Journal of Industrial Microbiology & Biotechnology (1999) 22, 600–607

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Engineering endoglucanase-secreting strains of ethanologenic *Klebsiella oxytoca* P2

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Recombinant *Klebsiella oxytoca* P2 was developed as a biocatalyst for the simultaneous saccharification and fermentation (SSF) of cellulose by chromosomally integrating *Zymomonas mobilis* genes (*pdc*, *adhB*) encoding the ethanol pathway. This strain contains the native ability to transport and metabolize cellobiose, eliminating the need to supplement with β-glucosidase during SSF. To increase the utility of this biocatalyst, we have now chromosomally integrated the *celZ* gene encoding the primary endoglucanase from *Erwinia chrysanthemi*. This gene was expressed at high levels by replacing the native promoter with a surrogate promoter derived from *Z. mobilis* DNA. With the addition of *out* genes encoding the type II protein secretion system from *E. chrysanthemi*, over half of the active endoglucanase (EGZ) was secreted into the extracellular environment. The two most active strains, SZ2(pCPP2006) and SZ6(pCPP2006), produced approximately 24 000 IU L^{-1} of CMCase activity, equivalent to 5% of total cellular protein. Recombinant EGZ partially depolymerized acid-swollen cellulose and allowed the production of small amounts of ethanol by SZ6(pCPP2006) without the addition of fungal cellulase. However, additional endoglucanase activities will be required to complete the depolymerization of cellulose into small soluble products which can be efficiently metabolized to ethanol.

Keywords: endoglucanase; ethanol; *Klebsiella*; *Erwinia*; lignocellulose; biomass

Introduction

Bioconversion of lignocellulosic biomass into fuel ethanol offers the potential to reduce our dependence on imported petroleum and improve the quality of the environment [13,16]. Although many aspects of this technology have been demonstrated, the current challenge is to develop a cost-effective process for commercialization [15,31,37]. Considerable progress has been made by the genetic engineering of enteric bacteria for ethanol production from hemicellulose-derived sugars [18,21,35]. However, additional improvements which reduce the cost of enzymatic saccharification are needed for industrial ethanol production [23].

The Gulf Oil Company patented a process in 1976 for the production of ethanol from cellulose using simultaneous saccharification and fermentation (SSF) [9]. In this process, fungal cellulase preparations and yeasts were added to a slurry of the cellulosic substrate in a single vessel. Ethanol was produced concurrently during cellulose hydrolysis. A variation of this process, direct microbial conversion, has been proposed to reduce the cost of ethanol production [4,14]. For direct microbial conversion, a single microorganism or a consortium must be developed which produces all of the enzymes necessary for cellulose saccharification and metabolizes the resulting sugars to ethanol [4]. Although this has yet to be achieved, two approaches are being pursued to develop a suitable biocatalyst: (1) functional insertion of genes encoding the ethanol pathway into microorganisms which produce cellulase enzymes [3]; (2) functional insertion of genes encoding cellulase production into strains which contain an efficient ethanol pathway [19,22,29,32]. Both approaches require secretion of high levels of multiple cellulase activities (endoglucanase, exoglucanase, and β-glucosidase) and efficient ethanol production in a robust biocatalyst.

We have focused on the incremental improvement of enteric bacteria as promising microbial platforms for the direct microbial conversion process [16]. *Klebsiella oxytoca* M5A1, a hardy prototrophic bacterium, was selected for modification. This organism has the native ability to metabolize cellobiose, cellotriose, xylobiose, xylotriose, sucrose, and all monomeric sugars which are constituents of lignocellulose. The fermentation pathway in M5A1 has been redirected to produce ethanol efficiently by chromosomally integrating the *pdc* and *adhB* genes from *Zymomonas mobilis* [35]. The resulting strain, P2, produced ethanol from soluble saccharides at 95% of the maximum theoretical yield. This strain contains a native phosphoenolpyruvate-dependent phosphotransferase system and phosphocellobiase for cellobiose metabolism, eliminating the need for supplemental β-glucosidase [21,35]. *K. oxytoca* P2 produces ethanol efficiently from a variety of cellulosic substrates in SSF tests using fungal enzymes [8,15,35]. For direct microbial conversion, further improvements are needed such as the addition of secreted endoglucanase and exoglucanase activities.

The most abundant endoglucanase from *Erwinia chrysanthemi* P86021, EGZ, has been shown to function with commercial fungal cellulase and P2 to improve ethanol production from mixed waste office paper [34]. The *celZ* gene encoding this enzyme has been cloned and expressed at levels of approximately 3000 IU L^{-1}. However, the recom-

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Received 14 December 1998; accepted 4 March 1999

binant EGZ protein accumulated as a periplasmic product. Additional treatments, harvesting of culture and cell disruption (ultrasound, detergent, solvent, or heat), were required to release EGZ into the extracellular environment containing insoluble cellulose [34].

Current studies were undertaken to increase *celZ* expression and eliminate the requirement for additional treatments to release EGZ. Expression of *celZ* was increased eight-fold by replacing the native promoter and chromosomal integration. Half of the recombinant endoglucanase activity was secreted into the surrounding medium by adding the *out* genes encoding the type II protein secretion system from *E. chrysanthemi* EC16 [12].

