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Gene Integration and Expression and Extracellular Secretion of *Erwinia chrysanthemi* Endoglucanase CelY (*celY*) and CelZ (*celZ*) in Ethanologenic *Klebsiella oxytoca* P2†

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The development of methods to reduce costs associated with the solubilization of cellulose is essential for the utilization of lignocellulose as a renewable feedstock for fuels and chemicals. One promising approach is the genetic engineering of ethanol-producing microorganisms that also produce cellulase enzymes during fermentation. By starting with an ethanologenic derivative (strain P2) of *Klebsiella oxytoca* M5A1 with the native ability to metabolize cellobiose, the need for supplemental β-glucosidase was previously eliminated. In the current study, this approach has been extended by adding genes encoding endoglucanase activities. Genes *celY* and *celZ* from *Erwinia chrysanthemi* have been functionally integrated into the chromosome of P2 using surrogate promoters from *Zymomonas mobilis* for expression. Both were secreted into the extracellular milieu, producing more than 20,000 endoglucanase units (carboxymethyl cellulase activity) per liter of fermentation broth. During the fermentation of crystalline cellulose with low levels of commercial cellulases of fungal origin, these new strains produced up to 22% more ethanol than unmodified P2. Most of the beneficial contribution was attributed to CelY rather than to CelZ. These results suggest that fungal enzymes with substrate profiles resembling CelY (preference for long-chain polymers and lack of activity on soluble cello-oligosaccharides of two to five glucosyl residues) may be limiting in commercial cellulase preparations.

The production of fuels and chemicals from cellulosic substrates using microbial biocatalysts offers the potential to reduce the use of fossil fuels and improve the environment (11, 18, 22, 29–31). However, the low activity of cellulase enzymes (15, 30) and the resulting cost of hydrolysis represent major barriers for the use of lignocellulosic feedstocks for fuels, bulk chemicals, and plastics (15, 29, 30, 41). The enzymatic hydrolysis of cellulose has been extensively studied but remains poorly understood (2, 3, 5, 21, 32). Hydrolysis results from the combined action of at least three classes of β-1,4-glucanase activities (2, 5, 26, 28): endoglucanases, exoglucanases, and cellobiases that complete the hydrolysis of soluble products (from two to six glucosyl residues) to monomeric glucose. Cellobiose and soluble cellobiosides are potent competitive inhibitors of endo- and exoglucanases that must be removed to prevent autoinhibition. Fungi such as *Trichoderma reesei* (19, 27, 39) secrete soluble β-1,4-glucosidases (cellobiase) to complete the hydrolysis process. In some bacteria, however, hydrolysis of soluble cellobiosides is completed intracellularly. Cellobiose and cellotriose are actively transported by a β-glucoside-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) (18, 20, 40). Intracellular cellobiose-phosphate is subsequently hydrolyzed to glucose and glucose-6-phosphate by a cytoplasmic phospho-β-glucosidase for immediate entry into glycolysis.

One approach to reduce the costs of cellulase enzymes for bioprocessing is to develop ethanologenic biocatalysts that provide a portion of the cellulolytic activity. This approach is being pursued with several naturally ethanologenic microorganisms such as *Saccharomyces cerevisiae* (8, 9, 33, 34), *Zymomonas mobilis* (7, 24, 31), and cellulolytic bacteria such as *Clostridium thermocellum* and *C. thermosaccharolyticum* (16, 35). Our laboratory is pursuing an alternative approach: the addition of cellulase activities to enteric bacteria (*Escherichia coli* B and *Klebsiella oxytoca* M5A1) that have been previously engineered to produce ethanol by adding the *Z. mobilis pdc* and *adhB* genes encoding the pyruvate-to-ethanol pathway (18). *K. oxytoca* M5A1 contains a native cellobiose-specific PTS and phospho-β-glucosidase (20, 25, 40), eliminating the need for extracellular β-glucosidase. The *K. oxytoca* operon containing the cellobiose utilization genes (*casAB*) has been cloned (20) and expressed in ethanologenic *E. coli* KO11 (18, 25) to construct a second, analogous biocatalyst. Subsequent studies have overexpressed the *celZ* endoglucanase (CelZ; formerly EGZ) from the plant saprophyte, *Erwinia chrysanthemi* (6, 10), in both *K. oxytoca* M5A1 (42) and *E. coli* B (44). By adding accessory genes (*out* genes) from *E. chrysanthemi*, high levels of endoglucanase activity were effectively secreted into the extracellular milieu by both organisms (13, 42, 44).

CelZ represents 95% of the total carboxymethyl cellulase (CMCase) activity in *E. chrysanthemi* (6). The remaining 5% is attributed to a second endoglucanase, CelY (formerly EGY). The *celY* gene has also been cloned and sequenced (13). Recent investigations have shown that CelY and CelZ differ in substrate preference (43). When mixed at ratios similar to those produced by cultures of *E. chrysanthemi*, these two enzymes function synergistically, indicating a potential need for

