Furfural and hydroxymethylfurfural tolerance in *Escherichia coli* Δ*acrR* regulatory mutants

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Abstract

The presence of the highly toxic furfural and hydroxymethylfurfural (HMF) in the hydrolysate of lignocellulosic biomass prompted the investigation of the *Escherichia coli* Δ*acrR* regulatory mutant for higher tolerance to these compounds, to facilitate the production of biofuels and biochemicals, and further biocatalytic conversions. In comparison with the parental strain, the regulatory mutant with the upregulated efflux pump AcrAB-TolC produced moderately better growth and higher tolerance to concentrations of furfural and HMF between 1 and 2 g L^{-1}. Volume 00, Number 0, Pages 1–5, 2014

Keywords: *furfural and HMF tolerance, efflux pump*

1. Introduction

For most biofuel and biochemical programs, lignocellulosic biomass is a major feedstock that is dominated by cellulose, hemicelluloses, and lignin. To depolymerize cellulose and hemicelluloses to C5 and C6 sugars, pretreatment of lignocellulosic biomass with the diluted acid/alkali followed by enzymatic hydrolysis is routinely practiced [1]. However, the pretreatment with diluted acid/alkali typically leads to the formation of phenolic compounds, furfural, and hydroxymethylfurfural (HMF), which are highly toxic to microbial hosts [2–4]. The removal of these toxic compounds from the hydrolysate of lignocellulosic biomass is difficult and economically unfeasible. The identification of cellular components that enhance the tolerance of these compounds and the fermentation of the resulting hydrolysate may contribute toward this endeavor.

Escherichia coli is known to have a natural bacterial efflux system consisting of a number of single or multicomponent drug transporters that secrete toxins, antibodies, and foreign compounds to enable it to survive under harsh environments [5–7]. Among them, the efflux pump AcrAB-TolC is capable of transporting a wide range of compounds including oxacillin, chloramphenicol, tetracycline, erythromycin, and solvent as demonstrated by a previous study [5]. However, recent studies indicated that the overexpression of this efflux pump increased *E. coli*'s tolerance to *n*-hexane and cyclohexane [8], but paradoxically decreased its tolerance to *n*-butanol and isobutanol [9–12], although exposure to butanol was previously demonstrated to upregulate the expression of AcrAB-TolC [8, 13]. This variability in solvent tolerances prompted the investigation on the effects of AcrAB-TolC on exporting furfural and HMF to determine whether the overexpression of AcrAB-TolC had a similar effect on them as with butanol.

To investigate the effect on cells' tolerance to furfural and HMF, *E. coli* regulatory mutants were created by the deletion of the repressor gene (*acrR*) of AcrAB-TolC. It revealed that the overexpression of AcrAB-TolC produced a positive effect on cells' tolerance to furfural and HMF, and the resulting regulatory mutant *E. coli* Δ*acrR* demonstrated a moderately higher tolerance to these toxic compounds than the parental strain.

Abbreviations: *AcrA, acridine efflux pump/membrane fusion protein/acriflavine resistance protein A; AcrB, RND-type permease/acriflavine resistance protein B; LB, lysogeny broth/Luria-Bertani media; NADH, nicotinamide adenine dinucleotide-reduced; NADPH, nicotinamide adenine dinucleotide phosphate-reduced; OD_{600}, optical density at 600 nm wavelength; RT-PCR, reverse transcription-quantitative polymerase chain reaction; TolC, outer membrane protein TolC.*

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2. Materials and Methods

2.1. Microorganisms, plasmids, and culture medium

E. coli MC4100 Δ*ara714* was the parental strain [14] used in this study. Plasmids pkD46, pkD4, and pCP20 were used for the deletion of genes from the genomic DNA of the *E. coli*, as described elsewhere [15]. *E. coli* cells were cultivated in lysogeny broth/Luria-Bertani (LB) broth, and an appropriate antibiotic was supplied where necessary. Furfural and HMF were purchased from Fluka and SAFC, respectively (Sigma-Aldrich, St. Louis, MO, USA).

