Cloning and Expression of *Clostridium acetobutylicum* Endoglucanase, Cellobiase and Amino Acid Biosynthesis Genes in *Escherichia coli*

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Clostridium acetobutylicum P262 endoglucanase and cellobiase genes, cloned on a 4.9 kb DNA fragment in the recombinant plasmid pHZ100, were expressed from their own promoter in *Escherichia coli*. Active carboxymethylcellulase and cellobiase enzymes were produced, but there was no degradation of Avicel. The endoglucanase activities observed in cell extracts of *E. coli* HB101(pHZ100) differed in their pH and temperature optima from those previously reported for *C. acetobutylicum* P270. Complementation of *E. coli* *arg* and *his* mutations by cloned *C. acetobutylicum* DNA was also observed.

INTRODUCTION

Acetone and butanol can be produced from a variety of carbohydrate substrates using *Clostridium acetobutylicum*. At present the economic feasibility of producing solvents by fermentation is limited by the high cost of starch- and sugar-based substrates (Lenz & Moreira, 1980). The use of microbial strains which can utilize cellulosic substrates provides an alternative for the production of chemical feedstocks (Zeikus, 1980). Allcock & Woods (1981) reported that *C. acetobutylicum* P270, an industrial strain which is a subculture of *C. acetobutylicum* P262, degraded amorphous cellulose substrates and showed low levels of carboxymethylcellulase (CMCase) and cellobiase activity, but no detectable activity against filter paper or Avicel. Since *C. acetobutylicum* was unable to degrade the crystalline cellulose substrate Avicel, it was concluded that the strain produced an inducible endoglucanase [endoglucanase is endo-1,4- β -glucanase or 1,4-(1,3;1,4)- β -D-glucan 4-glucanohydrolase (EC 3.2.1.4)] and a cellobiase [cellobiase is β -D-glucosidase or β -D-glucoside glucohydrolase (EC 3.2.1.21)]. Lee *et al.* (1985) reported that two additional *C. acetobutylicum* strains, NRRL B527 and ATCC 824, exhibited endoglucanase and cellobiase activities.

To characterize further and to improve the cellulase system of *C. acetobutylicum*, we constructed a gene bank of *C. acetobutylicum* DNA in *Escherichia coli*, and here we describe the cloning of a DNA fragment which contains an endoglucanase gene and a cellobiase gene.

METHODS

Bacteria, plasmids and growth conditions. *C. acetobutylicum* P262, which has weak CMCase activity, has been described previously (Allcock & Woods, 1981; Allcock *et al.*, 1982). *E. coli* HB101 (*leuB6 trpE38 metE70 recA13 supE44*) (Boyer & Roulland-Dussoix, 1969) and JC1553 (*leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 malA1* (λ^R) *xyl-7 rpsL104 tonA2 tsx-1* λ^- *supE44*) (Clark & Margulies, 1965) were used as recipient strains for recombinant plasmids. Plasmid pEcoR251, a gift from M. Zabeau, Plant Genetic Systems, Ghent, Belgium, is a positive selection vector containing the *E. coli* *EcoRI* gene under the control of the λ rightward promoter, the ampicillin (Ap) resistance gene and the pBR322 origin of replication. It was derived from the pCL plasmids described by Zabeau & Stanley (1982). The *EcoRI* gene product expressed at high levels by the λ promoter on pEcoR251 is lethal unless insertionally inactivated or regulated by plasmid pCI857, which contains a temperature

Abbreviations: CMCase, carboxymethylcellulase; CMC, carboxymethylcellulose; Ap, ampicillin; CBM, *Clostridium* basal medium.

sensitive λ repressor gene (Remaut *et al.*, 1983). The *EcoRI* gene has a single *BglII* cloning site. *C. acetobutylicum* P262 was grown in *Clostridium* Basal Medium (CBM) (O'Brien & Morris, 1971) as described by Allcock *et al.* (1982). *C. acetobutylicum* was grown and protoplasts were prepared under stringent anaerobic conditions (Allcock *et al.*, 1982). *E. coli* was grown in Luria medium and in minimal medium supplemented with specific carbon sources and amino acids (Maniatis *et al.*, 1982). For β -galactosidase assays, cells were grown in broth containing 1 mM-isopropyl thio- β -D-galactoside (IPTG). For alkaline phosphatase assays, cells were grown in the phosphate-limiting medium of Garen & Levinthal (1960).

