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Mutated *fabG* gene encoding oxidoreductase enhances the cost-effective fermentation of jasmine rice vinegar in the adapted strain of *Acetobacter pasteurianus* SKU1108

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A low-nutrient adapted strain, *Acetobacter pasteurianus* G-40, was successfully obtained by repetitive cultivation of *A. pasteurianus* 7E-13 under selective pressure. The adapted strain could grow well and produce 3.45-fold higher amounts of acetic acid than 7E-13 in jasmine rice wine containing 6% ethanol at 37 °C in a shaking flask. The G-40 strain also exhibited higher amounts of acetic acid (5.16%) in 2-L jar fermentor compared with 7E-13, where the bio-conversion yield to acetic acid from ethanol was 71% and 55.5% in the adapted strain and parental strain, respectively. In addition, genome sequence analysis of G-40 revealed that the strain has mutations in the 6 genes, of which the *fabG* gene encoding oxidoreductase is largely mutated by the partial recombination with a highly homologous *fabG* homolog present in the large plasmid of the strain. Over-expression of the mutated *fabG* gene and also the replacement of the original *fabG* gene in the chromosome with the mutated one obviously enhanced growth and acetic acid production of 7E-13 in the rice wine without any nutrient supplementation, indicating that the mutation in the *fabG* gene is mainly involved in higher fermentation ability under low-nutrient conditions. Thus, the results suggest that the adapted G-40 strain has proven useful for the cost-effective fermentation of rice vinegar.

[**Keywords:** *Acetobacter pasteurianus*; Rice vinegar fermentation; Low-nutrient adaptation; *fabG* gene; Oxidoreductase]

Rice vinegar is widely used in several countries, and especially in China, Japan and Korea (1). It has been commonly consumed as a food ingredient, for acidic seasoning and as a preservative. Moreover, rice vinegar has been considered for use for medical purposes such as antioxidant activity, anticancer risk and promoting blood circulation (2–4). Rice vinegar is produced from a two-step fermentation process involving alcoholic fermentation by koji mold (*Aspergillus oryzae*) and yeast (*Saccharomyces cerevisiae*), and subsequently, acetous fermentation by acetic acid bacteria (AAB) (5). The latter process is also known as oxidative fermentation which is performed *via* two sequential catalytic processes; ethanol oxidized by pyrroloquinoline quinone-dependent alcohol dehydrogenase and followed by aldehyde dehydrogenase. These enzymes are located on the periplasmic side of the cytoplasmic membrane and linked to the respiratory chain (6). The dominant AAB employed in rice vinegar are *Acetobacter* species, especially *Acetobacter pasteurianus* (1,7). This strain is largely used in traditional vinegar fermentation, in which the highest concentration of acetic acid is 6% (v/v).

Rice vinegar is industrially produced by two main methods: traditional static surface and submerged fermentation processes. Due to the significant increase in global demand for rice vinegar, the submerged fermentation technology is of interest. It provides an advantage over the traditional static surface method such as a higher fermentation rate and a higher acetic acid yield. The submerged fermentation method for rice vinegar fermentation employing *Acetobacter* species is usually undertaken at 30 °C. However, heat generated from oxidation metabolism increases the temperature in the system to over 30 °C, which leads to a decrease in both productivity rate and the acetic acid yield. Therefore, a cooling system is required to maintain the optimum temperature for growth and for acetic acid production during the fermentation process, especially under global warming era. Thermotolerant strains of AAB which could grow and efficiently produce acetic acid at least at 37 °C are isolated from various kinds of fruit and flowers in tropical countries (8–10). Thermotolerant *A. pasteurianus* SKU1108 (NBRC 101655) isolated from Thailand is one of the most promising strains in that it can grow well at high temperatures, even at 41 °C in the medium without ethanol supplementation. However, it exhibited the low efficiency in terms of acetic acid production in YPGD medium containing 6% ethanol at temperatures of more than 37 °C (8,10). In order to improve the efficiency of fermentation at high temperatures, the thermo-adapted strain, *A. pasteurianus* TH-3, having a higher fermentation ability than the

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wild-type strain at temperatures of up to 41 °C, was obtained by *in vitro* thermal adaptation of *A. pasteurianus* SKU1108 (11).

In addition to the thermotolerant mechanism, AAB strains which tolerate high concentrations of ethanol substrate, and which require low quantities of nutrients to produce high yields of acetic acid, would be useful for industrial vinegar fermentation (12). The ethanol-adapted strain, 7E-13, was obtained by sequential adaptation of the thermo-adapted strain, TH-3, to high ethanol concentrations of up to 7%. This strain exhibited an ability to grow and produce rice vinegar from jasmine rice wine adjusted to 6% ethanol containing YPGD medium in a fermentor under natural temperatures without the need for a cooling system (Theeragool et al., unpublished data). However, while this strain can grow in jasmine rice wine supplemented with YPGD medium, the yeast extract gives an unfavorable smell, changes the color of the vinegar, and increases the cost of production (7).

