Molecular study and overexpression of the *Clostridium cellulolyticum* *celF* cellulase gene in *Escherichia coli*

Corinne Reverbel-Leroy,¹ Anne Belaich,¹ Alain Bernadac,²
Christian Gaudin,¹ Jean-Pierre Belaich^{1,3} and Chantal Tardif^{1,3}

Author for correspondence: Chantal Tardif. Tel: +33 91 16 43 40. Fax: +33 91 71 33 21.
e-mail: gaudin@ir1cbm.cnrs-mrs.fr

^{1,2} Bioénergétique et
Ingénierie des protéines¹
and Laboratoire
d'Ingénierie et
Dynamique des Systèmes
Membranaires², Centre
National de la Recherche
Scientifique, 31 chemin
Joseph Aiguier, BP71,
13402 Marseille Cedex 20,
France

³ Université de Provence,
Marseille, France 3

The *CelF*-encoding sequence was isolated from *Clostridium cellulolyticum* genomic DNA using the inverse PCR technique. The gene lies between *cipC* (the gene encoding the cellulosome scaffolding protein) and *celC* (coding for the endoglucanase C) in the large *cel* cluster of this mesophilic cellulolytic *Clostridium* species. Comparisons between the deduced amino acid sequence of the mature *CelF* (693 amino acids, molecular mass 77 626) and those of other β -glycanases showed that this enzyme belongs to the recently proposed family L of cellulases (family 48 of glycosyl hydrolases). The protein was overproduced in *Escherichia coli* using the T7 expression system. It formed both cytoplasmic and periplasmic inclusion bodies when induction was performed at 37 °C. Surprisingly, the protein synthesized from the cytoplasmic production vector was degraded in the *lon* protease-deficient strain BL21(DE3). The induction conditions were optimized with regard to the concentration of inductor, cell density, and temperature and time of induction in order to overproduce an active periplasmic protein (*CelFp*) which was both soluble and stable. It was collected using the osmotic shock method. The enzymic degradation of various cellulosic substrates by *CelFp* was studied. *CelFp* degraded swollen Avicel more efficiently than substituted soluble CM-cellulose or crystalline Avicel and was not active on xylan. Its activity is therefore quite different from that of endoglucanases, which are most active on CM-cellulose.

Keywords: *Clostridium cellulolyticum*, cellulase, periplasmic inclusion bodies, overproduction

INTRODUCTION

Cellulose is the major component of plant cell walls and is consequently the most abundant biopolymer in the world. *Clostridium cellulolyticum* strain ATCC 35319 was isolated from compost (Petitdemange *et al.*, 1984) and the physio-

logical properties of this organism have been studied (Giallo *et al.*, 1983). This Gram-positive, mesophilic, anaerobic *Clostridium* species is a true cellulolytic organism, which can use cellulose as its sole source of carbon and energy (Giallo *et al.*, 1985).

It has recently been established that *Clost. cellulolyticum* possesses a macromolecular cellulolytic system, which has been called a cellulosome (Faure *et al.*, 1987; Madarro *et al.*, 1991). Cellulosomes were first described in *Clostridium thermocellum* (Lamed *et al.*, 1983a), a Gram-positive, thermophilic, anaerobic bacterium. SDS-PAGE analysis of these multiprotein complexes from two strains of *Clost. thermocellum* (YS and ATCC 27405) revealed the existence of two major components called S1 (210 kDa) and S8 (75 kDa) (Lamed *et al.*, 1983b), or S_L (250 kDa) and S_S

Abbreviation: p-NP, para-nitrophenyl

Gene nomenclature. In our previous work, the genes coding for the cellulosome components of *Clost. cellulolyticum* were referred to as *celCC*. In accordance with the standard rules of bacterial genetic nomenclature, these have been renamed *cel* in the present paper. For example, *celCCF* is now referred to as *celF*.

The GenBank accession number for the nucleotide sequence reported in this paper is U30321.

(82 kDa) (Wu & Demain, 1988), respectively. Most of the cellulosomal subunits in *Clostr. thermocellum* have been found to be cellulases which exhibit endoglucanase activity (Lamed *et al.*, 1983a,b), except for one true xylanase (Grépinet *et al.*, 1988), one lichenase (Schimming *et al.*, 1991) and the major components S1 and S8. S8 and S₈ were shown to be cellobiohydrolases (Morag *et al.*, 1991; Kruus *et al.*, 1995b).

The largest component, S1 (or S_L), recently renamed CipA (for cellulosome integrating protein) (Fujino *et al.*, 1993a), has been found to be a scaffolding protein (Salamitou *et al.*, 1992; Gerngross *et al.*, 1993) around which the various catalytic components are anchored, and also to promote the binding of the cellulosome to cellulose. The determinant involved in the attachment of the catalytic subunits to CipA has been identified as a duplicated segment of 22 residues, which is conserved in all the cellulosome subunits identified so far (Tokatlidis *et al.*, 1991).

To date, seven genes coding for components of the *Clostr. cellulolyticum* cellulosome have been identified. *celA* (Faure *et al.*, 1989) and *celD* (Shima *et al.*, 1991) were described as monocistronic transcription units. More recently, a cluster of *cel* genes was described (Bagnara-Tardif *et al.*, 1992) including ORF1, *celC*, *celG* and *celE*, with *celC* and *celG* associated in a polycistronic transcription unit. The *celA* and *celC* gene products have been extensively characterized (Bélaich *et al.*, 1992; Fierobe *et al.*, 1993; Fierobe *et al.*, 1991) and the three-dimensional structure of CelA has been established (Ducros *et al.*, 1995; Roig *et al.*, 1993). A very similar cluster has been described for the cellulase genes of *Clostridium josui*, in which the order and sequence of genes corresponding to ORF1, *celC* and *celG* are highly conserved (Fujino *et al.*, 1993b). *Clostr. josui* and *Clostr. cellulolyticum* are probably two strains of the same species.

In the present paper, we describe the cloning, sequencing and expression of the *celF* gene corresponding to ORF1 in the *cel* gene cluster. When *celF* was expressed in *Escherichia coli* under standard conditions, the recombinant protein aggregated and formed inclusion bodies. After optimizing the induction conditions, a mature form of CelF was produced, which was both soluble and stable. CelF hydrolysed cellulose (not xylan), but was poorly active on substituted cellulose. CelF may be the equivalent, in the *Clostr. cellulolyticum* cellulosome, of the S8/CelS component of *Clostr. thermocellum*, and probably corresponds to the major 72 kDa Avicel-adsorbed subunit described by Madarro *et al.* (1991).

In addition, another ORF located upstream from *celF* was identified as a *cip* gene of the *Clostr. cellulolyticum* cellulosome.

