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BIOENERGY/BIOFUELS/BIOCHEMICALS

Partial deletion of *rng* (RNase G)-enhanced homoethanol fermentation of xylose by the non-transgenic *Escherichia coli* RM10

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Abstract Previously, a native homoethanol pathway was engineered in *Escherichia coli* B by deletions of competing pathway genes and anaerobic expression of pyruvate dehydrogenase (PDH encoded by *aceEF-lpd*). The resulting ethanol pathway involves glycolysis, PDH, and alcohol dehydrogenase (AdhE). The *E. coli* B-derived ethanologenic strain SZ420 was then further improved for ethanol tolerance (up to 40 g l^{-1} ethanol) through adaptive evolution. However, the resulting ethanol tolerant mutant, SZ470, was still unable to complete fermentation of 75 g l^{-1} xylose, even though the theoretical maximum ethanol titer would have been less than 40 g l^{-1} should the fermentation have reached completion. In this study, the *cra* (encoding for a catabolite repressor activator) and the HSR2 region of *rng* (encoding for RNase G) were deleted from SZ470 in order to improve xylose fermentation. Deletion of the HSR2 domain resulted in significantly increased mRNA levels (47-fold to 409-fold) of multiple glycolytic genes (*pgi*, *tpiA*, *gapA*, *eno*), as well as the engineered ethanol pathway genes (*aceEF-lpd*, *adhE*) and the transcriptional regulator Fnr (*fnr*). The higher *adhE* mRNA level resulted in increased AdhE activity (>twofold). Although not measured, the increase of other mRNAs might also enhance expressions of their encoding proteins. The increased enzymes would then enable the resulting strain, RM10, to achieve increased cell growth and complete fermentation of 75 g l^{-1} xylose with an 84% improved ethanol titer (35 g l^{-1}), compared to that (19 g l^{-1}) obtained by the parent, SZ470. However, deletion of *cra* resulted in a negative impact on cell growth and xylose fermentation, suggesting that Cra is important for long-term fermentative cell growth.

Keywords Biomass · Cellulosic ethanol · *E. coli* · Xylose fermentation · RNase G

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Introduction

Although bioethanol is a promising renewable biofuel, it is not feasible to use starch-based bioethanol as a primary transportation fuel due to limited starch resources available for bioethanol production. On the other hand, cellulosic biomass is a well-suited alternative for the expansion of fuel ethanol production because of its large-scale availability, environmentally benign production, and non-competition with food resources [2, 24]. One of the key issues, however, is the development of a biocatalyst that efficiently utilizes biomass-derived C5 sugars (xylose and arabinose) for ethanol production. This is a limiting factor in natural commercial ethanol-producing strains such as *Saccharomyces cerevisiae* and *Zymomonas mobilis*, which are unable to ferment C5 sugars [9–12, 43].

Development of C5 fermenting biocatalysts has been one of the hot research areas of cellulosic ethanol technology. The majority of previous efforts have focused on transgenic approaches, either by cloning homoethanol pathway genes into C5-fermenting hosts [7, 36, 42], or by inserting

the C5 sugar catabolic pathway genes into homoethanol-fermenting hosts [9, 10, 12, 21, 43]. Few studies have been devoted to development of a non-transgenic strain for homoethanol fermentation from C5 sugars [3, 19, 40, 44, 45]. Non-transgenic strains may have fewer concerns than the transgenic ones, at least in certain regions of the world, regarding public perceptions and/or governmental regulations of the genetically modified organisms (GMO) when applied to large-scale fermentation.

In our previous studies, a non-transgenic *Escherichia coli* strain was engineered by establishing a native homoethanol pathway (1.2 xylose [glycolysis] => 2 pyruvate + 2 NADH; pyruvate[pyruvate dehydrogenase] => acetyl-CoA + NADH; acetyl-CoA + 2 NADH [alcohol dehydrogenase] => ethanol; summary stoichiometry 1.2 xylose => 2 ethanol) through deletions of the competing fermentation pathway genes and anaerobic expression of the pyruvate dehydrogenase operon [20, 41, 44, 45]. The resulting non-transgenic strain, SZ420, fermented xylose to ethanol to greater than 90% of the theoretic yield (1.2 molecule of xylose converted to 2 molecules of ethanol). Nevertheless, SZ420 was unable to complete fermentation of 50 g l^{-1} xylose [44]. Subsequently, we selected a fast-growing mutant derived from SZ420 through metabolic evolution [3]. This mutant, KC01, was able to complete 50 g l^{-1} xylose fermentation. Nevertheless, over 120 h was required for KC01 to complete the fermentation due to its limited ethanol tolerance (up to 15–20 g l^{-1} of ethanol). We then enhanced alcohol tolerance of KC01 through adaptive evolution by selection of ethanol tolerant mutants in screw-cap tubes containing gradually increasing concentrations of ethanol [40]. Ultimately, the adaptively evolved mutant SZ470 was able to grow anaerobically in LB medium containing 40 g l^{-1} ethanol and completed 50 g l^{-1} xylose fermentation in 72 h. However, when xylose concentrations were increased to 75 g l^{-1}, SZ470 was unable to complete the fermentation despite the maximum ethanol titer being less than 40 g l^{-1} had the fermentation run to completion. Moreover, SZ470 produced 24% less ethanol in 75 g l^{-1} xylose than in 50 g l^{-1}, even though 75 g l^{-1} xylose should not have caused restrictive osmotic pressure on the strain [3, 42].