Preparation of DNA. Plasmid DNA was prepared by the alkali-hydrolysis method of Ish-Horowicz & Burke (1981). *C. acetobutylicum* chromosomal DNA was prepared by the method of Marmur (1961), modified to overcome the high nuclease activity exhibited by *C. acetobutylicum* (Urano *et al.*, 1983). *C. acetobutylicum* cultures (1 litre) at OD₆₀₀ 0.5–0.6 were harvested by centrifugation and resuspended in 40 ml CBM containing 10% (w/v) sucrose, 12.5 mM-MgCl₂, 12.5 mM-CaCl₂ and 5 mg lysozyme ml⁻¹ (Allcock *et al.*, 1982). The development of protoplasts was monitored microscopically until approximately 90% protoplasts were obtained. SDS (2%, w/v, final concentration) was added under aerobic conditions and the lysed culture was extracted twice with phenol equilibrated with 10 mM-Tris and 1 mM-EDTA, pH 7.5 (TE). The aqueous phase was then extracted twice with ether and dialysed against TE at 4 °C. Ribonuclease treatment and further purification steps were as previously described by Marmur (1961).

Restriction enzymes. These were obtained from Boehringer Mannheim, and used according to the manufacturer's specifications.

Construction of a *C. acetobutylicum* P262 genomic library and cloning of cellulase genes. *C. acetobutylicum* chromosomal DNA was partially digested with *Sau3A* and fractionated on a sucrose density gradient (Maniatis *et al.*, 1982). Chromosomal *Sau3A* fragments (4–7 kb) were ligated (T4 DNA ligase, Boehringer) with pEcoR251 which had been previously digested with *BglII*. The ligated DNA was used to transform competent (Dagert & Ehrlich, 1979) *E. coli* HB101 cells, and transformants containing recombinant pEcoR251 plasmids were selected on Luria agar containing Ap (50 μ g ml⁻¹). *E. coli* HB101 Ap^r colonies were pooled and recombinant pEcoR251 plasmid DNA was isolated and used to retransform *E. coli* HB101 and JC1553. *E. coli* JC1553 transformants were selected on minimal medium with Ap and appropriate nutritional supplements. Transformed *E. coli* HB101 cells were selected on Luria agar containing low viscosity carboxymethylcellulose (CMC) (5 g l⁻¹) (Sigma no. C-8758; degree of substitution 0.7) and Ap. Colonies were replica plated, and the original plates were washed free of any remaining bacteria before staining with Congo red (0.1%, w/v), followed by destaining with 1 M-NaCl (Teather & Wood, 1982). Colonies showing CMCase activity were identified by a clear zone beneath the colony. The pHZ100 recombinant plasmid containing the *C. acetobutylicum* P262 cellulase genes was characterized by restriction mapping using standard procedures (Maniatis *et al.*, 1982).

Southern blot hybridization. Chromosomal DNA from *C. acetobutylicum* was digested to completion with *PstI*, *HindIII*, *EcoRI* and *BglII*. The digested DNA fragments were fractionated by electrophoresis in 0.7% (w/v) agarose gels in Tris/acetate buffer and transferred to Gene Screen nitrocellulose filters (New England Corp.) (Southern, 1975). pHZ100, nick-translated with [α -³²P]ATP, was used as a hybridization probe (Rigby *et al.*, 1977).

Preparation of cell-free extracts and periplasmic proteins. Cell-free extracts were prepared from 200 ml overnight *E. coli* cultures containing pHZ100. The cells were harvested, washed and resuspended in 15 ml PC buffer (50 mM-K₂HPO₄, 12.5 mM-citric acid, pH 6.3) (Cornet *et al.*, 1983b). The cells were freeze-thawed using liquid nitrogen and disrupted further by sonication on ice (10 s bursts for a total of 100 s). The preparation was clarified by centrifugation for 15 min at 27000 *g* and samples were stored at -70 °C. Periplasmic protein fractions were prepared by the chloroform treatment method of Ames *et al.* (1984).

Cellulase enzyme assays. Enzyme activity against CMC, acid-swollen cellulose and filter paper was determined by the release of glucose equivalents, as detected by the dinitrosalicylic acid method for reducing sugars (Miller, 1959). Activity against medium-viscosity CMC (Sigma no. C4888; degree of substitution 0.7) was assayed by incubating 50 μ l cell-free extract with 1 ml 1.5% (w/v) CMC in PC buffer at 50 °C for 10 min. Acid-swollen cellulose (Whatman CF11) was prepared by the method of Tansey (1971) and was resuspended in PC buffer. Enzyme activity was assayed by incubating 0.8 ml cellulose suspension (20 mg dry weight ml⁻¹) with 0.2 ml enzyme solution for 30 min at 50 °C. Activity against filter paper (Whatman no. 1) was determined by incubating a 50 mg strip of filter paper in 0.8 ml PC buffer with 0.2 ml enzyme solution for 0.5–24 h at 40 and 50 °C. Activity against Avicel was determined by incubating 0.2 ml enzyme solution with 1 ml 0.5% Avicel PH-102 (FMC Corp.) in PC buffer for 24 h at 40 and 50 °C.

