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Increased Furan Tolerance in *Escherichia coli* Due to a Cryptic *ucpA* Gene

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Expression arrays were used to identify 4 putative oxidoreductases that were upregulated (>3-fold) by furfural (15 mM, 15 min). Plasmid expression of one (*ucpA*) increased furan tolerance in ethanologenic strain LY180 and wild-type strain W. Deleting *ucpA* decreased furfural tolerance. Although the mechanism remains unknown, the cryptic *ucpA* gene is now associated with a phenotype: furan resistance.

Furfural is an inhibitory side product formed by the dehydration of pentose sugars during dilute acid pretreatment of lignocellulosic biomass (1, 3, 14, 20). An analogous compound, 5-hydroxymethylfurfural (5-HMF), is produced from hexose sugars such as fructose. These furans inhibit the growth and fermentation of biocatalysts such as yeasts (2, 9–13) and ethanologenic *Escherichia coli* (7, 14, 15, 28, 29), complicating fermentation processes.

Furan addition to *E. coli* fermentations results in an initial period of slow growth or lag, during which furans are reduced to alcohols and remain in the broth (11). Furfural-resistant mutants of ethanologenic *E. coli* LY180 have been isolated and characterized (17, 18, 26). Partial resistance to low concentrations of furfural resulted from the silencing of *yqhD*, a furfural-induced NADPH-dependent furfural oxidoreductase (17, 18, 26). Although multiple NADPH-dependent furfural reductases are present in *E. coli* and conversion of furfural to the less toxic alcohol is generally regarded as beneficial, the unusually low K_m of YqhD for NADPH (9 μM) is proposed to inhibit growth by depleting NADPH (17, 18). Furfural tolerance was improved by expression of *fucO* (propanediol oxidoreductase), an NADH-dependent furfural reductase (27) that normally functions during fucose catabolism (4). The use of NADH as the electron donor is of particular interest, because NADH is more abundant during fermentation (6, 27) and because its use for furfural reduction would not compete with biosynthesis.

The expression of all known furfural reductases in *E. coli* (YqhD, DkgA, and FucO) is upregulated by furfural (17). To identify additional NADH-dependent furfural reductases, mRNA expression levels in control cells (LY180) were compared to cells exposed to furfural (15 mM) for 15 min as previously described (17). Of the 261 genes with a 3-fold or higher expression, four were oxidoreductases (*aldA*, *xdhABC*, *yeiTA*, and *ucpA*) with defined or putative NADH binding domains. Expression vectors containing these candidate genes were constructed in pTrc99A (pLOI4320, pLOI4317, pLOI4855, and pLOI4856). Amplified regions included the ribosomal binding site, open reading frame, and terminator region. Cell lysates of plasmid-containing strains (LY180) were assayed as previously described (27) for furan reductase (furfural and 5-HMF). None appeared to encode a furan reductase. Activities in all were low and similar to that of the vector control (<0.02 U mg protein^{-1} for NADH and <0.10 U mg protein^{-1} for NADPH).

Derivatives of LY180 containing these plasmids were tested for furfural tolerance with (0.1 mM IPTG [isopropyl-β-D-thiogalactopyranoside]) and without IPTG induction using an MIC assay (18, 19, 27). Only pLOI4856 (*ucpA*) was beneficial (Fig. 1A), increasing the MIC of furfural by 50% (15 mM) compared to that of the vector control and the three other constructs (10 mM). IPTG provided little further benefit, indicating that high levels of UcpA are not needed. Expression of *ucpA* in LY180(pLOI4856) also increased the MIC for 5-HMF from 16 mM for the control to 20 mM for LY180(pLOI4856) (data not shown).

