AEM
Journals.ASM.org

Improving *Escherichia coli* FucO for Furfural Tolerance by Saturation Mutagenesis of Individual Amino Acid Positions

Huabao Zheng, Xuan Wang, Lorraine P. Yomano, Ryan D. Geddes, Keelnatham T. Shanmugam, Lonnie O. Ingram

Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida, USA

Furfural is an inhibitory side product formed during the depolymerization of hemicellulose with mineral acids. In *Escherichia coli*, furfural tolerance can be increased by expressing the native *fucO* gene (encoding lactaldehyde oxidoreductase). This enzyme also catalyzes the NADH-dependent reduction of furfural to the less toxic alcohol. Saturation mutagenesis was combined with growth-based selection to isolate a mutated form of *fucO* that confers increased furfural tolerance. The mutation responsible, L7F, is located within the interfacial region of FucO homodimers, replacing the most abundant codon for leucine with the most abundant codon for phenylalanine. Plasmid expression of the mutant gene increased FucO activity by more than 10-fold compared to the wild-type *fucO* gene and doubled the rate of furfural metabolism during fermentation. No inclusion bodies were evident with either the native or the mutated gene. mRNA abundance for the wild-type and mutant *fucO* genes differed by less than 2-fold. The K_m (furfural) for the mutant enzyme was 3-fold lower than that for the native enzyme, increasing efficiency at low substrate concentrations. The L7F mutation is located near the FucO N terminus, within the ribosomal binding region associated with translational initiation. Free-energy calculations for mRNA folding in this region (nucleotides −7 to +37) were weak for the native gene (−4.1 kcal mol^{-1}) but weaker still for the *fucO* mutant (−1.0 to −0.1 kcal mol^{-1}). The beneficial L7F mutation in FucO is proposed to increase furfural tolerance by improving gene expression and increasing enzyme effectiveness at low substrate levels.

The enzyme L-1,2-propanediol oxidoreductase (encoded by *fucO*) is an NADH-linked, iron-dependent group III dehydrogenase. Typically, this enzyme functions only during the catabolism of deoxy sugars, where it catalyzes the reduction of lactaldehyde (1–3). FucO is a homodimer in which each subunit contains an active site (4). This enzyme has a broad substrate range (5) that includes furfural (6), a toxic side product in dilute acid hydrolysates of hemicellulose and an important inhibitor of microbial fermentations (7–9). Expression of *fucO* from plasmids has been used to improve furfural tolerance in *Escherichia coli*-based fermentations for ethanol and lactic acid (6). The reduction of furfural to the less toxic alcohol seems essential for growth and fermentation of dilute acid hydrolysates of hemicellulose (10–13).

Deoxy sugars such as fucose seldom dominate in the natural environment of *E. coli* (14). The *fuc* genes encoding deoxy sugar metabolism remain silent unless induced by fucose or other related sugars in the absence of competing substrates (1, 2). Although a natural product, furfural is unlikely to be an important natural substrate for this enzyme.

In this study, we have used site-specific mutagenesis and growth-based selection to identify a *fucO* mutation that confers a further increase in furfural tolerance.

MATERIALS AND METHODS

Strains, media, and genetic manipulations. The strains, plasmids, and primers used in this study are listed in Table 1 (6, 15, 16). LB medium containing xylose was used for the construction of ethanol strains. AM1 minimal salts medium with xylose (17) was used for the maintenance and growth of ethanologenic strains. Solid medium contained 20 g liter^{-1} xylose. Broth cultures contained 50 g liter^{-1} xylose. Batch fermentations contained 100 g liter^{-1} xylose. Cultures were incubated at 37°C unless stated otherwise. Plates streaked with ethanologenic strains were incubated under argon.

Standard genetic methods were used for the isolation of DNA and plasmids, digestion with restriction enzymes, PCR amplification of DNA, and plasmid constructions (18). Enzymes were purchased from New England BioLabs (Ipswich, MA) and used as directed by the vendor. Plasmid constructions were confirmed by Sanger sequencing.

Design and construction of *fucO* libraries. AutoDock (19) was used to position furfural into the active site of FucO (4). This docked enzyme model served as a guide for library constructions (Fig. 1A). The first group of libraries was designed for the 5-Å region near furfural (designated Lib1 T143NNK, Lib2 N150NNK, Lib3 V152NNK, Lib4 K161NNK, Lib5 V163NNK, Lib6 F253NNK, Lib7 V165NNK, Lib8 T206NNK, Lib9 G257NNK, Lib10 C361NNK, Lib11 G363NNK, and Lib12 G364NNK). A second group of libraries was designed for the interface region of the homodimers (designated Lib13 I6NNK and Lib14 L7NNK). All 14 single-residue libraries were constructed by using QuikChange EZ (Agilent Technologies, Santa Clara, CA) with plasmid pLOI4319 as the template. pLOI4319 (pTrc99A derivative) contained a 1,302-bp fragment downstream from the *trc* promoter consisting of the 3′ end of *fucA*, the intergenic sequence, the complete *fucO* ribosomal binding and coding regions, and an additional 71 bp downstream of the native transcriptional terminator. Following PCR, template DNA was digested with DpnI, leaving only the PCR product. Resulting plasmids were transformed into *E. coli* TOP10F′ (Life Technologies, Grand Island, NY) by electroporation. Plasmid libraries of more than 1,000 colonies were prepared for each mutated amino acid.

