Addition of Genes for Cellobiase and Pectinolytic Activity in *Escherichia coli* for Fuel Ethanol Production from Pectin-Rich Lignocellulosic Biomass[▽]

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Ethanologenic *Escherichia coli* strain KO11 was sequentially engineered to contain the *Klebsiella oxytoca* cellobiose phosphotransferase genes (*casAB*) as well as a pectate lyase (*pelE*) from *Erwinia chrysanthemi*, yielding strains LY40A (*casAB*) and JP07 (*casAB pelE*), respectively. To obtain an effective secretion of PelE, the Sec-dependent pathway *out* genes from *E. chrysanthemi* were provided on a cosmid to strain JP07 to construct strain JP07C. Finally, oligogalacturonide lyase (*ogl*) from *E. chrysanthemi* was added to produce strain JP08C. *E. coli* strains LY40A, JP07, JP07C, and JP08C possessed significant cellobiase activity in cell lysates, while only strains JP07C and JP08C demonstrated extracellular pectate lyase activity. Fermentations conducted by using a mixture of pure sugars representative of the composition of sugar beet pulp (SBP) showed that strains LY40A, JP07, JP07C, and JP08C were able to ferment cellobiose, resulting in increased ethanol production from 15 to 45% in comparison to that of KO11. Fermentations with SBP at very low fungal enzyme loads during saccharification revealed significantly higher levels of ethanol production for LY40A, JP07C, and JP08C than for KO11. JP07C ethanol yields were not considerably higher than those of LY40A; however, oligogalacturonide polymerization studies showed an increased breakdown of biomass to small-chain (degree of polymerization, ≤6) oligogalacturonides. JP08C achieved a further breakdown of polygalacturonate to monomeric sugars, resulting in a 164% increase in ethanol yields compared to those of KO11. The addition of commercial pectin methylesterase (PME) further increased JP08C ethanol production compared to that of LY40A by demethylating the pectin for enzymatic attack by pectin-degrading enzymes.

Ethanol is the most prevalent renewable fuel, with the United States producing an estimated 13.2 billion gallons in 2010 (http://www.ethanolrfa.org/). Currently, the majority of ethanol is produced from corn; however, limited supply will force ethanol production from other sources of biomass, of which the United States produces 1 billion tons annually—enough to produce 80 billion gallons of renewable fuel (11). Moreover, the use of waste biomass for fuel production positively affects greenhouse gases and carbon debt without causing land use changes (10, 27).

Unlike corn grain, where the major component is starch, other sources of biomass are composed of 40 to 50% cellulose, 25 to 35% hemicellulose, and 15 to 20% lignin on a dry weight basis (11). In some biomass types, such as sugar beet pulp (SBP) and citrus peel, pectin can also comprise a significant portion of the lignocellulose structure (7, 12, 13, 20). This highly complex structure has necessitated the development of sequential processes for the production of fuel ethanol from lignocellulosic substrates, including thermochemical and/or mechanical pretreatment to allow enzymatic access, enzymatic degradation to reduce substrates to fermentable sugars, and,

finally, the fermentation of those sugars by microorganisms. Biomass from residues, like SBP, does not require thermochemical or mechanical pretreatments because it is already partially processed. However, for the entire process to become economically feasible, an optimization of the enzymatic degradation of lignocellulose to fermentable sugars is required, as is the development of ethanologens capable of fermenting those sugars (9).

Most ethanol fermentations in the United States today use the yeast *Saccharomyces cerevisiae* to convert starch glucose into ethanol and CO_2; however, lignocellulosic biomass contains many hexose and pentose sugars that *S. cerevisiae* is unable to ferment (24). Thus, *Escherichia coli*, which is capable of using these hexoses and pentoses, was engineered as a biocatalyst for ethanol production by the integration of the pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adhB*) genes from *Zymomonas mobilis* into the chromosome of *E. coli* to generate strain KO11 (23). Although *E. coli* strain KO11 is capable of producing high concentrations of ethanol, the microorganism still requires the addition of costly enzymes (including cellulases, β-glucosidase, hemicellulases, and pectinases) to degrade the biomass into fermentable monomeric sugars. *E. coli* is also unable to ferment cellobiose (a glucose dimer formed from cellulose during enzymatic saccharification), which can build up and inhibit cellulose degradation (15).

In this study we report the improvement of *E. coli* strain KO11 for the partial saccharification and cofermentation of

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TABLE 1. *E. coli* strains and plasmids used in this study