METHODS

Bacterial strains and vectors. *Clostr. cellulolyticum* strain ATCC 35319 was used as the source of genomic DNA. The *E. coli* strains used in this study were DH5 α (Bethesda Research Laboratories), which was used as the host for cloning plasmid pUC18, and BL21(DE3) [*F*⁻ *ompT* *hsdS* (τ_B m_B) *gal* *dcm* (DE3)],

Fig. 1. Map of the *cel* gene cluster from *Clostr. cellulolyticum*. Shaded areas indicate previously determined *cel* gene sequences. Thick bars and arrows indicate the fragments and primers used for inverse PCR. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pvu*II.

which was used as the host for the expression vector pET22b(+) (Novagen).

Growth conditions. *Clostr. cellulolyticum* was grown anaerobically at 32 °C on basal medium supplemented with cellobiose as the carbon and energy source (Giallo *et al.*, 1983).

E. coli DH5 α was grown at 37 °C in Luria Bertani medium supplemented with ampicillin (100 μ g ml⁻¹) when required.

E. coli BL21(DE3) was grown under the same conditions, except that the growth temperature and IPTG concentration were modulated to provide optimal conditions for heterologous protein production.

DNA isolations and manipulations. *Clostr. cellulolyticum* genomic DNA was purified as described by Quiviger *et al.* (1982). Small-scale plasmid purification from *E. coli* was performed using the alkaline lysis method (Engebrecht *et al.*, 1991). Plasmids for DNA sequencing reactions were purified by using the Qiagen kit.

PCR technique used in cloning experiments. The *celF* gene was amplified from *Clostr. cellulolyticum* genomic DNA by performing inverse PCR (Ochman *et al.*, 1990) in the presence of *Taq* DNA polymerase (Boehringer Mannheim). Divergent primers for amplification (Fig. 1) were derived from the previously determined sequence of the 3' region of the gene (Bagnara-Tardif *et al.*, 1992). PCR fragments were cloned into pUC18.

DNA sequence analysis. Nested deletions of recombinant plasmids were prepared as described previously (Bagnara-Tardif *et al.*, 1992). DNA was sequenced using the dideoxy chain termination method (Sanger *et al.*, 1977), with [³⁵S]dATP α S and a Sequenase 2.0 sequencing kit (US Biochemicals). Two different clones from two independent PCR events were sequenced to ensure that PCR did not induce any base exchange. The entire sequence described was determined on both strands. Analysis of the sequence was carried out using the DNASIS program (Pharmacia). Protein sequences were compared with the data-banks using the BLASTP program from NCBI (Altschul *et al.*, 1990).

Expression of *celF* in *E. coli*. The structural gene was amplified by PCR with restriction-site-containing primers (*Nco*I at the start of the gene and *Xho*I at the end) to facilitate in-frame cloning into the expression vector. The oligo primer sequences were 5'catg**ccatg**GCTTCAAGTCCTGCAAAC3' (5' end) and 5'ccg**ctcgag**TTGGATAGAAAGAAGTGC3' (3' end), with sequences complementary to the target DNA given in upper case letters and restriction sites in bold face type. After a digestion stage with *Nco*I and *Xho*I, the PCR-amplified fragment

was ligated into *Nco*I- and *Xho*I-digested pET22b(+). The coding sequence of *celF* was fused in-frame with an upstream sequence encoding the PelB leader peptide, and with a downstream sequence encoding six histidine residues (His-Tag). This gene fusion was under the control of the T7 promoter. The resulting phasmid was named pETFp. Another expression vector (pETFc) was obtained by deleting the sequence encoding PelB leader peptide. pETFp was digested with *Nco*I and *Nde*I and repaired with the Klenow fragment of DNA polymerase I. Since the phasmid was not stably maintained in BL21(DE3), *E. coli* DH5 α was transformed with the recombinant phasmids prior to being stored. The pETFp and pETFc constructs were verified by performing restriction site and DNA sequence analyses. Recombinant proteins were expressed in *E. coli* BL21(DE3).

To determine the solubility of the expressed proteins and their subcellular localization (i.e. cytoplasmic and/or periplasmic), cells were grown at 37 °C to a cell density equivalent to an OD₆₀₀ of 1 and IPTG was then added to a final concentration of 0.4 mM. Two hours after starting induction, the cells were harvested by centrifugation and washed in 20 mM Tris/HCl, pH 7.5. Aliquots of cell suspensions were used for electron microscopy and the remainder were disrupted in a French press. The suspensions obtained were centrifuged for 20 min at 14000 *g*. The pellets were washed twice in 20 mM Tris/HCl, pH 7.5. Aliquots of insoluble fractions were washed with 6 M urea before being dissolved in 8 M urea. All fractions were analysed by SDS-PAGE.

Aliquots of insoluble fractions were suspended in 6 M guanidinium chloride; 100 mM sodium phosphate; 10 mM Tris/HCl, pH 8. After 1 h, solubilized proteins bearing the His-Tag were purified by affinity chromatography on Ni-NTA resin (Novagen) according to the manufacturer's instructions.

To isolate soluble periplasmic proteins, cells were grown at 37 °C to an OD₆₀₀ of 1.6–2.4. Cultures were then left to equilibrate at 17 °C for 30 min before adding 40 μ M IPTG and then shaken overnight at 17 °C. Cells were centrifuged and resuspended in ice-cold buffer I [20 mM Tris/HCl, pH 8; 20% (w/v) sucrose] at an OD₆₀₀ of 5, and incubated on ice for 10 min. Cells were then pelleted by centrifugation at 16300 *g* at 4 °C for 2 min and osmotically shocked by resuspending in the same volume of ice-cold buffer II (20 mM Tris/HCl, pH 8). After incubating for 10 min on ice with gentle shaking, cells were centrifuged for 10 min at 10400 *g* (4 °C). The supernatant, which constituted the periplasmic fraction, was concentrated in a 400 ml Amicon concentrator before performing precipitation in the presence of ammonium sulfate at 80% saturation.

Electron microscopy of inclusion bodies. Cells were fixed in 2.5% (v/v) glutaraldehyde and 2% (w/v) osmium tetroxide, dehydrated in ethanol and embedded in Epon. Ultrathin sections were contrasted with aqueous uranyl acetate followed by lead citrate and examined with a Siemens Elmiskop 1A electron microscope.

N-terminal amino acid sequence analysis. Recombinant proteins were analysed by SDS-PAGE (7%, w/v, acrylamide) and transferred onto a modified fibreglass membrane (Biometra). The membrane was stained with Ponceau red for protein band detection. The recombinant protein band on the membrane was excised and the N-terminal amino acid sequence of the protein was determined using an Applied Biosystems 470 A sequence analyser.

