Characterization of hybrid proteins consisting of the catalytic domains of *Clostridium* and *Ruminococcus* endoglucanases, fused to *Pseudomonas* non-catalytic cellulose-binding domains

Debbie M. POOLE,* Alastair J. DURRANT,* Geoffrey P. HAZLEWOOD† and Harry J. GILBERT*‡

*Department of Agricultural Biochemistry and Nutrition, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K., and †Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, U.K.

The *N*-terminal 160 or 267 residues of xylanase A from *Pseudomonas fluorescens* subsp. *cellulosa*, containing a non-catalytic cellulose-binding domain (CBD), were fused to the *N*-terminus of the catalytic domain of endoglucanase E (EGE') from *Clostridium thermocellum*. A further hybrid enzyme was constructed consisting of the 347 *N*-terminal residues of xylanase C (XYLC) from *P. fluorescens* subsp. *cellulosa*, which also constitutes a CBD, fused to the *N*-terminus of endoglucanase A (EGA) from *Ruminococcus albus*. The three hybrid enzymes bound to insoluble cellulose, and could be eluted such that cellulose-binding capacity and catalytic activity were retained. The catalytic properties of the fusion enzymes were similar to EGE' and EGA respectively. Residues 37–347 and 34–347 of XYLC were fused to the *C*-terminus of EGE' and the 10 amino acids encoded by the multiple cloning sequence of pMTL22p respectively. The two hybrid proteins did not bind cellulose, although residues 39–139 of XYLC were shown previously to constitute a functional CBD. The putative role of the *P. fluorescens* subsp. *cellulosa* CBD in cellulase action is discussed.

INTRODUCTION

Microbial degradation of cellulose and xylan is effected by a consortium of hydrolytic enzymes which act co-operatively to convert these substrates into their constituent simple sugars. Multiple isoenzymic forms of endoglucanase (EC 3.2.1.4), which differ subtly with respect to substrate specificity and are encoded by multiple genes, are a major component of bacterial cellulase systems (see, for example, Béguin, 1990; Hazlewood *et al.*, 1988; Fukumori *et al.*, 1989); other activities such as β -glucosidase (EC 3.2.1.21) and cellobiohydrolase also form an integral part. Exoglucanase (cellobiohydrolase) (EC 3.2.1.91), a principle component of fungal cellulase, has been described only occasionally in bacteria (Creuzet *et al.*, 1983; Gardner *et al.*, 1987).

Biochemical and genetic studies have provided a detailed insight into the properties and molecular architecture of microbial cellulases and, to a lesser extent, xylanases. Some cellulase-related proteins, such as the endoglucanases of *Bacillus* sp. (Fukumori *et al.*, 1989) and *Ruminococcus albus* (Poole *et al.*, 1990) are composed of a single catalytic domain. In contrast, other cellulolytic organisms such as *Fibrobacter succinogenes* (McGavin & Forsberg, 1989), *Cellulomonas fimi* (Ong *et al.*, 1989), *Thermomonospora fusca* (Ghangas & Wilson, 1988), *Butyrivibrio fibrisolvens* (Berger *et al.*, 1989), *Clostridium thermocellum* (Durrant *et al.*, 1991), *Trichoderma reesei* (Van Tilbeurgh *et al.*, 1986) and *Pseudomonas fluorescens* subsp. *cellulosa* (Hall & Gilbert, 1988; Gilbert *et al.*, 1990), each produce at least one cellulase-related protein which contains two distinct functional domains, a catalytic domain and a non-catalytic cellulose-binding domain (CBD).

The role of CBDs in the catalytic mechanism of cellulases differs according to the organism studied. Exoglucanase and endoglucanase from *T. reesei* require a CBD for efficient hydrolysis of crystalline cellulose (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986), while in *Cl. thermocellum* a CBD increases

the affinity of endoglucanase E (EGE) for its substrate, barley β -glucan (Durrant *et al.*, 1991). In contrast, deletion of the CBD present in endoglucanases A (EGA; L. M. A. Ferreira, unpublished work) or B (EGB; Gilbert *et al.*, 1990) from *P. fluorescens* subsp. *cellulosa* had no significant effect on the catalytic properties of either protein. Furthermore the highly conserved *P. fluorescens* subsp. *cellulosa* CBD was shown to be present, not just in cellulases, but also in two xylanases (XYLA and XYLB) and an arabinofuranosidase (XYLC) (Ferreira *et al.*, 1990; Kellett *et al.*, 1990), casting considerable doubt on the assumption that the CBD has a specific role in the cellulase catalytic mechanism in the *Pseudomonas*.

