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High-temperature sorbose fermentation with thermotolerant *Gluconobacter frateurii* CHM43 and its mutant strain adapted to higher temperature

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Abstract We succeeded in obtaining a strain adapted to higher temperature from a thermotolerant strain, *Gluconobacter frateurii* CHM43, for sorbose fermentation. The adapted strain showed higher growth and L-sorbose production than original CHM43 strain at higher temperature around 38.5–40 °C. It was also shown to be useful even with the fermentation without temperature control. To understand the sorbose fermentation ability of the adapted strain at higher temperature, D-sorbitol-oxidizing respiratory chain was compared with the CHM43 strain and the adapted strain. We found that the activity of pyrroloquinoline quinone (PQQ)-dependent glycerol dehydrogenase (GLDH), which is a primary dehydrogenase of the respiratory chain and responsible for L-sorbose production, was decreased when the temperature increased, but the decreased activity of GLDH was recovered by the addition of PQQ. Since the adapted strain was found to produce more PQQ than the CHM43 strain, it was suggested that the adapted strain keeps GLDH as holoenzyme with the increased PQQ production, and thus produces more L-sorbose and grows better under higher temperature.

Keywords Quinoprotein glycerol dehydrogenase · PQQ · Sorbose fermentation · Thermotolerance · Adaptation · *Gluconobacter*

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Introduction

Gluconobacter is a genus of acetic acid bacteria and well known to have a strong ability to oxidize a broad range of sugars and sugar alcohols. These oxidations called oxidative fermentations are uniquely carried out by membrane-bound enzymes located on the outer surface of the cytoplasmic membrane, and the oxidation products are accumulated in the culture medium. These features of the microorganisms lead to the applications in industry for fermentation of valuable products such as L-sorbose, dihydroxyacetone, D-gluconate, and keto-D-gluconates (Adachi et al. 2003). However, since these fermentations by *Gluconobacter* are normally performed below 30 °C, the optimum growth temperature, a large amount of energy for cooling is needed in the industrial fermentation processes. Thus, the development of thermotolerant acetic acid bacteria would release us from the strict temperature control for such fermentation.

In a previous study, eight thermotolerant acetic acid bacteria belonging to the genus *Gluconobacter* were isolated in Thailand and named CHM strains (Moonmangmee et al. 2000, 2002). They can grow and perform oxidative fermentation at 37 °C, which is the temperature that mesophilic strains cannot grow. Actually, by using such thermotolerant strains isolated from Thailand, acetic acid fermentation (Saeki et al. 1997) and L-erythrulose production (Moonmangmee et al.

2002) have been performed successfully at 37 °C. In this study, we focused attention on L-sorbose fermentation of CHM strains to establish a system to carry out the fermentation at higher temperature and also to investigate the mechanism of thermotolerance. L-Sorbose is an important intermediate in the industrial production of vitamin C (Hancock 2009). In *Gluconobacter* strains, two different membrane-bound enzymes have been considered to play a main role in L-sorbose production from D-sorbitol (Soemphol et al. 2008). One is a glycerol dehydrogenase (GLDH) which has been purified from *Gluconobacter suboxydans* IFO3255, having pyrroloquinoline quinone (PQQ) as the prosthetic group (Sugisawa and Hoshino 2002). This enzyme has been shown later to oxidize many other sugar alcohols, for example, glycerol to dihydroxyacetone, D-gluconate to 5-keto-D-gluconate, D-mannitol to D-fructose, and D-arabitol to D-xylulose (Matsushita et al. 2003). Another sorbitol-oxidizing enzyme is a sorbitol dehydrogenase (SLDH) containing FAD and heme *c* as the prosthetic groups, which has been purified from *G. suboxydans* IFO3254 (Shinagawa et al. 1982). Both the dehydrogenases function to produce L-sorbose by linking to terminal ubiquinol oxidases via ubiquinone in the respiratory chain and consequently generating a proton-motive force (Soemphol et al. 2008).

Here, in order to establish a high-temperature fermentation system for sorbose production, we selected the most useful strain and further obtained an adapted strain able to grow and produce L-sorbose at higher temperature. Then, we compared the enzyme activities in the D-sorbitol-oxidizing respiratory chain to obtain information on the mechanism of thermotolerance. The results indicated the possibility that PQQ in the culture contributes to keep active GLDH holoenzyme at higher temperature. In addition, our results supply a system to carry out sorbose fermentation at higher temperature, and also to perform the fermentation without temperature control.

Materials and methods

Culture media and growth conditions

Gluconobacter frateurii CHM43 strain (NBRC 101659) and other CHM strains (Moonmangmee et al. 2000) and *G. frateurii* IFO3264^T were used in this study. These strains were maintained on agar plate that was prepared by adding 1.7 % agar and 0.5 % $CaCO_3$ to a 5 % sorbitol medium consisting of 50 g of D-sorbitol, 3 g of yeast extract (Oriental Yeast, Tokyo, Japan), and 3 g of polypeptone (Nihon Pharmaceuticals Co. Ltd., Osaka, Japan) filled to 1 L with tap water. The preculture was cultivated in 5 mL of 5 % sorbitol medium at 30 °C and transferred to 50 or 100 mL of appropriate medium in a 500-mL Erlenmeyer flask or a 500-mL buffle flask. Then, it was grown with rotary shaking at 200 rpm at different temperatures in an air incubator (Bioshaker BR-43FM or BR-43FL, TAITEC, Saitama, Japan) of which the temperature was controlled at a range ±0.3 °C with suitable proportional, integral, and differential (PID) parameters (P 3.7, I 108, and D 27). Bacterial growth was monitored with a Klett-Summerson photoelectric colorimeter with red filter.

