Expression of two xylanase genes from the rumen cellulolytic bacterium *Ruminococcus flavefaciens* 17 cloned in pUC13

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Two distinct xylanase genes (designated *xynA* and *xynB*) were subcloned in pUC13 from non-homologous restriction fragments of *Ruminococcus flavefaciens* 17 DNA originally isolated in λ EMBL3. The products of the two genes showed similar pH optima for hydrolysis of oat spelt xylan (around 5.5) and had little or no activity against carboxymethylcellulose. Trace activities against *p*-nitrophenyl (pNP) cellobioside and pNP-xyloside were detected in clones containing *xynA*, but not in one harbouring *xynB*. The xylanase associated with clones carrying *xynA* produced mainly xylobiose and xylose from xylan and did not give hydrolysis of xylobiose, while that encoded by *xynB* produced mainly xylobiose and higher xylo-oligosaccharides from xylan. There was evidence of increased expression, at the RNA level, of these two genes, and of another cloned region encoding multiple activities including xylanase, in *R. flavefaciens* 17 grown with xylan, as compared with cellobiose, as energy source. Total cell-associated xylanase and β -xylosidase activities, and supernatant xylanase activity, were shown to be similarly induced in xylan-grown *R. flavefaciens*, 17.

Introduction

Two species of obligately anaerobic ruminococci, *Ruminococcus flavefaciens* and *R. albus*, are thought to play important roles in the degradation of plant cell wall polysaccharides in the rumen (Bryant, 1986). Strains of both species have been shown to degrade the hemicellulose and cellulose components of plant cell wall material, although the extent of such degradation can vary widely for different strains and substrates (Dehority & Scott, 1967; Halliwell & Bryant, 1963). Recent molecular approaches have indicated a multiplicity of cellulase genes in strains of *R. albus* (Howard & White, 1988; Ware *et al.*, 1989; Romaniec *et al.*, 1989) and *R. flavefaciens* (Barros & Thomson, 1987; Huang *et al.*, 1989). Ruminococci rely on a range of other polysaccharidases in addition to cellulases for their ability to degrade complex substrates such as plant cell walls, however (Pettipher & Latham, 1979a; Greve *et al.*, 1984). In a previous report we screened a λ EMBL3 library of *R. flavefaciens* 17 DNA for clones expressing xylanase or endoglucanase activities and found that these activities were recovered most often on separate DNA fragments (Flint *et al.*, 1989). Clones isolated by their activity

against xylan showed no detectable carboxymethylcellulase (CMCase) activity and belonged to four different DNA homology groups (Flint *et al.*, 1989). The present report describes the characterization of two of these xylanase genes and their products through subcloning in pUC13. Additional experiments described here aim to demonstrate the potential roles of these and other genes concerned with hemicellulose breakdown in *R. flavefaciens*.

Methods

Strains and growth conditions. *R. flavefaciens* 17 was grown anaerobically at 39 °C following the methods of Bryant (1972) as described previously (Flint *et al.*, 1989). Cells used for RNA extraction were grown in 800 ml of the defined medium of Hungate & Stack (1982) containing 0.2% larch wood xylan or 0.2% cellobiose for 24 h from a 16 ml inoculum derived from a cellobiose-grown culture. *Escherichia coli* HB101 and DH5 α were grown on LB medium at 37 °C unless stated otherwise. 2YTP medium contained (per l) 16 g Bacto-tryptone, 10 g Bacto yeast extract, 5 g NaCl and 5 g KH₂PO₄. Ampicillin (30–100 μ g ml⁻¹) was included for transformants carrying pUC13 derivatives.

Cloning procedures. Subcloning using restricted and alkaline phosphatase treated pUC13 followed standard procedures (Maniatis *et al.*, 1982) except that transformation was achieved by electroporation, using a Bio-Rad Gene pulser fitted with a pulse controller, following the methods of Dower *et al.* (1988). Transformed cells were plated on to LB agar plates in an overlay of LB medium containing 0.5% agar and 100 μ g ampicillin ml⁻¹. Xylanase-positive colonies were detected either

Abbreviations: CMC, carboxymethylcellulose; CMCase, carboxymethylcellulase; pNP, *p*-nitrophenyl; RBB, Remazol Brilliant Blue R.

using a substrate overlay (0.1% larchwood xylan/0.4% agarose in 25 mM-potassium phosphate buffer pH 6.8/100 µg ampicillin ml⁻¹) which was subsequently stained with Congo red (Teather & Wood, 1982), or using plates containing 0.2% 4-O-methyl-D-glucurono-D-xylan Remazol Brilliant Blue R (RBB-xylan).

