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Short communication

Combined overexpression of genes involved in pentose phosphate pathway enables enhanced D-xylose utilization by *Clostridium acetobutylicum*

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ABSTRACT

D-Xylose utilization by *Clostridium acetobutylicum*, an important industrial microorganism used in ABE (*A*cetone, *B*utanol and *E*thanol) production, has attracted increasing interests. We demonstrated previously that co-overexpression of genes, encoding D-xylose symporter, D-xylose isomerase and xylulokinase, improved D-xylose utilization by *C. acetobutylicum* (Xiao, H., et al., 2011. Applied and Environmental Microbiology 77, 7886–7895). Here, we further identified genes involved in PPP (*P*entose *P*hosphate *P*athway) in *C. acetobutylicum* and evaluated their contribution to D-xylose utilization. Among all the candidate genes, the CAC1347, CAC1348, CAC1730 and CAC2880 were validated to encode genes *tal*, *tkl*, *rpe* and *rpi*, four key genes involved in PPP, respectively. The following combined overexpression of these genes conferred a significantly improved xylose-utilizing ability to the recombinant strain, reaching a solvent titer 42% higher than that of the wild-type strain. This finding offers a useful strategy to optimize D-xylose utilization by *C. acetobutylicum*.

Clostridium acetobutylicum is an important member of solventogenic clostridia, which offer hope toward sustainable and economical ABE solvent production through biological route (Gu et al., 2011). Butanol is not only a bulk chemical but also considered as a potential biofuel (Dürre, 2007). Compared to the food crop, lignocellulosic resources have been regarded as preferred substitutes to be used in ABE fermentation. D-Xylose metabolism is normally a key issue when lignocellulosic hydrolysates were used as the substrate of fermentation (Aristidou and Penttilä, 2000). Although *C. acetobutylicum* is naturally capable of utilizing D-xylose, its efficiency is undesired (Ounine et al., 1985). The recombinant *C. acetobutylicum* strain with increased TAL activity by overexpressing *Escherichia coli talA* gene was found to exhibit improved xylose-utilizing ability (Gu et al., 2009), suggesting that PPP genes may be essential for D-xylose metabolism in *C. acetobutylicum*. It is known that four key enzymes, namely transaldolase (TAL), transketolase (TKL), ribose-5-phosphate isomerase (RPI) and ribulose-5-phosphate 3-epimerase (RPE), normally catalyze PPP reactions responsible for xylose metabolism in microbes. According to genome annotation (Nölling et al., 2001), *C. acetobutylicum* only harbors one *tal* gene (CAC1347) and *rpe* gene (CAC1730), while has two putative *tkl* genes (CAC0944 and CAC1348) and three putative *rpi* genes (CAC0762, CAC1341 and CAC2880). To date, the genes involved in PPP have not yet been experimentally identified in this anaerobe.

In this study, we identified four genes (*tal*, *tkl*, *rpe* and *rpi*) involved in PPP and then examined the contribution of their combined overexpression to D-xylose utilization by *C. acetobutylicum*. All bacterial strains, plasmids and primers used in this study were listed in Table S1. Heterologous-host complementation in *E. coli* was carried out to examine whether the gene CAC0944 or CAC1348 is sufficient to perform the predicted transketolase function. They were separately introduced into *E. coli* BJ502 (Table S1), a mutant that is defective in transketolase expression. MacConkey agar supplemented with D-xylose was chosen as a selective culture medium.

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E. coli background	Plasmids carrying *C. acetobutylicum* genes	Xylose utilization
WT	—	
BJ502(Δ*tkl*)	pUC118	
BJ502(Δ*tkl*)	pUC118::CAC0944	
BJ502(Δ*tkl*)	pUC118::CAC1348	
JW3349-2(Δ*rpe*)	pUC118	
JW3349-2(Δ*rpe*)	pUC118::CAC1730	
JW5475-1-rpiB(Δ*rpiA* Δ*rpiB*)	pUC118	
JW5475-1-rpiB(Δ*rpiA* Δ*rpiB*)	pUC118::CAC0762	
JW5475-1-rpiB(Δ*rpiA* Δ*rpiB*)	pUC118::CAC1341	
JW5475-1-rpiB(Δ*rpiA* Δ*rpiB*)	pUC118::CAC2880	

Fig. 1. Complementation of *E. coli* mutants deficient in PPP genes by *C. acetobutylicum* genes. The empty vector was expressed in the same strain as a negative control. The colonies of cells capable of utilizing D-xylose showed red color, whereas those unable to utilize D-xylose keep yellow.