In this study, we aimed to develop the procedure for the cost-effective fermentation of commercial jasmine rice vinegar. The adapted 7E-13 strain was further improved by the repeated cultivation in 6% ethanol diluted jasmine rice wine without any nutrient supplementation at 37 °C. The low nutrient-adapted strain which can grow and produce higher yield of acetic acid was selected. The adaptive mutational sites were investigated at the whole genome level thorough the use of next-generation sequencing technology. The vinegar fermentation of the adapted strain was undertaken using a lab-scale fermentor. Furthermore, the effect of the mutated gene responsible for growth and for acetic acid production under low-nutrient conditions was studied.

MATERIALS AND METHODS

Bacterial strains, culture media and growth condition *A. pasteurianus* 7E-13, a thermo-ethanol adapted strain of *A. pasteurianus* SKU1108 (NBRC 101655) (Theeragool et al., unpublished data), *A. pasteurianus* G-40, a nutrient-adapted strain of the 7E-13 strain, and *Escherichia coli* were used in this study (Table S1). The stock culture of *A. pasteurianus* was maintained in rich YPGD medium (10 g/L of yeast extract, 10 g/L of polypeptone, 20 ml/L of glycerol, 5 g/L of D-glucose) containing 50% (v/v) glycerol and stored at −80 °C. *E. coli* was cultivated in Luria−Bertani (LB) broth (5 g/L of yeast extract, 10 g/L of polypeptone, 5 g/L of NaCl), supplemented with appropriate antibiotics. The conjugant strains of *A. pasteurianus* were selected on YPGD medium (5 g/L of yeast extract, 5 g/L of polypeptone, 5 g/L of glycerol, 5 g/L of D-glucose) supplemented with appropriate antibiotics. A pre-culture of *A. pasteurianus* 7E-13 and its adapted strains were generally grown in 5 ml of rich YPGD medium, and incubated at 37 °C with a shaking speed of 200 rpm overnight in an air incubator (JSSI-300C, JSR, Republic of Korea). The pre-culture was subsequently transferred to a 250-ml Erlenmeyer flask with 50 ml of rich YPGD broth containing 4% (v/v) ethanol, and incubated under the same conditions as previously. The pre-culture was used when the optical density of 540 nm reached 1.

Jasmine rice wine preparation Jasmine rice wine containing 16.7% (v/v) ethanol and 0.56% (v/v) acetic acid was kindly supplied by Kewpie Co., Ltd., Bangkok, Thailand. For rice vinegar fermentation, the jasmine rice wine (RW) was diluted with sterile distilled water to obtain RW-6E medium which contained a final concentration of 6% (v/v) ethanol, 0.2% (v/v) acetic acid, 0.19% (v/v) reducing sugar and 0.24% (v/v) protein content. The reducing sugar was measured using the Nelson Somogyi method (13) using glucose as standard. The protein content was measured using a modified Lowry's method with bovine serum albumin as the standard (14).

Low-nutrient adaptation procedure The thermo-ethanol adapted strain, *A. pasteurianus* 7E-13, was repeatedly cultivated in the RW-6E medium which allowed this strain to adapt to a low-nutrient condition. The 5 ml pre-culture was transferred to a 500-ml Erlenmeyer flask with 100 ml of the RW-6E medium, and incubated on a rotary shaker at 200 rpm at 37 °C (the first cultivation). When 2−3% acidity was produced, 5 ml of the cultured broth was transferred to 100 ml of a fresh RW-6E medium (the second cultivation). The cultivation procedure was sequentially performed 40-times until stable growth and acetic acid production was observed. The adapted strains were selected on rich YPGD agar containing 4% (v/v) ethanol at 37 °C for 2 days.

Determination of growth and acetic acid production The 5 ml pre-culture of the 7E-13 and G-40 strains was transferred to a 500-ml Erlenmeyer flask with 100 ml of the RW-6W medium with and without 0.5% (w/v) yeast extract supplementation. The cultivation was performed on a rotary shaker at 200 rpm at 37 °C. All these experiments were performed in triplicate. The bacterial growth was monitored by measuring the optical density at 540 nm (GENESYS 20, Thermo Fisher Scientific Inc., Waltham, MA, USA). The acidity (%) was determined by alkaline-titration with 0.8N NaOH in the presence of 0.2% (w/v) phenolphthalein as a pH indicator.

Submerged vinegar fermentation Batch fermentation was performed in a 2-L jar fermentor (B.E. Marubishi Co., Ltd., Tokyo, Japan) with 1-L working volume. The operating conditions were as follows: temperature, 37 °C; agitation rate, 400 rpm; aeration rate, 0.75 vvm. The 5% pre-culture of the 7E-13 and G-40 strains was separately inoculated in the fermentative medium, RW-6E medium, in the fermentor. The determination of growth and acetic acid production as previously described, was monitored every 6 h for the entire fermentation cycle. The ethanol consumption was performed as described previously (15), using capillary gas chromatography (GC-2010 plus, Shimadzu, Kyoto, Japan). The yield of acetic acid was converted into percentages based on the ratio of final acidity and initial ethanol concentration. One g/L of ethanol was completely transformed to 1.304 g/L acetic acid. (16,17).