RNA extractions from *R. flavefaciens*. Cell pellets (wet weights 5–7 g) were washed in 20 ml ice-cold 0.1 M-sodium phosphate buffer (pH 6.8), resuspended in 5 ml distilled water and frozen at –20 °C. Upon thawing, cells were disrupted using a French pressure cell and collected in guanidinium thiocyanate (4 M). RNA was then pelleted from CsCl at 48 000 r.p.m. in a Beckman Ti80 rotor for 16 h at 15 °C following the methods of Chirgwin *et al.* (1979) and its concentration estimated by measurements of A₂₆₀ and by agarose gel electrophoresis.

Nucleic acid hybridizations. Southern transfers were performed as described previously using Genescreen Plus (NEN) filters (Flint *et al.*, 1989). DNA:DNA hybridizations were at 42 °C in the presence of 50% (v/v) deionized formamide, 1% SDS, 10% (w/v) dextran sulphate, 1 M-NaCl and 10 µg denatured salmon sperm DNA ml⁻¹. Moderately stringent washing conditions (60 min at 60 °C in 0.3 M-sodium chloride/0.03 M-sodium citrate) were used. For 'dot blot' hybridizations, RNA was denatured at 50 °C in 50% deionized formamide/6% (v/v) formaldehyde and loaded onto Genescreen Plus Filters (NEN) using the manufacturer's recommended procedures. Hybridization in the presence of ³²P-labelled probe DNA was for 40 h at 42 °C as described previously (Flint & Wilkenning, 1986).

Enzyme assays. *E. coli* cell extracts were obtained as follows. A 5 ml culture was grown for 7 h, and used to inoculate five further tubes containing 5 ml medium (0.1 ml inoculum per tube) which were incubated for a further 17 h. Cells were recovered by centrifugation (5000 g 10 min), resuspended in 0.08 times the original culture volume of ice-cold 50 mM-sodium phosphate buffer (pH 6.5) containing 2 mM-dithiothreitol and disrupted by sonication (5 × 1 min, MSE Soniprep). The use of milder sonication regimes, or of osmotic shock procedures, resulted in less total xylanase activity being released. Reducing sugar release was determined by the method of Lever (1977), using 1% of the appropriate polysaccharide substrate in 50 mM-sodium phosphate buffer (pH 6.5) containing 2 mM-dithiothreitol. In a comparison using HB101(X103) cell sonicates the Lever method gave values for xylanase activity approximately twofold lower than the Somogyi–Nelson method (Somogyi, 1952). Assays with pNP derivatives were as described previously (Flint *et al.*, 1989). Protein was determined by the Lowry method.

For the assay of activities in *R. flavefaciens* cultures, samples were collected under 100% CO₂ and centrifuged in sealed bottles at 3000 g for 15 min at 4 °C. Supernatants were assayed directly, while cells were resuspended in 0.08 vols ice-cold 0.05 M-sodium phosphate buffer, treated with 0.1% toluene and used immediately for enzyme assays.

The oat spelt and larchwood xyans used here were obtained from Sigma. Analysis showed the oat spelt xylan comprised at least 75% xylose and 7% arabinose and around 1% glucose, while the corresponding figures for larchwood xylan were 51%, 6% and 12%.

Analysis of products of xylan hydrolysis. Incubations were performed as above, with 0.1% oat spelt xylan as substrate, in the presence of approximately 0.2 units xylanase activity ml⁻¹. After treatment with acid ethanol and concentration, as described by Whitehead & Hespell (1990), hydrolysis products were analysed by thin-layer chromatography using Whatman K5 silica plates, run with chloroform/methanol/acetic acid/distilled water (50:50:15:5, by vol.), and spots visualized by spraying with 0.2% orcinol as described by Fontana *et al.* (1988).

