The Isolation and Comparison of Cellulase Genes from Two Strains of
Ruminococcus albus

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Endo-1,4-β-glucanase genes have been cloned from two strains of Ruminococcus albus recently isolated in this laboratory. Although the strains were phenotypically similar, cross-hybridization studies between them showed significant genetic differences, with only 20% of the genome forming DNA heteroduplexes. Heteroduplexes displayed an average dissociation temperature 9 °C lower than that of the homoduplex. Consistent with this, restriction maps of the two endoglucanase genes showed no similarity, and hybridization work using the endoglucanase genes as probes revealed that neither gene was present in the genome of the other isolate of R. albus. Comparative enzyme characterization showed differences between the enzymes in their response to temperature, pH and substrate preference.

INTRODUCTION

There is a growing body of evidence to show that bacterial strains which are phenotypically similar may be genetically quite distinct (Salyers et al., 1983; Dellaglio & Torriani, 1986; Hood et al., 1987; Roberts et al., 1987). Where such genetic diversity occurs, it is reasonable to expect that genes coding for proteins with similar functions may also be quite diverse, possibly encoding proteins with markedly different properties.

The wide range of potential uses for cloned cellulase genes (Smith & Hespell, 1983; Zappe et al., 1986; Kim et al., 1987) makes it highly desirable to isolate a variety of genes from different sources, so that variable characteristics of the enzyme (such as temperature and pH optima) can be matched to a specific task. In this laboratory the principal aim is to increase the rate of cellulose degradation in the rumen. Therefore we wish to examine the properties of cellulase genes from as many different rumen micro-organisms as is practicable, in order to select those best suited for introduction into non-cellulolytic species.

In recent years cellulase genes from several species of rumen bacteria have been cloned into Escherichia coli (Taylor et al., 1987; Barros & Thomson, 1987), including an endoglucanase gene from Ruminococcus albus (Kawai et al., 1987). However, evidence of extensive genetic variability within rumen bacterial species (J. F. Hudman & K. Gregg, unpublished; Mannarelli & Lee, 1987) suggests that the cloning of additional cellulase genes from distinct isolates of these species may be of benefit. For this reason, we chose to clone cellulase genes from two recently isolated strains of R. albus, which appeared to be genetically dissimilar when judged by the restriction endonuclease digestion patterns of their genomic DNA.

This paper reports the cloning and characterization of endoglucanase genes from the R. albus isolates AR67 and AR68, and presents a comparison of the genes and the enzymes they encode.

Abbreviations: CMC, carboxymethylcellulose; LMP, low melting point.
Isolation and culture of *R. albus*. Rumen samples were obtained from a sheep fed once daily with oaten chaff. *R. albus* was isolated by means of serial dilution of strained rumen contents in anaerobic medium containing clarified rumen fluid in roll-tubes using the methods of Hungate (1966, 1969). The gas phase was CO₂/H₂ (95:5, v/v). Salt solutions A and B used in media preparation were according to Hungate (1969). The isolation medium used had the following composition: solution A, 16.5 ml; solution B, 16.5 ml; cell-free ovine rumen fluid, 17 ml; cellulose suspension, 10 ml; distilled water, 50 ml; NaHCO₃, 0.5 g; agar, 1.5 g; cysteine.HCl, 0.02 g; and resazurin, 0.0001 g. Inoculated tubes were incubated at 39 °C. The cellulose suspension consisted of Whatman no. 1 filter paper which was ball-milled and made up to 2% (w/v) in water.

When well-isolated cellulosytic colonies were obtained they were transferred to cellulose broth medium (isolation medium with agar omitted). *R. albus* was identified on the basis of cellular morphology, culture characteristics and fermentation products.

