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A novel polysaccharide hydrolase cDNA (*celD*) from *Neocallimastix patriciarum* encoding three multi-functional catalytic domains with high endoglucanase, cellobiohydrolase and xylanase activities

GANG-PING XUE,* KARI S. GOBIUS and COLIN G. ORPIN

CSIRO Division of Tropical Crops and Pastures, 306 Carmody Road, St Lucia, Qld 4067, Australia

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A plant polysaccharide hydrolase cDNA, designated *celD*, was isolated from a cDNA library of the rumen fungus *Neocallimastix patriciarum*. The enzyme encoded by *celD* had endoglucanase, cellobiohydrolase and xylanase activities. Deletion analysis revealed that *celD* cDNA can be truncated to code for three catalytically active domains. Each domain had the same substrate specificity as the enzyme produced by the untruncated *celD* and also possessed cellulose-binding capacity. Substrate competition studies showed that carboxymethylcellulose and xylan appear to compete with methylumbelliferyl cellobioside for the same active site within each domain. Expression of *celD* transcript in the rumen fungus was constitutive and was not affected by the presence of cellulose in the culture medium.

Introduction

Cellulose is one of the most abundant polysaccharides in nature and consists of a polymer of glucose linked by β-1,4-glucosidic bonds. Conversion of cellulose to simple sugars (cellobiose and glucose) involves at least two types of hydrolases: endoglucanases, which hydrolyse internal β-1,4-glucosidic linkages in less ordered regions of cellulose, and exoglucanases (mainly cellobiohydrolases), which cleave cellobiosyl units from non-reducing ends of cellulose chains. Xylan, similar in structure to cellulose, consists of a backbone of β-1,4-linked xylose units. The enzymic cleavage of β-1,4-xylosidic linkages is performed by endo-β-1,4-xylanases (xylanases). These three types of enzymes usually exist separately as individual proteins with unique substrate specificity.

Many endoglucanases cleave only internal β-1,4-glucosidic linkages, producing rapid depolymerization of a model substrate, carboxymethylcellulose (CM-cellulose); whereas cellobiohydrolases are able to hydrolyse crystalline cellulose and methylumbelliferyl cellobioside (MUC) and have no or little depolymerizing activity against CM-cellulose. Similarly, many xylanases exclusively attack β-1,4-xylosidic linkages. However, not all polysaccharide hydrolases have strict substrate specificity. Due to the similarity in the chemical nature of the substrates, cross-specificity occurs not only between two types of cellulases, but also between cellulases and xylanases. A large number of cloned cellulases from bacteria have been reported to possess some residual xylanolytic activity (usually $<1\%$) or vice versa (Saarilahti *et al.*, 1990; Yague *et al.*, 1990; Hazlewood *et al.*, 1990; Flint *et al.*, 1991; Taylor *et al.*, 1987). These hydrolases may not be defined as truly multi-functional enzymes. A few polysaccharide hydrolases possessing considerable levels of activities of both endoglucanases and exoglucanases or xylanases have also been described from gene cloning studies (Gilkes *et al.*, 1984; Hamamoto *et al.*, 1990; Foong *et al.*, 1991). The cross-specificity of a polysaccharide hydrolase is sometimes due to the presence of two different catalytic domains. Saul *et al.* (1990) have unambiguously shown that a cellulase from *Caldocellum saccharolyticum* contains one catalytic domain for hydrolysis of CM-cellulose and another for MUC. This type of cellulase is likely to have evolved from the gene fusion of an endoglucanase and an exoglucanase. Recently, Foong *et al.* (1991) reported a bifunctional enzyme of which endoglucanase and xylanase activities are located in the same region of the gene. However, whether the enzyme contains different active sites within a catalytic domain has yet to be determined.

* Author for correspondence. Tel. (07) 3770403; fax (07) 3713946.

Abbreviations: CM-cellulose, carboxymethylcellulose; MUC, methylumbelliferyl cellobioside; MUG, methylumbelliferyl β-D-glycopyranoside; pNPC, *p*-nitrophenyl β-D-cellobioside; pNPG, *p*-nitrophenyl glucopyranoside.

