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Homo-D-Lactic Acid Fermentation from Arabinose by Redirection of the Phosphoketolase Pathway to the Pentose Phosphate Pathway in L-Lactate Dehydrogenase Gene-Deficient *Lactobacillus plantarum*▽

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Optically pure D-lactic acid fermentation from arabinose was achieved by using the *Lactobacillus plantarum* NCIMB 8826 strain whose L-lactate dehydrogenase gene was deficient and whose phosphoketolase gene was substituted with a heterologous transketolase gene. After 27 h of fermentation, 38.6 g/liter of D-lactic acid was produced from 50 g/liter of arabinose.

Polylactic acid (PLA) can be produced from biomass and stereocomplex PLA, which is composed of both poly-L- and poly-D-lactic acid, has recently attracted attention due to its high thermostability. Therefore, D-lactic acid production, in addition to L-lactic acid production, is of significant importance.

Lignocellulose is inedible and represents one of the most abundant biomass sources in the world, consisting of three major components: cellulose, hemicellulose, and lignin (13). Cellulose consists of glucose that is available for microbial lactic acid fermentation, while hemicellulose consists primarily of pentose sugars such as xylose and arabinose, which are unavailable for most microorganisms (5). Therefore, efficient utilization of pentose sugars should be also developed.

Some lactic acid bacteria, such as *Lactobacillus pentosus* (2), *Lactobacillus brevis* (3), *Lactobacillus plantarum* (4), and *Leuconostoc lactis* (11), are known to ferment either or both arabinose and xylose. In these microorganisms, arabinose is converted to xylulose-5-phosphate (X5P) by arabinose isomerase (AraA), ribulokinase (AraB), and ribulose 5-phosphate 4-epimerase (AraD) (4) (Fig. 1). X5P is further converted to equimolar amounts of lactic acid and acetic acid (14). This low yield of lactic acid by heterolactic acid fermentation is an undesirable feature for industrial mass production of lactic acid as a raw material for production of PLA.

In this study, we successfully demonstrated homo-D-lactic acid fermentation from arabinose by engineering a metabolic pathway in L-lactate dehydrogenase gene-deficient *Lactobacillus plantarum* NCIMB 8826 (*L. plantarum* Δ*ldhL1*). *L. plantarum* Δ*ldhL1* strain can produce optically pure D-lactic acid (12) from arabinose. However, this strain produces both D-lactic acid and acetic acid from arabinose. As shown in Fig. 1, the phosphoketolase (PK) pathway produces both lactic acid and acetic acid, but the pentose phosphate (PP) pathway produces only lactic acid as a final product. Therefore, we promoted redirection of the PK pathway to the PP pathway in *L. plantarum* Δ*ldhL1* by substituting an endogenous PK gene (*xpk1*) with the heterologous transketolase gene (*tkt*) from *Lactococcus lactis* IL 1403 (1), thereby shifting heterolactic acid fermentation to homolactic acid fermentation.

The bacterial strains, oligonucleotides, and plasmids used in this study are listed in Table 1. The plasmid for disruption of the PK gene (*xpk1*) was constructed as follows. The 520-bp upstream region from the start codon of *xpk1* and the 1,000-bp downstream region from the stop codon of *xpk1* were amplified by PCR from the genome of *L. plantarum* NCIMB 8826 using oligonucleotide primers xpk1-up_F plus xpk1-up_R and xpk1-down_F plus xpk1-down_R, respectively. The resulting fragments were digested with SalI and ligated. Using the ligated fragment (1,520 bp) as a template, the same fragment was amplified by PCR using oligonucleotide primers xpk1-up_F and xpk1-down_R. The amplified fragment was digested with XhoI and NotI and was subsequently inserted into the XhoI and NotI sites of the plasmid pG$^+$host9 (8). The resulting plasmid was designated pGh9-Δxpk1. The plasmid to substitute *xpk1* with the heterologous transketolase gene from *L. lactis* IL 1403 (*tkt*) was constructed as follows. The open reading frame of *tkt* was amplified by PCR from the genome of *L. lactis* IL 1403 using oligonucleotide primers tkt_F and tkt_R. The amplified fragment was digested with BglII and SalI and was subsequently inserted into the BglII and SalI sites of plasmid pGh9-Δxpk1. The resulting plasmid was designated pGh9-xpk1::tkt. Disruption and substitution of the *xpk1* gene of *L. plantarum* Δ*ldhL1* using pGh9-Δxpk1 and pGh9-xpk1::tkt were carried out by pG$^+$host plasmid-based double-crossover homologous integration, as described previously (12). The resulting *xpk1*-disrupted or *xpk1*-substituted strains of *L. plantarum* Δ*ldhL1* were designated *L. plantarum* Δ*ldhL1*-Δ*xpk1* and *L. plantarum* Δ*ldhL1-xpk1*::*tkt*, respectively. Deletion and substitution of *xpk1* were confirmed by PCR, and the stability of a deleted or substituted *xpk1* gene is maintained after several times of passage cultivation.