2.2. DNA manipulation

The standard protocol for the deletion of *E. coli* genes from their chromosomal locations and the subsequent curing of the antibiotic-resistant marker at the deletion sites were practiced using the λ-red-mediated gene deletion method [14]. Briefly, the cassette for the deletion of the *acrR* gene sequence was PCR-amplified from *E. coli* containing pKD4 using oligo pair *del-acrR-Fwd* and *del-acrR-Rev* (5′-TAA ATC TAA CGC CTG TAA ATT CAC GAA CAT ATG GCA CGA TGT AGG CTG GAG CTG CTT CG-3′ and 5′-CCA GGA AAA ATC CTG GAG TCA GAT TCA GGG TTA TTC GTT CAT ATG AAT ATC CTC CTT AG-3′) by a colony PCR. The PCR product (kanamycin-resistant gene flanked by *FRT* sites) was electroporated into *E. coli* strain bearing pKD46, which carried genes encoding λ-red recombinase. The resulting kanamycin-resistant colonies were purified, and the integration of a Kan marker gene cassette was verified by the colony PCR. With this strain, phage P1 lysate was created, and the deletion of *acrR* gene sequence was transduced into *E. coli* MC4100 Δ*ara714* background, which resulted in the strain *E. coli* MC4100 Δ*ara714* Δ*acrR::kan*. The transformation of plasmid pCP20 containing *FRT*-mediated recombinase into this strain looped out the kanamycin resistance gene, leaving a copy of *FRT* sequence in the genome of this strain, which was designated as *E. coli* MC4100 Δ*ara714* Δ*acrR*. Subsequently, colony PCRs were performed with Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA) for the verification of deletion of the target gene.

2.3. Profiling gene expression by reverse transcription-quantitative polymerase chain reaction RT-qPCR

An overnight cell culture of *E. coli* was subcultured at 37 °C until optical density at 600 nm wavelength (OD_{600}) reached about 0.6. Cells were harvested, and total RNA was isolated by following the protocol for the total RNA isolation from Invitrogen (Carlsbad, CA, USA) with TRIzol reagents (catalog #16096020). The isolated mRNAs were reversely transcripted to cDNAs using random hexamers and the SuperScript® III reverse transcriptase from Invitrogen (catalog #18080-044) at 50 °C for 1 H.

Relative quantification of the target gene expression was performed by real-time PCR on the StepOne real time system (Applied Biosystems, Waltham, MA, USA) using Power SYBR Green. The comparative C_T method was chosen, and a housekeeping gene (*ftsL*) was included for the comparison. Primers for the target genes were designed with the Primer Express Software (Applied Biosystems) to give amplicons of 150 bp. The PCR mixture consisted of 2× Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher Scientific) (SYBR Green I Dye, AmpliTaq Gold DNA Polymerase, LD, dNTPs, and optimized buffer components), 0.25 μM of each primer, and 5 ng of cDNA template. Water was added to make up a total volume of 20 μL per well. The thermal cycling conditions were as default on the StepOne system: 10 Min at 95 °C followed by 40 cycles of 15 Sec at 95 °C and 1 Min at 60 °C. All reactions were performed in triplicate to ensure accuracy. The results of the real-time PCR including the fold change in gene expression measured by the comparative C_T method were analyzed by the system.

2.4. Furfural and HMF tolerance testing

Furfural and HMF tolerance testing was performed in the LB medium. In brief, the overnight cell cultures of those mutants were inoculated to a starting OD_{600} of 0.25 in 5 mL of the LB medium containing the individual inhibitors at various concentrations in 14 mL Falcon tubes (Fisher). The growth of *E. coli* mutants was monitored by the analysis of OD_{600} of the resulting cell culture. Each trial was repeated thrice, and the parental *E. coli* strain MC4100 Δ*ara714* was used as a control. One-tailed *t*-test analyses were carried out on the differences of OD_{600} ratios at 6/0 and 24/0 H. *P* values of less than 0.05 were used to indicate statistically significant differences between the two strains.

3. Results and Discussion

Overexpression of efflux pump AcrAB-TolC in *E. coli* is known to facilitate the export of a wide range of various compounds. In this study, the effect of overexpression of this efflux pump on furfural and HMF tolerance was investigated. To upregulate the expression of AcrAB-TolC, the known repressor gene *acrR* of AcrAB-TolC was deleted from the genomic DNA of *E. coli* by using the standard protocol [15]. The deletion of *acrR* gene sequence was confirmed by PCR amplification and further by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis [16]. The resulting regulatory mutant was designated as *E. coli* Δ*acrR*.

Using quantitative PCR analysis of cDNA produced from mRNA transcript from the mutant, the gene expression levels of *acrA*, *acrB*, and *tolC* were profiled. All three genes in the *E. coli* Δ*acrR* mutant produced an increase of about 1.2-fold of their transcription levels, relative to the parental strain (Fig. 1). The RT-qPCR analysis clearly indicated the overexpression of AcrAB-TolC in the Δ*acrR* mutant, consistent with previous literature [5, 16].

