Isolation of an Endoglucanase Gene from *Bacteroides ruminicola* subsp. *brevis*

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A gene coding for endo-1,4- β -glucanase activity has been isolated from *Bacteroides ruminicola* subsp. *brevis* by cloning in *Escherichia coli*. After restriction mapping of a 6.4 kb insert, a 2.2 kb DNA fragment was sub-cloned in pUC19 to produce the enzymically active clone pJW3. Recloning of the gene fragment in the reverse orientation in pUC18 (clone pJW4) indicated that a gene promoter was present in the cloned fragment and was able to function in *E. coli*. The clone pJW4 displayed increased activity which was attributed to expression from the *lac* promoter of pUC18. The enzyme encoded by pJW4 was optimally active at pH 5.5–6.0, and in the temperature range 37–42 °C. The preferred substrate was carboxymethylcellulose, but the enzyme displayed 50–60% of maximal activity on both acid-swollen cellulose and soluble xylan. No significant activity was detected on ball-milled filter paper or particulate xylan. Deletion experiments confirmed that both cellulase and xylanase activities were altered to a similar extent by deletion of DNA from the 3' end of the gene, suggesting that both are a function of the same polypeptide product.

INTRODUCTION

Digestion of cellulose within the rumen could be made more efficient by the use of genetic engineering techniques to introduce cellulase genes into non-cellulolytic rumen bacteria (Teather, 1985; Gregg, 1989; Russell & Wilson, 1988). The non-cellulolytic bacterium *Bacteroides ruminicola* has been proposed as a suitable recipient of cloned cellulase genes, because it is able to grow well under conditions of low pH, which inhibit the growth of the major cellulolytic bacterial species (Russell & Wilson, 1988).

We have selected an isolate of *B. ruminicola* subsp. *brevis* (AR20) as a suitable recipient of cloned cellulase genes. However, before attempting to introduce novel genes into this organism, it is important to know more about the genes that contribute to its natural, fibre-digesting capabilities. In addition to allowing us to define the extra genes required to achieve digestion of crystalline cellulose, this work will provide information on gene regulatory sequences that may be of use later, in regulating introduced foreign genes.

We describe here the cloning and characterization of a gene from *B. ruminicola* subsp. *brevis* AR20 which encodes an enzyme capable of digesting both soluble xylan and acid-swollen cellulose.

METHODS

Bacterial culture. *B. ruminicola* subsp. *brevis* strain AR20 was isolated from the ovine rumen using the roll-tube method of Hungate (1966) and was grown anaerobically at 39 °C in the medium described by Hudman & Glenn (1984), reduced with cysteine.HCl and sodium sulphide (0.25 g l⁻¹ each). Species identification was based on the

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criteria of Gram stain, morphology, motility, carbohydrate fermentation and volatile fatty acid production (Holdeman *et al.*, 1977; Ogimoto & Imai, 1981). All manipulations were performed anaerobically in an atmosphere of CO₂/H₂ (96:4, v/v).

Escherichia coli strain JM109 (F' *supE44 thi traD36 proA,B Δ(lac-proA,B) lacIqZΔM15 recA1 relA1 λ⁻ endA1 gyrA96 hsdR17*) was grown with shaking at 37 °C in Luria-Bertani medium (LB medium). Plate culture was on LB medium plus 1.5% (w/v) agar, with ampicillin (50 µg ml⁻¹) added for selection of plasmid-transformed colonies.

DNA extraction. Late exponential phase cultures of *B. ruminicola* subsp. *brevis* AR20 (100 ml) were centrifuged at 12000 g (Beckman JA20 rotor) for 30 min, washed in TE buffer (10 mM-Tris/HCl pH 7.5, 1 mM-EDTA) and recentrifuged as before. The pellet was resuspended in 4 ml TE buffer to which was added 8 mg solid lysozyme (Boehringer Mannheim). After incubation at 37 °C for 30 min, 10 µl of 10 mg ml⁻¹ ribonuclease (Sigma) was added together with 0.2 ml of 10% (w/v) SDS. After a further 30 min at 37 °C, 10 µl of 10 mg ml⁻¹ proteinase K (Boehringer-Mannheim) was added and the mixture was incubated for 1 h at 50 °C.