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli		
DH5α	*lacZ*ΔM15 *recA*	Bethesda Research Laboratory
TOP10F′	*hsdR mcrA lacZ*ΔM15 *endA recA*; F′ *tet lacI*	Invitrogen
HB101	*recA lacY*	ATCC 37159
S17-1	*thi pro recA hsdR* RP4-2-*tet*::Mu *aphA*::Tn7 λ*pir*	12
Z. mobilis CP4	Prototrophic	18
K. oxytoca		
M5A1	Prototrophic	40
P2	*pfl*::*pdc adhB cat*	40
SZ6	*pfl*::*pdc adhB cat*; integrated *celZ tet*	42
SZ12	*pfl*::*pdc adhB cat*; integrated *celZ celY kan*	This study
SZ21	*pfl*::*pdc adhB cat*; integrated *celZ celY*	This study
SZ22	*pfl*::*pdc adhB cat*; integrated *celY celZ*::*aac*	This study
Plasmids		
pUC18	*bla* cloning vector	New England Biolabs
pUC19	*bla* cloning vector	New England Biolabs
pCR2.1-TOPO	TA cloning vector, *bla kan*	Invitrogen
pMH18	*bla celY* from *E. chrysanthemi* 3937	13
pHPΩ45aac	*bla aac* source of apramycin gene	4
pBR322	*bla tet* cloning vector	New England Biolabs
pRK2013	*kan*, mobilizing plasmid	American Type Culture Collection
pCPP2006	*spm*, ~40-kbp fragment containing *out* genes from *E. chrysanthemi* EC16	14
pFT-A	*bla flp* low-copy vector containing recombinase and temperature-conditional pSC101 replicon	23
pLOI2224	*kan* integration vector containing conditional R6K replicon and two FRT sites	23
pLOI2302	pUC19 containing *Asc*I linkers inserted into blunt *Nde*I and *Sap*I sites	42
pLOI2307	*bla celZ* gene and a surrogate promoter from *Z. mobilis* DNA	44
pLOI2311	PCR fragment containing *celY* gene cloned into pCR2.1-TOPO, expressed from *lac* promoter	43
pLOI2316	pUC18 containing the *celY* gene on a Klenow-treated *Eco*RI fragment from pLOI2311 inserted into a *Hin*cII site; *celY* expressed from the *lac* promoter	This study
pLOI2317	*Eco*RI-*Hin*dIII fragment from pLOI2316 inserted into the corresponding sites of pLOI2302	This study
pLOI2318	*Sau*3A1 fragment of *Z. mobilis* DNA exhibiting promoter activity inserted into the *Bam*HI site of pLOI2317	This study
pLOI2319	*Sau*3A1 fragment of *Z. mobilis* DNA exhibiting promoter activity inserted into the *Bam*HI site of pLOI2317	This study
pLOI2320	*Sau*3A1 fragment of *Z. mobilis* DNA exhibiting promoter activity inserted into the *Bam*HI site of pLOI2317	This study
pLOI2323	*Sau*3AI fragment of *Z. mobilis* DNA exhibiting promoter activity inserted into the *Bam*HI site of pLOI2317	This study
pLOI2342	*Sau*3A1 fragment of *Z. mobilis* DNA exhibiting promoter activity inserted into the *Bam*HI site of pLOI2317	This study
pLOI2348	Random *Eco*RI fragment of *K. oxytoca* M5A1 DNA cloned into *Eco*RI site of pLOI2323	This study
pLOI2349	*Eco*RI linker inserted into the Klenow-treated *Sph*I site of pLOI2307	This study
pLOI2350	*Eco*RI fragment (*celZ* and surrogate promoter) from pLOI2349 inserted into the *Eco*RI site of pLOI2224	This study
pLOI2352	*Asc*I fragment (*K. oxytoca* fragment, *Z. mobilis* promoter fragment and *celY*) from pLOI2348 inserted into the *Asc*I site of pLOI2350	This study
pLOI2353	*Eco*RI-*Ava*I fragment (*tet* gene) from pBR322 inserted into the *Cla*I site of pFT-A	This study
pLOI2354	pUC19 derivative in which the multiple cloning sites from *Hin*dIII to *Sma*I were deleted by digestion, Klenow treatment, and self-ligation	This study
pLOI2355	*Eco*RI fragment (*celZ* gene) from pLOI2349 inserted into the *Eco*RI site of pLOI2354	This study
pLOI2356	*Sma*I fragment containing the apramycin resistance gene (*aac* gene) from pHPΩ45aac inserted into the T4 DNA polymerase-treated *Pst*I site of pLOI2355, disrupting the *celZ* gene	This study
pLOI2357	*Eco*RI fragment (*aac* and disrupted *celZ*) from pLOI2356 inserted into the *Eco*RI site of pLOI2224	This study
pLOI2358	Subclone of pLOI2323 in which the internal *Pst*I fragment was deleted, used for sequencing	This study
pLOI2359	Subclone of pLOI2323 in which the *Cla*I-*Hin*dIII fragment was deleted, used for sequencing	This study

both enzymes for optimal cellulose hydrolysis during fermentation.

In the current study, we have integrated the *celY* and *celZ* genes from *E. chrysanthemi* into the chromosome of *K. oxytoca* P2, an ethanologenic derivative of M5A1 (40). The secreted cellulase enzymes functioned together with commercial fungal cellulase to increase the production of ethanol during the simultaneous saccharification and fermentation (SSF) of crystalline cellulose.

MATERIALS AND METHODS

Bacteria, plasmids, and culture conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* DH5α and TOPO10F′ were used as hosts during plasmid constructions. The *celZ* gene was cloned from *E. chrysanthemi* P86021 (42, 44). The *celY* gene was cloned by Guiseppi et al. (13) from *E. chrysanthemi* 3937. The *out* genes were cloned by He et al. (14) from *E. chrysanthemi* EC16.

E. coli cultures were grown at 37°C in Luria-Bertani (LB) broth (1) containing (per liter) 10 g of Difco tryptone, 5 g of Difco yeast extract, and 5 g of sodium chloride or on solid LB medium containing agar (1.5%). Sugar was always included in broth (5% glucose or sorbitol) and solid media (2% glucose) used for the growth of ethanologenic strains. Clones were screened for endoglucanase production using the Congo red method (38, 40). Endoglucanase indicator plates were prepared by supplementing LB agar with 0.3% low-viscosity carboxymethyl cellulose (CMC). Ampicillin (50 μg/ml), apramycin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (40 μg/ml), and spectinomycin (100 μg/ml) were used for selection. Ethanologenic strains of *K. oxytoca* were maintained at 30°C on solid LB medium containing glucose (2%) and chloramphenicol (600 μg/ml).