Cells of AR67 and AR68 were spherical, with adjoining sides of diplococci flattened. Cells occurred as pairs, with smaller numbers occurring singly, and only occasional chains of up to four cells. The mean cell diameter was 1.0 μm for AR67 and 1.3 μm for AR68. Strain AR67 was Gram-variable and AR68 was Gram-negative. Colonies of both strains were white. Surface colonies on cellobiose rumen fluid agar were circular and convex; deep colonies in cellulose agar had the form of multiple lenses radiating from the centre of the colony. Both strains were obligately anaerobic, isolated at high dilution from rumen contents on rumen fluid cellulose agar medium. They were highly active digesters of cellulose and also grew on xylan and cellobiose. Growth in rumen fluid cellobiose broth was evenly turbid within 1 d at 39 °C.

Ethanol and fermentation acid products were determined by the method of Playne (1985) using a Gow-Mac series 580 gas chromatograph with a flame ionization detector. Hydrogen was determined using a Gow-Mac series 150 thermal conductivity detector. Major fermentation products were acetate, formate, ethanol and hydrogen. Succinate was not produced.

For isolation of DNA, strains AR67 and AR68 were grown in Wheaton bottles containing 100 ml broth prepared anaerobically, inoculated with 1 ml of an actively growing culture, and incubated overnight at 39 °C. This medium had the following composition: solution A, 16.5 ml; solution B, 16.5 ml; cell-free ovine rumen fluid, 33 ml; distilled water, 33 ml; peptone (Difco), 0.1 g; yeast extract (Difco), 0.1 g; NaHCO₃, 0.5 g; glucose, 0.2 g; cellobiose, 0.2 g; cysteine.HCl, 0.02 g; resazurin, 0.0001 g. The gas phase was CO₂/H₂ (95:5).

*Escherichia coli* strains. *E. coli* strains JM109 (Hanahan, 1985) and ED8767 (Murray et al., 1987) were grown in Luria-Bertani medium (LB medium: 5 g yeast extract l⁻¹, 10 g tryptone l⁻¹, 5 g NaCl l⁻¹), containing 50 μg ampicillin ml⁻¹ where necessary.

Isolation of DNA. Chromosomal DNA was extracted from *R. albus* using the following procedure. Late-exponential-phase cells were removed from the growth medium by centrifugation (12000 g for 30 min) and resuspended in 10 mM-Tris/HCl, 1 mM-EDTA (pH 7.5). Lysozyme was added (1 mg ml⁻¹; Boehringer Mannheim) and the cell suspension was incubated at 37 °C for 1 h. (Note: We have found that fresh supplies of lysozyme from this supplier will readily lyse *R. albus*, but aged preparations will not.) Sodium dodecyl sulphate (SDS, final concentration 0.2%, w/v) was added to lyse the cell membranes and the lysate was incubated with 10 μl ribonuclease A (RNAsae, 10 mg ml⁻¹) at 37 °C for 30 min. Finally 10 μl Proteinase K (10 mg ml⁻¹) was added and the solution was incubated at 50 °C for 1–2 h. The solution was extracted sequentially with an equal volume of phenol/chloroform (1:1, v/v), chloroform, and diethyl ether, before precipitating the DNA with 2.5 vols ethanol. Plasmid and phage DNA was prepared according to the procedures described by Maniatis et al. (1982).

Construction of gene libraries. *R. albus* DNA was subjected to partial digestion with the restriction endonuclease *Sal*3AI to produce an approximately random distribution of fragments. The resulting cohesive termini were partially end-filled using 100 μM-concentrations of the deoxynucleoside triphosphates dGTP and dATP, and 10 units of the Klenow fragment of DNA polymerase I (Bresatec) in a buffer of 20 mM-Tris/HCl, pH 7.9, 10 mM-MgCl₂ and 1 mM-dithiothreitol. The end-filling reaction was incubated at 37 °C for 15 min, and the enzyme was inactivated by heating to 70 °C for 5 min. Fragments of 4–10 kb were selected by separation on a 0.7%, low-melting-point (LMP) agarose gel (Pharmacia) in TAE buffer (0.08 M-Tris, 0.04 M-acetate, 2 mM-EDTA, pH 8). DNA in the chosen size range was extracted from the gel by adsorption to silica particles (Geneclean, Bio 101).

