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Isolation and Molecular Characterization of High-Performance Cellobiose-Fermenting Spontaneous Mutants of Ethanologenic *Escherichia coli* KO11 Containing the *Klebsiella oxytoca casAB* Operon†

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***Escherichia coli* KO11 was previously constructed to produce ethanol from acid hydrolysates of hemicellulose (pentoses and hexoses) by the chromosomal integration of *Zymomonas mobilis* genes encoding pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*). *Klebsiella oxytoca* P2 was constructed in an analogous fashion for the simultaneous saccharification and fermentation of cellulose and contains PTS enzymes for cellobiose. In this study, KO11 was further engineered for the fermentation of cellulose by adding the *K. oxytoca casAB* genes encoding Enzyme II^{cellobiose} and phospho-β-glucosidase. Although the two *K. oxytoca* genes were well expressed in cloning hosts such as DH5α, both were expressed poorly in *E. coli* KO11, a derivative of *E. coli* B. Spontaneous mutants which exhibited more than 15-fold-higher specific activities for cellobiose metabolism were isolated. The mutations of these mutants resided in the plasmid rather than the host. Three mutants were characterized by sequence analysis. All contained similar internal deletions which eliminated the *casAB* promoter and operator regions and placed the *lacZ* Shine-Dalgarno region immediately upstream from the *casA* Shine-Dalgarno region. KO11 harboring mutant plasmids (pLOI1908, pLOI1909, or pLOI1910) rapidly fermented cellobiose to ethanol, and the yield was more than 90% of the theoretical yield. Two of these strains were used with commercial cellulase to ferment mixed-waste office paper to ethanol.**

Development of efficient microbial biocatalysts for the conversion of renewable lignocellulose into liquid fuels and bulk chemicals offers the potential for reducing our dependence on petroleum (for automotive fuels) while improving the environment (9). The carbohydrates in lignocellulose (cellulose and hemicellulose) must be converted to soluble sugars prior to fermentation. Hemicellulose can be readily hydrolyzed to a mixture of hexose and pentose sugars by using dilute acids and can be efficiently fermented to ethanol by using *Escherichia coli* KO11, a derivative of *E. coli* B (= ATCC11303) in which the *Zymomonas mobilis pdc* and *adhB* genes have been chromosomally integrated (1, 13). Although concentrated mineral acids can be used for cellulose hydrolysis (9), our laboratory has focused on the use of fungal and bacterial cellulases for the solubilization of cellulose (5, 16).

Klebsiella oxytoca P2 was developed as a biocatalyst for cellulose fermentation by integrating *Z. mobilis* genes encoding the ethanol pathway (17). This organism contains a native phosphoenol-dependent phosphotransferase (PTS) system for cellobiose utilization (10), which eliminates the need for one class of cellulase enzymes. However, P2 does not function as effectively as KO11 for the fermentation of mixed hexose and pentose sugars (4, 11).

Cryptic genes for cellobiose metabolism have been described in some strains of *E. coli* (7, 14), and we have previously isolated cellobiose-positive mutants of KO11 by using MacConkey agar for selection (unpublished data). However, no mutant was capable of rapid growth and fermentation with cellobiose. Recently, genes encoding PTS enzymes for cellobiose metabolism were cloned from seven different organisms by screening libraries with 4-methylumbelliferyl-β-D-glucopyranoside (MUG), a chromogenic analog of cellobiose (10). Although three of these cloned operons allowed *E. coli* DH5α to grow in minimal medium containing cellobiose, all were expressed poorly in *E. coli* KO11.