Protein concentrations in cell-free extracts were determined by the Lowry method. Activities of β -galactosidase and alkaline phosphatase in periplasmic fractions were assayed by the methods of Pardee *et al.* (1959) and Garen & Levinthal (1960) respectively.

Cellobiase activity. The presence of cellobiase was determined by the production of acid and gas in peptone water containing cellobiose (10 g l⁻¹), and by growth on minimal medium containing cellobiose (2 g l⁻¹) as the sole carbon source.

RESULTS

Complementation of *E. coli* amino acid auxotrophs. *E. coli* JC1553 Arg⁺ and His⁺ transformants were isolated on supplemented minimal medium lacking either arginine or histidine, and containing Ap. Plasmid DNA was isolated from each transformant and used to retransform the *E. coli* auxotroph. In the retransformation experiments complementation of either the Arg⁻ or His⁻ phenotype was always associated with transformation of the recipient strain to Ap^r. The *C. acetobutylicum* P262 origin of the insert was confirmed by Southern hybridization (data not shown).

Cloning of *C. acetobutylicum* P262 cellulase genes. *E. coli* HB101 was transformed with recombinant pEcoR251 plasmid pools and six transformants were isolated which produced a clear zone beneath the colony on CMC Luria agar. Each contained a pEcoR251 recombinant plasmid, and one of these plasmids, designated pHZ100, was selected for further study. The plasmid origin of the CMCase activity was confirmed by retransformation of *E. coli* HB101. CMCase activity was always associated with transformation to Ap^r.

Origin of DNA inserts. The origin of the cloned fragment on pHZ100 was determined by Southern blotting with ³²P-labelled pHZ100 as the probe (Fig. 1). Chromosomal DNA from *C. acetobutylicum* was digested to completion with *Pst*I, *Hind*III, *Eco*RI or *Bgl*II. pHZ100 was nick-translated and hybridized to a nitrocellulose filter prepared from the agarose gel depicted in Fig. 1(a). A *Pst*I digest of λ was used for size markers (Fig. 1a, lane 1). The 14 kb fragment was a combination of the 11.5 kb fragment, which contains the λ rightward promoter, and the 2.56 kb fragment linked by the λ cos site. Therefore both the 14 and 11.5 kb fragments hybridized with the pEcoR251 region of pHZ100 due to the presence of the λ rightward promoter on pEcoR251 (Fig. 1b, lane 1). The number of hybridization bands in lanes 2–5 (Fig. 1b) confirmed the restriction map of the insert shown in Fig. 2. The smallest hybridization fragment present in the *Eco*RI digest (Fig. 1b, lane 3) corresponded to the internal fragment generated by *Eco*RI digestion of pHZ100. The *Pst*I site mapped at the extreme end of the insert, resulting in a very weak second hybridization fragment (Fig. 1b, lane 5). pEcoR251 did not show any homology with *C. acetobutylicum* DNA (data not shown).

Cellulase and cellobiase activities. *E. coli* HB101(pHZ100) produced acid and gas in peptone water containing cellobiose, and grew on minimal medium with cellobiose as the sole carbon source. *E. coli* HB101 and *E. coli* HB101(pBR322) were unable to utilize cellobiose. *E. coli* HB101(pHZ100) gave no reaction in peptone water lacking cellobiose.

Cell extracts were prepared from *E. coli* HB101(pHZ100) and assayed for activity against CMC, acid-swollen cellulose and filter paper. Enzyme activities of 287, 17 and 12 μ g glucose equivalents released (mg protein)⁻¹ min⁻¹ were obtained respectively (means of six experiments; standard errors of means were less than 20% of the reported values). Although activity against filter paper was detected initially, no further degradation and release of glucose equivalents occurred after 2 h at either 40 or 50 °C. No hydrolysis of Avicel was detected over 24 h at 40 or 50 °C.

Localization and characterization of CMCase activity in *E. coli* HB101(pHZ100). Localization of the CMCase activity in cell extracts indicated that periplasmic samples contained 75% of the total CMCase activity. Control experiments showed that 1.3% of the total β -galactosidase activity but 33% of the total alkaline phosphatase activity were present in the periplasmic preparations (means of three experiments; standard errors of means were less than 5% of the reported values). Supernatant samples showed no detectable CMCase or filter paper activity.

The optimum pH for CMCase activity of cell extracts was measured in different citrate (pH 3.0–6.5) and phosphate (pH 7.0–8.0) buffers. CMCase activity was totally inhibited at pH 3.75. The optimum pH for enzyme activity showed a plateau between pH 5.0 and 7.0; the activity decreased rapidly above pH 7.0, and <50% of the optimum activity remained at pH 8.0. The optimum temperature for CMCase activity was 50 °C at pH 6.3. The CMCase activity in cell extracts was completely stable for 4 months at –70 °C.