Strain, plasmid, or oligonucleotide	Relevant characteristic(s) or sequence[a]	Source or reference
Escherichia coli strains		
KO11	pdc^+ $adhB^+$; Cm^r	23
LY40A	KO11 with *casAB*; Cm^r	This study
JP07	LY40A with *pelE*; Cm^r Ap^r	This study
JP07C	JP07 with pCPP2006; Cm^r Ap^r Spc^r	This study
JP08C	JP07C with pTOGL; Cm^r Ap^r Spc^r Kn^r	This study
Plasmids		
pLOI1998	*casR'AB*	17
pST76-K	Cm^r	25
pLOI2707	pST76-K derivative; *lacY::casAB::lacA*	This study
pLOI2708	pLOI2707 derivative; *lacY::casAB::lacA*; *Zm* promoter	This study
pPEL748	*pelE*	16
pLOI2090	*pelE*; Ap^r	This study
pCPP2006	*out* genes; Spc^r	14
pDMA160	Mini-Tn7; *mob*; Em^r Kn^r	E. V. Stabb
pEDH24	pDMA160 derivative, consensus promoter	This study
pEDH25	pEDH24 derivative; *pelE*; Ap^r	This study
pUXBF13	R6K ori; *tns* genes; Ap^r	2
pEVS104	pRK2013 derivative; conjugal *tra* and *trb* genes	29
pTOGL	*ogl*; Kn^r	This study
Oligonucleotides		
LPY1	5′-GAGATCTTAAGGAAAAACAGCATGGA-3′	This study
LPY2	5′-ATAGCCGGCGTCCAGAAT-3′	This study
LacYF	5′-TTGCTCTTCCATGTACTATTTAAAAAACACAAAC-3′	Sigma Genosys
LacYR	5′-TTGCTCTTCGTTAAGCGACTTCATTCACCTGAC-3′	Sigma Genosys
LacAF	5′-TTGCTCTTCCATGCCAATGACCGAAGAATAAGAG-3′	Sigma Genosys
LacAR	5′-TTGCTCTTCGTTAAACTGACGATTCAACTTTATA-3′	Sigma Genosys
LacZ	5′-GGTGAAGTGCCTCTGGATGT-3′	This study
CasA	5′-CGCCTACCCGAGTGAGAATA-3′	This study
CasB	5′-GCAAAGCGGAAGTCTACCAG-3′	This study
CynX	5′-ATGCCTTCGGTGATTAAACG-3′	This study
Promoter	5′-CTAGTTGACATGATAGAAGCACTCTACTATATT-3′	E. V. Stabb
	3′-AACTGTACTATCTTCGTGAGATGATATAACTAG-5′	
EDH160	5′-TGCTCAACGGGAATCCTGCTCT-3′	This study
EDH2090F	5′-GCGCATGGGCCCCACACAGGAAACAGCTATGACC-3′	This study
EDH2090R	5′-GCATGCGGGCCCGTTACCAATGCTTAATCAGTGAGG-3′	This study
EDHPelB	5′-TCAGCACGAACACGAACCGTCTTA-3′	This study
EDHPelE	5′-TGTGCTGCAAGGCGATTAAGTTGG-3′	This study
OglF	5′-GCGCACAGCTGTTGACATGATAGAA-3′	This study
OglR	5′-GATCGCATATGGGCACGGTTGCAGG-3′	This study

[a] Cm, chloramphenicol; Ap, ampicillin; Spc, spectinomycin; Kn, kanamycin; Em, erythromycin.

lignocellulosic biomass for ethanol production, working toward a strain capable of achieving consolidated bioprocessing, where the saccharification and fermentation of biomass are combined into a single step. Furthermore, this approach can also be used to decrease commercial enzyme loading during the production of other biofuels and biochemicals.

SBP, a lignocellulosic biomass that can contain as much as 25% pectin, 24% cellulose, and 36% hemicellulose (6), was used to test the efficacies of the engineered strains. The breakdown of cellobiose, and pectin (a polymer composed primarily of galacturonic acid), which interacts with cellulose, hemicellulose, and lignin, was targeted. The addition of these degrading capabilities to *E. coli* strain KO11 through genetic engineering decreases the amount of commercial enzymes that must be used during partial saccharification.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains, plasmids, and oligonucleotides used in this study are listed in Tables 1 and 2. *E. coli* strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with 2% (wt/vol) glucose for etha-

TABLE 2. Summary of phenotypes of bioengineered strains of *E. coli* used in this study

E. coli strain	Description	Phenotype added	Reference
KO11	*Z. mobilis pet* operon Δ*frd*	Increased ethanol production	22
LY40A	*K. oxytoca casAB* operon	Cellobiose uptake and metabolism	This study
JP07	*E. chrysanthemi pelE*	Degradation of polygalacturonic acid to oligogalacturonides	This study
JP07C	*E. chrysanthemi out* operon	Secretion of PelE	This study
JP08C	*E. chrysanthemi ogl*	Degradation of oligogalacturonides to 4,5-unsaturated galacturonate and galacturonate	This study

nologenic strains. Antibiotics were used at the following concentrations unless otherwise stated: chloramphenicol (Cm) at 40 mg/liter, ampicillin (Ap) at 50 mg/liter, kanamycin (Kn) at 40 mg/liter, erythromycin (Em) at 150 mg/liter, and spectinomycin (Spc) at 50 mg/liter. For enzyme assays, ethanologenic *E. coli* cells were grown in minimal medium (MM) (1) [0.02 M (NH$_4$)$_2$SO$_4$, 0.01 M sodium citrate, 8 mM Na$_2$PO$_4$, 2 mM MgSO$_4$ · 7H$_2$O, 1 mM KCl, 30 nM FeSO$_4$ · 7H$_2$O] with 0.5% (wt/vol) glucose and either 0.5% (wt/vol) polygalacturonic acid or cellobiose. All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs (Ipswich, MA). DNA sequencing reactions were performed at the Sequencing and Synthesis Facility at the University of Georgia (Athens, GA).

Genetic procedures and recombinant techniques. Standard methods were employed to construct plasmids and transfer DNA (26). PCR was performed by using either Platinum *Taq* (Invitrogen, Carlsbad, CA) or a Phusion high-fidelity DNA polymerase kit (New England BioLabs, Ipswich, MA) according to the manufacturer's recommendations for reaction programs.

Chromosomal insertion of *K. oxytoca casAB* genes in *E. coli* strain KO11. The *casAB* genes from *Klebsiella oxytoca* (17) were chromosomally integrated into *E. coli* strain KO11 (23) between the *lacY* and *lacA* genes after the addition of a strong surrogate promoter (32). The DNA fragment constructed for integration was deposited in the GenBank database (accession no. EU848570). Primers used for construction are listed in Table 1 (LPY1, LPY2, LacYF, LacYR, LacAF, and LacAR).