In this study, we describe the isolation and molecular characterization of spontaneous mutants of KO11(pLOI1906) containing the *K. oxytoca casAB* operon which ferment cellobiose efficiently. Two of these mutants were used as effective biocatalysts for the simultaneous saccharification and fermentation of mixed-waste office paper to ethanol.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* KO11 (13) and *K. oxytoca* P2 (17) were used in all fermentation studies. These strains are derivatives of *E. coli* B and *K. oxytoca* M5A1, respectively, and contain the chloramphenicol acyl transferase gene (*cat*) and the *Z. mobilis* genes for ethanol production (*pdc*, *adhB*). Stock cultures of KO11 and P2 were maintained on modified Luria-Bertani (LB) agar containing (per liter) 5 g of NaCl, 5 g of yeast extract, 10 g of tryptone, 20 g of glucose, 15 g of agar, and 600 mg of chloramphenicol. Strains KO11 and P2 are prototrophic and recombination proficient. In liquid cultures and fermentation experiments, chloramphenicol (40 mg/liter) and cellobiose (60 or 90 g/liter) were added. Cellobiose stocks were filter sterilized to minimize hydrolysis.

The following three plasmids containing genes encoding PTS cellobiose uptake and cleavage enzymes were used in this study (10): pLOI1903 containing genes from *Bacillus subtilis*, pLOI1905 containing genes from *Butyrivibrio fibrisolvens*, and pLOI1906 containing the *K. oxytoca casAB* operon. A series of mutant plasmids derived from pLOI1906 were developed and analyzed in this study; these plasmids were pLOI1908, pLOI1909, and pLOI1910. *E. coli* DH5α was used as a host for genetic studies. Expression of plasmid-borne *casAB* genes in DH5α was compared to expression in KO11 derivatives.

Utilization of cellobiose was screened with MacConkey agar (20 g/liter), M9 minimal agar (20 g/liter) (2), modified LB agar containing MUG (20 mg/liter), and LB broth containing cellobiose (60 g/liter). Ampicillin (50 mg/liter) was added for plasmid selection.

In vitro assay of PTS activity. The combined activity of the *casAB* phosphotransferase system and phospho-β-glucoside cleavage enzyme was determined by

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using *p*-nitrophenyl-β-D-1,4-glucopyranoside (PNPG) as a substrate (10). Overnight cultures (grown for 18 h) were harvested by centrifugation (5,000 × *g*, 5 min, 4°C), washed twice, and resuspended in 50 mM $NaKHPO_4$ buffer (pH 7.2) to an approximate optical density at 550 nm of 50. Cells were disrupted by two passages through a French pressure cell at 20,000 lb/in^2. Lysates were assayed at 37°C in 50 mM $NaKHPO_4$ buffer (pH 7.2) containing 5 mM $MgCl_2$, 2 mM PNPG, and 2 mM phosphoenolpyruvate. Reactions were terminated by adding an equal volume of 1 M Na_2CO_3. After centrifugation (5,000 × *g*, 5 min) to remove cell debris, *p*-nitrophenol (PNP) content was measured at 410 nm. Protein content was estimated by using the Bradford reagent (Bio-Rad Laboratories, Richmond, Calif.) with bovine serum albumin as a standard. Activities are expressed below, as micromoles of PNP released per minute per milligram of cell protein.

Genetic methods and DNA sequencing. Standard methods were employed for plasmid isolation, transformation, and analyses (15). DNA was sequenced by the dideoxynucleotide chain termination method by using fluorescent M13 primers (forward primer 5′-CACGACGTTGTAAAACGAC-3′ and reverse primer 5′-CGATAACAATTTCACACAGG-3′) purchased from LI-COR (Lincoln, Nebr.). Fluorescently labeled ATP (LI-COR) was used with deoxynucleoside triphosphates and custom primers. A forward custom primer spanning CasA amino acid residues Phe-56 to Ser-50 (AAAGAAGAACAGCGCATCGC) was used to confirm the sequence at the 5′ junction between *K. oxytoca* and pUC18. A reverse custom primer spanning CasB amino acid residues Asn-312 to Leu-318 (AACAAAAAAGCGCGCGGCAA) was used to sequence the 3′ end of *casB* and downstream region. Extension reactions were performed as previously described (10) by using a Perkin-Elmer GeneAmp PCR System 9600 apparatus (Norwalk, Conn.) and a SequiTherm Long-Read LC cycle sequencing kit (Epicentre Technologies, Madison, Wis.). Products were separated and read with a LI-COR model 4000L DNA sequencer. Sequences were analyzed by using the Wisconsin Genetics Computer Group software package (6).