In this study, we test the effects of deleting the HSR2 region (110 bp) of *rng* and *cra* (**c**atabolite **r**epressor **a**ctivator) from SZ470 on fermentation of 75 g l^{-1} xylose. The HSR2 region encodes for the DNase I subdomain of endoribonuclease RNase G [34], which participates in mRNA turnover of *adhE* (alcohol dehydrogenase) and in the maturation of the 5′ terminal end of 16s rRNA [15, 37–39]. Deletion of *rng* HSR2 enabled the resulting strain, RM10, to complete 75 g l^{-1} xylose (and arabinose) fermentation in 72–84 h, with an 84% increased ethanol titer (35 g l^{-1}) compared to the parent SZ470 (19 g l^{-1}). On the other hand, deletion of *cra* resulted in a negative impact on cell growth and xylose fermentation; although prior studies indicated that *cra* deletion increased *adhE* expression [17, 30].

Materials and methods

Strains, media, and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. Bacterial cultures were grown at 37°C in Luria–Bertani (LB) broth (g l^{-1}: tryptone 10, yeast extract 5, and NaCl 5) supplemented with 50 g l^{-1} xylose (or arabinose), or on LB plates (agar 15 g l^{-1}) containing 50 g l^{-1} xylose (or arabinose) [40]. During strain construction, antibiotics were added as needed at the following concentrations (μg ml^{-1}): kanamycin, 50; ampicillin, 50.

Genetic methods

Standard methods were used for transformation, electroporation, PCR, and RT-qPCR [27, 33]. Chromosomal gene deletions were constructed using previously described procedures [5, 31, 44, 45]. The chromosomal deletion was verified by analysis of PCR product size. The antibiotic marker (*kan*) was then removed from the chromosome with FLP recombinase by using a temperature-conditional helper plasmid (pFT-A) [31]. The resulting kanamycin sensitive strain was used for fermentation analysis.

Fermentation

Seed cultures were prepared by inoculating colonies from fresh LB xylose plates into a 250-ml flask containing 20 ml of LB broth with 50 g l^{-1} xylose. After incubating for 11 h (35°C, 155 rpm), 2 ml of the culture was used to inoculate a 500-ml fermentation vessel (Fleaker™, Corning) containing 350 ml of LB broth with 75 g l^{-1} xylose (inoculum: 16.5 mg l^{-1} of cell dry wt). Fermentations were maintained at 35°C, 100 rpm mixing, and pH 6.0 by automatic addition of 2 N KOH. All fermentations have three or more replicates.

Quantitative real-time PCR

Escherichia coli SZ470 and RM10 were grown in fermentation vessels containing 75 g l^{-1} xylose, and 5-ml cultures were taken at 60-h time points. Bacterial cells were pelleted. After removing the residue LB medium, the cell pellets were resuspended by vortexing in 90 μl Tris–EDTA buffer (10 mM, pH 7.4, 0.1 mM EDTA, 0.9 mg of lysozyme), and were mixed with 10 μl of 10% SDS at 25°C. Total RNA was isolated using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) as described for bacterial cells, and was treated with RQ1 RNase-Free