For chromosomal insertion, the *casAB* genes were amplified from pLOI1998 (17) and ultimately engineered into pST76-K to produce pLOI2707, with *lacY* and *lacA* flanking the *casAB* genes. A surrogate promoter from *Z. mobilis* genomic DNA was placed upstream of *casAB*, as previously described (32), to produce pLOI1708. After the electroporation of *E. coli* strain KO11 with pLOI2708, *casAB* recombinants were selected by growing the culture on M9 minimal medium with 5% (wt/vol) cellobiose and 40 mg/liter Cm. Cells were later screened for a red colony color on MacConkey agar containing 2% (wt/vol) cellobiose and selected for high levels of expression of *casAB* and *Z. mobilis pdc* and *adhB* on LB agar containing 2% (wt/vol) glucose and 600 mg/liter Cm. An active strain was selected and designated *E. coli* strain LY40A. Integration by double homologous recombination was verified by using primers (LacZ, CasA, CasB, and CynX) that included the *lacZ* and *cynX* genes flanking the genomic insertion site.

Chromosomal insertion of *E. chrysanthemi pelE* and addition of the *out* cosmid in *E. coli* strain LY40A. A double-stranded *E. coli* consensus promoter sequence was constructed by the heating of two complementary single-stranded DNA oligonucleotides with overhangs at 98°C for 10 min (Table 1). The oligonucleotides were cooled to room temperature, cloned into the AvrII site of pDMA160 to make pEDH24, and transformed into *E. coli* strain BW23474 by using a standard heat shock protocol (26). The directionality of the promoter was confirmed via DNA sequencing using primer EDH160. The BsrBI fragment carrying the *pelE* gene from pPEL748 (16) was inserted into the SmaI-PstI site of pUC18, which was made blunt by using DNA polymerase I, to generate pLOI2090. The *pelE* and *bla* genes were amplified from pLOI2090 via PCR using primers EDH2090F and EDH2090R with engineered ApaI sites and cloned into pEDH24 at the ApaI site. Subsequent clones were investigated for the directionality of the *pelE-bla* fragment, and the plasmid with *pelE-bla* in the correct orientation with regard to the consensus promoter was named pEDH25. A triparental mating of *E. coli* strain LY40A with *E. coli* strain BW23474/pUXBF13 (2), *E. coli* strain BW23473/pEVS104 (29), and *E. coli* strain BW23474/pEDH25 was performed to insert the mini-Tn7 transposon with *pelE* and *bla* into the chromosome, yielding *E. coli* strain JP07 after selection on LB agar containing Cm and Ap. Strain verification was accomplished by sequence analyses using primers EDHPelB and EDHPelE. Cosmid pCPP2006, containing the *out* genes from *Erwinia chrysanthemi* for the secretion of *pelE* (14), was transformed into *E. coli* strain JP07 according to a standard heat shock protocol (26), giving *E. coli* strain JP07C.

Construction of *E. coli* strain JP08C. To construct pTOGL, the oligogalacturonide lyase gene, *ogl*, was PCR amplified from *Erwinia chrysanthemi* 3937 by using primers OglF and OglR and cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). OglF contained the consensus *E. coli* promoter sequence employed in previous experiments. pTOGL was then transformed into JP07C via heat shock (26), giving *E. coli* strain JP08C.

Cellobiase assay. Assays for cellobiase activity were performed essentially as described previously (22). Briefly, ethanologenic *E. coli* cells were grown in LB medium with 2% (wt/vol) cellobiose for 24 h at 37°C with shaking. Cells were harvested via centrifugation at 10,000 × *g* for 10 min and lysed by sonication in 50 mM phosphate buffer (pH 7.2). Lysates were assayed for 15 min in 50 mM

phosphate buffer with 2 mM *p*-nitrophenyl-β-D-1,4-glucopyranoside (PNPG). The reaction was terminated by the addition of 1 M Na$_2$CO$_3$ to the mixture, and the *p*-nitrophenol content was measured at 410 nm. Units are defined as μmol product formed per min per ml. Protein assays were performed on the supernatant according to the Bradford method (3), and enzyme activity is reported as specific activity in U/mg protein. Data represent the means of three separate experiments.

Pectate lyase assay. Assays for pectate lyase activity were performed as described previously (5). Briefly, ethanologenic *E. coli* cells were grown in MM with 0.5% (wt/vol) glucose and 0.5% (wt/vol) polygalacturonic acid for 48 h at 37°C with shaking. The culture supernatant was harvested via centrifugation at 10,000 × *g* for 10 min. The supernatant was assayed by rapid mixing with the substrate (60 mM Tris-HCl [pH 7.2], 0.6 mM CaCl$_2$, 0.24% [wt/vol] polygalacturonic acid), both previously equilibrated to 37°C, and monitoring the formation of 4,5-unsaturated products at 232 nm for 5 min with a linear rate of reaction for at least 30 s. Units are defined as μmol product formed per min per ml. Protein assays were performed on the supernatant by the Bradford method (3), and enzyme activity is reported as specific activity in U/mg protein. Data represent the means of at least four separate experiments.