Cellulase activity assays. Cellulase activity was assayed by mixing 1 ml of an appropriate enzyme dilution with 4 ml of 1% (w/v) CM-cellulose (medium viscosity; Sigma-Aldrich), Avicel

cellulose (Fluka), swollen cellulose [phosphoric-acid-swollen Avicel cellulose (Wood, 1988)] or xylan (oat spelts), in 25 mM potassium phosphate, pH 7.0. The amount of reducing sugars released in 1 ml was measured at various incubation times using the method described by Park & Johnson (1949) after removing solids by centrifugation, if required. One unit (IU) of cellulase activity corresponds to 1 μ mol D-glucose equivalent released min⁻¹.

p-Nitrophenyl (*p*-NP)-cellobioside, *p*-NP-lactoside, *p*-NP-glucoside and *p*-NP-xyloside (Sigma) were also tested. Samples (900 μ l) of a solution containing 1 g chromophoric substrate l⁻¹ in 25 mM potassium phosphate, pH 7.0 were mixed with 100 μ l diluted enzyme and incubated at 37 °C. The release of *p*-nitrophenol was followed by monitoring *A*₄₀₀. One unit of activity was defined as that corresponding to 1 μ mol *p*-nitrophenol liberated min⁻¹.

The protein concentration was determined using the Lowry method with BSA as the standard.

RESULTS

Cloning of the *celF* gene

In a previous study (Bagnara-Tardif *et al.*, 1992), we characterized part of a *cel* gene cluster of *Clostridium cellulolyticum*. A truncated ORF preceding the *celC* gene encoded a polypeptide whose C-terminal amino acid sequence included the reiterated segment typical of the *C. cellulolyticum* proteins involved in cellulolysis. The missing part of the ORF, now termed *celF*, was cloned in two steps of inverse PCR (Fig. 1). Flanking DNA (1009 bp) was recovered from the first 1695 bp *Bam*HI fragment. The 847 bp region upstream from this fragment was obtained by the same method from a 1641 bp *Kpn*I fragment.

Sequence analysis of the *celF* gene

The *celF* coding sequence with a length of 2169 nt was found to be present within a 3 kb fragment beginning with a *Kpn*I site and ending in a *Hind*III site (Fig. 2). The ORF encodes a polypeptide containing 722 amino acids with a calculated molecular mass of 80550 Da. The coding sequence is preceded by a typical ribosome-binding site. As expected, the N-terminal peptide sequence exhibits the characteristics of a 29 residue leader peptide. The sequence Ile₂₇-X₂₈-Ala₂₉-Ala₃₀ might constitute the consensus motif for leader peptide excision (Perlman & Halvorson, 1983). The mature protein consisting of 693 amino acids was found to have a calculated molecular mass of 77626 Da. Comparisons with sequences in the EMBL-GenBank database revealed that CelF (and the polypeptide encoded by *Clostridium josui* ORF1) were very similar to three proteins: (i) the cellobiohydrolase CelS of *Clostridium thermocellum* ATCC 27405 (Wang *et al.*, 1993; GenBank accession member L06942), which is probably identical to the S8 subunit of the cellulosome of *Clostridium thermocellum* YS (Morag *et al.*, 1993); (ii) the C-terminal domain of *Caldocellum saccharolyticum* endoglucanase CelA (Lüthi *et*