The mutant overexpressing AcrAB-TolC is next subjected to furfural and HMF stress by including various concentrations of those compounds individually in LB medium. The growth of the mutant and the parental *E. coli* strain was examined under 1, 2, and 3 g of furfural per liter. As indicated in Fig. 2,

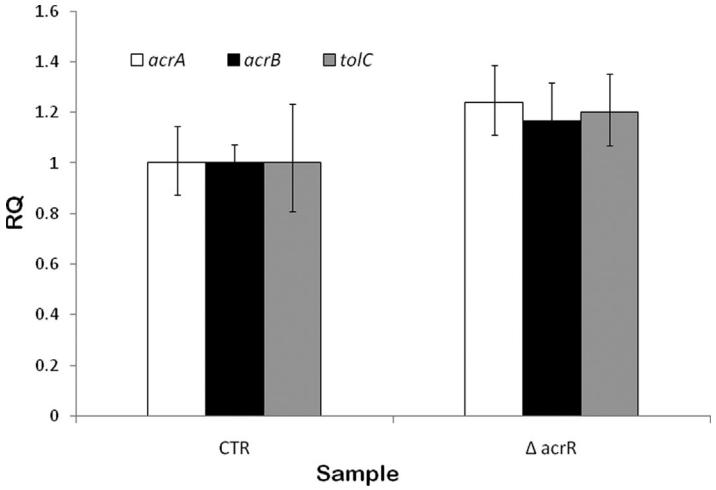

FIG. 1 *RT-qPCR analysis of acrA, acrB, and tolC transcripts in the E. coli ΔacrR regulatory mutant. RQ indicates relative quantity.*

the mutant showed increased but moderate growth relative to the parental strain at 1 and 2 g of furfural per liter. At 1 g of furfural per 1iter, the ratio of OD_{600} at 6 and 0 H was 1.15-fold higher than the parental strain. Likewise, at 2 g of furfural per liter, the mutant showed the increased growth as evident from its ratio of OD_{600} at 24 and 0 H, which was 1.3-fold higher than the parental strain, both results being statistically significant. However, at 3 g of furfural per liter, the OD_{600} ratios (6/0 and 24/0 H) for the mutant were lower than that for the parental strain. Because both mutant and parental strains barely grew past the log phase (see the Supporting Information), it may be assumed that 3 g of furfural per liter was too toxic for both strains to survive, and thus is not an accurate gauge of the tolerance levels of both strains.

The HMF tolerance of the *E. coli* Δ*acrR* regulatory mutant was further analyzed. Both the mutant and the parental strain were incubated with different concentrations of HMF in LB medium, and their growth was monitored. Similar to furfural tolerance, the Δ*acrR* mutant was observed to produce better growth than the parental strain under HMF stress at 2 g L^{-1}. Consistent with the furfural tolerance testing, the results of HMF tolerance testing revealed that the Δ*acrR* mutant was slightly more tolerant to HMF than the parental strain. The OD_{600} ratios (6/0 and 24/0 H) for the three concentrations of HMF tested showed a modest increase in the growth of the mutant compared with the parental strain (Fig. 3). This implies that the mechanisms of furfural and HMF stress response may be similar, and an increase in AcrAB-TolC transcription correlates with the increase to tolerance of these compounds.

An increased *acrAB* expression was previously demonstrated to correlate with the increased survival in hexane [8, 9] and the decreased hexane accumulation in *E. coli* [8]. Using hexane as a model compound, the overexpression of AcrAB-TolC efflux pump in Δ*acrR* mutant was further confirmed. Cells were treated with 1 and 3 g of hexane per liter in LB medium, and their densities were recorded and analyzed. The Δ*acrR* mutants were less sensitive to hexane than the parental strain as surmised. The OD ratios (6/0 and 24/0 H) of the Δ*acrR* mutant were 1.1- and 1.2-fold higher than those of the parental strain (Fig. 4). This indicated that the enhancement of the export of hexane was due to the upregulation of AcrAB-TolC in the Δ*acrR* mutant. Combined with the previous transcript analysis of *acrA*, *acrB*, and *tolC* by RT-qPCR (Fig. 1), the AcrAB-TolC was overexpressed in Δ*acrR* mutant relative to the parental strain. These results together suggested that the overexpression of AcrAB-TolC increased furfural and HMF tolerance in the Δ*acrR* regulatory mutant, to a slight extent and at concentrations of 1–2 g L^{-1}.