Adaptation of the CHM43 strain at 38.5 °C

During adaptation process, all cultivations were performed in 100 mL of 5 % sorbitol medium at 38.5 °C. When the first culture reached a Klett unit of 65, the culture (5 mL) was transferred into 100 mL of the fresh medium. Then, the second culture was transferred into a fresh medium after reaching 120 Klett units. Thus, the culture was repeated five more times, where each culture was transferred after reaching 180–240 Klett units. The final culture was spread on an agar plate, and ten colonies were picked up to check the growth and sorbose production. The growth of the mutants ranged from 60 to 105 Klett units, and sorbose conversion rate was from 25 to 48 %. Finally, a single colony that exhibits the highest growth (105 Klett units) and a higher sorbose conversion rate (46 %) at 38.5 °C was selected as an adapted strain, CHM43AD.

Jar fermentor cultivation

A laboratory-scale fermentor (total volume, 5 L; MDIAC-S5, B.E. Marubishi Co, Ltd. Tokyo Japan) was used for cultivation with 2 L of working volume (10 % sorbitol medium) at agitation rate of 500 rpm and aeration rate of 0.5 vvm. Antifoam 204 (Sigma, St. Louis, USA) was added to take out the foam produced. Cultivation in jar fermentor without temperature control was also conducted at the same condition by monitoring growth and temperature automatically.

Measurement of L-sorbose and D-sorbitol

L-Sorbose was measured routinely by resorcinol reagent that reacts with total ketohexose as described previously (Kulka 1956). L-Sorbose was also measured, together with D-sorbitol, by HPLC equipped with Gelpack GL-C610-S (7.8×300 mm, Hitachi High-Tech, Tokyo, Japan). The mobile phase was deionized water and column temperature was 60 °C. Elution was monitored by RI. When analyzed at a flow rate of 0.3 mL/min, L-sorbose and D-sorbitol were detected at 23.8 and 24.4 min, respectively.

Preparation of membrane fraction

Cells grown in jar fermentor were harvested at the mid-log and late-log phases by centrifugation at 9,000×*g* for 10 min

and washed twice with 10 mM piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (PIPES)–sodium hydroxide (NaOH) buffer (pH 6.5) containing 5 mM $MgCl_2$ and 2 mM $CaCl_2$. The washed cells were resuspended with 10 mM PIPES–NaOH buffer at a concentration of grams wet cells per 4 mL, and passed twice through a French pressure cell press (American Instrument Co., Silver Spring, MD, USA) at 16,000 psi. After centrifuged at 4,700×*g* for 10 min to remove intact cells, the supernatant was ultracentrifuged at 233,000×*g* for 30 min to separate membrane fraction from the soluble fraction. The membrane fraction was resuspended with four times volume of 10 mM PIPES–NaOH buffer per gram (wet weight) of the membrane.

Enzyme assay

All measurements were carried out at 25 °C. D-Sorbitol oxidase activity was measured by oxygen uptake using Clark-type oxygen electrode as previously described (Matsushita et al. 1992). The reaction mixture (1.5 mL) was composed of appropriate amounts of membrane fractions, 100 mM D-sorbitol, and 50 mM sodium acetate buffer (pH 5.0). The amount of enzyme reducing 1 natom O/min was taken as 1 unit, considering the amount of O dissolved in the buffer to be 498 nmol/mL.

GLDH activity was measured spectrophotometrically, as phenazine methosulfate (PMS) reductase activity, by coupling with 2,6-dichlorophenolindophenol (DCIP). The reduction of DCIP was measured by tracing the decrease in the absorbance at 600 nm. The reaction mixture (1 mL) contained 100 mM D-sorbitol, 0.2 mM PMS, 0.11 mM DCIP, 1.2 mM NaN_3, the appropriate amount of membrane fraction, and 50 mM sodium acetate buffer (pH 5.0). Holoenzyme formation was performed by the addition of 10 μM PQQ to the membrane suspension in 10 mM PIPES–NaOH buffer (pH 6.5) containing 5 mM $MgCl_2$ and 2 mM $CaCl_2$, and incubation at 25 °C for 30 min. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of substrate per minute under these assay conditions. The extinction coefficient of DCIP (pH 5.0) is 3.43 mM^{-1} cm^{-1}.

SLDH activity was measured using potassium ferricyanide as the electron acceptor, as described previously (Matsushita and Ameyama 1982). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of substrate per minute under the above conditions, which is equivalent to 4.0 absorbance at 420 nm.

Ubiquinol (ubiquinol-2, Q_2H_2) oxidase activity was measured with membrane fractions by following the absorption increase at 275 nm, as described previously (Matsushita et al. 1992). The reaction mixture (1 mL) contained 30 μM Q_2H_2, 0.02 % Tween 20, the appropriate amount of membrane fraction, and 50 mM potassium phosphate buffer (pH 6.5).