KpnI			
1	GGTACCGACTTTACAGTGTCAAATAATGTTGTAACAATCTCAAGAGCATTATTTGAGCACT	1981	AAAACCCCTGTATATGCTGACCCAGGTAGTAACACTTGGTTTGGTATGCAGGTATGGTCAA
	G T D F T V S N N V V T I S K S Y L S T	428	E N P V Y A D P G S N T W F G M Q V W S
61	TTAGCAGTTGGTTCAAAGACACTGACATTTGATTTTGGTTTACAAATAATCCAGTTCTG	2041	TGCAGCGTGTAGCTGAATTGTACTATAAGACTGGCGATGCCAGAGCTAAGAACTCTTAG
	L A V G S K T L T F D F G V T N N P V L	448	M Q R V A E L Y Y K T G D A R A K K L L
121	ACTTTAAACATCACTGACTCTACACCTGTTGTTACAGGACTTGGAGTAAAGATTGCTTCA	2101	ACAAATGGGCAAAATGGATTAAATGGCGAAATCAAGTTCAATGCTGACGGAACATTCCAGA
	T L T I T D T S T P V V T G L G V I A S	468	D K W A K W I N G E I K F N A D G T F Q
181	GTAACAGTAAAACCTGGTGATATAACAGTACCTGTAACCTGAGCAATGTTGTTAAA	2161	TTCTAGCACAAATTGATTGGGAAGGACAGCCGATCTTGGAAATCCACACAGGGATACA
	V T G K T G D T I T V P V T L S N V V K	488	I P S T I D W E G Q P D T W N P T Q G Y
241	TCAGGTAATGTAGGAACATGTAATTTCTATATTACATACGATGCAAGCATGCTGCAGGCA	2221	CCGGAATGCAAACTTGCAATGTTAAAGTTGTTAACTATGGTACTGACCTAGGTTGTGCTT
	S G N V G T C N F Y I T Y D A S M L Q A	508	T G N A N L H V K V V N Y G T D L G C A
301	GTATCAGCAACAGCTGGTGACATGACTCAATGCACCACTTAACCTCTCAAGCAGCATC	2281	CTTCACTTGCAAAACATGACTTACTATGCTGCTAAATCAGGAGATGAACTTCAAGGC
	V S A T A G D I V L N A P V N F S S S I	528	S S L A N T L T Y Y A A K S G D E T S R
361	AACGCAACAACTGGTACAATCAGTATCTTTTCTGGATAACACTATAGGTGATCAGCTC	2341	AGAATGCACAGAAATTAATGACGCTATGTGGAATACTATAGCGATTCAAAGGTTATAT
	N A T T G T I S I L F L D N T I G D Q L	548	Q N A Q K L L D A M W N N Y S D S K G I
421	ATTACCAGCGATGGAGTAGTTGCTAATCTTACATTTAAAGTAGTAGGAATCTCAAGCACA	2401	CAACTGTTGAACAGCTGGTGATTACCATAGATTCCTTGATCAGGAAGTTTGTACCAG
	I T S D G V V A N L T F K V V G T S S T	568	S T V E Q R G D Y K R F L D Q E A V F V P
481	ACAACCTCATTCGCATTTAAAGCAGCGGAGCTTTCCGTAATGGAACATGTCTAAATTT	2461	CTGGTTGGACTGGAAAAATGCCTAACGGCGACGTAATCAAACTGTTGTCAAGTTCATAG
	T T P I A F A G F G A F G N G N M S K I	588	A G W T G K M P N G D V I K S G V K F I
541	TCTGATATTACATTACAAACGGAAGTGCAAACTTAATTAATTATTGAATTTAAATTTT	2521	ACATTCGTTCCAAGTACAAGCAGGATCTGTAATGGCAGACAAATGGTTGCTGATTACAGG
	S D I T F T N G S A K L N *	608	D I R S K Y K Q D P E W Q T M V A A L Q
601	CCATACTTTATGGCATCAAGCCATAAAGTATGGAGTAAAAAATATGAGCAGATTTTA	2581	CAGGACAGGTTCCAACCTCAGAGATTACACCGTTTCTGGGCTCAGAGTGAATTTGCAGTG
		628	A G Q V P T Q R L H R F W A Q S E F A V
661	CTCTGCCATAAAATTCAGGAATAAGGAAGGTGTAAGTGAAGATTTTAAAGAG	2641	CAATGGAGTTTATGCAATCTCTCCAGATCAAGGTCAGAAAAATTTATGGGTGATG
1		648	A N G V Y A I L F P D Q G P E K L L G D
	-30 kcal/mol		
721	TAGGAGCAGTTGCAGTTGCTGCTGCAATGTCTATATGCAACCAACAGCATAA	2701	TAAACGGTGACGAACTGTAGACGCTATTGACCTTGCTATACTTAAAAATATCTTTAA
	V G A V A V A A A M S L S I M A T T S I	668	V N G D E T V D A I D L A I L K K Y L L
781	ATGCAGCTTCAAGTCTGCAACCAAGGTGTACCAAGATCGTTTGAATCCATGTACAGCA	2761	ACAGCAGTACTACAATAAATGCTGCAATGCGATATGAATAGTGATAATGCTATTGACG
	N A A S S P A N K V Y Q D R F E S M Y S	688	N S S T T I N T A N A D M N S D N A I D
841	AGATTAAAGGATCTGCAACAGGATACTTCAAGTGAACGGAATTCCTTACCCTCAATTG	2821	CTATTGACTATGCTCTTTTAAAGAAAGCACTTCTTCTATCCAATAGTATTTAATACTAT
	K I K D P A N G Y F S E Q G I P Y H S I	708	A I D Y A L L K K A L L S I Q *
901	AAACACTGATGGTGAAGCTCTGACTACGGACATGTTACAACAAAGTGAAGCTATGCTCT	2881	GACGCATATGTAACCTTAAAGTCCGGACAGTATTTGGTTTATTAAATTAATCTATTCTTG
	E T L M V E A F D Y G H V T T S E M A S	2941	TACTGTCCGGCTTATGAGTTACAAAGAAAAAGAAAGGATTAAGGTGAAGAACATGAT
961	ATTATATGTTGGTGAAGCAATGCACGAAGATTTTCAAGGATTTTACAGGTTTGTATA	1	
	Y Y M W L E A M H G R F S G D F T G F D		HindIII
1021	AGTCTTGGTCTGTACCAGCAAGTATTTGATCCCAACAGAAAGGATCAGCCCAATACAA	3001	CAAAGGTTCAAGCTT
	K S W S V T E Q Y L I P T E K D Q P N T	3	K G S S
1081	GTATGAGCAGATATGATGCTAACAACCGGCTACATACGCTCCGGAATTTTCAGGACCCAA		
	S M S R Y D E A K P A T Y A P E F Q D P		
1141	GCAAGTATCCTTCTCCGTTGGATACTAGTCAACCTGTTGGTAGAGATCCAATCAATCAC		
	S K Y P S P L D T S Q P V G R D P I N S		
1201	AATTGACTTCAGCATACGGAACAAGCATGCTGTATGGAATGCACCTGGATCTTGCAGTTG		
	Q L T S A Y G T S M L Y G M H W I L D V		
1261	ATAACTGGTATGATTGTTGAGCAAGAGCTGATGGAACCTCCAAGCCATCATATATCAATA		
	D N W Y G F G A R A D G T S K P S Y I N		
1321	CTTTCCAAAGAGGTGAGCAGGAGTCAACTTGGGAACCATACCTCAACATGTTGGGATG		
	T F Q R G E Q E S T W E T I P Q P C W D		
1381	AACATAAATTTGGTGGACATCGGATTCCTGGATCTCTTACAAGGATACAGGTACTC		
	E H K F G G Q Y G F L D L F T K D T G T		
1441	CGGCAAGCAATTCAAATATACAAATGCTCCAGATGCTGATGCTGCTGAGTTCAAGCAA		
	P A K Q F K Y T N A P D A D A R A V Q A		
1501	CTTACTGGGCTGATCAGTGGGCAAAAGCAAGGTAAGAGCGTAAGTACTAGCGTAGGTA		
	T Y W A D Q W A K E Q G K S V S T S V G		
1561	AGGCAACAAAGATGGGTGATTACCTTAGATATTCATTCTTTGATAAGTATTTAGAAAAGA		
	K A T K M G D Y L R Y S F F D K Y F R K		
1621	TCGGACAACCTTCTCAGGCTGGTACCGGATATGATGCAGCACATATGCTGTTTACGTT		
	I G Q P S Q A G T G Y D A A H Y L L S W		
1681	ACTATGCATGGGTTGGTGAATGACTCTACCTGGTCTTGGATAATCGGTAGCAGTCATA		
	Y Y A W G G I D Y T W S W I I G S H		
1741	ATCATTTCGGTTACCAAGAACCATTTGCAGCTTGGGTACTTTCAACAGATGCAAACTTCA		
	N H F G Y Q N P F A A W V L S T D A N F		
1801	AGCCTAAGTCATCAATGTCATCAGCTGGGCAAGAGTTTGGACAGACGCTTGAAT		
	K P K S S N G A S D W A K S L D R Q L E		
1861	TCTATCAGTGGTGCAGTCAGCAGAAGGTGCTATGCGGTTGGAGCTCAAACTACGGA		
	F Y Q W L Q S A E G A I A G G A T N S W		
1921	ACGGACGTTATGAAGCAGTTCCTTCAGGTACATCAACATTCATGGAATGGGTTATGTAG		
	N G R Y E A V P S G T S T F Y G M G Y V		

Fig. 2. Nucleotide sequence and the predicted translation product of the *Clostr. cellulolyticum celF* gene, including the upstream and downstream flanking regions (3' end of *cipC* and 5' end of *celC*). Shine-Dalgarno sequences have been underlined. Restriction sites are shown above the nucleotide sequence. The inverted repeated sequence in the intergenic region *cipC-celF* is indicated by arrows. The upward arrow indicates the predicted signal sequence cleavage site.

