Folia Microbiol. 39 (4), 251–254 (1994)

Cloning of Endoglucanase Genes from *Cellulomonas biazotea* into *E. coli* and *S. cerevisiae* Using Shuttle Vector YEp24

S. PARVEZ, M.I. RAJOKA, F. FARIHA and K.A. MALIK

National Institute for Biotechnology and Genetic Engineering, P.O. Box 577, Faisalabad, Pakistan

Received November 9, 1993
Revised version March 31, 1994

ABSTRACT. We constructed a *Sma*I genomic library of *Cellulomonas biazotea* DNA in *E. coli* and in the *S. cerevisiae* shuttle vector, YEP 24. Three clone were identified that conferred the ability for *E. coli* or *S. cerevisiae* transformants to produce carboxymethylcellulase (CMCase). Cells transformed with these clones were compared with one another and with nontransformed cells for hyper-production of CMCase. *In vivo* and *in vitro* studies indicated that the CMCase genes were fully expressed and the enzyme activity was located extracellularly. The optimum pH and temperature for the CMCase thus cloned were pH 7 and 50 °C, respectively, as was the case for the donor.

Enzymic hydrolysis of cellulose to metabolizable sugars is important for carbon recycling and could be the basis for eventual development of biomass conversion systems for alternative fuel production. Microbial cellulolytic enzyme systems involve synergistic action of multiple components including endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Marsden and Gray 1986).

In recent years cellulase genes from a wide variety of microorganisms have been cloned (Nakamura *et al.* 1986; Teeri *et al.* 1983; Cornet *et al.* 1983; Johson *et al.* 1986; Penttila *et al.* 1989; Knowles *et al.* 1987; Presutti *et al.* 1991; Gilkes *et al.* 1991). Structural genes for different cellulases from *Cellulomonas* spp. have been cloned in *E. coli* and *S. cerevisiae* (Penttila *et al.* 1989); the transformants secrete the gene product very efficiently *in vivo* as well as *in vitro*. This study adds to our previous work (Rajoka *et al.* 1992). All these efforts are made toward the development of cellulolytic yeast strains for single step ethanol production from agricultural waste materials.

MATERIALS AND METHODS

The restriction enzyme *Sma*I and T4 DNA ligase were from *New England Biolab* (USA). Lysozyme, carboxymethyl cellulose, ampicillin, 2-deoxy-D-glucose and agar were from *Sigma* (USA). All other chemicals were of analytical grade.

Strains and plasmid. Cellulomonas biazotea NIAB 442 was isolated from a bagasse heap (Rajoka and Malik 1986), plasmid YEP24 (*New England BioLabs*) from *E. coli* HB101 was supplied by *New England BioLabs*, the Cir^0 strain of *Saccharomyces cerevisiae* FAS-21 was a gift from the *International Centre for Genetic Engineering and Biotechnology*, Trieste (Italy).

Culture media. C. biazotea was grown in Dubos salts minimal medium consisting of (g/L) $NaNO_3$ 1, KCl 0.5, K_2HPO_4 1, $MgSO_4$ 0.5, and $FeSO_4$ 0.1, adjusted to pH 7.3. For isolation of chromosomal DNA, the medium was supplemented with CMC (0.5 g/L). *E. coli* cells were grown in LB medium or Dubos salts – 0.4 % yeast extract – CMC medium. All above media were supplemented with ampicillin (50 mg/L) where ever needed.

Isolation of DNA. C. biazotea cultures (100 mL) were grown for 20 h and harvested by centrifugation. Chromosomal DNA was extracted from the cell pellet using hexadecyltrimethylammonium bromide according to Ausubel *et al.* (1990). Recombinant and other plasmids were isolated by the method of Birnboim and Doly (1979). Large scale recombinant plasmids were isolated by the method of Ausubel *et al* (1990) and purified on a cesium chloride – ethidium bromide density gradient.

