous DNA, in the rumen.

GENETIC MANIPULATION OF NON-RUMINANT LIVESTOCK

The application of recombinant DNA technology to manipulate the germline of livestock provides us with the possibility of using transgenic techniques to manipulate the complement of enzymes secreted into the gastrointestinal (GI) tract of farm animals, with the object of improving nutrient utilization by simple-stomached animals. These animals depend on a consortium of cellulolytic and xylanolytic anaerobic bacteria, present in the hind-gut, to digest plant structural carbohydrates. Sugars released are fermented to volatile fatty acids (VFA) which are absorbed by the animal and utilized as C and energy sources. From the nutritional standpoint, hind-gut fermentation of sugars is inefficient because dietary energy and C are lost in the heat of fermentation and through methanogenesis. Furthermore, microbial biomass synthesized from dietary components in the hind-gut is lost to the animal. Finally, VFA, when absorbed, have only 60% of the energy value of an equivalent quantity of the monosaccharides from which they have been derived (Blaxter, 1962). From the foregoing discussion, it is apparent that substantially more energy would be available to non-ruminants, if plant structural carbohydrates could be digested in the small intestines and the sugars released absorbed without previous fermentation to VFA.

Existing barley-based diets for simple-stomached animals contain β-D-glucans and hemicellulose. Support for the notion that improved animal performance could result from introducing cellulases and xylanases into the GI tract of these animals is provided by Pettersson & Åman (1988, 1989) and Hesselman & Åman (1986), who showed increased performance of broiler chickens fed on cereal-based diets which were supplemented with β-1,4-glucanase.

To achieve the objective described previously requires that heterologous xylanase and cellulase genes are expressed in the exocrine pancreas and peptic cells, with the subsequent secretion of active enzymes in the GI tract. In this context, Swift *et al.* (1984) have shown that, in transgenic mice, the rat elastase gene is expressed specifically in the exocrine pancreas, and the enhancer sequence of this gene has been used to direct the expression of rat and human growth hormones in acinar cells of the pancreas (Swift *et al.* 1984). Furthermore, regulatory sequences of the chymotrypsin B gene will direct expression of reporter enzymes in exocrine pancreatic cell lines (Walker *et al.* 1983). Thus, appropriate enhancer sequences can be used to direct heterologous gene expression in the exocrine pancreas.

There have, to date, been no reports on the expression of cellulases and xylanases in transgenic animals, thus it must be established whether genes encoding these enzymes, when fused to the elastase enhancer and a polyadenylation sequence will be expressed exclusively in the pancreas. The role of introns in higher eukaryotic gene transcription is particularly pertinent to this question since prokaryotic genes lack introns. Using transgenic mice, Ornitz *et al.* (1985) showed that intervening sequences were essential for efficient expression of the rat growth hormone gene when controlled by elastase or metallothionin enhancers. However, the role of introns in prokaryotic gene expression in

mammals remains to be elucidated. Secretion of active cellulase and xylanase in the GI tract is a prerequisite for effective digestion of plant fibre. The importance of signal peptides in transporting proteins across cell membranes is well established, but although their structure is similar in both eukaryotic and bacterial cells (Carne & Sheele, 1984), it remains to be established whether the prokaryotic sequence will allow secretion of enzymes into the GI tract.

Recently, Hall *et al.* (1990) have shown that EG E from *C. thermocellum* is efficiently expressed and secreted in a mammalian cell line (Chinese hamster ovary) when fused to a prokaryotic or eukaryotic signal peptide. In addition, the enzyme was post-translationally modified, presumably in the endoplasmic reticulum and golgi apparatus.

In addition to improving the utilization of existing diets, the genetic modification could provide the opportunity for increasing the proportion of forage and decreasing the cereal content of diets fed to simple-stomached animals. This would improve the efficiency of pig and poultry production, while freeing cereals for human consumption.

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