Engineering furfural tolerance in *Escherichia coli* improves the fermentation of lignocellulosic sugars into renewable chemicals

Xuan Wang, Lorraine P. Yomano, James Y. Lee, Sean W. York, Huabao Zheng, Michael T. Mullinnix, K. T. Shanmugam, and Lonnie O. Ingram[1]

Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611

Edited by Arnold L. Demain, Drew University, Madison, NJ, and approved January 28, 2013 (received for review October 17, 2012)

Pretreatments such as dilute acid at elevated temperature are effective for the hydrolysis of pentose polymers in hemicellulose and also increase the access of enzymes to cellulose fibers. However, the fermentation of resulting syrups is hindered by minor reaction products such as furfural from pentose dehydration. To mitigate this problem, four genetic traits have been identified that increase furfural tolerance in ethanol-producing *Escherichia coli* LY180 (strain W derivative): increased expression of *fucO*, *ucpA*, or *pntAB* and deletion of *yqhD*. Plasmids and integrated strains were used to characterize epistatic interactions among traits and to identify the most effective combinations. Furfural resistance traits were subsequently integrated into the chromosome of LY180 to construct strain XW129 (LY180 Δ*yqhD ackA::*P_{yadC}*-fucO-ucpA*) for ethanol. This same combination of traits was also constructed in succinate biocatalysts (*Escherichia coli* strain C derivatives) and found to increase furfural tolerance. Strains engineered for resistance to furfural were also more resistant to the mixture of inhibitors in hemicellulose hydrolysates, confirming the importance of furfural as an inhibitory component. With resistant biocatalysts, product yields (ethanol and succinate) from hemicellulose syrups were equal to control fermentations in laboratory media without inhibitors. The combination of genetic traits identified for the production of ethanol (strain W derivative) and succinate (strain C derivative) may prove useful for other renewable chemicals from lignocellulosic sugars.

lignocellulose | metabolic engineering

The carbohydrate component of lignocellulose represents a potential feedstock for renewable fuels and chemicals (1–3), an alternative to food crops and petroleum. However, the cost-effective use of lignocellulosic sugars in fermentation remains challenging (4, 5). Unlike starch, lignocellulose has been designed by nature to resist deconstruction (2, 6). Crystalline fibers of cellulose are encased in a covalently linked mesh of lignin and hemicellulose. Steam pretreatment with dilute mineral acids is an efficient approach to depolymerize hemicellulose (20–40% of biomass dry weight) into sugars (hemicellulose hydrolysate, primarily xylose) and to increase the access of cellulase enzymes (2, 3, 6). However, side-reaction products (furfural, 5-hydroxymethylfurfural, formate, acetate, and soluble lignin products) are formed during pretreatment that hinder fermentation (7, 8). Furfural (dehydration product of pentose sugars) is widely regarded as one of the most important inhibitors (6–8). The concentration of furfural is correlated with the toxicity of dilute acid hydrolysates (9). Although overliming to pH 10 with $Ca(OH)_2$ can be used to reduce the level of furfural and toxicity, inclusion of this step increases process complexity and costs (9, 10).

Escherichia coli and yeasts have proven to be excellent biocatalysts for metabolic engineering (11, 12). However, both are inhibited by furan aldehydes (7, 8, 13–15) and both contain NADPH-dependent oxidoreductases that convert furfural and hydroxymethylfurfural (dehydration product of hexose sugars) into less toxic alcohols (15–17). It is this depletion of NADPH by oxidoreductases such as YqhD (low K_m for NADPH) that has been proposed as the mechanism for growth inhibition in *E. coli* (Fig.1) (18–20). Growth resumed only after the complete reduction of furan aldehydes (19). A similar furan aldehyde-induced delay in growth has been reported for fermenting yeasts (14, 15). The NADPH-intensive pathway for sulfate assimilation was identified as an early site affected by furfural (18). Addition of cysteine (18), deletion of *yqhD* (19) or increased expression of *pntAB* (transhydrogenase for interconversion of NADH and NADPH) increased tolerance to furan aldehydes (18, 21) (Fig. 1). Furfural tolerance was also increased by overexpression of an NADH-dependent propanediol (and furfural) oxidoreductase (FucO) normally used for fucose metabolism (17) and by overexpression of a cryptic gene (*ucpA*) adjacent to a sulfur assimilation operon (22) (Fig. 1). However, none of these traits alone fully eliminated the problem of furfural toxicity.

Little is known about the interactions among these four genetic traits. In this study, combinations of traits were constructed and tested for furfural tolerance in a derivative of *E. coli* W (LY180). Optimal combinations identified for ethanol production by LY180 derivatives were subsequently reengineered into strain KJ122, strain C engineered to produce succinate (precursor for solvents and plastics) (17). Resulting strains exhibited an increase in furfural tolerance and an increase in tolerance to toxins in hemicellulose hydrolysates.

Results

Epistatic Interactions Among Four Furfural Resistance Traits in Ethanologenic LY180. Previous studies have shown that deletion of *yqhD* and increased expression of *fucO*, *ucpA*, or *pntAB* from plasmids each improved growth of ethanologenic *E. coli* LY180 in the presence of 10 mM furfural (17–19, 22). Further constructions (*SI Text* and Table S1) were made to allow a comparison of all combinations of these genetic traits using pTrc99a-based plasmids for expression of target genes (*fucO*, *ucpA*, and *fucO-ucpA*). Three new derivatives of LY180 were constructed for use as host strains: Δ*yqhD*, *adhE::pntAB*, and Δ*yqhD adhE::pntAB*. Integration of *pntAB* behind the *adhE* promoter in LY180 provided furfural tolerance equivalent to pTrc99a expressing *pntAB*

Author contributions: X.W., K.T.S., and L.O.I. designed research; X.W., L.P.Y., J.Y.L., S.W.Y., H.Z., and M.T.M. performed research; X.W., L.P.Y., J.Y.L., S.W.Y., H.Z., M.T.M., K.T.S., and L.O.I. analyzed data; and X.W., K.T.S., and L.O.I. wrote the paper.