Genome sequencing and mapping analysis of the adapted strain The genomic DNA of the 7E-13 and G-40 strains were isolated by the method previously described (18), and investigated by Illumina sequencing using the Illumina HiSeq 1000 and Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA), respectively (19). The previously reported complete genome sequence of *A. pasteurianus* SKU1108 (GenBank accession numbers: AP014881−AP014885) was used as the reference sequences for genome mapping analysis (19). The Illumina sequence reads of the 7E-13 and G-40 strains were independently mapped onto the reference sequences using BWA with default parameters (20). The mutation sites were determined by using the Genome Analysis Toolkit (GATK) (21). To confirm newly identified nucleotide mutations in the adapted strain, PCR was performed using the genomic DNA of the 7E-13 and G-40 strains as a template, and PCR products were sequenced by the First Base Laboratory (Selangor, Malaysia).

Over-expression of *fabG* gene in 7E-13 and G-40 strains The *fabG* gene was amplified from the genomic DNA of the SKU1108 and G-40 strains by using specific primer sets, 1108-fabG-5 (5′- TGC TGC CTT TGG TTC CAA GG -3′) for the forward primer and 1108-fabG-3-RI (5′- GAA TTC GTG CTG GCA ATA ATA GC -3′) for the reversed primer. The 2.09 kb PCR product was purified using a MagExtracter DNA fragment purification kit (Toyobo, Tokyo, Japan). The purified PCR product was then introduced into pT7blue plasmid to obtain pTfabG-SKU1108 and pTfabG-G40 plasmids (Table S1). These were individually transformed to *E. coli* DH5α by heat shock (22). The transformants were selected on LB medium containing 50 μg/ml ampicillin and 10 μg/ml X-gal, which were confirmed by PCR amplification and nucleotide sequencing. Then, the *Xba*I and *Eco*RI-digested *fabG* genes obtained from both recombinant plasmids were separately ligated to the *Xba*I and *Eco*RI sites of a broad host range plasmid pCM62 (23), yielding the *fabG*-expressing vectors. The pCMfabG-SKU1108 or pCMfabG-G40 in *E. coli* DH5α was individually transferred to the 7E-13 and G-40 strains by triparental conjugation using the helper plasmid pRK2013 in *E. coli* HB101 (24). The conjugants were selected on YPGD agar containing tetracycline (50 μg/ml, final concentration), and were verified by plasmid pattern analysis. The *fabG*-overexpressing strains were further determined for growth and for acetic acid production in the RW-6E medium containing tetracycline (50 μg/ml, final concentration) as previously described.

Construction of a mutated *fabG* gene in 7E-13 strain A 7E-13 strain harboring *fabG*-mutated was constructed by the replacement of the *fabG* gene from the G-40 strain (*fabG*-G40) to the 7E-13 strain using the markerless deletion system of the pKOS6b plasmid (25). The purified 2.09 kb *fabG*-G40 was obtained by PCR as previously described in the overexpression section. The *Xba*I and *Eco*RI-digested *fabG*-G40 gene was inserted into the corresponding site in the pKOS6b vector. The resultant plasmid, pKOSfabG-G40 (Table S1), was transferred into the 7E-13 strain using the triparental conjugation method as previously described. The first recombinant strain, 7E-13/pKOSfabG-G40, was selected by spreading it on YPGD medium containing 50 μg/ml kanamycin. Then, the positive clones were spread on YPGD medium containing 60 μg/ml 5-fluorocytosine, which was used as a negative selection marker to drive the directed loss of the plasmid. Next, the second recombinant strain was selected from a 5-fluorocytosine resistant and kanamycin sensitive strain. The nucleotide sequence of the *fabG* gene in the second recombinant strain was confirmed by PCR using a specific primer set, oxidoF (5′- TCG CCT CTC ACA AAC CTA CC -3′) and oxidoR (5′- TGT TGT CTC GCA CCA ATC CA -3′), followed by the Sanger method of the First Base Laboratory. The second recombinant strain with allele replacement to *fabG-G40* was referred to as the F-1 strain and further determined for growth and acetic acid production in the RW-6E medium.

Sequence data deposition Illumina sequence reads from 7E-13 and G-40 strains were deposited in the DDBJ Sequence Read Archive (DRA) under accession numbers DRA007379 and DRA007380, respectively.