Recent studies, based on partial enzyme purification, showed that rumen anaerobic fungi such as *Neocallimastix frontalis* might produce multi-functional polysaccharide hydrolases (Gomez de Segura & Fèvre, 1991; Li & Calza, 1991). Multi-functional polysaccharide hydrolases are of particular interest in genetic manipulation of rumen bacteria to enrich for the lignocellulose-degrading capacity. Simultaneous enhancement of endoglucanase, cellobiohydrolase and xylanase activities would facilitate the disruption of the complex structure of lignocellulose, of which cellulose and xylan are the major components. It may also circumvent the rate-limiting problem which often occurs when only one of a complex of enzymic reactions is enhanced.

We are investigating the existence of multi-functional polysaccharide hydrolases in the rumen fungi and attempting to elucidate the molecular structure which confers the multiple substrate specificity of the hydrolases in the fungi by a molecular biological approach. Here we report isolation of a cDNA from the rumen fungus, *Neocallimastix patricarium*, encoding a highly active plant polysaccharide hydrolase. The cDNA contains sequences coding for three catalytic domains and each domain possesses endoglucanase, cellobiohydrolase and xylanase activities.

Methods

Microbial strains, vectors and culture media. The anaerobic fungus *Neocallimastix patriciarum* (type species) was isolated from a sheep rumen by Orpin & Munn (1986). Two different media, medium A (a semi-defined medium) and medium B (containing rumen fluid) were used for the growth of *N. patriciarum* as described previously (Xue *et al.*, 1992). Host strains for cDNA cloning were *Escherichia coli* PLK-F′ and XL1-Blue (Stratagene). *E. coli* strains were grown in L-broth (Sambrook *et al.*, 1989). λZAPII vector was obtained from Stratagene and the recombinant phage were grown in *E. coli* strains according to the supplier's instructions.

General recombinant DNA techniques. Isolation of RNA from *N. patriciarum* and purification of poly(A)$^+$ were described previously (Xue *et al.*, 1992). DNA isolation, restriction endonuclease digestion, ligation, transformation and preparation of RNA probes were performed according to the procedures described by Sambrook *et al.* (1989). Northern and Southern blot analyses were described previously (Xue & Morris, 1992; Xue *et al.*, 1992).

Construction and screening of the N. patriciarum cDNA library. Double-stranded cDNA was synthesized from mRNA isolated from *N. patriciarum* grown for 48 h on medium B containing 1% (w/v) Avicel and ligated with λZAPII using a ZAP-cDNA synthesis kit, according to the manufacturer's instructions (Stratagene). A cDNA library of 10^6 recombinants was obtained. Recombinant phage were screened for polysaccharide hydrolase activity by plating in 0·7% (w/v) soft agar overlays containing one of the following substrates: 0·5% (w/v) CM-cellulose, 1 mM-MUC or 0·1% (w/v) xylan. Isopropyl β-D-thiogalactopyranoside (IPTG; an inducer for *lacZp* controlled gene expression) was also included in the soft agar, at a concentration of 10 mM. Hydrolysis of CM-cellulose and xylan was detected by the Congo-red staining procedure (Teather & Wood, 1982). MUC hydrolysis was examined for fluorescence under UV light. The cDNA inserts in polysaccharide-hydrolase-positive phage were recovered in the form of pBluescript SK(−) by *in vivo* excision, according to Stratagene's instructions.

Construction of deletion mutants. Deletion of *celD* cDNA was achieved either by removing a cDNA fragment with restriction enzymes or by exonuclease III digestion (Sambrook *et al.*, 1989). The truncated *celD* cDNA was checked either by restriction mapping or by partial nucleotide sequencing at the insert terminals using the dideoxynucleotide method (Tabor & Richardson, 1987).