The effects of UcpA on growth, ethanol production, and furfural metabolism were investigated in more detail during pH-controlled batch fermentation in mineral salts medium (AM1 medium containing 100 g xylose liter^{-1}, 0.1 mM IPTG and 12.5 μg ml^{-1} ampicillin for all cultures harboring plasmids, furfural as indicated, and inoculum of 22 mg dry cell weight [dcw] liter^{-1}) as previously described (27). Ethanol (retention time of 1.1 min) and furfuryl alcohol (retention time of 6.2 min) were measured using an Agilent 6890N gas chromatograph (Santa Clara, CA) (18). Furoic acid (retention time of 51.2 min) and sugars were measured by high-performance liquid chromatography (7). Furfural was measured using a Beckman-Coulter DU 800 spectrophotometer (16).

Plasmid pLOI4856 containing *ucpA* increased furfural tolerance for growth and ethanol production in LY180 compared to those of the control containing pTrc99A (Fig. 1B and C) with IPTG induction. The vector control was substantially inhibited by 10 mM furfural for over 72 h (Table 1), while only a modest initial inhibition was observed for LY180(pLOI4856). During the initial slow phase, LY180(pLOI4856) quantitatively converted furfural to the less toxic furfuryl alcohol (Fig. 1D). No furoic acid was detected. Ethanol production and growth followed similar trends. After furfural was metabolized, the rate of growth and ethanol

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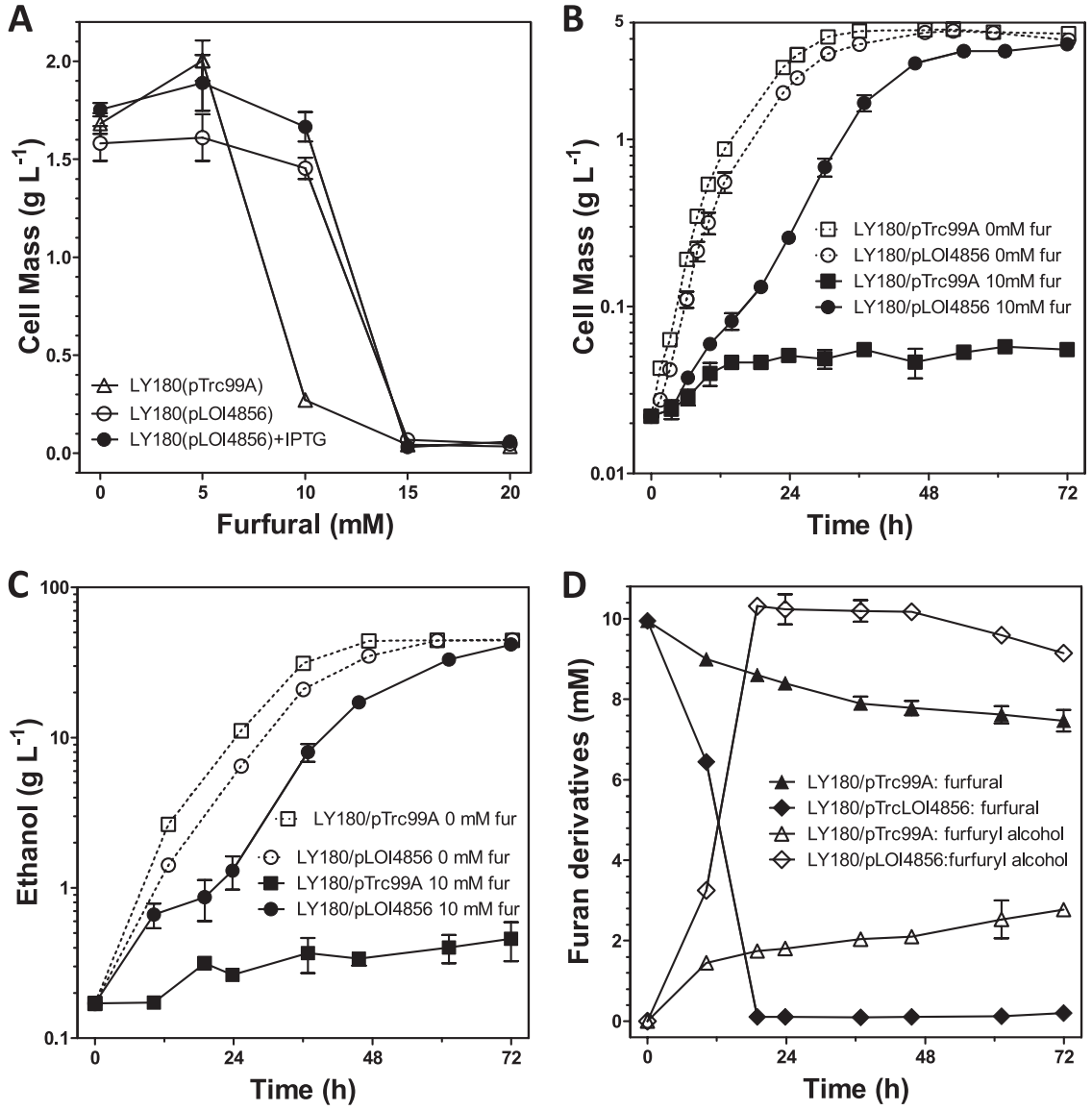