Table 1 *Escherichia coli* strains, plasmids, and primers used in this study

Strains	Relevant characteristics	Sources
SZ470	*ΔfrdBC*, *ΔldhA*, *ΔackA*. *ΔfocA-pflB*, *pflBp6-aceEF-lpd*. *Δmgs* ethanol tolerant mutant of KC01	[40]
RM03	SZ470, *Δcra*	This study
RM10	SZ470, Δ*rng* HSR2	This study
RM07	SZ470, Δ*cra*, Δ*rng* HSR2	This study
Plasmids		
pKD4	*bla frt*-kan-*frt*	[5]
pKD46	*bla*, red recombinase, temperature-dependent replication	[5]
pFT-A	*bla*, *flp*, temperature-dependent replication	[31]
Primers		
Δ*cra*-P1	GTGAAACTGGATGAAATCGCTCGGCTGGCGGGAGTGTCGCGGACCGTGTAGGCT GGAGCTGCTTC	This study
Δ*cra*-P2	TTAGCTACGGCTGAGCACGCCGCGGCGATAGAGATTACGTTTAATCATATGAATA TCCTCCTTAG	This study
Verify-*cra*-P1	TTTACGCAAGGGGCAATTGT	This study
Verify-*cra*-P2	TCACCTGGCGCGATTTTTTG	This study
Δ*rng*HSR2-P1	ACCACCGTGGACATCAATACCGGAGCGTTTGTCGGTCATCGCAATG TGTAGGC TGGAGCTGCTTC	This study
Δ*rng*HSR2-P2	GCCTGCTCCAGCGAGTGCAGCACTCGGCGGCGGTGATCTTCATTAC ATATGAATATCCTCCTTAG	This study
Verify-*rng*HSR2-P1	ATGACGGCTGAATTGTTAGTAAACG	This study
Verify-*rng*HSR2-P2	TTACATCATTACGACGTCAAACTGC	This study
RT-qPCR Primers		
rrsA primer 1	CGGTGGAGCATGTGGTTTAA	This study
rrsA primer 2	GAAAACTTCCGTGGATGTCAAGA	This study
pgi primer 1	GACGCTGGCGAAATTACAGGATCTGGCGAAAGAGTG	This study
pgi primer 2	GTGCATGTTCAGGTGGTTTTTGTACGGACGCAGAGC	This study
tpiA primer 1	AGACATCGGCGCACAGTACATCATCATCGGCCACTC	This study
tpiA primer 2	TCAGTTTTGCCCGCTTCGTTTTCAGCTTCGGTTTCA	This study
gapA primer 1	CGGCGCTTCCCAGAACATCATCCCGTCCTC	This study
gapA primer 2	GTTGCAGCTTTTTCCAGACGAACGGTCAGGTCAACT	This study
eno primer 1	GCACATCGCTGAACTGAACGGTACTCCGGGCAAATA	This study
eno primer 2	TCGGCGCATAGCCACCTTCGTCACCA	This study
fnr primer 1	CATCAGCCAGCTTTGCATCCCGTTCACACTCAA	This study
fnr primer 2	ACCAGTGATTTGCTCGTCGCCTTGCTCAGTG	This study
aceF primer 1	CAGGGCGGTTGCTTCACCATCTCCA	This study
aceF primer 2	GCGGCACGAACTCTTTACCATTCCACACC	This study
adhE primer 1	GGTGCAGAACTGGCAAACTCCTTCAAACCAGACGTG	This study
adhE primer 2	TCATTTTCGCTTTCACGCCCATTTTCGGGAACTTGT	This study

DNase (Promega Corp., Madison, WI) to remove residual chromosomal DNA.

cDNA was synthesized using the Superscript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) as follows: 1 μl (0.3–0.9 μg) of the DNase-treated total RNA, 1 μl random decamer primers (1 μg/μl), and 10 μl water were mixed, heated at 65°C for 5 min, and cooled to room temperature, then mixed with 12.5 μl of 2 × SYBR Green reaction mix and 0.5 μl of SYBR enzyme mix containing SuperScript III Reverse Transcriptase and Platinum *Taq* DNA polymerase. The cDNA was synthesized by incubating this reaction mixture in a thermocycler at 25°C for 10 min, 50°C for 50 min, and 95°C for 5 min.

The synthesized cDNAs (template) and the *E. coli* gene-specific primer pairs (Table 1) designed by using Primer3 software [32] were used for quantitative real-time PCR (RT-qPCR) analysis of gene expression using an Mx3000P system (Stratagene) as follows: A mastermix was prepared

by mixing 1.25 μl of each primer (10 ng/μl), 6.75 μl water, and 0.25 μl diluted (1×) reference dye R4526 (Sigma). The RT-qPCR was performed by mixing 9.5 μl mastermix, 3 μl tenfold diluted cDNA, and 12.5 μl SYBR Green JumpStart Taq ReadyMix (Sigma) using the following reaction conditions: initial denaturing (94°C for 2 min), 40 cycles of amplification (94°C for 30 s, 60°C for 30 s, 72°C for 30 s), and final denaturing (72°C for 1 min). Data were collected at the end of the annealing step. The cycle threshold (Ct) for each sample was generated by MxPro RT-qPCR software (Stratagene). The *E. coli* 16s ribosomal gene (*rrsA*) was used as the normalizing gene [29]. Each RT-qPCR reaction was run twice.