Genetic methods. Standard methods were used for plasmid construction, analyses, and sequencing (1). The ribosome-binding site and promoterless coding region of *celY* were amplified by the PCR using pMH18 as the template with the

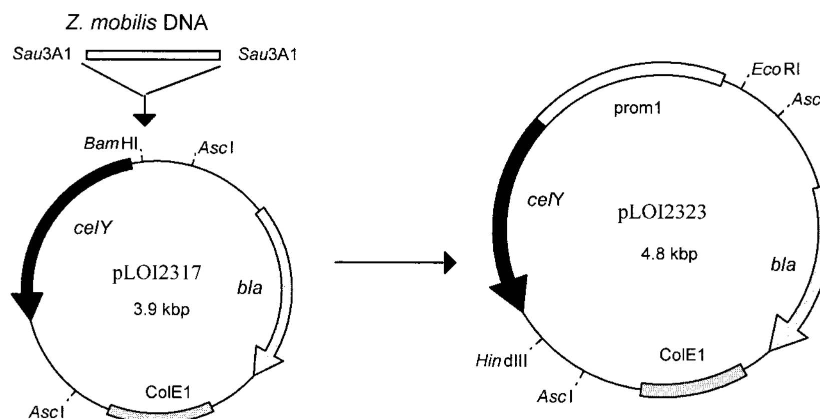

FIG. 1. Construction of promoter-probe vector for *celY. Sau*3A1 fragments of *Z. mobilis* chromosomal DNA were ligated into the *Bam*HI site of pLOI2317 to provide a strong, surrogate promoter for the *celY* coding region (solid segments). The *Z. mobilis* DNA fragment (promoter 1) is shown as an open segment. Replicons and antibiotic resistance genes are stippled; other vector DNA is shown as thin connecting lines. The arrows indicate the direction of transcription.

following primer pair: N terminus, 5′-CTGTTCCGTTACCAACAC-3′, and C terminus, 5′-GTGAATGGGATCACGAGT-3′. The *E. chrysanthemi out* genes (pCPP2006) were transferred by conjugation using pRK2013 for mobilization (42). Constructions were confirmed by sequencing using the dideoxy method and a LI-COR Model 4000-L DNA sequencer with fluorescent primers. The *E. chrysanthemi celY* and *celZ* genes were introduced into *K. oxytoca* P2 by electroporation using a Bio-Rad Gene Pulser. Recombinants were selected on solid medium containing kanamycin (50 mg/liter) as previously described (23, 42).

Primer extension analysis. Promoter regions were identified by mapping the transcriptional start sites using IRD41-labeled fluorescent primers within the coding regions (44): 5′-ACCATCAGCATCAACGCCCAACAACG-3′ for *celY* and 5′-GACTGGATGGTTATCCGAATAAGAGAGAGG-3′ for *celZ*. Extension products were dissolved in loading buffer and compared to parallel dideoxy sequences (42) using the LI-COR Model 4000-L DNA sequencer (LI-COR, Inc., Lincoln, Nebr.).

Enzyme assay. Endoglucanase activity was determined in vitro using 2% CMC as the substrate. Appropriate dilutions of cell-free culture broth (extracellular activity) or broth containing cells that had been disrupted by ultrasound (total activity) were assayed at 35°C in 50 mM citrate buffer (pH 5.2). Reactions were terminated by heating to 100°C for 10 min. Reducing sugars were measured using 3,5-dinitrosalicylic acid reagent with glucose as a standard (42, 44). Endoglucanase activity (CMCase) is expressed as micromoles of reducing sugar per minute (IU). Results are an average of two or more determinations.

Fermentation. SSF tests were conducted in unbaffled, 500-ml flasks containing 200 ml of broth. Flasks were fitted with a rubber stopper and vented with an 18-gauge needle. Fermentations were conducted at 35°C (120 rpm) in LB medium containing 10% Sigmacell 50 (crystalline cellulose). Inocula were grown for 12 h in LB medium containing 5% glucose. Cells were harvested by centrifugation and resuspended in LB medium. Each flask was inoculated with approximately 64 mg of cells (dry weight).

Materials and chemicals. Tryptone and yeast extract were products of Difco (Detroit, Mich.). Antibiotics, low-viscosity CMC, and Sigmacell 50 were obtained from the Sigma Chemical Company (St. Louis, Mo.). The IRD41-labeled fluorescent primers were purchased from LI-COR, Inc.

RESULTS

Construction of a promoter-probe vector for *celY*. Although the *celY* gene from *E. chrysanthemi* was cloned previously, it was poorly expressed in *E. coli* from the original plasmid (13). To increase expression, a promoter-probe vector was constructed as follows using *celY* as the reporter (Fig. 1). The promoterless *celY* coding region with a native ribosome-binding site (1.2 kbp) was amplified by PCR using pMH18 as the template and inserted into the topoisomerase vector, PCR2.1-TOPO. A clone oriented to express *celY* from the *lac* promoter was selected and designated pLOI2311 (5.2 kbp). An *Eco*RI fragment containing the promoterless *celY* gene was isolated from pLOI2311. The ends of this fragment were blunted using Klenow polymerase prior to ligation into the *Hin*cII site of pUC18. A clone oriented to express *celY* from the *lac* promoter was selected (3.9 kbp), and expression was confirmed using endoglucanase indicator plates (pLOI2316). The promoterless *celY* gene was isolated from pLOI2316 as a 1.2-kbp fragment using *Eco*RI and *Hin*dIII and inserted into the corresponding sites of pLOI2302 (pUC19 derivative) to reverse the direction of the *celY* gene. As expected, the resulting construct DH5α(pLOI2317) was inactive on endoglucanase indicator plates due to the lack of a promoter. To facilitate the insertion of DNA fragments containing promoter regions, plasmid pLOI2317 (3.9 kbp) contains a *Bam*HI site in the polylinker region, immediately upstream from the *celY* gene (Fig. 1).

Construction of plasmids with increased expression of *celY* in *E. coli* DH5α. *Sau*3A1 fragments of *Z. mobilis* chromosomal DNA were used to provide a heterologous promoter that would not be subject to native regulatory mechanisms in *K. oxytoca* or interfere with subsequent integration into the *K. oxytoca* chromosome (42). Fragments of 0.5 to 1.5 kbp were isolated and randomly ligated into the *Bam*HI site of pLOI2317 to generate a library of surrogate promoters (Fig. 1). Approximately 7,500 colonies were screened on endoglucanase indicator plates. One-third of the clones actively produced *celY*. The most active 100 colonies were identified by zone size, purified, and retested. The 30 clones with the largest zones of activity were grown overnight in LB medium and assayed for CMCase activity. The five most active clones are listed in Table 2 and exhibited approximately sevenfold-higher activity than the original clone, pMH18. Plasmid pLOI2323 was selected for further investigation.

