Isolation and Characterization of *Escherichia coli* Clones Expressing Cellulase Genes from *Cellulomonas fimi*

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A sensitive immunoassay developed previously was used to isolate a series of *Escherichia coli* clones expressing cellulase genes from *Cellulomonas fimi*. The clones fell into three groups. Clones in the first group contained plasmids with a 6.6 kb insert of *C. fimi* DNA, were strongly antigenic, and contained low levels of CM-cellulase activity. Those in the second group contained plasmids with a 5.0 kb insert, were weakly antigenic, and contained high levels of CM-cellulase activity. Those in the third group contained plasmids with a 5.6 kb insert, were weakly antigenic, and contained low levels of CM-cellulase activity. Restriction analysis showed that the three groups carried different cellulase genes. All of these CM-cellulase activities were exported to the periplasm in *E. coli*, but with different efficiencies. These activities represented important components of the *C. fimi* cellulase complex. Their properties indicated that they were different from each other and that they probably had complementary actions.

INTRODUCTION

Cloning appropriate structural genes is one approach to increasing cellulase production. We are attempting to clone the genes determining the components of the cellulase system of *Cellulomonas fimi* into *Escherichia coli*. To facilitate screening, we developed a simple, sensitive immunoassay for the detection of recombinant plasmids carrying cellulase genes from *C. fimi*. The assay was used to isolate a recombinant plasmid which directs the synthesis in *E. coli* of detectable levels of cellulase activity (Whittle *et al.*, 1982). We report here on the identification and characterization of additional clones expressing our previously reported activity as well as two new classes of clones producing different components of the *C. fimi* cellulase system.

METHODS

Bacterial strains and media. Except for *E. coli* CSR603, the bacterial strains and the media used for their cultivation were described previously (Whittle *et al.*, 1982). *Escherichia coli* CSR603 was the strain used to identify plasmid-coded proteins by the maxi-cell method (Sancar *et al.*, 1979).

DNA. The isolation of chromosomal DNA from *C. fimi* and of plasmid DNA from *E. coli*, digestion of DNAs with restriction endonuclease *Bam*HI, cloning of fragments of *C. fimi* DNA into pBR322, and the transformation of *E. coli* were described previously (Whittle *et al.*, 1982). Restriction digests of DNAs were fractionated by agarose gel electrophoresis (Dunn *et al.*, 1979); and plasmid DNA was isolated by the alkaline lysis procedure (Maniatis *et al.*, 1982).

Screening of recombinant clones for cellulases. The procedures for the selection of recombinant plasmids and their immunological screening for cellulase were described previously (Whittle *et al.*, 1982).

Enzyme assays. Cellulase activity was measured colorimetrically (Miller, 1959) using CM-cellulose as substrate (Miller *et al.*, 1960). One unit of enzyme released 1 µg glucose equivalents min⁻¹ as determined by reference to a standard curve. β-Lactamase activity was measured with nitrocefin as the substrate (O'Callaghan *et al.*, 1972). β-Galactosidase activity was measured with o-nitrophenyl-β-D-galactoside as the substrate (Miller, 1972).

PAGE. The procedures used for PAGE of proteins are described in the accompanying paper (Langsford *et al.*, 1984).

Analysis of cells for the location of CM-cellulase activity. Total cell extracts were prepared by rupturing the cells with a French press (Whittle *et al.*, 1982). Periplasmic proteins were obtained from spheroplasts by osmotic shock (Neu & Heppel, 1965; Nossal & Heppel, 1966). Zones of hydrolysis around colonies on CM-cellulose plates were detected with Congo red (Teather & Wood, 1982).

Identification of plasmid-coded proteins. Plasmid-coded proteins were identified by the maxi-cell technique (Sancar *et al.*, 1979), after transformation of *E. coli* CSR603 with the appropriate plasmid.

Enzymes and reagents. Enzymes were purchased from New England Biolabs, and ¹²⁵I-labelled protein A was prepared as described previously (Whittle *et al.*, 1982).

RESULTS

Isolation of further clones determining cellulase activity

The first plasmid obtained, pDW1, contained a 20.2 kb insert from a partial *Bam*HI digest of *C. fimi* DNA. Exhaustive digestion of pDW1 with *Bam*HI released 1.6, 6.6 and 12.0 kb fragments of *C. fimi* DNA (Whittle *et al.*, 1982).