The detailed experimental steps are as previously described (Gu et al., 2010). As shown in Fig. 1, expression of the gene CAC1348 completely restored the ability of *E. coli* BJ502 to utilize D-xylose (red color), whereas the CAC0944 could not. Actually, the expression patterns of these two genes were also quite different in the presence of different carbon resources (Grimmler et al., 2010; Servinsky et al., 2010). The CAC1348 was strongly induced by D-xylose and L-arabinose, whereas the CAC0944 was not; in the presence of glucose, the CAC1348 expression was repressed while the CAC0944 was not. These results indicated that the CAC1348 is involved in the pentose metabolism of *C. acetobutylicum*.

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This strategy was also used to identify the *rpi* gene from three candidates (*i.e.*, the CAC0762, CAC1341 and CAC2880) and to confirm the role of the CAC1730 in encoding RPE enzyme in *C. acetobutylicum*. PCR-targeting method (Datsenko and Wanner, 2000) was used for gene *rpiB* (b4090) deletion in *E. coli* JW5475-1, yielding the JW5475-1-rpiB. The results showed that the CAC1730 and CAC2880 were capable of restoring the ability of the *E. coli* JW3349-2 (Table S1) and *E. coli* JW5475-1-rpiB mutant (Table S1) to utilize D-xylose (Fig. 1), demonstrating their functions in encoding RPE and RPI enzyme, respectively.

As to the putative *tal* gene (CAC1347), the method above was not used because there were no suitable *E. coli* mutants for functional complementation. (Even the *E. coli* Δ*talA* Δ*talB* mutant still retained appreciable D-xylose-utilizing ability and thus showed red color on MacConkey agar plates; data not shown.) Therefore, we chose *in vitro* activity assessment to confirm the role of the CAC1347 in encoding TAL enzyme, namely overexpressing this gene in the *E. coli* Δ*talA* Δ*talB* mutant (PCR-targeting method was used for gene *talB* deletion) and then measuring the TAL activity of cell extracts. As expected, the overexpression strain showed approximate 50% higher TAL activity than the control strains (wild type and *E. coli* Δ*talA* Δ*talB* mutant) (Table 1). In addition, this gene was found to be part of an *araR*-containing genetic loci (CAC1339–CAC1349) that is responsible for L-arabinose metabolism and PPP reactions in *C. acetobutylicum* (Zhang et al., 2012), and moreover, was strongly induced by both D-xylose and