Conflict of interest statement: L.O.I. has less than 4% stock ownership in a company currently producing monomers for renewable plastics from edible sugars. This company hopes to use less expensive, wood-derived sugars that do not compete with food in the future.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: ingram@ufl.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1217958110/-/DCSupplemental.

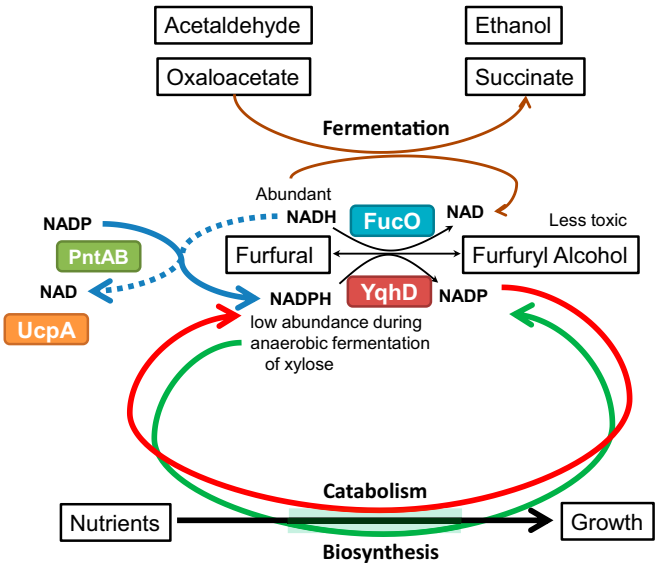

Fig. 1. Model showing relationships of furfural resistance traits, metabolism, and reducing cofactors. NADPH-linked reduction of furfural by YqhD is proposed to compete with biosynthesis, starving key steps in biosynthesis such as sulfate assimilation (18, 19). Deletion of *yqhD* or increased expression of *pntAB* (NADH/NADPH transhydrogenase) mitigated this problem by increasing the availability of NADPH. Overexpression of *fucO* increased the rate of furfural reduction and used NADH, an abundant cofactor during sugar fermentation (17). The cryptic gene *ucpA* is required for native furfural tolerance and further increased furfural resistance when overexpressed (22).

(uninduced). Higher levels of *pntAB* expression with inducer were inhibitory in the absence or presence of furfural (18).

Ethanol production from 100 g/L xylose was complete after 48 h in control cultures lacking furfural (Fig. 2*A*). Ethanol production at this time point was selected as a comparative measure of tolerance to 15 mM furfural. All individual traits except *fucO* improved ethanol production in the presence of 15 mM furfural (Fig. 2*A*). Combinations of two traits (Fig. 2*B*) were more effective than single traits with two exceptions: (*i*) Δ*yqhD* with *pntAB* integration and (*ii*) Δ*yqhD* with the *ucpA* plasmid (pLOI4856). All binary combinations with *fucO* were beneficial. Because growth and ethanol production were also inhibited by excess *pntAB* expression (18), the negative interactions between *pntAB* (increased NADPH production) and Δ*yqhD* (reduced NADPH consumption) could result from a similar problem. The poor performance of LY180 Δ*yqhD* containing the *ucpA* plasmid suggests that this cryptic gene may be associated with a similar action. Among ternary combinations, the combination of Δ*yqhD adhE::pntAB* and *ucpA* plasmid was particularly sensitive to furfural inhibition. Ethanol titer was low (13 g/L) when all four genetic traits were combined, comparable to strains with a single resistance trait (Fig. 2*B*). The most effective combinations were plasmid expression of *fucO-ucpA* in a host strain with either Δ*yqhD* or *adhE*::*pntAB*. Both constructs produced close to 30 g/L ethanol after 48 h in medium with 15 mM furfural, about 70% of the ethanol titer in control fermentations without furfural (Fig. 2*B*).

Constructing Plasmid-Free Strains for Ethanol Production (Integration of *fucO-ucpA*). The use of plasmids, antibiotics, and expensive inducers allowed an investigation of gene interactions but is unlikely to provide the desired genetic stability needed for commercial strains. Chromosomal integration of *fucO-ucpA* behind a strong promoter such as *ackA* (highly expressed in mRNA arrays) (18, 20, 22) was tested as a replacement for plasmid pTrc *fucO-ucpA* in LY180 *adhE*::*pntAB* and LY180 Δ*yqhD*. However, FucO activity of the integrated strains was low (Fig. 3*A*) and furfural tolerance (12.5 mM) was unchanged (Fig. 3*B*). Integration behind the strong *pflB* promoter (18, 20, 22, 23) also did not provide