Enzyme assays, cellulose-binding studies and product identification. E. coli cells harbouring the recombinant plasmids were grown to the end of the exponential phase in the presence of 1 mM-IPTG. Crude cell lysates prepared according to Schwarz *et al.* (1987) were used for enzyme assays. For quantitative assays, enzyme activities on various cellulosic substrates and xylan were measured at 39 °C in 50 mM-sodium citrate buffer (pH 5·7) as described previously (Xue *et al.*, 1992). The cell lysate prepared from *E. coli* harbouring non-recombinant pBluescript was used as control. Protein concentrations were determined by a dye-binding assay using a Bio-Rad protein assay kit II, according to the supplier's instructions. Qualitative assays were performed using 0·8% (w/v) agarose gel plates containing 0·2% (w/v) CM-cellulose, lichenan, laminarin or xylan or 1 mM-MUC in 50 mM-sodium citrate pH 5·7. Hydrolysis zones were detected as described above.

For assays of cellulose-binding capacity of the cloned cellulase, cell lysates were incubated with 200 µl of pre-washed 5% (w/v) Avicel in 50 mM-sodium citrate (pH 5·7) at 0 °C with continuous shaking for 1 h. The unbound protein was removed after centrifugation and the Avicel pellet was washed three times with 50 mM-sodium citrate (pH 5·7). The bound cellulase was assayed for enzyme activity as above.

Analysis of hydrolysis products of cellulosic substrates was performed using spin-dialysed enzyme preparations as described previously (Xue *et al.*, 1992). The dialysed enzyme was incubated at 39 °C in 50 mM-sodium citrate (pH 5·7) with 1% (w/v) Avicel for 20 h or with 2 mg ml^{-1} cellodextrins containing 3–5 glucose units for various times in order to examine the intermediate and end hydrolysis products. Hydrolysis products of cellulosic substrates were identified by thin-layer chromatography (TLC) using a silica gel plate and a solvent system of ethyl acetate/water/methanol (8 : 3 : 4, by vol.). The positions of sugars on the plate were visualized by spraying with the diphenylamine reagent as described by Lake & Goodwin (1976) and authentic cellodextrins (Merck) were used for identification.

Substrate competition assay. An agarose gel (0·8%, w/v) plate containing 0·005% (w/v) MUC in 50 mM-sodium citrate (pH 5·7) was used as control. Competing substrates (1%, w/v, CM-cellulose or 1%, w/v, xylan) were incorporated into MUC-containing agarose plates. An aliquot of enzyme extract was placed in a small well cut in the substrate plates. After incubation at 37 °C for 20 min, the intensity of MUC hydrolysis between the substrate plates with or without competing substrates was compared under UV light.

Results and Discussion

Isolation of a multi-functional polysaccharide hydrolase cDNA from a N. patriciarum cDNA expression library

A cDNA library was prepared from poly(A)$^+$ RNA isolated from *N. patriciarum* grown on Avicel as the sole carbohydrate source and was constructed in *E. coli* using