The DNA serving as a surrogate promoter in pLOI2323 was sequenced in both directions (GenBank accession no. AF305919). Based on database comparisons, this fragment appears to be derived from two pieces, an 890-bp fragment from the *Z. mobilis* chromosome that corresponds to a previously sequenced region plus a stray 41-bp fragment from

TABLE 2. Expression of *celY* in *E. coli* DH5α using *Sau*3A1 fragments of *Z. mobilis* chromosomal DNA as surrogate promoters[a]

Plasmids expressing *celY* or *celZ*	Endoglucanase activity		
	Extracellular (IU/liter)	Total (IU/liter)	% Extra-cellular
pMH18 (native *celY* promoter)	151	184	82
pLOI2317 (promoterless *celY* vector)	0	0	0
celY expressed from surrogate promoters			
pLOI2318	1,123	1,257	89
pLOI2319	888	1,023	87
pLOI2320	1,023	1,056	97
pLOI2323	1,257	1,291	97
pLOI2342	1,224	1,257	97
pLOI2349 (*celZ*)	3,414	16,234	21

[a] All plasmids are pUC derivatives. Endoglucanase activity was measured using cultures grown at 37°C for approximately 16 h.

pLOI2311 which was inadvertently ligated during the construction of pLOI2316. This 41-bp fragment alone did not serve as a functional promoter since no activity was observed prior to addition of the *Z. mobilis* DNA fragment. A BLAST search of the translated *Z. mobilis* sequence did not reveal identity to known genes. Four putative sites of transcriptional initiation were identified in DH5α(pLOI2323) by primer extension analysis. Upstream sequences share some similarity with three different sigma factors: σ^{32}, σ^{38}, and σ^{70} (Table 3). Although the differences in intensity were <2-fold, the sequence resembling the σ^{32} (*rpoH*) consensus appeared to be the most intense (36, 37).

Construction of a vector for the integration *celY* and *celZ* into the chromosome of *K. oxytoca* P2. Plasmid pLOI2307 (7.2 kbp) was previously constructed and expressed *celZ* from a surrogate *Z. mobilis* promoter at high levels in recombinant *E. coli* DH5α (44) and *K. oxytoca* M5A1 (42). To facilitate subcloning of this hybrid *celZ* gene and promoter (4.5 kbp), an *Eco*RI linker was inserted into the T4 DNA polymerase-treated *Sph*I site of pLOI2307 to provide flanking *Eco*RI sites for convenient excision (pLOI2349). Prior to constructing a plasmid containing *celY* and *celZ*, a random 3-kbp fragment of *Eco*RI-digested *K. oxytoca* M5A1 chromosomal DNA was inserted into pLOI2323 containing *celY* (and surrogate promoter) to serve as a guide for homologous recombination (pLOI2348; 8 kbp). This 3-kbp M5A1 fragment was partially sequenced and appears to encode the complete M5A1 *glgP* gene. In pLOI2348 (8 kbp), flanking *Asc*I sites allowed the excision of a single 5.5-kbp fragment containing the M5A1 *glgP* gene, the *Z. mobilis* surrogate promoter, and *E. chrysanthemi celY*.

Figure 2 summarizes the construction of the *celY*, *celZ* integration vector from pLOI2349, pLOI2224, and pLOI2348. The recombinant *celY* and *celZ* genes containing surrogate promoters and the guide fragment were sequentially inserted into the core integration vector, pLOI2224 (23) using *E. coli* S17-1 as the host, to produce pLOI2352 (11.7 kbp). The 4.5-kbp *Eco*RI fragment from pLOI2349 containing *celZ* and promoter 2 was inserted into pLOI2224 using an *Eco*RI site to make pLOI2350 (6.4 kbp). The 5.5-kbp *Asc*I fragment from pLOI2348 containing *celY*, promoter 1, and the guide fragment for integration was inserted into the *Asc*I site of pLOI2350 to make pLOI2352 (11.7 kbp). The fragments containing *cel* genes were oriented such that expression from the surrogate promoters was divergent. The resulting vector contained an R6K replicon that does not function in DH5α or M5A1. The two FRT sites in pLOI2352 facilitate removal of the kanamycin gene and replicon after integration (23).

Functional integration of *celY* and *celZ* into the *K. oxytoca* P2 chromosome. Plasmid pLOI2352 was introduced into P2 by electroporation, followed by selection for kanamycin resistance. Approximately 150 colonies were recovered, and all were positive on endoglucanase indicator plates. Ten clones with the largest zones of activity were purified, grown in broth, and assayed for endoglucanase activity. These produced 5 to 6 IU of endoglucanase activity per ml. One clone was selected for further study and designated SZ12.

Due to the natural resistance of *K. oxytoca* to ampicillin, an additional antibiotic resistance marker (*tet*) was added to plasmid pFT-A containing the *flp* recombinase gene to facilitate selection. The tetracycline gene was isolated as a 1.4-kbp *Eco*RI-to-*Ava*I fragment from pBR322. After treatment with Klenow polymerase, this fragment was ligated in the Klenow-treated *Cla*I site of pFT-A to produce pLOI2353 (7.0 kbp). This plasmid encodes resistance to both ampicillin and tetracycline, the FLP recombinase (*flp*) under the control of the tetracycline promoter, and a temperature-conditional pSC101 replicon.

