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ORIGINAL RESEARCH PAPER

Re-engineering *Escherichia coli* for ethanol production

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Abstract A lactate producing derivative of *Escherichia coli* KO11, strain SZ110, was re-engineered for ethanol production by deleting genes encoding all fermentative routes for NADH and randomly inserting a promoterless mini-Tn5 cassette (transpososome) containing the complete *Zymomonas mobilis* ethanol pathway (*pdc*, *adhA*, and *adhB*) into the chromosome. By selecting for fermentative growth in mineral salts medium containing xylose, a highly productive strain was isolated in which the ethanol cassette had been integrated behind the *rrlE* promoter, designated strain LY160 (KO11, Δ*frd*::*celY*$_{Ec}$ Δ*adhE* Δ*ldhA*, Δ*ackA lacA*::*casAB*$_{Ko}$ *rrlE*::(*pdc*$_{Zm}$-*adhA*$_{Zm}$-*adhB*$_{Zm}$-*FRT-rrlE*) *pflB*$^{+}$). This strain fermented 9% (w/v) xylose to 4% (w/v) ethanol in 48 h in mineral salts medium, nearly equal to the performance of KO11 with Luria broth.

Keywords Betaine · *E. coli* · Ethanol · Lactate · Osmotic stress

Introduction

Lignocellulosic biomass represents a renewable source of carbohydrate for biological conversion into fuels and chemicals, an alternative to petroleum-based technology (Arntzen and Dale 1999; Hahn-Hagerdal et al. 2006). Commodity production of ethanol from biomass will require high rates and efficiencies with biomass sugars, simple processes, and inexpensive media (Ingram et al. 1998; Zhang and Greasham 1999). Bacteria, such as *Escherichia coli*, have the native ability to metabolize all sugar constituents in lignocellulose. Environmental hardiness, broad substrate range, and ability to grow well in mineral salts media were criteria for selection of *E. coli* as a platform organism for metabolic engineering (Alterthum and Ingram 1989; Zhou et al. 2006a). Strain KO11 was engineered for ethanol production by integrating two *Zymomonas mobilis* genes (*pdc*, *adhB*) behind the *pflB* promoter of *E. coli* (Ohta et al. 1991). Despite the absence of clear auxotrophic requirements, complex nutrients were needed to rapidly and efficiently produce high ethanol titers with KO11 (Asghari et al. 1996; Martinez et al. 1999; Underwood et al. 2004; York and Ingram 1996). Efforts to develop improved media and genetic modifications have been unsuccessful in eliminating the requirement for complex nutrients, although betaine was helpful (Underwood et al. 2004).

Previously, we re-engineered KO11 for lactate production by constructing SZ110 (Zhou et al. 2005, 2006a, b). This strain rapidly and efficiently ferments sugars to D(−)-lactate at high yields in mineral salts media. The performance of strain SZ110 suggested that the specific construction of KO11 rather than a more fundamental problem may limit ethanol

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production in mineral salts media. Potential problems were identified in KO11 such as suboptimal expression from the *pflB* integration site of the *Z. mobilis* genes (*pflB adhB*), absence of a complete set of alcohol genes from *Z. mobilis* (*pdc* and *adhB* but lacking *adhA*), and the presence of an antibiotic resistance gene (*cat*). In this paper, we report the elimination of these three potential problems which resulted in a highly productive strain of *E. coli* for ethanol production.

Materials and methods

Organisms, media and growth conditions

Strains and plasmids used in this study are listed in Table 1. Luria broth was used for plasmid constructions (Miller 1992). Ampicillin (50 mg l^{-1}), kanamycin (50 mg l^{-1}) and apramycin (50 mg l^{-1}) were added as needed for selection. Temperature-conditional plasmids were grown at 30°C, all others at 37°C. Re-engineered ethanol strains were grown in NBS mineral salts medium (Causey et al. 2003) supplemented with xylose. Betaine (1 mM) and MOPS (100 mM, pH 7.4) were added where indicated (Underwood et al. 2004). Aldehyde indicator plates were used to screen for alcohol dehydrogenase activity (Conway et al. 1987). [Note that strain KO11 is a derivative of *E. coli* W (ATCC9637), not *E. coli* B as originally reported (Ohta et al. 1991).]

