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Expression of an endoglucanase gene from *Clostridium cellulolyticum* in *Escherichia coli*

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SUMMARY

A gene coding for an endoglucanase from the anaerobic cellulolytic bacterium *Clostridium cellulolyticum* has been cloned by direct selection in *Escherichia coli*, using the carboxymethyl cellulose–Congo Red assay. The cloned gene has been subcloned in the two possible orientations in pUC plasmids. One of the two resulting constructs exhibited a higher level of expression, which was associated with a high level of plasmid instability. The enzyme synthesized in *E. coli* from the cloned gene has been characterized by two procedures, maxicells and gel filtration chromatography, as a polypeptide of approximately 40 kilodaltons.

INTRODUCTION

The development of improved methods for the biological degradation of cellulose is an active field of biotechnology. The industrial significance of cellulose degradation is twofold: the production of fuels and chemicals from an abundant, cheap substrate, and the utilization of municipal and industrial wastes. Fungi and bacteria can be used either directly to ferment a cellulosic substrate, or indirectly as sources of cellulolytic enzymes.

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The genus *Clostridium* comprises species of anaerobic bacteria, several of which have remarkable cellulolytic capabilities. Cellulose degradation by *C. thermocellum* occurs by the synergistic action of different enzymes which are assembled in a structure, the cellulosome, consisting of at least 14 polypeptide subunits, many of which are cellulases [2,3]. As a necessary approach for the characterization of the individual components of the cellulosome, many cellulase-encoding genes have been cloned [10,11, 15,19,24] and their nucleotide sequences have been determined [4,14]. The availability of the cloned genes represents a great asset in designing new strategies for cellulose hydrolysis. New enzyme complexes can be obtained by combination of the pro-

teins encoded by these genes. Increased cellulolytic capabilities can be achieved by using molecular techniques to manipulate the cloned genes.

Besides its cellulolytic capability, *C. thermocellum* possesses the property of being thermophilic. The possibility of using temperatures above 60°C for cellulose hydrolysis is associated with higher enzyme activity, enhanced product recovery and shorter retention times, factors of considerable interest from an industrial standpoint. This explains the large amount of work that has been carried out with this species. Other species of the genus, while having a high cellulolytic potential, have been much less well characterized. In this paper we report on the cloning and expression in *Escherichia coli* of an endoglucanase gene from *C. cellulolyticum* H10 (ATCC 35319), a mesophilic species isolated from decayed grass [23].

MATERIALS AND METHODS

Bacterial strains and plasmids

C. cellulolyticum H10 [23], obtained from E. Petitdemange, Université de Nancy, France, was used as the source of DNA for the isolation of the endoglucanase gene. Plasmids pBR322 [6], pUC12 and pUC13 [30] were used for cloning in *E. coli*. The strains of *E. coli* used were HB101 [7] for pBR322 and JM83 [30] for pUCs.

Culture conditions

The cultures of *C. cellulolyticum* used for DNA purification were grown anaerobically in CM3 medium [31] with carboxymethylcellulose (CMC) as the carbon source, supplemented with 0.3% trisodium citrate dihydrate and 0.036% 2-mercaptoethanol. The medium was dispensed in 1 liter bottles, approximately 900 ml per bottle, reduced by heating under N_2 flow, stoppered and autoclaved. Each bottle was inoculated with 50 ml of a culture grown in CM3 medium with filter paper as the carbon source. Cultures used as inoculum were grown in this medium until the filter paper was disaggregated. This served as a control for the cellulolytic capability of the culture from which DNA was extracted. *E. coli* was cultivated following standard procedures [18].

DNA isolation

Total DNA from *C. cellulolyticum* was isolated from 2–4 liters of culture. Cells were collected by centrifugation, washed with TE (10 mM Tris, 1 mM EDTA, pH 7.5) and resuspended in 10 ml GETL (50 mM glucose, 25 mM Tris, 10 mM EDTA, 3 mg/ml lysozyme, pH 8.0). After 1–2 h of incubation at room temperature, proteinase K was added to a final concentration of 50 μg/ml. Lysis was carried out by adding sodium dodecyl sulfate to 1% final concentration. The preparation was incubated for 1 h at 37°C and 20 min at 60°C, and then centrifuged to remove cell debris. The aqueous phase was collected and treated with phenol to remove proteins. Nucleic acids were precipitated with ethanol and the DNA was purified by banding in a cesium chloride gradient with ethidium bromide [18].