Polyacrylamide gel electrophoresis. SDS-PAGE was carried out according to the method of Laemmli (1970) using Dalton VII molecular

mass markers (Sigma). Zymogram techniques using RBB-xylan overlays followed the methods of Biely (1988). For the application of zymogram techniques to SDS gels, sample pretreatment was for 15 min at 45 °C and proteins were renatured as described by Morris & Cole (1987).

Results

*Isolation of two xylanase genes from *R. flavefaciens* 17*

DNA fragments encoding xylanases were subcloned from two λ EMBL3 clones, X4 (14 kb) and X10 (13 kb) isolated previously (Flint *et al.*, 1989). The xylanase genes isolated from these are designated *xynA* (from X4) and *xynB* (from X10). Phage DNA was partially restricted with *Hind*III and ligated with phosphatase-treated *Hind*III-cut pUC13. Ligation mixtures were used to transform *E. coli* HB101 by electroporation and clones expressing xylanase activity were identified as described in Methods. Two transformants (X4129 and X469) harboured recombinant plasmids in which the same 3.6 kb *Hind*III fragment carrying *xynA* was inserted in opposite orientations in pUC13, indicating the presence of a *Ruminococcus* sequence acting as a promoter in *E. coli* (Fig. 1). A shorter clone (X4509, insert 2.5 kb) retaining xylanase activity was subsequently obtained after treating X4129 DNA with *Pst*I, religating and transforming *E. coli* DH5α. Both X4509 and X469 showed xylanase activities more than 10-fold lower than X4129 (Table 1) when assayed in sonicated cells (results not shown). A single xylanase-positive transformant (X103) containing the *xynB* xylanase gene from X10 (Fig. 1) was isolated.

DNA hybridization

Hybridization of ³²P-labelled DNA from plasmids carrying *xynA* or *xynB* to *Hind*III-digested chromosomal DNA from *R. flavefaciens* 17 confirmed that *Hind*III fragments of sizes similar to those in the cloned insert (3.6 kb for X4129, 1.1 and 0.9 kb for X103) were present (Fig. 2). In addition several other chromosomal restriction fragments hybridized in each case. Repeat experiments showed that these did not result from partial digestion, and it therefore appears that sequences homologous with regions of each clone are present at more than one location in the *R. flavefaciens* genome. The same multiple banding pattern was obtained when X4509 DNA was used as a probe in place of X4129 (not shown), indicating that the sequences involved in this case lie within the 2.5 kb fragment subcloned in X4509 (Fig. 1).

Fig. 1. Restriction enzyme maps of the pUC13 clones X4129 (carrying *xynA*) and X103 (carrying *xynB*) (see text). X4509 was derived from X4129 by deletion of a 1.1 kb *Pst*I fragment. Regions shown in black represent flanking vector DNA. E, *Eco*RI; H, *Hind*III; P, *Pst*I; B, *Bam*HI; Bg, *Bgl*II; Sc, *Sca*I. No sites were present within the insert for *Sma*I, *Sal*I or *Bsc*I.

Fig. 2. Hybridization of ³²P-labelled plasmid DNA from X4129 (*xynA*) (a) and X103 (*xynB*) (b) to 1 µg *Hind*III-cut chromosomal DNA from *R. flavefaciens* 17 (track 2 in each case). Track 1 contained approximately 8 ng *Hind*III-cut X4129 (a) or X103 (b) DNA as a control. Fragment sizes are given in kb.

Substrate specificities

Enzyme activities shown in Table 1 were determined on sonicated cell extracts. *xynA* (X4129) and *xynB* (X103) cell extracts both showed appreciable activity against larchwood and oat spelt xylan, with little or no associated activity against CMC or lichenan. Very low levels of activity against crystalline cellulose (Avicel) were detected in extended incubations [around 0.4 and 0.1 nmol min⁻¹ (mg protein)⁻¹ for *xynA* and *xynB*, respectively].

Table 1. Substrate specificities of two xylanase genes from *R. flavefaciens* 17 expressed in *E. coli* HB101(pUC13)

Activities were determined on sonicated extracts of whole cells as described in Methods, and represent the means of duplicate assays.