Materials and methods

Bacteria, plasmids and culture conditions

Strains and plasmids used are summarized in Table 1. *Escherichia coli* and *K. oxytoca* M5A1 were grown in Luria-Bertani broth (LB) containing per liter: 10 g Difco (Detroit, MI, USA) tryptone, 5 g Difco yeast extract, and 5 g sodium chloride, or Luria agar (LB supplemented with 15 g L^{-1} of agar) [28]. CMC-plates (Luria agar plates containing 3 g L^{-1} carboxymethyl cellulose) were used to screen colonies for endoglucanase activity [36]. For growth of ethanologenic strains, glucose was added to solid media (20 g L^{-1}) and broth (50 g L^{-1}). Glucose was replaced with sorbitol (50 g L^{-1}), a non-reducing sugar, in broth used for the determination of endoglucanase activity. Modified SOC medium was used to grow cultures for electroporation (20 g L^{-1} Difco tryptone, 5 g L^{-1} yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 50 g L^{-1} glucose) [28]. Ampicillin (50 mg L^{-1}), spectinomycin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), tetracycline (6 or 12 mg L^{-1}), and chloramphenicol (40, 200 or 600 mg L^{-1}) were added as appropriate for selection. Unless stated otherwise, cultures were grown at 37°C. Ethanologenic strains and strains containing plasmids with a temperature-sensitive pSC101 replicon were grown at 30°C.

Genetic methods

All plasmids were constructed using *E. coli* DH5α as the host. Standard methods were used for plasmid construction and transformation [1,28]. Plasmid pLOI2306 and the circular DNA fragment lacking a replicon (Figure 1) were electroporated into the ethanologenic *K. oxytoca* P2 using a Bio-Rad (Hercules, CA, USA) Gene Pulser with the following conditions: 2.5 kV and 25 μF [5] with a measured time constant of 3.8–4.0 ms. The *E. chrysanthemi* EC16 secretion system (pCPP2006) was conjugated into *K. oxytoca* using pRK2013 for mobilization [20]. Small-scale and large-scale plasmid isolations were performed using the TELT procedure [1] and a Promega (Madison, WI, USA) Wizard Kit, respectively. DNA fragments were isolated from gels using a Qiaquick Gel Extraction Kit from Qiagen (Hilden, Germany). Chromosomal DNA from *K. oxytoca* M5A1 and *Z. mobilis* CP4 were isolated as described by Cutting [6] and Yomano [38], respectively. DNA was sequenced using a LI-COR Model 4000-L DNA sequencer [34].

Chromosomal integration of celZ

Two approaches were investigated for chromosomal integration of *celZ*, selection with a temperature-conditional plasmid (pLOI2183) using a procedure described previously for *E. coli* [11] and direct integration of circular DNA fragments lacking a functional replicon. This latter method has been used successfully for chromosomal integration of *Z. mobilis* genes encoding the ethanol pathway in *E. coli* B [21] and *K. oxytoca* M5A1 [35]. Circular DNA was transformed into P2 by electroporation using a Bio-Rad Gene Pulser. Transformants were selected on solid medium containing tetracycline (6 mg L^{-1}) and tested on CMC plates to confirm endoglucanase activity.

Endoglucanase activity

Expression of *celZ* with different promoters was evaluated by staining CMC plates for activity [36]. The diameters of the yellow zones were used as a relative measure of *celZ* expression. Clones with the largest zones were further evaluated for endoglucanase activity at 35°C using carboxymethyl cellulose as the substrate (20 g L^{-1} dissolved in 50 mM citrate buffer, pH 5.2) [36]. Intracellular endoglucanase was released from cultures by treatment with ultrasound for 4 s (Model W-220F cell disruptor, Heat System-Ultrasonics, Plainview, NY, USA). Endoglucanase activity is expressed as μmol reducing sugar released per min (IU). Reducing sugar was measured as described by Wood [36] using glucose as a standard.

Substrate depolymerization

Endoglucanase activity was determined using different substrates (20 g L^{-1} suspended in 50 mM citrate buffer, pH 5.2). Acid-swollen and ball-milled cellulose were prepared as described by Wood [36]. *K. oxytoca* SZ6(pCPP2006) was grown at 30°C for 16 h in LB supplemented with sorbitol, a nonreducing sugar, as a source of EGZ. Dilutions of cell-free broth were added to substrates and incubated at 35°C for 16 h. Several drops of chloroform were added to prevent the growth of adventitious contaminants during incubation. Samples were removed before and after incubation to measure reducing sugars by the DNS method [36]. The degree of polymerization (DP) was estimated by dividing the total calculated sugar residues present in the polymer by the number of reducing ends.

Fermentation

Fermentations were carried out in 250-ml flasks containing 100 ml of Luria broth supplemented with 50 g L^{-1} of carbohydrate. Carbohydrates were sterilized separately and added after cooling. To minimize substrate changes, acid-swollen cellulose, ball-milled cellulose and xylan were not autoclaved. Chloramphenicol (200 mg L^{-1}) was added to prevent the growth of contaminating organisms. Flasks were inoculated (10% v/v) with 24-h broth cultures (50 g L^{-1} glucose) and incubated at 35°C with agitation (100 rpm) for 24–96 h. Samples were removed daily to determine the ethanol concentration by gas chromatography [7].