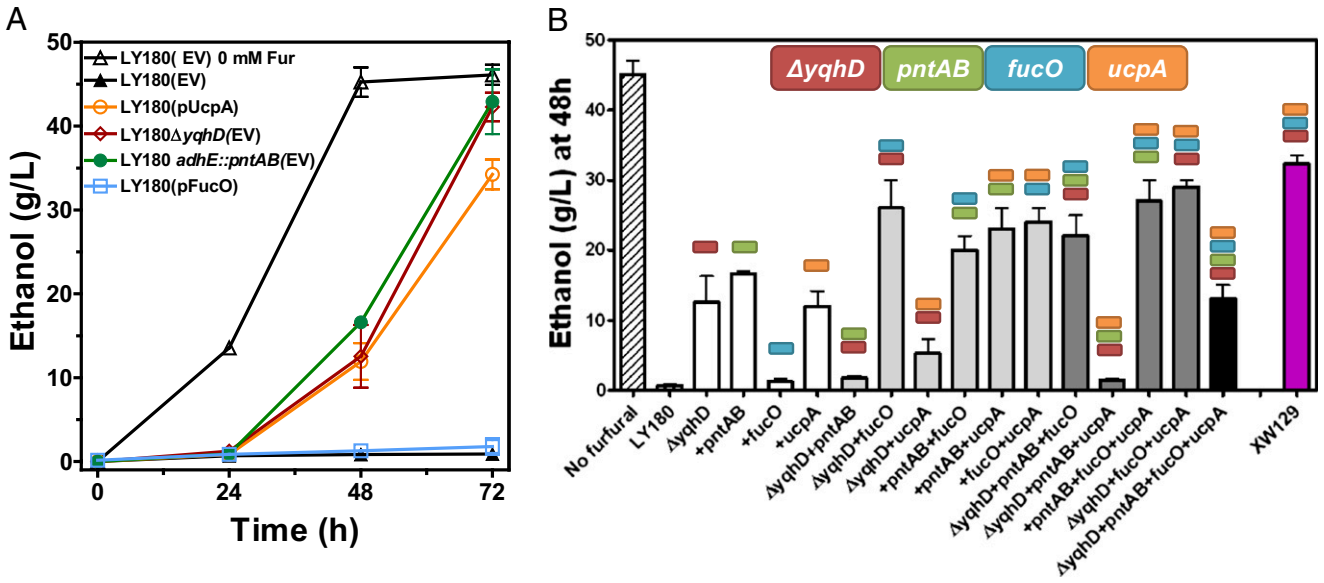

Fig. 2. Epistatic interactions of furfural resistance traits during ethanol production. Fermentations were conducted in isopropyl-β-D-1-thiogalactopyranoside mineral salts medium (100 g/L xylose, 0.1 mM IPTG, and 12.5 mg/L ampicillin) with 15 mM furfural. (*A*) Single furfural-resistant traits. LY180 containing empty vector pTrc99a (EV) was included as a control with and without furfural. LY180 Δ*yqhD* and LY180 *adhE::pntAB* also contained an empty vector to reduce differences related to plasmid burden. (*B*) Comparison of furfural tolerance for ethanol production (48 h). Test strains contain either empty vector or plasmids for expression of *fucO*, *ucpA*, or *fucO-ucpA*. Ethanol titers of parent strain LY180 (hatched bars) were included with or without furfural for comparison. Modified strains contain a single trait (open bars), two traits (light gray bars), three traits (dark gray bars), or four traits (black bar). Strain XW129 (LY180 Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA*) was obtained after promoter engineering and chromosomal integration (purple bar). The four-color boxes at the top represent a key to genetic traits. Stacked boxes correspond to traits in each respective strain. Data represent averages of at least two experiments with SDs.

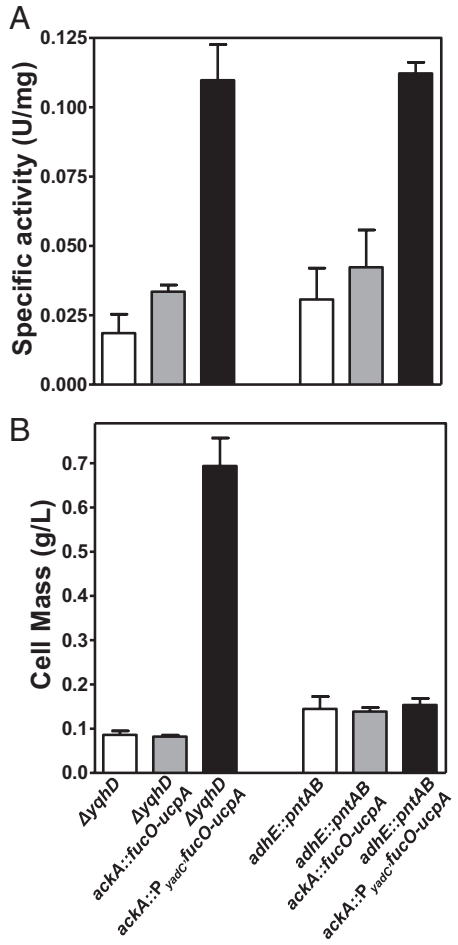

Fig. 3. Comparison of in vitro furfural reductase activity and furfural resistance. NADH-linked furfural-dependent reductase activity (*A*) and furfural tolerance for growth (*B*) are shown for plasmid-free strains containing predicted optimal combinations of furfural resistance traits. Cell mass was measured from tube cultures ($n = 3$) grown for 48 h in AM1 minimal media containing 50 g/L xylose with 12.5 mM furfural. Data represent averages of at least two experiments with SDs.

sufficient expression of *fucO-ucpA* for furfural tolerance. Clearly, a more efficient approach was needed.

A function-based selection was used to identify a useful promoter. A promoter probe vector was constructed for *fucO-ucpA* as a derivative of pACYC184 (low copy) with an appropriately engineered upstream BamH1 site (Fig. S1*A* and *SI Text*). Random Sau3A1 fragments (*E. coli* W chromosome) were ligated into this site, and resulting plasmids transformed into LY180 Δ*yqhD*. After selection for large colonies on furfural (12 mM) plates and further screening, the most effective promoter was identified by sequencing as a 600-bp internal fragment of the *E. coli yadC* gene, designated $P_{yadC'}$, in plasmid pLOI5259 (Fig. S1*B*). With this promoter, constitutive expression of *fucO* on a low-copy plasmid (pACYC184) was equal to induced expression of *fucO* from a high-copy plasmid (pTrc99a) (Fig. S1). The nucleotide sequences for this promoter and more details concerning isolation are included in *SI Text*.

The expression cassette from pLOI5259 (*ackA′*::P_{yadC}*fucO-ucpA -ackA′*) was amplified by PCR (Table S1) and integrated into the chromosomes of LY180 Δ*yqhD* and LY180 *adhE*::*pntAB* by precisely replacing the *ackA* coding region including 22 bp immediately upstream. Resulting strains were designated XW129 and XW131, respectively. Although both integrated strains produced fourfold to sixfold higher FucO activity than the respective parent strain (Fig. 3*A*), furfural tolerance was only improved in XW129 (Fig. 3*B*). It is possible that the higher level of FucO produced with plasmids (0.7 U/mg protein; Fig. S1*D*) is required to increase tolerance in the *adhE::pntAB* strain (XW131), where *yqhD* remains functional. Further studies have focused on the useful combination of genetic traits assembled in XW129 (Δ*yqhD* and increased expression of *fucO-ucpA*).

