Molecular Cloning of *Clostridium thermocellum* DNA and the Expression of Further Novel Endo- β -1,4-glucanase Genes in *Escherichia coli*

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*Sau*3A fragments of *Clostridium thermocellum* (NCIB 10682) DNA were ligated into the *Bam*HI site of pBR322 and expressed in *Escherichia coli* HB101 and a Lac[−] mutant thereof. Twenty-eight clones with carboxymethylcellulase (CMCase) activity were selected from two libraries by means of the Congo Red plate assay. Restriction enzyme analysis indicated that the CMCase⁺ clones contained a total of 13 unique DNA inserts. Hybridization of recombinant plasmids with chromosomal DNA confirmed the physical maps in all but one case and was further used to demonstrate the absence of homology between the *Hind*III restriction fragments of similar size which occurred in many of the clones. Without exception, CMCase⁺ *E. coli* clones expressed endoglucanase activity, but differed with respect to the amount and nature of the enzyme activity produced; additionally, some clones had exoglucanase activity which, in at least one case, was not attributable to the production of a second enzyme. For a few selected clones, the partially purified CMCase was analysed by electrophoresis. A temperature profile characteristic of a thermostable enzyme was demonstrated for the endoglucanase of one of the most active clones. Based on the evidence presented here, it is probable that the 13 unique DNA fragments described do not contain any of the *C. thermocellum* endoglucanase genes previously cloned.

INTRODUCTION

Micro-organisms able to break down cellulose have great economic potential for increasing the utilization of renewable plant biomass as animal feed and for the production of fuels and chemicals by fermentation. Thermophilic bacteria are of particular interest in this respect, both in their own right, and as a source of desirable genes encoding highly active thermostable proteins for genetic constructions in mesophiles (Sonnleitner, 1983). Some 100 species of bacteria and fungi are known to produce cellulases (Ljungdahl & Eriksson, 1985) but comparatively little information is available regarding the biochemistry and genetic regulation of these important enzymes.

The presence of thermostable cellulolytic activity capable of completely degrading crystalline cellulose (Johnson *et al.*, 1982) has generated considerable interest in the anaerobic thermophile *Clostridium thermocellum*. The activity resides in a complex (the cellulosome) which is secreted into the culture supernatant during growth in batch culture and is composed of at least 14 polypeptides, having endoglucanase (1,4- β -D-glucan 4-glucanohydrolase, EC 3.2.1.4) and exoglucanase (cellobiohydrolase; 1,4- β -D-glucan cellobiohydrolase, EC 3.2.1.91) activities, and includes a 210 kDa non-enzymic cellulose-binding factor (Lamed *et al.*, 1983*a, b*).

The molecular mass of the complex is commonly quoted as 2.1 MDa but values calculated from electron micrographs of cellulosomes varied from 4 to 102 MDa, depending on the degree

Abbreviations: CMC, carboxymethylcellulose; CMCase, carboxymethylcellulase; DS, degree of substitution; MUC, 4-methylumbelliferyl β -D-cellobioside; MUG, 4-methylumbelliferyl β -D-glucoside.

of dissociation of the complex (Coughlan *et al.*, 1985). A recent ultrastructural study has confirmed the existence of large extracellular complexes in *C. thermocellum* and has also provided evidence for the likely mechanism of cellulolysis (Bayer & Lamed, 1986). Biochemical analysis of the cellulosome has achieved some success through the purification and characterization of two endoglucanases with molecular sizes of 56 kDa (Petre *et al.*, 1981) and around 90 kDa (Ng & Zeikus, 1981). The objective of complementary genetic studies has been to dissect and characterize the cellulase complex by cloning and expressing cellulase-encoding genes in a suitable host. To date, cloning and characterization of both the genes and their products has been reported for the *celA*, *celB*, *celC* and *celD* genes encoding different endoglucanase activities (Beguin *et al.*, 1983; Cornet *et al.*, 1983*a, b*; Schwarz *et al.*, 1986; Petre *et al.*, 1986; Joliff *et al.*, 1986*b*); more recently, the *celA*, *celB* and *celD* genes have been sequenced (Beguin *et al.*, 1985; Grepinet & Beguin, 1986; Joliff *et al.*, 1986*a*) and mRNA encoding endoglucanase A has been mapped (Beguin *et al.*, 1986). Endoglucanase D, overproduced in *Escherichia coli* JM101, has been crystallized and studied by X-ray diffraction (Joliff *et al.*, 1986*c*).

The different cloning approaches employed by two independent groups have resulted in further apparently novel *C. thermocellum* clones expressing largely uncharacterized endoglucanase activity (eight clones), β -glucosidase activity (two) and putative cellobiohydrolase activity (three) (Millet *et al.*, 1985; Schwarz *et al.*, 1985).