RESULTS AND DISCUSSION

Selection of low-nutrient adapted strain To obtain an adapted strain for cost-effective industrial vinegar production, the

thermo-ethanol adapted strain, *A. pasteurianus* 7E-13, was repeatedly cultivated in RW-6E medium without nutrient supplementation at 37 °C. In the early repeated cultivation (50 days), this strain exhibited a long lag phase in terms of growth and delayed acetic acid production. However, after 17 repeated cultivations, the lag phase of growth became shorter, and the acetic acid productivity higher than that of the early repeated cultivation (Fig. S1). More stable growth and rapid acetic acid production rate were obtained by further repeated cultivations up to 40 times in RW-6E medium at 37 °C. Finally, the culture was spread on rich YPGD medium containing 4% ethanol, and incubated at 37 °C for 2 days. We found two types of colony, smooth and rough colonies, on the plate, of which the appearance has been known to occur spontaneously in *A. pasteurianus* (26–28). Moreover, the rough to smooth colony conversion has been shown to easily occur by a single nucleotide deletion and/or insertion in capsular polysaccharide synthesis gene cluster in *Acetobacter tropicalis* SKU1100 (29). Thus, both the representative colonies were determined for the growth and acetic acid production in RW-6E medium at 37 °C. It was found that the smooth colony exhibited better growth and higher level of acid productivity than that of the rough colony (data not shown). Thus, the smooth colony was selected as the potential strain, entitled G-40, for acetic acid fermentation in low-nutrient RW-6E medium at 37 °C. The isolated G-40 strain was maintained in the deep-frozen stock, and usually the strain was used for a couple of cultivation directly from the frozen stock culture. The phenotype of G-40 strain was not changed at least after such the cultivations. The results showed that the adaptation succeeded in terms of improving the efficiency of acetic acid production under a limiting nutrient condition at 37 °C, suggesting that the experimental adaptation allows *A. pasteurianus* to be adapted to various stressors not only higher temperatures (11) and higher ethanol concentrations (Theeragool et al., unpublished data) but also low-nutrient conditions. These results confirmed that bacterial cells can survive shifts in environmental conditions between their natural habitat and various culture media in the laboratory (30).

The growth and acetic acid production of the 7E-13 strain and the adapted strain, G-40, were further compared in RW-6E medium with and without 0.5% yeast extract supplementation at 37 °C. As a result, in the medium supplemented with yeast extract, the 7E-13 and the G-40 strains grew well; the former exhibited a high growth rate, but the latter showed a high total growth, and exhibited rapid oxidation of ethanol into acetic acid accumulated in the culture media; the maximum acidity of 4.64% and 5.04% were obtained from the 7E-13 and G-40 strains, respectively, within the 10 day cultivation period without any acetic acid overoxidation phase (Fig. 1). In the medium without yeast extract supplementation, the 7E-13 strain exhibited delayed growth (with no difference in the total growth) and decreased amounts (1.68%) of acetic acid production. On the other hand, the G-40 strain displayed a decreased total growth but increased acetic acid production. In the G-40 strain, the production of acetic acid (5.80%) was 3.45-fold higher than that of the 7E-13 strain (1.68%) after 10 days' cultivation. Furthermore, the G-40 strain produced a higher amount of acetic acid (5.80%) in RW-6E medium than that obtained from RW-6E medium supplemented with yeast extract (5.04%), even though better growth was observed in RW-6E medium supplemented with yeast extract (Fig. 1). Moreover, the adapted G-40 strain was able to produce acetic acid concomitant with no acetate overoxidation in both media at 37 °C. These results revealed that the adapted strain had the ability to produce high amounts of acetic acid in an alternative low-cost medium. This indicated that this adapted strain was a promising one that might be useful for industrial rice vinegar fermentation because it tolerates high temperatures and high concentrations of ethanol and requires low nutrients in order to grow and produce high yields of acetic acid. These unique characteristics of the adapted strain are necessary for industrial vinegar fermentation (12).

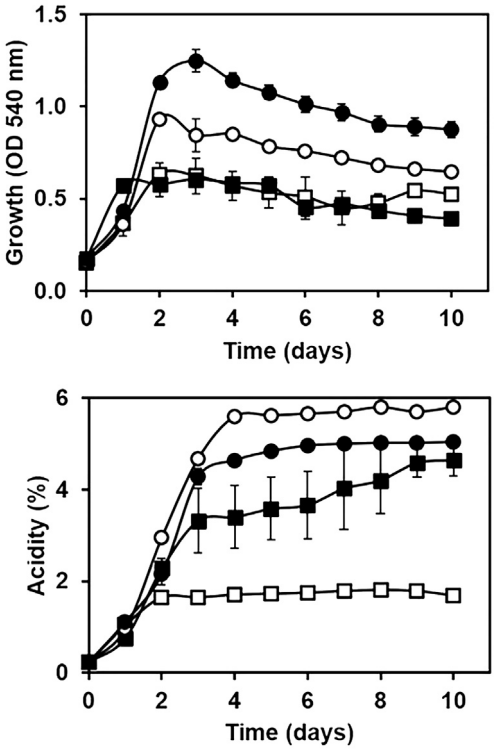

FIG. 1. Time course of growth and acetic acid production of *A. pasteurianus* 7E-13 (closed and open squares) and its adapted strain G-40 (closed and open circles) in flask shaking culture. Both strains were cultured in RW-6E without (open squares and open circles) and with 0.5% yeast extract supplementation (closed squares and closed circles) at 37 °C and shaken at 200 rpm for 10 days.