Affinity for cellulose also differs according to the source of the CBD. *T. reesei* and *Cl. thermocellum* CBDs bind weakly to cellulose and are relatively easy to displace. In contrast, those present in cellulases and xylanases from *P. fluorescens* subsp. *cellulosa* exhibit high affinity for various forms of cellulose and can be quantitatively eluted from the polysaccharide only by boiling in 10% (w/v) SDS.

The purpose of this study was to examine the role of the *Pseudomonas* CBD in cellulase action by fusing this region to the catalytic domain of heterologous endoglucanases, and investigating what effect this has on the catalytic properties of the enzymes. We also altered the location of the CBD in these enzymes and assessed its affinity for cellulose. Data presented show that the high-affinity CBD did not affect the action of the cellulases, and required a free *N*-terminal amino group to interact with cellulose.

MATERIALS AND METHODS

Plasmids, microbial strains and culture conditions

The *Escherichia coli* strain employed in this study was JM83 (Norlander *et al.*, 1983). The vectors used were pMTL22p (Chambers *et al.*, 1988) and pUC19 (Norlander *et al.*, 1983). The

Abbreviations used: CBD, cellulose-binding domain; EGE, endoglucanase E; EGA, endoglucanase A; EGB, endoglucanase B; XYLC, xylanase C; XYLA, xylanase A; XYLB, xylanase B; LB, Luria broth.

‡ To whom correspondence and reprint requests should be sent.

plasmids from which the hybrid genes were derived were as follows: pJH1, pJH2 (both described by Durrant *et al.*, 1991), pLE7, pLE15 (Kellett *et al.*, 1990), pRS4 (Hall *et al.*, 1989) and pD5 (Poole *et al.*, 1990). *E. coli* strains were cultured in Luria broth (LB) or on LB solidified with 2% (w/v) agar. Ampicillin (100 µg/ml) and 5-bromo-4-chloroindol-3-yl β-D-galactoside (2 µg/ml) were incorporated into medium to select for transformants and recombinants respectively. To select for *E. coli* strains expressing endoglucanase activity, bacterial colonies were grown on LB agar supplemented with CM-cellulose (5 mg/ml) and the medium was stained with 1% (w/v) Congo Red and destained with 1 M-NaCl to reveal zones of hydrolysis.

General recombinant DNA procedures

Agarose-gel electrophoresis, transformation of *E. coli* and modification of DNA using restriction enzymes and T4 DNA ligase were as described by Gilbert *et al.* (1987, 1988). Plasmid DNA was extracted from *E. coli* by 'Brij lysis' and purified by subsequent CsCl density-gradient centrifugation (Clewell & Helinski, 1969). The methods of Holmes & Quigley (1981) and Birnboim & Doly (1979) were employed for small-scale plasmid isolation and for restriction analysis and nucleotide sequencing respectively. DNA from recombinant plasmids was sequenced by the dideoxy-chain-termination method of Sanger *et al.* (1980) with the following modifications: T7 DNA polymerase (Tabor & Richardson, 1987) was used instead of the Klenow fragment of DNA polymerase, plasmid DNA was denatured in 1 M-NaOH for 5 min, the alkali was removed by passing the nucleic acid through a Sepharose 6B spun column and the DNA was annealed with either -40 universal primer, or reverse primer, at 42 °C for 15 min.

Construction of fusion proteins

Genes encoding the fusion proteins used in this study were constructed as follows.