The cell lysate was extracted with an equal volume of phenol/chloroform (1:1, v/v), followed by extractions with chloroform, and then diethyl ether, before being precipitated by the addition of 2.5 vols ethanol. The DNA was collected by centrifugation (24000 g, Beckman JA20 rotor) and the precipitate was washed for 10 min by gently overlaying it with 0.5 ml of 70% (v/v) ethanol at room temperature. After thorough draining and brief desiccation to remove ethanol, the DNA pellet was redissolved in sterile distilled water. The purity and concentration of the DNA were estimated by spectral analysis.

Plasmid DNA was extracted from *E. coli* using the alkaline-SDS method described by Maniatis *et al.* (1982).

Gene library construction. Genomic DNA from *B. ruminicola* subsp. *brevis* was partially digested with the restriction endonuclease *EcoRI* (Pharmacia), using the *Eco** activity [20 mM-Tris/HCl pH 7.9, 10 mM-MgCl₂, 1 mM-dithiothreitol (DTT) and 10% (v/v) glycerol]. From a series of partial digests, fragments in the range 3–10 kb were selected by separation on a low-melting-point agarose gel (0.8%, w/v, agarose in TAE buffer: 40 mM-Tris base, 20 mM-sodium acetate, 1 mM-EDTA, adjusted to pH 8.2 with glacial acetic acid). Size-selected DNA was extracted from gel slices by electro-elution and was ligated to *EcoRI*-digested arms of the bacteriophage vector λgtWES. λB (Bethesda Research Laboratories) in a 10 µl reaction volume containing 20 mM-Tris/HCl pH 7.5, 10 mM-MgCl₂, 10 mM-DTT, at 15 °C for 2 h. Ligation was confirmed by agarose gel electrophoresis and the products were packaged in λ-phage coats using the Packagene system (Promega-Biotec). Packaged, recombinant phage were infected into *E. coli* strain ED8767 (*recA56 supE supF HsdS⁻ R⁺ M⁺ Met⁻*) and plated onto agar in LB medium containing 0.2% (w/v) maltose.

Screening for xylanase and endoglucanase activity. Bacteriophage clones were screened using agar plates containing minimal medium plus 0.5% (w/v) tryptone, with either 0.2% (w/v) xylan (from oat spelts, Sigma) or 0.2% (w/v) carboxymethylcellulose (CMC, low viscosity, Sigma). These were overlaid with 3 ml LB medium plus 0.7% (w/v) agar to which had been added 0.4 ml of a suspension of *E. coli* ED8767 grown to OD₅₅₀ 0.7 in LB medium plus 0.2% (w/v) maltose.

Plaques were picked using sterile toothpicks and were transferred to the xylan and CMC plates and also to a replica LB plate to act as a reservoir for screened clones. After 2 d incubation at 37 °C, the top-agar overlay was removed by gentle scraping from the xylan and CMC plates, which were stained by flooding for 15–30 min with a solution of congo-red dye (1 mg ml⁻¹; Teather & Wood, 1982). After destaining with several changes of 1 M-NaCl, non-staining haloes were detectable around clones bearing a functional xylanase or endoglucanase gene.

Plasmid sub-cloning. The *B. ruminicola* subsp. *brevis* DNA insert, excised with *EcoRI* from the bacteriophage clone, was sub-cloned into the *EcoRI* site of the plasmid expression vector pUC19 (Yanisch-Perron *et al.*, 1985) and transferred to *E. coli* JM109 by the simple transformation procedure of Hanahan (1985). Plasmid sub-clones were screened for enzyme activity by spotting bacterial colonies onto CMC and xylan plates and allowing them to grow for 24–48 h before washing off the bacteria and staining the plates as above.

Restriction endonuclease mapping. Digestion with restriction endonucleases was performed using the conditions specified by the manufacturer (Pharmacia). Products were analysed by electrophoresis on agarose gels (0.8–1%, w/v, agarose in TBE buffer; Maniatis *et al.*, 1982), against size markers consisting of λ bacteriophage digested with *HindIII*.

DNA hybridization. Genomic DNA from strain AR20 was digested with *EcoRI* for electrophoresis on a 1% (w/v) agarose gel. The DNA was transferred by Southern blotting (Southern, 1975) to a nylon membrane (Hybond-N, Amersham) and hybridized with nick-translated DNA of the clone pJW4, using the method of Wahl *et al.* (1979). The radioactive probe was prepared as described previously (Ware *et al.*, 1989).