Table 1 Strains and plasmids used in this study

Strains/plasmids	Properties	Sources/references
Strains		
Zymomonas mobilis CP4	prototrophic	[17]
Escherichia coli		
DH5α	*lacZ* M15 *recA*	Bethesda Research Laboratory
HB101	*recA lacY recA*	ATCC 37159
Klebsiella oxytoca		
M5A1	prototrophic	[35]
P2	*pfl::pdc adhB cat*	[35]
SZ1	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ2	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ3	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ4	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ5	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ6	*pfl::pdc adhB cat*; integrated *celZ*	This study
SZ7	*pfl::pdc adhB cat;* integrated *celZ*	This study
SZ8	*pfl::pdc adhB cat;* integrated *celZ*	This study
SZ9	*pfl::pdc adhB cat;* integrated *celZ*	This study
SZ10	*pfl::pdc adhB cat;* integrated *celZ*	This study
Plasmids		
pUC19	*bla* cloning vector	New England Biolab
pBR322	*bla tet* cloning vector	New England Biolab
pLOI1620	*bla celZ*	[34]
pRK2013	*kan* mobilizing helper plasmid (mob$^+$)	ATCC
pCPP2006	Sp^r, 40-kbp fragment containing *out* genes from *E. chrysanthemi* EC16	[12]
pST76-K	*kan* low copy vector containing temperature-sensitive pSC101 replicon	[24]
pLOI2164	*bla celZ* (*Bam*HI eliminated from pLOI1620)	This study
pLOI2173	*kan celZ* (native *celZ* promoter)	This study
pLOI2177	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2178	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2179	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2180	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2181	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2182	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2183	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2184	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2185	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2186	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2187	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2188	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2189	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2190	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2191	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2192	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2193	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2194	*kan celZ* (surrogate promoter from *Z. mobilis*)	This study
pLOI2301	*Asc*I linker inserted into *Nde*I site of pUC19	This study
pLOI2302	*Asc*I linker inserted into *Sap*I site of pLOI2301	This study
pLOI2303	*Ava*I-*Eco*RI fragment from pBR322 inserted into *Pst*I site of pLOI2302 after Klenow treatment	This study
pLOI2305	*Eco*RI DNA fragment of *K. oxytoca* M5A1 genomic DNA (*ca.* 2.5 kb) cloned into the *Sma*I site of pLOI2303	This study
pLOI2306	*Eco*RI-*Sph*I fragment from pLOI2183 cloned into *Eco*RI site of pLOI2305	This study

Results

Construction of a vector for the cloning of promoters

Random fragments of *Z. mobilis* DNA previously served as an excellent source of surrogate promoters for enhancing heterologous gene expression in *E. coli* [17]. The same approach was also pursued for *celZ* expression in *K. oxytoca* M5A1.

A suitable vector was constructed using plasmid pLOI1620 as a source of the *celZ* gene [34]. The *Bam*HI site in this plasmid was eliminated by digestion, Klenow treatment, and self-ligation to produce pLOI2164. A DNA fragment containing a promoterless *celZ* gene was isolated from pLOI2164 by digestion with *Nde*I. After Klenow treatment, this fragment was further digested with *Hind*III to remove downstream vector DNA, and ligated into pUC19 (*Hind*III to *Hinc*II) to produce pLOI2170. The *Bam*HI-*Sph*I fragment from pLOI2170 containing a promoterless *celZ* was ligated into the corresponding sites of pST76-K, a low copy vector with a temperature-sensitive pSC101 replicon [24]. The resulting plasmid, pLOI2171, contains a single *Bam*HI site suitable for the insertion of promoter fragments, immediately upstream from the *celZ* ribosomal binding region. For comparison, the *Eco*RI-*Sph*I