Determination of PQQ concentrations

Enzymatic determination of PQQ contents was conducted with apo-form of soluble glucose dehydrogenase (GDH) as described previously (Ameyama et al. 1985). The holomerization mixture contained 3 mM $CaCl_2$, apo-GDH (~1 unit), appropriate amount of PQQ standard or sample, and 50 mM PIPES–NaOH buffer containing 0.1 % TritonX-100 (pH 6.5). After a 30-min incubation at 25 °C, GDH activities were measured as PMS reductase activity by coupling with DCIP as shown in GLDH assay (100 mM D-glucose was used as substrate instead of D-sorbitol). The amount of PQQ in the sample was estimated by comparing the results obtained with standard PQQ.

16S rDNA analysis

Polymerase chain reaction (PCR) amplification was performed using Ready-to-Go/PCR Beads kit (GE Healthcare, UK) with the specific primers 27F (5′-AGAGTTTGATCCTGGC AG-3′) and 1525R (5′-AAAGGAGGTGATCCAGCC-3′), which were selected from highly conserved regions of the nucleotide sequence of 16S rDNA of α-proteobacteria. The amplified PCR product (ca 1.5 kb) was cloned into a pGEM-T Easy vector (Promega Corporation, USA) and transformed into *Escherichia coli* DH5α cells. The recombinant plasmids containing the amplified fragments were extracted and sequenced by using ABI PRISM310 genetic analyzer (Applied Biosystem, USA). The nucleotide sequence has been deposited in the DNA Data Bank of Japan and National Center for Biotechnology Information nucleotide sequence databases under accession number AB678443 (16S rDNA of CHM43).

Sequence analysis of the genes encoding GLDH subunits

Illumina/Solexa sequencing using the GAII platform was performed with CHM43 genome at the Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The sequence data were assembled and protein coding regions of GLDH were predicted using Glimmer 3 with self-training data set, as described in a previous paper (Matsutani et al. 2012; Soemphol et al. 2011; Zerbino and Birney 2008; Salzberg et al. 1998; Delcher et al. 2007). The draft genome assembly of CHM43 is available at GenBank accession BADZ01000001–BADZ01000145. GLDH gene (*sldBA*) is present in nucleotide number from 55430 to 58032 in the contig (BADZ0100008). The *sldBA*-PCR sequencing was performed to confirm the sequence in the draft genome, and also with CHM43AD genome to compare the *sldBA* sequence with that of the CHM43. For this PCR, two sets of PCR primers were

designed, based on the genome data, to be the first forward (CHM43-sld fwd 1) and reverse (CHM43-sldAB rev 1) primers, and the second forward (CHM43-sldAB fwd 2) and reverse (CHM43-sldAB rev 2) primers. The CHM43-sldAB fwd 1 (5′-GTGACTATTCCGGCTG GGTA-3′) and fwd 2 (5′-GCTGTTGTGAACCACGAAGT-3′) are located in nucleotide number of the contig from 55258 to 55277 and 56641 to 56660, respectively, while the CHM43-sldAB rev 1 (3′-TTCTCTTCACCCGTCTTGAC-5′) and rev 2 (5′-GGCGGAA AACCATTCTGAAC-3′) to be 56966 to 56947 and 58205 and 58186. PCR products were purified by using a MagExtractor kit (Toyobo, Tokyo, Japan) and then sequenced by using BigDye ® Terminator Cycle sequencing kit (Applied Biosystems, USA).

Results

Isolation and characterization of thermotolerant *Gluconobacter* sp. adapted to higher temperature

In the previous study, many thermotolerant *Gluconobacter* species have been isolated from Thailand (Moonmangmee et al. 2000). In order to select thermotolerant strains able to perform sorbose fermentation well at high temperature, we examined the growth temperature of isolates on sorbitol medium (Fig. 1). Among eight thermotolerant *Gluconobacter* CHM strains, CHM43 strain showed the best growth at 37 °C and could grow even at 39 °C, where the other strains could not grow at all. Thus, we selected the CHM43 strain as the best strain for further analysis. Of the CHM strains examined, CHM16 and CHM54 had already been identified as *G. frateurii*, based on 16S rDNA sequence similarity to that of the type culture (99.63 and 99.79 %, respectively; Moonmangmee et al. 2000). CHM43 strain was also shown to have a high similarity to *G. frateurii* with the identity (rDNA) of 99.66 % in this study.

As shown in Fig. 2a, the growth in shaking culture of the CHM43 strain was decreased as the growth temperature increased; it was obviously decreased at 38.5 °C and stopped completely at 39 °C. Mesophilic *G. frateurii* IFO3264, a type strain of *G. frateurii*, exhibited much more temperature sensitivity at both plate and shaking cultures (Figs. 1 and 2a). To obtain a more thermostable strain, the CHM43 strain was grown successively at 38.5 °C, which is the upper limited temperature for the growth (Fig. 2a). However, in order to do such an adaptation experiment, we needed severe temperature control for the growth at high temperature because the temperature is very much fluctuated every time the door was opened and closed. Thus, as described in the "Materials and methods" section, we searched the best PID parameters of air incubator and set up the temperature control around the setting temperature (±0.3 °C). Thus, the adapted strain, CHM43AD, was obtained after a seven-time repetition of shaking cultures at 38.5 °C, as described in the "Materials and methods" section.