The major inhibitors present in the hydrolysate of lignocellulosic biomass, furfural, and HMF are highly toxic to microbial hosts, thus affecting the economy of most biofuel and biochemical programs. It is pivotal to have microbial hosts capable

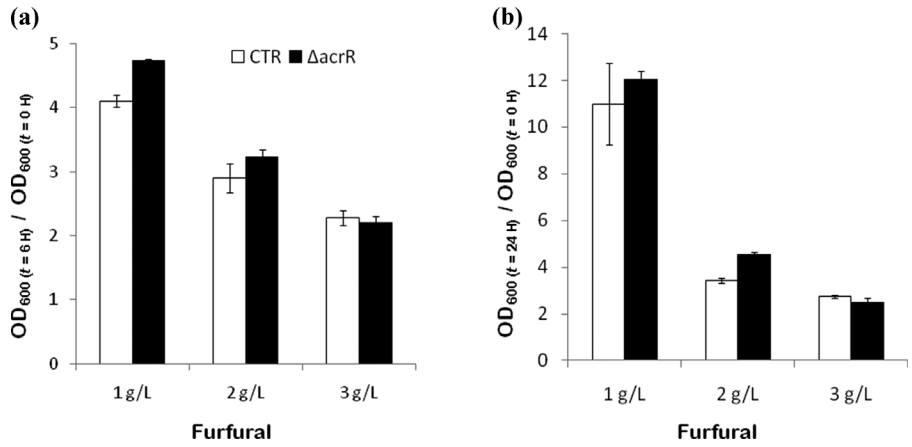

FIG. 2 *Furfural tolerance testing of the E. coli ΔacrR mutant. The y-axis indicates the ratio of OD_{600} at 0 and 6 H (a), and 0 and 24 H (b) of the parental strain and ΔacrR mutant.*

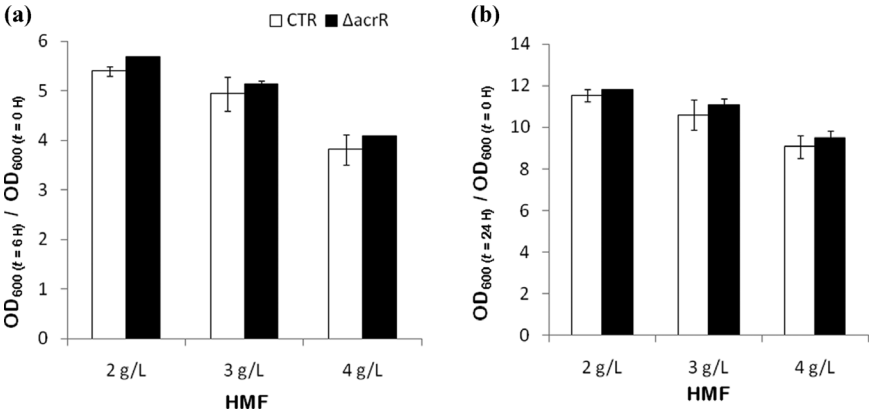

FIG. 3 *HMF tolerance testing of the E. coli ΔacrR mutant. The y-axis indicates the ratio of OD_{600} at 0 and 6 H (a), 0 and 24 H (b) of the parental strain and ΔacrR mutant.*

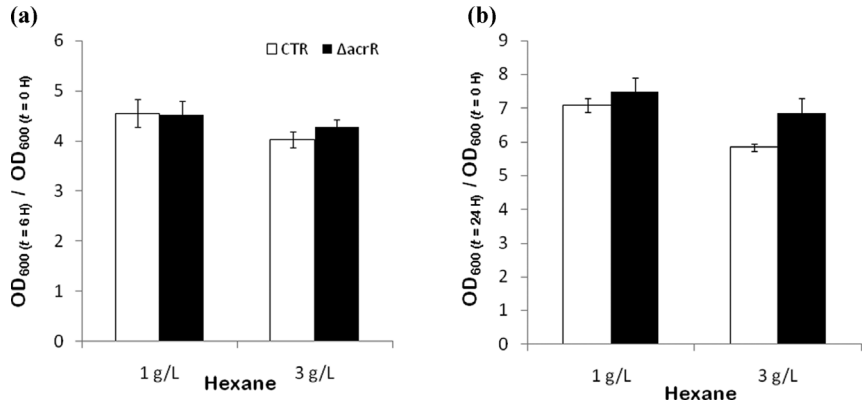

FIG. 4 *Hexane sensitivity testing of the E. coli ΔacrR regulatory mutants. The y-axis indicates the ratio of OD_{600} at 0 and 6 H (a) and 0 and 24 H (b) of the parental strain and the ΔacrR mutant.*

of tolerating relatively high concentrations of those inhibitors in the hydrolysate of lignocellulosic biomass. Because of its well-documented genetic backgrounds and well-developed molecular tools, *E. coli* is a popular host typically utilized for many biofuel and biochemical programs [9, 17].