al., 1991; GenBank accession number L32742); and (iii) the cellobiohydrolase CbhB (formerly termed CenE) of *Cellulomonas fimi* (Shen *et al.*, 1995; Genbank accession number L38827). Apart from the signal peptides, CelS and CelF had 60% identical residues. The C terminus of both proteins contained the conserved, duplicated region typical of many clostridial cellulases and hemicellulases. Excluding the duplicated region, the sequences of CelF and of the C-terminal domain of *Cald. saccharolyticum* CelA were 65% identical, whereas the sequences of CelF and of CbhB of *Cell. fimi* were only 57% identical. Fig. 3 illustrates the similarity between CelF, CelS and the C-terminal domain of CelA. Similarities were also observed with the available N-terminal sequence of *Clostridium stercorarium* Avicelase II (Bronnenmeier *et al.*, 1991) (45% identity among 33 residues). The similarity between residues 578–604 of CelF and residues 49–76 of an endoglucanase of *Bacteroides ruminicola* (Matsushita *et al.*,

Fig. 3. Protein sequence comparisons between CelA from *Cald. saccharolyticum*, and CelF and CelS from *Clost. thermocellum*. The amino acid sequence of the CelA protein from *Cald. saccharolyticum* was obtained from the GenBank database (accession number L32742). Amino acids which are identical in both the mature proteins and CelF are denoted by shaded boxes. Amino acids belonging to the signal sequences are given in italics. The C-terminal clostridial conserved reiterated sequences are boxed.

1991) has been pointed out previously (Bagnara-Tardif *et al.*, 1992).

Sequence analysis of the region preceding *celF*

The 3' region of an ORF was found upstream from *celF*. The intergenic 116 bp region includes an inverted repeated sequence extending over 35 nt [$\Delta G = -30$ kcal mol⁻¹ (-126 kJ mol⁻¹)]. This structure might constitute either a terminator or an intercistronic regulatory element. The predicted translational product of the preceding ORF showed 45% identity with the C-terminal sequence of CbpA, the cellulosome scaffolding protein from *Clostridium cellulovorans* (Shoseyov *et al.*, 1992) (Fig. 4), where the same organization of hydrophilic and hydrophobic domains was observed. This ORF was called *cipC*, by analogy with the *cipA* gene encoding the cellulosome-

integrating protein of *Clost. thermocellum*. The reiterated sequence which is present at the C terminus of CipA from *Clost. thermocellum* NCIB 10682 (Gerngross *et al.*, 1993) was not observed at the C terminus of CipC.

Overproduction of the CelF protein in *E. coli*

The expression phasmids pETFc and pETFp encoding CelFc and CelFp, respectively, were constructed to study the cytoplasmic and periplasmic forms of CelF in *E. coli* (Fig. 5). Both polypeptides carried an eight amino acid C-terminal extension including six histidine residues, and could be purified by performing Ni²⁺ affinity chromatography. A methionine residue occurs prior to the native sequence in the N-terminal sequence of the periplasmic mature CelFp protein.

In order to analyse the expression, the solubility and the localization of the recombinant proteins, crude cell extracts, and soluble and insoluble fractions were examined by SDS-PAGE. As shown in Fig. 6(a), CelFc and CelFp were both produced in soluble (lanes 3 and 8) and insoluble (lanes 4 and 9) forms. The inclusion bodies could be dissolved in 8 M, but not in 6 M urea. CelFc, but not CelFp, was degraded to a smaller peptide with a molecular mass of about 45 kDa. Like CelFp and CelFc, the 45 kDa species could be purified by Ni²⁺ affinity chromatography, indicating that it was derived from the C-terminal region of CelFc. Transmission electron microscopy analysis of thin sections of induced BL21(DE3)[pETFc] and BL21(DE3)[pETFp] showed in both cases the presence of inclusion bodies (Fig. 7). This was quite surprising, since periplasmic inclusion bodies have rarely been reported (Bernadac *et al.*, 1987). Ribosomes were excluded from some parts of the cytoplasm (Fig. 7b) because of the presence of aggregated heterologous proteins. Interestingly, these showed up in the form of less electron-dense aggregates than the periplasmic inclusion bodies or than the BL21(DE3)[pETFc] cytoplasmic inclusion bodies. The composition of the CelFp aggregates was analysed by protein sequencing. Insoluble material from strain BL21(DE3)[pETFp] was fractionated by SDS-PAGE (10% acrylamide) and the N-terminal amino acid sequence of the band corresponding to CelF was determined. Two different N-terminal sequences were obtained. The first sequence corresponded to the expected N-terminal sequence of the mature protein (approx. 50% of the proteins analysed) and the second sequence corresponded to the N-terminal sequence of the PelB signal sequence. This indicated that the signal peptide was correctly removed from a portion of the proteins, but that a large fraction of the CelF (approx. 50%) was not processed and probably not exported into the periplasmic space. It could therefore be assumed that under these induction conditions, the export machinery was overloaded, which led the precursor form of CelFp to accumulate inside the cytoplasm as observed in Fig. 7(b). The different appearance of cytoplasmic aggregates of CelFp and CelFc might be correlated with the lack of CelFp degradation.

Fig. 4. Amino acid identities between the C-terminal hydrophobic domains of the scaffolding proteins CbpA from *Clostridium cellulovorans*, CipC from *Clostridium cellulolyticum* and CipA from *Clostridium thermocellum*. The hydrophilic domains of CbpA from *Clostridium cellulovorans* and CipC are shaded light grey. Gaps have been introduced to show the similarities between the proteins. Identical amino acids are denoted by dark grey shaded boxes.

Fig. 5. Construction of expression phasmids pETFp and pETFc. Only the restriction sites used for the construction are shown. Boxes contain details of the upstream and downstream in-frame fusions. Amino acids in bold characters are from CelF and those in light characters originate from the construction. Details are given in Methods.

The culture conditions were optimized so that mature CelFp could be purified in soluble form from BL21(DE3)[pETFp] using the osmotic shock procedure.