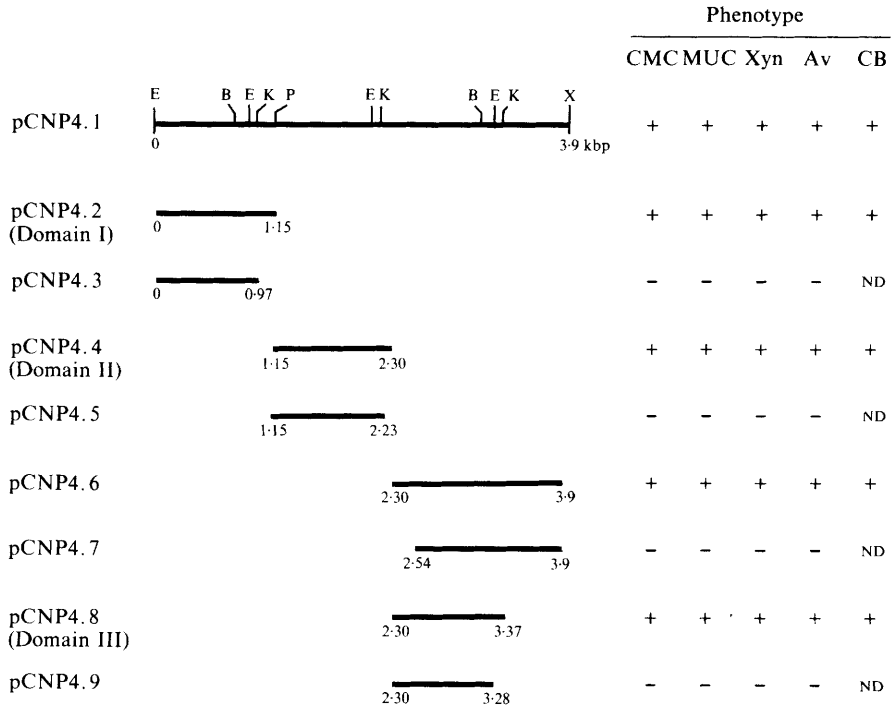

Fig. 1. Restriction map of *celD* cDNA and its deletion mutants. The positions of the cleavage sites of *Eco*RI (E), *Bgl*II (B), *Kpn*I (K), *Pvu*II (P) and *Xho*I (X) are shown. The positions of deletion mutants of *celD* are indicated by solid bars and numbers in kbp corresponding to the positions in pCNP4.1. The enzyme activity of the clones was determined on substrate-containing agarose gel plates and cellulose-binding capacity was determined with Avicel: +, active; −, inactive; ND, not determined. CMC, CM-cellulose; Xyn, xylan; Av, Avicel; CB, cellulose-binding.

a λZAPII vector. In an attempt to obtain multi-functional polysaccharide-degrading clones with endoglucanase, cellobiohydrolase and xylanase activities, the library was initially screened for expression of endoglucanase activity on CM-cellulose plates. Two hundred CM-cellulose-positive plaques were identified after screening 4×10^5 plaques from the library. These CM-cellulose positive clones were then screened for cellobiohydrolase activity on MUC plates and were further tested for the ability to hydrolyse micro-crystalline cellulose by assaying the reducing sugar released after adsorption of cellulase in the supernatant of the recombinant bacteriophage lysates to Avicel followed by incubation at 39 °C for 3 h (see cellulose-binding assay in Methods). Eleven bacteriophage clones which exhibited large hydrolysis zones on both CM-cellulose and MUC plates, as well as activity towards Avicel, were selected. These eleven clones were then tested for xylanolytic activity on xylan plates and all were positive.

Analysis of the selected clones by restriction mapping revealed that ten of the eleven clones (the size of cDNA inserts ranging from 1·6 to 3·9 kbp) shared the same restriction pattern. A restriction map of the longest cellulase cDNA sequence, designated *celD* (pCNP4.1) is shown in Fig. 1. The remaining clone possessed an insert of 7·0 kbp and also had a similar restriction pattern to *celD*, but it contained two additional 1·15 kbp internal *Eco*RI-*Eco*RI fragments and a 1·7 kbp *Eco*RI-*Eco*RI fragment at the 5′ region of the cDNA. Southern blot hybridization analysis showed that *celD* hybridized to all of the selected shorter-length cDNAs as well as to the 7·0 kbp cDNA (data not shown), using a nucleic acid probe prepared from the 3′-region-deleted *celD* cDNA insert (a 0·65 kbp *Kpn*I-*Xho*I fragment at the 3′ region was deleted). Thus, it is most likely that the ten clones originated from the same gene and the 7·0 kbp clone is a related cDNA to *celD* (the 7·0 kbp cDNA was not further characterized as the activity of enzyme it codes for was about fourfold lower than that encoded by *celD*).