Unfortunately, *E. coli* is naturally unable to tolerate high concentrations of furfural and HMF. The addition of thymine, related precursors, and the upregulation of pyrimidine deoxyribonucleotide synthesis has previously been shown to increase the tolerance of furfural [18]. Detoxification of furfural to furfuryl alcohol was achieved by the upregulation of the nicotinamide adenine dinucleotide phosphate-reduced (NADPH)-dependent *yqhD* oxidoreductase [19] at the expense of sulfur assimilation [20] during biosynthesis. This limitation is further circumvented by either the addition of sulfur-containing amino acids cysteine and methionine, the upregulation of the nicotinamide adenine dinucleotide-reduced (NADH)/NADPH transhydrogenase *puntAB*, or the use of the nicotinamide adenine dinucleotide-reduced-dependent propanediol oxidoreductase *fucO* [19, 21] catalyzing the same detoxification reaction mentioned above.

By exploring the *E. coli* AcrAB-TolC efflux pump, this study investigates its effect on the furfural and HMF tolerance phenotypes. To overexpress AcrAB-TolC efflux pump, the *E. coli* Δ*acrR* regulatory mutant was created, and it was demonstrated that the resulting mutant with the overexpressed AcrAB-TolC was only moderately more tolerant to furfural and HMF than the parental strain. The results suggest that overexpression of AcrAB-TolC facilitates the export of furfural and HMF in the culture medium.

The *E. coli* AcrAB-TolC efflux pump is known to have a wide substrate range that includes a number of antibiotics and toxic compounds [5, 22, 23]. An overexpression of this efflux pump increases cells' capability of exporting those compounds. On the basis of this observation, the Δ*acrR* regulatory mutant overexpressing the AcrAB-TolC efflux pump was hypothesized to increased furfural and HMF tolerance. Recent studies indicated that *E. coli acrA* mutants with the disrupted AcrAB-TolC efflux pumps had starkly different responses between ethanol (C2) and higher alcohols (C4–5). The deletion of the *acrA* gene led to the abolishment of AcrAB-TolC [9, 24], and the resulting *acrA* mutant was less tolerant to the water-miscible ethanol, but more tolerant to the more hydrophobic solvents

butanol, isobutanol, and pentanol than the parental *E. coli* [9, 11, 12].

The AcrR transcription repressor has been extensively reported to repress AcrAB-TolC transcription [25], as a secondary response to global stress. No other mechanisms have been reported to be effected by AcrR. In addition, the protein structure of AcrR reveals two organic ligand binding sites and a DNA binding site. An evidence suggests the following palindromic sequence: 5′-TACATACATTTGTGAATGTATGTA-3′ in the promoter sequence of *acrAB* to be the binding site for AcrR [26]. A sequence search on the *E. coli* MC4100 genome reveals this sequence to be unique to *acrAB*, suggesting the specificity of AcrR transcription factor.

A recent sequence enrichment study [27] identified several genes conferring increased resistance to 0.75 g L^{-1} furfural tolerance, AcrAB-TolC was not among these upregulated genes. This may be explained by the insensitivity of AcrR, MarR, and AcrS/EnvR transcription inhibitors to furfural and related phenolic compounds. In this study, the artificial overexpression of *AcrAB-TolC* produced a slight increase in tolerance to furfural and HMF at higher concentrations. This might be enhanced by engineering or directed evolution of the AcrB efflux unit, which has been demonstrated to improve *n*-butanol tolerance [28].

4. Conclusions

The results of the AcrAB-TolC efflux pump overexpression were presented and discussed with respect to its effects on *E. coli* tolerance to furfural and HMF. The results collectively demonstrated a modest increase in tolerance of *E. coli* Δ*acrR* mutant to those compounds at 1–2 g L^{-1} concentrations in comparison with the parental strain. However, an additional investigation would be required to determine other factors contributing to the response of *E. coli* to those toxic compounds, so as to further enhance its tolerance levels and to facilitate its use in biochemical production programs.

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