Batch fermentations of cellobiose. Fermentations (350-ml working volume) were conducted in modified LB medium essentially as described previously (3) except that cellobiose (90 g/liter) was used as the fermentable sugar. KOH was automatically added to prevent the broth pH from declining below 6.0 (35°C, 100 rpm). Increases in pH were not controlled. Ethanol content was monitored by gas chromatography (3, 12). Cell mass was estimated by determining the optical density at 550 nm. When Embden-Meyerhof glycolysis and the *Z. mobilis* ethanol pathway were used, the maximum theoretical yield was 4 mol of ethanol per mol of cellobiose (0.538 g of ethanol per g of cellobiose).

Simultaneous saccharification and fermentation of mixed-waste office paper. Autoclaved mixed-waste office paper (100 g/liter) was fermented to ethanol essentially as described previously (5) in agitated (60 rpm), 800-ml vessels (35°C, pH 4.9 to 5.2). Spezyme CP cellulase was generously provided by Genencor International (South San Francisco, Calif.). The enzyme preparation was diluted 1:100 to provide approximately 1,000 filter paper units of cellulase activity per liter of fermentation broth (10 filter paper units/g of mixed-waste office paper).

RESULTS AND DISCUSSION

Isolation of cellobiose-positive mutants of KO11. The following three plasmids which allowed DH5α to utilize cellobiose (10) were transformed into KO11: pLOI1903, pLOI1905, and pLOI1906 (10). The resulting transformants exhibited weak activity with the fluorescent cellobiose analog MUG, but were negative on MacConkey agar containing cellobiose and minimal medium containing cellobiose. Plasmids were reisolated from these KO11 recombinants and transformed back into DH5α. All of the resulting DH5α recombinants had the original cellobiose-positive phenotype.

Enrichment cultures were established for KO11 harboring each of these plasmids by using cellobiose (60 g/liter) as the fermentable carbohydrate for selection (10 ml of LB broth in 18- by 150-mm culture tubes; 30°C; no agitation). The cultures were diluted 100-fold every 24 h for a 3-week period, and we continued to incubate ancestral cultures. Only KO11(pLOI1906) enrichment cultures became dense and produced abundant gas, indicative of cellobiose fermentation. Two cultures were streaked for isolation on MacConkey agar containing cellobiose. Approximately one-half of the colonies from each enrichment culture were raised, dark red, and strongly positive for cellobiose utilization. Ten positive clones (strains MM101 to MM110) were selected and tested in tubes containing LB broth supplemented with cellobiose (Table 1). All of these clones grew to four times the cell density of and produced four times as much ethanol as the unmutated strains KO11(pLOI1906) and KO11(pUC18). With all mutants, the broth pH declined to approximately pH 5.6 (due primarily to carbonic acid) while the pH of the parent culture remained nearer neutrality, consistent with the absence of carbohydrate metabolism.

TABLE 1. Comparison of cellobiose-positive mutants of KO11(pLOI1906)

Strain or mutant	Broth pH after 24 h	Optical density at 550 nm	Ethanol concn (g liter^{-1})	Colony uniformity on solid medium
MM101	5.7	4.74	7.9	Yes
MM102	5.6	4.85	7.7	No
MM103	5.7	5.00	8.0	No
MM104	5.6	4.20	7.3	No
MM105	5.6	4.05	7.0	No
MM106	5.7	4.74	7.9	Yes
MM107	5.7	4.80	7.7	No
MM108	5.6	4.42	7.4	Yes
MM109	5.7	4.64	7.4	Yes
MM110	5.5	4.05	6.8	No
KO11(pUC18)	6.3	1.20	1.7	Yes
KO11(pLOI1906)	6.2	1.20	1.8	Yes