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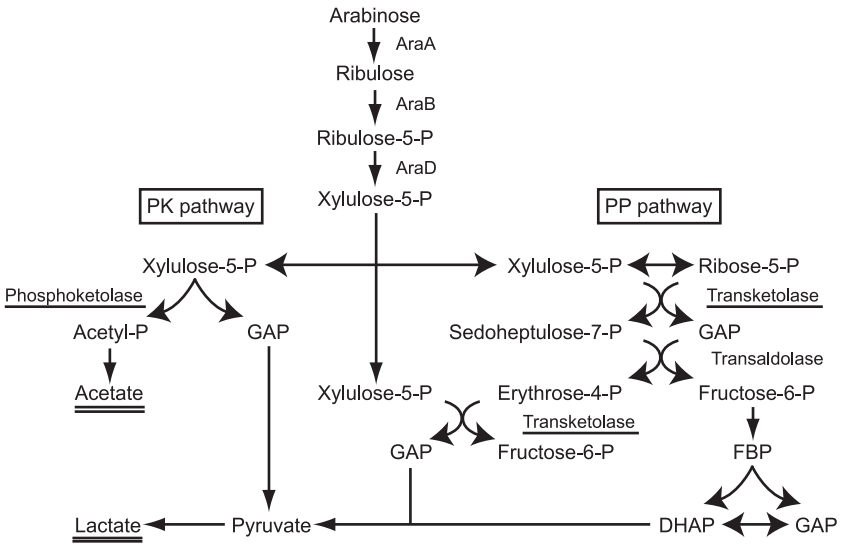

FIG. 1. Predicted pathway for arabinose metabolism in *L. plantarum* mutant strains. The key enzymes phosphoketolase and transketolase are underlined, and the key products lactic acid and acetic acid are double-underlined. P, phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate.

Cell growth with arabinose was evaluated using modified MRS medium (10 g/liter of peptone, 5 g/liter of yeast extract, 2 g/liter of K_2HPO_4, 2 g/liter of ammonium citrate, 0.58 g/liter of $MgSO_4 \cdot 7H_2O$, and 0.28 g/liter of $MnSO_4 \cdot 5H_2O$; all reagents are purchased from Nacalai Tesque, Inc., Kyoto, Japan) containing 2.0% (wt/vol) arabinose and lacking sugar at 37°C. Figure 2 shows the optical density at 600 nm (OD_{600}) of each strain after 12 h of cultivation. The *L. plantarum* Δ*ldhL1* strain was efficiently grown using arabinose as the sole carbon source (OD_{600} of 1.48). In the case of control cultivation (without arabinose), growth decreased markedly (OD_{600} of 0.59). Alternatively, *L. plantarum* Δ*ldhL1*-Δ*xpk1* showed poor growth with arabinose (OD_{600} of 0.26), even compared with control cultivation (OD_{600} of 0.45). This lack of growth was restored by

TABLE 1. Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant phenotype and description or sequence (5′ to 3′)[a]	Source or reference
Strains		
Escherichia coli		
VE7108	Kanamycin resistance marker; containing the wild-type *repA* plasmid gene (not thermosensitive); host for pG$^+$host9 DNA manipulation	9
VE6838	Kanamycin resistance marker; VE7108 carrying pG$^+$host9	9
Lactobacillus plantarum		
WT	NCIMB 8826 wild-type strain	NCIMB
Δ*ldhL1*	NCIMB 8826 Δ*ldhL1* strain	12
Δ*ldhL1*-Δ*xpk1*	NCIMB 8826 Δ*ldhL1* Δ*xpk1* strain	This study
Δ*ldhL1-xpk1*::*tkt*	NCIMB 8826 Δ*ldhL1* strain with *xpk1* gene replaced with *tkt*	This study
Plasmids		
pG+host9	Thermosensitive replicon; erythromycin resistance marker	8
pGh9-Δxpk1	Vector for deletion of *xpk1* gene of Δ*ldhL1*; erythromycin resistance marker	This study
pGh9-xpk1::tkt	Vector for replacement of *xpk1* gene of Δ*ldhL1* with *tkt*; erythromycin resistance marker	This study
Oligonucleotide primers		
xpk1-up_F	CCGCTCGAGGCTGCTGGTCATTCGGAATT	
xpk1-up_R	AACGAGCTCGTCGACAGATCTTTCAAAAACACTCCTTAATTTGCTTTTC	
xpk1-down_F	ACGCGTCGACGAGCTCGCAGTTAAAATCTTAATTTGAAAATAGCGT	
xpk1-down_R	GTGGCGGCCGCATGAGCTTGCATGGTCGG	
xpk1-up_seq	AAGCACTGAACTGGTTGCC	
xpk1-down_seq	ATAATTAAAAACCAATTAAAATATGCTCACAATTC	
tkt_F	GGAAGATCTATGTTTGATACTACTGATCAATTGGCTG	
tkt_R	ACGCGTCGACTTAGTCAAGCTCTTTGTATGCTTTAACTA	