DNA from the isolate AR67 was ligated to the vector pgtWES.JB (Leder et al., 1977). Vector was prepared by digestion with the restriction endonuclease *Xho*I and partial end-filling with dCTP and dTTP to produce cohesive ends complementary to those of the size-selected AR67 DNA. The vector DNA was then extracted with phenol/chloroform to remove proteins and concentrated by ethanol precipitation.

Ligation was performed with 1 μg of vector arms and 0.15 μg of size-selected AR67 DNA using conditions specified by the enzyme manufacturer (Bresatec). The ligated DNA was packaged into phage particles using the Packagene kit (Promega Biotec) and infected into the *E. coli* strain ED8767, according to the instructions provided with the Packagene kit.
AR68 DNA was prepared in the same manner as that from AR67, but was ligated to the plasmid pUC19 (Yanisch-Perron et al., 1985) which had been digested with the restriction endonuclease SalI, and partially end-filled with dCTP and dTTP, giving single-stranded ends complementary to those of the AR68 DNA. The products of the ligation were used to transform E. coli strain JM109 by electroporation as follows. Bacteria were grown to OD_{550} = 0.7 in LB medium, harvested by centrifugation at 1000 g and washed in 0.3 vol. electroporation buffer (272 mM-sucrose, 7 mM-sodium phosphate, pH 7.4, and 1 mM-MgCl₂). After recentrifugation as above, cells were resuspended in 0.05 vol. electroporation buffer and stored on ice for 10–30 min before use. DNA was mixed with a 0.8 ml volume of bacteria before pulsing with 2.5 kV from a 25 μF capacitor, across a cuvette with 4 mm path length. The treated bacteria were transferred to a sterile tube containing 2 ml LB medium and incubated with shaking for 60 min, before adding 1.5 ml 2% (w/v) agar in LB medium and spreading onto agar plates containing LB medium and 50 μg ampicillin ml⁻¹.

Screening for endoglucanases. Plasmid-borne gene clones coding for endoglucanases were selected by their ability to produce active enzyme. Enzyme activity was detected on plates containing carboxymethylcellulose (CMC; low viscosity, Sigma) using the Congo red staining method (Teather & Wood, 1982). A clear 'halo' was observed around clones which had digested CMC.

The &gt;WES.λB gene library was screened by spotting recombinant phage onto CMC plates which had been overlaid with 3 ml LB medium containing 0.7% agar and 400 μl of an ED8767 suspension grown to OD_{550} = 0.7 in LB medium + 0.2% (w/v) maltose. Overnight incubation at 37 °C produced a lawn of bacteria, containing plaques at the positions where phage had been spotted. After removal of bacterial lawns by gentle scraping and washing, the plates were stained with Congo red. Recombinant phage containing a functional endoglucanase gene produced plaques containing the enzyme from that gene. As a result a non-staining halo could be observed directly, as for colony screening.

Hybridization. Plasmid DNA was digested to isolate the foreign DNA insert, which was separated from the plasmid on a 0-7% (w/v) LMP agarose gel in TAE buffer, and extracted from the gel using the GeneClean method. The insert DNA was then radioactively labelled with [α-³²P]dATP (1·1 × 10⁶ MBq mmol⁻¹; 3.7 MBq per reaction) using the oligo-labelling kit supplied by Bresatec.

Genomic DNA was labelled by nick-translation, using 100 μM-concentrations of dGTP, dCTP and dTTP, 5 units of DNA polymerase I (Bresatec) and 0·06 μg deoxyribonuclease I (Sigma). [²³P]-dATP (Bresatec) was added as above and the reactions incubated at 15 °C for 90 min.

Radioactively labelled DNA was separated from the remaining precursors on a Sephadex G-50 mini-column, using a buffer of 10 mM-Tris, 1 mM-EDTA, pH 7.0.

DNA was heat-denatured by boiling for 5–10 min before being added to hybridization solution (Wahl et al., 1979).