The substrate specificity of the enzyme encoded by *celD* was further characterized by quantitative measurement of its activity on various cellulosic substrates and xylan. As shown in Table 1, the *celD* enzyme was very active on CM-cellulose, but it also possessed cellobiohydrolase-like properties, as it was highly active on crystalline cellulose (Avicel), MUC and *p*-nitrophenyl cellobioside (pNPC) as well as amorphous cellulose. The enzyme showed no activity on methylumbelliferyl β-D-glucopyranoside (MUG) and *p*-nitrophenyl β-D-glucopyranoside (pNPG), substrates for β-glucosidase. Other

Table 1. *Activity of the cloned celD enzyme on various substrates*

Crude cell lysate preparations were used for the measurement of enzyme activities as described in Methods. The values given are means of three assays from different enzyme preparations.

Substrate	Specific activity [nmol product* min^{-1} (mg protein)$^{-1}$]	Percentage†
CM-cellulose	4929	100
Avicel	179	3·6
Amorphous (H_3PO_4-swollen) cellulose	812	16·5
Xylan	466	9·5
Lichenan	14312	290
pNPG	0	
pNPC	169	3·4
MUG	0	
MUC	944	19·2

*Reducing sugar or *p*-nitrophenol released after hydrolysis was measured.

† Values expressed as percentages of the activity on CM-cellulose.

cellulosic substrates tested were lichenan (a mixed glucan containing β-1,4 and β-1,3 glucosidic linkages) and laminarin (a glucan containing predominantly β-1,3 glucosidic linkages). The *celD* enzyme had very high activity towards lichenan (Table 1) and produced a large hydrolysis zone on the lichenan-containing agarose gel plate, but did not produce a hydrolysis zone on the laminarin plate (data not shown). This indicates that cleavage on lichenan is at the β-1,4-linkages. Interestingly, a high xylanase activity was also present in the *celD* enzyme. It appears that it is a truly multi-functional plant-polysaccharide-degrading enzyme. Analysis of hydrolysis products by TLC showed that the *celD* enzyme was able to hydrolyse cellodextrins (containing 3–5 glucose units) to glucose and cellobiose. Its catalytic mode on these cellulosic substrates was that of a typical endoglucanase (i.e. it cleaved β-1,4-glucosidic linkages at random positions, as shown in Fig. 2). However, the hydrolysis products of crystalline cellulose were mainly cellobiose with a trace amount of glucose (Fig. 2), indicative of cellobiohydrolase activity. The ability of the *celD* enzyme to degrade crystalline cellulose is of particular interest in selecting genetic material for transfer to rumen bacteria to improve plant fibre digestion, since most cellulases from rumen bacteria possess no or low activity on this substrate (Robson & Chambliss, 1989; Hazlewood *et al.*, 1990; Berger *et al.*, 1989; Romaniec *et al.*, 1989; Flint *et al.*, 1989).

Structural studies have revealed that many cellulases consist of at least two distinct functional domains: a catalytic domain and a cellulose-binding domain (Gilkes *et al.*, 1991; Goyal *et al.*, 1991; Béguin, 1990). The cellulose-binding capacity of the *celD* enzyme was assessed by a comparative assay of the enzyme activity