Fig. 1. (a) Agarose gel (0.7%, w/v) electrophoresis of total digests of *C. acetobutylicum* DNA cleaved with *Bgl*II (lane 2), *Eco*RI (lane 3), *Hind*III (lane 4) and *Pst*I (lane 5). Lane 1, *Pst*I-digested λ DNA as size standards. (b) Autoradiograph of 32 P-labelled pHZ100 hybridized to a DNA blot prepared from (a).

Fig. 2. Restriction and deletion map of pHZ100. The *Bgl*II sites were lost during cloning. pHZ100 and pHZ101 both expressed endoglucanase activity.

Restriction endonuclease mapping of the cloned C. acetobutylicum DNA fragment. The restriction endonuclease map of pHZ100 was obtained by digestion with the following 6 bp recognition restriction enzymes: *Ava*I, *Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Hind*III, *Hpa*I, *Kpn*I, *Pst*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, *Xba*I and *Xho*I. The 4.9 kb *C. acetobutylicum* DNA fragment was cleaved by only five of the restriction enzymes tested; it contained single restriction sites for *Sal*I, *Pst*I and *Eco*RV, two sites for *Eco*RI and three sites for *Hind*III (Fig. 2).

A deletion plasmid, pHZ101, was constructed by digestion of pHZ100 with *Eco*RI. This resulted in the removal of the λ rightward promoter and 1.8 kb of the *C. acetobutylicum* insert. The expression of the endoglucanase gene in pHZ101 was not affected and was identical to that observed in pHZ100.

DISCUSSION

Allcock & Woods (1981) reported that *C. acetobutylicum* P270, a subculture of P262, had inducible, extracellular CMCase and cellobiase activity but did not degrade filter paper. In comparison with other cellulolytic bacteria and fungi, the levels of endoglucanase and cellobiase obtained in *C. acetobutylicum* P270 were low, and hydrolysis of insoluble cellulose substrates was not demonstrated in extracellular or intracellular fractions. We have cloned and characterized cellulase genes from *C. acetobutylicum* P262. The *E. coli* extracts were able to hydrolyse filter paper, but only at low levels for the first 2 h of the assay, suggesting that the cloned cellulase genes were only able to hydrolyse the amorphous cellulose in the filter paper and not the crystalline cellulose. The presence of endoglucanase activity and absence of exoglucanase activity were confirmed by the lack of hydrolysis of Avicel over 24 h.

The conditions for optimum activity of the endoglucanase from *C. acetobutylicum* P270 and *E. coli* HB101(pHZ100) differed. The optimum pH and temperature for the *C. acetobutylicum* P270 extracellular CMCase activity was pH 4.6 and 37 °C, whereas for the CMCase of *E. coli* HB101(pHZ100) periplasmic extracts it was pH 5.0–7.0 and 50 °C. This difference could be due to the possibility that *C. acetobutylicum* contains more than one endoglucanase gene. Cloning of endoglucanase genes from *Clostridium thermocellum* has revealed seven distinct DNA fragments coding for endoglucanases and three further fragments coding for cellobiasehydrolases (Millet *et al.*, 1985). In *C. acetobutylicum* P270 the CMCase activity was inducible and a small molecule present in molasses was required for induction. The *C. acetobutylicum* P262 CMCase activity in cell-free extracts from *E. coli* containing pHZ100 did not require molasses for induction and was expressed constitutively. The cellulase genes from *C. acetobutylicum* in *E. coli* were expressed from their own promoter and were not regulated by the λ rightward promoter situated on pEcoR251.

Localization experiments indicated that the CMCase activity occurred predominantly in periplasmic fractions in *E. coli* cells containing the cloned gene. Cornet *et al.* (1983a) reported that the cloned *C. thermocellum* CMCase activity in *E. coli* was about equally distributed between the periplasmic and cytoplasmic compartments. The appearance of zones of hydrolysis on CMC plates was presumably due to the release of the endoglucanase by cell lysis.

Although the expression of other *Clostridium* genes in *E. coli* has been reported (Cornet *et al.*, 1983b; Ishii *et al.*, 1983), this is the first report that amino acid synthesis genes and cellulase genes from *C. acetobutylicum* can be expressed from their own promoters in *E. coli*. Since the transfer of plasmids by transformation (Lin & Blaschek, 1984) and conjugation (Oultram & Young, 1985) to *C. acetobutylicum* has been demonstrated, the opportunity exists to transfer the cloned cellulase genes back into *C. acetobutylicum*. This will enable a study of the expression of the cloned cellulase genes in this solvent-producing bacterium.

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