At present, a number of questions remain unanswered. (1) Are distinct and different genes present for each of the many cellulases shown to be produced by *C. thermocellum* in culture, and is their expression co-ordinately controlled? (2) Is cellobiohydrolase activity in *C. thermocellum* encoded by a separate gene or is it a property of certain endoglucanases? (3) Is the multiplicity of endoglucanase genes a consequence of gene reiteration? In order to address these questions, we have constructed genomic libraries from *C. thermocellum* and, in common with others, have found clones with carboxymethylcellulase activity (CMCase⁺) occurring at high frequency. The clones have been characterized with respect to the level of activity expressed in *E. coli* and their substrate specificity, and after restriction enzyme analysis and Southern hybridization of the cloned DNA fragments, it appears that a further 13 unique clones with endoglucanase or both endoglucanase and cellobiohydrolase activities have been found.

METHODS

Bacterial strains and media. For the isolation of DNA, *C. thermocellum* NCIB 10682 was cultured anaerobically in CM3 medium (Weimer & Zeikus, 1977). Basal medium (75 ml volumes) adjusted to pH 7.8 with NaOH and containing, per litre, (NH₄)₂SO₄ (1.3 g), K₂HPO₄·3H₂O (2.9 g), KH₂PO₄ (1.5 g), MgCl₂·6H₂O (1.0 g), CaCl₂ (0.15 g), FeSO₄ (1.25 mg), yeast extract (2.0 g), resazurin (2 mg), cellobiose (2.0 g) and carboxymethylcellulose (CMC, sodium salt, low viscosity, DS > 0.4, BDH) (2.0 g), was autoclaved (121 °C; 15 min), gassed with N₂ and, after the addition of filter-sterilized reducing solution (5%, v/v) containing (g l⁻¹) cysteine hydrochloride (10.0) and NaHCO₃ (100.0), was equilibrated with O₂-free CO₂. Freshly-prepared medium was inoculated (1%, v/v) with a 2 d culture of *C. thermocellum* in the same medium and the culture bottle, with a slightly loosened cap, was placed in an anaerobe jar which was flushed three times with O₂-free CO₂ before being incubated at 60 °C for 2–5 d. *E. coli* HB101 and *E. coli* MR65 were cultured overnight at 37 °C in Luria broth (LB) adjusted to pH 7.5 with NaOH and containing, per litre, tryptone (10.0 g), yeast extract (5.0 g) and sodium chloride (10.0 g). Agar (1.5%, w/v) and filter-sterilized ampicillin (50 µg ml⁻¹) were added as required.

Isolation of DNA. Total DNA from *C. thermocellum* cultured in CM3 medium was isolated according to the method of Marmur (1961), modified to include treatment with proteinase K (1 mg ml⁻¹; BRL) and RNAase (100 µg ml⁻¹; type II-A; Sigma). Large-scale preparations of plasmid DNA were made from *E. coli* HB101 by alkaline lysis (Maniatis *et al.*, 1982). The method of Birnboim & Doly (1979) was used for small-scale extraction and rapid screening of plasmid-containing clones.

In vitro genetic recombination. Purified pBR322 DNA (75 µg) in a total volume of 300 µl was linearized by incubating for 1 h at 37 °C with *Bam*HI (100 U) under the conditions recommended by the enzyme supplier. Calf intestinal phosphatase (Boehringer Mannheim) was added to the reaction mixture at a final concentration of 0.02 U (µg DNA)⁻¹. The linearized plasmid DNA was washed twice with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), twice with chloroform/isoamyl alcohol (24:1, v/v) and precipitated with ethanol before resuspending in 10 mM-Tris/HCl, 1 mM-EDTA (TE buffer), pH 7.6.

For the construction of genomic library 1, *C. thermocellum* DNA was partially digested with *Sau3A* [$0.07 \text{ U}(\mu\text{g DNA})^{-1}$] for 1 h at 37°C and, after horizontal agarose gel electrophoresis in borate buffer (Maniatis *et al.*, 1982), fragments in the 4–9 kbp size range were recovered by electroelution. For genomic library 2, digestion of chromosomal DNA was carried out under similar conditions in four tubes, but the *Sau3A* concentration was varied from 0.001 to $0.025 \text{ U}(\mu\text{g DNA})^{-1}$. Digests were pooled and DNA fragments 4–9 kbp in size were recovered from a gel by electroelution.

For both libraries, ligation of *C. thermocellum* DNA fragments ($0.25 \mu\text{g}$) to linearized vector ($0.5 \mu\text{g}$) was carried out at 12.5°C for 36 h under the buffering conditions specified for T4 DNA ligase by the supplier.