Genetic methods

Standard methods were used for plasmid construction, transformation, electroporation, conjugation, and PCR amplification (Ausubel et al. 1987; Miller 1992;

Table 1 Strains and plasmids

Designation	Relevant genotype	Source or citation
Strains		
DH5α	*lacZ*ΔM15 *recA*	Invitrogen
S17-1*λpir*	*thi pro recA hsdR* RP4-2-*tet*::Mu *aph*A::Tn78*pir*, *spc tet*	de Lorenzo et al. 1990
TOP10F′	F′ {*lac*I^q Tn10 *tet*)}	Invitrogen
KO11	W Δ*frd pflB*::pdc_{Zm} $adhB_{Zm}$ $ldhA^+$ *cat*	Ohta et al. 1991
SZ110	KO11, Δ *frd*::$celY_{Ec}$ Δ*adhE* Δ*ackA* Δ*focA-pflB lacA*::$casAB_{Ko}$	Zhou et al. 2005
LY158	*SZ110* Δ*ldhA*, *rrlE*::(pdc_{Zm}-$adhA_{Zm}$-$adhB_{Zm}$-*FRT-kan-FRT*) $pflB^+$(*pflA-FRT-aac-FRT-ycaK*)	This study
LY160	LY158 *rrlE*::(pdc_{Zm}-$adhA_{Zm}$-$adhB_{Zm}$-*FRT-rrlE*) $pflB^+$(*pflA*-FRT-*ycaK*)	This study
Plasmids		
pCR2.1-TOPO	ColE1, TOPO T/A cloning vector, *bla kan*	Invitrogen
pKD46	pSC101^{ts}, *repA101 γβ exo*, (Red recombinase), *bla*	Datsenko and Wanner, 2000
pFT-A	pSC101^{ts}, *flp* (FLP recombinase), *bla*	Posfai et al. 1997
pLOI3421	ColE1, with *FRT* sites flanking the *aac* gene, *bla aac*	Wood et al. 2005
pLOI3469	R6K, *oriT*, *PacI* site within the Tn5 I and O ends, *tnp*, *bla*	Purvis et al. 2005
pUC18	ColE1, *bla*	New England Biolabs
ldhA deletion		
pLOI3497	*SmaI/EcoRI FRT-aac-FRT* from pLOI3421 in *MfeI/SalI* of *ldhA*, *ldhA′-FRT-aac-FRT-ldhA′ bla*, *kan*, *aac*	This study
pfB restoration		
pLOI3901	*ycaO-focA-pflB-pflA-FRT-aac-FRT*-trm-*ycaK-ycaN′*, colE1, *bla aac*	This study
Ethanol cassette		
pLOI135	*Z. mobilis adhA*, ColE1, *bla*	Keshav et al. 1990
pLOI295	*Z. mobilis pdc-adhB*, ColE1, *bla*	Ingram et al. 1998
pLOI3491	*pdc-adhA-adhB-FRT-kan-FRT*, R6K, oriT, *tnp*, *bla kan*	This study
pLOI3951	*Eco*RI fragment of LY160, ColE1, *bla* (integration site of ethanol genes)	This study

Table 2 Primers

Primers	Sequence
ldhA removal	N-terminus 5′-TTGCTCTTCCATGAAACTCGCCGTTTATAGCACA
	C-terminus 5′-TTGCTCTTCGTTAAACCAGTTCGTTCGGGCAGG
ycaN-pflB′ restoration	Forward 5′-GGCGCAATCGTTCATAGAGA-3′
	Reverse 5′-ATATGGCCGTGGCCGTATCA-3′
ycaO-pflA′ restoration	Forward 5′-AATGACGATGTGCCAGAAGG-3′
	Reverse 5′-GGTGTCGCGGTTATGACAAT-3′
Z. mobilis adhA	N-terminus 5′-ACTAGTGATCGTAATCGGCTGGCAAT-3′
	C-terminus 5′-ACTAGTGTTTATGCTTCCGCCTTCAC-3′
rrlE-pdc site of integration	Forward 5′-GGACGGAGAAGGCTATGTTG-3′ (within *rrlE*)
	Reverse 5′-TGCGAAGTGATGCTTGAGAC-3′ (within *pdc*)

Sambrook and Russell 2001). Primers are listed in Table 2. *Taq* PCR Master Mix (Qiagen Inc., Valencia, CA) was used to construct deletions and for analyses. Platinum *Pfx* DNA polymerase (Invitrogen, Carlsbad, CA) and Easy-A High-Fidelity PCR Cloning Enzyme (Stratagene, La Jolla, CA) were used to amplify functional genes for chromosomal integration. Integration of linearized DNA was facilitated by using plasmid pKD46 (Datsenko and Wanner 2000). *FRT*-flanked antibiotic genes were used to facilitate subsequent removal with recombinase (Martinez-Morales et al. 1999; Posfai et al. 1997). Chromosomal constructions were verified by PCR analyses and phenotype.