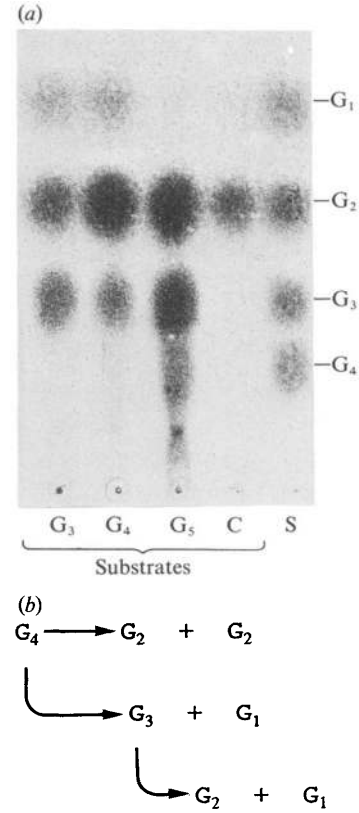

Fig. 2. Analysis of products of cellulosic compounds hydrolysed by the *celD* enzyme. (*a*) Crude cell lysate was prepared from *E. coli* harbouring plasmid pCNP4.1 and low-molecular-mass compounds were removed by spin-dialysis using a Centricon-10 tube (Amicon). The enzyme preparation was incubated with cellodextrins: [cellotriose (G_3), cellotetraose (G_4) and cellopentaose (G_5), each 2 mg ml^{-1}] or with 1% (w/v) Avicel (C) as described in Methods. Partial hydrolysis of cellodextrins is shown to illustrate the intermediate products. Hydrolysis products were identified by their R_F values on a TLC plate. Authentic cellodextrins (S) are shown in the rightmost lane and the positions of glucose (G_1), cellobiose (G_2), G_3 and G_4 are indicated. (*b*) Illustration of the catalytic mode of the *celD* enzyme on cellotetraose.

with or without prior adsorption to crystalline cellulose (Avicel). The amount of reducing sugar released from Avicel after adsorption of the enzyme to Avicel followed by extensive washing of the enzyme–substrate complex was 23·3 μg glucose equivalents min^{-1} (mg protein)$^{-1}$ (mean of duplicate assays, using one crude cell lysate preparation), compared to 24·5 μg min^{-1} mg^{-1} for the enzyme added without prior adsorption. Addition of bovine serum albumin at a final concentration of 10 mg ml^{-1} during adsorption to Avicel did not reduce this high recovery, indicating that the cellulose-binding of the *celD* enzyme is unlikely to be due to non-specific adsorption. This high recovery (95%) of the enzyme activity after adsorption and washing suggests that the *celD* enzyme possesses a strong cellulose-binding capacity. Presumably, the cellulose-binding domain is important for the close contact of the enzymes with an insoluble substrate, such as crystalline cellulose.

Functional domains of celD enzyme

To investigate the locations of catalytic and cellulose-binding domains of the *celD* enzyme and to elucidate whether the multiple substrate specificity of the enzyme is due to the presence of different catalytic domains, a series of deletion analyses of *celD* cDNA was conducted. As shown in Fig. 1, *celD* cDNA could be truncated to code for three catalytically active domains, when each domain was fused in-frame with the vector's *lacZ* translation initiation codon. These are designated domain I (pCNP4.2), domain II (pCNP4.4) and domain III (pCNP4.8), respectively. The subclone construction of domain I was obtained by deletion of a 2·75 kbp fragment at the 3′ region of *celD* cDNA (the *Pvu*II–*Xho*I fragment). Domain II contained sequence from the position 1·15 kbp to 2·3 kbp of *celD* cDNA and domain III from 2·3 kbp to 3·37 kbp. The subclone construction of domain II (pCNP4.4) was achieved by deletion of a 1·15 kbp *Eco*RI–*Pvu*II fragment at the 5′ region and exonuclease III digestion at the 3′ region of *celD* cDNA, and domain III by exonuclease III digestion from both the 5′ region and the 3′ region of *celD*. Interestingly, all three domains possessed the same pattern of substrate specificities as the enzyme produced by the untruncated *celD* cDNA. The specific activity of domain I and domain II on CM-cellulose, pNPC, xylan and Avicel was only slightly lower than the activity of the untruncated *celD* enzyme. However, the activity of the enzyme prepared from the pCNP4.8 clone (domain III) on all these substrates was about 10-fold lower than the untruncated enzyme. Moreover, all three domains had cellulose-binding capacity. Recovery of the enzyme activity after adsorption to Avicel and subsequent washing ranged from 70% to 80%, slightly lower recovery than for the enzyme from the untruncated *celD* cDNA. Further deletion of 90 bp from the 3′ region of domain III resulted in complete loss of all three types of enzyme activities (Fig. 1). Similarly, further deletion of 249 bp from the 5′ region of domain III abolished all enzyme activities (the position of the deletion was determined by nucleotide sequencing of the 5′ region of pCNP4.7, which was still fused in-frame with the *lacZ* translational initiation codon, as compared to the 5′ region sequence of pCNP4.6). These data suggest that endoglucanase, cellobiohydrolase and xylanase activities are all located within a single functional domain. It appears that both 90 bp from the 3′ region and 249 bp from the 5′ region of domain III are required to encode amino residues which are essential for the production of a catalytically active enzyme. Furthermore, retention of cellulose-binding capacity of domain III indicates that the sequence for cellulose-binding may be located within the catalytic domain. However, the possibility of the presence of a short sequence for a discrete cellulose-binding domain flanking the catalytic core of domain III can not be excluded. For most other cellulases, the enzyme activity and cellulose-binding capacity are located in two discrete domains, which are usually separated by sequences rich in proline and hydroxy-amino residues (Gilkes *et al.*, 1991; Goyal *et al.*, 1991; Béguin, 1990). Deletion of the cellulose-binding domain results in a dramatic reduction in adsorption of *Trichoderma reesei* cellobiohydrolases to crystalline cellulose (Van Tilbeurgh *et al.*, 1986; Tomme *et al.*, 1988) and complete loss of cellulose-binding capacity of two cellulases from *Cellulomonas fimi* (Gilkes *et al.*, 1988). More recently, Meinke *et al.* (1991) reported that the catalytic domain of an endoglucanase B from *Cellulomonas fimi* also has cellulose-binding capacity. However, it has an additional domain for cellulose-binding with no catalytic function, demonstrated by fusing the cellulose-binding sequence of the endoglucanase B with the catalytic domain of an exoglucanase. It remains to be determined whether *celD* cDNA also contains an extra sequence for a cellulose-binding domain.