DNA transfer to a nitrocellulose filter was performed by the method of Southern (1975) and hybridization was carried out according to Maniatis et al. (1982).

Melting curves. The method for measurement of homoduplex and heteroduplex melting kinetics was a modification of the method described by De Ley et al. (1973). In brief, filter-bound AR67 and AR68 genomic DNA (2 μg) was hybridized to labelled AR67 or AR68 DNA (Wahl et al., 1979). Hybridization was carried out for three periods of 24 h at 42 °C, 37 °C and 30 °C, to encourage formation of duplexes even where sequence homology was poor. Filters were washed at very low stringency (5 x SSC + 0·1% SDS at room temperature, approx. 22 °C; 1 x SSC = 0·15 m-sodium chloride, 0·015 m-sodium citrate); then incubated in successive 2 ml volumes of 2 x SSC, 30% (v/v) dimethylsulphoxide at a range of temperatures between 50 °C and 95 °C.

The amount of radioactivity released from the filters at each temperature was measured by Cerenkov radiation in a Packard liquid scintillation counter using the ³H discriminators.

The degree of similarity between DNA sequences from the two strains was assessed by calculating the difference in the T_m value between homologous and heterologous duplexes (melting temperature, T_m, is defined as the temperature at which 50% of the radioactive probe remains bound to the filter). The total number of counts bound by formation of heteroduplexes, compared to counts bound by homoduplex formation, was used as an indication of the proportion of the two genomes that are sufficiently similar to allow duplexes to form.

Preparation of cell lysates. Enzyme extracts were prepared by growing bacteria containing either pTCl or pMH1 (see Results) to stationary phase in LB medium + ampicillin. Cells were washed in 25 mM-Tris, 10 mM-EDTA, 15% (w/v) sucrose (pH 7·5) and resuspended in 10 mM-Tris, 1 mM-EDTA (pH 7) to 10% of the original volume. Cells were disrupted by sonication to release enzyme (Branson sonifier; 4 min on power level 5, 50% duty cycle).

Trigon X-100 (0·1%, w/v) was added in some cases. Cell debris was removed by centrifugation at 27300 g for 10 min.

Measurement of enzyme activity. Enzyme activity in cell lysates was measured by incubating 200 μl lysate with 500 μl substrate (usually 1%, w/v, low-viscosity CMC) in reaction buffer (170 mM-Na₂HPO₄, 14 mM-citric acid, adjusted to pH 7 with HCl). Mixtures were incubated for 30 min at 38 °C unless otherwise stated, after which the
reactions were stopped by chilling on ice. The amount of reducing sugar produced during the incubation time was estimated by the method of Nelson (1944).

Xylan (from oat spelts, Sigma) was prepared as an enzyme substrate by dissolving in 1 M-NaOH overnight then neutralizing to pH 7 in enzyme reaction buffer. Undissolved xylan was removed by centrifugation before estimating xylan concentration using the anthrone reaction (Ashwell, 1957).

Phosphoric-acid-swollen cellulose was prepared as described by Wood (1971). Crystalline cellulose (Sigmacell, Sigma) was suspended in enzyme reaction buffer (pH 7).

When comparing the rate of enzyme activity on different substrates, all substrates were standardized to a concentration of 0.34% (w/v). Concentrations were determined using the anthrone method (Ashwell, 1957). By comparing the estimates of sugar content obtained from the anthrone test with the number of reducing ends determined by the method of Nelson (1944), a mean chain length of 175 glucose units was calculated for the CMC, and a maximum mean of 260 monosaccharide units for the xylan. The proportion of sugar residues present as side-chains in this xylan preparation was not determined.

RESULTS

Examination of genetic inter-relationships

The isolates AR67 and AR68 were classified as the same species, Ruminococcus albus, on the basis of phenotypic characteristics. However, a comparison of the banding patterns produced by digestion of their genomic DNA with the restriction endonuclease HindIII revealed major differences (Fig. 1). This indication of genetic variation between the two strains was examined more closely by measuring the level of cross-hybridization, and the change in melting temperature of the heteroduplex in comparison to the homoduplex.