The ligation mixture for the first library was transformed into *E. coli* HB101, and for the second library into *E. coli* MR65, a Lac^- mutant of strain HB101 generated in this laboratory to facilitate the selection of clones expressing β -glucosidase activity from the *C. thermocellum* genomic library. Recombinant plasmid was transformed into both *E. coli* strains with an efficiency of about $3 \times 10^3 (\mu\text{g total DNA})^{-1}$. Competent cells were prepared using a modified CaCl_2 procedure (Cohen *et al.*, 1973) and stored at -70°C until required. Transformants were initially plated on LB medium containing ampicillin ($50 \mu\text{g ml}^{-1}$) and the frequency of occurrence of the $\text{Ap}^R \text{Tc}^S$ phenotype ($>95\%$ of clones) was subsequently determined and taken as a measure of the rate of insertion. The presence of recombinant plasmid in mini-preparations from $\text{Ap}^R \text{Tc}^S$ clones was confirmed by gel electrophoresis after treatment of extracted plasmid DNA with *EcoRI* under conditions specified by the enzyme supplier.

For the selection of CMCase⁺ clones, transformants were replicated on LB with ampicillin ($50 \mu\text{g ml}^{-1}$) using sterile nitrocellulose filters (Millipore HATF085), and after overnight incubation at 37°C , colonies were screened by means of the Congo Red plate assay as described below.

Restriction digests. Single and double enzyme digestion of recombinant plasmids extracted from CMCase⁺ clones were carried out using the restriction endonucleases *Bam*HI, *Hind*III, *Pst*I, *Eco*RI, *Bgl*II and *Sal*I. Incubations were of 16 h duration with an enzyme concentration of $1\text{--}3 \text{ U}(\mu\text{g DNA})^{-1}$. Conditions used were those specified by the supplier of each enzyme. DNA fragments were fractionated by electrophoresis in 1% (w/v) agarose gels and their sizes estimated by reference to the migration of standards.

Southern blot hybridization. Chromosomal DNA from *C. thermocellum* or recombinant plasmid DNA from CMCase⁺ *E. coli* clones was digested with restriction endonucleases as required and fractionated on 1% (w/v) agarose gels. DNA was then blotted on Hybond N (Amersham). ^{32}P -labelled DNA probes (recombinant plasmids or restriction fragments therefrom) were prepared by nick translation (Rigby *et al.*, 1977) using $\alpha\text{-}^{32}\text{P}$ -dCTP (Amersham) and hybridization was carried out as described by Southern (1975). Autoradiography was performed at -70°C using Kodak X-S1 film and two intensifying screens.

Preparation of cell-free extracts. CMCase⁺ *E. coli* clones containing recombinant plasmids were cultured with vigorous aeration for 16 h at 37°C from a 1% (v/v) inoculum in LB medium (100 ml) containing ampicillin ($50 \mu\text{g ml}^{-1}$). Cells were collected by centrifugation ($4000 g$, 4°C , 10 min), washed once with ice-cold PC buffer ($50 \text{ mM-K}_2\text{HPO}_4$, $12 \text{ mM-citric acid}$, pH 6.5) and resuspended in the same buffer to a final volume of 4 ml. Cell suspension, cooled on ice, was ultrasonicated for 3 min (model W-225 ultrasonic processor; Heat Systems-Ultrasonic Inc., Farmingdale, NY, USA; microtip; output control 7; 50% duty cycle) and then centrifuged for 30 min at up to $40000 g$ to remove debris. Cell-free extract was stored at -20°C .

Assay of endo- β -1,4-glucanase activity. (i) **Congo Red assay.** Cellulase activity in *E. coli* HB101 transformants containing recombinant plasmids was assayed by means of the Congo Red plate assay, which is based on the strong interaction occurring between the dye Congo Red and polysaccharides containing more than six contiguous β -1,4-linked glucose residues (Teather & Wood, 1982). Colonies cultured overnight at 37°C on LB with ampicillin ($50 \mu\text{g ml}^{-1}$) were overlaid with PC buffer containing 0.5% (w/v) CMC (sodium salt; low viscosity; DS > 0.4 , BDH) and 1% (w/v) molten agar. After 3 h incubation at 60°C , plates were flooded with a 1% (w/v) aqueous solution of Congo Red (Sigma) for 15 min. Excess Congo Red solution was poured off and plates were washed with 1 M-NaCl. CMCase⁺ colonies were surrounded by a pale yellow halo or zone of clearing against a red background.