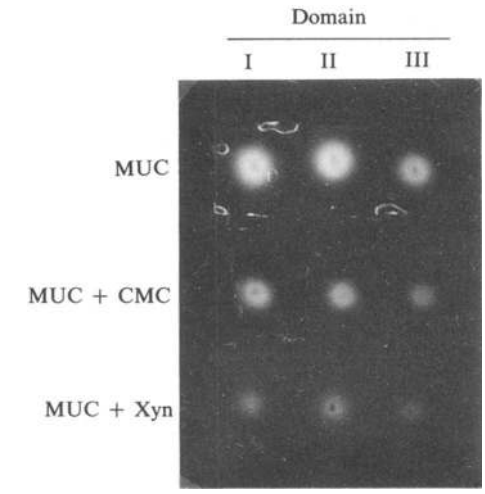

Fig. 3. CM-cellulose and xylan inhibiting MUC hydrolysis of three truncated forms of the *celD* enzyme. Crude enzyme extracts prepared from clones containing pCNP4.2 (domain I), pCNP4.4 (domain II) and pCNP4.8 (domain III) were used in the assay. Five microlitres of 10-fold diluted enzyme extracts (approximately 0·3 mg protein ml^{-1}) were loaded into wells cut in agarose gel plates containing 0·005% (w/v) MUC, 0·005% (w/v) MUC plus 1% (w/v) CM-cellulose or 0·005% (w/v) MUC plus 1% (w/v) xylan. MUC hydrolysis was detected under UV light. CMC, CM-cellulose; Xyn, xylan.

To explore further whether separate active sites for individual substrates exist within a functional domain, substrate competition assays were performed. Extremely sensitive detection for MUC hydrolysis allowed us to use high ratios of competing substrates (1%, w/v, CM-cellulose or xylan) to MUC (0·005%, w/v). As shown in Fig. 3, both CM-cellulose and xylan strongly inhibited

hydrolysis of MUC by enzyme extracts from the clones containing domain I, II or III. Thus, it was clearly demonstrated that CM-cellulose or xylan can compete with MUC for the same active site. Concurrent loss of all three types of enzyme activities, resulting from deletion of a small fragment of amino residues from each domain (Fig. 1), is also indicative of only one active site within each domain.