***xynA*'480-*celE*' and *xynA*'801-*celE*'.** Plasmid pRS4 (Hall *et al.*, 1989) was cleaved with *EcoRV* (cleaves at nucleotide 858 of *xynA*), digested with *Bal31*, and blunt-ended with T4 DNA polymerase. The modified plasmid was then restricted with *HindIII*, which cleaves within the multiple-cloning region of pUC19, upstream of *xynA*, and the DNA fragments consisting of the 5' region of *xynA* were cloned into *SmaI*/*HindIII*-digested pUC19. The extent of the deletions within the xylanase gene was determined by nucleotide sequencing, and two derivatives of the gene, containing 480 bp (pPD1) and 801 bp (pPD2), of the 5' region respectively were retained for further use. The plasmid pJH1 (Durrant *et al.*, 1991) was then digested with *KpnI*, which cleaves in the multiple-cloning region of pMTL22p downstream of *celE*', blunt-ended with T4 DNA polymerase and then restricted with *SphI*. The 1 kb restriction fragment generated, which contains *celE*', was cloned into *SphI*/*StuI*-digested pMTL22p to create pPD3. The 5' regions of *xynA* were excised from pPD1 and pPD2 on *HindIII*/*KpnI* restriction fragments and cloned into *HindIII*/*KpnI*-digested pPD3 to generate pPD4 and pPD5 respectively. The *celE*' gene was in-phase with the *xynA* derivatives and thus pPD4 and pPD5 encode XYLA'-EGE' fusion proteins.

***celE*'-*xynC*' and *lacZ*'-*xynC*'.** The plasmid pLE7 (Kellett *et al.*, 1990) was cleaved upstream of *xynC* with *SmaI*. After *Bal31* digestion, the DNA was blunt-ended with T4 DNA polymerase and then restricted with *BamHI* (*BamHI* cleaves *xynC* at nucleotide 1040). DNA fragments containing the 5' region of *xynC* were cloned into pMTL22p cleaved with *EcoRV* and *BamHI*. The extent of the *xynC*' deletions was determined and the

plasmids containing nucleotides 108-1040 (pPD6) and 98-1040 (pPD8) of *xynC*' were retained for further use. In pPD8, *xynC*' was in frame with the translational start of *lacZ*'; thus the plasmid encoded a β-GAL-XYLC' fusion protein. A 0.94 kb *NaeI*/*BamHI* restriction fragment, containing the 5' region of *xynC*, was excised from pPD6 and cloned into *BglII*/*SmaI* digested pJH1 to generate pPD7. In pPD7, the *celE*' gene was in-phase with *xynC*'; thus the plasmid encoded an EGE'-XYLC' fusion protein.

***xynC*'.** The plasmid pLE7 was restricted with *EcoRV*, digested with *Bal31* and finally cleaved with *EcoRI*. The 1.6 kb restriction fragment, containing nucleotides 1-1017 of *xynC*, was cloned into pUC18 to generate pPD9.

***xynC*'-*celA*'.** The 1.3 kb *EcoRI*/*BamHI* restriction fragment, derived from pLE7, containing nucleotides 1-1040 of *xynC*', was cloned into *BglII*/*EcoRI*-digested pD5, creating a *xynC*'-*celA*' fusion gene. The resultant plasmid (pPD10) encodes 346 residues of XYLC fused to the N-terminus of EGA.

Purification of EGE' and EGA

Ruminococcus albus EGA and the catalytic domain of *Cl. thermocellum* EGE (EGE') were purified, as described by Poole *et al.* (1990) and Durrant *et al.* (1991) from *E. coli* JM83 harbouring pD12 (Poole *et al.*, 1990) and pMST1 (Hall *et al.*, 1990) respectively.

Cellulose-binding studies and purification of fusion proteins containing XYLC' and XYLA'

The preparation of cell-free extracts from *E. coli* strains containing appropriate recombinant plasmids, the incubation of the resultant *E. coli* proteins with crystalline cellulose (Avicel) and the elution of Avicel-bound polypeptides were as described by Ferreira *et al.* (1990). To determine the size of cellulose-bound proteins, the polysaccharide was incubated with 10% SDS (w/v) at 95 °C for 5 min. The *M_r* of eluted polypeptides was determined by SDS/PAGE (Laemmli, 1970) using a 10% (w/v) acrylamide gel. Functional fusion proteins were eluted from Avicel with 5 ml of 6 M-guanidinium chloride (EGE' derivatives) or pure ethylene glycol (EGA) and were dialysed twice against 1000 vol. of 100 mM-Tris/HCl, pH 8.0.

Assays

Derivatives of EGE' and EGA were assayed for cellulase activity as described by Hazlewood *et al.* (1990) and Poole *et al.* (1990) respectively. One unit of enzyme activity generates 1 µmol of glucose equivalent/min. Protein was measured as described by Lowry *et al.* (1951), with BSA as standard.