(ii) **Viscometric assay.** As a consequence of their mode of action, endoglucanases decrease the degree of polymerization of cellulosic substrates, causing a drop in viscosity. A rapid but nevertheless valid estimate of endoglucanase activity can therefore be obtained by measuring the time taken for a 50% reduction in the viscosity of CMC on incubation with the enzyme. Cell-free extract from CMCase⁺ *E. coli* clones containing 1 mg total protein was diluted to 0.25 ml with PC buffer, and was incubated at 60°C with 16 ml substrate solution containing CMC (0.54%, w/v). Reaction was stopped by placing on ice or by boiling for 10 min and the efflux time was determined for the passage of the reaction mixture through a capillary-type viscometer (size C; viscosity range, 6–45 cS, Baird & Tatlock). Specific viscosity (η) was calculated as described by Schwarz *et al.* (1986). In place of enzyme, controls contained either water or cell-free extract from a CMCase⁻ *E. coli* clone containing recombinant plasmid.

(iii) **Reducing sugar assay.** Samples (4 ml) were removed from reaction mixtures set up for the viscometric assay and the reducing sugar present was measured colorimetrically using 3,5-dinitrosalicylic acid (Miller, 1959). Results were expressed as glucose equivalents, and in order to comply with other published work, one unit of

enzyme activity was defined as that which released 1 μmol glucose equivalent per hour from CMC, under the assay conditions.

Assay of cellobiohydrolase and β -glucosidase activity. Cell-free extract (50 μl) was added to 200 μl PC buffer, pH 6.5, containing 4-methylumbelliferyl β -D-cellobioside (MUC; 0.5 mg ml^{-1}), a substrate held to be specific for cellobiohydrolase (Gilkes *et al.*, 1984; Van Tilbeurgh *et al.*, 1982), or 4-methylumbelliferyl β -D-glucoside (MUG; 0.5 mg ml^{-1}). After 3 h incubation at 60 $^{\circ}\text{C}$, a positive result was indicated by fluorescence under UV illumination at 310 nm.

Thin-layer chromatography (TLC). Products formed from CMC incubated to completion in reaction mixtures described previously were fractionated by TLC on precoated silica-gel plates (F₂₅₄; Merck) using chloroform/methanol/6 M-ammonia (48:60:16, by vol.) as developing solvent. To locate products, plates were sprayed with 50% (v/v) sulphuric acid, followed by 10% (w/v) resorcinol in acetone/water (1:1, v/v) before heating at 160 $^{\circ}\text{C}$ for 5 min. Glucose and cellobiose standards (up to 25 μg) were run on each plate.

Partial purification of endoglucanases. Cell-free extracts were prepared as previously from cultures (up to 11 litres) of CMCase⁺ *E. coli* clones in LB medium containing ampicillin (50 $\mu\text{g ml}^{-1}$). After heating at 60 $^{\circ}\text{C}$ for 3 min, heat-agglutinated protein was removed by centrifugation (2500 *g*, 4 $^{\circ}\text{C}$, 10 min) and $(\text{NH}_4)_2\text{SO}_4$ (0.7 g ml^{-1}) was added to the supernatant. Precipitated protein was dissolved in 200 mM-phosphate buffer, pH 6.8 (20–30 ml), and applied to a column (3.4 $\times$ 150 cm) of Sephacryl S-200, which was eluted with the same phosphate buffer. CMCase activity in column fractions was monitored by spotting samples (12 μl) onto CMC agar films and incubating at 60 $^{\circ}\text{C}$ for 30 min before staining with Congo Red as described previously. Active fractions producing a clear zone in the agar were combined and the protein was recovered by ammonium sulphate precipitation (0.6 g ml^{-1}) and centrifugation (10000 *g*, 20 $^{\circ}\text{C}$, 10 min). Typically, CMCase activity emerged from the Sephacryl column after passage of 3 column volumes of elution buffer and had a 10-fold higher specific activity than the protein initially applied to the column. Partially purified proteins in the minimum volume of phosphate buffer were diluted with denaturing buffer and were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (1970).

RESULTS

Screening of libraries

The two genomic libraries prepared by ligating the 4–9 kbp *Sau*3A fragments of *C. thermocellum* DNA into the *Bam*HI site of plasmid pBR322 had an insertion rate of >90%. Five thousand Ap^RTc^S *E. coli* clones from the first library and 3000 clones from the second library were screened by means of the Congo Red plate assay and a total of 28 CMCase⁺ clones were recovered: RV1.1 to RV1.10 from the first library and RV2.1 to RV2.18 from the second. Plasmid was extracted from each CMCase⁺ clone and it was confirmed that CMCase activity cotransformed with the Ap^R character in *E. coli* HB101 with 100% frequency.

