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Cloning of Multiple Egl Genes Conferring Production of Endoglucanase on *Escherichia Coli*

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Abstract

Genes for endo-glucanase (Egl) isolated from a genomic library of the cellulolytic bacterium, *Cellulomonas biazotea,* were cloned in pUC18 in its *Sac*1 cloning site and transformed to *E. coil.* Six clones showed yellow zones of hydrolysis on Carboxy Methyl Cellulose (CMC) plates with Congo red, in liquid culture, and on native polyacrylamide gel electrophoresis activity gels. They belonged to three distinctly different groups. Three representative *E. coil* clones carrying recombinant palsmids were designated pRM2, pRM13 and pRMC28. The genes were located on 1.1, 2.1 and 4.6 kb fragments respectively. Their location was obtained by deletion analysis which revealed that 0.65, 1.1 and 3.2 kb fragments were essential to code for EgIA, EgIB and EgIC, respectively and conferred intracellular production of endoglucanase on *E. coli.* Expression of the Egl genes resulted in hyperproduction of endo-glucanase in all clones. The secretion occurred into the periplasmic fractions. The endo-glucanases produced by recombinant *E. coli* resemble the native endo-glucanases with respect to temperature optima, pH optima, and effect of metal ions on enzyme activities but differ with respect to location of the enzyme and some other enzyme properties. The cloned genes can be used as selection markers for introducing recombinant plasmids in wild strains of *E. coll.*

Key words: Cloning, endo-glucanase,, multiple genes, pUC18, production

Introduction

Endo-β-1, 4-glucanase (EC 3.2.1.4) or carboxymethylcellulase (CMCase) randomly cleaves β-1, 4-glucosidic bonds of cellulose into oligosaccharides and could be used in efficient biomass conversion (Lindner, 1988). It is desirable that the activity of this enzyme be enhanced for its application in several industrial processes and in research (Esterbauer *et al.,* 1991). *Aspergillus niger* is the best producer of this enzyme but because of low specific activity of the enzyme, a large amount of enzyme is required for completing the process needs (Gadjil *et al.,* 1995). Furthermore, biosynthesis of cellulases is subject to catabolite repression and end-product inhibition, caused by the hydrolysis products, especially glucose (Rajoka *et al.,* 1998a). Mutagenesis has been applied to improve gene expression and some hypersecretive mutants have been isolated (Gadjil *et al.,* 1995). Cloning the relevent genes under the influence of a strong promoter is an other approach for hyper-production of a product (Rajoka *et al.,* 1998a).

Cellulomonas biazotea NIAB 442 (Rajoka and Malik, 1997), like many cellulolytic organisms, produces all components of the cellulase complex namely endo-glucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21). Studies have indicated that endo-glucanase is secreted in the form of differtent isozymes present in extra-cellular fractions of *Cellulomonas* spp. (Gilkes *et al.,* 1991; Sami and Akhtar, 1993). Endo-glucanase genes from different organisms have been cloned and expressed in *E. coli* (Nakamura *et al.,* 1986; Moser *et al.,* 1989; Gilkes *et al.,* 1991; Kim and Pack, 1993). We already have shown that β-glucosidase production in the donor is coded by three distinct genes (Rajoka *et al.,* 1998b). In this study, structural genes from *C. biazotea* have been cloned in *Sac*1 site of pUC18, to see the number of genes essential for expression of endoglucanase by restricting chromosomal DNA with *Sac*1.

Materials and Methods

Bacterial strains and vectors: *Cellulomonas biazotea* (Rajoka and Malik, 1997) was used as a source for isolation and cloning of cellulase genes. *E. coli* strain HB101 (Ausubel *et al.,* 1996) was used as the host for plasmid pUC18 for identifying recombinants on the medium having 50 μg/mL ampicillin (Amp).

Enzymes and reagents, culture media, culture conditions, isolation of chromosomal and plasmid DNA/cloning procedures, subcloning, determination of minimum size of insert necessary for coding the proteins, protein characterization procedures in native gels were essentially the same as described previously (Rajoka *et al.,* 1992, 1998b; Ausubel *et al.,* 1996; Siddiqui *et al.,* 1997).

Growth studies: The ability of the *E. coil* recombinants harboring pUC18 with reference to the donor to utilize CMC as a sole carbon source was examined in basal Dubos salts medium containing (g/L), $NaNO_3$, 0.50 g; $K_2HPO_4.7H_2O$, 0.50; $MgSO_4$, 0.5 g; KCl, 1 g; $FeSO_4$, 0.1 g and yeast extract, 2 g as described earlier (Rajoka *et al.,* 1992). Carbon source (10 g/L), or promoter inducer namely IPTG

l5 mM/L) was added individually to batches of basal medium to give a desired level of carbohydrate or promoter inducer; and was added to autoclaved medium after filter-sterilization. All media were adjusted to pH 7.0 with 1N-KOH or 1N-H_3PO_4, were dispensed in 200 mL aliquots into 1-L Erlenmyer flasks in triplicate.