Characterization of the higher temperature-adapted strain

Compared with the original CHM43 strain, CHM43AD strain grew well at 38.5 °C, but not at 39 °C in a flask shaking culture (Fig. 2a), which is the maximum growth temperature for the agar plate having D-sorbitol as a carbon source (Fig. 1). In the culture at 38.5 °C, cell tended to exhibit elongated and/or abnormal shape under a microscope, especially with the wild strain, which is contrast to the culture at 37 °C where cells of both wild and adapted

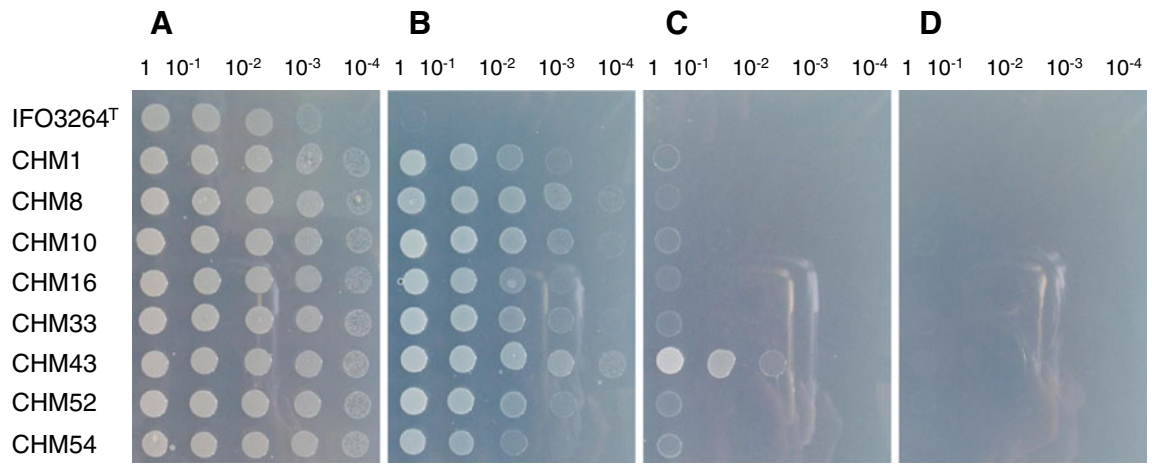

Fig. 1 Growth of *G. frateurii* IFO3264^T and *Gluconobacter* sp. CHM strains at various temperatures. The bacterial cultures (Klett unit of 137–197) were diluted first with water to adjust to the Klett unit of 30. Then, the diluted cultures were further diluted serially four times, and each 8 μL of the diluted bacterial solution was spotted on the agar plate of 5 % sorbitol medium and incubated at 30 (**a**), 37 (**b**) 39 (**c**), and 41 °C (**d**) for 19 h