Cloning procedures. Two samples, each containing 5 µg *C. biazotea* DNA, were digested with *Sma*I at 25 °C partially (for 1 h) or completely (2 h). The DNA samples were purified by extraction with phenol – chloroform, phenol – chloroform – 3-methyl-1-butanol solutions and subsequently precipitated with 2-propanol and dissolved in TE buffer according to Ausubel *et al.* (1990). Plasmid YEP24 was digested completely with *Sma*I and purified. The partially and completely digested chromosomal DNA (5 µg) were ligated with 5 µg of *Sma*I cut-purified preparation of YEP24 using 4 U of

T4 DNA ligase. The ligation mixture was maintained at 4 °C for approximately 2 d after which it was transformed to competent cells of *E. coli* following the protocol described by Ausubel *et al.* (1990). The transformants were selected on Dubos – CMC – ampicillin – agar medium. The transformants converted CMC to oligosaccharides which reacted with NaCl to produce yellow halos (Teather and Wood 1982). The diameter of the halos (in mm) was taken as measure of endoglucanase secretion and compared with *in vivo* production of endoglucanase.

Subcloning in the Cir^0 yeast strain. The recombinant plasmids, prepared as above, were transformed to competent cells of Cir^0 yeast by the method of Ausubel *et al.* (1990); the yeast transformants were compared as above.

Preparation of enzyme extracts. *E. coli* strains were grown at 37 °C in Dubos salts – 0.4 % yeast extract supplemented with 50 µg Amp/mL using 0.25 – 1.0 % CMC as carbon source. Yeast recombinants were grown in Del Rosario's medium with 50 mg/L ampicillin. The *E. coli* and yeast cultures were grown to late exponential phase using 1 % (*V/V*) inoculum from overnight cultures grown in the above media. Extracts were harvested by centrifuging cells in 5 mmol/L acetate buffer after sonicating on ice for two 3-min bursts; cell debris was removed by centrifugation for 5 min and the supernatant solution was preserved for enzyme assay.

Enzyme assays. Cell extracts were assayed for CMCase activity by the methods of Nakamura and Kitamura (1982) and Rajoka and Malik (1986). One mL of 1.0 % (*W/V*) carboxymethyl cellulose (CMC) solution in 50 mmol/L phosphate buffer (pH 7) was incubated with 50 µL diluted enzyme solution at 50 °C for various periods of time. The release of reducing sugars from CMC was measured by the DNS method of Miller (1959). One unit of activity was defined as the amount of enzyme liberating 1 µmol of reducing sugar as glucose per min under the assay conditions. The viscosity of the CMC reaction mixture was measured with an Ostwald viscosimeter and specific fluidity was determined.

RESULTS

Cloning of endoglucanase genes. A total of 300 Amp^r transformants were obtained and three showed detectable endoglucanase activity in *E. coli* recombinants. The recombinant plasmids isolated from these clones were named pPR9-1, pPR14-1 and pPR38-1.

Expression of endoglucanase genes. Results of *in vivo* screening of endoglucanase secretion in *E. coli* and yeast recombinants are shown in Table I and Fig. 1. (These assays were performed as described in *Material and Methods*.) The zone of yellow hallows measured in mm was taken as measure of enzyme secretion. The yeast recombinants produced 3 – 4 fold more endo-glucanase than that produced by the donor (*results not shown*).

Table I. Production of CMCase by *E. coli* and *S. cerevisiae* recombinants harboring chromosomal genes from *C. biazotea* in *Sma*I site of YEP24 after growth on 0.5 % CMC

Recombinants harboring plasmids	*E. coli*		*S. cerevisiae*	
	A_{610}[a]	CMCase IU mL^{-1} h^{-1}	A_{610}[a]	CMCase IU mL^{-1} h^{-1}
pPR9-1	1.74	2.20	1.45	2.90
pPR14-1	1.81	6.16	1.85	6.20
pPR38-1	1.68	9.78	1.76	9.85
Donor[b]	1.97	23.0	1.97	23.0
Control	0	0	0	0

[a]On 5-fold dilution culture.
[b]Under optimum conditions of growth after 20 h.

Expression of endoglucanase genes in E. coli. Results of endoglucanase assays of extracts obtained from *E. coli* transformants (Table I) indicate that the yield was approximately one-half that produced by the donor though these grew to the same cell concentration and supported the work of Ghangas and Wilson (1987).