Plasmid pLOI2353 was transformed into SZ12 and plated at 30°C with selection for tetracycline resistance. The presence of tetracycline also induced *flp* expression, resulting in a deletion of the kanamycin gene and the R6K replicon from the chromosomally integrated pLOI2352. Of 307 tetracycline-resistant colonies tested, >99% retained expression of the endoglucanase genes and were sensitive to kanamycin. Clones were purified, grown in broth, and assayed for endoglucanase activ-

TABLE 3. Transcriptional initiation sites and putative promoter regions for the *celY* promoter in DH5α(pLOI2323)[a]

Sequence[b]	RNA start	Proposed σ factors	σ Factor consensus sequence	
			−35	−10
ATATTTTTGATTTTTCAAGAAAAGCCTGATATCTTCCAACA**T**CTT	**T**	σ^{70}	TTGACA	TATAAT
GATTTGATCCTCTAGAGTCAACCTGCTTGTTACTCGTGATCCC**A**T	**A**	σ^{70}	TTGACA	TATAAT
GAGTCAACCTGCTTGTTACTCGTGATCCCATTCACAAGGG**C**GAA	**C**	σ^{32}	CTTGAAA	CCCCAT
TTACTCGTGATCCCATTCACAAGGGCGAATTAATTCGCC**C**TT	**C**	σ^{38}	CCGCCT	TATACT

[a] Transcriptional starts for *celY* were identified by primer extension analysis. Four putative promoters are shown.
[b] Upstream regions with similarity to *E. coli* −35 (left) and −10 (right) regions are underlined. RNA start sites are shown in boldface.

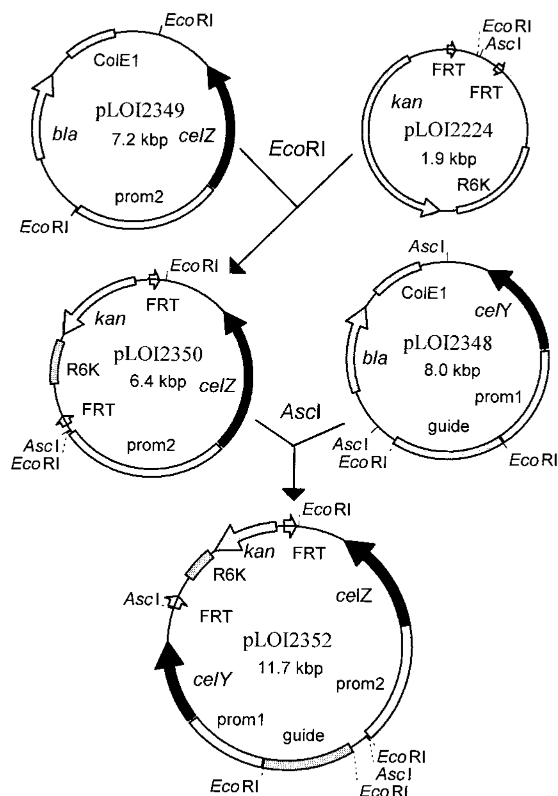

FIG. 2. Construction of pLOI2352 for the functional integration of *celY* and *celZ* into the chromosome of ethanologenic *K. oxytoca* P2. Coding regions for *celY* and *celZ* are shown as solid segments. Fragments of *Z. mobilis* DNA that serve as promoters (prom1 and prom2) are shown as open segments. The *K. oxytoca* DNA fragment which serves as a guide for chromosomal integration (guide) is stippled. Replicons and antibiotic resistance genes are stippled; other vector DNA is shown as a thin connecting line. The arrows on segments indicate the direction of transcription. The small open arrows represent the FRT sites which are recognized by the *flp* recombinase. FRT sequences are asymmetrical and are arranged to allow the deletion of plasmid DNA (replicon and selectable marker) after chromosomal integration.

ity. All were similar, and one was designated SZ21(pLOI2353). The helper plasmid was eliminated from SZ21 by overnight growth at 37°C.

Results from primer extension analysis of *celY* and *celZ* in SZ21 were similar to those observed in DH5α. A single major transcriptional start was identified for *celZ* that corresponded precisely to the most prominent start site in DH5α(pLOI2183) which contains the same promoter fragment (42, 44). DNA immediately upstream from this site resembles the recognition sequence for a σ^{70} promoter (36, 37). As observed with DH5α (pLOI2323) (Table 3), primer extension analysis of *celY* indicated the presence of multiple putative transcriptional start sites in SZ21. Although localized in the same regions as the start sites in DH5α(pLOI2323), all bands were of near equal intensities.

Construction of a *celZ* knockout mutation. To confirm the presence of a functional *celY* in SZ21, a knockout mutation of the chromosomally integrated *celZ* was constructed by double, homologous recombination using plasmid pLOI2357 (Fig. 3). Plasmid pUC19 was digested with *Sma*I and *Hin*dIII, treated with Klenow polymerase, and self-ligated to eliminate many of the polylinker sites (pLOI2354). The remaining unique *Eco*RI site was used to insert a 4.5-kbp *Eco*RI fragment containing the promoter and *celZ* gene from pLOI2349 to make pLOI2355 (7.2 kbp). The 1.8-kbp *Sma*I fragment from pHPΩ45aac containing the apramycin resistance gene (*aac*) was then ligated into the central region of *celZ*, replacing a small internal *Pst*I fragment (after treatment with T4 DNA polymerase) to produce pLOI2356 (9 kbp). The 6.3-kbp *Eco*RI fragment from this plasmid was isolated and inserted into the core integration vector, pLOI2224, to produce pLOI2357 (8.2 kbp). This plasmid contains a conditional R6K replicon and kanamycin resistance gene in addition to a *celZ* gene that is interrupted by an apramycin resistance gene.

Plasmid pLOI2357 was electroporated into SZ21 with selection for apramycin. Approximately 10% of the recombinants were apramycin resistant and kanamycin sensitive, indicating a double homologous recombination event. These clones exhibited low levels of endoglucanase production on indicator plates (Table 4). One was selected and designated SZ22. Loss of CelZ and retention of CelY in SZ22 was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using the Pharmacia Phast Gel system (data not shown).