Integration of Traits Restored Ethanol Fermentation in 15 mM Furfural. Strain XW129(LY180 Δ*yqhD ackA*::P_{yadC}*fucO-ucpA*) was compared with the parent LY180 during batch fermentation in AM1 mineral salts medium (100 g/L xylose) with and without 15 mM furfural (Fig. 4 *A* and *B*). In the absence of furfural, ethanol yields for both strains were equal. In the presence of 15 mM furfural, growth and fermentation of LY180 was completely blocked. Only 5 mM furfural was metabolized (reduced to furfuryl alcohol) by LY180 after 72 h. Addition of 15 mM furfural delayed the growth of strain XW129 by 24 h, during which time furfural was fully reduced. However, the time required to complete fermentation was extended by only 6 h. The final ethanol yield for strain XW129 with 15 mM furfural was equal to the control without added furfural, 90% of the theoretical yield. Despite being sixfold lower in FucO activity (Fig. 3*A* and Fig. S1*D*), ethanol titers (32 g/L after 48 h) for strain XW129 (LY180 Δ*yqhD ackA*::P_{yadC}*fucO-ucpA*) with integrated *fucO-ucpA* were equivalent to LY180 Δ*yqhD* with induced expression of *fucO-ucpA* from plasmids (Fig. 2*B*). This suggests that the metabolic burden of plasmid maintenance and producing larger amounts of target protein (FucO, UcpA) may have countered any benefit from the additional activities.

Furfural-Resistance Traits also Increased Resistance to Hemicellulose Hydrolysate. The inhibitory role of furfural in dilute acid hydrolysates of hemicellulose was confirmed in part by a comparison of batch fermentations containing sugarcane bagasse hemicellulose hydrolysate (Fig. 4*C*). The onset of rapid ethanol production was delayed in hydrolysate, similar to the delay with 15 mM furfural in AM1 medium containing 10% xylose (Fig. 4*B*). The onset of rapid ethanol production in AM1 medium with furfural and in hydrolysate medium (LY180 and XW129) again coincided with the depletion of furfural. Although total fermentation time in hydrolysate medium and final ethanol titers were similar for both the parent LY180 and the engineered strain XW129, the furfural-resistant XW129 reduced furfural at twice the volumetric rate of LY180. This more rapid reduction of furfural by XW129 shortened the initial delay in ethanol production by 24 h, half that of the parent (Fig. 4*C*).

Reengineering *E. coli* KJ122 for Conversion of Hemicellulosic Hydrolysates to Succinate. Strain LY180 is derived from *E. coli* KO11, a sequenced strain that has acquired many mutations during laboratory selections for growth in mixed sugars, high sugars, lactate resistance, and other conditions (24–26). It is possible that some of the mutations in KO11 or the heterologous genes encoding ethanol production in this strain may be critical for engineering furfural tolerance and improving resistance to hemicellulose hydrolysate. To address this concern, we have reconstructed the optimal traits for furfural resistance in KJ122, a succinate-producing derivative of *E. coli* C (27). Initially, strain KJ122 was unable to effectively ferment 100 g/L xylose (Fig. 5*A*). Mutants with fivefold improvement of succinate titer were readily selected after 40 generations of serial cultivation in xylose AM1 medium. A clone was isolated and designated XW055 with a succinate yield from xylose of 0.9 g/g, equivalent to the yield reported previously for glucose (27).

The same genetic tools used to construct furfural tolerance in ethanol-producing biocatalysts were used to engineer XW055 (Fig. S2 and Fig. 5*B*). As with ethanol biocatalysts, combining a

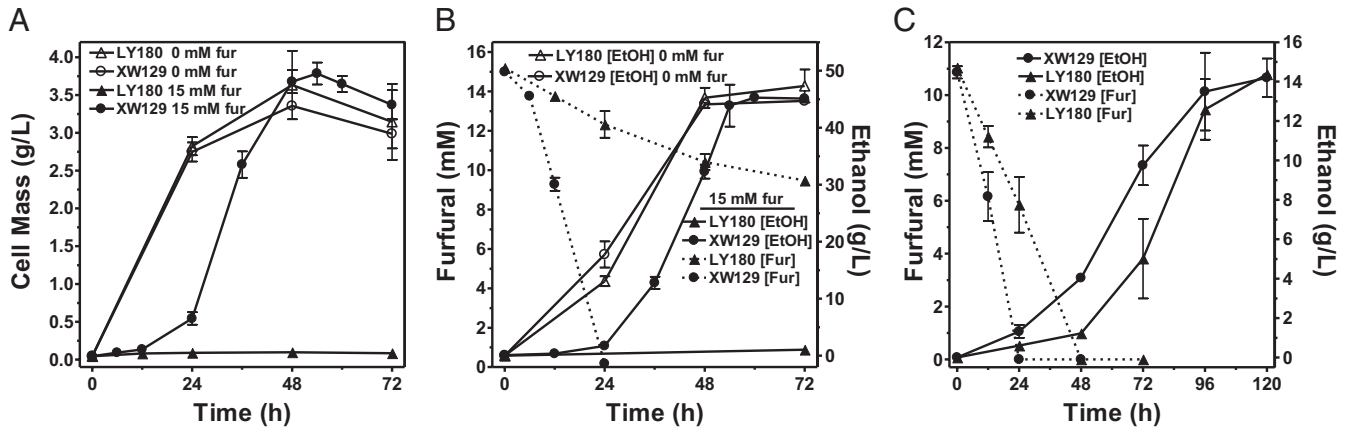

Fig. 4. Comparison of batch fermentations for the parent LY180 and the plasmid-free, furfural-resistant strain XW129. Furfural resistance traits in XW129 (LY180 Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA*) improved fermentation with furfural in AM1 medium and also improved the fermentation of hemicellulose hydrolysate. For *A* (cell mass) and *B* (ethanol and furfural), fermentations were conducted in mineral salt medium AM1 (100 g/L xylose and 15 mM furfural). Control fermentations without furfural were also included. Fermentations (*C*) were also conducted using hemicellulose hydrolysate containing 36 g/L total sugar, supplemented with components of AM1 medium and 0.5 mM sodium metabisulfite. Data represent averages of at least two experiments with SDs.

yqhD deletion with integration of *pntAB* was not helpful (Fig. S2). The most effective combination for succinate production was Δ*yqhD* and *ackA*::P_{yadC}-*fucO-ucpA*, resulting in strain XW120 (Fig. S2 and Fig. 5*B*). These genetic changes increased the minimal inhibitory concentration of furfural from 7.5 mM (XW055) to 15 mM (XW120). Plasmid derivatives of pTrc99a expressing *fucO* alone and *ucpA* alone were tested in XW120. Addition of a *fucO* plasmid further increased furfural tolerance (Fig. S3). The benefit of this plasmid was supplied by another chromosomal integration, replacing the coding region of *adhE* with the coding region of *fucO* to make XW136. The additional expression of *fucO* from the *adhE* promoter increased furfural tolerance to 17.5 mM (Fig. 5*B*).