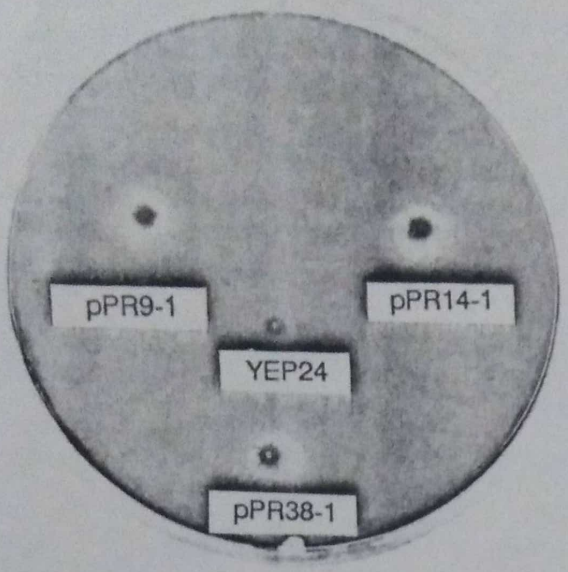

Fig. 1. Selection of CMCase-positive clones; these clones produce a *yellow* zone (appearing *white* in picture) around the colony when stained with Congo red and fixed with NaCl after Teather and Wood (1982).

Plasmids from *E. coli* recombinants were isolated after Birnboim and Dolly (1979) and were transformed to competent cells of the Cir^0 strain of *S. cerevisiae*. *In vivo* culture screening tests on yeast indicated that the genes were expressed at high level in yeast as well (3–4 fold improvement). When cells were grown in 0.5 % CMC added to yeast fermentation medium (Del Rosario *et al.* 1979); *E. coli* and yeast recombinants grew to an absorbance of 1.876 measured on 5-fold diluted cultures at 610 nm.

Results of *in vitro* screening of *S. cerevisiae* recombinants after production of enzyme in 0.5 % CMC are shown in Table I. The maximum enzyme activity produced by the best recombinant was one-half the activity produced by the donor.

DISCUSSION

In our previous paper (Rajoka *et al.* 1992) we showed the cloning of β-glucosidase gene from *C. biazotae* and the cloned genes expressed well in host cell. The same procedure has been used for the cloning of the endoglucanase gene in *E. coli* as well as in *S. cerevisiae*. Structural genes for endoglucanase from *C. uda* CB4 have been cloned in *E. coli* (Nakamura *et al.* 1986). These authors reported that the cloned genes expressed very well in the host; the clones produced twice the activity of the donor. The enzyme activity produced was mainly extracellular. In the present study, *E. coli* recombinants produced 3–4 times more endoglucanase *in vivo*; *in vitro* yeast recombinants produced a low level of activity, comparable with that produced by the endoglucanase genes from *Ruminococcus albus* cloned in *E. coli* and *S. cerevisiase* (Honda *et al.* 1988). The enzyme was secreted extracellularly in yeast transformants and produced a level of enzyme activity similar to the *E. coli* transformants. Endoglucanase gene from *Aspergillus niger* and *Clostridium thermocellum* have been cloned in *S. cerevisiae* (Knowles *et al.* 1987; Silva *et al.* 1991). Sacco *et al.* (1984) reported the expression of an endoglucanase gene in yeast from *C. thermocellum*. Similarly, the endoglucanase structural gene from *C. fimi* has also been cloned in yeast (Skipper *et al.* 1985). In some cases, a low expression of genes has been reported and support our finding.

REFERENCES

AUSUBEL F.M., BRENT R., KINGSTON R.E., MOORE D.D., SEIDMAN J.G., SMITH J.A., STRUHL, K.: *Current protocols is Molecular Biology*. Greene Publishing Associates and Wiley – John Wiley and Sons, New York 1990.

BIRNBOIM H.C., DOLLY J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl.Acids Res.* **7**, 1513–1523 (1983).

CORNET P., TRONIK D., MILLET K., AUBERT J.P.: Cloning and expression of *Clostridium thermocellum* genes coding for amino acid synthesis and cellulose hydrolysis. *FEMS Microbiol.Lett.* **16**, 137–141 (1983).