Alcohol dehydrogenase assays

Bacterial cells were grown in LB broth containing 5% xylose at 37°C anaerobically for 12 h in 18-ml screw cap tubes. Next, 30 ml of the culture was pelleted, resuspended in 10 ml 1× Tris buffer (100 mM Tris, 1 mM dithiothreitol, pH 6.5), cooled on ice for 20 min, and sonicated three times (10 s each time) using a Sonifier Cell Distributor W-350 (Branson Sonic Power Inc.). The sonicated cells were centrifuged at 4°C. The collected supernatant was used for the alcohol dehydrogenase assay as follows [4, 28]: 100 μl of Tris buffer (1 M, pH 6.5), 500 μl of crude enzyme (cell free extract), 150 μl of NADH (1.5 mM) and 600 μl of H_2O were added to a cuvette (1.5 ml, 1 cm, quartz). The reaction was initiated by adding 150 μl of acetaldehyde (50 mM) to the above mixture, and the absorbance was read at 340 nm for 5 min using a UV-2401PC UV–VIS recording spectrophotometer (Shimadzu). All components without 150 μl of acetaldehyde (50 mM) were used as the blank. One unit of enzyme activity was calculated as nanomoles of NADH used per minute per mg of cell dry mass.

Analyses

Cell mass was estimated by optical density (1.0 ml of cells at 1.0 OD_{550} equals approximately 0.33 mg dry cell weight) using a Unico1100 spectrophotometer with a round culture tube (diameter: 1.0 cm) as a cuvette [40]. Ethanol concentrations were measured by gas chromatography (Varian CP3800 equipped with a flame ionization detector and a capillary column) with 1-propanol used as an internal standard for ethanol measurements. The concentrations of sugars and organic acids were determined by high-pressure liquid chromatography (Waters) equipped with a refractive index detector and a UV detector (210 nm). Products were separated using a Bio-Rad HPX 87H column with 4 mM H_2SO_4 as the mobile phase (10 μl sample injection volume, 0.4 ml min^{-1} of mobile phase running speed, 45°C column temperature).

Results

Deletion of *cra* and the HSR2 region of *rng*

In previous studies, a non-transgenic homoethanol strain *E. coli* SZ420 was engineered from *E. coli* B by chromosomal gene deletions of competing fermentation pathway genes (*ldhA*, *pflB*, *ackA*, *frdBC*) and anaerobic expression of the pyruvate dehydrogenase operon (*pflBp6-aceEF-lpd*) [44, 45]. The cell growth and ethanol production rates of SZ420 were low in glucose and even lower in xylose fermentation [44]. KC01, a fast-growing mutant selected from SZ420 through metabolic evolution, had better cell growth and ethanol production rates [3], but was still unable to grow anaerobically with 15–20 g l^{-1} of ethanol. An ethanol-tolerant mutant, SZ470, selected from KC01 through adaptive evolution, was able to grow anaerobically at 40 g l^{-1} of ethanol, but failed to complete fermentation of 75 g l^{-1} xylose or glucose in multiple trials [40]. Apparently, ethanol tolerance was not the reason for incomplete fermentation of 75 g l^{-1} xylose because the theoretical maximum ethanol titer would be less than 40 g l^{-1} had the fermentation reached completion. Our hypothesis is that the conversion of acetyl-CoA to ethanol catalyzed by alcohol dehydrogenase (AdhE) is a rate-limiting step responsible for incomplete fermentation of 75 g l^{-1} xylose.

To test our hypothesis, *cra* (encoding for Cra, a transcriptional regulator that negatively regulates *adhE* expression) and the HSR2 region of *rng* (encoding for RNase G, an endoribonuclease that plays a role in *adhE* mRNA turnover) were deleted from SZ470 to improve *adhE* expression and xylose fermentation. The resulting strains were designated as RM03 (Δ*cra*), RM10 (Δ*rng* HSR2) and RM07 (Δ*cra*, Δ*rng* HSR2).

50 g l^{-1} xylose fermentation

Small-scale fermentations (500 ml) in pH and temperature-controlled vessels containing 350 ml of LB supplemented with 50 g l^{-1} xylose were initially used to evaluate the fermentation performance of RM03, RM07, and RM10, with SZ470 as the control strain. As shown in Fig. 1a, during the 96-h fermentation, the cell growth was similar during the first 36 h for SZ470 and RM10. However, deletion of the *rng* HSR2 domain enabled RM10 to continuously grow to a maximum cell mass of 6.86 g l^{-1} at 60 h, achieving a 29% higher cell mass than that of SZ470 (5.33 g l^{-1}), which was unable to gain additional cell mass after 36 h. On the other hand, deletion of the *cra* gene and double deletions of *cra* and *rng* HSR2 significantly inhibited fermentative cell growth of RM03 (3.76 g l^{-1}) and RM07 (1.95 g l^{-1}). Neither strain reached the same maximum cell mass of the parent strain SZ470 (5.33 g l^{-1}).