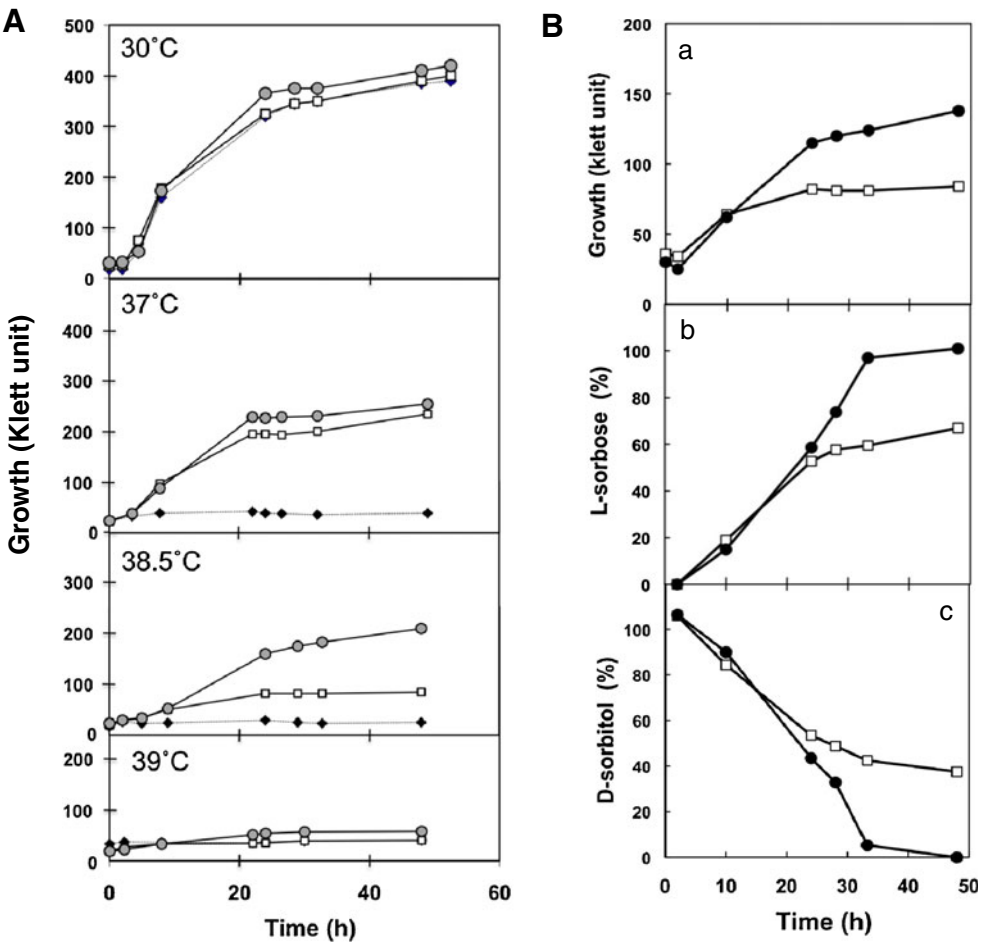

Fig. 2 Comparison of the growth at various temperatures among *G. frateurii* IFO3264^T, CHM43, and CHM43AD strains and of L-sorbose production at 38.5 °C between CHM43 and CHM43AD strains. **a** The *G. frateurii* IFO3264^T (*black diamond*), CHM43 (*white square*), and CHM43AD (*gray circle*) were cultured at 30, 37, 38.5, and 39 °C in 100 mL of 5 % sorbitol medium. **b** Cell growth at 38.5 °C (*a*) on 100 mL of 5 % D-sorbitol medium was monitored. L-Sorbose production (*b*) and D-sorbitol consumption (*c*) were also measured with HPLC as described in "Materials and methods" section. CHM43 (*white square*), CHM43AD (*black circle*). The result shown is typical data for the growth and sorbose production of experiments performed more than three times, of which all showed similar growth and production patterns

strains exhibited normal short-rod shape (data not shown). Even after several repeated shaking culture at 30 °C, CHM43AD strain kept the ability to grow well at 38.5 °C (data not shown). This indicated that some mutations occur in the process of adaptation under high temperature. L-Sorbose production from D-sorbitol in the flask culture was also compared between the wild and CHM43AD strains at 38.5 °C (Fig. 2b). The production rate was decreased around 25 h with the wild strain but kept increasing with CHM43AD strain up to 35 h. Thus, CHM43AD strain could convert almost all the substrates to L-sorbose, but the wild strain did only 60 %. When compared between 30 and 38.5 °C, despite of the growth rate being very much different, both the strains exhibited almost the same sorbose production rate, at least in the initial phase of the production (data not shown). Only in the case of CHM43 cultivated at 38.5 °C was the production rate very much decreased at the later phase (Fig. 2b).

We also conducted the cultivation in a jar fermentor at higher aeration condition as described in the "Materials and methods" section. As shown in Fig. 3, the cell growth was much higher in the jar fermentor than in the flask culture. Concomitant with the increased cell growth of CHM43AD strain, cell elongation observed in the flask culture at 38.5 °C was not seen in the jar fermentor (data not shown). L-Sorbose production was also much higher in the jar fermentor, and 10 % D-sorbitol was completely converted even at 38.5 °C. Different from the flask culture, CHM43AD strain showed a significant growth and converted all the substrates even at 39 °C in the jar fermentor. The adapted strain exhibited some growth even at 40 °C and converted about 60 % of D-sorbitol to L-sorbose.

Comparison of enzyme activities related to sorbose fermentation between CHM43 and the adapted CHM43AD strains

To understand the thermotolerant mechanism of the CHM43AD strain, enzyme activity involved in the D-sorbitol-oxidizing respiratory chain was compared between wild and the adapted strains. The respiratory chain consists of two primary dehydrogenases, GLDH and SLDH, ubiquinone, and cytochrome bo_3 ubiquinol oxidase, where SLDH has been shown not mainly to be involved in the sorbose production (Soemphol et al. 2008). Terminal ubiquinol oxidase activity of the respiratory chain in the membrane fraction was rather high, more than 3.5 units/mg of Q_2H_2

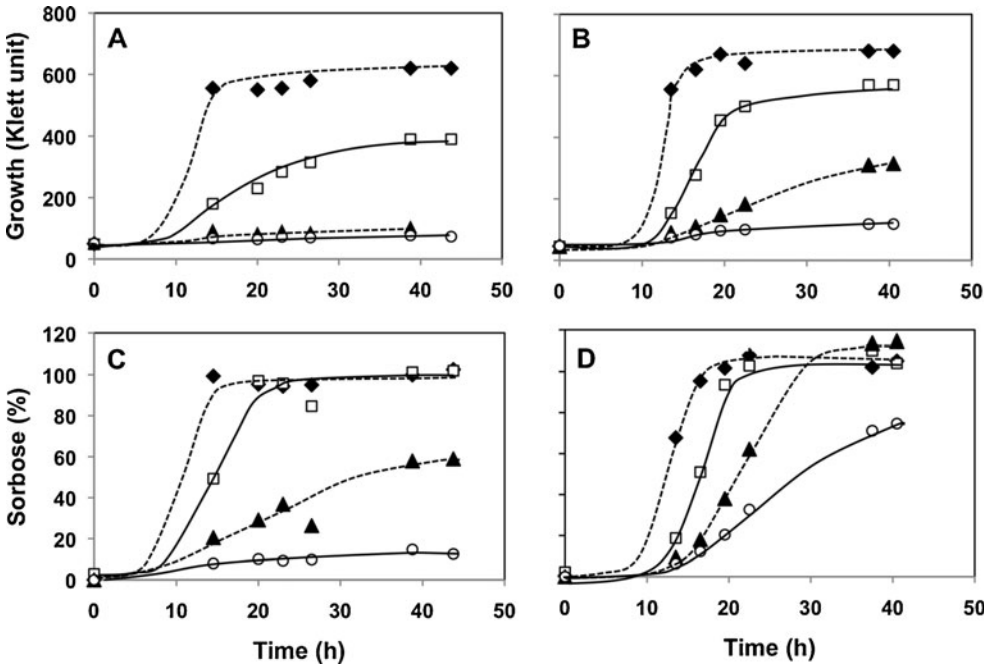

Fig. 3 Cell growth and L-sorbose production of *G. frateurii* CHM43 and CHM43AD in jar fermentor at various temperatures. Cell growth (**a** wild strain, **b** CHM43AD strain) on 10 % D-sorbitol medium was monitored in a jar fermentor at 37 (*black diamond*), 38.5 (*white square*), 39 (*black triangle*), and 40 °C (*white circle*), and L-sorbose production (**c** wild strain, **d** CHM43AD strain) was measured with resorcinol method. The result shown is also typical data for the growth and sorbose production of several similar fermentation experiments