Model sugar fermentations and analysis of ethanol production and sugar utilization. Fermentations were performed essentially as described previously (8). Model sugars for fermentation consisted of a mixture of pure sugars representative of the composition of SBP, with roughly equal parts cellulose, hemicellulose, and pectin (20). The model sugar contained 17% (wt/vol) glucose, 17% (wt/vol) cellobiose (both representing cellulose), 33% (wt/vol) arabinose, and 33% (wt/vol) galacturonic acid (representing hemicellulose and pectin, respectively). Fermentations were conducted with 500-ml bioreactors placed into a water bath at 35°C and mixed with magnetic stirrers. The pH was adjusted to 6.7 by use of a Jenco (San Diego, CA) 3671 pH controller, 10 M KOH, and 6 N HCl as necessary. Fermentation mixtures contained 10% (wt/vol) model sugars, 50 ml 2× LB liquid medium, and appropriate antibiotics and were brought up to a final volume of 100 ml with sterilized water. Fermentation mixtures were inoculated with 10^8 to 10^9 cells collected via centrifugation (10,000 × *g* for 10 min) from cultures of *E. coli* strains KO11, LY40A, JP07, JP07C, and JP08C grown overnight (18 h) (the inoculation concentration did not affect the results [data not shown]). Fermentations continued for 120 h with samples collected at specific time intervals.

To quantify ethanol production, gas chromatography (GC) was performed by using a Shimazdu (Columbia, MD) GC-8A instrument (8). Fermentation supernatant samples were filtered with a 0.22-μm filter prior to analysis. Ethanol concentrations were normalized based on control fermentations conducted without pure sugar.

Sugar utilization was quantified by use of high-performance liquid chromatography with refractive index detection (HPLC-RID). Approximately 200 μl of sample was removed after filtration and transferred into an autosampler vial (screw top, fixed insert; Agilent) before HPLC-RID analysis. The analysis of glucose, cellobiose, arabinose, and galacturonic acid was performed by using a Shimadzu Prominence LC-20AT liquid chromatographic system (Shimadzu Scientific Instruments, Columbia, MD). Chromatographic separation was achieved by using a Bio-Rad (Hercules, CA) Aminex HPX-87H 300-mm by 7.8-mm column with a Bio-Rad Cation H guard column. An isocratic run at the flow rate of 0.6 ml/min was performed with 5 mM H$_2$SO$_4$ solution as a mobile phase with a 60°C column temperature.

SBP (5%, wt/vol) fermentations and analysis of ethanol production and sugar utilization. Fermentations were performed essentially as described previously (8). The SBP dry weight was calculated by using a Denver Instrument (Denver, CO) IR 35 moisture analyzer. Each fermentation mixture contained 5% (wt/vol) SBP (based on dry weight), 100 ml 2× LB medium, and water to a final volume of 200 ml. Fermentation mixtures were blended at 10,000 rpm for 10 s by using a Grindomix 200 food processor (Retsch, Inc., Newtown, PA) and then autoclaved in a 500-ml bioreactor prior to inoculation; blending was necessary to reduce the particle size when very low fungal enzyme loads were used. Bioreactors were placed into a water bath at 45°C and mixed with magnetic stirrers. The pH was adjusted to 4.5. Partial saccharification was conducted for 24 h by using cellulase (Spezyme CP; Genencor, Copenhagen, Denmark) and pectinase (Pectinex P2736) from *Aspergillus niger* (Novozymes, Franklinton, NC) at concentrations of 0.5 filter paper units (FPU) of cellulase/g (dry weight) SBP and 4 polygalacturonase units (PGU) of pectinase/g (dry weight) SBP. After 24 h, the pH was increased to 6.7 by using 10 M KOH, and the temperature was decreased to 35°C; both conditions were maintained throughout fermentation. Appropriate antibiotics were added to each bioreactor, and they were inoculated with 10^8 cells. Fermentations continued for 120 h with samples collected every 24 h.

Ethanol was quantified by using GC (8). Ethanol concentrations were normal-

TABLE 3. Cellobiase and extracellular pectate lyase specific
activities for *E. coli* strain KO11 and derivative strains[a]

E. coli strain	Mean sp act (IU/mg protein) ± SD	
	Cellobiase	Pectate lyase
KO11	0	0
LY40A	7.3 ± 0.4	0
JP07	6.7 ± 0.2	0.2 ± 0.3
JP07C	7.5 ± 0.4	18.9 ± 1.2
JP08C	6.5 ± 0.3	49.3 ± 0.9

[a] $n = 3$.

ized to zero to correct for ethanol added from antibiotic stocks. Reducing sugar
analysis was performed by using the dinitrosalicylic acid assay method (21).

Examination of oligogalacturonides. To quantify oligogalacturonides with a
degree of polymerization (dp) of less than 6, the fermentation supernatant was
diluted 1:3 in water, and ethanol was added to a final concentration of 11%
(vol/vol). The solution was incubated with agitation for 16 h at 4°C and then
centrifuged at 7,500 × *g* for 15 min. The supernatant was diluted, and the
absorbance at 235 nm was measured (28). The absorbance of the fermentation
supernatant preparation of *E. coli* strain KO11 at 72 h was used as the baseline.
Data represent the averages of data from two experiments.

**SBP (10%, wt/vol) fermentations with pectin methylesterase and analysis of
ethanol production and sugar utilization.** Fermentations were conducted as
described above, with the key changes noted. A higher concentration of SBP
(10%, wt/vol) was added to the fermentation mixtures. The 24-h partial sacchar-
ification was conducted with 10 FPU of cellulase/g (dry weight) SBP. A set of
fermentation mixtures contained 1.4 pectin methylesterase (PME) units (PMU)
of purified PME/g (dry weight) SBP (Rapidase PEP purified from *A. niger*; DSM
Food Specialties B.V., the Netherlands) during the 24-h partial saccharification.
The purified PME had no cellulolytic or pectinolytic activities (data not shown).
Ethanol and methanol were quantified by using GC, and sugar utilization was
analyzed by HPLC.