We first checked that 40 μ M IPTG was sufficient to achieve complete induction. Both secretion efficiency and protein solubility have been found to depend on cell

Fig. 6. (a) SDS-PAGE analysis of induced *E. coli* BL21(DE3)[pETFc] and *E. coli* BL21(DE3)[pETFp]. Proteins from 80 μ l culture were separated by SDS-PAGE (10% acrylamide) and stained with Coomassie Blue. Lanes: 1, cell extract of strain BL21(DE3); 2–5, strain BL21(DE3)[pETFc]; 7–10, strain BL21(DE3)[pETFp]; 2 and 7, cell extracts; 3 and 8, soluble fractions; 4 and 9, insoluble fractions resuspended in an equivalent volume of water; 5 and 10, granules washed in 6 M urea and dissolved in an equivalent volume of 8 M urea. (b) SDS-PAGE analysis of 10 μ l 50-fold concentrated periplasmic fraction (lane 1). Lanes 6 (a) and 2 (b) contain marker proteins with molecular masses indicated on the right. The arrow indicates the position of CelF at 78/80 kDa.

density and induction temperature (LaVallie *et al.*, 1993; Stader *et al.*, 1990; Totsuka & Fukazawa, 1993). To investigate the influence of induction temperature and stage of growth at the time of induction, cultures were incubated at 37 °C until the desired OD₆₀₀ was reached. When necessary, the culture was allowed to equilibrate at the desired induction temperature for 30 min before adding 40 μ M IPTG. The culture was then incubated for 2 h at 37 °C, or overnight when expression was performed at 17 °C. To assess the expression and solubility of CelFp, the cell pellets were subjected to osmotic shock treatments (see Methods). After the final shock, the supernatants and pellets were analysed by SDS-PAGE, and the cellulase activity tested. The soluble activity released by osmotic shock from cultures induced at 17 °C was four- to ninefold greater than from those induced at 37 °C (Table 1). At 17 °C, the total activity released increased in proportion to the OD₆₀₀ at the time of induction, probably due to the fact that a larger number of cells produced the heterologous protein. In fact, *E. coli* cells do not grow satisfactorily at 17 °C and after overnight induction, the biomass obtained had increased only twofold. Cell lysis occurred during longer induction times. The low cellulase activity in the 37 °C shock supernatant might be attributable to the fact that most of the protein probably partitioned into large insoluble inclusion bodies which were not expelled from the periplasm during the osmotic shock procedure.

Enzymic activity of soluble CelFp

The expression of pETFp in *E. coli* BL21(DE3) after overnight incubation at 17 °C in the presence of 40 μ M IPTG yielded soluble active CelFp. As expected, the BL21(DE3) transformed with the expression vector

pET22b(+) did not contain any detectable cellulase activity when grown in either the presence or absence of IPTG.

Fiftyfold concentrated final shock supernatant (Fig. 6b) was used to test the ability of the enzyme to hydrolyze various β -1,4 glycans. CelFp was able to hydrolyse various cellulose types, especially amorphous cellulose (Fig. 8). This enzyme showed very poor activity however on CM-cellulose, a substituted amorphous cellulose substrate, which clearly differentiates it from the classical endoglucanases (Fierobe *et al.*, 1991, 1993). Whereas measures performed after 24 h in the presence of Avicel showed that a linear increase in the reducing sugar released had occurred (data not shown), the hydrolysis reaction appeared to be blocked after only a few minutes in the case of CM-cellulose. No activity was observed on *p*-NP-cellobioside, *p*-NP-glucoside or *p*-NP-lactoside (less than 10⁻⁵ IU ml⁻¹), or on xylan (less than 10⁻⁴ IU ml⁻¹).

DISCUSSION

Madarro *et al.* (1991) have recently established that the Avicel-adsorbed fraction of the *Clostridium cellulolyticum* complex consists mainly of three major components. Two of them (the 160/170 kDa and the 72 kDa components) do not show any detectable activity on CM-cellulose and xylan, and the third one (95 kDa) is strongly active on CM-cellulose. The largest component is assumed to be the scaffolding protein, whose gene lies upstream from *celF* and has been partially cloned. Its molecular mass is in good agreement with those of similar clostridial scaffolding proteins. CelF might constitute the 72 kDa major component of the *Clostridium cellulolyticum* cellulosome, which did not exhibit any detectable activity on CM-cellulose or xylan (Madarro *et al.*, 1991).

Fig. 7. Electron micrographs of thin sections of strain BL21(DE3)[pETFc] (a) and BL21(DE3)[pETFp] (b). Cells were grown at 37 °C in LB medium containing 100 µg ampicillin ml⁻¹. cIB, cytoplasmic inclusion body; pIB, periplasmic inclusion body; cAP, cytoplasmic aggregated proteins. Bar, 0.2 µm.

CelF is similar to the catalytic core of several enzymes belonging to the cellulase family L (Shen *et al.*, 1994) [family 48 of glycosyl hydrolases (Henrissat & Bairoch, 1993; B. Henrissat, personal communication)]. The cellulases of this family whose properties have been investigated to date include the following three proteins: *Clostridium stercorarium* Avicelase II (Bronnenmeier *et al.*, 1991), *Clostridium thermocellum* CelS/S8 (Morag *et al.*, 1991; Kruus *et al.*, 1995b), and *Cellulomonas fimi* CbhB (formerly termed CenE)

(Shen *et al.*, 1995). The activity of these three enzymes is highest on amorphous cellulose, and much lower if detectable on CM-cellulose and not detectable at all on aryl- β -D-cellobiosides. The same pattern was observed with CelF, whose activity on CM-cellulose appeared to be blocked after a few minutes. This might be due to CelF being inhibited by the presence of carboxymethyl groups on the substituted cellulose. It is therefore plausible that CelF might be a recurrently acting enzyme.

Table 1. Effect of induction temperature and stage of growth at the time of induction on soluble periplasmic CelfP production

Parameter	Induction temp. (°C)			
	37	17	17	17
OD ₆₀₀ of culture at time of induction	2.2	1.6	2.0	2.4
OD ₆₀₀ of harvested culture	4.0	3.0	4.0	5.0
Cellulase activity [IU (1 broth) ⁻¹]*	0.5	2.0	3.9	4.3

* Activity of the periplasmic fraction, obtained by osmotic shock from 1 l broth, on swollen Avicel (see Methods).

Fig. 8. Activity of Celf on various cellulosic substrates. In all the assays, 300 μ l 50-fold concentrated periplasmic fraction (1.5 mg ml⁻¹) diluted in 1 ml was used. Each data point represents the mean of at least three values which did not vary more than 15% from the mean. ●, Swollen Avicel; ▲, Avicel; □, CM-cellulose; ○, xylan. Activity is expressed as μ mol D-glucose equivalent released (ml diluted enzyme)⁻¹.

Avicelase II and S8 has been reported to show significant xylanase activity (Bronnenmeier *et al.*, 1991; Morag *et al.*, 1991), whereas CelS, CbhB and Celf did not hydrolyse xylan. In the case of CelS, which was purified from aggregated recombinant protein (Wang *et al.*, 1994; Kruus *et al.*, 1995b), the possibility that a loss of xylanase activity may have occurred in the course of renaturation cannot be ruled out. However, Avicelase II and S8 were both purified from whole cellulase systems including xylanases, and the xylanolytic activity of the preparation may have been due to contamination with high specific activity xylanases. The absence of xylanase activity in Celf obtained in soluble form from BL21(DE3)[pETFP] supports this interpretation.

The possibility of obtaining Celf in undenatured form from a recombinant clone should therefore open the way to characterizing the true properties of the enzyme in detail. In particular, if the identity of Celf with one of the

three major cellulosome components is confirmed, it will be of interest to test its ability to degrade crystalline cellulose synergistically with the purified endoglucanases and/or CipC.