Growth-based screening procedures. Libraries were transformed into strain XW92 and spread onto AM1 plates containing xylose and ampicillin (100 μg ml^{-1}). A total of 100 colonies from each library were

Received 25 January 2013 Accepted 6 March 2013
Published ahead of print 8 March 2013
Address correspondence to Lonnie O. Ingram, ingram@ufl.edu.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/AEM.00149-13.

doi:10.1128/AEM.00149-13

TABLE 1 Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence[a,b,c]	Reference or source
Strains		
TOP10F′	F′ [*lacI*^{q} Tn*10*(Tet^r)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lacX74 deoR nupG recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL*(Str^r) *endA1* λ$^-$	Life Technologies
BL21λ(DE3)	F$^-$ *ompT gal dcm lon hsdS$_B$*(r$_B$$^-$ m$_B$$^-$) λ(DE3 [*lacI lacUV5*-T7 gene 1 *ind1 Sam7 nin5*]	Promega
XW92	LY180 Δ*yqhD*	16
LY180[a]	Δ*frdBC*::(*frg$_{Zm}$ celY$_{Ec}$*) Δ*ldhA*::(*frg$_{Zm}$ casAB$_{Ko}$*) *adhE*::(*frg$_{Zm}$ estZ$_{Pp}$* FRT) Δ*ackA*::FRT *rrlE*::(*pdc adhA adhB* FRT) Δ*mgsA*::FRT	15
Plasmids		
pCR2.1-TOPO	P$_{lac}$, *bla*, *kan*	Life Technologies
pTrc99A	P$_{trc}$, *bla*, *lacI*^{q}	Pharmacia
pLOI4319	*fucO* in pTrc99a	6
pET-15b	T7 expression vector	Novagen
pLOI5535	*fucO*(*L7FA47V*) in pTrc99a	This study
pLOI5536	*fucO*(*L7F*) in pTrc99a	This study
pLOI5537	*fucO*(*A47V*) in pTrc99a	This study
pLOI5538	*fucO* with His tag in pET15b	This study
pLOI5539	*fucO*(*L7F*) with His tag in pET15b	This study
Primers		
Thr143-lib1-For	CAATCCCCACCACAGCAGGCNNKGCGGCAGAAGTGACCATTAAC	This study
Thr143-lib1-Rev	CACGTAGTTAATGGTCACTTCTGCCGCMNNGCCTGCTGTGGTG	This study
Asn150-lib2-For	CTGCGGCAGAAGTGACCATTNNKTACGTGATCACTGACGAAGA	This study
Asn150-lib2-Rev	CTTCGTCAGTGATCACGTAMNNAATGGTCACTTCTGCCGCAGTG	This study
Val152-lib3-For	CAGAAGTGACCATTAACTACNNKATCACTGACGAAGAAAAAC	This study
Val152-lib3-Rev	GCGCCGTTTTTCTTCGTCAGTGATMNNGTAGTTAATGGTCAC	This study
Lys161-lib4-For	GACGAAGAAAAACGGCGCNNKTTTGTTTGCGTTGATCCGC	This study
Lys161-lib4-Rev	GATATCATGCGGATCAACGCAAACAAAMNNGCGCCGTTTTTC	This study
Val163-lib5-For	CGGCGCAAGTTTNNKTGCGTTGATCCGCATGATATCCCGCAGG	This study
Val163-lib5-Rev	CCTGCGGGATATCATGCGGATCAACGCAMNNAAACTTGCGCCG	This study
Phe253-lib6-For	GCAGTATGTTGCGGGTATGGGCNNKTCGAATGTTGGGTTAGG	This study
Phe253-lib6-Rev	CACCAACCCTAACCCAACATTCGAMNNGCCCATACCCGCAAC	This study
Val165-lib7-For	CGGCGCAAGTTTGTTTGCNNKGATCCGCATGATATCCCG	This study
Val165-lib7-Rev	CGGGATATCATGCGGATCMNNGCAAACAAACTTGCGCCG	This study
Thr206-lib8-For	GCTATTGAGGGGTATATTNNKCGTGGCGCGTGGGCGCTA	This study
Thr206-lib8-Rev	TAGCGCCCACGCGCCACGMNNAATATACCCCTCAATAGC	This study
Gly257-lib9-For	ATGGGCTTCTCGAATGTTNNKTTAGGGTTGGTGCATGGT	This study
Gly257-lib9-Rev	ACCATGCACCAACCCTAAMNNAACATTCGAGAAGCCCAT	This study
Cys361-lib10-For	GCGGCACTGGATGATGTTNNKACCGGTGGCAACCCGCGT	This study
Cys361-lib10-Rev	ACGCGGGTTGCCACCGGTMNNAACATCATCCAGTGCCGC	This study
Gly363-lib11-For	CTGGATGATGTTTGTACCNNKGGCAACCCGCGTGAAGCA	This study
Gly363-lib11-Rev	TGCTTCACGCGGGTTGCCMNNGGTACAAACATCATCCAG	This study
Gly364-lib12-For	GATGATGTTTGTACCGGTNNKAACCCGCGTGAAGCAACG	This study
Gly364-lib12-Rev	CGTTGCTTCACGCGGGTTMNNACCGGTACAAACATCATC	This study
Ile6-lib13-For	ATGATGGCTAACAGAATGNNKCTGAACGAAACGGCATGGTTTGG	This study
Ile6-lib13-Rev	CCAAACCATGCCGTTTCGTTCAGMNNCATTCTGTTAGCCATCAT	This study
Leu7-lib14-For	ATGATGGCTAACAGAATGATTNNKAACGAAACGGCATGGTTTGG	This study
Leu7-lib14-Rev	CCAAACCATGCCGTTTCGTTMNNAATCATTCTGTTAGCCATCAT	This study
F-L7F-fucO	GCTAACAGAATGATTTTTAACGAAACGGCATGGTTTGGT	This study
R-L7F-fucO	ACCAAACCATGCCGTTTCGTTAAAAATCATTCTGTTAGC	This study
F-A47V-fucO	CTGGTGCAATGCGGCGTGGTGGTGAAAGTGACCGATAAGATGG	This study
R-A47V-fucO	CCATCTTATCGGTCACTTTCACCACCACGCCGCATTGCACCAG	This study
fucO-F-EcoRI	CCATGGAATTCGATTGCCGTAGTGCTG	This study
fucO-R-BamHI	GCGGATCCTGCGGTTGGTACGGTAACGGCGA	This study
fucO-F-6His-NcoI	CATGCCATGGCGATGGCTAACAGAATG	This study
fucO-R-6His-BamHI	GCGGGATCCTTAATGATGATGATGATGCCAGGCGGTATGGTAAAGCTCTACAATAT	This study
bla-RT-F	GCTATGTGGCGCGGTATTAT	This study
bla-RT-R	AAGTAAGTTGGCCGCAGTGT	This study
fucO-RT-F	ACGCCGTGGTTATCAGAAGG	This study
fucO-RT-R	CAGGTAATCCGCGCCGCTAT	This study