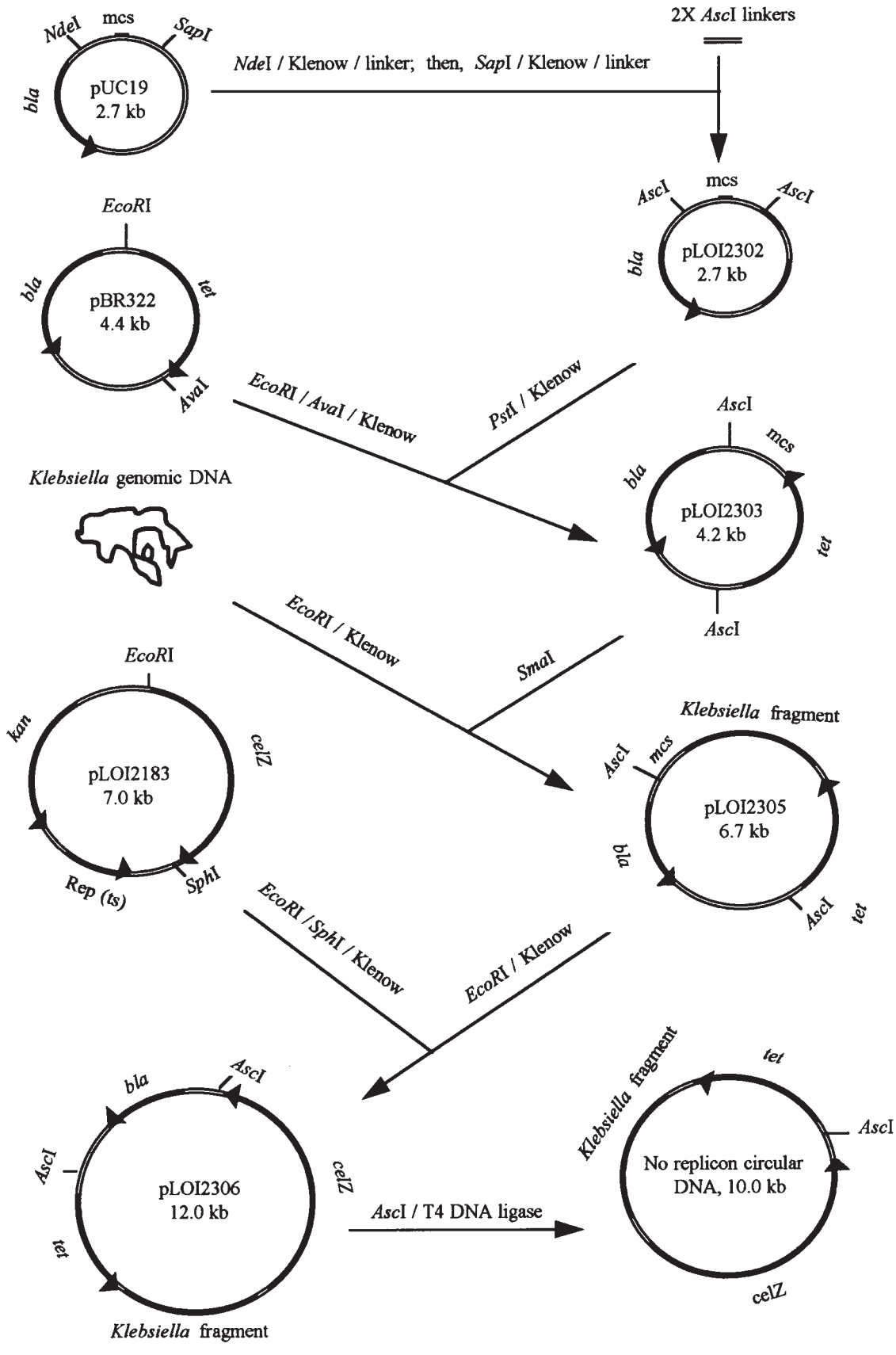

Figure 1 Construction of the *celZ* integration vector pLOI2306. mcs, multiple cloning site.

fragment containing the native *celZ* gene (with promoter) from pLOI2164 was also cloned into pST76-K (*Eco*RI to *Sph*I) to produce the control plasmid, pLOI2173.

Screening promoters for enhancing celZ expression

*Sau*3AI-digested *Z. mobilis* DNA fragments were ligated into the *Bam*HI site of pLOI2171 to generate a library of potential promoters. These plasmids were transformed into *E. coli* DH5α for initial screening. Of the 18 000 colonies individually tested on CMC plates, 75 clones produced larger yellow zones than the control (pLOI2173). Plasmids from these 75 clones were then transformed into *K. oxytoca* M5A1, retested, and found to express high levels of *celZ* in this second host.

The 18 M5A1 clones (pLOI2177–pLOI2194) with the largest zones were grown in LB broth and assayed for endoglucanase activity (Table 2). Activities with these plasmids were up to eight-fold higher than with the control plasmid containing a native *celZ* promoter (pLOI2173). The four plasmids which produced the largest zones (pLOI2177, pLOI2180, pLOI2182 and pLOI2183) also produced the highest total endoglucanase activities (approximately 20 000 IU L^{-1}) in broth. One of these plasmids, pLOI2183, was selected for chromosomal integration.

Chromosomal integration of celZ with a temperature-sensitive plasmid

Gene integration using a temperature-sensitive pSC101 replicon has been successfully used in *E. coli* [11,21] but has not been tested in *K. oxytoca* M5A1 or P2. Our attempts to integrate *celZ* into the chromosome of strain P2 using pLOI2183 were unsuccessful but resulted in the production of a fusion plasmid. This plasmid may result from fusion with a native cryptic plasmid. It was readily transformed into *E. coli* DH5α but was not studied further.

Table 2 Evaluation of promoters for *celZ* expression and secretion in *K. oxytoca* M5A1

Plasmids[a]	No secretion genes		Secretion genes present (pCPP2006)	
	Total activity (IU L^{-1})[b]	Secreted activity (IU L^{-1})	Total activity (IU L^{-1})	Secreted activity (IU L^{-1})
pLOI2173	2450	465	3190	1530
pLOI2177	19 700	3150	32 500	13 300
pLOI2178	15 500	2320	21 300	11 500
pLOI2179	15 400	2310	21 400	12 000
pLOI2180	21 400	3210	30 800	13 600
pLOI2181	15 600	2490	21 000	11 800
pLOI2182	19 600	3130	31 100	14 000
pLOI2183	20 700	3320	32 000	14 000
pLOI2184	15 500	2480	21 200	11 900
pLOI2185	15 100	2420	24 600	11 500
pLOI2186	17 000	2380	25 700	13 400
pLOI2187	15 800	2210	24 500	12 200
pLOI2188	18 200	2180	25 600	12 000
pLOI2189	14 800	2360	27 100	12 700
pLOI2190	16 100	2410	26 500	12 500
pLOI2191	15 800	2210	25 000	12 400
pLOI2192	15 100	1810	24 900	12 500
pLOI2193	16 700	2010	24 600	12 800
pLOI2194	15 400	2770	21 500	11 900

[a]pLOI2173 contains the *celZ* gene with the original promoter, all others contain the *celZ* gene with a *Z. mobilis* DNA fragment which serves as a surrogate promoter.
[b]Endoglucanase (CMCase) activities were determined after 16 h of growth at 30°C.