Rice vinegar fermentation using the adapted strain Scale-up is the most important challenge for industrial vinegar fermentation, so the relevant models and parameters have been studied. Thus, the vinegar fermentation of 7E-13 and its adapted G-40 strain was performed in a 2-L fermentor containing 1-L of RW-6E medium at 37 °C at an agitation speed of 400 rpm and an aeration rate of 0.75 vvm. The 7E-13 strain produced 4.32% acidity concomitant with the complete consumption of 5.47% of ethanol at 24 h (Fig. 2A) while the G-40 strain produced 5.04% acidity which was 1.2-fold higher than that produced by the 7E-13 strain at 24 h, at which time the ethanol was completely consumed (Fig. 2B). The G-40 strain exhibited the highest acidity at 5.16% which was obtained of 5.31% initial ethanol. Thus, the bio-conversion yield of acetic acid using this adapted strain was increased by up to 71%. This was 20% higher than that of the 7E-13 strain. The results suggest that the adapted strain, G-40, could grow under a limiting nutrient and could efficiently oxidize ethanol to acetic acid. In addition, this strain showed no acetate overoxidation during the stationary growth phase after 24 h. The results revealed that the G-40 strain would be useful for the cost-effective fermentation of rice vinegar because it could rapidly produce high amounts of acetic acid at 24 h in RW-6E medium without nutrient supplementation in a 2-L scale-up fermentor at high temperatures.

There have been many trials for submerged vinegar fermentation with *Acetobacter* species using a variety of raw materials

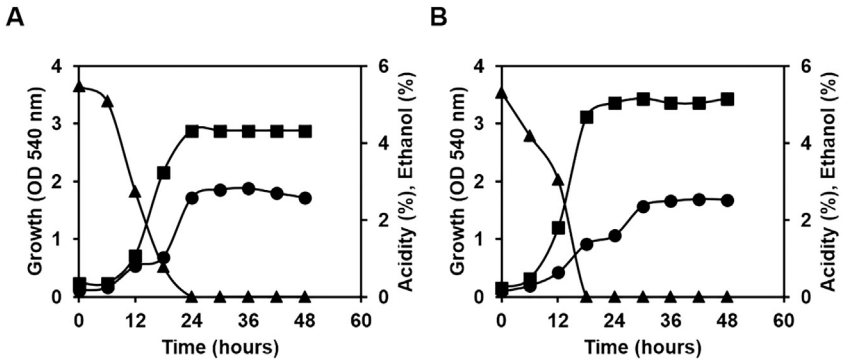

FIG. 2. Time course of growth (closed circles), acetic acid production (closed squares) and ethanol consumption (closed triangles) by *A. pasteurianus* 7E-13 (A) and its adapted strain, G-40 (B). These strains were grown in RW-6E medium without yeast extract supplementation in 2-L of jar fermentor (1-L working volume) at 37 °C, with a 400 rpm agitation speed and 0.75 vvm aeration rate.

supplemented with some nutrient compounds. Using rice wine to which was added 0.5 g/L nutrient salts including diammonium phosphate, potassium sulfate, magnesium sulfate, concentrated bacterial growth factors and vitamins, 6.85% (w/v) acetic acid was produced by *Acetobacter* sp. in a FRINGS Acetator (Germany) at 30 ± 0.3 °C (16). Onion vinegar of 4.5% acidity was obtained from *A. tropicalis* using onion extract added ethanol and yeast extracts by fed-batch culture in a pilot-scale fermentor (31). The cider vinegar of 5.67 ± 0.05% (w/v) acidity was produced by *A. pasteurianus* CICIM B7003 from cider mash supplemented with some amino acids and vitamins in a Frings 9-L Pilot-Acetator at 30 °C (7). Furthermore, 4.26% acetic acid was produced by *A. pasteurianus* JST-S using a purple sweet potato wine containing 9% initial ethanol concentration at 30 °C (32). As compared with these acetic acid fermentations, the G-40 strain could be proven to be the strain with highest potential in terms of low-cost vinegar production. It exhibited superior properties when it came to not only producing high yields using low-nutrient, but also by reducing the cooling costs with regard to temperature control.

Genome sequencing and mutational site analysis of the adapted strains Genetic instability is an important trait for investigation in the case of *A. pasteurianus*, because genomic substitutions, genomic deletions or transposon insertions would often occur during its adaptation under various pressure conditions (33). In this study, we proposed an in-depth genomic mutation information during a low-nutrient adaptation of the adapted G-40 strain through whole genome analysis using Illumina sequencing. The genome mapping was analyzed using the previously-reported referential genomes, *A. pasteurianus* SKU1108 (NBRC 101655), the TH-3 strain (11,19) and the 7E-13 strain. The mutated positions with the contig number, their annotations and mutation modes are shown in Table 1. As a result, 26 mutation sites were detected in the genome of the G-40 strain when compared to SKU1108. However, 11 and 2 mutational sites occurred during the thermo-adaptation of the TH-3 strain and the ethanol adaptation of the 7E-13 strain, respectively. Thus, 13 mutational regions comprised of 12 single nucleotide mutations and 1 insertion (14 bp), were additionally observed after the low-

TABLE 1. List of experimentally validated mutations or insertion regions in adapted strain, G-40 of *A. pasteurianus* SKU1108 compared to TH-3, 7E-13 strain.