Radioactively labelled genomic DNA from each strain was hybridized to filter-bound DNA of both AR67 and AR68. The proportion of DNA probe bound by heteroduplex formation was about 20% of the amount bound by formation of homoduplexes (data not shown), suggesting that only 20% of the genome sequence was sufficiently similar to allow cross-hybridization. The stability of DNA hybrids was tested by heat-denaturation, and Fig. 2 shows the melting curves produced by dissociation of labelled DNA from both homologous and heterologous hybrids. The stability of the heteroduplexes was reduced in comparison with the homoduplexes, with a 9 °C reduction in $T_m$. This indicated that the 20% of genome sequence able to form a heteroduplex attained only a poor degree of complementarity.

The considerable degree of genetic disparity between these two isolates of R. albus suggested that it would be valuable to isolate cellulase genes from both strains, as there may be important differences in the properties of the encoded proteins.

Cloning of endoglucanase genes

Three bacteriophage clones from the AR67 gene library (ATC1, ATC2 and ATC3) produced a zone of digestion on CMC and a single, active plasmid clone (pMH1) was obtained from AR68.

Of the AR67 clones, ATC1 was chosen for further study because it produced the highest level of activity, judged by the size of the clearing zone produced on a CMC plate. More detailed studies of ATC2 and ATC3 will form the basis of a later report. The R. albus DNA insert of ATC1 was removed from the phage DNA, by digestion with the restriction endonucleases BamHI and SalI, and separated on a 0.7% (w/v) LMP agarose gel. The 4.5 kb fragment was ligated to a pUC19 plasmid which had been digested with BamHI and SalI, and the ligation products were transformed into E. coli JM109. The resulting plasmid, pTCl, contained about 1.2 kb of DNA. The majority of this was removed by digestion with ClaI and HindIII, end-filling the cohesive termini of the major fragment and religating (see Fig. 3).

Difficulties were experienced in obtaining reproducible expression of endoglucanase activity from pTCl in broth culture. In an attempt to overcome this problem the 2.5 kb ClaI–PstI fragment was subcloned into pUC18. This clone, pTCl1.1, produced good yields of enzyme from overnight cultures, and was used in obtaining enzyme for characterization work.

By asymmetric digestion with the restriction endonucleases PstI and XbaI, the R. albus DNA was removed from pMH1 and ligated into pUC18, such that the orientation of the endoglucanase gene relative to the lac promoter was opposite to that in pUC19. Endoglucanase
Cellulase genes from two strains of *R. albus*

Fig. 1. (a, b) Autoradiographs of a nitrocellulose filter prepared by a Southern blot of gel (c), an agarose gel (1% w/v) electrophoresis of genomic *R. albus* DNA, digested to completion with *HindIII*. Lane 1, AR67; lane 2, AR68. Probes were (a) pTC1 DNA, and (b) pMH1 DNA.

![Autoradiographs](image)

Fig. 2. Melting curves of homoduplex and heteroduplex DNA hybrids (*R. albus* strains AR67 and AR68). Homoduplex melting kinetics for the two strains (○) were essentially identical and the mean of the two results is shown. Heteroduplex hybridization (▲) was performed in two reciprocal experiments and the results were averaged.

![Melting curves](image)

Activity was expressed from both pUC18 and pUC19 clones, suggesting that the gene was being transcribed from its own promoter.

Similarly, pTC1.1 showed endoglucanase activity in both pUC18 and pUC19 clones, and it was concluded that the natural endoglucanase gene promoter is also present and operational in pTC1.1.