Chromosomal integration of celZ with a circular DNA

A second vector was designed for the integration of *celZ* into P2. This vector (pLOI2306) was constructed to facilitate the isolation of a DNA fragment which lacked all replication functions but contained the *celZ* gene with surrogate promoter, a selectable marker, and a homologous DNA fragment for integration (Figure 1). Two *Asc*I sites were added to pUC19 by inserting a linker (GGCGCGCC) into Klenow-treated *Nde*I and *Sap*I sites which flank the polylinker region to produce pLOI2302. A blunt fragment containing the *tet* gene from pBR322 (*Eco*RI and *Ava*I, Klenow treatment) was cloned into the *Pst*I site of pLOI2302 (*Pst*I, Klenow treatment) to produce pLOI2303. A blunt fragment of *K. oxytoca* M5A1 chromosomal DNA (*Eco*RI, Klenow treatment) was ligated into the *Sma*I site of pLOI2303 to produce pLOI2305. The *Eco*RI–*Sph*I fragment (Klenow treated) containing the surrogate *Z. mobilis* promoter and *celZ* gene from pLOI2183 was ligated into the *Eco*RI site of pLOI2305 (*Eco*RI, Klenow treatment) to produce pLOI2306.

Digestion of pLOI2306 with *Asc*I produced two fragments, the larger of which contained the *celZ* gene with surrogate promoter, *tet* gene and chromosomal DNA fragment for homologous recombination. This larger fragment (10 kbp) was purified by agarose gel electrophoresis, circularized by self-ligation, and electroporated into P2 with selection for tetracycline resistance. The resulting 21 tetracycline-resistant colonies were purified and tested on CMC plates. All were positive with large zones indicating functional expression of *celZ*. Clones were tested for the presence of plasmids by transforming DH5α with plasmid DNA preparations and by gel electrophoresis. No transformants were obtained with 12 clones. However, two of these strains were subsequently found to contain large plasmid bands which may contain *celZ* and were discarded. Both strains with large plasmids contained DNA which could be sequenced with T7 and M13 primers confirming the presence of multicopy plasmids. The remaining 10 strains contain integrated *celZ* genes and could not be sequenced with either primer.

Endoglucanase production by the integrated strains

The ten integrated strains (SZ1–SZ10) were investigated for endoglucanase production in LB-sorbitol broth (Table 3). All produced 5000–7000 IU L^{-1} of active enzyme. Although this represents twice the activity expressed from plasmid pLOI2173 containing the native *celZ* promoter, the integrated strains produced only 1/3 the endoglucanase activity achieved by P2(pLOI2183) containing the same surrogate *Z. mobilis* promoter (Table 2). The reduction in endoglucanase expression upon integration may be attributed to a decrease in copy number (ie high copy plasmid *vs* single integrated copy).

Table 3 Comparison of culture growth, endoglucanase production and secretion from ethanologenic *K. oxytoca* strains containing integrated *celZ*

Strains	Growth on solid medium (600 mg L^{-1} Cm)	Endoglucanase production and secretion (IU L^{-1})			
		No secretion system		Adding secretion system (pCPP2006)	
		Total activity	Secreted activity	Total activity	Secreted activity
P2	++++	0	0	0	0
SZ1	++	6140	1600	26 100	14 300
SZ2	++++	6460	1160	23 700	11 400
SZ3	+++	5260	1320	18 400	8440
SZ4	+++	7120	1070	23 200	9990
SZ5	+	6000	1080	29 300	15 500
SZ6	++++	7620	1520	24 300	11 900
SZ7	+	6650	1330	28 800	15 500
SZ8	+++	7120	854	28 700	14 900
SZ9	++	7530	1130	26 700	12 800
SZ10	+++	4940	939	17 000	6600

Endoglucanase (CMCase) activities were determined after 16 h of growth at 30°C.

Secretion of endoglucanase EGZ

K. oxytoca contains a native Type II secretion system for pullulanase secretion [25], analogous to the secretion system encoded by the *out* genes in *Erwinia chrysanthemi* which secrete pectate lyases and endoglucanase EGZ [2,12]. Type II secretion systems are typically very specific and function poorly with heterologous proteins [12,26,30]. Thus, as expected, recombinant *celZ* was expressed primarily as a cell-associated product with either M5A1 (Table 2) or P2 (Table 3) as the host. Less than 1/4 (12–26%) of the total recombinant EGZ activity was recovered in the broth. With *E. coli* DH5α, however, even lower levels of extracellular EGZ (8–12% of total) were present (data not shown). Thus the native secretion system in *K. oxytoca* may facilitate partial secretion of recombinant EGZ.