Restriction enzyme mapping

Recombinant plasmids extracted from each of the 28 CMCase⁺ clones were digested by *Bam*HI, *Eco*RI, *Hind*III, *Pst*I and *Sal*I; no sites were found for the enzyme *Bgl*II. Analysis of the restriction data indicated that the 28 clones carried a total of 13 different DNA inserts. Cloned fragments occurring more than once were present in either identical or inverted orientation (Table 1). The sizes of the restriction fragments obtained by digesting the DNA insert of each of the 13 different recombinant plasmids with either *Eco*RI or *Hind*III are shown in Table 1. The inserted DNA fragment varied in size from 3.0 to 12.8 kbp but for 10 of the 13 unique clones fell within the range 4.0 to 5.6 kbp.

Southern blot hybridization

To establish that the different DNA inserts in CMCase⁺ *E. coli* clones had been cloned intact and did not arise as a result of DNA rearrangements and deletions during cloning, ³²P-labelled probes were prepared from each recombinant plasmid, including those cloned in duplicate, and were examined for hybridization with *C. thermocellum* chromosomal DNA which had been digested with *Bam*HI, *Eco*RI, *Hind*III, *Pst*I or *Sal*I. With the exception of pRV2.4, the pattern of hybridization confirmed the physical map obtained for each insert (results not shown). According to restriction enzyme analysis, plasmid pRV2.4 was expected to hybridize with seven *Hind*III genomic fragments and three *Eco*RI fragments (Table 1). In reality hybridization took

Fig. 1. Southern hybridization of ^{32}P -labelled pRV2.4 with *Bam*HI (B), *Sal*I (S), *Pst*I (P), *Eco*RI (E) and *Hind*III (H) digests of *C. thermocellum* chromosomal DNA. *Hind*III fragments of λ phage DNA were run as standards (Std) and the procedure was as described in Methods.

Table 1. Restriction enzyme analysis of the different cloned DNA fragments from *CMCase*⁺ *E. coli* clones containing *C. thermocellum* DNA in pBR322, and the duplication of *CMCase*⁺ clones in two genomic libraries

Plasmid	Size (kbp)	Insert size (kbp)	Size of <i>Eco</i> RI restriction fragments (kbp)	Size of <i>Hind</i> III restriction fragments (kbp)	No. of similar clones	
					Identical orientation	Inverted orientation
pRV1.1	8.7	4.4	1.9, 1.9, 0.5	1.8, 1.6, 0.7, 0.3	4	1
pRV1.5	8.3	4.0	2.0, 1.1, 0.6, 0.3, 0.05	2.4, 1.6	4	—
pRV1.6	8.3	4.0		3.0, 0.8, 0.2	—	1
pRV1.7	8.3	4.0	2.8, 1.2	2.3, 1.4, 0.3	2	—
pRV2.1	9.6	5.3	4.5, 0.8	2.7, 2.0, 0.6	—	—
pRV2.2	9.8	5.5	4.4, 1.1	2.2, 2.0, 0.7, 0.5	1	—
pRV2.3	17.1	12.8	7.0, 2.1, 1.9, 1.5, 0.6	4.9, 2.4, 2.2, 0.8, 0.8, 0.5, 0.4, 0.3, 0.25, 0.2	—	—
pRV2.4	9.9	5.6	4.7, 0.4, 0.4, 0.1	1.4, 0.8, 0.8, 0.75, 0.65, 0.6, 0.6	—	—
pRV2.5	9.4	5.1	4.8, 0.3	4.5, 0.4, 0.2	—	—
pRV2.6	7.3	3.0	2.7, 0.3	1.5, 1.3, 0.2	—	—
pRV2.9	8.7	4.4		3.5, 0.5, 0.2	1	1
pRV2.10	13.2	8.9	6.5, 1.9, 0.5	5.0, 1.4, 1.1, 0.7, 0.3, 0.2, 0.2	—	—
pRV2.18	9.2	4.9	3.8, 1.1	2.7, 1.9, 0.3	—	—

Table 2. *Enzyme activity in cell-free extracts of CMCase⁺ E. coli clones containing C. thermocellum DNA in pBR322*

Clone	CMCase activity		Substrates hydrolysed		
	Reducing sugar assay*	Viscometric assay†	CMC	MUC	MUG
RV1.1	53.9	12.5	+	+	—
RV1.5	0.4	0.3	+	—	—
RV1.6	3.4	1.1	+	—	—
RV1.7	0.3	0.3	+	—	—
RV2.1	1.7	0.5	+	+	—
RV2.4	22.5	10.0	+	+	—
RV2.18	2.0	1.6	+	+	—
RV2.2	0.5	0.3	+	—	—
RV2.3	2.7	0.7	+	—	—
RV2.5	3.4	7.1	+	—	—
RV2.6	ND	0.2	+	—	—
RV2.9	60.6	16.7	+	—	—
RV2.10	5.0	6.3	+	—	—

ND, Not detected.

* Units (mg protein)⁻¹: 1 unit = 1 μ mol glucose equivalent liberated h⁻¹.