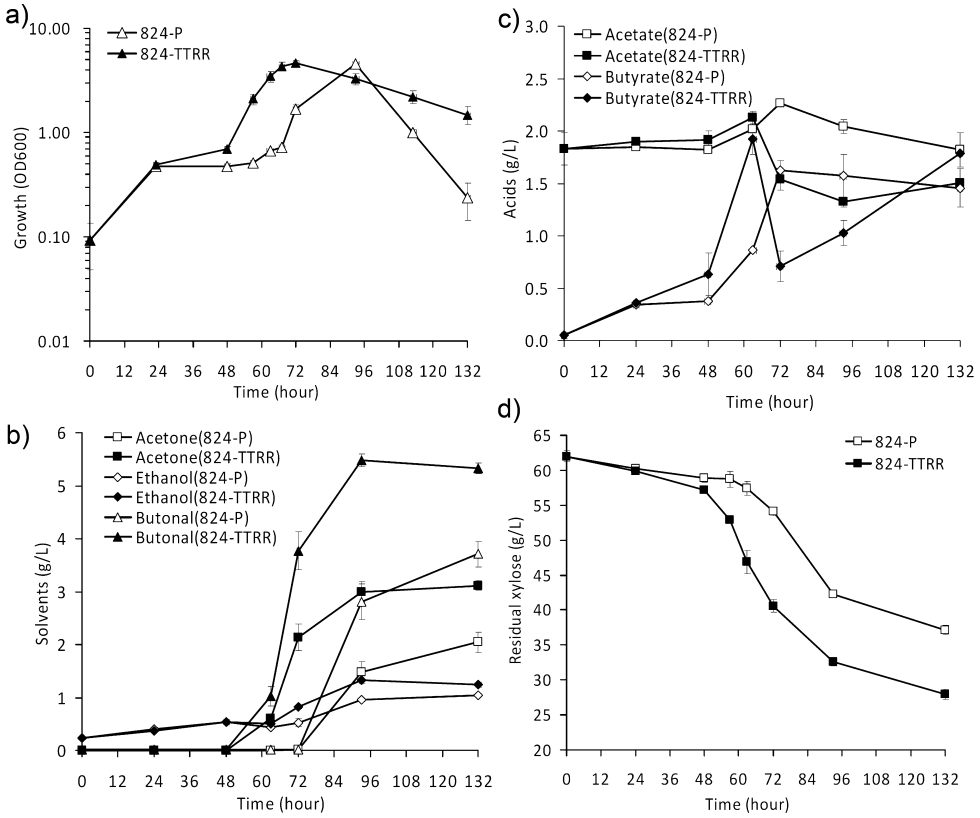

Fig. 2. Comparative fermentation performance of *C. acetobutylicum* 824-P and 824-TTRR: (A) cell growth; (B) solvents; (C) acids; and (D) residual sugars. The CGM (Wiesenborn et al., 1988) and P2 medium (Baer et al., 1987) were used for pregrowth and routine fermentation, respectively. Cell growth was monitored by A_{600} with a Hitachi U-1800 spectrophotometer. The concentrations of solvents and sugar in the medium were determined using gas chromatography (GC) and high-pressure liquid chromatography (HPLC) system, respectively. Conditions of gas and liquid chromatographs were the same as described previously (Ren et al., 2010).

Table 1
Specific transaldolase activity in crude cell extracts of *E. coli* strains.

E. coli strains[a]	Activities (U/mg protein)[b]
K12 (wild type)	0.099 ± 0.003
JW2448-4-talB-P (Δ*talA* Δ*talB*, pUC118)	0.084 ± 0.005
JW2448-4-talB-1347(Δ*talA* Δ*talB*, expressing CAC1347)	0.146 ± 0.003

[a] Samples were harvested at 24 h of fermentation.
[b] Transaldolase (TAL) activity was measured spectrophotometrically as previously described (Banki et al., 1996). One unit of enzyme activity was defined as the amount of enzyme that oxidizes 1 mol of NADH per min under these conditions. Values represent the average of duplicate experiments with a standard deviation between samples. The average and the standard deviation were determined from duplicate independent experiments.

L-arabinose (Servinsky et al., 2010). Thus, it can be concluded that the CAC1347 is a TAL-encoding gene.

Then, these four genes were cloned into the plasmid pIMP1 (Mermelstein and Papoutsakis, 1993) and driven by the promoter P_{thl} and P_{ptb} (Tummala et al., 1999), yielding the plasmid pIMP1-TTRR (Table S1). For combined overexpression of these four genes, the plasmid pIMP1-TTRR was firstly methylated (Mermelstein and Papoutsakis, 1993) and then transformed into *C. acetobutylicum* ATCC 824, yielding the recombinant strain 824-TTRR.

The 824-TTRR was examined in utilizing D-xylose compared to the plasmid control strain 824-P. D-Xylose (60 g/L) was used as the sole carbon source and 10 μg/mL of erythromycin was supplemented in the medium for stable plasmid maintenance. As expected, the 824-TTRR strain grew much faster than the 824-P during the fermentation (Fig. 2A). Accordingly, much more xylose was used by the 824-TTRR strain compared to the control. At 132 h of fermentation, about 34 g/L D-xylose was consumed by the strain 824-TTRR, while that of the strain 824-P was only 24 g/L (Fig. 2D). In addition, more acids were reassimilated by the 824-TTRR against the 824-P (Fig. 2C). Finally, overexpression of these four genes enabled the 824-TTRR to produce 3.11 g/L of acetone, 5.32 g/L of butanol and 1.24 g/L of ethanol, which were much higher that those (2.05 g/L of acetone, 3.71 g/L of butanol and 1.04 g/L of ethanol) of the 824-P (Fig. 2B). The transcriptional level of these four genes of the 824-TTRR and the 824-P were also compared. RNA preparation and real-time PCR were performed as described previously (Xiao et al., 2011). The gene CAC0905 (encoding dehydrogenase) was used as an internal control (Servinsky et al., 2010). The results showed that the 824-TTRR had significant increase in transcript of all four genes over the 824-P, reaching 116, 46, 43 and 96-fold, respectively. This suggested a sufficient plasmid-carried gene expression here. Next, this engineering strategy, combined with our previous method (Xiao et al., 2011), might yield a new recombinant strain superior over its predecessors in D-xylose utilization.

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