Plasmid DNA was isolated from *E. coli* following standard procedures [18].

Cloning procedures

DNA from *C. cellulolyticum* was partially digested with *Sau*3A to an average fragment size of 5 kb and ligated to pBR322 cut with *Bam*HI and dephosphorylated with calf alkaline phosphatase. The ligation mixture was used to transform *E. coli* HB101 to ampicillin resistance. Transformants were tested for cellulolytic activity on agar plates containing CMC which were stained with Congo Red [10,29].

Enzyme activity

Assays of carboxymethylcellulase (CMCase) activity were carried out at 55°C in citrate-phosphate buffer (pH 6.8) containing 1.6% CMC (low viscosity carboxymethylcellulose sodium salt, Sigma Chemical Co.). CMCase activity was monitored using the Nelson-Somogyi method [28], by measuring reducing sugars released from CMC. One unit of CMCase activity corresponds to 1 μmol of glucose equivalent per min. Protein concentration was determined with the Bio-Rad assay, by measuring absorbance at 595 nm, using bovine serum albumin as a standard.

Periplasmic enzyme activity was determined in the supernatant of an osmotically shocked cell suspension prepared as described by Aït et al. [1]. Intracellular enzyme was determined using cells disrupted by sonication.

β-Galactosidase activity, measured as a control for intracellular activity, was determined according to Pardee et al. [22].

Assays on petri dishes were carried out as follows. Colonies were grown on complete medium (Luria broth) supplemented with ampicillin. The cells were lysed by flooding the plates with chloroform, which was subsequently evaporated; the colonies were overlaid with soft agar containing 0.5% CMC in citrate-phosphate buffer pH 6.8. The plates were incubated at 50°C for several hours and then stained with 0.5% Congo Red and washed with 1 M NaCl. CMC^+ colonies were identified by a clear halo (Fig. 1).

Maxicells

Plasmid-encoded polypeptides were identified by the procedure described by Sancar et al. [25]. The maxicells were prepared from the *recA E. coli* strain SE5000 [27] transformed with plasmid pC1. After UV irradiation to inactivate the bacterial chromosome, the newly (plasmid-encoded) synthesized proteins were labeled with [^{35}S]methionine at a final concentration of 30 μCi/ml and electrophoresed in a 10% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). Radioactive bands were identified in an autoradiogram [25].

Chromatography

E. coli JM83 harboring a plasmid with the cloned endoglucanase gene was grown to early stationary phase in Luria broth supplemented with 50 μg/ml ampicillin. Cells were disrupted by sonication and the lysate was treated for 30 min at 50°C. Heat treatment removed about 50% of the total protein without significant loss of activity. Partial purification was carried out in a 16 × 400 mm Bio-Gel P100 gel filtration column (Bio-Rad). The column was eluted with 50 mM Tris-HCl, 50 mM NaCl, pH 7.5 at a flow rate of 4 ml/h. Fractions of 1 ml were collected and assayed for protein concentration and CMCase activity.

RESULTS

Expression of the endoglucanase gene in E. coli

HB101 colonies transformed to ampicillin resistance with *C. cellulolyticum* DNA ligated to pBR322 were tested for cellulolytic capability. One colony among 3000 screened showed endoglucanase activity, as revealed by a halo surrounding the colony on CMC plates stained with Congo Red (Fig. 1). This transformant harbors a plasmid designated pC1 which contains a 2 kb fragment of DNA from *C. cellulolyticum* inserted at the *Bam*HI site of pBR322. Fig. 2 shows the physical map of pC1.