Substrate	Enzyme activity [nmol min ⁻¹ (mg protein) ⁻¹]	
	Clone:	
	X4129 (<i>xynA</i>)	X103 (<i>xynB</i>)
Xylan (larchwood)	870	210
Xylan (oat spelt)	690	150
CMC	5	2
Avicel	<1	<1
Phosphoric acid-swollen cellulose	<1	<1
Lichenan	<1	1
pNP-cellobioside	7.3	0.05
pNP-glucoside	0.3	0.2
pNP-xyloside	1.4	0.1

xynA, but not *xynB*, also showed a low level of activity against pNP-cellobioside and pNP-xyloside. It has not been possible to establish whether these trace activities are similarly associated with extracts from the other clones harbouring *xynA* (X4509, X469) because of the much lower levels of activity (mentioned above) obtained from these clones.

Hydrolysis products from oat spelt xylan were analysed by thin-layer chromatography, as described in Methods. Extracts of *xynA* cells gave only two distinct spots, corresponding to xylose and xylobiose/arabinose, after 24 h digestion (Fig. 3). Xylobiose was not hydrolysed (not shown). In contrast, *xynB* cell extracts gave rise to xylo-oligosaccharides equal to, or larger than, xylobiose in size, with little or no xylose being produced after either 3 h or 24 h incubation. The final, asymptotic level of reducing sugar release in these incubations was attained after 3 h with *xynB* and was approached at 24 h with *xynA*. The asymptotic value for *xynA* was approximately four times that for *xynB* (not shown); this may be largely due to the longer chain length of the end-products generated by *xynB* hydrolysis.

As shown in Fig. 4, the pH optimum for xylan hydrolysis was similar for *xynA* and *xynB*, at around 5.5, although *xynA* gave activity over a slightly broader pH range. Xylanase activities were approximately 50% higher at pH 7.5 in sodium borate (18 mM) buffer than with sodium phosphate or phosphate/citrate buffers (not shown).

Xylanase activities were similar for both clones whether cultures were grown in LB or 2YTP medium. On the other hand, enzyme activities were reduced (eight fold or more) in cultures harbouring *xynA* grown for 24 h

Fig. 3. Products of the hydrolysis of oat spelt xylan by sonicated extracts of HB101(X4129) (*xynA*) (lanes 4 and 5: 3 h and 24 h incubation) and HB101(X103) (*xynB*) (lanes 6 and 7: 3 h and 24 h incubation) analysed by thin-layer chromatography. Lanes 1, 2 and 3 were loaded with standard solutions of xylose, arabinose and xylobiose, respectively.

Fig. 4. pH dependence of hydrolysis of oat spelt xylan by sonicated extracts of HB101(X4129) (*xynA*) (●) and HB101(X103) (*xynB*) (○). Assays were performed as described in Methods, but using a citrate (114 mM)/Na₂HPO₄ (160 mM) buffer system.

in LB medium containing 0.2% glucose. This effect occurred with the gene in either orientation (in X4129 or X469) but was not observed in exponentially growing cultures, or in cultures grown in 2YTP/glucose medium (results not shown).

Fig. 5. SDS-PAGE separation of total cell proteins from *E. coli* HB101 carrying pUC13 (lane 2), X4129 (lane 3) or X103 (lane 4). Lane 1 contained molecular mass markers (sizes in kDa). Arrows indicate the positions of additional bands in HB101(X4129) (see text).

Fig. 6. 'Dot blot' hybridization of total RNA from *R. flavefaciens* 17 grown for 24 h in defined medium with 0.2% larchwood xylan (X) or 0.2% cellobiose (C) as substrate. Loadings were (from top to bottom) 5, 1, 0.2, 0.04, 0.008 and 0 μg RNA, and approximately 50 ng unlabelled probe DNA. Probes were X103 DNA (a) and an 11.5 kb *SalI* fragment from the λ EMBL3 clone L9 (b).