Enzyme production: Solid culturing and screening was performed essentially as described earlier (Rajoka *et al.,* 1998a). Shake-flask batch cultivations were carried out with 200 mL of medium as above at 30°C for donor or at 37°C for *E. coli* cultures and shaking at 100 rev/min in a rotary shaker. Cultivations were for predetermined time intervals after inoculation of 20 mL of an overnight inoculum of 1.0 OD adjusted at 610 nm. The amount of growth was measured as dry cell mass. The enzyme activity present in the extracellular (cell-free supernatant) or cellular fraction (cell extract) was assayed

Cell fractionation: Culture samples of *C. biazotea,* and *E. coli* were centrifuged (15000 x g at 4°C for 30 min) and the cell-free supernatants were used as extracellular fraction. One portion of the cells (50 mL) was suspended in biological saline and used for gravimetric cell mass determination, The cells in the second fraction (100 mL) were fractionated after Barron *et al.* (1986) for enzyme assays.

Enzyme assays: All CMCase assays were performed as described previously (Siddiqui *et al.,* 1997) with the modification that assay solution contained 1.5% (w/v) carboxymethylcellulose-Na salt (CMC) as the substrate whose pH was adjusted to 7.0 with MES. Appropriate amounts of enzyme were added and after 30 min of incubation at 40°C, the reaction was stopped by adding 3 mL of dinitrosalicylic acid (DNS) reagent. The mixture was boiled for 15 min, cooled in ice and its optical density at 550 nm was determined. One unit of CMCase activity is defined as μmol glucose equivalents liberated/min.

Protein estimation: Total proteins were estimated by Bradford method using bovine serum albumin as standard (Bradford, 1976).

Ezyme characterization: Native and recombinant CMCases were characterized as described earlier (Siddiqui *et al.,* 1997).

Results and Discussion

Chromosomal DNA (50 μg) prepared from *C. biazotea* was partially digested with *Sac*1 to generate fragments in the range 1-7 Kb. These fragments were ligated in *Sac*1 linearized pUC18 in 200 μL to 25 μg *Sac*1 digested pUC18 in its polycloning site. The ligation mixture was transformed to competent cells of *E. coli* HB101 to generate a bank of egl^+ Amp clones. The transformation frequency ($2.1x10^4$ Amp transformants/μg DNA) was very high. A few strongly growing colonies were picked from the recombinants growing on CMC-Dubos salt-yeast extract Arnpicilin-IPTG-medium plates which changed the color of hydrolysis zone yellow on fixing with NaCI (Rajoka *et al.,* 1998a; Teather and Wood, 1982) to isolate endo-glucanase (Egl) recombinants. The recombinants harboring the wild-type plasmid did not change the color of the media. Out of 1000 recombinants, 20, Egl^+ recombinants were selected from replicaplatings for characterization for inductive as well as constitutive production of endoglucanase (results not shown) in plate tests as described earlier (Rajoka *et al.,* 1998a). Six recombinants produced two fold more endoglucanase than that by the donor. The recombinants were fast growing organisms on Dubos salt medium than the donor and were selected for further characterization in liquid culture.

Quantitative photometric analyses of the endoglucanases were done to verify the plate-test results. On screening, 6 recombinants showed remarkably high endoglucanase production in Dubos-CMC liquid medium (Table 1). The gene expression levels from these recombinants are higher than those from the donor and gene products accumulated intracellularly within 24 h while donor produced maximum activity after 72 h of fermentation. An average yield of enzyme is a several-fold improvement over that produced by *E. coli* and *Z. mobilis* recombinants (Brestic *et al.,* 1989; Su *et al.,* 1993; Kim *et al.,* 1987; Lejeune *et al.,* 1988; Gilkes *et al.,* 1991). The recombinant proteins also showed mild activity towards filter paper.

Table 1: Production of CMCase from the recombinants after 20 h growth in Dubos medium[a] of pH 6.8

	CMCase	
E. Coli HB101 with	------(μ mole/mg protein)-----	
recombinant plasmid	-IPTG	+IPTG
pUC 18 (control)	0.0	0.0
PRM2	0.49	2.30
pRM3	0.51	2.25
pRM13	0.60	2.36-
pRM15	0.57	2.29
pRMC28	1.90	3.90
pRMC22	1.56	3.67

All cultures were grown at 30°C at 100 rpm shaking speed in orbital shaker.

A) All grown on CMC-DSYE-Amp medium

b) Donor under optimum conditions produced FPase = 0.85 and CMCase = 2.45IU/mg protein after 72 h of fermentation.