[a] LY180 is an ethanologenic derivative of *E. coli* W containing integrated genes from other organisms and various chromosomal mutations. Note that the *celY* gene (endoglucanase) from *Erwinia carotovora* is expressed from a *Zymomonas mobilis* surrogate promoter and integrated into the *frd* region. The *Klebsiella oxytoca casAB* operon is expressed from a *Z. mobilis* surrogate promoter and integrated into the *ldhA* region. The *Z. mobilis pdc adhA adhB* artificial operon for ethanol production is integrated and expressed from the native *rrlE* promoter. The *Pseudomonas putida estZ* (acetyl esterase) is integrated into *adhE* and expressed from a surrogate promoter from *Z. mobilis*.

[b] Restriction enzyme sites used in construction are underlined.

[c] NNK and MNN were used as codon degeneracy in forward and reverse primers, respectively.

screened individually. Colonies were inoculated into 100-well plates for seed culture growth (AM1 plus 5% xylose, 50 μg ml^{-1} ampicillin, and 0.025 mM isopropyl-β-D-thiogalactopyranoside [IPTG]) with the Bioscreen C growth curve analyzer (Growth Curves USA, Piscataway, NJ). Stationary-phase seed cultures (15 μl) were diluted into wells containing 0.39 ml screening medium (AM1 plus 10% xylose, 12.5 μg ml^{-1} ampicillin, 0.025 mM IPTG, and 12.5 mM furfural). Bioscreen C plates were incubated at 37°C (with shaking for 10 s at 30-min intervals). Cell density

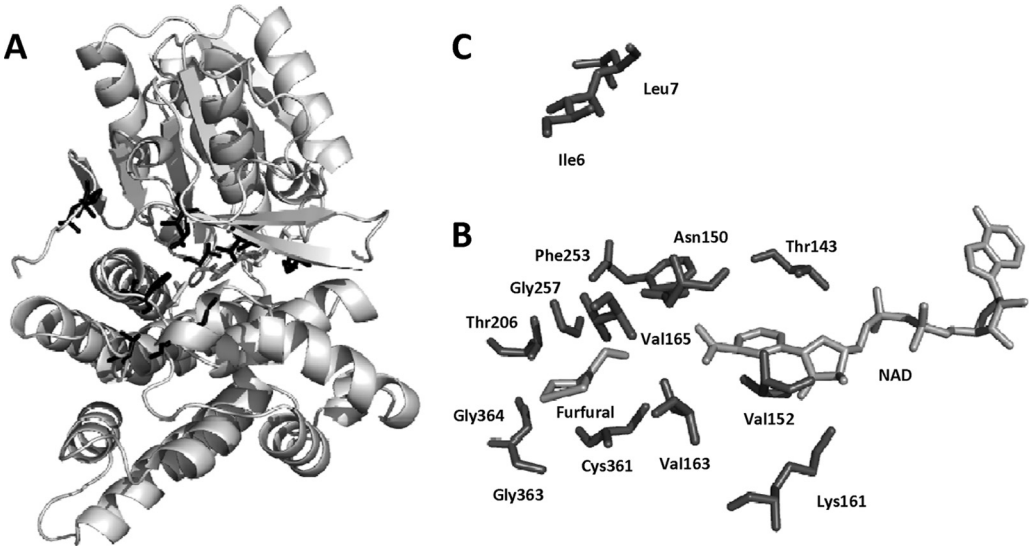

FIG 1 Crystal structure of FucO. (A) The complete FucO structure. Twelve amino acid residues residing around the active site (B) and two residues at the homodimer interface (C) were targeted for mutagenesis. Libraries: Lib1 Thr143, Lib2 Asn150, Lib3 Val152, Lib4 Lys161, Lib5 Val163, Lib6 Phe253, Lib7 Val165, Lib8 Thr206, Lib9 Gly 257, Lib10 Cys361, Lib11 Gly363, Lib12 Gly364, Lib13 Ile6, and Lib14 Leu7.

was recorded automatically. Beneficial mutations were identified by improved growth. These were confirmed by back transformation, subcloning, retesting, and sequencing.

Tube assays for furan tolerance (MIC) and batch fermentations. Furfural toxicity was examined at 37°C by using culture tubes (13 by 100 mm) containing 4 ml of AM1 minimal salts medium (50 g liter^{-1} xylose, 12.5 μg liter^{-1} ampicillin, 0.025 mM IPTG, and furfural). Tube cultures were inoculated to an initial density of 43 mg (dry cell weight) liter^{-1}. Conditions for MIC determinations and batch fermentation procedures were as previously described (15). Furfural was measured with a Beckman Coulter DU 800 spectrophotometer (20).