RESULTS AND DISCUSSION

Construction of *E. coli* strain LY40A. Previous research
identified cellobiose phosphoenolpyruvate-dependent phos-
photransferase genes (*casAB*) from *K. oxytoca* that allowed the
rapid growth of *E. coli* strain DH5α with cellobiose as the sole
carbon source. The *casAB* operon encodes a cellobiose per-
mease and a phospho-β-glucosidase, which uptake cellobiose
and hydrolyze the dimer to glucose, respectively (17). When a
plasmid containing *casAB* was transferred into *E. coli* strain
KO11, expression was poor; previously reported mutational
studies of this plasmid in KO11 suggested that the native pro-
moter was more tightly controlled in this strain (22). In the
present study, to create a stable cellobiose-fermenting strain
from *E. coli* strain KO11, the *casAB* genes were inserted into
the chromosome with a strong surrogate promoter from *Z.
mobilis*.

The *casAB* recombinants were screened for the ability to
ferment cellobiose and selected for high levels of expression of
Z. mobilis pdc and *adhB*. A single strain was selected and
named *E. coli* strain LY40A. The recombinant strain demon-
strated cellobiase activity, while the parent strain lacked cello-
biase activity (Table 3). The ability of LY40A to degrade cel-
lobiose increases the amount of fermentable sugars and
decreases the inhibition of cellulose degradation caused by
cellobiose end product inhibition (15).

While the chromosomal insertion of *casAB* improves *E. coli*
strain KO11 by enabling the breakdown of cellobiose without
supplemental cellobiase (which is present in low concentra-

tions in cellulase mixtures), the complexity of lignocellulosic
substrates necessitates other types of enzymes for breakdown.
Further engineering of *E. coli* strain LY40A with additional
enzymes should allow the decreased use of exogenous en-
zymes.

Construction of *E. coli* strains JP07, JP07C, and JP08C. In
lignocellulosic substrates, pectin interacts with lignin, hemicel-
lulose, and cellulose; the degradation of pectin is necessary for
the disintegration of these cell wall components. Therefore, a
pectate lyase, which cleaves the polygalacturonate repeating
chains of pectin, producing short-chain oligogalacturonides,
was engineered into *E. coli* strain LY40A with a surrogate
promoter.

The chromosomal integration of *pelE* was conducted as de-
scribed above. Sequencing was conducted to verify the pro-
moter directionality. pEDH25 was conjugated into *E. coli*
strain LY40A, and *pelE* was transposed into the *att*Tn7 site,
resulting in *E. coli* strain JP07.

Since polygalacturonate is too large to enter the cell, PelE
must be secreted. Previous studies with *E. chrysanthemi* pectate
lyases showed that a Sec-dependent pathway, encoded by the
out genes, was necessary for the secretion of these enzymes
(14). A cosmid with a 40-kb fragment of the *E. chrysanthemi*
genome containing the *out* genes, pCPP2006 (14), was electro-
porated into strain *E. coli* JP07 to give strain *E. coli* JP07C. The
oligogalacturonide lyase of *E. chrysanthemi* (4) was trans-
formed into *E. coli* strain JP07C to give strain JP08C, where *ogl*
is maintained on plasmid pTOGL. Ogl degrades the short-
chain oligogalacturonide products of PelE into monomeric
fermentable sugars. The engineering of KO11 into JP08C is
summarized in Table 2.

Enzyme assays with *p*-nitrophenyl-β-D-1,4-glucopyranoside
were performed to ensure that the cellobiase activities in *E.
coli* strains JP07, JP07C, and JP08C were not affected by the
addition of *pelE* (Table 3). Subsequently, assays were per-
formed with polygalacturonic acid to demonstrate extracellular
pectate lyase activity (Table 3). *E. coli* strains KO11 and
LY40A demonstrated no activity. The JP07 pectate lyase ac-
tivity varied greatly, reaching 0.5 U/mg protein at most; the
occasional presence of activity could be attributed to cell lysis.
E. coli strain JP07C exhibited 18.9 U/mg protein of extracel-
lular pectate lyase activity, demonstrating the functionality of
the *out* gene secretion system. Ogl from JP08C cleaves polyga-
lacturonate, producing 4,5-unsaturated galacturonate. Enzyme
assays with JP08C demonstrated a large increase in the level of
production of 4,5-unsaturated products, indicating oligogalac-
turonide activity in addition to pectate lyase activity (Table 3).

**Fermentation of model sugars to examine the utilization of
sugars characteristic of SBP fermentations.** The fermentations
of model sugars confirmed that the engineered microorgan-
isms maintained the fermentation capabilities of the parent
strain, *E. coli* strain KO11.