Table 2. *Effect of growth substrate on polysaccharidase activities in R. flavefaciens 17*

Cultures were grown on defined medium (Hungate & Stack, 1982) containing the carbohydrates indicated at 0.1%. Inocula were from a culture grown in medium containing 0.2% cellobiose. The 17 h and 40 h values are based on samples taken from single 500 ml cultures (see Methods), and represent means of duplicate assays. Duplicate values differed by less than 20%.

Growth period	Growth substrate	Cell protein [$\mu\text{g (ml culture)}^{-1}$]	Cell-associated activities [$\text{nmol min}^{-1} (\text{mg cell protein})^{-1}$]					Total culture xylanase
			Xylanase	CMCase	pNP-cellobiosidase	pNP-glucosidase	pNP-xylosidase	
17 h	Xylan	18	515	51	32	26	24	1480
	Cellobiose	16	128	80	37	37	3	480
	Xylan + cellobiose	27	191	112	76	41	3	690
40 h	Xylan	30	624	69	36	36	42	1860
	Cellobiose	50	78	68	23	23	0.7	360
	Xylan + cellobiose	66	407	100	97	32	44	1940

Electrophoresis of cloned gene products

Additional polypeptide bands of around 30 kDa and over 100 kDa were detected repeatedly in SDS-PAGE comparisons of total cell proteins from HB101(X4129) (*xynA*), compared with HB101(pUC13) (Fig. 5). No additional bands were detected for HB101(X103) (*xynB*). Zymograms derived from SDS-PAGE gels revealed a single diffuse activity band of molecular mass between 20 and 30 kDa for X103 (*xynB*). X4129 (*xynA*) gave a band of activity at around 30 kDa, but activity was also associated with material of higher molecular mass (results not shown). This may indicate proteolytic cleavage or incomplete denaturation of the cloned gene product, but the possibility that a second xylanase gene is present in X4129 cannot be excluded.

Regulation of xylanase gene expression in *R. flavefaciens* 17

R. flavefaciens 17 grew well with larchwood xylan as sole energy source. Xylanase activity was three to five-fold higher and β -xylosidase activity at least eightfold higher in cultures grown with xylan compared to cellobiose, whereas CMCase activity showed little change (Table 2). Supernatant and cell-associated xylanase activity responded similarly, with between 66% and 80% of the activity being present in the culture supernatant, while β -xylosidase activity was not detected in supernatants. Addition of cellobiose to xylan-containing medium resulted in delayed induction, particularly of β -xylosidase activity (Table 2). The regulatory changes seen in Table 2 occurred despite the presence of some glucose-containing material in the larchwood xylan used in these experiments (see Methods).

Total RNA was prepared from cells grown on xylan or on cellobiose and probed with ^{32}P -labelled plasmid DNA carrying *xynA* or *xynB* to examine the expression of these cloned genes. As shown in Fig. 6, RNA homologous with *xynB* (X103) DNA was present at a higher concentration in cells of *R. flavefaciens* grown on xylan than in cells grown on cellobiose. Similar results were obtained using hybridization probes carrying *xynA* (not shown) and an 11.5 kb insert from the multifunctional λ EMBL3 clone L9 which was shown previously to encode xylanase, β -xylosidase and β -(1,3-1,4)-glucanase activities (Flint *et al.*, 1989) (Fig 6). Analysis of RNA hybridizing with the L9 probe after Northern blotting showed a size range from just over 3000 to around 300 bases (not shown), indicating mRNA degradation *in vivo* or *in vitro*.

Discussion

Among rumen bacteria, genes coding for xylanases have been isolated previously from the cellulolytic species *Bacteroides succinogenes* (Sipat *et al.*, 1987) and from *Bacteroides ruminicola* (Whitehead & Hespell, 1989) and *Butyrivibrio fibrisolvens* (Mannarelli *et al.*, 1990), while a β -xylosidase gene has been recovered from *Butyrivibrio fibrisolvens* (Sewell *et al.*, 1989). Genes encoding these activities were shown to be present in *R. flavefaciens* by cloning in bacteriophage λ (Flint *et al.*, 1989) but had not previously been isolated in plasmid vectors. The present work identifies two distinct xylanase genes whose products differ in their substrate specificities and hydrolysis products. The *xynA* enzyme showed a low level of associated activity against pNP-cellobioside and pNP-xyloside, and produced xylose in addition to