A 2 kb *Hin*dIII-*Sal*I fragment containing most of the pC1 insert was subcloned in the two possible orientations in pUC12 and pUC13 (Fig. 3). Transformants containing these constructions, designated pLM25 and pLM26 respectively, were selected among white colonies on X-Gal plates, by the β-galactosidase assay used with pUC plasmids [30], and subsequently identified as halo-forming colonies on CMC plates. In this assay, CMCase activity was

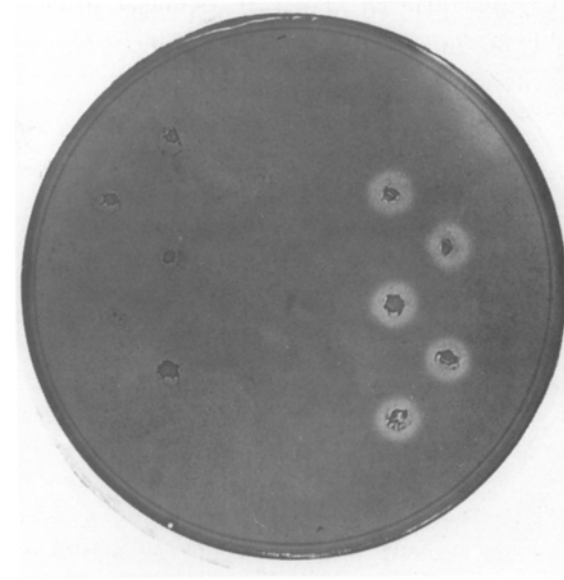

Fig. 1. Petri dish assay of β-endoglucanase activity produced by transformed colonies of *E. coli*. Colonies harboring plasmid pC1, which contains a cloned gene from *C. cellulolyticum*, appear surrounded by a halo on plates containing CMC. Colonies carrying pBR322 do not show a halo and are included as a negative control.

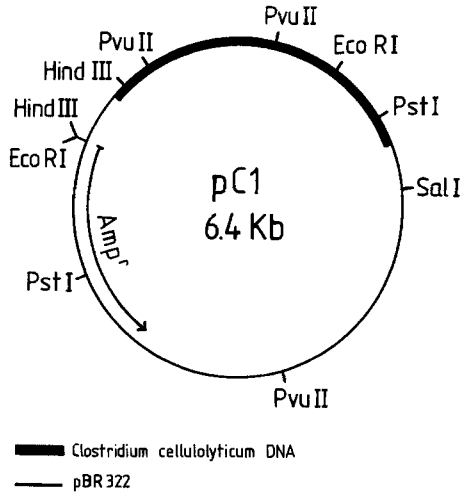

Fig. 2. Physical map of plasmid pC1. This plasmid contains a 2 kb DNA fragment, including an endoglucanase gene from *C. cellulolyticum*, cloned at the *Bam*HI site of pBR322.

considerably higher for pLM25 than for pLM26, as revealed by the size of the halos. However, pLM25 clones were very unstable, losing their activity at a high frequency without the concomitant loss of the resistance to ampicillin. CMCase activity of pLM26 clones was higher than that of pC1 clones (7.6 and 1.1 U/mg of protein, respectively). In these cases activity was stably maintained.

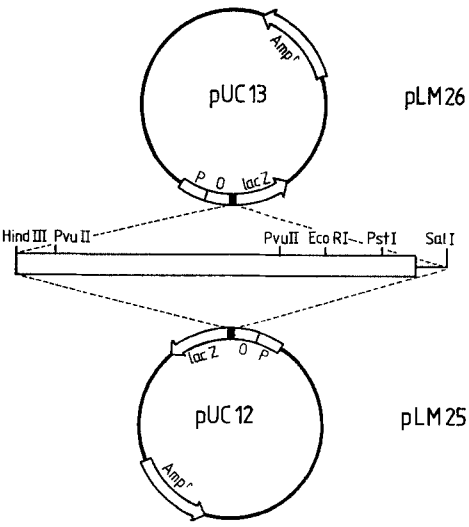

Fig. 3. Subcloning of a 2 kb *Hin*dIII-*Sal*I fragment of pC1 containing the endoglucanase gene from *C. cellulolyticum* in pUC vectors.