The Type II secretion genes (*out* genes) from *E. chrysanthemi* EC16 have been cloned and reconstituted in *E. coli*, enabling the secretion of recombinant pectate lyases encoded by genes from the same strain [12]. We have tested the ability of the *out* genes (pCPP2006) to facilitate secretion of the recombinant EGZ (from strain P86021) in ethanologenic strains of *K. oxytoca* (Table 2 and Table 3). For most strains containing plasmids with *celZ*, addition of the *out* genes resulted in a five-fold increase in extracellular EGZ and a two-fold increase in total endoglucanase activity. For strains with integrated *celZ*, addition of the *out* genes resulted in a 10-fold increase in extracellular EGZ and a four-fold increase in total endoglucanase activity. In both cases, the *out* genes facilitated secretion of approximately half of the total endoglucanase activity. The increase in EGZ activity resulting from addition of *out* genes may reflect improved folding of the secreted product in both plasmid and integrated *celZ* constructs. The smaller increase observed with the pUC-based derivatives may result from plasmid burden and competition for export machinery during the production of periplasmic β-lactamase from the *bla* gene on this high copy plasmid.

Two criteria were used to identify the best integrated strains of P2, growth on solid medium containing high levels of chloramphenicol (a marker for high level expression of the upstream *pdc* and *adhB* genes) and effective secretion of endoglucanase with the *out* genes. Two recombinant strains were selected for further study, SZ2 and SZ6. Both produced 24 000 IU L^{-1} of endoglucanase activity, equivalent to approximately 5% of the total cellular protein [27].

Substrate depolymerization

The substrate range of recombinant EGZ was surprisingly narrow (Table 4). Excellent activity was observed with CMC. However, activity with acid-swollen cellulose was less than 10% of CMC activity and barely measurable with ball-milled cellulose, Avicel, or xylan. Overnight digestion with EGZ, resulted in a measurable reduction in average polymer length for all substrates. CMC and acid-swollen cellulose were depolymerized to an average length of seven residues. Cellulose polymers of seven residues are marginally soluble and too large to be effectively metabolized without further digestion [35]. The average chain length of ball-milled cellulose and Avicel was reduced to 1/3 of the original length while less than a single cut was observed per xylan polymer.

Fermentation

To be useful, addition of *celZ* and *out* genes to strain P2 must not reduce the fermentative ability of the resulting biocatalyst. A comparison was made using glucose and cellobiose (Table 5). All strains were equivalent in their ability to ferment these sugars indicating a lack of detrimental effects from the integration of *celZ* or addition of pCPP2006. These strains were also examined for their ability to convert acid-swollen cellulose directly into ethanol. The most active construct SZ6(pCPP2006) produced a small amount of ethanol (3.9 g L^{-1}) from amorphous cellulose. Approximately 1.5 g L^{-1} ethanol was present initially at the time of inoculation for all strains. This decreased with time to zero for all strains except SZ6(pCPP2006). Thus the 3.9 g L^{-1} ethanol observed with SZ6(CPP2006) may represent an underestimate of total ethanol production. However, at best, this represents conversion of only a fraction of the polymer present. It is likely that low levels of

Table 4 Depolymerization of various substrates by EGZ from cell-free broth of strain SZ6(pCPP2006)

Substrates	Enzyme activity (IU L^{-1})	Estimated degree of polymerization	
		Before digestion	After digestion
Carboxymethyl cellulose	13 175	224	7
Acid-swollen cellulose	893	87	7
Ball-milled cellulose	200	97	28
Avicel	41	104	35
Xylan from oat spelts	157	110	78

Strain SZ6(pCPP2006) was grown in LB-sorbitol broth for 16 h as a source of secreted EGZ.

Table 5 Ethanol production by strain SZ6 containing *out* genes (pCPP2006) and integrated *celZ* using various substrates (50 g L^{-1})

Strains	Ethanol production (g L^{-1})		
	Glucose	Cellobiose	Acid-swollen cellulose
P2	22.9	22.7	0
P2 (pCPP2006)	22.6	21.3	0
SZ6	21.5	19.7	0
SZ6(pCPP2006)	22.7	21.2	3.9

Initial ethanol concentrations at the time of inoculation were approximately 1.5 g L^{-1} for all cultures.
With acid-swollen cellulose as a substrate, these levels declined to 0 after 72 h of incubation for all strains except SZ6(pCPP2006).

glucose, cellobiose and cellotriose were produced by EGZ hydrolysis of acid-swollen cellulose and fermented. These compounds can be metabolized by the native phosphoenolpyruvate-dependent phosphotransferase system in *K. oxytoca* [21,35].

