Transcriptional regulation of an endoglucanase and a cellobextrinase gene in *Ruminococcus flavefaciens* FD-1

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A gene which encodes a 35 kDa protein with both carboxymethylcellulase and xylanase activity was cloned from *Ruminococcus flavefaciens* FD-1 and the nucleotide sequence determined. The FD-1 gene, *celE*, and the *celA* gene, which encodes a cellobextrinase, were used as probes to analyse transcription in *R. flavefaciens* grown under different conditions. Transcription of both genes was induced when cellulose was added to cells growing in cellobiose. This induction continued after cellulose depletion and after cell division had ceased. Transcription of both genes was also induced by cellotriose, although the effect was not as pronounced as induction by cellulose and was greater for the *celA* gene than for the *celE* gene.

Introduction

*Ruminococcus flavefaciens* is one of the most important cellulolytic bacterial species in the rumen (Bryant, 1973; Halliwell & Bryant, 1963). It degrades crystalline cellulose by the combined action of endo-β-1,4-glucanase, exo-β-1,4-glucanase and cellobextrinase activities (Pettipher & Latham, 1979; Gardner et al., 1987; Rasmussen et al., 1988). In an early report on the regulation of cellulases in *R. flavefaciens* and *R. albus*, Fusee & Leatherwood (1972) concluded that cellobiose appeared to repress enzyme synthesis. However, further studies reported that cellulases were produced constitutively and that cellobiose inhibited enzyme activity but did not repress enzyme synthesis (Hiltner & Dehority, 1983; Pettipher & Latham, 1979; Smith et al., 1973). More recently, Gardner et al. (1987) reported that cellobiose inhibited the catalytic activity of an exoglucanase from *R. flavefaciens* FD-1. Rasmussen et al. (1988) also showed that methylcellulose, which is resistant to biological degradation in anaerobic environments, acts as a competitive inhibitor of endo- and exocellulase but not of cellobextrinase activity.

Flint et al. (1991) have shown that expression of two xylanase genes from *R. flavefaciens* 17 is induced by xylan. Recently, Doerner et al. (1992) reported that cellulose induced the expression in *R. flavefaciens* FD-1 of an endoglucanase and a bifunctional cellulase/xylanase gene but not of a cellobextrinase gene or a gene encoding an enzyme with carboxymethylcellulase (CMCase) and xylanase activity. However, it is unclear how insoluble cellulose induces the transcription of cellulase genes. We were interested to investigate whether cellobiose and cellotriose, the main products of cellulose degradation by cellulases of *R. flavefaciens* FD-1 (Rasmussen et al., 1988), affected the transcription of cellulase genes. We therefore cloned a gene which encoded an enzyme with CMCase and xylanase activity and used it, together with a previously cloned cellobextrinase gene (Wang & Thomson, 1990), as probes to investigate RNA levels in cells grown on cellobiose, cellotriose and cellobiose. Our choice of cellobiose was based on the findings that *R. flavefaciens* appears to lack an extracellular β-glucosidase, is unable to metabolize extracellular glucose, but grows well on cellobiose (Rasmussen et al., 1988).

Methods

Bacterial strains, plasmids and growth conditions. *R. flavefaciens* FD-1 was grown anaerobically at 39 °C in non-rumen fluid medium as
described by Caldwell & Bryant (1966), except that 0.4 ml trace element solution and 1 ml vitamin solution were added per 100 ml. In addition, the resazurin and cysteine. HCl, H2O were replaced by indigo carmine (0.0005%, w/v) and dithiothreitol (0.002%, w/v) as indicator and reducing agents respectively. The carbon source was cellulbiose (0.1%, w/v) or ball-milled filtered paper (0.1%, w/v). The Escherichia coli strains MM294 (supE44 hsdR endA1 pro thi) and JM105 (supE endA ble: B15 hsdR4 rpsL1 thi Δlac-proAB), and the E. coli Bacillus subtilis shuttle vector pEBl (Lin et al., 1990) were used. The suicide bacteriophage vector λd47T: Tn5 (b221 rex: Tn5 CI857 Oam29 Pam80) was used for Tn5 mutagenesis (de Bruijn, 1984). DNA digested with Sau3A and fragments of approximately 3 to 10 kb (% HsdR4 rpsLI thi A1ac-proAB), and the R. flavefaciens growth measurements. Cells were counted using a counting chamber under phase contrast microscopy. Optical density readings at 600 nm were taken on a Corning colorimeter 252 under anaerobic conditions.