XW055 and the furfural-resistant XW136 (XW055, Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA adhE*::*fucO*) were compared during batch fermentation using hemicellulose hydrolysate as a source of sugar (Fig. 5*C*). Hydrolysate medium contained 12 mM furfural and completely inhibited growth and fermentation of the parent. During 96 h of incubation, the parent reduced only 3 mM furfural and was unable to grow or effectively ferment hemicellulose sugars. In contrast, furfural (12 mM) was completely reduced within 24 h by the furfural-resistant strain XW136. With this strain, fermentation of hemicellulose sugars (primarily xylose) into succinate was complete after 96 h with a yield of 0.9 g/g. This succinate yield from hemicellulose sugars was equivalent to that of the parent organism (KJ122) during the fermentation of glucose in AM1 mineral salts medium without furfural (27).

Discussion

Importance of Furfural As an Inhibitor in Hemicellulose Hydrolysate. Microbial biocatalysts can be used to produce renewable chemicals from lignocellulosic sugars. Large-scale implementation of

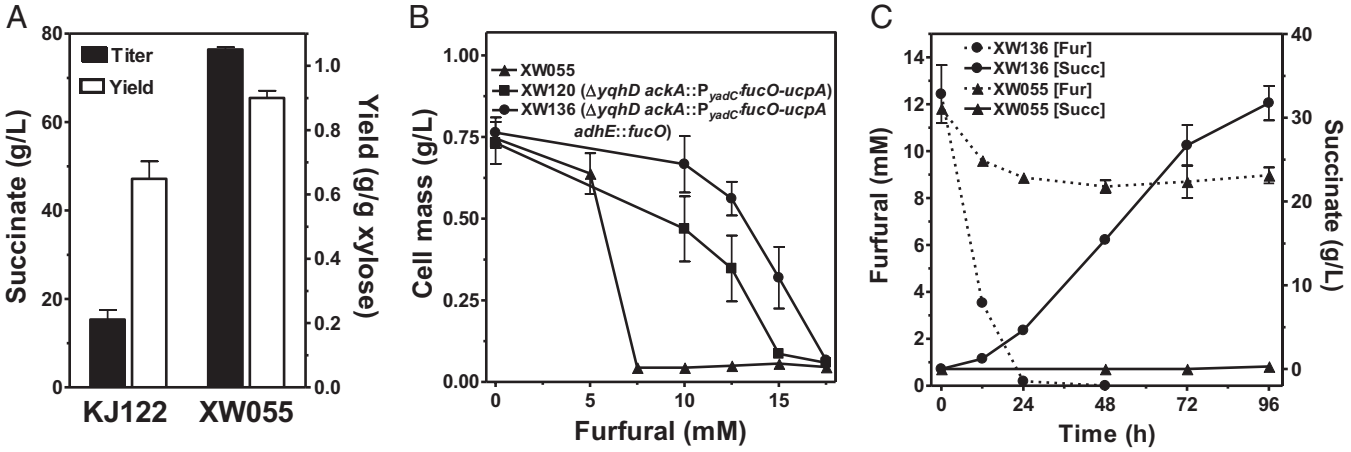

Fig. 5. Engineering furfural-resistant derivatives of *E. coli* C for hemicellulose conversion to succinate. (*A*) Fermentation titer and yield (96 h) for parent KJ122 and mutant XW055 selected for improved xylose metabolism. Strains were grown in AM1 medium containing 100 g/L xylose as described previously (27) using KOH/K_2CO_3 to automatically maintain pH 7. Yield was calculated as grams of succinate produced per grams of xylose metabolized. (*B*) Comparison of furfural tolerance in tube cultures containing AM1 medium (50 g/L xylose, 100 mM MOPS, and 50 mM $KHCO_3$). Strain XW055 was compared with strains XW120 and XW136 containing chromosomally integrated traits for furfural resistance. Cell mass was measured after incubation for 48 h. (*C*) Fermentation of hemicellulose hydrolysate (components of AM1 medium, 0.5 mM sodium metabisulfite,100 mM potassium bicarbonate, and 36 g/L total sugar). Strain XW136 (XW055 Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA* adhE*::*fucO*) completed the reduction of furfural in 24 h, coincident with the onset of rapid fermentation. Strain XW055 was unable to completely metabolize furfural or ferment sugars in hemicellulose hydrolysate. Data for furfural and succinate are shown by broken lines and solid lines, respectively. All data represent averages of at least two experiments with SDs.

biobased processes has the potential to replace petroleum for solvents, plastics, and fuels without disrupting food supplies or animal feed. Costs for such processes remain a challenge and can be reduced by developing biocatalysts that are tailored for specific feedstocks. Inhibitors formed during the deconstruction of lignocellulose such as furfural are part of this challenge. Previous studies have shown that furfural was unique in binary combinations of inhibitors, increasing the toxicity of other compounds (soluble lignin products, formate, acetate, etc.) in hemicellulose hydrolysates (13). Our studies demonstrate that removal of furfural is essential before rapid growth and metabolism of sugars by *E. coli* biocatalysts (Fig. 4 *B* and *C* and Fig. 5*C*). The starting strain for ethanol production, LY180, was more resistant to furfural, as well as hemicellulose hydrolysates, than the starting strain for succinate production, XW055 (Fig. S4, Fig. 4*C*, and Fig. 5*C*). However, the same combination of furfural-resistance traits was optimal for furfural tolerance with both strains. Genetic changes that increased furfural tolerance also increased resistance to hemicellulose hydrolysate, establishing the importance of furfural for toxicity and the generality of this approach. Although furfural is not the only inhibitor present in hydrolysate, enzymatic reduction of this compound should allow further studies to identify additional genes that confer resistance to remaining toxins. By developing biocatalysts that are resistant to furfural and other hemicellulose toxins, remaining toxins in hydrolysates can reduce the cost of fermentations by serving as a barrier that prevents the growth of undesirable contaminants.