Discussion

Cellulase is a critical component for the development of a cost-effective lignocellulose-to-ethanol technology [13]. Despite its commercial application in the food processing, detergent and paper industries [23], fungal cellulase remains too expensive for use in a bioethanol process without further improvements [13,31]. Development of cellulase-producing and -secreting strains of ethanologenic biocatalysts represents a promising approach to reduce the cost of bioconversion. By introducing *Z. mobilis* genes encoding ethanol production, *K. oxytoca* P2 was previously engineered as a biocatalyst for the simultaneous saccharification and fermentation of cellulose to ethanol [8,21,35]. This organism has the native ability to use cellobiose (and cellotriose), which eliminates the requirement for supplementation with β-glucosidase [35]. However, the complete saccharification of cellulose requires two types of extracellular enzymes, endoglucanases and exoglucanases. The *E. chrysanthemi* endoglucanase EGZ functions with fungal cellulase to improve ethanol production during SSF [34]. However, EGZ alone does not appear to provide all of the endoglucanase activities needed for the digestion of amorphous cellulose despite a large increase in functional expression, chromosomal integration, and secretion. We have achieved excellent endoglucanase production by SZ6(pCPP2006), 24 000 IU L^{-1}, which is roughly equivalent to the CMCase activity provided by commercial cellulase in SSF tests of mixed waste office paper containing 1000 filter paper units of cellulase per liter [16]. However, the degree of depolymerization of acid-swollen cellulose was insufficient to allow effective metabolism of the products. Additional endoglucanase activities appear to be required. Genes encoding two candidate glucohydrolases have been previously isolated from *E. chrysanthemi: celY* [10] and *bgxA* [33]. EGY (*celY*) is a minor endoglucanase in *E. chrysanthemi* and represents approximately 5% of total endoglucanase activity [10]. The *bgxA* gene encodes β-glucosidase and β-xylosidase activities but is not present in all strains of *E. chrysanthemi* [33]. One or both of these gene products may function with EGZ in *E. chrysanthemi* to depolymerize the amorphous regions of cellulose and provide soluble substrates for growth.

Acknowledgements

The authors thank Dr Collmer for sharing plasmid pCPP2006 and Dr Posfai for sharing plasmid pST76-K. This research was supported in part by the grants from the US Department of Agriculture, National Research Initiative (98–35504–6177), the US Department of Energy (DE–FG02–96ER20222), and the Florida Agricultural Experiment Station (Publication No. R-06652).

References

1 Ausubel FM, R Brent, RE Kingston, DD Moore, JG Seigman, JA Smith and K Struhl. 1998. Current Protocols in Molecular Biology. John Wiley & Sons, New York, NY.
2 Barras F, F Gijsegem and AK Chatterjee. 1994. Extracellular enzymes and pathogenesis of soft-rot *Erwinia*. Annu Rev Phytopathol 32: 201–234.
3 Beall DS and LO Ingram. 1993. Genetic engineering of soft-rot bacteria for ethanol production from lignocellulose. J Ind Microbiol 11: 151–155.
4 Christakopoulos P, BJ Macris and D Kekos. 1990. On the mechanism of direct conversion of cellulose to ethanol by *Fusarium oxysporum*: effect of cellulase and β-glucosidase. Appl Microbiol Biotechnol 33: 18–20.
5 Comaduran LF, F Lara and M Soberon. 1998. Increased respiration through cytochrome *d* enhances microaerobic N_2 fixation in *Klebsiella pneumoniae*. Biotechnol Lett 20: 489–493.
6 Cutting SM and PBV Horn. 1990. Genetic analysis. In: Molecular Methods for *Bacillus* (Harwood CR and SM Cutting, eds), pp 61–74. John Wiley & Sons, New York, NY.
7 Dombek KM and LO Ingram. 1986. Magnesium limitation and its role in the apparent toxicity of ethanol during yeast fermentation. Appl Environ Microbiol 52: 975–981.
8 Doran JB, HC Aldrich and LO Ingram. 1994. Saccharification and fermentation of sugar cane bagasse by *Klebsiella oxytoca* P2 containing chromosomally integrated genes encoding the *Zymomonas mobilis* ethanol pathway. Biotechnol Bioeng 44: 240–247.
9 Gauss WF, S Suzuki and M Takagi. 1976. Manufacture of alcohol from cellulosic material using plural ferments. US Patent No. 3 990 944.
10 Guiseppi A, JL Aymeric, B Cami, F Barras and N Creuzet. 1991. Sequence analysis of the cellulase-encoding *celY* gene of *Erwinia chrysanthemi*: a possible case of interspecies gene transfer. Gene 106: 109–114.
11 Hamilton CM, M Aldea, BK Washburn, P Babitzke and SR Kushner. 1989. New methods for generating deletions and gene replacements in *Escherichia coli*. J Bacteriol 171: 4617–4622.
12 He SY, M Lindeberg, AK Chatterjee and A Collmer. 1991. Cloned *Erwinia chrysanthemi out* genes enables *Escherichia coli* to selectively secret a diverse family of heterologous proteins to its milieu. Proc Natl Acad Sci USA 88: 1079–1083.
13 Himmel ME, WS Adney, JO Baker, R Elander, JD McMillan, RA Nieves, JJ Sheehan, SR Thomas, TB Vinzant and M Zhang. 1997. Advanced bioethanol production technologies: a perspective. In: Fuels and Chemicals from Biomass (Saha BC and J Woodward, eds), pp 2–45, American Chemical Society, Washington, DC.
14 Hogsett DA, HJ Ahn, TD Bernardez, CR South and LR Lynd. 1992. Direct microbial conversion: prospects, progress, and obstacles. Appl Biochem Biotechnol 34–35: 527–542.
15 Ingram LO and JB Doran. 1995. Conversion of cellulosic materials to ethanol. FEMS Microbiol Rev 16: 235–241.
16 Ingram LO, PF Gomez, X Lai, M Moniruzzaman, BE Wood, LP Yomano and SW York. 1998. Metabolic engineering of bacteria for ethanol production. Biotechnol Bioeng 58: 204–214.