Recombinant DNA techniques. R. flavefaciens FD-1 DNA was isolated as described previously (Barros & Thomson, 1987), partially digested with Sau3A and fragments of approximately 3 to 10 kb isolated by sucrose gradient centrifugation. All other techniques were according to Sambrook et al. (1989).

Enzyme activity detection and assays. Strains were screened for carboxymethylcellulase (CMCase) and xylanase activity by plating on LB agar containing 0.5% (w/v) medium-viscosity CMC or 0.1% xylan (Sigma), and staining with Congo red according to the method of Teather & Wood (1982). An additional screen was performed by plating on LB and flooding the plates with 4-methylumbelleryl β-D-cellobioside (MUC; Sigma) according to the method of Van Tilbeurgh & Claeysens (1985). To screen liquid cultures, cell-free extracts, prepared by French press (pressure up to 600 p.s.i. (about 4-1 MPa)) from stationary phase cells, were applied to wells (0.4 mm in diameter) in LB agar containing 0.5% CMC or 0.1% xylan; the plates were stained with 0.1% (w/v) Congo red after incubation for 48 h at 37 °C, and destained with 1 M-NaCl. For liquid assays, cells were grown overnight at 37 °C in 200 ml LB, harvested and resuspended in 5 ml 0.2 M-potassium phosphate buffer (pH 6.8). The cell suspension was cooled on ice and extracts prepared in a French press (pressure up to 600 p.s.i.). The cell debris was removed by centrifugation for 15 min at 27,000 g and the extract stored at −20 °C. Cellulase and xylanase activities were determined by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 1959) using CMC (0.5%, w/v) or xylan (0.25%, w/v) as substrates. Activity on p-nitrophenyl β-D-cellobioside (pNPC) was as described previously (Wang & Thomson, 1990).

Analysis of proteins synthesized in vitro. Proteins synthesized in vitro were analysed using the prokaryotic DNA-directed translation kit (Amersham) according to the manufacturer’s instructions, except that half the recommended quantities of all the components were used. The proteins were separated by SDS-PAGE (Laemmli, 1970).

Ts5 mutagenesis. Ts5-mediated transposon mutagenesis was performed by the method of de Brijn & Lupski (1984), except that transposon-carrying JM105(pWFl) cells were selected on LB plates containing 50 μg Km ml−1 and 100 μg Ap ml−1. The cells were washed off, plasmids isolated and transformed into MM294 to screen for CMCase activity.

DNA sequencing. To obtain templates for nucleotide sequencing, the 1.2 kb HindIII fragment of pWFl was subcloned into Bluescript SK (Stratagene), and exonuclease III was used to generate two sets of overlapping deletions of opposite polarity (Henikoff, 1984). Sequencing was done by the chain termination method of Sanger et al. (1977) using a Sequenase kit (United States Biochemicals). The nucleotide and deduced amino acid sequences of the cellulase gene celE, and the encoded enzyme CelE, were analysed using the GenePro software package (Version 4.20; Riverside Scientific). The predicted amino acid sequence of CelE was compared with sequences in the GenEMBL data base using FASTA programs from the Genetics Computer Group sequence analysis package (Devereux et al., 1984).