Assay of FucO activity and SDS-PAGE analysis. Cell cultivation was as described for tube assays of furfural tolerance. Cells were harvested after 24 h. FucO activity was determined as previously described (6). One unit of activity is defined as the amount of enzyme that converts 1 μmol of NADH to NAD$^+$ per min (6).

The protein concentrations in cell extracts and purified enzyme preparations were determined with the Pierce BCA protein reagent (Thermo, Waltham, MA) with bovine serum albumin as the standard. Whole-cell proteins and soluble proteins were examined by 12% SDS-PAGE (Bio-Rad, Hercules, CA).

Construction and purification of His-tagged FucO. Wild-type FucO and the L7F mutant were purified by affinity chromatography. A tag of six histidine residues was added at the 3′ end of each gene. PCR products with this tag were ligated into pET15b and transformed into BL21λ(DE3). The resulting clones were designated pLOI5538 (wild type) and pLOI5539 (L7F). Expression and purification of recombinant FucO were done as previously described (6), except that the induction temperature was reduced to 30°C and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was included as a reducing reagent during purification. An Amicon ultra-15 centrifugal filter was used to remove imidazole and concentrate enzymes prior to kinetic studies. Since FucO is oxygen sensitive (21), cytoplasmic activities were notably more stable in the presence of TCEP.

Transcriptional analyses of mutant *fucO*(*L7F*). The transcript abundance of native *fucO* was compared with that of the mutant *fucO*(*L7F*) gene by using cultures grown as described for tube assays of tolerance. Cells were harvested at 24 h by centrifugation at 4°C and washed once with cold distilled water. RNA was extracted and purified using RNeasy Mini columns (Qiagen) and digestion with DNase I. cDNA was prepared from 50 ng total RNA using SuperScript II. Samples were analyzed by using a Bio-Rad iCycler with SYBR green reverse transcription-PCR. Transcript abundance was estimated by using the *bla* gene (vector) as an internal standard (22, 23).

mRNA folding and energy calculations. The minimum free energy of mRNA secondary structures was calculated for partial sequences of wild-type *fucO* and mutant *fucO*(*L7F*) by using the UNAFOLD web server (http://mfold.rna.albany.edu; 28) with the default parameters. The sequence window was set from nucleotide (nt) −7 through nt +37 with the A in ATG (start codon) being designated nt 0, as proposed by G. Kudla et al. (24). Energies were calculated for a series of seven segments in which the intergenic region was increased by increments of 10 bp.

RESULTS

FucO mutation L7F increases furfural tolerance. Two groups of libraries were designed on the basis of the X-ray structures of FucO and furfural (Fig. 1A) (4, 25). The first group included 12 libraries targeting amino acids associated with furfural docking (Fig. 1B). The second group targeted amino acids located at the interface between FucO homodimers (Fig. 1C). After all 14 libraries were tested for improvements in growth with 12.5 mM furfural, a single clone was recovered and designated pLOI5535. Sequencing revealed that the *fucO* gene in this plasmid contained two mutations, L7F from library 14 (homodimer interface) and an undirected PCR error, A47V. Site-specific mutagenesis was used to create corresponding single mutations in FucO, designated pLOI5536 and pLOI5537, respectively (Fig. 2A). Only plasmids that contained the L7F mutation (pLOI5535 and pLOI5536) improved growth in medium containing 12.5 mM furfural (Bioscreen C; see Fig. S1 in the supplemental material). All enhancement of furfural tolerance resided with the single L7F mutation, a conservative replacement of leucine with another hydrophobic amino acid (phenylalanine). The 10 amino acid residues at the N terminus are required for dimerization and essential for activity (4). Dimerization is dominated by hydrophobic interactions which should be retained by the replacement of leucine with phenylalanine.