Table 1

Localization of enzyme activity in *E. coli* clones transformed with pC1 and pLM26 plasmid

	pC1		pLM26
	% CMCase	% β-galactosidase	% CMCase
Supernatant	n.d.[a]	n.d.	n.d.
Periplasm	44	1	56
Cytoplasm	56	99	44

[a] n.d. = not detected

The localization of the enzyme synthesized by *E. coli* for both HB101 cells containing plasmid pC1 and JM83 cells containing plasmid pLM26 was investigated. In both cases we have found the CMCase activity equally distributed between the periplasm and the cytoplasm. In this experiment, β-galactosidase activity in HB101 cells containing plasmid pC1 was measured as a control (Table 1).

Protein characterization

The endoglucanase synthesized in *E. coli* from the cloned gene has been characterized by two different approaches.

Fig. 4A shows a polyacrylamide gel in which the proteins obtained by lysis of maxicells have been separated and stained with Coomassie Blue. Fig. 4B presents an autoradiogram of the gel. Bands correspond to plasmid-encoded polypeptides labeled with [^{35}S]methionine. In Fig. 4A and B, tracks A, E and I contain molecular weight standards. The three visible bands in these tracks correspond to 66, 45 and 29 kDa. Tracks B, D and G were loaded with increasing amounts of protein prepared from *E. coli* SE5000 maxicells carrying plasmid pC1. Tracks C, F and H were prepared in the same way but the maxicells did not contain any plasmid. Several intensely labeled bands observable in the autoradiogram correspond to β-lactamase (28 kDa), to the truncated product of the *tet* gene and probably to smaller peptides produced by proteolysis. A weaker band of about 40 kDa indicated by an ar-