**Comparison of cloned genes**

Restriction maps of the two plasmids pTC1 and pMH1 were constructed by digesting each with the following 6-base restriction enzymes: *AceI*, *AvaI*, *BamHI*, *BglII*, *ClaI*, *DraI*, *EcoRI*, *HindIII*, *PstI*, *PvuII*, *SalI*, *SmaI*, *XbaI* and *XhoI*. Fig. 3 shows the maps constructed as a result of these digests.
Fig. 3. Restriction maps of endoglucanase genes from three strains of *R. albus*, cloned into *E. coli*. pTC1 and pMH1 are genes from strains AR67 and AR68 respectively. pRA1 was cloned from the strain F-40 by Kawai *et al.* (1987). Three stages in the reduction of pTC1 are shown in the upper part of the diagram. A thin line denotes *R. albus* DNA, a thick line denotes vector DNA; an open line denotes plasmid, a solid line denotes λ phage DNA. Recognition sites shown are all of those detected for the 14 restriction endonucleases tested on pTC1 and pMH1. No recognition sites were found in pTC1 or pMH1 for the enzymes: AccI, BamHI, BglII, DraI, HindIII, Sall, SmaI, XbaI and XhoI.

Fig. 4. (a, b) Autoradiographs of a hybridization filter prepared from agarose gel (c), produced by electrophoresis of four CM-cellulase genes cloned from *R. albus*. Lane 1, pTC1 cleaved with *Ava*I; lane 2, *ATC2* cleaved with BamHI; lane 3, *ATC3* cleaved with BamHI; lane 4, pMH1 cleaved with *Ava*I. Probes were (a) pTC1 insert DNA and (b) pMH1 insert DNA. The faint band visible in the pTC1 track in (a) corresponds to a trace amount of linearized pTC1 (7.2 kb), resulting from incomplete digestion of the plasmid. Clones pTC1, ATC2 and ATC3 were from strain AR67; clone pMH1 was from strain AR68.
Table 1. Substrate preferences of the endoglucanases produced from pTC1 and pMH1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pTC1</th>
<th>pMH1</th>
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<tbody>
<tr>
<td>CMC</td>
<td>100*</td>
<td>100†</td>
</tr>
<tr>
<td>Acid-swollen cellulose</td>
<td>11.0 ± 4.6*</td>
<td>26.95 ± 9.6†</td>
</tr>
<tr>
<td>Crystalline cellulose</td>
<td>2.3 ± 1.2*</td>
<td>2.8 ± 2.1†</td>
</tr>
<tr>
<td>Soluble xylan</td>
<td>63.7 ± 9.9*</td>
<td>16.9 ± 9.9†</td>
</tr>
<tr>
<td>Particulate xylan</td>
<td>5.25‡</td>
<td>1.1‡</td>
</tr>
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* Results are the mean of five experiments.  
† Results are the mean of four experiments.  
‡ Results are the mean of two experiments.

The restriction maps of pTC1 and pMH1 show no similarity. Furthermore, neither of the maps bear any resemblance to that reported by Kawai et al. (1987) for an endoglucanase gene isolated from *R. albus* strain F-40.

These results indicated that the endoglucanase genes in pTC1 and pMH1 were quite distinct, and not merely the same gene cloned independently from two isolates. To determine whether there was any measurable degree of sequence homology between the two genes, a Southern blot was performed on all four of the CM-cellulase genes isolated from AR67 and AR68 (Fig. 4). When the DNA bound to the nitrocellulose filter was hybridized with radioactively labelled pTC1 insert DNA, hybridization was observed only to the track containing pTC1 DNA. Likewise the *R. albus* DNA from pMH1 did not hybridize to a detectable level with any other DNA on the filter. Clearly these two endoglucanase genes do not share any measurable sequence homology, nor are they closely related to the genes cloned in LTC2 and LTC3. Furthermore hybridization work using the insert from LTC3 as a probe showed that LTC2 and LTC3 also contain dissimilar genes (data not shown), thus the three CMC-degrading activities detected in the AR67 library are encoded by three separate genes.