The L7F FucO mutation in strain XW92(pLOI5536) increased

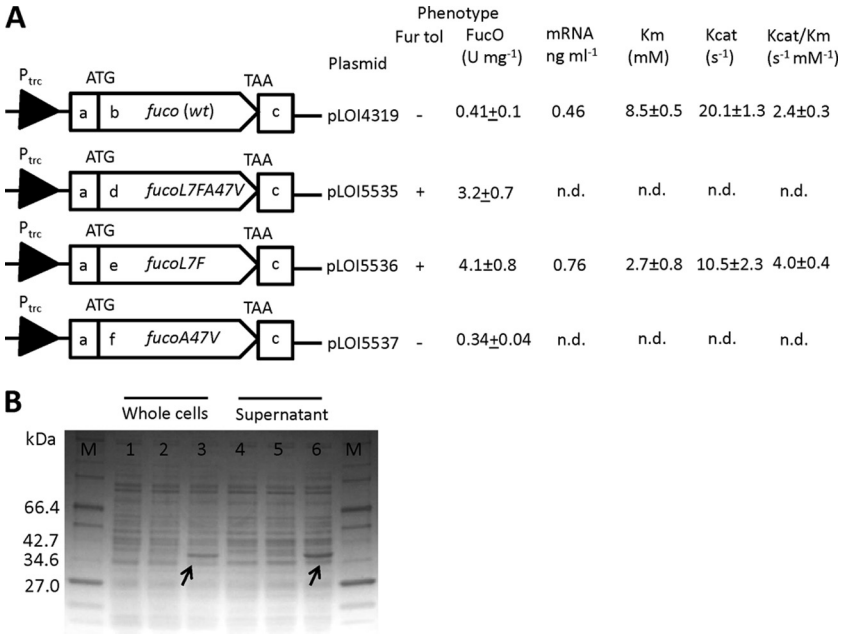

FIG 2 (A) Schematic representation plasmid constructs. Plasmid pLOI4319 contains the wild-type *fucO* gene. Plasmid pLOI5535 contains the original double mutant gene [*fucO*(*L7FA47V*)]. Plasmid pLOI5536 contains the *fucO*(*L7F*) gene. Plasmid pLOI5537 contains the *fucO*(*A47V*) gene. Assumed native ribosome binding site regions before ATG and the native terminator sequence after TAA were included while amplifying the *fucO* gene from *E. coli* W. P_{trc}, the promoter of vector pTrc99a. Segments: a, the 82 bp of chromosome sequence before starting codon ATG of the *fucO* gene; b, the wild-type *fucO* gene; c, the 71 bp of chromosome sequence after stop codon TAA of the *fucO* gene; d, the double mutant *fucO*(*L7FA47V*) gene; e, the mutant *fucO*(*L7F*) gene; f, the mutant *fucO*(*A47V*) gene. All cultures were grown under MIC conditions as described in Materials and Methods. Cells were harvested after 24 h. Samples used for transcriptional analysis and FucO assay were prepared as described in Materials and Methods. "Fur tol" means the furfural tolerance of the recombinant strain with the corresponding plasmid in the presence of 12.5 mM furfural. (B) SDS-PAGE of crude extract from strains XW92 and pTrc99a (lanes 1 and 4) and pTrc99a derivatives containing *fucO* genes. Plasmid pLOI4319 containing the wild-type *fucO* gene (lanes 2 and 5). Plasmid pLOI5536 containing the mutated *fucO*(*L7F*) gene (lanes 3 and 6). Lanes 1, 2, and 3 contained the indicated whole-cell samples at 24 h. Lanes 4, 5, and 6 contained the indicated supernatant samples at 24 h. The arrows indicate the putative FucO protein from plasmid expression. M, molecular mass marker lanes. n.d., not determined.

the MIC of furfural, in comparison to the wild-type gene (pLOI4319) or the empty vector (Fig. 3A). The L7F FucO mutation also improved the fermentation performance (strain XW92) in AM1 medium with 100 g liter^{-1} xylose and 15 mM furfural (Fig. 3B to D). With the mutant gene (pLOI5536), 15 mM furfural was completely metabolized in 12 h, compared to 24 h with the native *fucO* gene (pLOI4319) and 48 h with the empty vector. With all three strains, growth and ethanol production were delayed until furfural had been substantially metabolized to the corresponding alcohol.

Increased furfural reductase activity is responsible for improvement of furfural tolerance. The furfural reductase activity (Fig. 2A) of strain XW92 harboring the L7F mutant *fucO* gene (pLOI5535 and pLOI5536) was 10-fold higher than that of XW92 harboring the wild-type or *fucO*(*A47V*) mutant gene (pLOI4319 or pLOI5537). SDS-PAGE (12.5% acrylamide) was used to compare the protein profiles (soluble and whole cell) of the *fucO* mutant strains (Fig. 2B). A new dense band was observed in the FucO region (38.2 kDa) of the L7F mutant (pLOI5536). This band was estimated to have >10 times the intensity of a corresponding band in XW92(pLOI4319) containing the wild-type gene and was absent from strains harboring the empty vector. His-tagged plasmid constructs with wild-type FucO and L7F mutant FucO were purified and characterized. The K_m of L7F was 2.7 mM, 1/3 that of wild-type FucO (Fig. 2A). Thus, the increase in furfural tolerance associated with the L7F mutation in *fucO* appears to result from an increase in activity and an increase in enzyme effectiveness at low substrate concentrations.

The L7F mutation in *fucO* increases cytoplasmic FucO activity. Changes in transcription, translation, and enzyme stability were investigated as potential causes of the large increase in cytoplasmic FucO in the L7F mutant. FucO mRNA levels were less than 2-fold higher in XW92(pLOI5536) containing the mutant gene than in XW92(pLOI4319) containing the wild-type gene (Fig. 2A). This may contribute to increased expression but is insufficient to explain the 10-fold increase in FucO activity (Fig. 2A). Enzyme stability was also investigated by comparing activity in stationary-phase cells incubated at 37°C. After 96 h, wild-type furfural reductase activity in whole cells of XW92(pLOI4319) declined by 10% ± 9%, while that of the L7F mutant (pLOI5536) had declined by 20% ± 2%, eliminating differential protein stability as a basis for the increase in cytoplasmic FucO.

Folded mRNA structures have been shown to reduce protein synthesis by slowing translation or translational initiation (26, 27). The L7F mutation is near the N terminus of FucO and within the ribosomal binding region. The genetic region corresponding to this N terminus encodes a segment of protein essential for both quaternary structure and activity (4). Although the L7F mutation represents a conservative amino acid replacement and did not alter specific activity, the nucleotide changes could have a more dramatic effect on mRNA folding and translation.