The hybridization membrane was rinsed several times in 2 × SSC (0.3 M-NaCl, 0.03 M-trisodium citrate) with 0.1% (w/v) SDS at room temperature, and washed twice, for 30 min each, in 0.1 × SSC, 0.1% (w/v) SDS at 65 °C, before being autoradiographed with X-ray film (Fuji).

Preparation of cell lysates for enzyme studies. Enzymically active extracts were prepared by growing *E. coli* JM109, containing recombinant plasmids pJW3 or pJW4, to stationary phase overnight in LB medium. After centrifugation (1000 g) the cells were washed in 0.25 vol. TES buffer (25 mM-Tris/HCl pH 7.5, 10 mM-EDTA,

15%, w/v, sucrose), recentrifuged (2000 g) and resuspended in 0.04 vol. TE buffer. Cells were lysed by disruption with a Branson Sonifier for 4 min on power level 5, 50% duty cycle. Triton X-100 was added to a final concentration of 0.1% (v/v) and the cell debris was removed by centrifugation at 27500 g for 10 min.

Measurement of enzyme activity. Enzyme activity in cleared cell lysates was determined by incubating 100–250 µl of lysate with 500 µl of substrate dissolved in phospho-citrate buffer (0.17 M-Na₂HPO₄, 14 mM-citric acid, pH 7.0). Components were mixed in an ice-bath and transferred to the incubation water-bath for the duration of the reaction, which was terminated by returning the tubes to ice. The amount of reducing sugar released during the incubation time was estimated by the colorimetric method of Nelson (1944).

Xylan was prepared as an enzyme substrate by dissolving 2 g in 20 ml 1 M-NaOH overnight, then diluting fivefold in phospho-citrate buffer and neutralizing with HCl. Undissolved xylan was removed by centrifugation (12000 g, Beckman JA20 rotor).

Phosphoric-acid-swollen cellulose was prepared as described by Wood (1971). All substrate concentrations were measured by the anthrone method described by Ashwell (1957) and standardized to 0.34% (w/v) for determination of substrate specificity.

Protein estimation. The protein content of bacterial extracts was determined by the dye-binding method of Bradford (1976) using bovine serum albumin as a standard.

Preparation of deletion mutants from pJW4. A series of deletions were made, from the DNA at the 3' end of the gene, using the 'Erase-a-Base' kit (Promega-Biotec). In brief, the plasmid pJW4 was cleaved in the multiple cloning site, adjacent to the 3' end of the gene, with the restriction endonucleases *Pst*I and *Bam*HI. Aliquots of the sample were then incubated for a series of timed increments with exonuclease III. Using this method, the exonuclease III was able to digest DNA from the *Bam*HI terminus, into the *B. ruminicola* subsp. *brevis* insert, but was not able to digest the pUC vector from the 3' single-stranded terminus produced by *Pst*I. The single-stranded regions generated by exonuclease III digestion were hydrolysed with S1 nuclease, the termini were end-filled with the Klenow fragment of DNA polymerase I, and the plasmid was then recircularized by ligation.

Transformation of *E. coli* JM109 was followed by screening of the resultant colonies on xylan and CMC plates as described above. Plasmid mini-preparations were performed for analysis of the deletions which caused an alteration in expression of enzyme activity.

RESULTS

Detection of enzymically active gene clones, and plasmid sub-cloning

After screening 700 bacteriophage clones, a clone designated λJW1 was selected for its ability to produce a zone of digestion on CMC plates and on xylan plates. This clone, containing a 6.4 kb DNA insert, was purified, and phage DNA was prepared for sub-cloning, using the plate-lysis method described by Maniatis *et al.* (1982).

The 6.4 kb insert from λJW1 was removed intact, by cleavage with *Eco*RI, and was inserted into the plasmid pUC19. The resulting recombinant plasmid (pJW1) was analysed by digestion with a series of restriction enzymes to produce the map shown in Fig. 1. After cleavage with *Ava*I, the 4.2 and 2.2 kb sub-fragments were cloned separately into pUC19 to produce the sub-clones pJW2 and pJW3 respectively. When tested on xylan and CMC plates, *E. coli* JM109(pJW2) failed to display enzyme activity, while *E. coli* JM109(pJW3) showed activity on both substrates, with larger, clearer haloes being produced on CMC.