Some indications on the essential role of this kind of enzyme in the synergistic mechanism of cellulose degradation have been obtained by Wu & Demain (1988) and Kruus *et al.* (1995a), working on CipA (then termed S_L) and CelS (then termed S_S) of *Clostr. thermocellum*, and by Creuzet *et al.* (1983) with an endoglucanase and an exoglucanase of *Clostr. stercorarium*.

ACKNOWLEDGEMENTS

We thank the 'Service de Séquençage' (IFRC 1000, CNRS, Marseille) for performing the N-terminal Edman sequencing of CelfP. We are grateful to Vincent Méjean and Marc Rousset for helpful discussions and to Jessica Blanc for correcting the English manuscript.

This research was supported by grants from the Centre National de la Recherche Scientifique and the Université de Provence.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Bagnara-Tardif, C., Gaudin, C., Bélaich, A., Hoest, P., Citard, T. & Bélaich, J. P. (1992). Sequence analysis of a gene cluster encoding cellulases from *Clostridium cellulolyticum*. *Gene* **119**, 17–28.
- Bélaich, A., Fierobe, H. P., Baty, D., Busetta, B., Bagnara-Tardif, C., Gaudin, C. & Bélaich, J. P. (1992). The catalytic domain of endoglucanase A from *Clostridium cellulolyticum*, effects of arginine 79 and histidine 122 mutations on catalysis. *J Bacteriol* **174**, 4677–4682.
- Bernadac, A., Bolla, J. M., Lazdunski, C., Inouye, M. & Pages, J. M. (1987). Precise localization of an overproduced periplasmic protein in *Escherichia coli*, use of double immuno-gold labelling. *Biol Cell* **61**, 141–147.
- Bronnenmeier, K., Rucknagel, K. P. & Staudenbauer, W. L. (1991). Purification and properties of a novel type of exo-1,4- β -glucanase (Avicelase II) from the cellulolytic thermophile *Clostridium stercorarium*. *Eur J Biochem* **200**, 379–385.
- Creuzet, N., Berenger, J. F. & Frixon, C. (1983). Characterization of exoglucanase and synergistic hydrolysis of cellulose in *Clostridium stercorarium*. *FEMS Microbiol Lett* **20**, 347–350.
- Ducros, V., Czjzek, M., Bélaich, A., Gaudin, C., Fierobe, H. P., Bélaich, J. P., Davies, G. J. & Haser, R. (1995). Crystal structure of the catalytic domain of a bacterial cellulase belonging to family 5. *Structure* **3**, 939–949.
- Engbrecht, J., Brent, R. & Kaderbhai, M. A. (1991). In *Current Protocols in Molecular Biology*, pp. 1.6.1–1.6.10. Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith & K. Struhl. USA: Greene Publishing Associates & John Wiley.
- Faure, E., Bélaich, A., Bagnara, C., Gaudin, C. & Bélaich, J. P. (1987). Characterization of the cellulolytic system of *Clostridium cellulolyticum*. In *Biomass for Energy and Industry* (4th European Communities Conference), pp. 717–721. Edited by G. Grassi, B. Delmon, J. F. Molle & H. Zibetta. London & New York: Elsevier Applied Science.
- Faure, E., Bélaich, A., Bagnara, C., Gaudin, C. & Bélaich, J. P. (1989).