FIG 1 Plasmid expression of *ucpA* increases furfural tolerance (MIC) and ethanol production by LY180 (pH-controlled fermentations; 10% xylose). (A) Effect of *ucpA* plasmid (pLOI4856) on MIC for furfural; (B) effect of pLOI4856 on growth in 10 mM furfural; (C) effect of pLOI4856 on ethanol production in 10 mM furfural; (D) effect of pLOI4856 on furfural metabolism during fermentation. Controls were included without furfural (dotted lines).

production increased to near that of controls without furfural, with similar final yields for cell mass and ethanol.

UcpA appears to increase growth in the presence of furfural but does not directly metabolize furfural using NADH or NADPH as electron donors. Although the volumetric rate of furfural reduction was increased by plasmid copies of *ucpA* (Fig. 1D), whole-cell-specific activity (furfural reductase) (27) was similar to that of the vector control ($<$0.10 U mg dcw^{-1}). Deletion of chromosomal *ucpA* in an LY180 background (strain XW118) using Red recombinase technology (5) (Gene Bridges GmbH, Dresden, Germany) decreased furfural tolerance (Fig. 2 and Table 1), confirming that the chromosomally encoded UcpA is functional and beneficial.

Strain LY180 has been highly engineered for ethanol production and contains many mutations. Although this engineered strain was more sensitive to inhibition by furfural than the parent strain W, cell yields for LY180 were twice that of strain W with 0 mM and 8 mM furfural (Table 1). Both LY180 and strain W exhibited similar changes in furfural tolerance with regard to *ucpA*. The addition of plasmid pLOI4856 increased furfural tolerance in strain W (Table 1). Deletion of *ucpA* from strain W (strain XW137) lowered furfural tolerance. The furfural sensitivity of LY180 may be related to higher aldehyde levels in this homoethanol producer than in strain W (mixed acid fermentation). Mixtures of acetaldehyde and furfural were previously shown to exhibit more than additive toxicity for ethanologenic *E. coli* (29).

Very little is known about the *ucpA* gene other than its location, upstream from the *cysP* operon. UcpA homologues contain a proposed NAD-binding site with homology to short-chain alcohol dehydrogenases (24) and to human 3-hydroxybutyrate dehydrogenase (DHRS6) (8). Reed et al. (23) predicted that this gene may encode diacetyl reductase (acetoin dehydrogenase). These com-

TABLE 1 Summary of pH-controlled fermentations (10% xylose)[a]