Fermentation conditions

Fermentation tests were carried out in 500 ml fermentation vessels with automatic pH control (37°C, pH 6.5, and 150 rpm, 350 ml working volume) (Underwood et al. 2004) using NBS mineral salts medium containing 9% (w/v) xylose, and 1 mM betaine. Pre-inocula were grown in standing screw-capped tubes at 37°C for 24–48 h in NBS mineral salts medium containing 5% (w/v) xylose and 1 mM betaine, and transferred to small fermentors. After incubation for 24 h, this culture was used to provide an initial inoculum level of 10–16 mg dry cell wt (dcw)1^{-1}.

Analyses

Cell mass was estimated from the OD_{550} value (OD 1 = 333 mg cdw l^{-1}). Organic acids and sugars were determined by using HPLC (Grabar et al. 2006). Ethanol was determined by GLC (Ohta et al. 2001).

Results

Construction of ethanologenic strain from SZ110 (lactate producer)

Strain SZ110 was previously constructed from KO11 by deleting the *Z. mobilis* cassette encoding *Z. mobilis* ethanol genes (*pdc*, *adhB*, *cat*) and the native genes for *ackA* and *adhE* leaving D(−)-lactate dehydrogenase (*ldhA*) as the only fermentative pathway (Zhou et al. 2005, 2006a, b). Genes for cellobiose utilization (*Klebsiella oxytoca casAB*) and endoglucanse (*Erwinia carotovora celY*) were also added to expand substrate range. The resulting strain, SZ110, was further engineered for ethanol production by replacing the central region of *ldhA* with a FRT-flanked apramycin gene (Fig. 1a), restoring the *pflB* region (Fig. 1b; two primer sets), and randomly integrating a transpososome cassette from pLOI3491 (Fig. 1c) containing *Z. mobilis pdc adhA adhB* and a FRT-flanked kanamycin gene. Using direct selection of pooled recombinants for xylose fermentation in mineral salts medium, a highly productive strain was isolated and designated LY158 (Fig. 2). During selection, improvements were observed in growth rate and cell yield, accompanied by an increase in rate of ethanol production. A clone was isolated using solid media and designated LY158. FLP recombinase was used to simultaneously remove both antibiotic

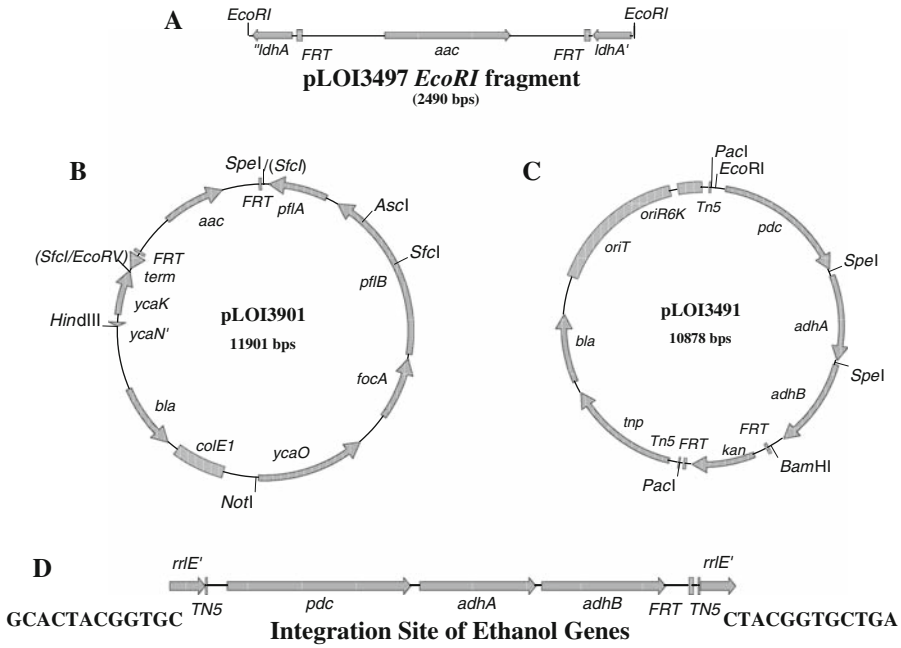

Fig. 1 Plasmids and DNA fragments used in strain construction. (**a**) pLOI3497, (**b**) pLOI3901, (**c**) pLOI3491, (**d**) ethanol genes and integration site