Preparation of antiserum

XYLC, purified from *E. coli* JM83 harbouring pLE7 as described by Kellett *et al.* (1990), was diluted in water to 200 µg/ml, emulsified with an equal volume of Freund's complete adjuvant, and 2 ml of the resultant emulsion was injected subcutaneously and intramuscularly into New Zealand White rabbits. Second and third injections using 100 µg of XYLC, mixed with Freund's incomplete adjuvant, were carried out at 2- and 5-week intervals. Serum was collected 3 weeks after the final injection.

Immunological detection of XYLC' fusion proteins

Proteins fractionated by SDS/PAGE were electrophoretically transferred to Hybond C (Amersham) as described by Towbin *et al.* (1979). The biotin-streptavidin system (Amersham) was used for immunological detection of XYLC' on Western blots as directed in the supplier's protocol. The dilutions of primary and secondary antisera and the biotinylated peroxidase-streptavidin

complex were 1/750, 1/500 and 1/400 respectively. Diaminobenzidine (1.3 mM) was the substrate used to detect bound peroxidase.

Protein sequencing

The N-terminal sequence of XYLC' was determined by automated Edman sequencing using a 470 A gas-phase sequencer equipped with a 120 A on-line phenylthiohydantoin analyser (Applied Biosystems) as described by Hunkapillar *et al.* (1983).

Enzymes and reagents

Restriction enzymes, bacteriophage T4 DNA ligase and polymerase were from Bethesda Research Laboratories. Bacteriophage T7 DNA polymerase and other reagents for DNA sequencing were from Cambridge Bioscience. Deoxyadenosine 5'-[α -thio-³⁵S]triphosphate (d[³⁵S]ATP; 2000 mCi/mmol) was supplied by Amersham International, as were the Western detection kit and Hybond C. *Bal31* was obtained from Boehringer. Unless specified, all other reagents were supplied by Sigma.

RESULTS

Binding to cellulose of hybrid forms of EGA and EGE'

Previous studies have shown that full-length EGE from *Cl. thermocellum* consists of two distinct functional domains: a catalytic domain (EGE') and a weak CBD (Durrant *et al.*, 1991). In contrast, EGA from *R. albus* constitutes a single-domain protein with no apparent affinity for insoluble cellulose (Poole *et al.*, 1990). To examine whether the acquisition of a CBD, which exhibits strong affinity for crystalline cellulose, altered the catalytic properties of EGE' and EGA, the hybrid genes *xynC'-celA*, *xynA'480-celE'* and *xynA'801-celE'* were constructed. The enzymes encoded by two of these genes (XYLC'-EGA and XYLA'160-EGE') consisted of CBDs derived from *P. fluorescens* subsp. *cellulosa* xylanases (Kellett *et al.*, 1990; Ferreira *et al.*, 1990), fused to the N-terminus of EGA and EGE' respectively. XYLA'267-EGE', encoded by *xynA'801-celE'*, consisted of the N-terminal 267 residues of XYLA, which constitutes both a CBD and extended linker sequences, joined to the N-terminus of EGE' (Fig. 1). To investigate whether XYLC'-EGA, XYLA'160-EGE' and XYLA'267-EGE' bind to crystalline cellulose, cell-free extracts prepared from *E. coli* JM83 harbouring *xynC'-celA*, *xynA'480-celE'* and *xynA'801-celE'* respectively were incubated with crystalline cellulose. Results (Fig. 2) showed that the recombinant strains expressed proteins with *M_r* values of 70000 (*xynC'-celA*), 49000 (*xynA'160-celE'*) and 60000 (*xynA'801-celE'*) which bound tightly to cellulose, at 4 °C or 60 °C, and could be eluted with 10 % (w/v) SDS at 95 °C. To confirm the identity of these proteins as XYLC'-EGA, XYLA'160-EGE' and XYLA'267-EGE', Avicel-bound polypeptides derived from the three recombinant *E. coli* strains were eluted with guanidinium chloride (EGE' derivatives) or ethylene glycol (CBD-EGA). Protein eluted from cellulose, under these milder conditions, exhibited endoglucanase activity and could be quantitatively rebound to Avicel (Table 1). *E. coli* JM83 harbouring pUC18 did not synthesize any proteins that bound to cellulose (results not shown). These data confirm that fusion of a *P. fluorescens* subsp. *cellulosa* CBD to the N-terminus of EGE' or EGA confers on the hybrid protein the capacity to bind insoluble cellulose. Furthermore, the hybrid enzymes can be eluted from the polysaccharide under conditions that did not affect either catalytic activity or cellulose-binding capacity.