A second Southern blot was performed to determine whether each of the two genes in pTC1 and pMH1 were represented at all in the genome of the other isolate. Fig. 1 shows the result of hybridizations, using labelled pTC1 and pMH1 DNA as probes to HindIII-digested genomic DNA from each strain of *R. albus*. There was no observable binding of pTC1 to AR68 DNA, although a faint cross-reaction of pMH1 to AR68 DNA was detectable at a site distant from that of the pTC1 band, in a fragment of about 4 kb. However, the amount of cross-reaction was small in comparison to the hybridization of pMH1 to AR68, becoming visible only when the autoradiograph was over-exposed. This indicated that pMH1 is not closely related to any gene in the AR67 genome, although sequences bearing some similarity are present. These results show that pTC1 and pMH1 represent alternative forms of endoglucanase genes rather than complementary genes. Each gene is located on only one restriction fragment in the genome, corresponding to a fragment size of about 8 kb in the HindIII-digested AR67 DNA, and greater than 20 kb in the AR68 DNA.

### Enzyme characterization

Having established that the genes contained in pMH1 and pTC1 are not closely related, it became of interest to compare some of the properties of the enzymes they encode.

Table 1 shows the relative activity of each enzyme on a variety of substrates. Substrate preferences appeared to be similar, in most respects, for the two enzymes. Both showed the highest activity on CMC, with a much lower but significant activity on acid-swollen cellulose. Activity on crystalline cellulose was barely detectable, suggesting that the rate of substrate breakdown is largely influenced by the accessibility of the substrate to the enzyme. Activity of pTC1 enzyme on solubilized xylan was about four times that of pMH1 enzyme. When
particulate xylan was suspended in enzyme buffer, the extent of digestion by both enzymes was very low. No activity was observed on the synthetic substrates 4-methylumbelliferyl $\beta$-D-glucopyranoside and 4-methylumbelliferyl $\beta$-D-cellobioside.

The relationship between temperature and enzyme activity was studied for each enzyme. Under the assay conditions described in Methods, pTCl enzyme possessed a temperature optimum centred around 38°C, whereas the pMH1 enzyme was more active at higher temperatures, with activity peaking at 46°C. A pH optimum of 6 was determined for the enzyme produced from pTCl, while the pMH1 enzyme showed highest activity over the range pH 6–7.5.

The effects of substrate concentration on enzyme activity were examined. Using concentrations in the range 0.07–0.7% (w/v), estimations of $K_m$ varied widely from one experiment to another. At substrate concentrations higher than 0.7% an apparent inhibition of pMH1 enzyme activity was observed. pTCl enzyme was slightly more resistant but also showed a decrease in activity at CMC concentrations above 1%, making it impossible to extend the range of concentrations used to estimate $K_m$. It was concluded that the $K_m$ could not be meaningfully determined on an ill-defined substrate, using impure preparations of the cloned proteins.

**DISCUSSION**

The results presented here are in agreement with previous studies on rumen bacteria, which show that bacterial isolates that were phenotypically classified as the same species may be genetically distinct from each other (K. Gregg & J. F. Hudman, unpublished; Mannarelli & Lee, 1987). This finding is important when cloning genes for a particular purpose, because the genes from one isolate may be quite different to the analogous genes from another isolate of the same phenotypic species. Indeed we have found this to be true of the strains of *R. albus* studied here.

The genes from AR67 and AR68, while both coding for endo-$1,4$-$\beta$-glucanases, showed no cross-hybridization. Therefore we conclude that the DNA sequences can, at best, be only distantly related. It was considered possible that they may represent complementary enzyme forms from within the one cellulase complex. However, neither of these genes showed significant levels of cross-hybridization with the genomic DNA of the other bacterial isolate. This demonstrated that the two genes are not merely two components of a gene complex for cellulose degradation, which is common to both isolates, but that they appear to be genetically distinct alternatives.