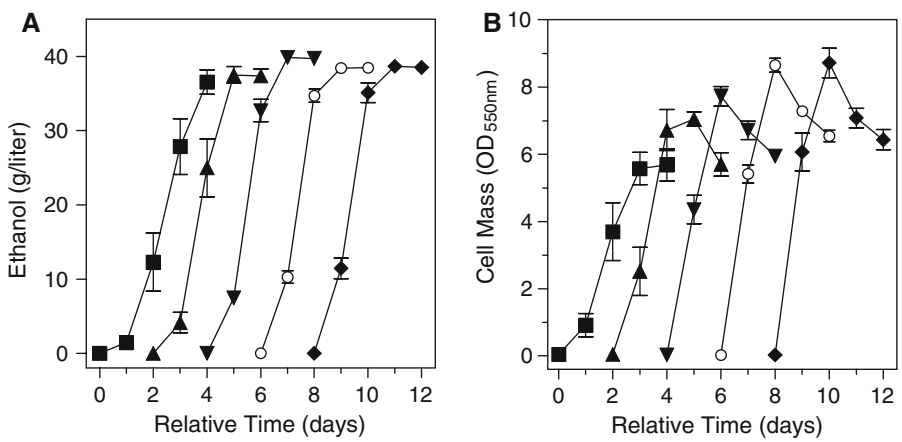

Fig. 2 Growth based selection. A population of random Tn5-based insertions (*Z. mobilis pdc-adhA-adhB-FRT-kan-FRT*) was serially transferred 38 times in NBS mineral salts medium (9% xylose) as a growth-based selection to enrich for exconjugates capable of rapid growth in NBS salts medium containing xylose. Initial transfers were made at 48-h intervals. As growth improved, transfers were made at 24-h intervals. Fermentations were monitored for 4 days. Transfers have been grouped and averaged: ■, transfers 1–9 (48 h intervals, inoculum of 17 mg dcw l^{-1}); ▲, transfers 10–14 (48-h intervals, 1 mM betaine, inoculum of 16 mg dcw l^{-1}); ▼, transfers 15–24 (24-h intervals, 1 mM betaine, inoculum of 10 mg dcw l^{-1}); ○, transfers 24–34 (24-h intervals, 1 mM betaine, inoculum of 10 mg dcw l^{-1}); ◆, transfers 35–38 (24-h intervals, 1 mM betaine, inoculum of 10 mg dcw l^{-1}). (**a**) Ethanol, (**b**) cell mass

markers (apramycin and kanamycin) from LY158. The resulting strain was designated LY160.

Integration site of ethanol cassette in LY160

A gene library of LY160 was constructed in pUC18 using *Eco*RI-digested DNA and screened for red colonies on aldehyde indicator plates to identify clones containing integrated *Z. mobilis* genes expressing alcohol dehydrogenase activity. One plasmid containing a 10.2 kb insert (pLOI3951) was selected for subcloning and partial sequencing. Sequencing revealed that the *pdc-adhA-adhB-FRT* cassette was integrated within the highly expressed *rrlE* (23S

ribosomal RNA) and oriented in the same direction of transcription (Fig. 1d). Primers were constructed and verified the site of chromosomal insertion. Discovery of a ribosomal gene as the optimal site for integration and expression of these foreign genes was unexpected. Although ribosomal genes are highly expressed in *E. coli* and represent a source of strong promoters, integration into a gene of such importance for protein synthesis represented a potentially detrimental event for growth.

Fermentation of 9% (w/v) xylose

The fermentation of strain LY160 was compared to that of KO11 in NBS mineral salts medium containing xylose and 1 mM betaine (Fig. 3). After 48 h, ethanol production and cell yield were more than 10-fold higher with LY160 than with KO11. A comparison of fermentation products revealed incomplete sugar utilization by KO11 and higher levels of formate and acetate (Fig. 4).

Growth and ethanol production by KO11 were dramatically improved by replacing NBS mineral salts with Luria broth. With xylose, cell yield and ethanol production by LY160 in NBS mineral salts medium containing betaine were similar to that of KO11 in Luria broth (Fig. 3).