Catalytic properties of EGE', EGA and their hybrid derivatives

In a previous report we demonstrated that the endogenous CBD of full-length EGE increased the affinity of the enzyme for its substrate (Durrant *et al.*, 1991). In this study we set out to examine the effect on the enzymes' catalytic properties of fusing a 'high-affinity' CBD to the catalytic domains of EGE' and EGA from *R. albus*. The hybrid proteins synthesized in *E. coli* were purified by Avicel affinity chromatography and their capacity to hydrolyse suitable substrates was evaluated. The data (Table 1) revealed that EGA and CBD-EGA exhibited similar catalytic activities. In contrast, XYLA'160-EGE' displayed considerably lower catalytic activity than EGE' or XYLA'267-EGE'. This latter observation may be a consequence of steric hindrance between the *P. fluorescens* subsp. *cellulosa* CBD and the catalytic domain of EGE, in XYLA'160-EGE', affecting the activity of the hybrid enzyme. The linker sequence which is interposed between these two domains in XYLA'267-EGE' could prevent

Plasmid Gene

Fig. 1. Derivatives of EGE', EGA, XYLA' and XYLC' encoded by pPD1-pPD5

The N- and C-terminal residues of each protein are shown. The position in the primary sequence of the appropriate enzymes of residues at the boundary of each protein domain are shown. EGE', EGA, XYLA', β -galactosidase and XYLC' are depicted by □, ▨, ▩, and ■ respectively. Construction of the plasmids encoding the various proteins is described in the Materials and Methods section. Nucleotide sequences represented as ∇ are multiple-cloning regions of pUC19 (19), pUC18 (18) or pMTL22p (22). The positions of the restriction sites *Bgl*II (Bg), *Bam*HI (B), *Eco*RI (R), *Eco*RV (RV), *Hind*III (H), *Nae*I (Na), *Nru*I (Nr), *Kpn*I (K), *Sma*I (Sm), *Sph*I (Sp), *Sst*I (Ss) and *Stu*I (Stu) are shown. Restriction sites in parentheses have been destroyed during construction of the hybrid genes.

Fig. 2. Binding of derivatives of EGE' and EGA to Avicel

Protein prepared from *E. coli* cells harbouring pPD4 (a), pPD5 (b) and pPD10 (c) were incubated with Avicel. After the Avicel had been washed, the bound protein was eluted with 10% SDS (w/v) at 95 °C as described in the Materials and Methods section. Proteins in each fraction were subjected to SDS/PAGE. Cell-free extracts of the recombinant strains are shown in lane 3. Lanes 2 and 1 contain material in the last Tris/HCl wash and protein eluted from cellulose with SDS respectively. The M_r values ($\times 10^{-3}$) of the polypeptides that bound to Avicel are shown.

Fig. 3. Binding of XYLC', EGE'-XYLC' and β -GAL-XYLC' to Avicel

Protein prepared from *E. coli* cells containing pPD9, pPD8 and pPD7 were incubated with Avicel, and bound protein was eluted with SDS. The polypeptides in each fraction were analysed by SDS/PAGE. Cell-free extracts of recombinant strains are shown in lanes 1 (pPD9), 3 (pPD8) and 5 (pPD7). Lanes 2 (pPD9), 4 (pPD8) and 6 (pPD7) contain protein eluted from the cellulose with SDS. The M_r value ($\times 10^{-3}$) of the protein that bound Avicel is shown.

the same phenomenon from occurring with this hybrid enzyme, hence its higher specific activity.

Kinetic analysis of the enzymes (Table 1) revealed that EGA and EGE' exhibited the same affinity for β -glucan as their derivatives containing *P. fluorescens* subsp. *cellulosa* CBDs. In contrast, a previous study showed that full-length EGE, containing its endogenous CBD, exhibited higher activity than EGE' at low substrate concentrations (Durrant *et al.*, 1991). The hybrid enzymes, in common with full-length EGE, EGE' and EGA, did not cleave either amorphous or crystalline cellulose (Table 1). These data suggest that the fusion of a high-affinity CBD to either EGA or the catalytic domain of EGE did not affect either of the enzymes' affinities for soluble substrates, or their ability to hydrolyse insoluble cellulose.