It is interesting that cell clumping in liquid culture, typical of

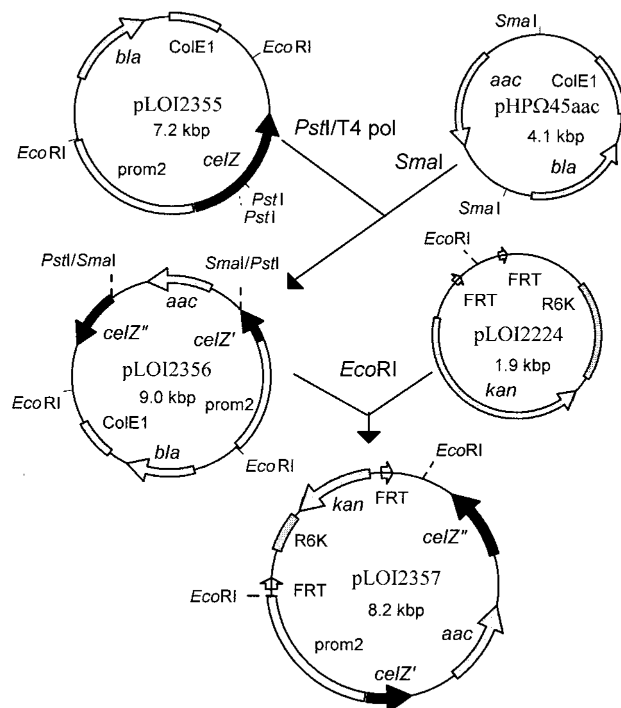

FIG. 3. Construction of pLOI2357 for the inactivation of *celZ* by double homologous recombination. Coding regions for *celY* are shown as solid segments. After disruption, the N-terminal and C-terminal segments are indicated by *celZ'* and *celZ"*, respectively. The fragment of *Z. mobilis* DNA that serves as a promoter (prom1) is shown as an open segment. Replicons and antibiotic resistance genes are stippled; other vector DNA is shown as a thin connecting line. The arrows on segments indicate the direction of transcription. The small open arrows represent the FRT sites which are recognized by the *flp* recombinase.

TABLE 4. Effect of *out* genes (pCPP2006) on endoglucanase production by derivatives of *K. oxytoca* P2

Strain or Spezyme additive	CMC zone[a] (mm)	OD_{550}[b]	CMCase activity[c]		
			Extra-cellular (IU/liter)	Total (IU/liter)	Secretion (%)
P2	0	10.5	0	0	0
SZ6	8.5	11.0	1,920	8,800	22
SZ21	6.7	11.0	1,620	7,800	21
SZ22	2.0	10.0	480	879	55
P2(pCPP2006)	0	10.0	0	0	0
SZ6(pCPP2006)	10.8	9.6	13,800	22,300	62
SZ21(pCPP2006)	11.5	10.2	20,100	26,900	75
SZ22(pCPP2006)	2.0	9.7	449	833	54
Spezyme CE (10 ml/liter)[d]				27,000	
Spezyme CP (10 ml/liter)[d]				33,400	

[a] Diameter of cleared zone on CMC indicator plates.
[b] Culture density after 24 h of incubation (30°C in LB medium containing 5% sorbitol).
[c] Endoglucanase activity was measured using cultures grown for 24 h.
[d] Dilution equivalent to the highest Spezyme level used in fermentation experiments (Table 5).

M5A1 and P2, was eliminated by the functional expression of *celZ* from integrated genes or from plasmids. Clumping was not affected by the functional expression of *celY* alone and provides a convenient marker for expression.

Effect of the *E. chrysanthemi out* genes (pCPP2006) on the extracellular secretion of CelY and CelZ in derivatives of *K. oxytoca* P2. Table 4 summarizes the endoglucanase activities exhibited by cellulolytic derivatives of ethanologenic *K. oxytoca* P2. Strain SZ6 (42) contains a chromosomally integrated hybrid *celZ* gene with the same promoter fragment used to construct SZ21. Despite the presence of two endoglucanase genes in SZ21, extracellular and total endoglucanase activities were 13% lower in this strain than in SZ6. Most of the endoglucanase activity produced by SZ21 can be attributed to *celZ*. SZ22, a *celZ* mutant of SZ21, expressed only 11% of the endoglucanase produced by the parent containing functional *celY* and *celZ* genes. In strains SZ6 and SZ21 containing a functional *celZ*, most of the endoglucanase activity (primarily CelZ) was cell associated. In strain SZ22, containing a functional *celY* alone, half of the endoglucanase activity was extracellular.

Previous studies have shown that the addition of the *out* genes (pCPP2006) to recombinant *E. coli* and *K. oxytoca* M5A1 harboring *celZ* caused a dramatic increase in the functional expression of *celZ* and in the fraction of CelZ that was secreted into the extracellular milieu (42, 44). The same effects were observed for ethanologenic *K. oxytoca* SZ21 containing *celZ* and *celY* (Table 4). Addition of the *out* genes to SZ22 (inactive *celZ*) had no effect on the functional expression of *celY* or the extent of CelY secretion. This *celZ* mutant, SZ22(pCPP2006), produced only 3% of the total endoglucanase activity (CelY) produced by SZ21(pCPP2006) containing functional *celY* and *celZ* genes. It is interesting that the secreted endoglucanase produced by SZ21(pCPP2006) with the *out* genes was substantially higher than the sum of the individual activities expressed from the same respective promoters in SZ6 (CelZ) and SZ22 (CelY), a finding consistent with synergism between these two enzymes (43). In this assay, synergy is estimated to be 1.4-fold the arithmetic sum of the individual activities [SZ6(pCPP2006) and SZ22(pCPP2006)] for the combination of extracellular enzymes produced by SZ21(pCPP2006).