17 Ingram LO and T Conway. 1988. Expression of different levels of ethanologenic enzymes from *Zymomonas mobilis* in recombinant strains of *Escherichia coli*. Appl Environ Microbiol 54: 397–404.
18 Ingram LO, T Conway, DP Clark, GW Sewell and JF Preston. 1987. Genetic engineering of ethanol production in *Escherichia coli*. Appl Environ Microbiol 53: 2420–2425.
19 Minamigochi K, T Ooi, T Kawagochi, K Okada, S Murao and M Arai. 1995. Secretive expression of the *Aspergillus aculeatus* cellulase (FI-CMCase) by *Saccharomyces cerevisiae*. J Ferment Bioeng 79: 363–366.
20 Murata H, M Fons, A Chatterjee, A Collmer and AK Chatterjee. 1990. Characterization of transposon insertion *out*$^-$ mutants of *Erwinia carotovora* subsp *carotovora* defective in enzyme export and a DNA segment that complements *out* mutants in *E. carotovora* subsp *carotovora*, *E. carotovora* subsp *atroseptica*, and *Erwinia chrysanthemi*. J Bacteriol 172: 2970–2978.
21 Ohta K, DS Beall, JP Mejia, KT Shanmugam and LO Ingram. 1991. Genetic improvement of *Escherichia coli* for ethanol production: chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase and alcohol dehydrogenase II. Appl Environ Microbiol 57: 893–900.
22 Okamoto T, S Yamano, H Ikeaga and K Nakamura. 1994. Cloning of the *Acetobacter xylinum* cellulase gene and its expression in *Escherichia coli* and *Zymomonas*. Appl Microbiol Biotechnol 42: 563–568.
23 Philippidis GP. 1994. Cellulase production technology: evaluation of current status. In: Enzymatic Conversion of Biomass for Fuel Production (Himmel ME, JO Baker and RP Overend, eds), pp 188–217, American Chemical Society, Washington, DC.
24 Posfai G, MD Koob, HA Kirkpatrick and FR Blattner. 1997. Versatile insertion plasmids for targeted genome manipulations in bacteria: isolation, deletion, and rescue of the pathogenicity island LEE of the *Escherichia coli* 0157:H7 genome. J Bacteriol 179: 4426–4428.
25 Pugsley AP. 1993. The complete general secretory pathway in Gram-negative bacteria. Microbiol Rev 57: 50–108.
26 Py B, GPC Salmond, M Chippaux and F Barras. 1991. Secretion of cellulase in *Erwinia chrysanthemi* and *E. carotovora* is species specific. FEMS Microbiol Lett 79: 315–322.
27 Py B, I Bortoli-German, J Haiech, M Chuppaux and F Barras. 1991. Cellulase EGZ of *Erwinia chrysanthemi*: structural organization and importance of His98 and Glu133 residues of catalysis. Protein Engin 4: 325–333.
28 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning: A Laboratory Manual, 2nd edn. pp 1–478, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
29 Saito T, T Suzuki, A Hayashi, H Honda, M Taya, S Iijima and T Kobayashi. 1990. Expression of a thermostable cellulase gene from a thermophilic anaerobe in *Saccharomyces cerevisiae*. J Ferment Bioeng 69: 282–286.
30 Sauvonnet N and AP Pugsley. 1996. Identification of two regions of *Klebsiella oxytoca* pullulanase that together are capable of promoting β-lactamase secretion by the general secretory pathway. Mol Microbiol 22: 1–7.
31 Sheehan JJ. 1994. Bioconversion for production of renewable transportation fuels in the United States: a strategic perspective. In: Enzymatic Conversion of Biomass for Fuel Production (Himmel ME, JO Baker and RP Overend, eds), pp 1–52, American Chemical Society, Washington, DC.
32 Su P, CQ Liu, RJ Lucas, SF Delaney and NW Dunn. 1993. Simultaneous expression of genes encoding endoglucanase and β-glucosidase in *Zymomonas mobilis*. Biotechnol Lett 15: 979–984.
33 Vroemen S, J Heldens, C Boyd, B Henrissat and NT Keen. 1995. Cloning and characterization of the *bgxA* gene from *Erwinia chrysanthemi* D1 which encodes a β-glucosidase/xylosidase enzyme. Mol Gen Genet 246: 465–477.
34 Wood BE, DS Beall and LO Ingram. 1997. Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivatives of *Escherichia coli* KO11. Biotech Bioeng 55: 547–555.
35 Wood BE and LO Ingram. 1992. Ethanol production from cellobiose, amorphous cellulose and crystalline cellulose by recombinant *Klebsiella oxytoca* containing chromosomally integrated *Zymomonas mobilis* genes for ethanol production and plasmids expressing thermostable cellulase genes from *Clostridium thermocellum*. Appl Environ Microbiol 58: 2103–2110.
36 Wood TM and KM Bhat. 1988. Methods for measuring cellulase activities. In: Methods in Enzymology, vol 160 (Wood WA and ST Kellogg, eds), pp 87–112, Academic Press, San Diego, CA.
37 Wyman CE. 1995. Economic fundamentals of ethanol production from lignocellulosic biomass. In: Enzymatic Degradation of Insoluble Carbohydrates (Saddler JN and MH Penner, eds), pp 272–290, American Chemical Society, Washington, DC.
38 Yomano LP, RK Scopes and LO Ingram. 1993. Cloning, sequencing and expression of the *Zymomonas mobilis* phosphoglycerate mutase gene (*pgm*) in *Escherichia coli*. J Bacteriol 175: 3926–3933.