Repeated streaking of mutant strains on solid medium revealed instability in 6 of the 10 clones. The four stable clones (MM101, MM106, MM108, and MM109) grew well on M9 minimal medium containing cellobiose. Figure 1 compares the growth of the parental strain and strain MM106 in shaken flasks containing LB broth supplemented with cellobiose. As is characteristic for ethanologenic KO11 with other sugars (12), the growth of MM106 with cellobiose was roughly linear at cell densities above an optical density at 550 nm of 2.

Initial characterization of cellobiose-positive mutants. Plasmids were isolated from strains MM101, MM106, MM108, and MM109 and transformed into fresh KO11 (and DH5α), and all of the recombinants utilized cellobiose, indicating that mutations responsible for the acquired phenotype resided in the plasmids rather than in the host. Restriction analysis revealed that all four plasmids were approximately 500 bp smaller than the original pLOI1906. Strains MM106 and MM108 were siblings, and only MM106 was retained for study. The plasmids from MM101, MM106, and MM109 were designated pLOI1908, pLOI1909, and pLOI1910, respectively. The stability of these plasmids in KO11 was examined by serially transferring broth

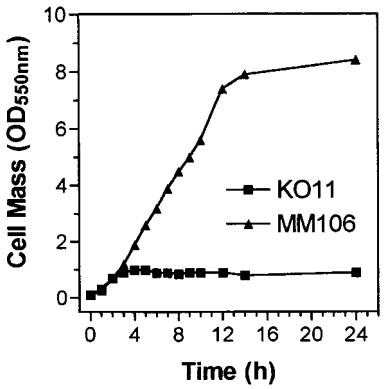


FIG. 1. Comparison of growth in LB broth containing cellobiose. Cultures were incubated at 35°C in shaken 250-ml flasks containing 50 ml of LB broth supplemented with 60 g of cellobiose per liter. OD_{550nm}, optical density at 550 nm.

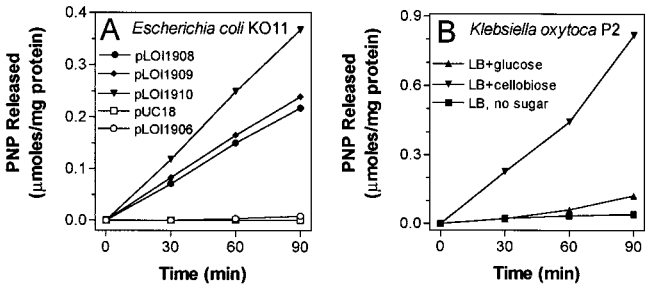


FIG. 2. Comparison of PTS cellobiose uptake and cleavage activity in vitro when *p*-nitrophenyl-β-D-glucoside was used as a model substrate. (A) Recombinant strains of *E. coli* KO11 harboring plasmids. Cells were grown in LB broth containing 60 g of glucose per liter. (B) *K. oxytoca* P2. Cells were grown in LB broth without added sugar, LB broth containing 60 g of glucose per liter, or LB broth containing 60 g of cellobiose per liter.

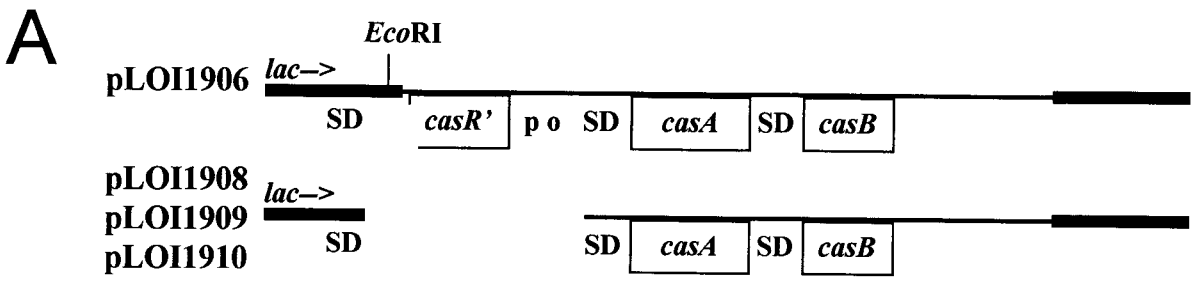
cultures (LB broth supplemented with glucose) in the absence of antibiotic selection. After 35 generations, 96% of the colonies which grew on LB agar supplemented with glucose were also positive on MacConkey agar containing cellobiose.