Synergism between recombinant *E. chrysanthemi* endoglucanase (CelZ and CelY) and fungal cellulase (Spezyme CE) during the fermentation of cellulose to ethanol. SSF experiments were performed with flasks without pH control to evaluate the combined effects of fungal cellulase (Spezyme) and cellulase enzymes produced by the biocatalysts on ethanol production from Sigmacell 50, a highly crystalline substrate (Table 5). Although very low levels of ethanol were produced by all strains in the absence of Spezyme, strains SZ6(pCPP2006) and SZ21(pCPP2006) containing functional *celZ* genes produced higher levels of ethanol ($P \leq 0.05$) than strain SZ22 (pCPP2006) containing only a functional *celY* gene and strain P2(pCPP2006) lacking both endoglucanase genes. In the absence of both Spezyme and Sigmacell 50, all strains produced 0.22 g of ethanol per liter. The additional increment of ethanol produced by SZ6(pCPP2006) and SZ21(pCPP2006) during incubation with Sigmacell 50 is attributed to hydrolysis of the small fraction of amorphous cellulose in the substrate by CelZ (42–44). Digestion of amorphous cellulose by CelY alone produces saccharides that are too large to be transported and metabolized without further hydrolysis (43).

Spezyme CE and Spezyme CP contain a commercially optimized combination of endoglucanase, exoglucanase, and cellobiase activities (3, 5, 27, 28). Despite this optimization, Spezyme-supplemented fermentations with two of the endoglucanase-producing biocatalysts, SZ21(pCPP2006) and SZ22 (pCPP2006), produced significantly higher levels of ethanol than the control P2(pCPP2006), which lacks endoglucanase genes. The combinations of Spezyme and SZ21(pCPP2006)

TABLE 5. Ethanol production from Sigmacell 50 (100 g/liter)

Strain	Genencor Spezyme			Fermentation[a]	
	Type	Addition (ml/liter)	*n*	Ethanol concn ± SD (g/liter)[b]	% Control ± SD
P2(pCPP2006)	None	0	3	0.23 ± 0.01	100 ± 2.0
SZ6(pCPP2006)	None	0	3	0.28 ± 0.02*	124 ± 8.5
SZ21(pCPP2006)	None	0	3	0.26 ± 0.02*	116 ± 8.5
SZ22(pCPP2006)	None	0	3	0.24 ± 0.01	107 ± 1.0
P2(pCPP2006)	CE	5.0	6	13.7 ± 0.3	100 ± 2.0
SZ6(pCPP2006)	CE	5.0	6	13.8 ± 0.3	101 ± 2.4
SZ21(pCPP2006)	CE	5.0	6	16.0 ± 0.5**	117 ± 3.5
SZ22(pCPP2006)	CE	5.0	6	15.2 ± 0.3**	112 ± 1.5
P2(pCPP2006)	CE	10.0	6	20.7 ± 0.5	100 ± 2.1
SZ6(pCPP2006)	CE	10.0	6	21.2 ± 0.1	103.4 ± 0.4
SZ21(pCPP2006)	CE	10.0	6	24.6 ± 0.5**	121 ± 2.3
SZ22(pCPP2006)	CE	10.0	6	25.2 ± 1.1**	122 ± 5.0
P2(pCPP2006)	CP	5.0	6	15.2 ± 0.3	100 ± 1.7
SZ21(pCPP2006)	CP	5.0	6	17.8 ± 0.4**	116 ± 2.2
P2(pCPP2006)	CP	10.0	6	25.3 ± 0.7	100 ± 2.6
SZ21(pCPP2006)	CP	10.0	6	27.2 ± 0.3**	107 ± 1.2

[a] Cultures grown without added cellulose or Spezyme produced 0.22 ± 0.01 g of ethanol per liter. Spezyme contained approximately 100 FPU/ml. The addition of 5 or 10 ml of Spezyme corresponds to 5 and 10 FPU/g of cellulose, respectively.
[b] Student *t* test shows that there is significant difference in ethanol production compared to the respective P2 controls at each Spezyme dilution. A *P* value of ≤0.001 is indicated by two asterisks; a *P* value of ≤0.05 is indicated by one asterisk.

and SZ22(pCPP2006) were synergistic in terms of ethanol production, which was up to 20% higher than the sum of ethanol produced by each individually ($P \leq 0.001$). Synergy was observed for both dilutions of Spezyme CE and for Spezyme CP. This synergistic effect can be attributed primarily to CelY since this is the only endoglucanase produced by SZ22(pCPP2006). No synergy was observed for SZ6(pCPP2006) that produces CelZ alone.

DISCUSSION

Although the development of a recombinant biocatalyst that can supply all of the enzymes needed for the hydrolysis of cellulose and ethanol production has yet to be achieved, incremental progress has been made with both bacterial and yeast systems. Plasmid-based strains of *Z. mobilis* have been developed that express low levels (estimated to be <100 U/liter) of both an endoglucanase and a cellobiase from *Xanthomonas albilineans* (31). Higher levels of activity (600 to 1,000 U/liter) were produced by plasmid-based strains of *Z. mobilis* expressing an endoglucanase gene from *Cellulomonas uda* (24) and the *celZ* gene (CelZ) from *E. chrysanthemi* (7). Although the *C. uda* endoglucanase remained cell associated in recombinant *Z. mobilis*, up to 40% of the CelZ was released from the periplasm in late stationary phase without apparent lysis (7). No data were presented concerning the utilization of cellobiose or β-1-4-linked glucosides for growth and ethanol production by this strain.

Cellobiase and three different cellulase genes have been coexpressed from a single plasmid in recombinant *Saccharomyces cerevisiae* (33): cellobiase (*bgl1*) from *Endomyces fibuliger*, endoglucanase (*end1*) from *Butyrivibrio fibrisolvens*, cellobiohydrolase (*cbh1*) from *Phanerochaete chrysosporium*, and cellodextrinase (*cel1*) from *Ruminococcus flavefaciens*. Secretion of the gene products was facilitated by adding various leader sequences. *URA3*-based autoselection in an auxotrophic host was used to ensure plasmid retention. The resulting strain expressed higher levels of cellobiase activity than cellulase activity, sufficient to allow slow growth of the recombinant on cellobiose. An analogous construct expressing two cellulase genes (34) produced higher levels of endoglucanase (approximately 1,200 U/liter). No data were presented regarding ethanol production by these strains or growth on polymeric β-1-4-linked glucosides.