Epistatic Interactions of Beneficial Traits for Furfural Tolerance. There is no established strategy to predict the epistatic interactions of target genes, and searching for the optimal combination of beneficial genetic traits is challenging (28). A general model is included to illustrate interactions among the four genetic traits for furfural tolerance (Fig. 1). Energy generation and growth require nutrients, intermediates from carbon catabolism, and balanced oxidation and regeneration of NADPH and NADH. YqhD has a low K_m for NADPH that competes effectively with biosynthesis, limiting growth by impeding NADPH-intensive processes such as sulfate assimilation (18). Increasing PntAB transhydrogenase partially restored this imbalance using NADH as a reductant (abundant during fermentation) (18). However, the combination of a *yqhD* deletion and increased expression of *pntAB* was more sensitive to furfural inhibition than either alone (Fig. 2*B*). NADPH-dependent furfural reductase YqhD may play a positive role for furfural tolerance in strains where *pntAB* expression has been increased. Pyridine nucleotide transhydrogenase activity of PntAB couples proton translocation and makes the reduction of NADP by NADH a costly energy process (29). This increase in energy demand during expression of *yqhD* and *pntAB* could reduce fitness, despite potential benefits of reducing furfural to the less toxic alcohol. FucO can serve as a more effective furfural reductase because it uses NADH (abundant during fermentation) as the reductant and does not compete for biosynthetic NADPH. Like *pntAB*, increased expression of *ucpA* in a *yqhD* deletion strain did not further increase furfural tolerance. This epistatic interaction suggests the UcpA-dependent furfural resistance may also involve NADPH availability (Fig. 2*B*).

Two furfural-resistant strains have been isolated and characterized previously, EMFR9 [selected for furfural tolerance (19)] and MM160 [selected for hydrolysate resistance (17)]. Each contains a mutation that improves furfural tolerance by silencing YqhD using completely different mechanisms, IS10 disruption of adjacent *yqhC* (transcriptional activator for *yqhD*) and a nonsense mutation in *yqhD*, respectively (17, 20). Silencing genes such as *yqhD* can be caused by a myriad of genetic changes (30). An increase in fitness by gene silencing would be expected to emerge early in populations under growth-based selection. No mutations were found in these strains that increased expression of *ucpA*, *pntAB*, or *fucO* (18–20). Genetic solutions for gain-of-function mutants can be very limited and much less abundant (30, 31). Also, recovery of mutants with increased expression of *ucpA* and *pntAB* would be prevented by their negative interactions with *yqhD* silencing. Very high levels of *fucO* expression were needed that may require multiple mutations, dramatically limiting recovery without deliberate genetic constructions.

Succinate Fermentation from Lignocellulose Sugars. Succinic acid is currently produced from petroleum derived maleic anhydride and can serve as a starting material for synthesis of many commodity chemicals used in plastics and solvents (32). Genetically engineered strains of *E. coli* (33) and native succinate producers such as *Actinobacillus succinogenes* (34–36) and *Anaerobiospirillum succiniciproducens* (37) have been tested for lignocellulose conversion to succinate. However, fermentation using these strains required costly additional steps (34), nutrient supplementation (33–37), and mitigation of toxins in hydrolysates by overliming or treating with activated charcoal (33, 36). Reengineering derivatives of KJ122 using known combinations of furfural resistance traits resulted in strain XW136 that now ferments hemicellulose hydrolysates in mineral salts medium without costly detoxification steps (32 g/L succinic acid with a yield of 0.9 g/g sugars; Fig. 5*C*). The ability to use defined genetic traits for furfural tolerance to improve tolerance to inhibitors in hemicellulose hydrolysates should prove useful as a starting point for many new biocatalysts and products.

Materials and Methods

Strains and Growth Conditions. Strains used in this study are listed in Table S1. Ethanologenic *E. coli* LY180 (a derivative of *E. coli* W; ATCC 9637) and succinate-producing *E. coli* KJ122 (a derivative of *E. coli* C; ATCC 8739) were developed previously in our laboratory (19, 27). Strains XW092 (LY180, Δ*yqhD*), XW103 (LY180, *adhE::pntAB*), XW109(LY180, Δ*yqhD adhE::pntAB*), XW115 (LY180, Δ*yqhD ackA::fucO-ucpA*), XW116 (LY180, *adhE::pntAB ackA::fucO-ucpA*), XW129 (LY180, Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA*), and XW131 (LY180, *adhE::pntAB ackA*::P_{yadC}-*fucO-ucpA*) were genetically engineered for furfural tolerance using LY180 as the parent strain. Strain KJ122 (succinate production from glucose) was serially transferred in pH-controlled fermenters (27) at 48-h intervals for ~40 generations to isolate a mutant with improved xylose fermentation (designated XW055). Strains XW120 (XW055, Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA*) and XW136 (XW055, Δ*yqhD ackA*::P_{yadC}-*fucO-ucpA adhE::fucO*) were genetically engineered using XW055 as the parent strain. Cultures were grown in low-salt xylose AM1 medium as described previously (38).

Genetic Methods. Methods for seamless chromosomal deletion, gene replacement, or integration were described previously using Red recombinase technology (12, 27). Plasmids, primers, and construction details are listed in Table S1. The Clone EZ PCR Cloning Kit from GenScript was used for gene replacement on the plasmid. Constructions were made in Luria broth containing 20 g/L xylose or 50 g/L arabinose (inducer for lambda Red recombinase; Gene Bridges) or 100 g/L sucrose (for counter selection of *sacB*). Antibiotics were added when required. Additional details are included in *SI Text* and Table S1.