Likewise, the endoglucanase gene from *R. albus* cloned by Kawai et al. (1987) appears to be quite different from the genes cloned in this laboratory, when judged by restriction mapping and enzyme characteristics. In view of the temporal and geographical separation between sources of these bacterial strains, such differences are unremarkable. It is rather more notable that two *R. albus* strains from the same laboratory, isolated at about the same time, are nevertheless genetically divergent. It is also interesting that after exhaustive screening of the gene library from *R. albus* F-40, Kawai et al. (1987) had found several copies of only a single endo-$1,4$-$\beta$-glucanase gene, whereas AR67 has yielded at least three CM-cellulase genes. Without a more extensive search in AR67 and AR68, the final number of endoglucanases in each remains uncertain. However, it is likely that significant variability may occur in the number of genes concerned with cellulose degradation within different *R. albus* strains. Our conclusions here are consistent with the data published by Morris & Cole (1987), who also found considerable variability among 23 isolates of *R. albus*, both in their total protein profiles and in the number and size of xylanase and CM-cellulase enzymes each contained. These results support the hypothesis that cloning the genes from a single isolate of a bacterial species may utilize only a small part of the available genetic resources. Screening a range of isolates for endoglucanase genes may uncover a useful diversity of genes encoding a correspondingly diverse group of enzymes. Thus when searching for a gene to perform a particular function, the screening of a range of isolates provides a considerable advantage in locating the most suitable gene/enzyme combination.
There may be an important secondary benefit arising from the genetic differences that exist among different strains within a species, in that it may provide an opportunity to study the structure of variant proteins and how this relates to function. By determining which regions of the protein are highly conserved among enzymes from related strains, it may be possible to deduce which parts of the protein are important to its catalytic and binding functions. This may contribute to an understanding of the mode of action of cellulase enzymes on their substrate.

Early work on enzyme characterization has already revealed certain differences in enzyme properties in the areas of temperature and pH optima and substrate preferences. We have considered that the introduction of these genes into a foreign bacterium may have altered some of these properties relative to those of the native form. Possible mechanisms by which functional variation may occur include the lack of post-translational modification, variable sites of initiation for transcription and/or translation, or incorrect folding of the protein.

It was observed that the characteristics of the pMH1 enzyme tended to vary from one enzyme preparation to another, particularly with respect to substrate preferences and pH optimum. This may be due to several conformational forms of the enzyme being present, with the dominant form varying amongst individual preparations of the enzyme. In the native bacterium, folding of the protein may be more precisely controlled, either enzymically or by environmental conditions. Tests on the activity of the pMH1 and pTC1 enzymes were conducted in parallel, and pTC1 showed more precise reproducibility in its response to pH and substrate type, eliminating the possibility that the variability observed with pMH1 may have been due to the experimental conditions.

Another point to arise out of the enzyme characterization work is the high activity of the two enzymes on solubilized xylan. Previous reports have demonstrated that both xylanase and cellulase activity may occur within a single protein complex (Pettipher & Latham, 1979; Williams & Withers, 1982). Our observations suggest that the two capabilities may even be present within the same polypeptide. However, it is possible that substrate specificity may differ between the cloned protein and the natural product. Other groups working with cloned endoglucanases have observed negligible activity on xylan (Kawai et al., 1987; Taylor et al., 1987), but these results are confounded by differing levels of solubility, making comparison difficult. Our results have shown that whereas high levels of digestion could be measured on fully-solubilized xylan, little activity was detected on insoluble xylan. Therefore the observed level of activity on xylan will be greatly influenced by the proportion of soluble xylan in the assay, which is determined by the type of xylan used, and what steps were taken to solubilize it. In most published reports, insufficient information has been given to permit valid comparison.

At present, further characterization of the gene products of pMH1 and pTC1 is in progress to determine which gene is more suitable for the transformation of non-cellulolytic rumen bacteria. In addition, further characterization will reveal the extent to which the differences between genes are reflected in differences in enzyme properties. Sequencing of the gene may then make it possible to correlate differences in protein sequence with observed differences in enzyme properties.

The cloning of two distinct endoglucanases from two strains of R. albus supports the view that, although grouped under a relatively few species headings, the rumen contains an enormous diversity of micro-organisms. This diversity represents a rich source of genetic material which may prove invaluable for the genetic engineering of rumen micro-organisms.

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REFERENCES