[a] For primers, restriction enzyme sites are underlined.

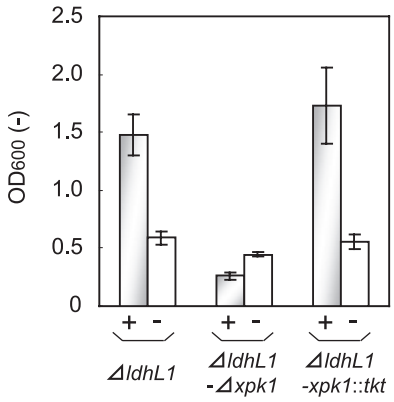

FIG. 2. Growth assay with arabinose using *L. plantarum* NCIMB 8826 Δ*ldhL1*, *L. plantarum* Δ*ldhL1*-Δ*xpk1*, and *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* strains. +, growth in medium with arabinose; −, growth in medium without arabinose. Each bar represents the mean and standard deviations for three independent experiments.

introducing *tkt* into the *xpk1* locus, and the resulting strain *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* showed a higher OD_{600} value, 1.73, than that for control cultivation (OD_{600} of 0.56). These results suggest that the PK pathway is the sole pathway responsible for arabinose assimilation in *L. plantarum* Δ*ldhL1* and that introduction of transketolase enables assimilation of pentose through the PP pathway. Although *L. plantarum* Δ*ldhL1* has another gene, *xpk2*, that encodes a putative PK (7), our results suggest that *xpk2* is silent or its gene product was inactivated in some way under the present cultivation conditions. And although there are several genes that encode presumed transketolases (*tkt2*, *tkt3*, and *tkt4*) and transaldolases (*tal1*, *tal2*, and *tal3*) in the genome of *L. plantarum* (7), these enzymes do not appear to be expressed in their active form, as *L. plantarum* Δ*ldhL1*-Δ*xpk1* could not utilize arabinose (Fig. 2).

Encouraged by these findings, we carried out lactic acid fermentation from 50 g/liter of arabinose using *L. plantarum* Δ*ldhL1* and *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* strains in a 2.0-liter bioreactor. Heat-sterilized, liquid-modified MRS medium and arabinose solution were added to the fermentor, 10 M H_2SO_4 was added to the medium in order to adjust the pH to 6.0, and 7 ml of inoculum (adjusted to an OD_{600} of 10 with sterile distilled water) was added. Prior to this addition, inoculum was grown in MRS medium and subcultured at regular intervals (12 h) to stabilize the growth rate. Temperature was maintained at 36°C, agitation speed was maintained at 100 rpm, and pH was kept at approximately 6.0 (±0.03) by the automatic addition of 10 M NH_3 solution. Arabinose and lactic acid concentrations were measured by high-performance liquid chromatography as described previously (6, 10). The optical purity of lactic acid was measured using a BF-5 biosensor (Oji Scientific Instruments, Hyogo, Japan) as described previously (12). Optical purity of lactic acid was defined as follows: optical purity (%) = (D-lactic acid concentration − L-lactic acid concentration)/(D-lactic acid concentration + L-lactic acid concentration) × 100.