The gene fragment was removed from pJW3, by cleavage at the *Eco*RI and *Hind*III sites in the multiple cloning site, and was inserted into pUC18 to reverse its orientation relative to the plasmid *lac* promoter. *E. coli* JM109 carrying the resulting plasmid (pJW4) produced a higher level of enzyme activity, yielding digestion haloes two to three times the diameter of those produced by cells carrying pJW3. The ability of the gene to produce active enzyme when cloned in either orientation was interpreted as an indication that the natural promoter of the gene was present in the 2.2 kb fragment and was functional in *E. coli*.

Origin of the gene in pJW4

Hybridization of pJW4 to a Southern blot of genomic DNA of strain AR20 showed clear and specific hybridization to an *Eco*RI fragment of around 7 kb (Fig. 2). This confirmed that the DNA fragment cloned within pJW4 was derived from the rumen bacterium. The discrepancy in size between the genomic fragment identified by hybridization and the DNA insert in pJW1

Fig. 1. Restriction map of (a) the plasmid clone pJW1 and (b) the plasmid sub-clone pJW4 derived from it. The broad lines represent vector DNA, the narrow lines represent DNA from *B. ruminicola* subsp. *brevis*, and the arrow indicates the direction of transcription of the endoglucanase gene. MCS, multiple cloning site. Restriction sites: Ac, *AccI*; Av, *AvaI*; B, *BamHI*; E, *EcoRI*; H3, *HindIII*; PI, *PstI*; PII, *PvuII*.

Fig. 2. A, Agarose gel showing *B. ruminicola* subsp. *brevis* genomic DNA digested with *EcoRI*, and B, the autoradiograph of a Southern blot of the same gel. The hybridization probe was nick-translated DNA of clone pJW4. Size markers are λ bacteriophage DNA digested with restriction endonuclease *HindIII*.

suggested that one (or both) of the terminal *EcoRI* sites on the clone pJW1 was (were) present in the genome as an *Eco** site(s), but became a full *EcoRI* recognition site(s) when ligated to the vector.

Gene orientation

It was hypothesized that the dramatic increase in enzyme activity following the reversal of gene orientation may have resulted from increased transcription of the endoglucanase gene under the control of the plasmid *lac* promoter. To confirm this, we made use of the ability of the

Table 1. Effect of *lac* promoter induction and repression on the diameter of digestion zones produced by *E. coli* JM109 containing pJW3 or pJW4

A constant volume of bacterial suspension (usually 5 µl) was spotted on to CMC plates with the designated additives, followed by incubation at 37 °C for 15 h. Results are means of two measurements.

Treatment	Relative halo diameter on CMC plates	
	pJW3	pJW4
Control	1.0	1.0
Glucose	1.12	0.87
IPTG	0.92	1.11

lac promoter to be stimulated by isopropyl β -D-thiogalactopyranoside (IPTG) and repressed by the presence of glucose. Colonies of *E. coli* JM109 containing plasmid pJW3 or pJW4 were grown on CMC or xylan plates, containing either 2% (w/v) glucose, or 0.1 mg IPTG ml⁻¹. To ensure that similar numbers of bacteria were present in each colony, a specific volume (usually 5 µl) of an exponential-phase bacterial suspension was spotted in triplicate on each plate. Comparison of the clearing zone produced on these plates, with that produced on a control plate, made it clear that the enzyme activity produced by pJW4 was increased in the presence of IPTG and decreased in the presence of glucose (Table 1). The converse results were observed for pJW3, with increased activity in the presence of glucose and decreased activity in the presence of IPTG.

It was concluded that the *B. ruminicola* subsp. *brevis* gene in pJW4 occupied the same orientation as the β -galactosidase gene. Attempts to make quantitative measurements of specific enzyme activity under these conditions were complicated by the presence of large amounts of reducing sugars within the bacterial cells which were grown in the presence of high levels of glucose. However, the qualitative results obtained by measuring clearing zone diameter were sufficient to confirm activation and repression of the gene.

Enzyme characterization

Under our assay conditions, the optimum temperature for activity of the enzyme on CMC (37–42 °C) is similar to that within the ovine rumen (39 °C). However, the optimum pH on this substrate of 5.5–6.0 is somewhat lower than that normally observed in the rumen (pH 6.5–7.0) and lower than that observed for a number of other cloned endoglucanases (Ware *et al.*, 1989; Taylor *et al.*, 1987; Kawai *et al.*, 1987; Barros & Thompson, 1987).