oxidase, and not much different between both strains (data not shown), whereas in the primary dehydrogenase side, GLDH activity was very much higher than SLDH activity (data not shown) and also different between both strains (Fig. 4). CHM43AD strain showed significantly higher GLDH activities than the wild strain, especially at 38.5 °C; ~2-fold higher before holoenzyme formation with PQQ. However, after the holoenzyme formation, GLDH activity of the wild strain was increased 1.6–1.7 times and close to that of CHM43AD strain which was not much increased with PQQ.

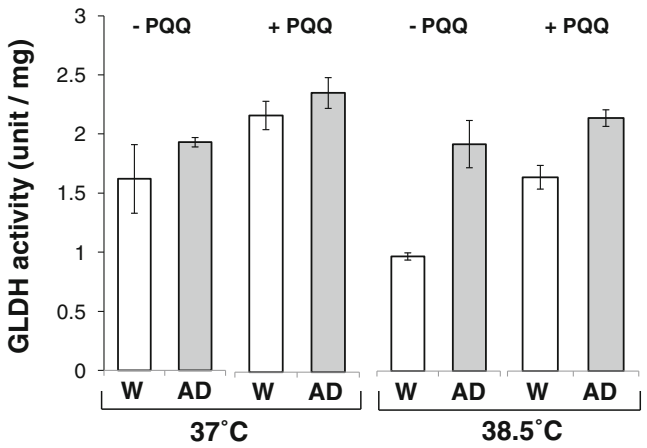

Fig. 4 GLDH activities in the membrane fractions of the cells grown at 37 or 38.5 °C in a jar fermentor. GLDH activity was measured with PMS-DCIP method as described in the "Materials and methods" section, before (−*PQQ*) and after holoenzyme formation (+*PQQ*). The membranes were prepared from the wild (*W*; *white bars*) and CHM43AD (*AD*; *gray bars*) cells harvested at late-log phase. The data shown represent mean values of the 3 different measurements

Sorbitol oxidase activity, whole respiratory activity, was also changed similarly to the change of GLDH activity (data not shown). Thus, in the wild CHM43 strain, GLDH was expected to easily lose PQQ when grown at higher temperature.

Thermostability of GLDH

Since the results described above suggest that CHM43AD strain has more stable GLDH than the wild strain at higher temperature, thermostability of GLDH was examined with the membrane of the wild and CHM43AD strains grown at 30 °C (Fig. 5). GLDH activity of the native membrane fractions was decreased 60 to 70 % and lost almost completely after heat treatment for 1 h at 38 and 40 °C, respectively. In contrast to these results, PQQ-treated membrane fractions (holoenzyme formation) kept the activity even after incubated at 40 °C. The results showed that there is no significant difference in the stability of GLDH between wild and CHM43AD strains. Though data are not shown, there was no clear difference between the wild and CHM43AD strains even when the time course of GLDH inactivation was examined at 38 °C. In addition, GLDH was shown not to be mutated by the adaptation because the sequence of *sldBA* gene, encoding small and large subunits of GLDH (Miyazaki et al. 2002; Matsushita et al. 2003), between both the wild and adapted strains was identical from each other (data not shown, see "Materials and methods" section). Thus, the results suggested that the thermostability of GLDH activity is not dependent on the origin of the strains, but on the holoenzyme formation which critically enhances the stability.

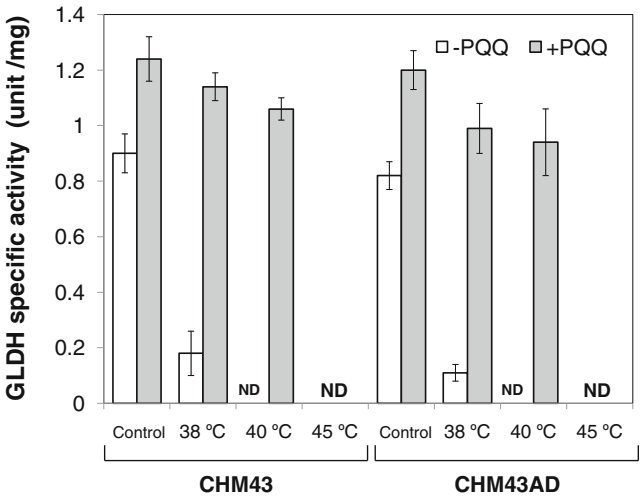

Fig. 5 Stability of GLDH at various temperatures. The membrane fractions prepared from the cells of wild strain CHM43 and the adapted strain CHM43AD were treated first with PQQ (holoenzyme formation) (*gray square*) or without PQQ (*white square*), as described in the "Materials and methods" section, and then incubated at each temperature (38, 40, and 45 °C) for 1 h. Then, GLDH activity was measured with PMS-DCIP method as described in the "Materials and methods" section. The data shown represent mean values of the three different measurements

PQQ production

To examine the effect of PQQ production on holo-GLDH formation, we measured PQQ concentration of each culture medium because large parts of PQQ synthesized have been shown to be released into culture medium (Hölscher and Görisch 2006). As shown in Fig. 6a, CHM43AD strain produced significantly higher amounts of PQQ than the wild strain in both cultures at 37 and 38.5 °C. The results indicated that the PQQ production contributes to keep GLDH as the holoenzyme and thus increases the thermostability of GLDH. Calculating the amount of PQQ based on the cell amounts, CHM43AD strain produced about two times more PQQ (data not shown). This calculation could be done only at 37 but not at 38.5 °C because the wild strain seemed to cause cell lysis and thus the cell proteins may not be measured correctly.