A recent study has shown that substantial levels of cellulases can be produced and secreted in recombinant *S. cerevisiae* by integrating up to 50 copies of the β-glucosidase gene from *Bacillus circulans* and an endo- or exocellulase gene from *Bacillus* sp. strain DO4 into multiple chromosomal δ sites of Ty1 retrotransposons (8, 9). Both genes contained added leader sequences to facilitate efficient secretion. In batch fermentations, 300 to 500 U of both activities were produced per liter. Recombinant strains achieved approximately 30% more cell mass and ethanol than the parent strain during growth on a mixture of glucose and soluble cellobiosides (9). A novel two-step process was developed using the recombinant in which high levels of cell mass and cellulase were initially produced aerobically from glucose (9). After supplementing this broth with additional commercial cellulase, crystalline cellulose substrate was added and fermented to ethanol. Although broth from the first step contained up to 700 filter paper units (FPU) of cellulase activity per liter (Whatman filter paper hydrolysis), no ethanol was produced without the further addition of commercial cellulase. Production of these enzymes by recombinant *S. cerevisiae* allowed a 40% reduction in the amount of commercial enzyme required, from 30 to 18 FPU/g of cellulose.

Our approach to reduce the requirement for fungal cellulase has been to functionally express genes encoding the *Z. mobilis* pathway in *K. oxytoca*, an abundant organism in pulp mill waste that has the natural ability to transport and metabolize cellobiose (20, 40). This recombinant can rapidly and efficiently produce 40 to 50 g/liter of ethanol from cellobiose (40). We have now added two endoglucanase genes from a closely related organism that macerates plant cell walls and tissues in nature, i.e., *E. chrysanthemi* (6, 10). High levels of endoglucanase activity were produced (over 20,000 U/liter), equivalent to 1% of the endoglucanase present in concentrated commercial cellulase products (Table 4 and reference 27). These activities are more than an order of magnitude higher than was previously reported for engineered strains of *S. cerevisiae* or *Z. mobilis*. To minimize some of the problems associated with the expression of heterologous genes in industrial strains, unregulated promoters were isolated from random fragments of *Z. mobilis* DNA using functional assays, hybrid genes were integrated into the chromosome to ensure stability, and the antibiotic resistance markers used in construction were deleted using the FLP recombinase system (23) to facilitate future genetic modifications. Approximately 95% of the CMCase activity produced by this strain was attributed to the *celZ* gene product, with the balance attributed to the *celY* product.

CelY and CelZ appear to be secreted by different mechanisms. Approximately 70% of the CelZ was secreted as an extracellular product when the *E. chrysanthemi out* genes were added on a plasmid (pCPP2006), consistent with a type II secretion system (16). Half of the CelY activity was secreted in the presence or absence of the *out* genes, consistent with a type IV secretion system (17). The integrated genes have been maintained without selection for over 6 months. However, pCPP2006 containing the *out* genes is less stable and requires antibiotic selection to ensure maintenance. In SSF experiments with added commercial cellulase, two of the new *K. oxytoca* recombinants, SZ21 and SZ22, produced more ethanol than the parent strain lacking *E. chrysanthemi* cellulases. Both of these strains also produced ethanol levels equivalent to the best yeast SSF experiments (9) using approximately one-third of the amount of added commercial cellulase (5 PFU/g of cellulose versus 18 FPU/g of cellulose for recombinant yeast [9]).

Previous studies have shown that endoglucanases CelY and CelZ function synergistically to affect the hydrolysis of CMC and amorphous cellulose (43). This synergy is based on differences in substrate range and mechanism of action. CelZ hydrolyzes cellotriose, cellotetraose, cellopentaose, amorphous cellulose, and CMC. CelY hydrolyzes polymeric substrates to products of approximately 10 glucosyl residues. Optimum synergy was observed with a high ratio of CelZ to CelY, similar to that produced in nature by *E. chrysanthemi* and by SZ21. Strains SZ6(pCPP2006) and SZ21(pCPP2006) expressing CelZ and CelY+CelZ, respectively, produced small amounts of ethanol from Sigmacell 50 even without the addition of fungal enzymes (Table 5). Both ethanol values were signifi-

cantly higher than strains grown in broth without added cellulose and strain P2(pCPP2006) with cellulose (no endoglucanase genes). This small amount of ethanol is attributed to the hydrolysis of a minor amorphous component of Sigmacell 50.

SSF experiments (Table 5) demonstrated that bacterial cellulases produced by ethanologenic *K. oxytoca* functioned synergistically with added commercial cellulase to increase ethanol production (7 to 22%) from crystalline cellulose (Sigmacell 50). Surprisingly, the beneficial effect was attributed almost exclusively to CelY, despite the fact that CelY activities were quite low in comparison to CelZ. Strain SZ22 expressing CelY was nearly equivalent to strain SZ21 expressing both activities. Strain SZ6 expressing only CelZ showed little increase in ethanol despite the production of over 20,000 U of endoglucanase activity per liter. Differences in the effectiveness of CelY and CelZ in combination with Spezyme may result from differences in substrate specificities and modes of action. Fungi such as *T. reesei* produce multiple endoglucanase activities which are presumed to function together with exoglucanases during the hydrolysis of crystalline cellulose (26, 32, 39). It is possible that the fungal activity resembling CelZ is not limiting hydrolysis in dilutions of Spezyme. In contrast to CelZ, CelY does not hydrolyze soluble cellobiosides but preferentially acts on longer-chain substrates, producing ends which can function as new sites for exoglucanase activity. Thus, endoglucanases with an activity profile resembling that for CelY may be limiting in fungal preparations supplied commercially.

In the absence of fungal cellulase additions, previous studies have shown that CelY and CelZ function synergistically to degrade amorphous cellulose (43). In nature, lignocellulosic substrates are depolymerized by mixtures of extracellular enzymes produced by consortia of fungi and bacteria. Thus, it is not surprising that a mixture *E. chrysanthemi* enzymes and *T. reesei* enzymes can improve the digestion of lignocellulosic substrates during bioconversion to ethanol.

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