In vitro expression of the *casAB* operon in KO11 was evaluated by measuring the combined PTS transport and cleavage activity by using PNPG as a model substrate (Fig. 2A). Low activities were present in the control strains, KO11(pUC18) and KO11 harboring unmutated pLOI1906. With the mutant plasmids, pLOI1908, pLOI1909, and pLOI1910, the PTS genes were expressed in KO11 at approximately one-third to one-half the level measured in *K. oxytoca* P2 grown with cellobiose (9 nmol/min per mg of protein). The PTS activity in KO11(pLOI1910) was equal to that previously observed in DH5α containing the original, unmutated pLOI1906. Interestingly, little activity was detected in *K. oxytoca* P2 after growth in LB medium supplemented with glucose or in LB medium without sugar, indicating that the native *casAB* operon requires cellobiose for induction (Fig. 2B).

Genetic analysis of mutations facilitating *casAB* expression. Mapping with restriction endonucleases identified a deletion at the 5′ end of the *K. oxytoca* insert in all three plasmids. The *Eco*RI site (vector) near the 5′ end of the insert was absent, while the 3′ end appeared to be unaltered (Fig. 3A). The lack of deletions at the 3′ ends of *casB* and the vector at this junction was confirmed by sequence analysis.

Two types of deletion were found in the 5′ end, and these deletions differed by only 2 bp (Fig. 3B). Plasmids pLOI1908 and pLOI1909 were shortened by 442 bp of *K. oxytoca* DNA and 37 bp of vector DNA compared to the original plasmid, pLOI1906. Plasmid pLOI1910 was shortened by 441 bp of *K. oxytoca* DNA and 38 bp of vector DNA. The two recombination events were quite similar and, deleted the incomplete *casR′*, putative *casAB* promoter, and operator regions and a stem-loop region described previously (10). In the deleted mutants, the *lacZ* Shine-Dalgarno sequence resided a few bases upstream from the *casA* Shine-Dalgarno region. Thus, in pLOI1908, pLOI1909, and pLOI1910, expression of *casAB* is dependent on the *lac* promoter (vector). Translation may be enhanced by the presence of tandem Shine-Dalgarno regions (*lacZ* and *casA*).

The similarity between the two independent deletions prompted an examination of sequence homology in this region (Fig. 3B). Aligning the respective pUC18 and *casA* sequences revealed 6 G+C identities immediately upstream from the putative crossover site, 4 G+C identities near the 5′ end of the *casA* coding sequence, and 19 A+T identities. These similarities in the sequences may have facilitated deletion events which, by coincidence, increased the expression of *casAB* in KO11. Although both deletion events eliminated the regulatory region and juxtaposed the *lacZ* and *casA* Shine-Dalgarno



B

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                                                          M  T  M  I  T  N  S  S  S  V  P  G-->
pUC18      TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGG(BamHI)
                 ||  |  |  ||||    |      | |    | *   * ** | * *   | |   |     |  |  |             |* ** *
1906       CTCGGGTTTTTTTATTTTTAATACTCAGTTAACGATGCCTGCGGGCAGCGGAAGTAAGGAAAAACAGCATGGAATATAAAGCACTCGCG
                                                                                  M  E  Y  K  A  L  A-->