† 1/t: t = time in h for a 50% reduction in viscosity of CMC under the standard assay conditions.

place between the probe and multiple *Hind*III and *Eco*RI fragments, as well as with a number of *Bam*HI and *Pst*I fragments (Fig. 1). This result was clearly not due to incomplete digestion of chromosomal DNA because, when the same filter was re-probed with other recombinant plasmids, the predicted result was obtained. It seems more likely that a particular reiterated sequence or fragment occurring in the genomic DNA is coincidentally carried on the insert of pRV2.4.

Restriction enzyme analysis of the recombinant plasmids indicated that *Bam*HI and *Sal*I sites occurred less frequently than *Eco*RI and *Hind*III sites. This view was supported by the hybridization experiments which further showed that, in general, inserts contained in the CMCase⁺ clones were part of very large *Bam*HI and *Sal*I chromosomal fragments.

By combining hybridization data with the previously determined sizes of restriction enzyme fragments, it was possible with certain clones to localize supplementary restriction sites on the chromosome adjacent to the cloned fragment, but there was no indication that any of the endoglucanase genes cloned in this work map close to each other.

Digestion of recombinant plasmid from CMCase⁺ clones with *Hind*III resulted in the production of numerous apparently similar restriction fragments less than 1 kbp in size. To determine whether these fragments were homologous, each recombinant plasmid was completely digested with *Hind*III and the fragments were fractionated on a 1% agarose gel and then transferred to Hybond N. ³²P-labelled probes prepared from the 0.65, 0.75 and 0.8 kbp *Hind*III fragments of pRV2.4 (Table 1) were used separately to probe each of the digested plasmids; hybridization occurred only between each fragment and the digest of the homologous insert. There was no evidence of homology with any of the other clones.

Characteristics of CMCase⁺ clones

CMCase⁺ clones differed with respect to the diameter of the halo produced on LB plates overlaid with CMC agar and stained with Congo Red; clones RV1.1, RV2.4 and RV2.9 produced large distinctive haloes with diameters of up to 10 mm, while other clones produced smaller, but nevertheless very definite zones of clearing. *E. coli* HB101 had no detectable CMCase activity. Cell-free extracts prepared from CMCase⁺ *E. coli* clones varied quite considerably in their ability to generate reducing sugar and reduce viscosity during the hydrolysis of CMC (Table 2). Under the assay conditions used, cell-free extract from the most

Fig. 2. Effect of temperature on CMCase activity of clone RV1.3. Viscometric assays were carried out as described in Methods, using 50 μ g cell-free extract protein for each incubation and CMC at 0.8% (w/v). CMCase activity (initial rate of reduction of η) is expressed as a percentage of the activity at 60 °C.

active clones (RV1.1, RV2.4, RV2.9) produced reducing sugar rapidly and reduced the viscosity of CMC by 50% in 5 min or less; for these clones, change in specific viscosity ($\Delta\eta$) per μ mol glucose equivalent produced varied from 0.7 to 1.6; other less active clones attacked CMC more slowly but produced a similar reduction in viscosity per μ mol glucose equivalent. In contrast, extract from clones such as RV2.5 and RV2.10 reduced the viscosity of CMC by 50% in around 10 min without producing such large quantities of reducing sugar, and for these clones, $\Delta\eta$ per μ mol glucose equivalent produced varied from 4.6 to 7.4, indicating a different type of enzyme activity.

Some properties of the enzyme activities expressed by clones RV1.1, RV1.5, RV1.6 and RV1.7

Based on enzyme activity, the four different CMCase⁺ clones of the first library that are listed in Table 2 appeared to be broadly representative of the two types of clone recovered from our libraries.

Analysis by TLC of the products formed from CMC by cell-free extracts prepared from these four clones revealed that the more active clone (RV1.1) produced glucose, cellobiose and longer-chain-length cellulose-oligosaccharides and their carboxymethyl derivatives, while extract from the less active clones (RV1.5, RV1.6, RV1.7) produced only cellulose-oligosaccharides bigger than cellobiose from CMC.

Polypeptides retaining enzymic activity after complete or partial denaturation of the purified CMCase activity of each of the four clones were separated by SDS-PAGE and located on the gel by Congo Red staining of a CMC agar overlay (Beguin, 1983). The major endoglucanase activity of clone RV1.1 was stable after boiling in denaturing buffer, and had an apparent molecular mass of 78 kDa. The purified CMCase from clone RV1.5 was not stable on boiling, but retained activity after heating at 60 °C in denaturing buffer and produced two major polypeptides with apparent molecular masses of 73 and 57 kDa. In contrast, CMCase activity purified from clones RV1.6 and RV1.7 was temperature labile, so these samples were applied to the gel in denaturing buffer without heating. Each of these clones produced a different pattern of three polypeptides with CMCase activity; for RV1.6 the molecular masses ranged from 55 to 90 kDa and for RV1.7 the range was 80 to 90 kDa.