In *E. coli*, the *fucO* gene is downstream from *fucA* and is part of

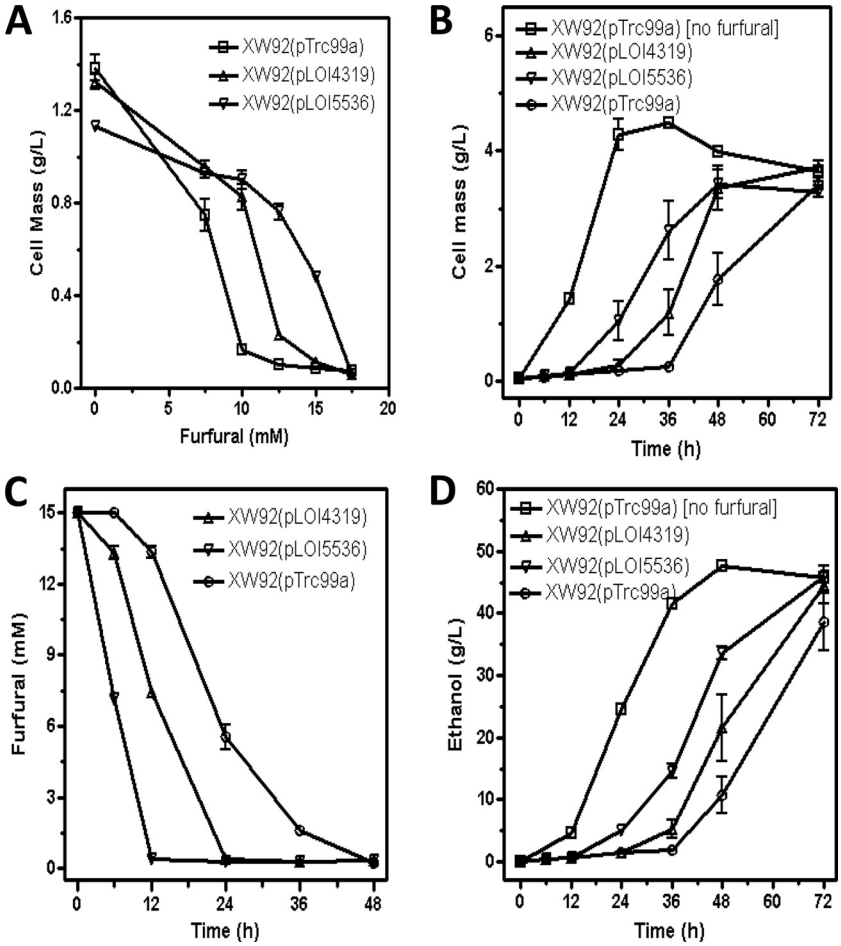

FIG 3 Increased expression of *fucO* increased the MIC of furfural and the furfural degradation rate in fermentation. (A) MIC of furfural. (B) Cell mass of fermentation on 15 mM furfural. (C) Furfural degradation of strains during fermentation with 15 mM furfural. (D) Ethanol production of strains during fermentation with 15 mM furfural.

the *fucA-fucO* operon. Using the UNAFOLD Web server (http://mfold.rna.albany.edu/; 28), minimal energy structures were predicted for a series of seven sequence segments that span the region from 38 bases of the *fucO* 5′ end through the 27-base intergenic region (segments 1 to 3) and into the *fucA* 3′ end (segments 4 to 7), increasing in length by 10-base increments. Calculated ΔG energies for all segments tested and all predicted structures for the wild-type gene were more stable (lower ΔG) than corresponding regions of the *fucO*(*L7F*) mutant (Table 2). The largest difference was observed for the shortest segment examined, the 5′ end of *fucO* plus 7 upstream bases. A single stem was predicted for this segment (4.3 kcal mol^{-1}), and this stem includes the codon (CUG) at amino acid residue 7. The mutation at position 7 to UUU eliminated this structure and was predicted to form a weaker structure. Weak mRNA structures in this region (−7 to +27 region) have been shown to correlate with increased translational initiation and improved expression (26, 29). Although predicted structures for both the native and mutant *fucO* segments were not strong, the mutant structures were consistently weaker.

The 10-fold increase in cytoplasmic activity caused by the L7F mutation appears to result from improved translation rather than increased transcription or changes in mRNA or protein stability.

TABLE 2 Predicted mRNA folding energies

mRNA[a] segment, region	Folding energy (kcal mol^{-1})[b]		Difference in energy (kcal mol^{-1})[c]
	Wild-type *fucO* gene	Mutant *fucO*(*L7F*) gene	
1, −7 to +37	−4.3 (1)	−0.38 ± 0.41 (6)	+3.92
2, −17 to +37	−4.2 ± 0.46 (3)	−2.85 ± 0.64 (2)	+1.35
3, −27 to +37	−8.0 (1)	−4.43 ± 0.42 (4)	+3.67
4, −37 to +37	−10.9 (1)	−7.05 ± 0.35 (2)	+3.85
5, −47 to +37	−10.33 ± 0.51 (3)	−8.47 ± 0.77 (3)	+1.86
6, −57 to +37	−13.48 ± 0.45 (5)	−12.0 ± 0.53 (3)	+1.48
7, −67 to +37	−15.84 ± 0.38 (5)	−14.0 ± 0.53 (3)	+1.84

[a] Segments were numbered by designating the adenosine nucleotide of the *fucO* start codon zero, increasing in the direction of translation. Negative numbers from −1 to −27 represent bases in the intergenic region between *fucA* and *fucO* (upstream) and proceeding further upstream into the 3′ end of *fucA* (−27 to −67).
[b] Minimal folding energies and structures were predicted by the UNAFOLD web server (http://mfold.rna.albany.edu; 28). Average values are presented with standard deviations. The values in parentheses are the numbers of predicted structures.
[c] Wild-type *fucO* gene minus mutant *fucO*(*L7F*) gene. Energies for all predicted structures were more positive (weaker) for the mutant *fucO*(*L7F*) than for the wild-type mRNA segments.

DISCUSSION

The crystal structure of FucO has been described previously and exhibits remarkable similarity to two other oxidoreductases in *E. coli*, YqhD (NADPH-linked furfural reductase) (30, 31) and AdhE (NADH-linked alcohol dehydrogenase) (32, 40). All three of these enzymes have broad substrate ranges. Although YqhD can effectively reduce furfural, the utility of this enzyme is severely limited by the low K_m for NADPH that competes with essential biosynthetic reactions (15, 33). NADH is abundant during fermentative metabolism, and the small amounts needed for furfural reduction by *fucO* should not adversely impact growth or product yields.