Cross-hybridization analysis showed that these three catalytic domains were highly homologous (data not shown). The detailed structural features of *celD* cDNA remain to be established by nucleotide sequencing, which could also provide some insights into the molecular evolution of this cellulase gene. Overall functional analysis has revealed the novel properties of the *celD* enzyme. Although some cellulases and xylanases consist of two mono-functional catalytic domains (Saul *et al.*, 1990; Gilbert *et al.*, 1992) or possess a single multi-functional domain (Foong *et al.*, 1991), there is no previous example of a polysaccharide hydrolase cDNA encoding three multi-functional catalytic domains, with each catalytic domain possessing cellulose-binding capacity. A multi-functional enzyme would be beneficial for the rumen fungus in its natural environment, where these polysaccharide substrates exist in a complex structure. Usually, several types of polysaccharide hydrolases are required to form a multi-enzyme complex acting co-operatively on these natural substrates.

Analysis of the celD transcript in N. patriciarum

Northern blot hybridization was conducted using a nucleic acid probe consisting of the 3′-region-deleted *celD* cDNA insert in order to look at the size and expression pattern of the *celD* transcript in *N. patriciarum*. As shown in Fig. 4, *celD* hybridized to a major transcript of 5·5 kb of *N. patriciarum* RNA, which appears to be longer than *celD* cDNA. Two minor transcripts of higher molecular mass were also detected after prolonged colour development during the enzymic detection of Northern blot analysis.

Regulation of cellulase gene expression is an interesting issue. It has been shown that expression of microbial cellulase genes is regulated by cellulose at the mRNA level (El-Gogary *et al.* 1989; Messner & Kubicek, 1991; Xue *et al.*, 1992). It is intriguing that an insoluble substrate such as cellulose can regulate cellulase synthesis without entering the cell. Evidence from the literature indicates that aerobic cellulolytic fungi grown on a medium without cellulosic substrates synthesize a low constitutive level of cellulases with undetectable levels of cellulase transcripts (El-Gogary *et al.*, 1989; Messner & Kubicek, 1991). It has been speculated that the constitutive level of cellulase synthesis, though low, generates

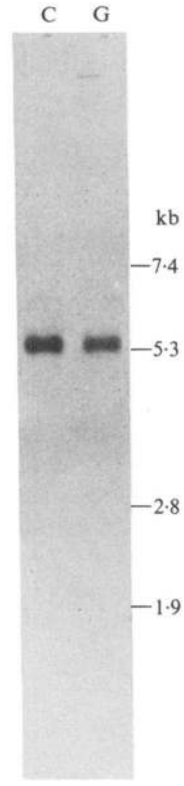

Fig. 4. Northern blot analysis of *celD* transcript expression in *N. patriciarum*. RNA was prepared from *N. patriciarum* grown at 39 °C for 4 d on medium A (rumen-fluid-free) with 0·5% (w/v) crystalline cellulose, Avicel (C) or 1% (w/v) glucose (G). One microgram of each poly(A)$^+$ RNA preparation was fractionated on 1·2% (w/v) agarose/2·2 M-formaldehyde gel. A digoxigenin-labelled RNA probe generated from the 3′-region-deleted *celD* cDNA was used for hybridization. Numbers (in kb) on the right represent RNA molecular markers (Boehringer).

soluble cellulosic molecules which may be able to trigger the expression of higher levels of cellulases (Béguin, 1990; Robson & Chambliss, 1989). We have previously demonstrated that the levels of *celA*, *celB* and *celC* transcripts in *N. patriciarum* are subject to dramatic induction by the presence of cellulose in the culture medium (Xue *et al.*, 1992). In contrast, the level of *celD* transcript was high when the fungus was grown in a glucose medium without cellulose and rumen fluid (Fig. 4). Growth of the fungus on a cellulose medium did not produce a significant increase in *celD* transcript level. To our knowledge, this is the first study to demonstrate a cellulase gene whose transcript level was not significantly affected by its substrate. This may explain the relatively high constitutive level of cellulase observed in *N. patriciarum* (Williams & Orpin, 1987; our unpublished data). Although the *celD* enzyme has been demonstrated to possess the novel properties of multiple multi-functional catalytic domains and a constitutive expres-

sion pattern of its transcript, how significant a role it plays in lignocellulolysis by this rumen fungus remains to be established.

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