Using cell-free extract as source of enzyme and a saturating concentration of CMC, the effect of temperature on enzyme activity was determined for the endoglucanase expressed by clone RV1.3; this clone contained the same DNA insert and endoglucanase gene as RV1.1. Under our assay conditions, the rate of reduction of specific viscosity (η) of the substrate was maximal around 65 °C, characteristic of a thermostable enzyme (Fig. 2).

DISCUSSION

Biochemical analysis has shown the cellulosome of *C. thermocellum* to be composed of in excess of 14 polypeptides having endo- or exo-glucanase activity (Lamed *et al.*, 1983a) and accordingly, one might expect the number of *cel* genes present on the genome to be high. It is therefore not particularly surprising that we, and another group (Millet *et al.*, 1985), have found apparently different CMCase⁺ clones with high frequency in genomic libraries prepared from the chromosomal DNA of this organism. In the present work, CMCase⁺ clones occurred with varying frequency in two libraries constructed from *Sau*3A-digested DNA ligated into the *Bam*HI site of plasmid pBR322. Although we have no information regarding the distribution of *Sau*3A sites in the DNA fragments encoding endoglucanase activity, it is possible that the higher frequency of occurrence of CMCase⁺ clones in the second library (1 per 167 clones compared with 1 per 500 clones in the first) may be attributable to the very gentle manner in which the chromosomal DNA was partially digested.

The Congo Red assay, using CMC as substrate, does not select cellobiohydrolase-producing clones (Gilkes *et al.*, 1984), so it seems likely that the 13 different DNA fragments cloned here bear genes encoding endoglucanase activity. The fact that cell-free extract prepared from each of the CMCase⁺ clones liberated reducing sugar from CMC while significantly reducing its viscosity reinforces this view. However, variation in the ratio of the reduction in viscosity per μ mol reducing sugar liberated suggests that genes encoding different types of endoglucanase have been cloned. Thus, the more active clones such as RV1.1, RV2.4 and RV2.9 encode enzymes which liberate more reducing sugar per unit change in viscosity by cleaving bonds close to, as well as remote from, the ends of a chain and possibly in shorter chain-length cellulooligosaccharides also, while other clones such as RV2.5 and RV2.10 encode enzymes which cleave bonds remote from the chain ends, producing a more dramatic reduction in viscosity for each reducing end group liberated. Using clones obtained from the first library, limited TLC analysis of the products formed from CMC strengthens this view, in that the clone RV1.1 which has the CMC⁺MUC⁺ phenotype, characteristic of both endoglucanase and exoglucanase activities, produced short- and long-chain cellulooligosaccharides and their carboxymethylated derivatives as well as glucose, while enzyme from clones RV1.5, RV1.6 and RV1.7 formed the long-chain products more characteristic of classical endoglucanase activity.

None of the clones described here had detectable β -glucosidase activity judging by their inability to hydrolyse MUG. Classification of the enzymes encoded by the 13 different DNA fragments as endoglucanases is slightly compromised by the ability which some have to attack MUC, a substrate thought to be specific for exoglucanase (Van Tilbeurgh *et al.*, 1982). We take the view that, rather than indicating cellobiohydrolase activity, the CMC⁺MUC⁺ phenotype is characteristic of certain endoglucanases with broad specificity which are able to cleave bonds close to the end of a CMC chain. This phenotype could result either from the production of two enzymes or, as has been previously suggested (Millet *et al.*, 1985), from a single protein. In further unpublished work, we have shown that clone RV1.2, which is homologous with clone RV1.1 but carries a 2.4 kbp DNA insert, has a promoter on a 0.5 kbp *Eco*RI fragment. The remaining 1.9 kbp *Eco*RI fragment is probably only large enough to code for one complete protein and yet the clone expressed enzyme activity which, like that of clone RV1.1, hydrolysed both CMC and MUC. For other clones with the same phenotype but larger inserts, production of more than one enzyme cannot be ruled out. At present, there is a clear need for further biochemical characterization of the cloned cellulases of *C. thermocellum*, but available evidence suggests that they differ considerably from those described for the fungal system (Ljungdahl & Eriksson, 1985).