The DNA from a total *Bam*HI digest of pDW1 was used to construct recombinant plasmids from pBR322. Those recombinants determining cellulase activity were detected by immunoassay of ampicillin-resistant, tetracycline-sensitive clones (Whittle *et al.*, 1982). All fifteen of the positive clones which were screened contained a 6.6 kb insert of *C. fimi* DNA (data not shown). This prompted us to attempt the cloning of further cellulase genes from total rather than partial *Bam*HI digests of *C. fimi* DNA.

Sixty-one immunopositive clones were obtained from 6500 ampicillin-resistant, tetracycline-sensitive clones; 38 of these exhibited cellulase activity. The cellulase-producing clones fell into three groups on the basis of antigenicity (as judged from the screening autoradiograms) and cellulase activity. One group (five clones) was strongly antigenic but gave low levels of enzyme activity; the second group (eight clones) was weakly antigenic but gave high levels of enzyme activity; the third group (25 clones) was weakly antigenic and gave very low levels of enzyme activity.

All of the clones in the first group contained a plasmid, designated pEC1, with a 6.6 kb insert of *C. fimi* DNA (Fig. 1). All those in the second group contained a plasmid, designated pEC2, with a 5.0 kb insert of *C. fimi* DNA (Fig. 1). The screening of the remaining clones is incomplete; of the 10 screened thus far, the plasmid in three of these, designated pEC3, contained a 5.6 kb insert of *C. fimi* DNA (Fig. 1). The 6.6 kb insert in pEC1 was identical to the cellulase-encoding insert in pDW1.

Comparison of pEC1, pEC2, and pEC3

The *C. fimi* inserts in pEC1, pEC2 and pEC3 gave different restriction patterns with *Sal*I and *Pst*I (Fig. 2). Cell-free extracts from cells carrying these plasmids gave single bands of CM-cellulase activity by non-denaturing PAGE (Fig. 3). Therefore, the genes encoding the enzymes were clearly different, but the enzyme mobilities in PAGE were very similar. They compared closely to the substrate-bound enzymes seen in normal *C. fimi* supernatants (Langsford *et al.*, 1984).

Polypeptides determined by pEC plasmids

Escherichia coli CSR603(pEC) strains were prepared by transformation with pEC1 and pEC2. Plasmid-determined polypeptides were labelled with [³⁵S]methionine after UV-irradiation of the cells to reduce the synthesis of chromosomally-determined polypeptides (Sancar *et al.*, 1979). The labelled polypeptides were analysed by SDS-PAGE (Fig. 4). *Escherichia coli* CSR603 clearly did not produce polypeptides corresponding to those encoded by pBR322 and the pEC plasmids. *Escherichia coli* CSR603(pBR322) produced the *amp* and *tet* polypeptides, as expected; *E. coli* CSR603(pEC1) and *E. coli* CSR603(pEC2) produced the *amp* but not the *tet* polypeptide because the *C. fimi* fragments in their plasmids were inserted in the *tet* gene at the *Bam*HI site. Polypeptides of 51 and 53 kDal were produced by *E. coli* CSR603(pEC1) and *E. coli*

Fig. 1

Fig. 2

Fig. 1. Cloned DNA fragments coding for *C. fimi* cellulases. Plasmid DNA was isolated from *E. coli* C600(pEC) strains, digested with *Bam*HI and separated on a 0.7% agarose gel containing 1 μ g ethidium bromide ml^{-1} . Lane 1, *Hind*III-cleaved fragments of λ phage DNA; lane 2, pEC1; lane 3, pEC2; lane 4, pEC3. The arrow indicates linear pBR322 DNA.

Fig. 2. Restriction endonuclease digest of pEC plasmids. Purified pEC plasmids were digested with the restriction endonucleases *Sal*I and *Pst*I. The fragments were separated on a 1.1% (w/v) agarose gel containing 1 μ g ethidium bromide ml^{-1} . Lanes 1–3, *Sal*I digests of pEC1, pEC2 and pEC3, respectively; lane 4, *Hpa*I-digested T7 phage DNA as a molecular weight marker; lanes 5–7, *Pst*I digests of pEC1, pEC2 and pEC3, respectively.