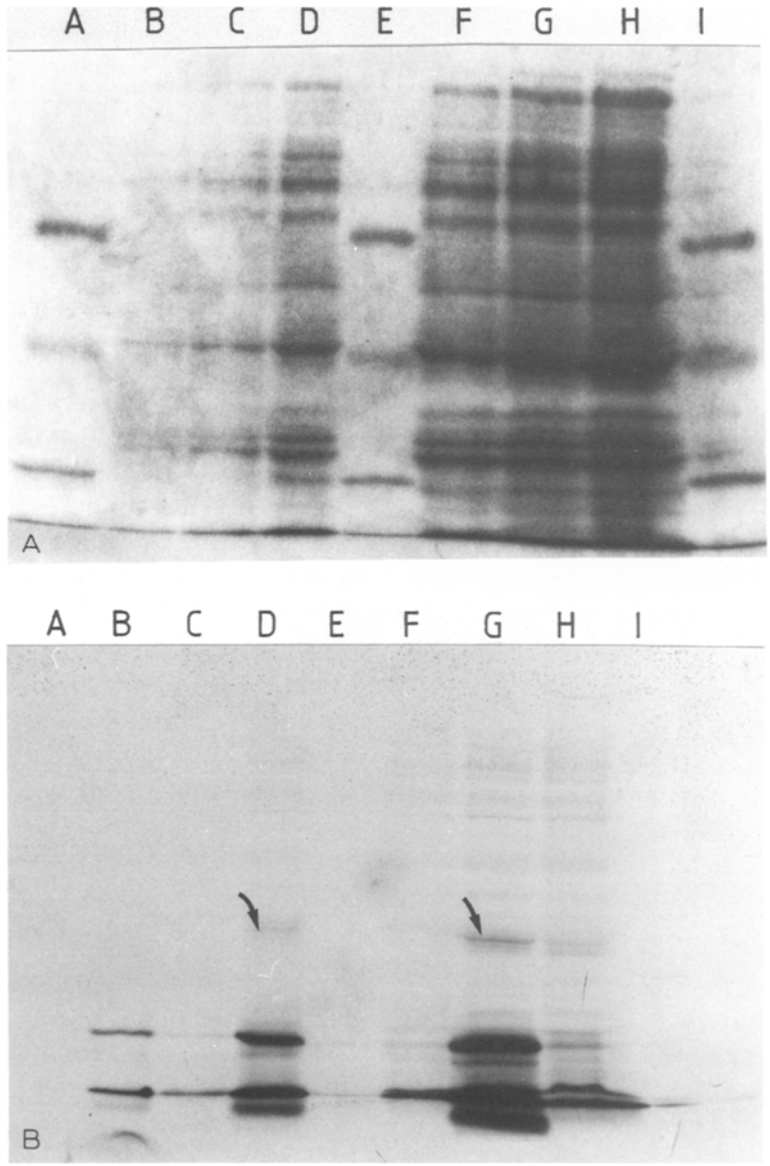

Fig. 4. Characterization of plasmid-encoded proteins synthesized by maxicells. (A) 10% SDS-PAGE of total proteins from *E. coli* maxicells. Tracks A, E, and I contain molecular weight markers: bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Tracks B, D, and G contain increasing amounts of protein prepared from maxicells carrying plasmid pC1. Tracks C, F, and H contain the same amounts of protein as tracks B, D, and G, respectively, but in this case were prepared from maxicells which did not contain any plasmid. (B) Autoradiogram of the gel shown in A. The arrows indicate a band of approximately 40 kDa which corresponds to the product of the cloned gene.

row in the figure, probably corresponds to the cloned endoglucanase.

The alternative approach used for the characterization of the protein was chromatography. Cell extracts were prepared as described in Materials and Methods from strain JM83 containing plasmid pLM26, and loaded onto a Bio-Gel P100 gel filtration column. The profile of CMCase activity in

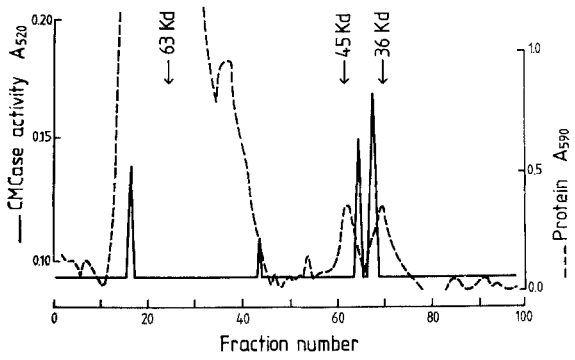

Fig. 5. Bio-Gel P100 gel filtration column of a cell extract prepared from *E. coli* JM83 containing plasmid pLM26.

fractions eluted from the column is shown in Fig. 5. Two contiguous peaks of activity in fractions 65 and 68 indicate a molecular weight of about 40 kDa. Other peaks of higher molecular weight present in fractions 17 and 44 might correspond to oligomers and/or complexes of the enzyme with other proteins of *E. coli*.

DISCUSSION

Results presented in this paper report on the cloning of an endoglucanase gene from *C. cellulolyticum* by direct selection of activity in *E. coli*. This simple strategy has been employed successfully in the cloning of cellulolytic genes from different bacteria [8,9,11,13,16,17,19,24]. Expression in *E. coli* of genes from gram-positive bacteria such as *Bacillus* and *Clostridium* is explained by the similarities found in their promotor and ribosome binding site sequences [4,5,14,20,21].

The differential expression of the cloned gene in the constructs made in pUC plasmids must be due to transcription from the *lacZ* promotor of pUC12 in pLM25 (Fig. 3). This effect resembles the finding of Joliff et al. [15] for the *celD* gene of *C. thermocellum* cloned in pUC8. Unfortunately, in our case the higher activity was associated with a very high instability of the plasmid responsible for such activity.

The CMCase activity synthesized by *E. coli* from the cloned gene is found intracellularly and in the periplasm in approximately equal amounts. Similar results have been reported for *C. thermocellum* and *Thermomonospora* endoglucanases cloned in *E. coli* [9,10]. This indicates that the signal responsible for the transport of these enzymes across the cell membrane is functional in *E. coli*.

The enzyme synthesized in *E. coli* has been characterized by two methods, maxicells and gel filtration chromatography, as a polypeptide of approximately 40 kDa. This size corresponds to one of the four bands of endoglucanase activity observable in the supernatant of *C. cellulolyticum* cultures [12]. In the chromatogram presented in Fig. 5, two major contiguous peaks of CMCase activity, corresponding to a molecular weight of about 40 kDa, are observable. Similar results in the literature are attributed to proteolysis [26].

The work reported in this paper represents a step towards the development of economically viable processes for the enzymatic hydrolysis of cellulosic substrates.

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