- Sequence analysis of the *Clostridium cellulolyticum* endoglucanase-A-encoding gene, *celCCA*. *Gene* **84**, 39–46.
- Fierobe, H. P., Gaudin, C., Bélaich, A., Loutfi, M., Faure, E., Bagnara, C., Baty, D. & Bélaich, J. P. (1991). Characterization of endoglucanase A from *Clostridium cellulolyticum*. *J Bacteriol* **173**, 7956–7962.
- Fierobe, H. P., Bagnara-Tardif, C., Gaudin, C., Guerlesquin, F., Sauve, P., Bélaich, A. & Bélaich, J. P. (1993). Purification and characterization of endoglucanase C from *Clostridium cellulolyticum* – catalytic comparison with endoglucanase A. *Eur J Biochem* **217**, 557–565.
- Fujino, T., Béguin, P. & Aubert, J. P. (1993a). Organization of a *Clostridium thermocellum* gene cluster encoding the cellulosomal scaffolding protein CipA and a protein possibly involved in the attachment of the cellulosome to the cell surface. *J Bacteriol* **175**, 1891–1899.
- Fujino, T., Karita, S. & Ohmiya, K. (1993b). Nucleotide sequences of the *celB* gene encoding endo-1,4- β -glucanase-2, ORF1 and ORF2 forming a putative cellulase gene cluster of *Clostridium josui*. *J Ferment Bioeng* **76**, 243–250.
- Gerngross, U. T., Romaniec, M. P. M., Kobayashi, T., Huskisson, N. S. & Demain, A. L. (1993). Sequencing of a *Clostridium thermocellum* gene (*cipA*) encoding the cellulosomal SL-protein reveals an unusual degree of internal homology. *Mol Microbiol* **8**, 325–334.
- Giallo, J., Gaudin, C., Bélaich, J. P., Petitdemange, E. & Caillet, F. (1983). Metabolism of glucose and cellobiose by cellulolytic mesophilic *Clostridium* sp. strain H10. *Appl Environ Microbiol* **45**, 843–849.
- Giallo, J., Gaudin, C. & Bélaich, J. P. (1985). Metabolism and solubilization of cellulose by *Clostridium cellulolyticum* H10. *Appl Environ Microbiol* **49**, 1216–1221.
- Grépinet, O., Chebrou, M. C. & Béguin, P. (1988). Nucleotide sequence and deletion analysis of the xylanase gene (*xynZ*) of *Clostridium thermocellum*. *J Bacteriol* **170**, 4582–4588.
- Henrissat, B. & Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **293**, 781–788.
- Kruus, K., Lua, A. C., Demain, A. L. & Wu, J. H. D. (1995a). The anchorage function of CipA (CelL), a scaffolding protein of the *Clostridium thermocellum* cellulosome. *Proc Natl Acad Sci USA* **92**, 9254–9258.
- Kruus, K., Wang, W. K., Ching, J. & Wu, J. H. D. (1995b). Exoglucanase activities of the recombinant *Clostridium thermocellum* CelS, a major cellulosome component. *J Bacteriol* **177**, 1641–1644.
- Lamed, R., Setter, E. & Bayer, E. A. (1983a). Characterization of a cellulose-binding cellulase-containing complex in *Clostridium thermocellum*. *J Bacteriol* **156**, 828–836.
- Lamed, R., Setter, E., Kenig, R. & Bayer, E. A. (1983b). The cellulosome, a discrete cell surface organelle of *Clostridium thermocellum* which exhibits separate antigenic, cellulose-binding and various cellulolytic activities. *Biotechnol Bioeng Symp* **13**, 163–181.
- LaVallie, E. R., DiBlasio, E. A., Kovacic, S., Grant, K. L., Schendel, P. F. & McCoy, J. M. (1993). A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Bio/Technology* **11**, 187–193.
- Lüthi, E., Bahna Jasmal, N., Grayling, R. A., Love, D. R. & Bergquist, P. L. (1991). Cloning, sequence analysis and expression in *Escherichia coli* of a gene coding for a β -mannanase from the extremely thermophilic bacterium '*Caldocellum saccharolyticum*'. *Appl Environ Microbiol* **57**, 694–700.
- Madarro, A., Pena, J. L., Lequerica, J. L., Vallés, S., Gay, R. & Flors, A. (1991). Partial purification and characterization of the cellulases from *Clostridium cellulolyticum* H10. *J Chem Technol Biotechnol* **52**, 393–406.
- Matsushita, O., Russel, J. B. & Wilson, D. B. (1991). A *Bacteroides rumicola* 1,4- β -D-endoglucanase is encoded in two reading frames. *J Bacteriol* **173**, 6919–6926.
- Morag, E., Halevy, I., Bayer, E. A. & Lamed, R. (1991). Isolation and properties of a major cellobiohydrolase from the cellulosome of *Clostridium thermocellum*. *J Bacteriol* **173**, 4155–4162.
- Morag, E., Bayer, E. A., Hazlewood, G. P., Gilbert, H. J. & Lamed, R. (1993). Cellulase S₈ (CelS) is synonymous with the major cellobiohydrolase (subunit S8) from the cellulosome of *Clostridium thermocellum*. *Appl Biochem Biotechnol* **43**, 147–152.
- Ochman, H., Medhora, M. M., Garza, D. & Hartl, D. L. (1990). Amplification of flanking sequences by inverse PCR. In *PCR Protocols*, pp. 219–227. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press.
- Park, J. T. & Johnson, M. S. (1949). A submicrodetermination of glucose. *J Biol Chem* **181**, 149–151.
- Perlman, D. & Halvorson, H. O. (1983). A putative signal peptidase recognition site and sequence in eucaryotic and procaryotic signal peptides. *J Mol Biol* **167**, 391–409.
- Petitdemange, E., Caillet, F., Giallo, J. & Gaudin, C. (1984). *Clostridium cellulolyticum* sp. nov. a cellulolytic mesophilic species from decayed grass. *Int J Syst Bacteriol* **34**, 155–159.
- Quiviger, B., Franche, C., Lutfalla, G., Rice, D., Haselkorn, R. & Elmerich, C. (1982). Cloning of nitrogen fixation (*nif*) gene cluster of *Azospirillum brasilense*. *Biochimie* **64**, 495–502.
- Roig, V., Fierobe, H. P., Ducros, V., Czjzek, M., Bélaich, A., Gaudin, C., Bélaich, J. P. & Haser, R. (1993). Crystallization and preliminary X-ray analysis of the catalytic domain of endoglucanase A from *Clostridium cellulolyticum*. *J Mol Biol* **233**, 325–327.
- Salamitou, S., Tokatlidis, K., Béguin, P. & Aubert, J. P. (1992). Involvement of separate domains of the cellulosomal protein S1 of *Clostridium thermocellum* in binding to cellulose and in anchoring of catalytic subunits to the cellulosome. *FEBS Lett* **304**, 89–92.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schimming, S., Schwarz, W. H. & Staudenbauer, W. L. (1991). Properties of a thermoactive β -1,3 glucanase (lichenase) from *Clostridium thermocellum* expressed in *Escherichia coli*. *Biochem Biophys Res Commun* **177**, 447–452.
- Shen, H., Tomme, P., Meinke, A., Gilkes, N. R., Kilburn, D. G., Warren, R. A. J. & Miller, R. C., Jr (1994). Stereochemical course of hydrolysis catalysed by *Cellulomonas fimi* CenE, a member of a new family of β -1,4-glucanases. *Biochem Biophys Res Commun* **199**, 1223–1228.
- Shen, H., Gilkes, N. R., Kilburn, D. G., Miller, R. C., Jr & Warren, R. A. J. (1995). Cellobiohydrolase B, a second exo-cellobiohydrolase from the cellulolytic bacterium *Cellulomonas fimi*. *Biochem J* **311**, 67–74.
- Shima, S., Igarashi, Y. & Kodama, T. (1991). Nucleotide sequence analysis of the endoglucanase-encoding gene, *celCCD*, of *Clostridium cellulolyticum*. *Gene* **104**, 33–38.
- Shoseyov, O., Takagi, M., Goldstein, M. A. & Doi, R. H. (1992). Primary sequence analysis of *Clostridium cellulovorans* cellulose binding protein A. *Proc Natl Acad Sci USA* **89**, 3483–3487.
- Stader, J. A. & Silhavy, T. J. (1990). Engineering *Escherichia coli* to secrete heterologous gene products. *Meth Enzymol* **185**, 166–187.

- Tokatlidis, K., Salamitou, S., Béguin, P., Dhurjati, P. & Aubert, J. P. (1991).** Interaction of the duplicated segment carried by *Clostridium thermocellum* cellulases with cellulosome components. *FEBS Lett* **291**, 185–188.
- Totsuka, A. & Fukazawa, C. (1993).** Expression and mutation of soybean β -amylase in *Escherichia coli*. *Eur J Biochem* **214**, 787–794.
- Wang, W. K., Kruus, K. & Wu, J. H. D. (1993).** Cloning and DNA sequence of the gene coding for *Clostridium thermocellum* cellulase Ss (CelS), a major cellulosome component. *J Bacteriol* **175**, 1293–1302.
- Wang, W. K., Kruus, K. & Wu, J. H. D. (1994).** Cloning and expression of the *Clostridium thermocellum* celS gene in *Escherichia coli*. *Appl Microbiol Biotechnol* **42**, 346–352.
- Wood, T. M. (1988).** Preparation of crystalline, amorphous, and dyed cellulase substrates. *Meth Enzymol* **160**, 19–23.
- Wu, J. H. D. & Demain, A. L. (1988).** Proteins of the *Clostridium thermocellum* cellulase complex responsible for degradation of crystalline cellulose. In *Biochemistry and Genetics of Cellulose Degradation* (FEMS Symposium no. 43), pp. 117–131. Edited by J. P. Aubert, P. Béguin & J. Millet. London & New York: Academic Press.
-
- Received 2 October 1995; revised 1 December 1995; accepted 7 December 1995.