Specific activity of CMCase was surprisingly high in cell-free extracts prepared from our most active clones (RV1.1, RV2.4, RV2.9), exceeding levels previously reported for three different *C. thermocellum* endoglucanases cloned in *E. coli* (Beguín *et al.*, 1983; Cornet *et al.*, 1983a; Petre *et al.*, 1986; Schwarz *et al.*, 1986) or bacteriophage λ (Schwarz *et al.*, 1986) and for endoglucanases from *Bacteroides succinogenes* (Crosby *et al.*, 1984), *Cellulomonas fimi* (Gilkes *et al.*, 1984) and *Thermomonospora* YX (Collmer & Wilson, 1983) cloned and expressed in *E. coli*. Since *C.*

thermocellum DNA is expressed well in *E. coli*, in some instances independently of orientation, differences in the CMCase activity of cell-free extract prepared under identical conditions from the same volume of *E. coli* culture may be attributable to the presence or absence of *C. thermocellum* regulatory sequences on the insert and the efficiency with which they function in *E. coli*. As an alternative, the high specific activity of our clones may be due in part to our assay procedure. The presence of substituents can affect the hydrolysis of adjacent linkages in CMC, so we have routinely used substrate with a low degree of substitution (0.4) which is likely to give higher rates of hydrolysis than the more heavily substituted CMC used in at least one other study (Schwarz *et al.*, 1986).

Restriction enzyme analysis showed that our original collection of 28 CMCase⁺ *E. coli* clones from two libraries contained 13 unique DNA inserts. With one exception, Southern hybridization with chromosomal DNA ratified the physical maps and confirmed that the inserts consisted of linear fragments cloned without deletions or rearrangements. The anomalous pattern of hybridization occurring between pRV2.4 and chromosomal DNA can be explained if a repeated sequence, such as might be associated with a transposon, is present in the insert of pRV2.4 and the chromosome (P. Beguin, personal communication). Eleven of the 13 unique DNA inserts described here contained internal *Eco*RI sites. By virtue of the cloning approach adopted, *celA*, *celB* and five of the other previously cloned endoglucanase genes of *C. thermocellum* are borne on *Eco*RI fragments which have no internal *Eco*RI sites (Cornet *et al.*, 1983a; Millet *et al.*, 1985). Excluding the possibility that some *cel* genes may be reiterated with different flanking sequences, it seems likely that the DNA fragments we have cloned do not contain any of the *cel* genes previously cloned by the French group. With the exception of pRV2.9, which has some similarity with the *celA* coding region, this conclusion has been reinforced by comparison of detailed restriction maps (J.-P. Aubert, P. Beguin & J. Millet, personal communication). *C. thermocellum* genes cloned in bacteriophage λ included the *celA* gene and another uncharacterized endoglucanase gene (clone LIC 17) borne on a large insert which had numerous *Eco*RI and *Hind*III restriction sites (Schwarz *et al.*, 1985). In the absence of detailed restriction maps or relevant hybridization data, it is impossible to conclude that this gene is absent from our inserts, but it may be significant that the proteins expressed by LIC 17 exhibited measurable activity against *p*-nitrophenyl β -D-glucoside, whereas our clones expressed no β -glucosidase activity.

In common with others (Millet *et al.*, 1985) we have found that digestion of *C. thermocellum* DNA encoding CMCase activity with *Hind*III resulted in numerous fragments of similar size, suggesting the possibility of homology. However, three such fragments from pRV2.4 failed to hybridize with any of the fragments produced by *Hind*III digestion of each of the other 12 inserts, indicating that homology, if it exists, is quite low.

The effect of temperature on activity of the endoglucanase expressed by clone RV1.3 (homologous with RV1.1) confirms that the enzyme is thermostable and that this particular property is unchanged by expression in *E. coli*.

Loss of activity on boiling in the presence of SDS has been previously reported for some of the endoglucanases of *C. thermocellum* (Beguin, 1983). During this work, polypeptides obtained by SDS treatment of the endoglucanases from a representative sample of clones behaved differently on electrophoresis. SDS-PAGE of the completely denatured endoglucanase purified from clone RV1.1 indicated a molecular mass of 78 kDa for the polypeptide produced. For clones RV1.5, RV1.6 and RV1.7 the appearance of more than one polypeptide band during electrophoresis could result from partial denaturation of protein, or from different gene products or from post-translational modification of a single protein, so values obtained for molecular mass should be treated with caution. Nevertheless, it is interesting that the apparent molecular masses of the constituent polypeptides of the cloned endoglucanases were within the size range reported for the partially denatured crude endoglucanases of *C. thermocellum* (Beguin, 1983).

Cloning and expression in *E. coli* of a further 13 apparently unique fragments of *C. thermocellum* DNA encoding CMCase activity brings the total of different endoglucanase genes reported to 21. In the absence of gene reiteration, which has been suggested but not adequately demonstrated, and excluding the possibility that enzymically active components of

the cellulosome have escaped detection, this confirms that the genome of *C. thermocellum* contains more *cel* genes than are apparently required.

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