Isolation of RNA from R. flavefaciens for Northern blot and dot-blot analysis. Late exponential phase cultures of R. flavefaciens, grown in non-rumen fluid medium (as described above) containing cellulbiose (0.1%, w/v) for 24 h, were diluted 1:100 in cellulbiose medium and grown for a further 36 h. Ball-milled filter paper (0.1%, w/v) was then added and the cultures grown for 40 h, by which time the cellulose had been depleted. Cellulbiose (0.1%, w/v) was then added. Control cells were grown in cellulbiose medium alone. Samples were taken at various time intervals. RNA was isolated from the samples according to the method of Aiba et al. (1981), with the following modifications. The buffer in which the RNA precipitate was collected contained DNAase I (final concentration 1 μl−1) and was incubated at 37 °C for 60 min, after which a phenol extraction and ethanol precipitation were performed. The RNA concentration was determined by measuring the absorbance at 260 nm. For Northern blotting, 20 μg samples of each RNA preparation were electrophoresed in 1% agarose/6% formamide gels at 20 to 40 mA with recirculation of running buffer. Gels were transferred onto Hybond-N+ membranes by alkaline blotting procedures with the appropriate RNA alkali transfer buffer (0.05 M-NaOH for 3 h). The sizes were estimated by reference to the migration of DNA molecular mass markers (λ HindIII). For dot-blotting, 5 μg samples were applied to Hybond-N+ membranes using a dot-blotting apparatus. The RNA was fixed onto the membrane by alkaline fixation (0.05 M-NaOH for 5 min). Prehybridization and hybridization were performed essentially according to the procedures recommended by Amersham. Prehybridization was performed in a shaking water-bath at 65 °C for 1 h. The endoglucanase gene probe was a 0.7 kb Psrl–PvuI fragment of pWFl (Fig. 1.). The cellobextrinase gene probe was a 1.2 kb HindIII fragment of pSk1 (Wang & Thomson, 1990). The probes were labelled with [32P]dCTP using a nick-translation kit (Amersham) and 10 d.p.m. labelled probe [6 x 105 d.p.m. (pmol DNA)−1] was hybridized with the membrane at 65 °C for 20 h. Blots were washed in 2 x SSC, 0.1% SDS at 65 °C for 10 min. After autoradiography, the films were scanned with a custom-built densitometer (Retief et al., 1987) for quantification.

Results and Discussion

Cloning and endoglucanase gene and restriction enzyme mapping of the plasmid pWFl

A gene library of R. flavefaciens FD-1 DNA was constructed by partial Sau3A digestion, ligation with BglII-digested pEBl and transformation into E. coli MM294. It was screened on CMC-containing plates and by flooding LB plates with MUC. Of the 6000 clones tested, nine had CMCase activity but none had activity on MUC. Preliminary restriction enzyme maps and xylanase assays showed that five of the inserts carried the celE gene previously cloned on pMEB200 (Wang & Thomson, 1990), three carried the same 3.6 kb insert and had xylanase activity and one carried a different insert
but had only CMCase activity. The CMCase/xylanase-expressing plasmid was called pWFl and was further characterized. A restriction map of the plasmid was prepared (Fig. 1).

Southern blot analysis

To ensure that the cellulase gene, designated celE, on pWFl was from R. flavefaciens FD-1 and to confirm that it was different from celA, FD-1 chromosomal DNA, pWFl and pMEB200 were analysed by Southern gel hybridization. A 32P-labelled 3-5 kb HindIII–PstI fragment of the insert on pWFl was used as a probe. Hybridization was performed in hybridization buffer containing 6× SSC, 0-5% SDS at 68 °C for 16 h (Maniatis et al., 1982). Following hybridization, Hybond-N+ membranes were washed at 68 °C for 30 min (Maniatis et al., 1982). The probe hybridized to the same sized fragment in FD-1 and pWFl DNA cut with the same two enzymes (results not shown). It did not hybridize to pMEB200 DNA, proving that it was a different gene which originated from FD-1. To determine whether the gene was present in other rumen anaerobes, DNA from R. albus 22.08.6A, Butyrivibrio fibrisolvens H17c and R. flavefaciens 17 was subjected to Southern blot analysis using the same probe and same conditions. No hybridization was found (results not shown). This is not too surprising as the relatedness between R. flavefaciens FD-1 and 17 has not been accurately determined. Moreover, there is often not sufficient similarity at the DNA level, even between enzymes from the same cellulase family, for hybridization to occur.