CSR603(pEC2), respectively. Neither polypeptide was produced by cells containing pBR322. The labelled polypeptides produced by cells containing pEC1 or pEC2 were analysed further by immune precipitation with antisera against the supernatants of *C. fimi* cultures grown on celluloses (Whittle *et al.*, 1982). The antigen–antibody complexes were adsorbed on *Staphylococcus aureus* (Ivarie & Jones, 1979) and the labelled proteins obtained were analysed by SDS-PAGE. Polypeptides of 51 and 53 kDa were obtained from pEC1-containing and pEC2-containing cells, respectively (Figs 5 and 6). These polypeptides corresponded closely in molecular weight to two of the cellulases in *C. fimi* culture supernatants. Thus, pEC1 and pEC2 encoded different components of the *C. fimi* cellulase complex.

Fig. 3. PAGE analysis of cellulases encoded by pEC plasmids. Whole cell extracts of *E. coli* C600(pEC) strains were run on 6% non-denaturing polyacrylamide gels. The gels were sliced in 2 mm fractions; these slices were placed directly into a CM-cellulase assay mixture (Langsford *et al.*, 1984) and incubated for 18–20 h at 37 °C. The reactions were terminated, and reducing sugars were determined colorimetrically (Miller, 1959). (a) Activity profiles of pEC1; (b) pEC2; and (c) pEC3. N.B. Only the top part of the gel is shown here; since no activity was seen in the remainder of the gel, the top part is plotted in an expanded form. The arrows in (a) show the relative positions of the three activities.

Fig. 5

Fig. 5. Immunoprecipitation of pEC1 polypeptide. The proteins encoded by pEC1 were labelled as described in Fig. 4. The labelled proteins then were precipitated with various antisera, and the precipitates were analysed by SDS-PAGE. Lane 1, the labelled proteins before treatment with antisera; lane 2, protein precipitated with antisera raised against CM-cellulose-induced *C. fimi* extracellular enzymes; lane 3, protein precipitated by antisera raised against Avicel-induced *C. fimi* extracellular enzyme. Numbers refer to sizes (kDa).

Fig. 6

Fig. 6. Immunoprecipitation of pEC2 polypeptide. The proteins encoded by pEC2 were labelled as described in Fig. 4. The labelled proteins then were precipitated with various antisera, and the precipitates analysed by SDS-PAGE. Lane 1, the labelled proteins before treatment with antisera; lane 2, protein precipitated with a monoclonal antibody, A2/23.11.32 (Langsford *et al.*, 1984) directed against *C. fimi* cellulase; lane 3, protein precipitated with antisera raised against CM-cellulose-induced *C. fimi* extracellular enzymes. Numbers refer to sizes (kDa).

Fig. 4. Polypeptides encoded by cloned DNA fragments. Strain CSR603 was transformed with plasmids pEC1 and pEC2. The proteins encoded by the plasmids were labelled according to the procedures of Sancar *et al.* (1979). The labelled proteins were analysed by SDS-PAGE. Lane 1, CSR603; lane 2, CSR603(pBR322); lane 3, CSR603(pEC1); lane 4, CSR603(pEC2). Numbers refer to sizes (kDa).

Table 1. *Specific activities of CM-cellulase and marker enzymes in E. coli(pEC) strains*

Plasmid	Enzyme	Specific activity* in:		
		Periplasmic fraction	Cytoplasmic fraction	Cell extract
pEC1	CM-cellulase	26	0.4	3.6
	β -Galactosidase	160	2722	1932
	β -Lactamase	14480	72.9	2200
pEC2	CM-cellulase	95	29	39
	β -Galactosidase	325	4048	2738
	β -Lactamase	9372	63	2604
pEC3	CM-cellulase	10.3	0.36	1.2
	β -Galactosidase	584	4909	2892
	β -Lactamase	67653	2556	6390

* Units: CM-cellulase, μ g glucose equiv. released min^{-1} (mg protein) $^{-1}$; β -galactosidase, nmol *o*-nitrophenol released min^{-1} (mg protein) $^{-1}$; β -lactamase, nmol nitrocefoic acid produced min^{-1} (mg protein) $^{-1}$.