Switching the position of the CBD

To determine whether an *N*-terminal CBD will function when fused to the *C*-terminus of a protein, the gene *celE'-xynC'* (Fig. 1) was constructed. This DNA sequence encodes a hybrid enzyme

consisting of the CBD derived from *P. fluorescens* subsp. *cellulosa* XYLC fused to the *C*-terminus of EGE'. To examine whether the enzyme bound to insoluble cellulose, the cell-free extract of *E. coli* JM83 harbouring *celE'-xynC'* was incubated with Avicel. Results (Fig. 3) revealed that none of the proteins in the extract bound to cellulose. To examine whether this was a consequence of steric hindrance by EGE' affecting the formation of CBD-Avicel complexes, two further genes, *xynC'* and *lacZ'-xynC'*, were constructed which encode residues 1–347 of XYLC (XYLC') and 10 amino acids encoded by the multiple cloning sequence of pMTL22p fused to the *N*-terminus of residues 34–347 of XYLC (β -GAL'-XYLC') respectively. It was expected that the signal peptide of XYLC' would be cleaved when the protein was secreted into the *E. coli* periplasm. This was confirmed by *N*-terminal sequence analysis of the secreted protein (ACTYTIDS), which exhibited 100% identity with the first eight residues of mature XYLC (Kellett *et al.*, 1990). In contrast, β -GAL'-XYLC' lacks most of the signal peptide and thus the *N*-terminus will not be processed. Of the two proteins, only XYLC' bound to cellulose (Fig. 3).

Table 1. Characteristics of native and hybrid enzymes

Results are means $\pm$ S.D. ND, not determined.

Protein	Avicel binding	Cellulase activity (units/mg of catalytic domain)				K_m (mM)
		β -Glucan	CM-cellulose	Acid-swollen cellulose	Crystalline cellulose	
XYLC'	+	0	0	0	0	0
β -GAL'-XYLC'	–	0	0	0	0	0
EGA	–	371 $\pm$ 13	112 $\pm$ 9	0	0	33 $\pm$ 7
EGE'	–	320 $\pm$ 23	121 $\pm$ 24	0	0	4.7 $\pm$ 1.2
XYLC'-EGA	+	393 $\pm$ 46	124 $\pm$ 16	0	0	31 $\pm$ 6
XYLA'160-EGE'	+	229 $\pm$ 26	48 $\pm$ 11	0	0	5.1 $\pm$ 1.7
XYLA'267-EGE'	+	354 $\pm$ 35	132 $\pm$ 29	0	0	5.3 $\pm$ 1.6
EGE'-XYLC'	–	ND	ND	ND	ND	ND

Fig. 4. Western-blot analysis of recombinant and native *E. coli* strains

Cell-free extracts of *E. coli* JM83 harbouring pPD8 (1), pPD7 (2) and pUC18 (3) were subjected to SDS/PAGE and the gel Western-blotted using anti-XYLC' antiserum as the probe. The M_r values ($\times 10^{-3}$) of the major proteins that bound to anti-XYLC' IgG are shown.

Before concluding that the CBD in EGE'-XYLC' and β -GAL-XYLC' is not functional it is important to verify that full-length forms of the hybrid proteins were synthesized in the *E. coli* recombinant strains. Consequently, we subjected proteins present in cell-free extracts of *E. coli* JM83 harbouring *celE'*-*xynC'* and *lacZ'*-*xynC'* to SDS/PAGE and subsequent Western analysis using antiserum raised against XYLC'. The results (Fig. 4) revealed that β -GAL-XYLC' and EGE'-XYLC' with M_r values of 33000 and 69000 respectively were present in the bacterial extracts. Some processing of EGE'-XYLC' had occurred as a polypeptide of M_r 28000 which cross-reacted with XYLC' antiserum was also present in *E. coli* containing *celE'*-*xynC'*. These data confirm that the XYLC CBD did not bind cellulose when only 10 residues were fused to its *N*-terminus. Thus an *N*-terminal CBD will not bind Avicel when switched to the *C*-terminus of a protein.