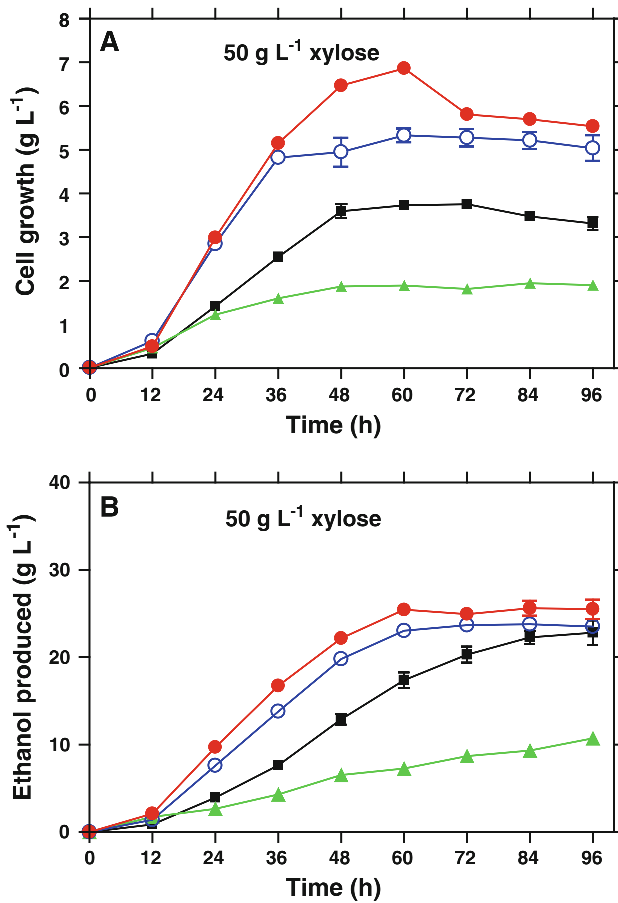

Fig. 1 Evaluation of *cra* and *rng* HSR2 deletion mutant with 50 g l^{-1} xylose fermentation. **a** Cell growth; **b** ethanol production. *Open circle* control strain SZ470; *filled square* RM03 (Δ*cra*); *filled circle* RM10 (Δ*rng* HSR2); *filled triangle* RM07 (Δ*cra*, Δ*rng* HSR2) (color figure online)

Along with improved cell growth, RM10 achieved a maximum ethanol titer of 25 g l^{-1} at 60 h, a 5% improvement over that of SZ470 (23.73 g l^{-1}) (Fig. 1b). However, RM03 and RM07 never reached the same ethanol titer of the control strain SZ470 at 50 g l^{-1} xylose. Therefore, neither RM03 nor RM07 was evaluated further at higher sugar concentration.

75 g l^{-1} xylose fermentation

As previously discussed, SZ470 was unable to complete fermentation of 75 g l^{-1} xylose, probably due to the limitation of AdhE activity. Deletion of the RNase G HSR2 domain enabled RM10 to improve *adhE* expression, cell growth, and ethanol production in 50 g l^{-1} xylose fermentation. To evaluate the potential of RM10, fermentation of 75 g l^{-1} xylose was evaluated in pH-controlled conditions. The results shown in Table 2 and Fig. 2 determined that RM10 grew better than SZ470 at all time points and reached its maximum cell density at 6.89 g l^{-1} after 60 h, whereas SZ470 had a maximum cell density of 5.44 g l^{-1} after 48 h. The growth curve of RM10 was consistent with an increased cell mass during the first 60 h before reaching its growth limit. The growth curve of SZ470 appeared to fluctuate within a small range after 48 h without ever achieving the same cell density as RM10 at the same time period (Fig. 2a). Also, RM10 achieved a significantly improved ethanol production over SZ470 after 12 h of fermentation (Fig. 2b). This improved ethanol production was maintained at a constant rate until fermentation was completed after 84 h, reaching a maximum ethanol titer of 35 g l^{-1} and a yield of 92% for RM10 (Table 2). In contrast, SZ470 was unable to complete fermentation and had a constant, but slower ethanol production rate up to 48 h before leveling off, achieving a maximum ethanol titer of 19 g l^{-1} and a yield of 52% based on initial sugar concentrations added before fermentation. Nevertheless, SZ470 still achieved 90% ethanol yield based on sugar consumed.