Table 2. *Characteristics of cellulase plasmids*

Plasmid	Insert size (kb)	Antigenicity	CM-cellulase activity in <i>E. coli</i> *	Zone of clearing on CM-cellulose-Congo red†
pEC1	6.6	Strong	3.6	—
pEC2	5.0	Weak	39	++
pEC3	5.6	Very weak	1.2	+

* See Table 1 for units of activity.

† Symbols: —, absent; +, moderate; ++, large.

The *C. fimi*-specific polypeptides(s) produced by *E. coli* CSR603(pEC3) were not clearly distinguishable by these methods. It seems that the cells produce very low levels of this polypeptide(s), so we are attempting to increase the level of expression of the gene(s) to facilitate identification.

Location of cellulase activity in E. coli(pEC) strains

Cells of *E. coli* carrying pEC1, pEC2 or pEC3 exported significant fractions of their cellulase activities to the periplasm (Table 1). The very low levels of β -galactosidase from the periplasmic fraction were consistent with its known cytoplasmic location in the cell and indicated that there was no significant contribution of cytoplasmic enzymes to the activities measured in the periplasmic fraction. Very low levels of cellulase activity were found in *E. coli*(pEC) supernatants, and these probably arose as a result of cell lysis (data not shown).

Colonies of *E. coli*(pEC2) or *E. coli*(pEC3) produced haloes on CM-cellulose plates, whereas *E. coli*(pEC1) did not produce haloes on these plates even though its CM-cellulase activity was higher than that of *E. coli*(pEC3) (Table 2). In our hands this procedure worked best and most reproducibly if the colonies were washed from the agar surface before the plates were flooded with the Congo red solution. Clearly, the enzymic activity of pEC1 was qualitatively different from those of pEC2 and pEC3. Similar results were obtained using a leaky strain of *E. coli* which released the enzymes into the culture medium. The isolation and characterization of this mutant are described elsewhere (Gilkes *et al.*, 1984).

DISCUSSION

The *C. fimi* cellulase system contains three or maybe four enzymically active components (Langsford *et al.*, 1984). The newly isolated plasmids described in this paper account for three of these components. The properties of these plasmids, together with the characteristics they confer on *E. coli*, are summarized in Table 2. The restriction enzyme analysis shows clearly that

the three plasmids are different as are the antigenic and enzymic properties of the cellulases they encode. The mobilities of these cellulases on native polyacrylamide gels are similar and the *C. fimi* polypeptides encoded by pEC1 and pEC2 are of similar molecular weight. Their electrophoretic mobilities are similar to but not identical with those of the major *C. fimi* cellulases. Since it is likely that the *E. coli* enzymes are not glycosylated whereas at least two of the *C. fimi* cellulases are, slight differences in mobility are to be expected.

The production of haloes on CM-cellulose plates by colonies of *E. coli*(pEC2) and *E. coli*(pEC3) allows direct selection of cellulase-producing clones and has been used successfully to select sub-clones of pEC2 (unpublished results). However, we still favour the use of the immunoassay because it detects clones with CM-cellulase activities which do not produce haloes on CM-cellulose plates. It will also detect components without CM-cellulase activity which could act synergistically with the active components present in *C. fimi* supernatants.

Halo formation on CM-cellulose plates results from cleavage of CM-cellulose into fragments smaller than cellohexaose to which Congo red does not bind (Wood, 1980). In addition, haloes could result from cleavage of CM-cellulose into fragments small enough to be washed out of the plates during the staining procedure. In either case, only endoglucanase activity would be expected to produce a zone of clearing. Consequently, the CM-cellulase activity encoded by *E. coli*(pEC1), which does not produce haloes on CM-cellulose plates, may be an exoglucanase. Exoglucanase would still generate new reducing groups detectable by the reducing sugar assay. Conversely, the pEC3 enzyme could be an endoglucanase which would cleave CM-cellulose infrequently. This would give low activity by the reducing sugar assay, but produce haloes on a CM-cellulose plate.

The CM-cellulase activities of the individual *E. coli*(pEC) strains are low compared to the activity of a *C. fimi* culture supernatant. However, it must be borne in mind that the supernatant contains multiple component(s) of the cellulase system, which probably act synergistically, whereas the *E. coli* strains produce only single components of the system. Cloning of the remaining component(s) will allow reconstitution of the system and elucidation of the synergism between the various products of the cloned genes.

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