Removal of furfural from lignocellulosic sugars is essential for effective fermentation (10, 39). The toxicity of hydrolysates is correlated with the concentration of furfural (9, 13). Cells typically initiate rapid growth only after furfural is fully metabolized (10). Plasmid-based expression of *fucO* improved furfural tolerance (6) and provided an opportunity for further improvement by site-specific mutagenesis. Growth-based selection for furfural tolerance provided a powerful screening method, recovering a single mutation (L7F) that increased cytoplasmic activity 10-fold and increased the affinity for furfural (3-fold lower K_m).

Many methods are available to create sequence diversity libraries, including chemical mutagenesis (34), error-prone PCR, saturation mutagenesis, and DNA shuffling (29, 35, 36, 41). However, effective screening often remains as a bottleneck. Recently, a Q263R transaldolase mutant was obtained by directed evolution with a 5-fold increase in activity. Almost 60,000 colonies were subjected to high-throughput TAL activity screening with a sensitive fluorescence assay (37). A similar success (site-specific mutagenesis and growth-based screening) has been reported previously for the expression of *Piromyces* sp. xylose isomerase (*xylA*) in *Saccharomyces cerevisiae* (38). Cytoplasmic activities were increased by 77%, and ethanol production from xylose was dramatically improved (38). In contrast, 14 single residues were targeted in FucO for saturation mutagenesis libraries based on a visual inspection of furfural docked in the FucO structure (Fig. 1). The *fucO*(*L7F*) mutant exhibited a 10-fold increase in cytoplasmic activity and was isolated after the screening of only 1,400 colonies. The site of this mutation was unexpected, with the contact region of homodimers distant from the active site. With the mutant gene, furfural was metabolized *in vivo* at twice the rate of the native enzyme during fermentation.

ACKNOWLEDGMENTS

L. O. Ingram is a consultant for the Myriant Corporation and is a minor shareholder.

This research was supported by the Myriant Corporation, the Department of Agriculture (grant 2011-10006-30358), and the Department of Energy (grant DE-PI0000031).

REFERENCES

1. **Boronat A, Aguilar J.** 1979. Rhamnose-induced propanediol oxidoreductase in *Escherichia coli*: purification, properties, and comparison with the fucose-induced enzyme. J. Bacteriol. **140**:320–326.
2. **Chen YM, Lin ECC.** 1984. Dual control of a common L-1,2-propanediol oxidoreductase by L-fucose and L-rhamnose in *Escherichia coli*. J. Bacteriol. **157**:828–832.
3. **Zhu Y, Lin ECC.** 1989. L-1,2-Propanediol exits more rapidly than L-lactaldehyde from *Escherichia coli*. J. Bacteriol. **171**:862–867.
4. **Montella C, Bellsolell L, Perez-Luque R, Badia J, Baldoma L, Coll M, Aguilar J.** 2005. Crystal structure of an iron-dependent group III dehydrogenase that interconverts L-lactaldehyde and L-1,2-propanediol in *Escherichia coli*. J. Bacteriol. **187**:4957–4966.
5. **Blikstad C, Widersten M.** 2010. Functional characterization of a stereospecific diol dehydrogenase, FucO, from *Escherichia coli*: substrate specificity, pH dependence, kinetic isotope effects and influence of solvent viscosity. J. Mol. Catal. B Enzym. **66**:148–155.
6. **Wang X, Miller EN, Yomano LP, Zhang X, Shanmugam KT, Ingram LO.** 2011. Increased furfural tolerance due to overexpression of NADH-dependent oxidoreductase FucO in *Escherichia coli* strains engineered for the production of ethanol and lactate. Appl. Environ. Microbiol. **77**:5132–5140.
7. **Mills TY, Sandoval NR, Gill RT.** 2009. Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. Biotechnol. Biofuels **2**:11. doi:10.1186/1754-6834-2-11.
8. **Parawira W, Tekere M.** 2011. Biotechnological strategies to overcome inhibitors in lignocellulose hydrolysates for ethanol production: review. Crit. Rev. Biotechnol. **31**:20–31.
9. **Zheng HB, Wang X, Yomano LP, Shanmugam KT, Ingram LO.** 2012. Increase in furfural tolerance in ethanologenic *Escherichia coli* LY180 by plasmid-based expression of *thyA*. Appl. Environ. Microbiol. **78**:4346–4352.
10. **Geddes CC, Nieves IU, Ingram LO.** 2011. Advances in ethanol production. Curr. Opin. Biotechnol. **22**:312–319.
11. **Martinez A, Rodriguez ME, Wells ML, York SW, Preston JF, Ingram LO.** 2001. Detoxification of dilute acid hydrolysates of lignocellulose with lime. Biotechnol. Prog. **17**:287–293.
12. **Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO.** 2000. Effects of $Ca(OH)_2$ treatments ("overliming") on the composition and toxicity of bagasse hemicellulose hydrolysates. Biotechnol. Bioeng. **69**:526–536.
13. **Zaldivar J, Martinez A, Ingram LO.** 1999. Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. Biotechnol. Bioeng. **65**:24–33.
14. **Mayer C, Boos W.** 29 March 2005, posting date. Chapter 3.4.1, Hexose/pentose and hexitol/pentitol metabolism. *In* Böck A (ed), EcoSal---*Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC. http://www.ecosal.org.
15. **Miller EN, Jarboe LR, Yomano LP, York SW, Shanmugam KT, Ingram LO.** 2009. Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. Appl. Environ. Microbiol. **75**:4315–4323.
16. **Wang X, Yomano LP, Lee JY, York SW, Zheng HB, Mullinnix MT, Shanmugam KT, Ingram LO.** 2013. Engineering furfural tolerance in Escherichia coli improves the fermentation of lignocellulosic sugars into renewable chemicals. Proc. Natl. Acad. Sci. U. S. A. **110**:4021–4026.
17. **Martinez A, Grabar TB, Shanmugam KT, Yomano LP, York SW, Ingram LO.** 2007. Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. Biotechnol. Lett. **29**:397–404.
18. **Sambrook J, Fritsch EF, Maniatis T.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
19. **Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ.** 2009. Autodock4 and AutoDockTools4: automated docking with selective receptor flexibility. J. Comput. Chem. **30**:2785–2791.
20. **Martinez A, Rodriguez ME, York SW, Preston JF, Ingram LO.** 2000. Use of UV absorbance to monitor furans in dilute acid hydrolysates of biomass. Biotechnol. Prog. **16**:637–641.
21. **Cabiscol E, Hidalgo E, Badia J, Baldoma L, Ros JQ, Aguilar J.** 1990. Oxygen regulation of L-1,2-propanediol oxidoreductase activity in *Escherichia coli*. J. Bacteriol. **172**:5514–5515.
22. **Su Y, Rhee MS, Ingram LO, Shanmugam KT.** 2011. Physiological and fermentation properties of *Bacillus coagulans* and a mutant lacking fermentative lactate dehydrogenase activity. J. Ind. Microbiol. Biotechnol. **38**:441–450.
23. **Wang QZ, Ingram LO, Shanmugam KT.** 2011. Evolution of D-lactate dehydrogenase activity from glycerol dehydrogenase and its utility for D-lactate production from lignocellulose. Proc. Natl. Acad. Sci. U. S. A. **108**:18920–18925.
24. **Kudla G, Murray AW, Tollervey D, Plotkin JB.** 2009. Coding-sequence determinants of gene expression *in Escherichia coli*. Science **324**:255–258.
25. **Miller EN, Jarboe LR, Turner PC, Pharkya P, Yomano LP, York SW, Nunn D, Shanmugam KT, Ingram LO.** 2009. Furfural inhibits growth by