Molecular characterization of CMCase from C. *biazotea* has indicated that three activity bands on staining were visible in the extracellular portion of the enzyme preparation (Siddiqui *et al.,* 1997). The enzymes in the culture supernatants are being converted by deglycosylation or proteolysis to derivatives which retain activity. To see the possible identity or otherwise of the cloned gene product in *E. coli,* it was of interest to compare the CMCase produced by recombinants with that of *C. biazotea.* Analysis by native gel and subsequent assaying activity of CMCase with

Congo red (Teather and Wood, 1982; Rajoka *et al.,* 1998a) revealed that the CMCase produced by the donor and recombinants unlike β-glucosidase (Rajoka *et al.,* 1998b) was not identical. Reports are available where one recombinant carrying endo-glucanase gene elaborated three heterologous endo-glucanases comparable to those secreted by the donor (Akhtar *et al., 1988;* Sami and Akhtar, 1993) but only one recombinant reported here, produced three bands in activity staining test. However, the recombinant proteins were substantially identical with respect to optimum temperature, pH and mineral requirements for enzyme activity. The enzymes from the donor reacted with the periodic acid-Schiff's reagent but unlike donor CMCase, neither recombinant enzymes appeared to be glycosylated. The molecular weight of the endo-glucanase produced in *E. coli,* carrying the smallest plasmid of 1.1 kb insert size was 26 kDa as determined on native gel. This is in good agreement with 23 kDa M, for one of the mature forms of the native enzyme, as determined by gel filteration on fast protein liquid chromatography (FPLC) and 20 and 30 kDa sub-unit M, as determined by denaturing-renaturing PACE (Siddiqui *et al.,* 1997).

Restriction analysis revealed that on representative recombinant plasmids pRMC28 (Fig.1) showed them to have insert size of 4.6 kb and produced three bands on activity staining gel like donor, all others (including pRM13 (Fig. 2) of 2.1 kb insert size respectively) showed a single yellow band with Congo red activity staining (results not shown). The plasmids carrying the inserts were subjected to deletion analysis (Rajoka *et al.,* 1998b) by treating with SI nuclease and *Bal*31 after various transformations made in *E. coil* to know the size of gene coding for endoglucanase. The deletion of 1.02 kb inactivated the gene in this plasmid, whereas 1.0 kb did not. Therefore more than 1.10 kb of insert is essential for endoglucanase expression to code for a protein of 40 kDa as visualized on native gel. The deletion of larger than 0.60 kb, with S1 nuclease and *Bal*31, inactivated the egl genes in the plasmids pRM2 (insert size 1.24) and pRM3 (insert size 1.10) whereas deletion of 0.560 kb did not. Therefore more than 0.68 kb of insert is essential for Egl expression to code for a protein of 20 and 25 kDa respectively. The data on insert sizes substatantiate the work of Akhtar *et al.* (1988). These plasmids and other plasmids retained the ability to confer egl^+ phenotype on egl^- strains of *E. coil* and, therefore, contain functional genes and could imply that the products were the expression products of different genes or a single protein subjected to post-translational modification to derivatives which retain enzymatic activities. Plasmid pRM13 and pRM15 were identical with respect to insert size and gene size as were plasmid pRM2 and plasmid pRM3; and pRMC22 and pRMC28. These data also suggest that the *C. biazotea* genome also may have three copies of this gene and that the genes may be located on different chromosomal loci. From these results, it became apparent that the six clones represent atleast three different *Cellulomonas* CM-cellulases as has been reported for other *Cellulomonas* spp. (Akhtar *et al.,* 1988; Moser *et al.,* 1989; Sami and Akhtar, 1993), alkalophilip *Bacillus sp.* (Kim *et al.,* 1987) and *Clostridium thermocellum* (Ali *et al.,* 1995). To help in answering the question of the identity and originality of egl genes, further work could include sequence analysis, DNA analysis of the restriction digests by Southern hybridization studies, preparation of antibodies against the *E. coil* enzymes and determination of their reactivity against the purified cellulases of the donor (Lejeune *et al.,* 1988). Recently reports are available where authors have used strong promoters and have obtained high expression of endo-glucanase. There is a possibility for further improvement by gene cloning technology as has been reported for cloning endo-glucanase gene in *Bacillus subtilis* under the influence of a strong promoter (Kim and Pack, 1993). With further enhancement of the gene expression level, the production of cellulase enzymes can become an economically viable process for industrial applidations like recombinant restriction enzymes already available in the market.

Fig. 1: Restriction analysis of rec-plasmid pRMC28 positive for carboxymethyl cellulase. Lanes 3 and 7 are lambda *Hindill* markers; Lane 1, uncut pRMC28; Lane 2, uncut pUC 18; lane 4, recplasmid pRMC28 cut with *BamH*I; Lane 5, Recplasrnid mRMC28 cut with *Sac*1*;* lane 6, pUC18 cut with *Sac*1*.*

Fig. 2: Restriction analysis of rec-plasmid pRM13 positive for carboxymethyl cellulase. Lanes 1 is lambda HindIII marker; Lane 2, pUC18 cut with *Sac*1*;* Lane 3, uncut pRM13; Lane 4, recplasmid pRM13 cut with *Sac*1.

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