DISCUSSION

The major purpose of this study was to establish whether the catalytic properties of two bacterial cellulases can be modified through the acquisition of a 'high-affinity' CBD, and thus provide an insight into the role of *P. fluorescens* subsp. *cellulosa* CBDs in cellulose hydrolysis. Specifically, we wished to examine (i) whether replacement of the endogenous low-affinity CBD of EGE with the corresponding pseudomonad protein domain increases the catalytic activity of the enzyme at low substrate concentration and (ii) if the fusion of a strong CBD to EGA, an endoglucanase that does not have an endogenous CBD, alters the properties of the cellulase. Data presented indicate that the fusion of the *P. fluorescens* subsp. *cellulosa* CBD does not affect the catalytic properties of EGA or EGE'. As Durrant *et al.* (1991) have shown that the weak CBD of EGE increases the enzyme's activity at low substrate concentrations, it could be argued that a high-affinity CBD would further improve the capacity of EGE to hydrolyse low concentrations of β -glucan. That the *P. fluorescens* subsp. *cellulosa* CBD did not affect cellulase activity suggests that its role in cellulolysis is distinct from that of the corresponding *Cl. thermocellum* sequence. It can be envisaged that the weak association between cellulose and enzyme, elicited by the EGE CBD, results in a high concentration

of substrate in the vicinity of the enzyme without preventing subsequent desorption of the enzyme from the substrate. In contrast, the high-affinity CBD, by binding tightly to cellulose, may prevent the enzyme from dissociating and then reassociating with the substrate at a different position, which would locate a different glycosidic bond at the active site on each reassociation. Because of the strong interaction between cellulose and the enzyme mediated by the high-affinity CBD, the active site of the endoglucanase in the hybrid protein would only be accessible to β -1,4-glycosidic linkages in other cellulose molecules. Thus the *P. fluorescens* subsp. *cellulosa* CBD would not alter the catalytic domain's capacity to hydrolyse low levels of β -glucan.

The acquisition of *P. fluorescens* subsp. *cellulosa* CBDs by EGE and EGA did not confer the capacity to hydrolyse insoluble cellulose. This is contrary to the view of West *et al.* (1989), who argued that the fusion of a CBD to an enzyme that hydrolyses β -1,4-glycosidic bonds alters the enzyme's substrate specificity. Support for their notion is provided by several reports which suggest that the capacity of *T. reesei* cellulases to hydrolyse crystalline cellulose is dependent on the presence of a CBD (Tomme *et al.*, 1988; Van Tilbeurgh *et al.*, 1986), and also by the observation that the CBDs of *F. succinogenes* (McGavin & Forsberg, 1989) and *Cl. thermocellum* (Durrant *et al.*, 1991) alter the affinity of endoglucanases for their substrates. The data presented here, in conjunction with previous studies, have shown that the role of CBDs in cellulolysis is dependent on the nature and origins of the protein domains. Those CBDs that exhibit low affinity for cellulose (*T. reesei* and *Cl. thermocellum*) exert a direct effect on the catalytic activity of cellulases. The 'high-affinity' *P. fluorescens* subsp. *cellulosa* CBD appears to have a more indirect role in cellulose hydrolysis. We envisage that in the normal ecosystem of soil pseudomonads the CBD provides a general mechanism by which a range of polysaccharide hydrolases can bind to the plant cell walls which are a potential source of carbon and energy. Close association with the plant cell walls is mediated by the CBD, ensuring that the appropriate enzymes are available to cleave the different substrates exposed during cell-wall digestion.

The data presented in this report also demonstrate that blockage of the *N*-terminal residue of the XYLC' CBD prevents the protein domain from binding to cellulose. This indicates that a free *N*-terminal region of the CBD is either essential for the correct folding of the domain or plays a direct role in its interaction with cellulose. It is interesting to note that, although the *C*-terminal CBD of *P. fluorescens* subsp. *cellulosa* EGA (Hall & Gilbert, 1988) exhibits a high degree of sequence conservation with XYLC, and with other CBDs that are located at the *N*-terminus of *P. fluorescens* subsp. *cellulosa* cellulases and xylanases (Kellett *et al.*, 1990), the domain still interacts with Avicel despite the lack of a free *N*-terminal amino group (D. M. Poole, unpublished work). This indicates that there are subtle differences in the way the *C*-terminal CBD either folds or interacts with its ligand when compared with *N*-terminal CBDs.

The present paper describes the use of hybrid enzymes to define the role of specific regions in multidomain cellulases. It is expected that these studies will form the basis for the future rational design of novel enzymes with enhanced plant-cell-wall-hydrolysing activity.

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