To know the effect of PQQ on the cell growth at 38.5 °C, we compared the cell growth in the absence and the presence of PQQ and $CaCl_2$, both of which are required to make a holo-GLDH by tight binding of PQQ to the enzyme. As shown in Fig. 6b, the wild strain grew better with cofactors, while the adapted strain exhibited a little growth inhibition. The addition of PQQ only did not affect these cell growths. The production rate of sorbose was also stimulated with PQQ and $CaCl_2$ in the wild strain but not in the adapted strain (data not shown).

Cultivation in a jar fermentor without temperature control

For applications of acetic acid bacteria to industrial fermentation, the huge cost of cooling system often becomes a problem. Therefore, we tried to examine the sorbose fermentation without temperature control in a jar fermentor. After setting up the medium and room temperatures at 25 °C, we started the cultivation without control of the medium temperature and monitored the growth and temperature. We also followed the temperature of the fermentor containing water as the control. Starting the cultivation, the temperature was increased immediately and reached to about 33 °C (Fig. 7). After the lag phase, the temperature

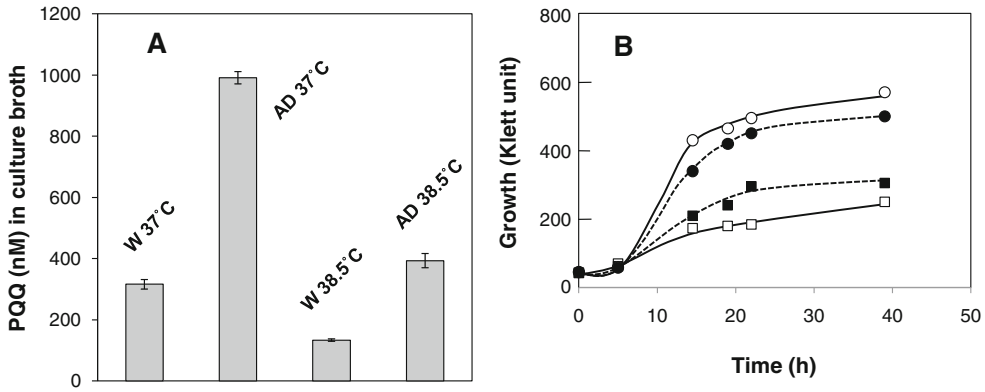

Fig. 6 PQQ production of wild and the adapted strains (**a**) and effect of PQQ addition on the growth of wild and adapted strains at 38.5 °C (**b**). Cultivations of CHM43 wild strain (*W*) and the adapted CHM43AD (*AD*) were performed in a 500-mL Erlenmeyer buffle flask filled with 50 mL of 10 % sorbitol medium at 37 or 38.5 °C. **a** PQQ concentrations with the culture supernatants obtained at mid-log phase of the culture were determined as described in the "Materials and methods" section. The results are shown as an average of the three different measurements from one typical culture of three different cultivation experiments. **b** The cell growth of CHM43 strain (*white* and *black squares*) and of CHN43AD strain (*white* and *black circles*) was carried out at 38.5 °C in the presence (*black square* and *black circle*) or the absence (*white circle* and *white square*) of 0.6 μM PQQ and 2 mM $CaCl_2$. This result is typical data from several different cultivation experiments, all of which showed the same PQQ effect

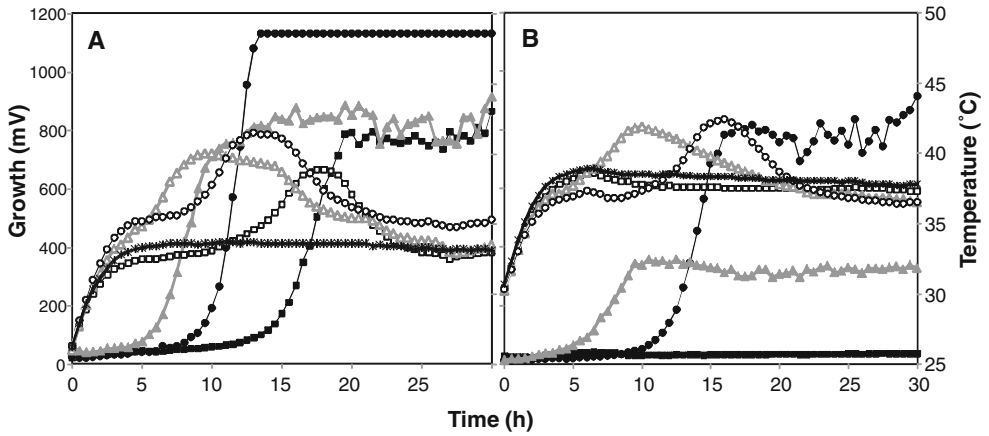

Fig. 7 Cultivation of *G. frateurii* IFO3264^T, CHM43 and CHM43AD strains in a jar fermentor without temperature control. Cell growth (IFO3264^T: *black square*, CHM43 *gray triangle*; CHM43AD *black circle*) and temperature (IFO3264^T *white square*; CHM43 *white triangle*, CHM43AD *white circle*, water *multiplication sign*) on 10 % D-sorbitol medium in a jar fermentor were automatically monitored as described in the "Materials and methods" section. The cultivations ware started from 25 (**a**) and 30 °C (**b**), and the room temperature was controlled at the same temperature. In 25 °C cultivation, the growth of CHM43AD strain proceeded over the monitor limit after 12 h