DEL ROSARIO E.J., LEE K.H., ROGERS P.L.: Kinetics of alcohol fermentation at high yeast levels. *Biotechnol.Bioeng.* **21**, 1477–1482 (1979).

GHANGAS G., WILSON D.B.: Expression of a *Thermonospora fusca* cellulase gene in *Streptomyces lividans* and *Bacillus subtilis*. *Appl.Environ.Microbiol.* **53**, 1470–1475 (1987).

GILKES N.R., KILBERN D.G., MILLER R.C. JR., WARREN R.A.J.: Bacterial cellulases. *Biores.Technol.* **36**, 21–35 (1991).

HONDA H., SAITO T., LIJIMA S. KOBAYASHI T.: Molecular cloning expression of β-glucosidase from *Ruminococcus albus* in *E. coli*. *Enzyme Microb.Technol.* **10**, 559–562 (1988).

JOHNSON J.A., WONG W.K.R., BEATTY J.T.: Expression of cellulase gene in *Rhodobacter capsulatus* by use of plasmid expression vectors. *J.Bacteriol.* **167**, 604 – 610 (1986).

KNOWLES J., LEHTOVAARA P., TEERI T.: Cellulase families and their genes. *Trends Biotechnol.* 5, 255 – 261 (1987).

MARSDEN W.L., GRAY P.P.: Enzymatic hydrolysis of cellulose in lignocellulosic material. *CRC Crit.Rev.Biotechnol.* 3, 235 – 276 (1986).

MILLER G.L.: Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal.Chem.* **31**, 426 – 428 (1959).

NAKAMURA K., MISAWA N., KITAMURA K.: Cellulase genes of *Cellulomonas* CB4. Cloning and expression of a CM-cellulose hydrolyzing enzyme (endoglucanase) gene in *E. coli*. *J.Biotechnol.* 3, 239 – 246 (1986).

NAKAMURA K., KITAMURA K.: Isolation and identification of crystalline cellulose hydrolysing bacterium and its enzymatic properties. *J.Ferment.Technol.* **60**, 343 – 348 (1982).

PENTTILA M., LEHTOVAARA P., KNOWLES J.: Cellulolytic yeast strains and their application, pp. 247 – 268 in *Yeast Genetic Engineering* (Barr *et al.*, Eds). Butterworths, Singapore 1989.

RAJOKA M.I., PARVEZ S., MALIK K.A.: Cloning of structural gene for β-glucosidase from *Cellulomonas biazotea* into *E. coli* and *S. cerevisiae* using shuttle vector pBLU-D. *Biotechnol.Lett.* **14**, 1001 – 1006 (1992).

PRESUTTI D.G., HUGHES T.A., STUTZENBERGER F.J.: Cloning of three endoglucanase genes from *Thermomonospora curvata* into *Escherichia coli*. *J.Biotechnol.* **17**, 177 – 188 (1991).

RAJOKA M.I., MALIK K.A.: Comparsion of different strain of *Cellulomonas* for production of cellulolytic and xylanlytic enzyme from biomass produced on saline lands. *Biotechnol.Lett.* **8**, 753 – 756 (1986).

SACCO M., MILLET J., AUBERT J.P.: *Ann.Inst.Pasteur (Microbiol.)* **135 A**, 485 – 488 (1984).

SILVA A., BENITEZ J., HOLLENBERG C.P.: Endoglucanase A gene fusion vectors for monitoring protein secretion and glycosylation in yeast. *Anal.Biochem.* **197**, 290 – 295 (1991).

SKIPPER N., SUTHERLAND M., DAVIES R.W., KILBURN D., MILLER R.C. Jr., WARREN A., WONG R.: Scretion of a bacterial cellulase by yeast. *Science* **230**, 958 – 960 (1985).

TEATHER R.M., WOOD P.J.: Use of Congo red – polysaccharide interactions in enumeration and characterization of cellulolytic bacteria from bovine rumen. *Appl.Environ.Microbiol.* **43**, 777 – 780 (1982).

TEERI T., SALOVUORI I., KNOWLES J.: The molecular cloning of the major cellulase gene from *Trichoderma reesei*. *Bio/-technology* **1**, 696 – 699 (1983).