In summary, compared to SZ470, RM10 achieved 29% more cell mass, had an improved final ethanol yield (from 52 to 92%) based on the initial concentration of sugar added, a 20% greater maximum volumetric productivity,

Table 2 Summary of 75 g l^{-1} pentose fermentation by RNase G mutant RM10

Strain	Substrate	Cell growth[a]		Ethanol produced[b]		Volumetric productivity[c] (g l^{-1} h^{-1})		Specific productivity[c] (g g^{-1} h^{-1})	
		Mass (g l^{-1})	Rate (h^{-1})	Titer (g l^{-1})	Yield (%)	Maximum	Average	Maximum	Average
SZ470	Xylose	5.44 ± 0.063	0.113 ± 0.001	19 ± 1.26	52	0.423 ± 0.063	0.392 ± 0.013	0.078 ± 0.012	0.072 ± 0.003
RM10	Xylose	6.89 ± 0.361	0.114 ± 0.006	35 ± 2.53	92	0.509 ± 0.044	0.407 ± 0.026	0.074 ± 0.004	0.059 ± 0.003
RM10	Arabinose	7.50 ± 0.537	0.125 ± 0.009	34.5 ± 1.19	90	0.565 ± 0.012	0.459 ± 0.002	0.075 ± 0.006	0.061 ± 0.008

[a] Cell mass was the maximum cell mass achieved (48 h for SZ470; 60 h for RM10). The growth rate was calculated from the time zero to the time point achieving maximum cell mass (0–48 h for SZ470; 0–60 h for RM10)

[b] Yield was calculated as percent of theoretical maximum (0.51 g ethanol per g of initial sugar added)

[c] Maximum volumetric and specific productivities were calculated from the most productive 24 h period (24–48 h for both SZ470 and RM10). Average volumetric and specific productivities were calculated from the time zero to the time culture ceased to produce product (0–48 h for SZ470, xylose; 0–84 h for RM10, xylose; 0–72 h for RM10, arabinose)

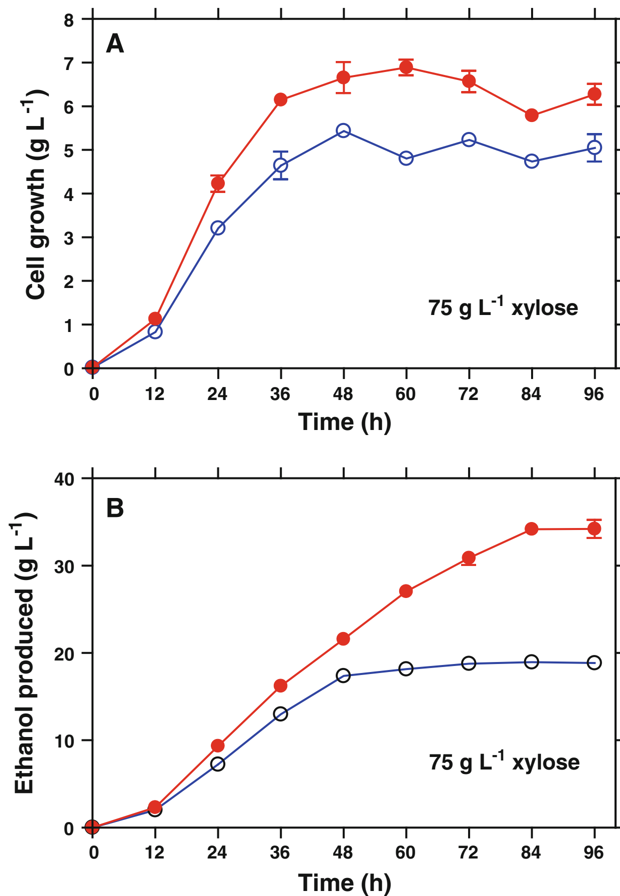

Fig. 2 Evaluation of RM10 (Δ*rng* HSR2) with 75 g l^{-1} xylose fermentation. **a** Cell growth; **b** ethanol production. *Open circle* control strain SZ470; *filled circle* RM10 (Δ*rng* HSR2) (color figure online)

and completed 75 g l^{-1} xylose fermentation with an 84% increased ethanol titer.

75 g l^{-1} arabinose fermentation

With the success of 75 g l^{-1} xylose fermentation by RM10, arabinose, the second-most abundant pentose sugar commonly found in cellulosic biomass, was measured for fermentative efficiency. The results are shown in Table 2 and Fig. 3. The fermentation performance of RM10 in 75 g l^{-1} arabinose was similar (if not identical) to the 75 g l^{-1} xylose fermentation. RM10 maintained cell growth for 60 h, reached a maximum cell mass of 7.50 g l^{-1}, and completed 75 g l^{-1} fermentation in 72 h, with a consistent ethanol production rate and a maximum ethanol titer of 34.5 g l^{-1}.