Substrate specificity of CelE and localization of the celE gene on pWFl

The enzyme activity of MM294(pWFl) was determined on a variety of substrates and maximal activity was found on CMC and xylan (Table 1). Various deletions of pWFl were made to determine the localization of the celE gene. pWFl-A, an AccI deletion removing 1.3 kb of the insert and 3.5 kb of the vector, gave activity on both CMC and xylan, albeit at somewhat reduced levels (Table 1). pWF1-SH, a Styl–HpaI deletion removing 1.65 kb of pWFl adjacent to the AP, promoter, resulted in the total loss of CMCase and xylanase activity. It would therefore appear that the celE gene is located in the AP, proximal region of the insert.

To confirm this, saturation Tn5 mutagenesis was performed on pWFl. A total of 33 CMCase+ mutants were isolated and mapped (results not shown). All were located within the AP, promoter of the vector or in the adjacent region of the insert. Thus the celE gene is probably being transcribed by the AP, promoter. We have found that a strong promoter in the vector is often required to obtain good expression in E. coli of genes from rumen anaerobes (Berger et al., 1990; Lin & Thomson, 1991; unpublished observations). It was of interest to note that no Tn5 insertions occurred in the opposite end of the insert. The reason for this is unknown. Further evidence for the involvement of AP, was obtained when a 3-8 kb HindIII fragment of pWFl
### Table 1. Enzyme activity of plasmid-containing E. coli strains

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<th>Plasmid</th>
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<tr>
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<td>pNPC†</td>
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ND, Not detectable.

* p-Nitrophenyl β-d-glucopyranosidase.
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that removed the λ promoter but retained most of the insert, was subcloned into pBluescript SK to generate the plasmid pSK-H. Reduced levels of CMCase activity were seen in strains carrying this plasmid (pSK-H) despite it having a higher copy number than pWF1 as the former is a pUC and the latter is a pBR322 derivative (Sambrook et al., 1989; Table 1).

### Identification of the CMCase encoded by pWF1

Analysis by SDS-PAGE of *in vitro* protein synthesis directed by pWF1 revealed the presence of three protein bands, with apparent molecular masses of ca. 45 kDa, 35 kDa and 24 kDa, which were not synthesized by the vector alone (Fig. 2, lanes 3 and 4). Plasmid pWF1-SH, in which a 3.5 kb fragment of pWF1 adjacent to the ilPr promoter had been deleted, directed the synthesis of the 35 kDa protein. As there was no CMCase or xylanase activity in cells carrying this plasmid, it would appear that the 35 kDa protein displays this activity. Plasmid pWF1-A, in which 1.3 kb of the insert and 3-5 kb of the vector have been deleted, that removed the λ promoter but retained most of the insert, was subcloned into pBluescript SK to generate the plasmid pSK-H. Reduced levels of CMCase activity were seen in strains carrying this plasmid (pSK-H) despite it having a higher copy number than pWF1 as the former is a pUC and the latter is a pBR322 derivative (Sambrook et al., 1989; Table 1).

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**Fig. 3.** Nucleotide sequence of the CMCase gene on pWF1. The derived amino acid sequence is given in the one-letter code from position 1 to 960 (320 residues). The Shine–Dalgarno (SD) sequence is underlined. The positively charged amino acids at the start of the putative signal sequence are indicated by plus signs, and the hydrophobic region is doubly underlined. The inverted repeat is indicated by arrows.**

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**Fig. 2.** SDS-PAGE analysis of proteins encoded *in vitro*. Lanes 1, pWF1-SH; 2, pWF1-A; 3, pWF1 and 4, pEB1. * Proteins encoded by pWF1.