limiting sulfur assimilation in ethanologenic *Escherichia coli* strain LY180. Appl. Environ. Microbiol. **75**:6132–6141.
26. **Jia M, Li Y.** 2005. The Relationship among gene expression, folding free energy and codon usage bias in *Escherichia coli*. FEBS Lett. **579**:5333–5337.
27. **Tsao D, Shabalina SA, Gauthier J, Dokholyan NV, Diatchenko L.** 2011. Disruptive mRNA folding increases translational efficiency of catechol-*O*-methyltransferase variant. Nucleic Acids Res. **39**:6201–6212.
28. **Zuker M.** 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. **31**:3406–3415.
29. **Labrou NE.** 2010. Random mutagenesis methods for i*n vitro* directed enzyme evolution. Curr. Protein Pept. Sci. **11**:91–100.
30. **Jarboe LR.** 2011. YqhD: a broad-substrate range aldehyde reductase with various applications in production of biorenewable fuels and chemicals. Appl. Microbiol. Biotechnol. **89**:249–257.
31. **Sulzenbacher G, Alvarez K, van den Heuvel RHH, Versluis C, Spinelli M, Campanacci V, Valencia C, Cambillau C, Eklund H, Tegoni M.** 2004. Crystal structure of *E. coli* alcohol dehydrogenase YqhD: evidence of a covalently modified NADP coenzyme. J. Mol. Biol. **342**:489–502.
32. **Conway T, Ingram LO.** 1989. Similarity of *Escherichia coli* propanediol oxidoreductase (*fucO* product) and an unusual alcohol dehydrogenase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*. J. Bacteriol. **171**:3754–3759.
33. **Turner PC, Miller EN, Jarboe LR, Baggett CL, Shanmugam KT, Ingram LO.** 2011. YqhC regulates transcription of the adjacent *Escherichia coli* genes yqhD and dkgA that are involved in furfural tolerance. J. Ind. Microbiol. Biotechnol. **38**:431–439.
34. **Kim Y, Ingram LO, Shanmugam KT.** 2008. Dihydrolipoamide dehydrogenase mutation alters the NADH sensitivity of pyruvate dehydrogenase complex of *Escherichia coli* K-12. J. Bacteriol. **190**:3851–3858.
35. **Reetz MT.** 2011. Laboratory evolution of stereoselective enzymes: a prolific source of catalysts for asymmetric reactions. Angew. Chem. Int. Ed. Engl. **50**:138–174.
36. **Wang M, Si T, Zhao HM.** 2012. Biocatalyst development by directed evolution. Bioresour. Technol. **115**:117–125.
37. **Chen SH, Hwang DR, Chen GH, Hsu NS, Wu YT, Li TL, Wong CH.** 2012. Engineering transaldolase in *Pichia stipitis* to improve bioethanol production. ACS Chem. Biol. **7**:481–486.
38. **Lee S-M, Jellison T, Alper HS.** 2012. Directed evolution of xylose isomerase for improved xylose catabolism and fermentation in the yeast *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **78**:5708–5716.
39. **Chundawat SPS, Vismeh R, Sharma LN, Humpula JF, Sousa LD, Chambliss CK, Jones AD, Balan V, Dale BE.** 2010. Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. Bioresour. Technol. **101**:8429–8438.
40. **Reid MF, Fewson CA.** 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. **20**:13–56.
41. **Roodveldt C, Aharoni A, Tawfik DS.** 2005. Directed evolution of proteins for heterologous expression and stability. Curr. Opin. Struct. Biol. **15**:50–56.