Identification of Promoter for *fucO-ucpA* Cassette. A genome-wide promoter library with more than 10,000 clones was constructed in plasmid pLOI4870 (pACYC184 derivative) by ligating Sau3A1 fragments of *E. coli* genomic DNA into a unique BamH1 site immediately upstream from a promoterless *fucO-ucpA* cassette (Fig. S1). The library was transformed into LY180 Δ*yqhD* cells with selection under argon for large colonies on AM1–xylose plates containing 12 mM furfural and 40 mg/L chloramphenicol. Of more than 10,000 transformants, 176 exhibited a large colony phenotype and were further compared using a BioScreen C growth curve analyzer (BioScreen).The most effective clone was identified and designated plasmid pLOI5237 containing a 1,600-bp insert. Subcloning reduced the size of this promoter fragment to 600 bp (pLOI5259). This smaller fragment was identified by sequencing as part of the *yadC* coding region. The BamH1-furfural resistance cassette in pLOI4870 and pLOI5259 (includes upstream promoter fragment) were bordered by segments of *ackA* for chromosomal integration. Additional details are provided in *SI Text*.

NADH-Dependent Furfural Reductase Assay and SDS/PAGE. The preparation of cell crude lysates and furfural reductase assay were as described previously

(17). Soluble protein lysates (15 μg of protein) were also analyzed on 12% (wt/vol) SDS/PAGE gels (Bio-Rad).

Furfural Tolerance in Tube Cultures. Furfural toxicity was measured in tube cultures (13 mm × 100 mm) as described previously for ethanol strains (17, 22). For succinate strains, tubes contained 4 mL AM1 medium with 50 g/L xylose, 50 mM $KHCO_3$, and 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) as a buffer. Tubes were inoculated with starting cell density of 44 mg/L. Cell mass was measured at 550 nm after incubation for 48 h (37 °C).

Fermentation of Ethanol or Succinate. Ethanol fermentations with xylose were carried out as described previously (17, 22), with and without furfural. For succinate production from xylose, seed precultures of strains were grown in sealed culture tubes containing AM1 medium (20 g/L xylose, 50 mM $KHCO_3$, and 100 mM MOPS). After incubation for 16 h, preinocula were diluted into 500-mL fermentation vessels containing 300 mL of AM1 media (100 g/L xylose, 1 mM betaine, and 100 mM $KHCO_3$) at an initial density of 6.6-mg dry cell weight. After 24 h of growth, these seed cultures were used to provide starting inocula for batch fermentations (AM1 medium, 100 g/L xylose, and 100 mM $KHCO_3$). Fermentations were maintained at pH 7.0 by automatic addition of base containing additional CO_2 (2.4 M K_2CO_3 in 1.2 M KOH) as described previously (27). Quantitative analyses of sugars, ethanol, furfural, and succinate were as described previously (17, 27, 39).

Preparation and Fermentation of Hemicellulose Hydrolysates. Hemicellulose hydrolysate was prepared as described previously (40, 41). Briefly, sugarcane bagasse (Florida Crystals) impregnated with phosphoric acid (0.5% of bagasse dry weight) was steam-treated for 5 min at 190 °C (40, 41). Hemicellulose syrup (hydrolysate) was recovered using a screw press, discarding solids. After removal of fine particulates with a Whatman GF/D glass fiber filter, clarified hydrolysate was stored at 4 °C (pH 2.0). Hydrolysate was adjusted to pH 9.0 (5 M ammonium hydroxide) and stored for 16 h (22 °C) before use in fermentations, declining to pH 7.5. Batch fermentations (300 mL) were conducted in pH-controlled vessels containing 210 mL hemicelluloses hydrolysate supplemented with 0.5 mM sodium metabisulfite, components of AM1 medium (38), and inoculum. Potassium bicarbonate (100 mM) was included for succinate production. Final hydrolysate medium contained 36 g/L total sugar (primarily xylose), furfural 1.2 g/L, hydroxymethylfurfural 0.071 g/L, formic acid 1.1 g/L, and acetic acid 3.2 g/L. Precultures and seed cultures were prepared as described above. After 20 h of incubation, seed cultures were used to provide a starting inoculum of 66 mg for hemicelluloses hydrolysate fermentations producing succinate or 13 mg for ethanol. Fermentations were maintained at pH 7.0 by the automatic addition of base (2.4 M K_2CO_3 in 1.2 M KOH for succinate or 2 M KOH for ethanol).

ACKNOWLEDGMENTS. This research was supported by US Department of Agriculture Grant 2011-10006-30358 and by the Myriant Corporation.