Genetic impacts of *rng* HSR2 deletion on the expression of *adhE* and other homoethanol pathway genes

To understand the genetic effects of the *rng* HSR2 deletion for improved xylose fermentation, the expression of *adhE*

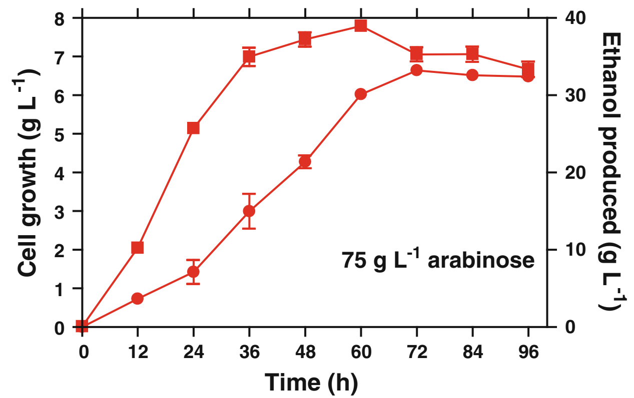

Fig. 3 Evaluation of RM10 (Δ*rng* HSR2) with 75 g l^{-1} arabinose fermentation. *Filled square* cell growth; *filled circle* ethanol production (color figure online)

and other glycolytic genes were analyzed by RT-qPCR and compared to those of the parent strain SZ470. As shown in Fig. 4, the *rng* HSR2 deletion significantly increased *adhE* mRNA levels by 409-fold and AdhE activity by greater than twofold (data not shown), when compared to the parent strain SZ470. Interestingly, the RNase G HSR2 deletion also increased mRNA expression of many other genes related to glycolysis and the homoethanol pathway, such as glucokinase (158-fold), phosphoglucose isomerase (88-fold), triose phosphate isomerase (78-fold), glyceraldehyde 3-phosphate (47-fold), enolase (164-fold), pyruvate dehydrogenase (*aceEF-lpd*) (64-fold), and transcriptional regulator Fnr (*fnr*) (88-fold) [25]. Enhanced expression of the *aceEF-lpd* operon was probably derived from the improved *fnr* expression, which enhances anaerobic expression of *pflB*, because *aceEF-lpd* was previously transcriptionally fused with a *pflB* promoter and Fnr binding box [44, 45].

Discussion

Ethanol is a minor fermentation product in wild-type *E. coli*. The expression of alcohol dehydrogenase (*adhE*) is regulated at the transcriptional and translational levels to accommodate this minor production. First, *adhE* is transcriptionally regulated by the Cra protein, a dual transcriptional regulator that directs carbon flow [30]. Previous research showed that Cra down-regulates *adhE* expression, and Cra mutants showed an increased production of the *adhE* transcript [17, 22, 26]. Second, the ribosomal binding site of *adhE* mRNA is blocked by its secondary structure. Removing the secondary structure by RNase III is required for *adhE* translation [1]. However, removing this secondary structure exposes a 5′-monophosphate terminal end of *adhE* mRNA, which is subjected to RNase G degradation [14]. RNase G, encoded by the *rng*, is a member of the RNase E/G family that acts as ribonucleases [6, 8, 13, 16].