E. coli strain LY40A produced 45% more ethanol from the
model sugars than did KO11 (Fig. 1A) due to the capability of
LY40A to ferment cellobiose (Fig. 1B and C). JP07, JP07C,
and JP08C produced concentrations of ethanol that were
higher than those produced by KO11 (approximately a 15%
increase) but lower than those produced by LY40A (Fig. 1A).
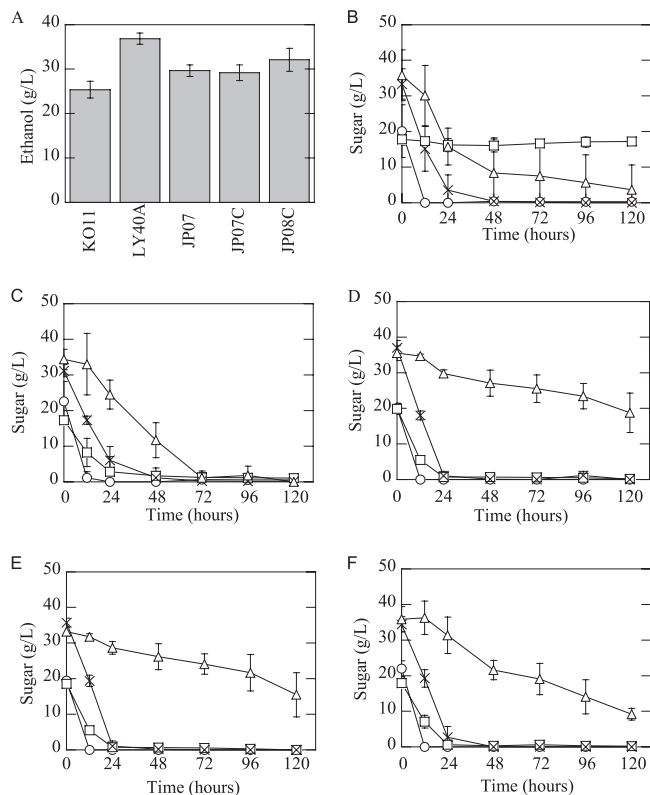All three strains maintained the ability to use cellobiose but did

FIG. 1. (A) Ethanol production at 72 h from 10% (wt/vol) model sugar fermentations for *E. coli* strains KO11, LY40A, JP07, JP07C, and JP08. (B to F) Sugar utilization (standard error; $n \geq 3$) (○, glucose; □, cellobiose; ×, arabinose; △, galacturonic acid) from 10% (wt/vol) model sugar fermentations for KO11 (B), LY40A (C), JP07 (D), JP07C (E), and JP08C (F).
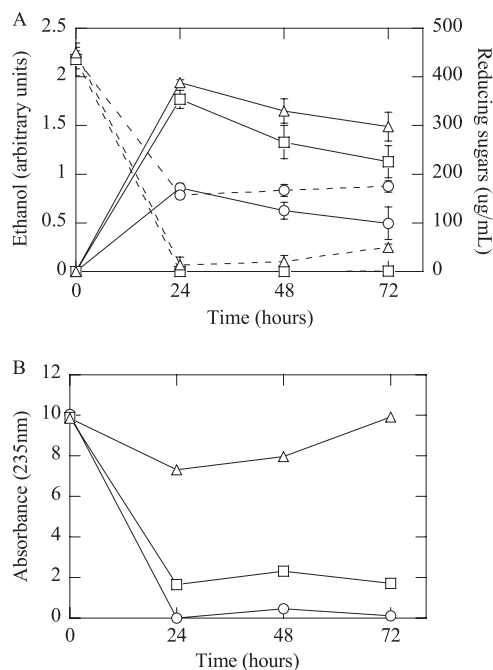

FIG. 2. (A) Ethanol production (solid lines) and reducing sugars (dashed lines) from 5% (wt/vol) SBP fermentations for *E. coli* strains KO11, LY40A, and JP07C (standard error; $n = 3$). (B) Absorbances at 235 nm of oligogalacturonides with a dp of ≤6 from the above-mentioned SBP fermentations (data represent average data from two experiments). ○, KO11; □, LY40A; △, JP07C.

not ferment all of the galacturonic acid present in the model sugars (Fig. 1D, E, and F).

Acetic acid, primarily a product of galacturonic acid fermentation, is known to be inhibitory to recombinant *E. coli* strains (18). JP07, JP07C, and JP08C may not have completely fermented galacturonic acid due to the presence of high concentrations of acetic acid produced during fermentation (data not shown). The inhibitory effect of acetic acid is pH dependent, decreasing with increasing pH (18). Model sugar fermentations (10%, wt/vol) conducted with a 0.1 M potassium phosphate buffer (pH 6.7) allowed JP07 and JP07C to ferment all of the galacturonic acid, although higher concentrations of acetic acid resulted instead of increased ethanol production (data not shown). In fermentations conducted with potassium phosphate buffer, the pH did not decrease as much as it did during unbuffered fermentations. These results further support the hypothesis that acetic acid caused a decrease in galacturonic acid use in model sugar fermentation mixtures inoculated with JP07, JP07C, and JP08C.

Comparison of *E. coli* strains KO11, LY40A, and JP07C in SBP fermentations. To demonstrate the utility of engineered *E. coli* strains LY40A and JP07C, SBP fermentations were performed with very low fungal enzyme loads and substrate concentrations (Fig. 2A). A total of 0.5 FPU cellulase/g (dry weight) SBP and 4 PGU pectinase/g (dry weight) SBP were added to the fermentation mixtures. Low concentrations of exogenous enzymes caused only a small portion of the lignocellulose structure to be degraded; therefore, most sugars available for conversion into ethanol remained in a polymeric form, leading to low ethanol yields. Higher enzyme concentrations typically used for maximum ethanol production (10 to 15 FPU cellulase/g [dry weight] SBP and 60 to 120 PGU pectinase/g [dry weight] SBP) would result in higher ethanol yields; however, this protocol allows the activities that the engineered *E. coli* contributes to the fermentation profile to be observed. These fermentations have not been optimized; therefore, the level of ethanol production is low. However, the results demonstrate the value of the addition of degradation enzymes to the ethanologen's enzymatic profile.