1908       TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGC//AAGGAAAAACAGCATGGAATATAAAGCACTCGCG
1909                                                                                M  E  Y  K  A  L  A-->

1910       TCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAG//TAAGGAAAAACAGCATGGAATATAAAGCACTCGCG
                                                                                  M  E  Y  K  A  L  A-->
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FIG. 3. Comparison of pLOI1906 and spontaneous deletions which facilitated expression of the *casAB* operon in recombinants of *E. coli* KO11. The thick lines represent vector DNA (pUC18). The thin lines represent DNA derived from *K. oxytoca* (10). G and C identities are indicated by asterisks. A and T identities are indicated by vertical dashes. Double shills indicate the site of the deletion. Abbreviations: *lac*→, *E. coli lacZ* promoter; *casR′*, putative regulatory gene; p o, putative *casAB* promoter-operator regions containing a conserved binding site for antiterminator protein; SD, Shine-Dalgarno region; *casA*, PTS Enzyme II; *casB*, phospho-β-glucosidase. In panel B, the SD region of *lacZ* is indicated by an overbar; the SD region of *casA* is indicated by underscoring.

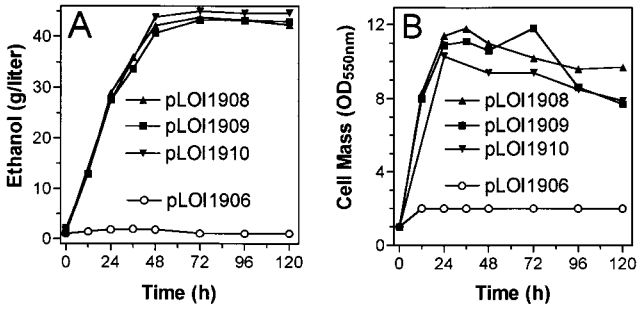

FIG. 4. Fermentation of cellobiose by recombinant strains of *E. coli* KO11. (A) Ethanol production. (B) Cell growth. OD_{550nm}, optical density at 550 nm.

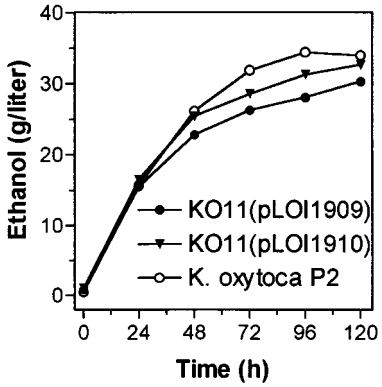

FIG. 5. Simultaneous saccharification and fermentation of mixed-waste office paper (100 g/liter) by *K. oxytoca* P2 and *E. coli* KO11 harboring various plasmids.

regions, it is likely that the frequency and specificity of deletion were provided by sequence similarity (Fig. 3B).

Our results also indicate that expression of *casAB* from the native promoter (and upstream *lac* promoter) is more tightly controlled in KO11, a derivative of *E. coli* B, than in DH5α. The basis for this control may be the palindromic sequence and operator region which are presumed to require binding of an antiterminator protein plus cellobiose for expression in *K. oxytoca* (10). Recent studies have identified surprising differences in sigma factors among K-12 strains of *E. coli* (8). It is possible that variations in sigma factors or other regulatory proteins may be responsible for the differences in *K. oxytoca casAB* expression between *E. coli* DH5α and KO11 (an *E. coli* B derivative).

Fermentation of cellobiose to ethanol. Ethanol production from cellobiose was examined by using recombinant KO11 harboring either native pLOI1906 or a mutant plasmid (pLOI1908, pLOI1909, or pLOI1910) with increased *casAB* expression (Fig. 4). KO11(pLOI1906) grew poorly and produced little ethanol. In contrast, KO11 harboring any of the mutant plasmids produced more than 6-fold-greater cell mass and 40-fold-higher ethanol concentrations and consumed significant amounts of base consistent with the neutralization of carbonic acid to maintain pH 6 (Table 2). Fermentations with KO11 harboring mutant plasmids rapidly reached completion, produced approximately 1 M ethanol, and achieved ethanol yields which exceeded 90% of the theoretical maximum yield from cellobiose.