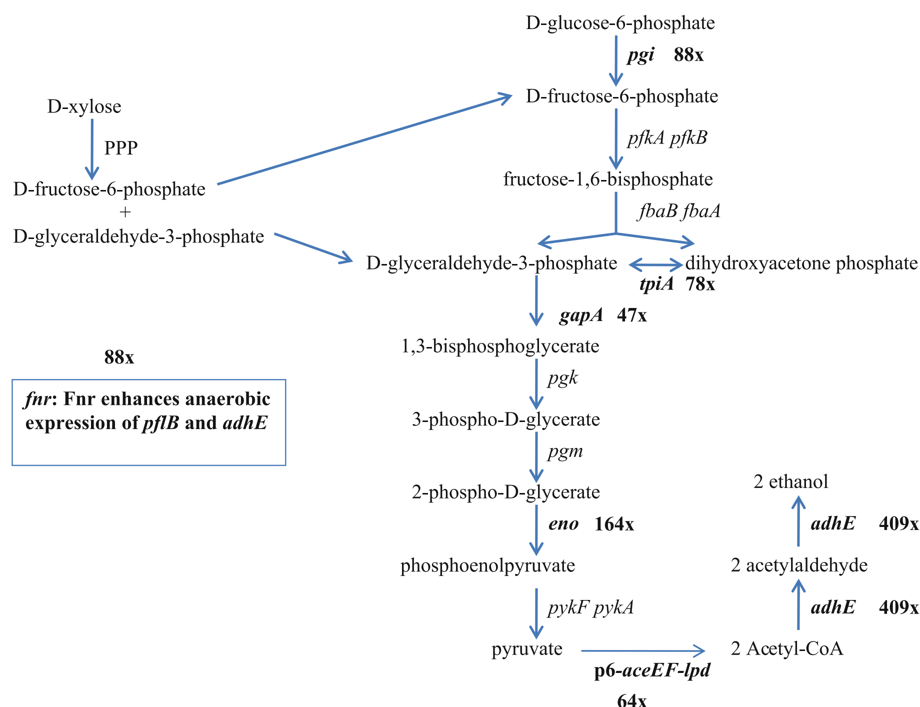

Fig. 4 Enhanced expressions of the homoethanol pathway genes by deletion of *rng* HSR2. The enhanced expressions of the genes encode for the following homoethanol pathway enzymes: *adhE* alcohol dehydrogenase; p6-*aceEF-lpd* anaerobically expressed pyruvate dehydrogenase with a *pflB* promoter; *eno* Enolase; *gapA*, glyceraldehyde-3-phosphate dehydrogenase; *tpiA* triose phosphate isomerase; *pgi*, phosphoglucose isomerase; *fnr* transcriptional regulator (that enhances expression of anaerobic genes). The number following the gene name was the fold increase in the mRNA level after *rng* HSR2 deletion (color figure online)

Both RNases were found to play an integral role in 16 s rRNA maturation [37] and mRNA turnover [18, 35, 37]. Nevertheless, RNase G plays a minor role in the RNase E/G family and is dispensable in wild-type *E. coli* [23, 38]. The defects in RNase G were found to up-regulate *adhE* expression [37]. Further research has shown that a defect in the C-terminal end of RNase G High Similarity Region II (HSR2) increased AdhE production while maintaining 16 s rRNA maturation [39].

In the engineered homoethanol strain SZ470, however, ethanol is the sole fermentation product. The negative regulation of Cra and RNase G on *adhE* expression may limit efficient ethanol fermentation. The effects of Cra and RNase G on SZ470 ethanol fermentation from xylose were, therefore explored via gene deletions of *cra* (RM03), *rng* HSR2 (RM10), and the double deletions of *cra* and *rng* HSR2 (RM07). Although, prior studies indicate that *cra* deletion increased *adhE* expression [17, 26], in this study, however, *cra* deletion or double deletions of *cra* and HSR2 of *rng* had negative impacts on cell growth and xylose fermentation. Even though RM03 nearly completed 50 g l^{-1} xylose fermentation, it did so with a 36-h-longer fermentation time when compared to SZ470 (Fig. 1). RM07 had nowhere near approached SZ470′s maximum cell mass and ethanol titer. These results led us to conclude that the Cra protein is vital to long-term fermentative growth, and the deletion of *cra* (RM03 & RM07) had a negative impact on cell growth and ethanol production.

RNase G HSR2's role in degrading *adhE* mRNA and decreasing AdhE expression, which subsequently regulated ethanol production, was also evident by RT-qPCR and enzyme assay data in our current study. As expected, the *adhE* mRNA had an extended half-life when *rng* HSR2 was deleted [37, 39], resulting in a 409-fold higher *adhE* mRNA expression level in RM10 compared to that of SZ470. Interestingly, the levels of the AdhE enzyme were not proportional to the amount of mRNA present in RM10, with slightly greater than a twofold increase in AdhE enzyme activity. This enhancement, however, is sufficient to enable RM10 to complete fermentation of 75 g l^{-1} xylose (or arabinose).

The significant improvement of xylose (and arabinose) fermentations by the *rng* HSR2 deletion may not only have been derived from the significantly increased *adhE* expression but also from the increased mRNA expressions of the genes active in glycolysis and the homoethanol pathway (Fig. 4). The greater mRNA expressions of glucokinase, phosphoglucose isomerase, triose phosphate isomerase, glyceraldehyde 3-phosphate, enolase, pyruvate dehydrogenase, alcohol dehydrogenase and Fnr resulted in faster glycolysis and ethanol production by RM10.

Lastly, 100 g l^{-1} xylose fermentation was attempted with RM10, but was unsuccessful in growth media used for the aforementioned fermentations. However, upon increasing the concentration of tryptone (enzymatic digest of casein, source of amino acids), RM10 was able to complete 100 and 120 g l^{-1} xylose fermentation with >90% efficiency in 120 h and 216 h, respectively (data not shown). Tryptone provides all the amino acids needed for *E. coli* growth, and determining which specific amino acids enabled RM10 to complete up to 120 g l^{-1} xylose fermentation is currently being analyzed.

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