Strain	Furfural concn (mM)	Furfural-dependent slow growth		μ max (h^{-1})	Cell yield (g $liter^{-1}$)
		μ (h^{-1})	Duration (h)[b]		
LY180/pTrc99A	0	No slow phase		0.23 ± 0.1	4.6 ± 0.1
LY180/pTrc99A	10	<0.04	>72	<0.04	0.06 ± 0.01
LY180/pLOI4856	0	No slow phase		0.22 ± 0.01	4.5 ± 0.2
LY180/pLOI4856	10	0.09 ± 0.01	22 ± 2	0.14 ± 0.01	3.6 ± 0.1
LY180	0	No slow phase		0.23 ± 0.01	3.6 ± 0.2
LY180	8	0.09 ± 0.01	21 ± 1	0.16 ± 0.01	4.0 ± 0.1
LY180	10	<0.04	>72	<0.04	0.08 ± 0.02
XW118[c]	0	No slow phase		0.22 ± 0.03	3.1 ± 0.2
XW118	8	0.01 ± 0.01	55 ± 2	0.14 ± 0.01	4.0 ± 0.1
XW118	10	<0.04	>72	<0.04	0.04 ± 0.01
E. coli W	0	No slow phase		0.23 ± 0.01	2.0 ± 0.1
E. coli W	8	0.18 ± 0.01	13 ± 1	0.19 ± 0.01	1.9 ± 0.1
E. coli W	10	0.06 ± 0.02	21 ± 1	0.20 ± 0.02	2.0 ± 0.1
XW137[d]	0	No slow phase		0.22 ± 0.01	2.0 ± 0.1
XW137	8	0.11 ± 0.02	16 ± 3	0.19 ± 0.01	1.9 ± 0.1
XW137	10	0.02 ± 0.01	37 ± 2	0.12 ± 0.02	1.9 ± 0.1
E. coli W/pTrc99A	0	No slow phase		0.23 ± 0.01	2.4 ± 0.2
E. coli W/pTrc99A	10	0.03 ± 0.01	26 ± 4	0.13 ± 0.01	2.0 ± 0.3
E. coli W/pLOI4856	0	No slow phase		0.22 ± 0.01	1.9 ± 0.1
E. coli W/pLOI4856	10	0.07 ± 0.01	16 ± 2	0.18 ± 0.02	2.5 ± 0.3

[a] Fermentations ($n \geq 2$) were performed in AM1 medium with 100 g xylose $liter^{-1}$ (27).
[b] The duration of furfural-induced slow growth was estimated as the time of intersection using extrapolated rates.
[c] XW118 (LY180 *ucpA::kan*).
[d] XW137 (*E. coli* W *ucpA::kan*).

FIG 2 Deletion of chromosomal *ucpA* (XW118) decreased furfural tolerance of LY180 during pH-controlled fermentations (10% xylose). (A) Cell mass; (B) furfural metabolism; (C) ethanol. Controls were included without furfural (dotted lines).

pounds (acetoin, diacetyl, 3-hydroxybutyrate, and acetoacetate) were tested as potential substrates using appropriate cofactors as described by Guo et al. (8). Additional alcohols and aldehydes (ethanol, glycerol, *n*-butanol, 2-propanol, methanol, 1,3-propanediol, methyglyoxal, dihydroxyacetone, acetaldehyde, butyraldehyde, malondialdehyde, and acrolein) were also tested for alcohol dehydrogenase or aldehyde reductase activities (22, 25). Although an IPTG-induced band corresponding to the predicted size for UcpA (28 kDa) was clearly evident (data not included), lysates of induced LY180 (pLOI4856) did not metabolize any of the substrates at a higher rate than control lysates from LY180(pTrc99A).

The mechanism of UcpA action remains unknown. UcpA does not directly metabolize furfural using NADH or NADPH as electron donors. In both LY180 and the parent strain W, furfural retarded fermentation by delaying growth until metabolism to the alcohol form was near completion. UcpA appears to partially restore growth and thereby decrease the time required to complete furfural metabolism. Growth (and fermentation) then resumes at near control rates and final yields for ethanol and cell mass.

Plasmid expression of *ucpA* was beneficial for both the native W strain and ethanologenic strain LY180. Homologues of UcpA are widely distributed in nature (8, 24) and may be generally useful to improve the furan tolerance in many microbial biocatalysts. Deletion of the chromosomal *ucpA* was detrimental for furfural tolerance, providing a clear phenotype for this cryptic gene.

Microarray data accession number. These new microarray data were deposited in the Gene Expression Omnibus (GEO) database at http://ncbi.nlm.nih.gov/geo (accession number GSE34956).

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