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See in strains carrying this plasmid (pSK-H) despite it having a higher copy number than pWF1 as the former is a pUC and the latter is a pBR322 derivative (Sambrook et al., 1989; Table 1).
Ruminococcus flavefaciens gene regulation

1.0
0.6
0.2
-1.0

12 24 36 48 60 72 84 96
Time (h)

Fig. 4. Growth of FD-1 cells measured as log cell numbers (○, ●) under phase contrast microscopy or OD₆₀₀ (□, ■) under anaerobic conditions. Arrows indicate the addition of 0·1 % cellulose (36 h) and 0·1 % celllobiose (76 h). Filled symbols, cells growing in celllobiose; open symbols, in cellulose. Samples were taken at the times indicated by the symbols.

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Nucleotide sequence of the insert on pWF1

The nucleotide sequence of the 3·6 kb insert of pWF1 carried an open reading frame (ORF) which, from the ATG start codon to the stop codon (TAA), contained 963 nucleotides encoding 320 amino acid residues (Fig. 3). The molecular mass of the predicted polypeptide coded for by this region was 35·9 kDa, which agrees with the observed value of 35 kDa. Although the sequence has a possible ribosomal binding site (GGAGGG) 5 bp upstream of the putative ATG initiation codon, it does not have any identifiable promoter sequences. The stop codon is followed by an inverted repeat sequence. The potential stem/loop structure that could be formed in the resulting mRNA had a ΔG = -33·5 kJ mol⁻¹ (calculated according to Salser, 1977). This is therefore unlikely to be a rho-independent transcription termination sequence. The predicted amino acid sequence of CelE was compared with sequences in the GenEMBL data base using FASTA programs from the Genetics Computer Group sequence analysis package (Devereux et al., 1984). It did not show any significant homology to other cellulases or xylanases.

Regulation of celA and celE gene expression in R. flavefaciens FD-1

R. flavefaciens FD-1 grew well in celllobiose as sole carbon source (Fig. 4). During the late exponential phase of growth (36 h), celllobiose was added and cell numbers increased from 8 × 10⁸ to 1·6 × 10⁹ until the celllobiose was depleted (76 h). At this stage, celllobiose was added and a further slight increase in cell numbers occurred to 2·1 × 10⁹ (88 h), followed by a slight decrease to 1·4 × 10⁹ (92 h). Northern blots of RNA isolated from cells grown in celllobiose for 36 h showed the absence of celA- and celE-specific transcripts (Fig. 5). However, after addition of celllobiose and growth for a further 40 h, celA- and celE-specific transcripts could be detected. The celA and celE probes hybridized to mRNA transcripts from R. flavefaciens FD-1 that were approximately 1100 bp long and were comparable to the sizes estimated from each sequence. RNA dot-blot hybridization showed that RNA homologous to celA and celE was present at very low levels during the first celllobiose growth phase (Fig. 6a, b). Upon addition of celllobiose there was a slow increase in the levels of both RNA transcripts, which reached a peak at 76 h when microscopic examination showed that the celllobiose had been depleted. Celllobiose was added at this point. Thereafter the levels of both RNA transcripts declined somewhat, but were still readily detectable for a further 16 h. In the culture grown only on celllobiose, both RNA transcripts remained at a very low level throughout the 48 h period, by which stage the cells had reached stationary phase (data not shown).

These results are in contrast to the conclusion drawn by Doerner et al. (1992) that celA is constitutively expressed in celllobiose-grown cultures. However, their

Fig. 5. Northern blot analysis using celA (a) and celE (b) as probes. RNA was isolated after 36 h growth in celllobiose (lane 1) and after addition of celllobiose and a further 40 h growth (lane 2). Molecular size markers are indicated on the left. Arrows indicate the presence of the mRNA transcripts. Times of exposure of autoradiographs were 78 h (a) and 136 h (b).
Fig. 6. Dot-blots (a) and relative intensities (b) of RNA extracted from FD-1 cells grown in 0.1% cellobiose with the addition at 36 h of 0.1% ball-milled filter paper (b) and probed with celA (■) or celE (□). RNA was extracted from cells at the indicated time intervals. Times of exposure of autoradiographs were 96 h (celA, ■) and 240 h (celE, □). Dot-blots (c, e) and relative intensities (d, f) of RNA extracted from FD-1 cells grown in 0.1% cellobiose with the addition at 36 h of 0.01% cellobiose (1, □), 0.01% cellotriose (2, □), 0.1% cellobiose (3, □), 0.1% cellotriose (4, ■), and probed with celA (c, d) and celE (e, f). RNA was extracted from cells at the indicated time intervals. Times of exposure of autoradiographs were 96 h (celA, c) and 240 h (celE, e).