1. Carole TM, Pellegrino J, Paster MD (2004) Opportunities in the industrial biobased products industry. *Appl Biochem Biotechnol* 115:871–885.
2. Gírio FM, et al. (2010) Hemicelluloses for fuel ethanol: A review. *Bioresour Technol* 101(13):4775–4800.
3. Saha BC (2003) Hemicellulose bioconversion. *J Ind Microbiol Biotechnol* 30(5): 279–291.
4. Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresour Technol* 101(13):4851–4861.
5. Harris EE (1949) Wood saccharification. *Adv Carbohydr Chem* 4:153–188.
6. Geddes CC, Nieves IU, Ingram LO (2011) Advances in ethanol production. *Curr Opin Biotechnol* 22(3):312–319.
7. Almeida JR, Bertilsson M, Gorwa-Grauslund MF, Gorsich S, Lidén G (2009) Metabolic effects of furaldehydes and impacts on biotechnological processes. *Appl Microbiol Biotechnol* 82(4):625–638.
8. Mills TY, Sandoval NR, Gill RT (2009) Cellulosic hydrolysate toxicity and tolerance mechanisms in *Escherichia coli*. *Biotechnol Biofuels* 2:26.
9. 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(5):526–536.
10. Martinez A, et al. (2001) Detoxification of dilute acid hydrolysates of lignocellulose with lime. *Biotechnol Prog* 17(2):287–293.
11. Abbott DA, Zelle RM, Pronk JT, van Maris AJ (2009) Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: Current status and challenges. *FEMS Yeast Res* 9(8):1123–1136.
12. Jarboe LR, et al. (2010) Metabolic engineering for production of biorenewable fuels and chemicals: Contributions of synthetic biology. *J Biomed Biotechnol* 2010:761042.
13. Zaldivar J, Martinez A, Ingram LO (1999) Effect of selected aldehydes on the growth and fermentation of ethanologenic *Escherichia coli*. *Biotechnol Bioeng* 65(1):24–33.
14. Taherzadeh MJ, Gustafsson L, Niklasson C, Lidén G (2000) Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 53(6):701–708.
15. Liu ZL, Blaschek HP (2010) Biomass conversion inhibitors and in situ detoxification. *Biomass to Biofuels: Strategies for Global Industries*, eds Vertès AA, Blaschek HP, Yukawa H (John Wiley and Sons, West Sussex, United Kingdom), pp 233–259.
16. Liu ZL, Moon J, Andersh BJ, Slininger PJ, Weber S (2008) Multiple gene-mediated NAD(P)H-dependent aldehyde reduction is a mechanism of in situ detoxification of furfural and 5-hydroxymethylfurfural by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 81(4):743–753.
17. Wang X, et al. (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(15):5132–5140.
18. Miller EN, et al. (2009) Furfural inhibits growth by limiting sulfur assimilation in ethanologenic *Escherichia coli* strain LY180. *Appl Environ Microbiol* 75(19): 6132–6141.
19. Miller EN, et al. (2009) Silencing of NADPH-dependent oxidoreductase genes (*yqhD* and *dkgA*) in furfural-resistant ethanologenic *Escherichia coli*. *Appl Environ Microbiol* 75(13):4315–4323.
20. Turner PC, et al. (2011) YqhC regulates transcription of the adjacent *Escherichia coli* genes *yqhD* and *dkgA* that are involved in furfural tolerance. *J Ind Microbiol Biotechnol* 38(3):431–439.
21. Miller EN, Turner PC, Jarboe LR, Ingram LO (2010) Genetic changes that increase 5-hydroxymethyl furfural resistance in ethanol-producing *Escherichia coli* LY180. *Biotechnol Lett* 32(5):661–667.
22. Wang X, Miller EN, Yomano LP, Shanmugam KT, Ingram LO (2012) Increased furan tolerance in *Escherichia coli* due to a cryptic *ucpA* gene. *Appl Environ Microbiol* 78(7): 2452–2455.
23. Sawers RG (2006) Differential turnover of the multiple processed transcripts of the *Escherichia coli focA-pflB* operon. *Microbiology* 152(8):2197–2205.
24. Turner PC, et al. (2012) Optical mapping and sequencing of the *Escherichia coli* KO11 genome reveal extensive chromosomal rearrangements, and multiple tandem copies of the *Zymomonas mobilis pdc* and *adhB* genes. *J Ind Microbiol Biotechnol* 39(4): 629–639.
25. Yomano LP, York SW, Zhou S, Shanmugam KT, Ingram LO (2008) Re-engineering *Escherichia coli* for ethanol production. *Biotechnol Lett* 30(12):2097–2103.
26. Jarboe LR, Grabar TB, Yomano LP, Shanmugan KT, Ingram LO (2007) Development of ethanologenic bacteria. *Adv Biochem Eng Biotechnol* 108:237–261.
27. Jantama K, et al. (2008) Eliminating side products and increasing succinate yields in engineered strains of *Escherichia coli* C. *Biotechnol Bioeng* 101(5):881–893.
28. Sandoval NR, et al. (2012) Strategy for directing combinatorial genome engineering in *Escherichia coli*. *Proc Natl Acad Sci USA* 109(26):10540–10545.
29. Clarke DM, Bragg PD (1985) Purification and properties of reconstitutively active nicotinamide nucleotide transhydrogenase of *Escherichia coli*. *Eur J Biochem* 149(3): 517–523.
30. Elena SF, Lenski RE (2003) Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. *Nat Rev Genet* 4(6):457–469.
31. Nam H, Conrad TM, Lewis NE (2011) The role of cellular objectives and selective pressures in metabolic pathway evolution. *Curr Opin Biotechnol* 22(4):595–600.
32. McKinlay JB, Vieille C, Zeikus JG (2007) Prospects for a bio-based succinate industry. *Appl Microbiol Biotechnol* 76(4):727–740.
33. Liu R, et al. (2012) Fermentation of xylose to succinate by enhancement of ATP supply in metabolically engineered *Escherichia coli*. *Appl Microbiol Biotechnol* 94(4): 959–968.
34. Li Q, et al. (2010) Efficient conversion of crop stalk wastes into succinic acid production by *Actinobacillus succinogenes*. *Bioresour Technol* 101(9):3292–3294.
35. Zheng P, Dong JJ, Sun ZH, Ni Y, Fang L (2009) Fermentative production of succinic acid from straw hydrolysate by *Actinobacillus succinogenes*. *Bioresour Technol* 100(8): 2425–2429.
36. Borges ER, Pereira N, Jr. (2011) Succinic acid production from sugarcane bagasse hemicellulose hydrolysate by *Actinobacillus succinogenes*. *J Ind Microbiol Biotechnol* 38(8):1001–1011.
37. Lee PC, Lee SY, Hong SH, Chang HN, Park SC (2003) Biological conversion of wood hydrolysate to succinic acid by *Anaerobiospirillum succiniciproducens*. *Biotechnol Lett* 25(2):111–114.
38. Martinez A, et al. (2007) Low salt medium for lactate and ethanol production by recombinant *Escherichia coli* B. *Biotechnol Lett* 29(3):397–404.
39. 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(4):637–641.
40. Geddes CC, et al. (2011) Simplified process for ethanol production from sugarcane bagasse using hydrolysate-resistant *Escherichia coli* strain MM160. *Bioresour Technol* 102(3):2702–2711.
41. Varga E, Réczey K, Zacchi G (2004) Optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. *Appl Biochem Biotechnol* 114:509–523.