xylobiose as a major hydrolysis product from oat spelt xylan. Since this xylanase did not cause hydrolysis of xylobiose, xylose must presumably be released by the action of the enzyme on larger oligosaccharides. There are several well-documented examples of single gene products from bacteria showing both xylanase and pNP-cellobiosidase activities (Gilkes *et al.*, 1984; Lüthi *et al.*, 1990; Grépinet *et al.*, 1988). In contrast to *xynA*, the *xynB* xylanase produced xylobiose and higher xylo-oligosaccharides but no xylose from oat spelt xylan. Thus the two enzymes may play distinct roles in xylan hydrolysis.

At least two further genes encoding xylanase activity and a separate β -xylosidase gene were identified previously through λ EMBL3 cloning. An 11.5 kb DNA fragment cloned in the recombinant phage L9 has been inferred to carry separate genes for xylanase, β -xylosidase and $\beta(1,3-1,4)$ -glucanase activity (Flint *et al.*, 1989). A role for this region in xylan degradation is supported by the observation made here that RNA homologous with L9 DNA is considerably increased in *R. flavefaciens* 17 cells grown with xylan as compared to cellobiose (Fig. 6), although it is not known which genes were mainly responsible for the RNA changes. Multiple xylanase genes have been demonstrated previously in *Pseudomonas fluorescens* subsp. *cellulosa* (Gilbert *et al.*, 1988).

The products of genes isolated from ruminococci on the basis of their CMCase activity have also been found to exhibit xylanase activity, but the activities that have been reported were at least 10-fold lower in *E. coli* than those observed here (Romaniec *et al.*, 1989; Huang *et al.*, 1989; Ware *et al.*, 1989). The enzymes examined by Ware *et al.* (1989) apparently gave appreciable activity only with xylan that had been solubilized with NaOH.

The reported effects of glucose supplementation of *E. coli* cultures on the expression of cloned genes have been variable, but include stimulation of expression of a cellulase gene from *R. flavefaciens* (Barros & Thomson, 1987). Reduced expression of the *xynA* xylanase was sometimes observed here in glucose-supplemented *E. coli* cultures. Glucose repression of transcription from the *lacZ* promoter cannot account for this effect entirely, because reduced expression occurred with *xynA* cloned in either orientation in pUC13. Furthermore, reduced expression occurred only during the later stages of growth on LB (not 2YPT) medium plus glucose. Involvement of regulatory elements in the *Ruminococcus* insert cannot be ruled out in view of our evidence that expression of these genes is regulated in *R. flavefaciens*. On the other hand, the reduced expression may result from changes in the stability or activity of the gene product, as suggested by Ohmiya *et al.* (1988), rather than changes in the rate of enzyme synthesis.

Hybridization of fragments carrying *xynA* and *xynB* to multiple bands in *Hind*III-cut *R. flavefaciens* DNA is reminiscent of observations reported with certain *Clostridium thermocellum* genes (Hazlewood *et al.*, 1988; Béguin *et al.*, 1988) and may reflect the presence of sequences either linked to or contained within each xylanase gene that are repeated several times in the genome. Little cross-homology was found here between clones carrying *xynA* or *xynB*, or previously between four xylanase-encoding regions from *R. flavefaciens* 17 cloned in EMBL3 (Flint *et al.*, 1989). On the other hand, it is possible that 'domain shuffling' (West *et al.*, 1989) may have led to certain homologous coding sequences being present in genes encoding different functions.

The two xylanase genes identified here seem likely to play a significant role in substrate degradation, since they both showed induced expression at the RNA level in *R. flavefaciens* 17 growing on xylan. Xylanase and β -xylosidase activities were shown here to be subject to induction in xylan-grown *R. flavefaciens* 17, whereas CMCase activity changed little. Such regulation does not appear to have been demonstrated previously in *R. flavefaciens*, although regulation of extracellular polysaccharidases in response to the growth substrate has been reported in *R. albus* 8 (Greve *et al.*, 1984) and there is evidence for a degree of catabolite repression of xylanase and CMCase activities in cellobiose-grown *R. flavefaciens* (Pettipher & Latham, 1979b).

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