E. coli strains LY40A and JP07C had significantly higher ethanol yields than *E. coli* strain KO11 (Fig. 2A). A reducing sugar assay supports the finding of the low ethanol yield of *E. coli* strain KO11 (Fig. 2A); large amounts of reducing sugars (140 to 185 μg/ml) present throughout the fermentation correspond to oligomeric substrates that KO11 is unable to metabolize. A comparison between *casAB*-containing *E. coli* strains LY40A and JP07C, whose reducing sugar concentrations decrease to near zero within 24 h, suggests that a major component of the *E. coli* strain KO11 reducing sugars is cellobiose, emphasizing the significance of the addition of *casAB* to the strain.

Ethanol yields for *E. coli* strain JP07C were not significantly higher than those for LY40A. However, the concentration of reducing sugars for *E. coli* strain JP07C continually increased after 24 h, while that of LY40A did not (Fig. 2A). If PelE
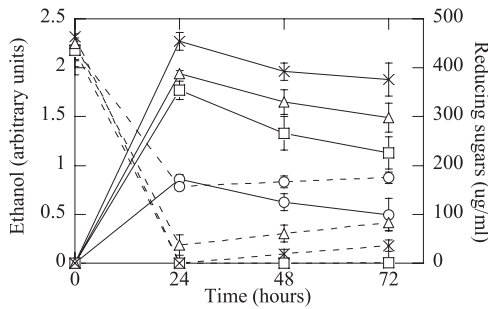
FIG. 3. Ethanol production and reducing sugars from 5% (wt/vol) SBP fermentations for *E. coli* strains KO11, LY40A, JP07C, and JP08C (standard error; *n* = 3). Solid lines indicate ethanol concentrations, and dashed lines represent reducing sugar concentrations. ○, KO11; □, LY40A; △, JP07C; ×, JP08C.

produced by *E. coli* strain JP07C is cleaving large polygalacturonate chains without releasing large amounts of monomeric sugars, the reducing sugar concentration would increase, while ethanol production would not. To test this hypothesis, oligogalacturonides with a degree of polymerization (dp) of greater than 6 were precipitated from fermentation samples, and the remaining oligogalacturonides with a dp of 6 or less were measured by the absorbance at 235 nm. The absorbance of *E. coli* strain JP07C samples was significantly higher than that of KO11 or LY40A throughout the fermentation (Fig. 2B). After the fermentation of sugars released during the partial saccharification, the absorbance of JP07C samples continued to increase from 24 to 72 h. These differences in absorbance correspond to an increase in concentrations of short-chain oligogalacturonides throughout the fermentation, demonstrating the enzymatic breakdown of polygalacturonate by PelE secreted from JP07C.

Comparison of JP07C and JP08C in SBP fermentations. SBP fermentations were performed to determine if the Ogl activity of JP08C leads to higher ethanol yields than those of the predecessor strains. The combination of *pelE* and *ogl* significantly increased ethanol production by 28% compared to that of LY40A (Fig. 3). An examination of the reducing sugar concentrations for JP08C showed that while the concentrations were decreased compared to those of JP07C, they continued to increase slightly throughout the fermentation; this suggests that polygalacturonic acid chains are being released from the SBP but are not being cleaved into di- and trigalacturonides,

which are subjected to Ogl activity. As SBP is highly methyl esterified (60%) (30), the activities of both PelE and Ogl may be partially inhibited, and the further addition of a pectin methylesterase could increase the activities of these two enzymes.

SBP fermentations with JP08C and commercial PME. The level of PelE activity is known to decrease with increasing levels of pectin methylation (31). Since SBP is highly methylated, the addition of PME could increase the pectinase activity of JP08C. When commercial PME was added during the partial saccharification, there was an increase in the level of methanol production, suggesting that PME is actively demethylating the pectin (Table 4). JP08C fermentation mixtures supplemented with PME produced more ethanol than did fermentation mixtures without PME (14.55 g/liter and 10.77 g/liter, respectively) (Table 4). The liberation of glucose and cellobiose after the 24-h partial saccharification increased when PME was added, even though there was no detectable cellulase activity in the commercial PME preparation (Table 4). However, fermentations conducted with LY40A supplemented with PME, showing an increased release of sugars after partial saccharification, did not result in elevated levels of ethanol production compared to those for JP08C fermentations (Table 4). Therefore, the increased ethanol production in JP08C fermentations conducted with PME is likely caused by the PME demethylation of SBP pectin, which allows the heterologous pectinase enzymes from JP08C to degrade the polygalacturonate and produce more ethanol. Without the pectinase enzymes of JP08C, supplementation with PME is ineffective.

Conclusions. The engineering of these ethanologenic *E. coli* strains to produce lignocellulose-degrading enzymes during fermentation can result in the decreased use of exogenous fungal enzymes in biomass saccharification steps, reducing the cost of the entire process. First, the addition of *casAB* for cellobiose utilization significantly impacts ethanol production from lignocellulosic biomass and drastically reduces the need for fungal cellobiases, possibly eliminating the need for this type of enzyme altogether. Second, the addition of *pelE* and the resulting secretion of PelE considerably increased the degradation of polygalacturonate. Third, the engineering of *E. coli* strain JP07C to produce Ogl allowed the breakdown of polygalacturonate to monomeric sugars during fermentation and increased the ethanol yield.