Locus_tag	Mutation in contigs			Gene	Product	Position	Mode of mutation or insertion
	TH-3	7E-13	G-40				
APT_00170	+	+	+	*marR*	MarR family transcriptional regulator	190842	R98H
APT_00228	+	+	+	–	Hypothetical protein	248856	Frameshift
APT_00229	+	+	+	*ansP*	L-Asparagine permease	250613	Frameshift
APT_00250	+	+	+	–	D-Alanyl-D-alanine serine-type carboxypeptidase	268640	Q292L
APT_00389	–	+	+	*nhaK*	Na+/H+ antiporter	417020	Silent
APT_00474	–	–	+	–	Hypothetical protein	505191	V79A
APT_00732	+	+	+	–	Hypothetical protein	777821	6-bp deletion
APT_00827	–	–	+	–	Adenylate/guanylate cyclase	863592	C440S
APT_00948	–	–	+	–	Transporter YjgP/YjgQ	993852	E302D
APT_00950	–	–	+	–	Multidrug transporter ATP-binding protein	998944	14-bp insertion
APT_01316	+	+	+	*asmA*	Lipopolysaccharide biogenesis periplasmic protein AsmA	1385142	Silent mutation
APT_01446	+	+	+	*dctA*	C4-dicarboxylate transporter	1565424	Tn
ATP_01497	–	+	+	*ispA*	Geranylgeranyl pyrophosphate synthase	1620736	M107I
APT_01579	–	–	+	*fabG*	Oxidoreductase	1718758	P109S
						1718763	D110E
						1718765	Q111L
						1718778	Silent
						1718781	Silent
						1718799	Silent
						1718844	Silent
						1718904	Silent
APT_01702	+	+	+	–	Hypothetical protein	1841392	G164S
APT_01763	+	+	+	*glnD*	Uridylyltransferase PII	1909918	6-bp deletion
APT_01785	+	+	+	*mucR*	MucR family transcriptional regulator	1935892	4-bp deletion
Upstream of APT_01848	+	+	+	–	Glycosyl transferase	2000030	Tn
APT_02410	–	–	+	–	UDP-*N*-acetylglucosamine 4-epimerase	2622522	F316V

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G40_01579  1718734  CATACTCACTTTGATGCGGCGACATCTGAACTGCTTGATAATCAATATAACATTCATTTC
APT_01579  1718734  CATACTCACTTTGATGCGGCGACACCTGACCAGCTTGATAATCAGTACAACATTCATTTC
APT_10189   171032  CACACTCGCTTTGACGCGGCGACATCTGAACTGCTTGATAATCAATATAACATTCATTTC
                    ** **** ****** ********* **** * ***********  ** ***********

G40_01579  1718794  AAGGGGCCTTACCTGTTAACTGTGGCCCTTCTGCCGCTGATCTGCGATGGTGGACGTATT
APT_01579  1718794  AAGGGACCTTACCTGTTAACTGTGGCCCTTCTGCCGCTGATCTGCGATGGCGGACGTATT
APT_10189   171092  AAGGGGCCTTACCTGTTAACTGTGGCCCTTCTGCCGCTGATCTGCGATGGTGGACGTATT
                    *****  ********************************************  *********

G40_01579  1718854  CTCAACATCACTTCGGCAGCCACACGCTTTTACCTGCCCGATCATGGCCCCTACTCCGCT
APT_01579  1718854  CTCAACATCACTTCGGCAGCCACACGCTTTTACCTGCCCGATCATGGCCCTTACTCCGCT
APT_10189   171152  CTCAACATCACTTCGGCAGCCACACGCTTTTACCTGCCCGATCATGGCCCCTACTCCGCC
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G40_01579  1718914  ATGAAAGGGGCTACCGAAGTAATCTCACTTTACATGGCCAAGGAACTGGGGGAACGCCGC
APT_01579  1718914  ATGAAAGGGGCTACCGAAGTAATCTCACTTTACATGGCCAAGGAACTGGGGGAACGCCGC
APT_10189   171212  ATGAAAGGGGCTACCGAAGTAATCTCACTGTACATGGCAAAGGAACTGGGGGAACGCCGC
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FIG. 3. Rearranged and boundary regions of APT_01579 in G-40 strain (partial alignment). Box portion indicates the rearranged region. The bold red color indicates nucleotide mutations that occurred during low nutrient adaptation.

nutrient adaption of the G-40 strain. The mutational sites were confirmed as existing in the genome of the G-40 strain by PCR and sequencing.