Discussion

There are no inherent barriers preventing the rapid and efficient fermentation of xylose to ethanol by recombinant *E. coli* in mineral salts medium. Improvements observed over previously engineered strains result from a combination of changes including random integration with growth based-selection to identify an optimal site for chromosomal integration of the *Z. mobilis* genes for ethanol production. Selection of optimal promoters and integration sites is problematic. The general approach described in this paper using transpososomes for the integration of key pathway

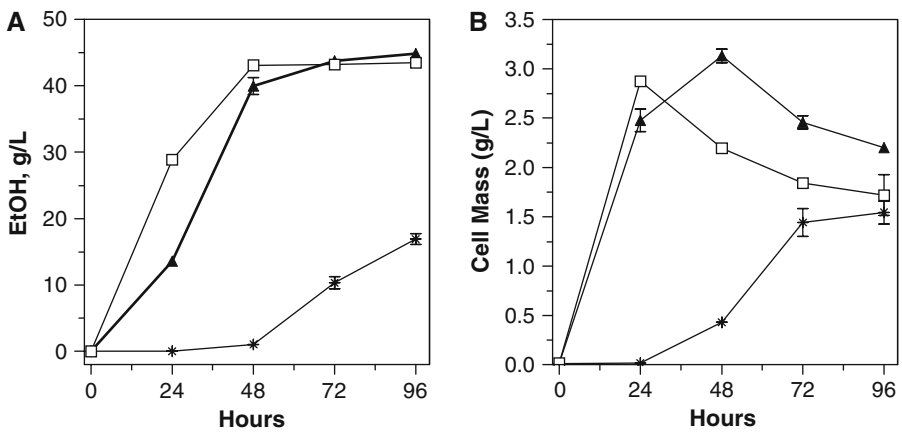

Fig. 3 Fermentation of 9% xylose (w/v) by recombinant *E. coli*. (**a**) Ethanol, (**b**) cell mass. Symbols: ▲, LY160 in NBS with 1 mM betaine; □, KO11 in Luria broth; *, KO11 in NBS with 1 mM betaine

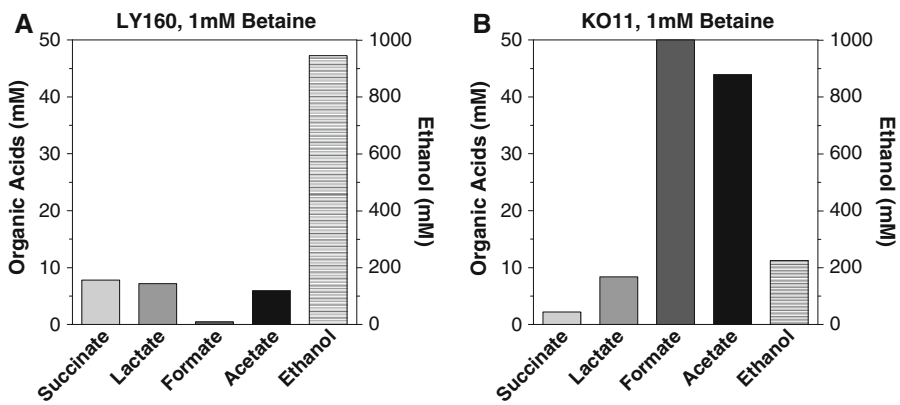

Fig. 4 Products of xylose fermentation in NBS mineral salts medium containing 9% xylose (w/v) and 1 mM betaine. (**a**) LY160, (**b**) KO11

genes coupled with growth-based selection offers the potential to rapidly develop new and improved biocatalysts. The inclusion of the full complement of *Z. mobilis* genes for ethanol production may also be beneficial. Activities of these two genes have been shown to vary in *Z. mobilis* with the *adhA* product dominating in stationary phase (Mackenzie et al. 1989). Both have been preserved during evolution.

High levels of sugar are required to produce high concentrations of products such as ethanol. Under fermentative conditions, *E. coli* is very limited in the production of protective osmolytes (Purvis et al. 2005; Underwood et al. 2004). The addition of betaine, a potent osmoprotectant, increased growth and improved performance with the high sugar concentrations needed for ethanol production (Underwood et al. 2004) and lactate production (Zhou et al. 2006a, b). Improvements in growth rate and cell yield observed with betaine are presumed to result in part from a decrease in the biosynthetic demand for native osmoprotectants such as glutamate and trehalose (Purvis et al. 2005; Underwood et al. 2004). With strain LY160, supplementing with 1 mM betaine was nearly as beneficial for growth as the combination of vitamins, amino acids, and other intermediates found in yeast extract and tryptone were for KO11. This problem of osmotic stress may contribute to the undefined need for complex nutrients in many commercial fermentations in the absence of clear auxotrophic requirements.

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