data were not quantitative and RNA was extracted 8 h after addition of cellulose. Our results show that induction by cellulose takes from 12 to 24 h and suggest that neither celA nor celE are constitutively expressed at high levels. However, there are presumably other constitutively expressed cellulases which, upon addition of cellulose, degrade it sufficiently to produce cello-dextrins which can be taken up by the cells and induce transcription of these genes. The uptake of cellotriose by *R. flavefaciens* FD-1 has been postulated by Helaszek & White (1991).

When cellotriose was substituted for cellulose, the increase in RNA homologous to celA and celE was detected after only 6 h, although the levels of induction were lower than those induced by cellulose (Fig. 6c, d, e, f). In addition, while induction of celA was detectable in cells grown in 0.01% cellotriose, increases in RNA homologous to celE were only detectable during...
growth in 0.1% cellotriose. This is in keeping with the hypothesis that induction by cellulose requires its breakdown by constitutive enzymes to cellobextrins, including cellotriose, which may then be taken up by cells to induce gene expression. Addition of cellobiore instead of cellulose allowed more rapid uptake of the dextrin and induction of celA and celE, but the lower levels of induction could have a number of explanations. These include the fact that cellobiore is a metabolizable substrate and the possibility that cellobextrins other than cellobiore can be taken up and act as inducers.

In similar studies on induction of cellulase gene expression in Cellulomonas fimi, Greenberg et al. (1987a) have shown that the cenA gene, which encodes an endoglucanase, is transcribed at low levels when the cells are grown in glycerol, at high levels in CMC, but not at all in glucose. In contrast, the cex gene, which encodes an exoglucanase, is transcribed only during growth in CMC. They concluded that the cenA gene was being expressed at a low constitutive level during growth on glycerol and its function, and that of other constitutively expressed endoglucanases, is presumably to generate low molecular mass cellulose-specific degradation products which can act as inducers once a suitable substrate is encountered. They ascribed the lack of transcription during growth in glucose to repression at the transcriptional level by this readily assimilated carbon source. In contrast to the cenA gene, the cenB gene, which also encodes an endoglucanase, was transcribed during growth in glucose, albeit at a low level (Greenberg et al., 1987b).

During growth in cellolbiose alone, levels of both RNA transcripts remained low. This is unlikely to be due to catabolite repression as cellolbiose and cellotriose are the final breakdown products of cellulose by \textit{R. flavefaciens} (Rasmussen et al., 1988), and cellolbiose will therefore be present throughout growth on cellulose. Moreover, addition of cellolbiose after depletion of cellulose did not result in a marked decrease in transcription of the two genes. It is likely that the RNA detected after cellulose depletion is due to continued induction of transcription by the celldextrins present in the medium. It is unlikely to represent stable RNA as, although mRNA turnover has not been measured in \textit{R. flavefaciens}, it would probably not be stable for 16 h. Thus induction of celA and celE expression continues after cessation of cell division.

A number of cellulase genes have been shown to be induced by sophorose (Mandels et al., 1962; Hrmova et al., 1986, 1991; El-Gogary et al., 1989). We have not tested this compound as, in order to produce sophorose from cellulose, a micro-organism must hydrolyse cellulose to glucose and then transglycosylate the glucose (Kubicek, 1987). As there are no reports of $\beta$-glucosidase production by \textit{R. flavefaciens} and the end-products of cellulose breakdown are cellolbiose and cellotriose (Rasmussen et al., 1988; Helaszek & White, 1991), it is unlikely that this bacterium could produce sophorose from cellulose.

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References