Of the mutation sites, since 8 nucleotide mutations identified in the *fabG* gene (APT_01579) were located on the narrow 147-bp region (genomic position: 1718758 to 1718904) (see Table 1), we looked for the homologous genes in the genome, and found a highly homologous *fabG* homolog, APT_10189, which is located on the large plasmid 1 in SKU1108 genome. These two genes have 93% nucleotide sequence identity with each other, and we aligned these sequences and mutants derived from G-40 (Fig. S2). As shown in Fig. 3, these regions in the two genes seem to be rearranged through the boundary region of the *fabG* gene (APT_01579) in the G-40 strain by a homologous recombination. Furuta and Kobayashi (34) have reported that such a genomic rearrangement occurred among the target recognition domain of DNA methyltransferase conserved in various *Helicobacter pylori* strains. These movements are mediated by DNA recombination machinery, which alters the methylation status of a genome, and leads to changes in global gene expression patterns and various phenotypes. As the genomic rearrangements through short-repeat regions sometimes occur in bacterial strains, it was thought that such a rearrangement also occurs through boundary regions in these two *fabG* homologs.

Thus, in the G-40 strain, a total of 6 genes were mutated, and these were categorized in the encoding proteins as adenylate/guanylate cyclase, transporter YjgP/YjgQ, multidrug transporter ATP-binding protein, oxidoreductase, UDP-*N*-acetylglucosamine 4-epimerase, and hypothetical protein. The identified mutated genes

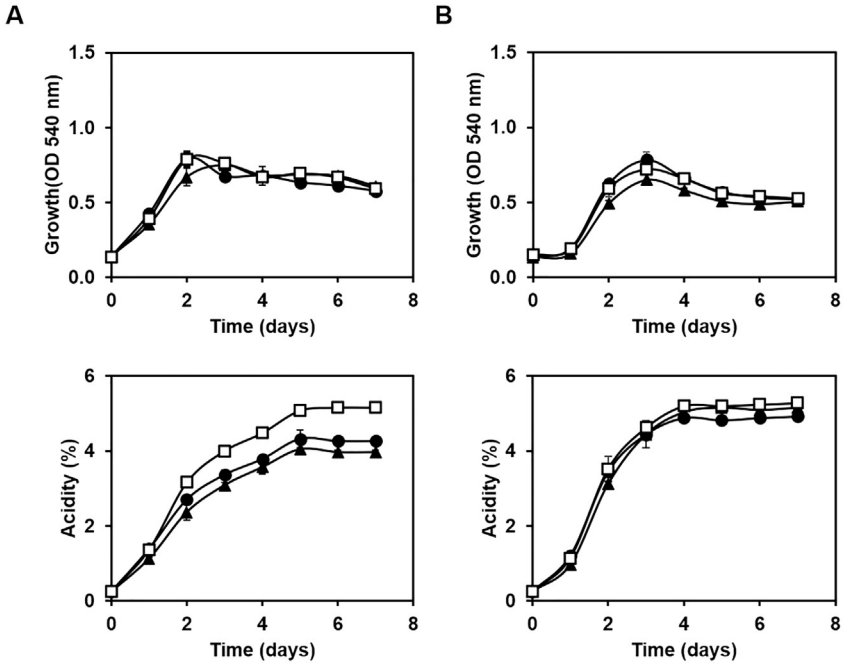

FIG. 4. Time course of growth and acetic acid production of *A. pasteurianus* 7E-13 (A) and G-40 strain (B) harboring pCM62 (closed triangles), pCMfabG-SKU1108 (closed circles) or pCMfabG-G40 (open squares). These strains were grown in RW-6E added 50 μg/ml tetracycline and incubated at 37 °C and shaken at 200 rpm for 7 days.

were found to be involved in gene regulation, cell membrane transportation and fatty acid biosynthesis. Adenylate/guanylate cyclase protein was found to have a mutated site on the GAF domain, which associates with gene regulation in bacteria, cyclic GMP (cGMP)-regulated cyclic nucleotide phosphodiesterase, adenylyl cyclase and two-component sensor histidine kinase (35,36). The transporter YjgP/YjgQ was previously reported to be an essential protein for the transportation of lipopolysaccharide to the outer membrane. The low level of this protein led to an increase in outer membrane permeability in *E. coli* (37). The multidrug transporter ATP-binding protein is known as the membrane proteins responsible for the translocation of a variety of compounds across membranes (38,39). The FabG protein or oxidoreductase is known as a member of the short-chain alcohol dehydrogenase/reductase (SDR) family. It is involved in bacterial type II fatty acid synthesis (FASII), with the role of core enzyme in the elongation step needed to catalyze the reduction of 3-ketoacyl-ACP that results in 3-hydroxyacyl-ACP at the concomitant expense of NADPH and H^+ (40–42). UDP-*N*-acetylglucosamine 4-epimerase is responsible for the conversion of UDP-*N*-acetylglucosamine into UDP-*N*-acetylgalactosamine, which is the composition of the exopolysaccharides (EPS) (43). These results suggest that at least one of the 6 mutations is involved in changing the phenotypic characteristics of *A. pasteurianus* adapted in a limitation of nutrient environment.