was increased as the cells started growing. Though IFO3264^T strain and CHM43 strain stopped growing at 38.9 and 40 °C, respectively, CHM43AD strain continued growing up well to the maximum cell growth (680 Klett units) and the medium temperature increased up to 41.5 °C. Furthermore, we conducted the same experiment at 30 °C, in which the cultivation was started and continued under atmosphere of 30 °C. In this case, the temperature increased up to 38 °C first and then up to 41.9 and 42.4 °C, where the growth of CHM43 strain and CHM43AD strain ceased, respectively. CHM43AD strain grew significantly higher than the CHM43 strain, but IFO3264^T strain could not grow after the temperature increased up to 38 °C. Compared with the fermentation with temperature control (Fig. 3), these data clearly indicate that both strains exhibit much bigger difference in the fermentation without temperature control, and thus that CHM43AD strain would be very useful for industrial fermentation without serious care of the temperature control.

Discussion

In this study, we successfully adapted a thermotolerant strain CHM43 to the growth-limited temperature, 38.5 °C, to get a thermo-adapted strain CHM43AD. This strain could grow better and produce more L-sorbose than the original CHM43 strain at higher temperature in shaking flask culture, or much more in a jar fermentor with high aeration. Since these abilities of the adapted strain were retained stably after isolation, adaptive mutations must be accumulated in the strain. Thus, in this study, we examined the enzymatic background of the adapted strain for the high sorbose production at higher temperature.

The enzyme activities in the D-sorbitol-oxidizing respiratory chain, consisting of GLDH, ubiquinone, and the terminal ubiquinol oxidase (Matsushita et al. 2003, 2004; Soemphol et al. 2011), were examined to understand the mechanism of thermotolerance in CHM43AD strain. Since the quinol oxidase activity was significantly higher than GLDH activity, it seems that the limited step of the D-sorbitol-oxidizing respiratory chain is in the primary dehydrogenase (GLDH) but not in the terminal oxidase. The results that sorbitol oxidase activity was changed parallel to GLDH activity also support our notion that GLDH limits the D-sorbitol-oxidizing respiratory chain. Furthermore, the wild strain, but not the adapted strain, was shown to have a significant amount of the apo-form of GLDH, especially at higher temperature (Fig. 4). The results led us to speculate that CHM43AD strain has more stable GLDH than CHM43 wild strain at higher temperature. However, there was no significant difference in the thermostability of GLDH between wild and CHM43AD strains, although the stability was largely increased in PQQ-treated membranes than native membranes, despite of the wild or adapted strains (Fig. 5). In addition, since no mutation has been shown to occur in the structural genes (*sldAB*) of GLDH (Miyazaki et al. 2002; Matsushita et al. 2003) by the adaptation, such thermostability may not be dependent on the enzyme protein itself but on the holoenzyme formation with PQQ. In fact, such the holoenzyme formation with PQQ has been shown to increase the thermostability in two quinoproteins, *Escherichia coli* GDH (Ameyama et al. 1986) and *Pyrobaculum* aldose sugar dehydrogenase (Sakuraba et al. 2010). Thus, together with the finding that CHM43AD strain actually produced significantly more PQQ than the wild strain (Fig. 6), it is suggested that the PQQ production contributes

to keep GLDH as the holoenzyme and thus increases the thermostability of GLDH. Therefore, it is expected that adapted strain may have mutations in genes involving in PQQ biosynthesis and/or its regulation, although mutations were not seen at least in ORFs of each gene for *pqqABCDE* (Hölscher and Görisch 2006; Yang et al. 2010) (data not shown). In addition, addition of PQQ with $CaCl_2$ to the culture medium stimulated the cell growth of the wild strain at 38.5 °C (Fig. 6b), providing additional evidence that PQQ in the culture contributes to keep holo-GLDH at higher temperature. However, the same addition of PQQ to CHM43AD strain caused the growth inhibition. Although the reason is not clear, we may speculate that the adapted strain may produce high enough PQQ and thus additional extra PQQ may disturb the cell growth. In the wild stain, even though the cell growth is increased by the PQQ addition, it does not reach to that level of CHM43AD strain, suggesting that high PQQ production in CHM43AD strain would be one of the factors, but not all, for the thermotolerance.

In this study, in addition to elucidating the physiological properties of the adapted strain CHM43AD, we performed more practical experiments, the sorbose fermentation without temperature control in jar fermentor, and found that CHM43AD strain could keep growing over the high temperature due to the mechanical heat and the fermentation heat generation under which conditions another strain stop growing, indicating that CHM43AD strain is useful for cutting cooling cost down. Thus, this study shows the possibility of a new style of L-sorbose fermentation and further the usefulness of such adapted strain in the applications to industry.

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