Maximum enzyme activity was observed using CMC as a substrate [equivalent to 20–40 nmol reducing sugar min⁻¹ (mg extracted protein)⁻¹]; activity on soluble xylan was 59% of maximum, and that on acid-swollen cellulose, 52%. No activity was detected on the synthetic substrates methylumbelliferyl xylopyranoside, methylumbelliferyl glucopyranoside or methylumbelliferyl cellobioside.

Evidence for the presence of a single gene

Progressive deletion of DNA from the 2.2 kb fragment, at the 3' end of the gene, resulted in the synchronous loss of both cellulase and xylanase activities. The number of nucleotides deleted from each sub-clone was determined by digestion with *Pvu*II and measurement of the reduction in size of the 2.0 kb fragment that spans the deletion point (refer to Fig. 1). Deletions of approximately 120 bp and 320 bp resulted in an increase in endoglucanase and xylanase activity, as judged by halo diameter. Removal of a further 270 bp resulted in the complete loss of both enzyme activities. It was concluded that both activities produced from this DNA fragment were derived from the same protein-coding sequence.

DISCUSSION

The current model for digestion of crystalline cellulose involves the co-ordinated action of three enzymes: endoglucanase, exoglucanase and cellobiase (Enari, 1983). This hypothesis was supported by the work of Wong *et al.* (1988), who inserted the endoglucanase and exoglucanase genes of *Cellulomonas fimi* into *Saccharomyces cerevisiae* and were able to demonstrate the digestion of filter-paper and treated wood-chips by the altered yeast. As predicted, the digestive efficiency was enhanced by the presence of cellobiase.

Bacteroides ruminicola can use cellobextrins as a carbohydrate source (displaying a form of exoglucanase activity) and can ferment cellobiose (cellobiase activity), but cannot digest cellulose (Russell, 1988). Isolate AR20 of *B. ruminicola* subsp. *brevis* is similar in this respect (J. F. Hudman, unpublished). Therefore, the presence of an endoglucanase gene in this species (Russell & Wilson, 1988) is intriguing, since it might be predicted that the presence of these three activities would impart the ability to digest cellulose. A possible explanation for this apparent paradox is that the exoglucanase which allows *B. ruminicola* to digest cellobextrins, and the endoglucanase detected here, may differ from those required for the degradation of crystalline cellulose in properties not yet determined. Important questions such as this may only be answered when it is possible to perform enzymic complementation tests by introducing novel genetic material into the bacterium.

Most reports dealing with cloned endoglucanases or xylanases from rumen bacteria (Taylor *et al.*, 1987; Sipat *et al.*, 1987; Kawai *et al.*, 1987) have described enzymes which digest cellulose or xylan, but not both. Nevertheless, the presence of both xylanolytic and cellulolytic capabilities within cloned enzymes, from rumen bacteria, has been reported previously (Ware *et al.*, 1989), and enzymes capable of both types of activity have been isolated from non-rumen species including *Clostridium acetobutylicum* (Lee *et al.*, 1987), *Clostridium thermocellum* (Hall *et al.*, 1988), and *Cellulomonas fimi* (Gilkes *et al.*, 1984).

The structural similarity of substrates composed of β -1,4-linked glucopyranose residues and β -1,4-linked xylopyranose residues suggests that an endoglucanase might cleave a xylan molecule if it contained sufficiently long chains of unsubstituted residues. In the present case, the ability to digest both substrates may be due to the use of oat-spelt xylan, which has a different type and extent of substitution from the more frequently used larch-wood xylan (Aspinall, 1980). For the purpose of studying ruminant digestion, it is possible that xylan derived from monocotyledon tissue may provide more relevant results.

It is envisaged that studies of enzymes cloned from fibre-digesting bacteria may contribute to our understanding of plant cell wall digestion, and to the practical aim of making that process more efficient. For the latter purpose, however, the introduction of novel genes into a rumen bacterium could impose a metabolic burden which would selectively disadvantage the organism when reintroduced to the rumen. To prevent this, it will be necessary to ensure that introduced genes will be expressed as they would in their native organism. It is unlikely that regulatory elements which control a gene from a cellulolytic species, such as *Ruminococcus albus*, will function correctly when introduced into *B. ruminicola*. Therefore, to ensure that novel genes are expressed in a manner that is advantageous to the recipient bacterium, it may be necessary to attach the coding sequences of foreign cellulase genes to appropriate regulatory sequences from the host cell. The gene described here may provide control sequences suitable for this purpose.

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