Effect of the *fabG* overexpression on acetic acid production in strains 7E-13 and G-40 In the case of *Ralstonia solanacearum*, two homologs of the *fabG* gene have been reported to be involved in fatty acid synthesis and growth. The first homolog of this gene was essential for cell growth, and the other homolog related to adaptation in the high concentration of salt and low pH, and the pathogenesis of disease in tomato plants (44). Furthermore, in the case of *Campylobacter jejuni*, the *fabG* gene was previously demonstrated to be involved in the growth at high temperatures. The deletion of this gene led to a decrease in the proportion of bacterial unsaturated fatty acids, and the defective *fabG* strain showed lower growth at 42 °C than at 37 °C, while the wild type exhibited almost similar growth at both temperatures (45). Therefore, we also considered two *fabG* homologs (APT_01579 and APT_10189) in *A. pasteurianus* that may be involved in the fatty acid composition and some stress responses, and that the mutated *fabG* recombined from the two homologs that occurred in the G-40 strain may change the fatty acid composition and directly affect their growth ability in a low-nutrient environment. Thus, the *fabG* gene of SKU1108 and the mutated *fabG* gene of G-40 were over-expressed in 7E-13 and its adapted G-40 strain. Growth and acetic acid production were compared in RW-6E medium containing 50 μg/ml tetracycline. As a result, the 7E-13 strain harboring pCMfabG-G40 exhibited a similar growth phase to the strain harboring pCMfabG-SKU1108 or pCM62 (empty vector), with regard to which the maximum growth was observed in the second day of cultivation. The stationary phase was then observed without any acetate overoxidation phase during the cultivation period. However, the strain harboring pCMfabG-G40 clearly exhibited higher amounts of acetic acid reaching a final acidity level of 5.16% compared with the two other over-expressing strains (Fig. 4A). In contrast, the adapted strain, G-40, harboring pCMfabG-SKU1108 or pCMfabG-G40, exhibited little better growth than the strain harboring pCM62 (empty vector). On the other hand, the G-40 strain harboring pCMfabG-G40 and pCM62 displayed 5.28% and 5.16% acidity respectively, after 7 days' cultivation, which was quite similar to the 4.92% acidity obtained from the strain harboring pCMfabG-SKU1108 (Fig. 4B). The result suggests that the over-expression of the single mutated *fabG* gene is obviously responsible for the increase in the ethanol oxidation in the 7E-13 strain. It indicates that the *fabG* gene is at least one of the genes involved in the low-nutrient response.

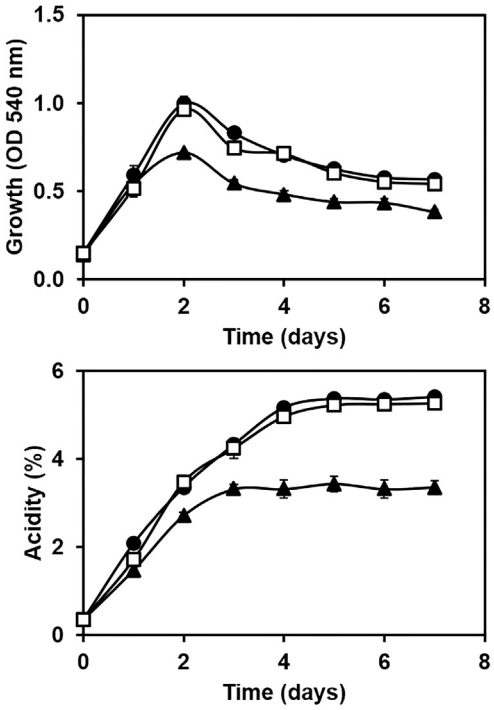

FIG. 5. Time course of growth and acetic acid production of *A. pasteurianus* 7E-13 (closed triangles), G-40 (closed circles) and 7E-13/fabG-G40 (open squares). These strains were grown in RW-6E medium incubated at 37 °C and shaken at 200 rpm for 7 days.

Determination of the mutated *fabG* gene replacement in the 7E-13 strain In order to confirm the above evidence more clearly, the gene replacement of the parental strain with the mutated *fabG* gene was studied. The *fabG* gene of the 7E-13 strain was successfully replaced with the *fabG G-40* allele by using the suicide vector pKOS6b, and the resultant strain was referred to as the F-1 strain. To determine the effect of the *fabG* gene on growth and on acetic acid production, the 7E-13, G-40 and F-1 strains were cultivated in RW-6E medium at 37 °C. As a result, the adapted G-40 and the F-1 strains showed almost identical growth patterns and acetic acid production levels. They produced high amounts of acetic acid to a final acidity level of 5.26% and 5.40% acidity, respectively. In contrast, the parental strain, 7E-13, obviously exhibited lower growth and a final 3.36% acidity when compared with both strains (Fig. 5). The results suggest that the mutated *fabG* gene is responsible for the increased growth and the ethanol oxidation ability of the adapted G-40 strain. This finding was the first attempt to elucidate the *fabG* gene involved in growth and acetic acid production in acetic acid bacteria under low-nutrient conditions. However, the function of the *fabG* gene in terms of the growth and acetic acid fermentation in the adapted G-40 strain under low-nutrient conditions will be further investigated. In addition, the effect of multi-gene responsibility on the growth and acetic acid fermentation of the adapted G-40 strain will also be determined in the future.

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