The engineering of *E. coli* strain JP08C demonstrates the

TABLE 4. Sugar utilization, methanol production, and ethanol production from 10% (wt/vol) SBP fermentations for *E. coli* strains LY40A and JP08C with or without PME supplementation[a]

E. coli strain + enzyme(s)	Mean utilization or production (g/liter) ± SD				
	Glucose[b]	Cellobiose[b]	GalA[b,c]	Methanol[d]	Ethanol[e]
LY40A + cellulase	7.56 ± 1.4	5.50 ± 1.1	0.72 ± 0.5	0.44 ± 0.1	10.66 ± 1.1
LY40A + cellulase + PME	9.51 ± 2.3	6.74 ± 1.0	0.91 ± 0.3	1.15 ± 0.09	10.43 ± 0.9
JP08C + cellulase	5.46 ± 1.0	3.75 ± 0.8	0.50 ± 0.2	0.44 ± 0.01	10.77 ± 0.3
JP08C + cellulase + PME	8.62 ± 0.2	5.51 ± 0.2	0.63 ± 0.2	0.97 ± 0.04	14.56 ± 1.1

[a] *n* ≥ 3.
[b] Yield at the time of inoculation (0 h).
[c] GalA, galacturonic acid.
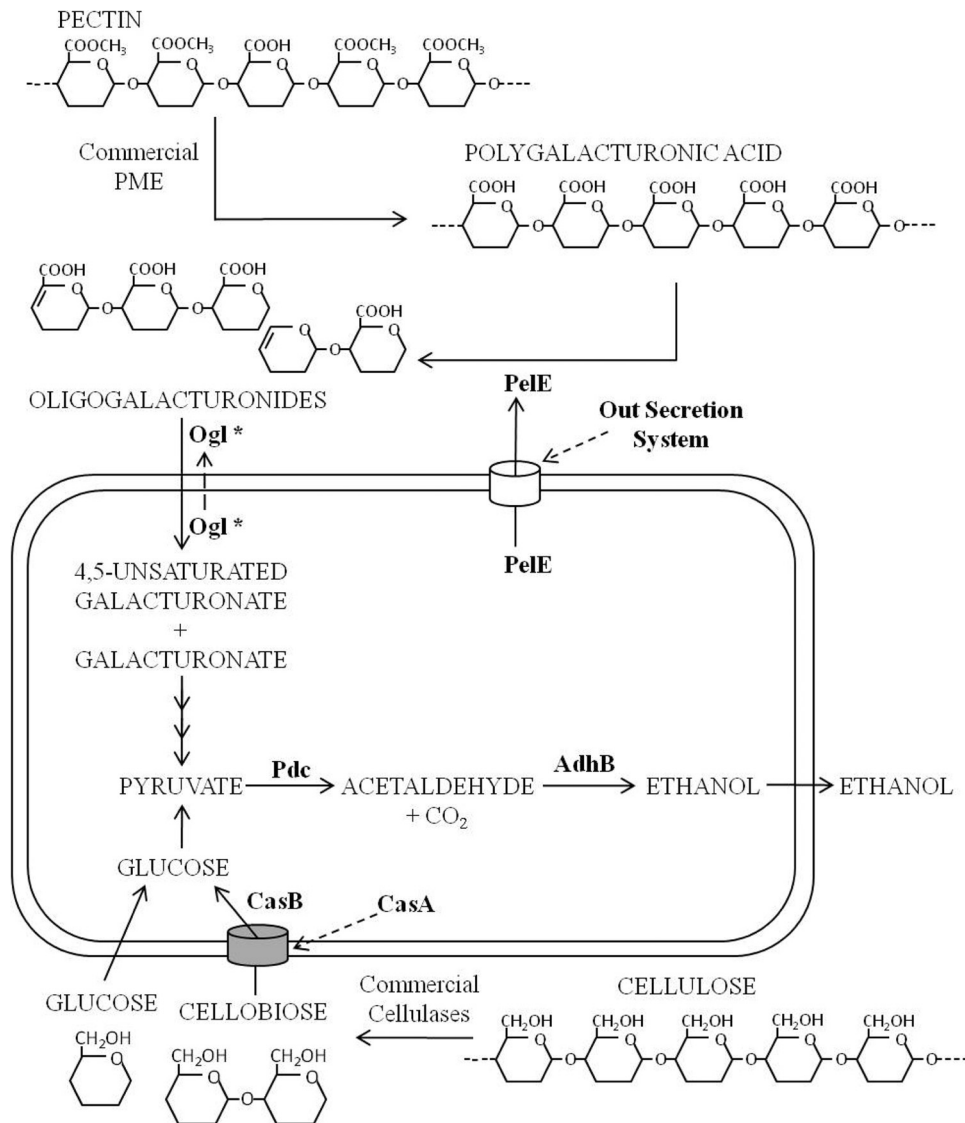[d] Yield at 24 h.
[e] Maximum ethanol yield at 72 h.

FIG. 4. Ethanol production pathway in *E. coli* strain JP08C. The metabolism of pectin and cellulose to ethanol is shown, with the heterologous enzymes of JP08C highlighted in boldface type. (∗, it is unknown whether Ogl acts intra- or extracellularly.)

addition of enzyme activities targeted toward the degradation of a specific component of lignocellulosic biomass, namely, pectin, thereby reducing or eliminating the need for exogenously supplied pectinases (Fig. 4) (although *ogl* and *pme* still need to be chromosomally integrated for stability). Fermentations conducted with these organisms have not been optimized. Therefore, ethanol production from these strains has yet to reach an economically feasible level. Additional fermentations using various concentrations of commercial enzymes need to be conducted to elucidate the enzyme loading needed to produce ethanol at industrially relevant concentrations. Furthermore, work to integrate other plant cell wall-degrading enzymes, such as cellulases and hemicellulases, will advance this organism toward a goal of a single microorganism that is capable of both the degradation and fermentation of lignocellulosic biomass (19).

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