It is possible to estimate a minimal in vivo rate for cellobiose uptake and hydrolysis by KO11 derivatives based on the rate of ethanol production and an estimate of cell mass. Assuming that an optical density at 550 nm of 4.0 represents approximately 1 mg of cell protein per ml, the rate of cellobiose metabolism by KO11(pLOI1910) is estimated to be approximately 0.030 μmol/min per mg of protein. This in vivo activity is fourfold higher than the in vitro activity (disrupted cells) measured in cellobiose-induced *K. oxytoca* P2 by using a chromogenic substrate (PNPG) and 10 times higher than the activity of the best *E. coli* construct, KO11(pLOI1910). Since previous studies with DH5α have clearly shown that a fully functional PTS with Hpr, EnzI, CasA, and CasB are all required for activity (10), we attribute the lower activity obtained with the surrogate substrate, PNPG, to poor uptake, phosphorylation, or hydrolysis.

Simultaneous saccharification and fermentation of mixed-waste office paper. Previous studies have demonstrated the effectiveness of *K. oxytoca* P2 for the conversion of cellulosic substrates into ethanol (5, 9). *E. coli* KO11 derivatives (pLOI1908 or pLOI1910) expressing the *casAB* operon were almost equivalent to *K. oxytoca* P2 for ethanol production from mixed-waste office paper (Fig. 5; Table 2). The initial rates of fermentation were similar, although with P2 a higher final ethanol concentration was obtained. KO11(pLOI1910), the construct with the highest functional expression of the *K. oxytoca casAB* operon, also appeared to be superior to KO11 (pLOI1908) for conversion of mixed-waste office paper to ethanol. These results suggest that improvements may be possible by further increasing *casAB* expression in KO11.

Significance. The general design for the conversion of native lignocellulose to ethanol involves the separation of a dilute

TABLE 2. Fermentation of cellobiose and mixed-waste office paper to ethanol[a]

Biocatalyst	Substrate	Substrate concn (g/liter)	Cell dry wt (g/liter)	Amt of base consumed (mmol/liter)	Amt of ethanol produced (g/liter)[b]	Yield (% of theoretical yield)[c]
KO11(pLOI1908)	Cellobiose	90	3.9	63	44.6	92
KO11(pLOI1909)	Cellobiose	90	3.4	54	44.4	92
KO11(pLOI1910)	Cellobiose	90	3.1	40	45.4	94
KO11(pLOI1906)	Cellobiose	90	0.3	0	1.0	2
KO11(pLOI1908)	Paper	100	ND[d]	6	30.4	67
KO11(pLOI1910)	Paper	100	ND	6	32.7	72
K. oxytoca P2[e]	Paper	100	ND	6	34.5	76

[a] Data are averages from two or more fermentations.
[b] The values are the total amounts of ethanol produced per liter of original fermentation broth and were adjusted for dilution by base additions.
[c] The theoretical yields are 0.538 g of ethanol per g of cellobiose and 0.568 g of ethanol per g of cellulose. Mixed-waste office paper (paper) contains approximately 80% cellulose (maximum theoretical yield from cellulose of 0.454 g of ethanol per g of mixed-waste office paper).
[d] ND, not determined.
[e] All of the other biocatalysts are derivatives of *E. coli* B.

acid hydrolysate of hemicellulose from the acid-resistant fiber (cellulose and lignin) (1, 5, 9, 16). Previously, these two streams were fermented separately by using different biocatalysts. Our newly engineered organism, which adds the cellobiose utilization system from *K. oxytoca* P2 to *E. coli* KO11 by using plasmid pLOI1910, offers the opportunity to use a single biocatalyst for both fermentations, which reduces the costs and complexity associated with the propagation of two different organisms.

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