As shown in Fig. 3a and b, *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* showed significantly higher growth, sugar consumption, and acid production rates than *L. plantarum* Δ*ldhL1*. The OD_{600} value for *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* increased with fermentation time and reached 14.9 after 24 h of fermentation, while that of *L. plantarum* Δ*ldhL1* was significantly lower, even after 36 h of fermentation (Fig. 3a). Differences in sugar consumption and acid production were also observed between the strains; *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* consumed all sugar within 27 h of fermentation and produced 39.2 g/liter of organic acid (Fig. 3b), while fermentation continued after 36 h with *L. plantarum* Δ*ldhL1*, with 19.6 g/liter of arabinose being consumed and 15.0 g/liter of organic acid being produced (Fig. 3b).

As shown in Fig. 3c and Table 2, *L. plantarum* Δ*ldhL1* produced both lactic acid and acetic acid (8.7 and 6.3 g/liter, respectively), while *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* produced predominantly lactic acid over acetic acid (38.6 and 0.4 g/liter, respectively). As a result, *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* achieved a high lactic acid yield of 0.82 (gram per gram of consumed arabinose) compared with that of *L. plantarum* Δ*ldhL1* (0.44) (Table 2). Moreover, the optical purity of lactic acid was very high at 99.9% (Table 2). These results strongly indicate that introduction of the PP pathway by substitution of *xpk1* with *tkt* was useful for homo-D-lactic acid fermentation from arabinose.

Surprisingly, substitution of *xpk1* with *tkt* provoked not only a shift to homolactic acid fermentation but also a significant improvement in cell growth and fermentation rate (Fig. 3) (Table 2). The high maximum volumetric productivity of lactic acid by *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* from arabinose (3.78 g/liter/h) is comparable to that from glucose (4.54 g/liter/h)

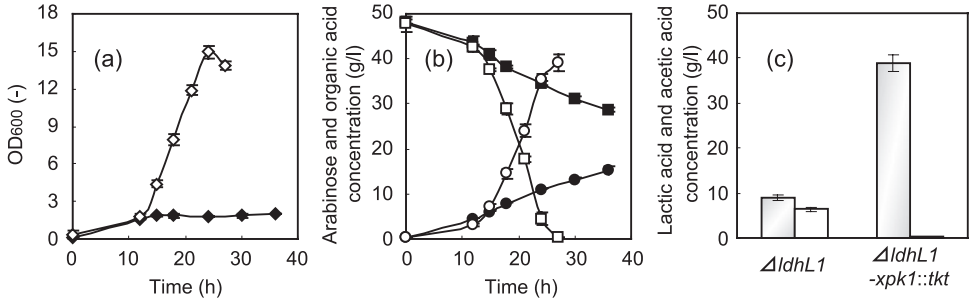

FIG. 3. Fermentation experiments with arabinose using *L. plantarum* NCIMB 8826 Δ*ldhL1* (closed symbols) and *L. plantarum* Δ*ldhL1*-*xpk1*::*tkt* (open symbols) strains. (a) OD_{600} of culture (diamonds). (b) Arabinose (squares) and organic acid (as sum of lactic acid and acetic acid) (circles) concentrations. (c) Lactic acid (gray bars) and acetic acid (white bars) concentrations. Data points and bars represent means and standard deviations for three independent experiments.

TABLE 2. Various parameters in arabinose fermentation[a]

Strain	Lactic acid produced (g/liter)	Acetic acid produced (g/liter)	Arabinose consumed (g/liter)	Yield (g lactic acid produced per g arabinose consumed)	Max lactic acid productivity (g/liter/h)	Optical purity of lactic acid (%)
Δ*ldhL1*[b]	8.7 ± 0.6	6.3 ± 0.5	19.6 ± 0.2	0.44 ± 0.03	0.37 ± 0.03	99.8 ± 0.1
Δ*ldhL1-xpk1::tkt*[c]	38.6 ± 1.8	0.4 ± 0.0	47.4 ± 1.7	0.82 ± 0.05	3.78 ± 0.12	99.9 ± 0.0

[a] Values represent means ± standard deviations for three independent experiments.
[b] Values after 36 h of cultivation.
[c] Values after 27 h of cultivation.

(12). As introduction of multicopy plasmids for overexpression of *xpk1* in *L. plantarum* Δ*ldhL1* did not lead to an increased cell growth or fermentation rate (data not shown), conversion of X5P by Xpk1 does not appear to be a rate-limiting step.

In conclusion, we successfully demonstrated homo-D-lactic acid production from arabinose using *ldhL1*-deficient and *xpk1*-substituted *L. plantarum*. Application of the findings obtained in this study to D- and L-lactic acid production from not only arabinose but also xylose, or even a mix of pentose and hexose sugars, is thus expected for lignocellulose utilization. This is the first report of homo-D-lactic acid fermentation from arabinose.

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