Title

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Three ATP-dependent phosphorylating enzymes in the first committed step of dihydroxyacetone metabolism in *Gluconobacter thailandicus* NBRC3255

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Abstract
Dihydroxyacetone (DHA), a chemical suntan agent, is produced by the regiospecific oxidation of glycerol with *Gluconobacter thailandicus* NBRC3255. However, this microorganism consumes DHA produced in the culture medium. Here, we attempted to understand the pathway for DHA metabolism in NBRC3255 to minimize DHA degradation. The two gene products, NBRC3255_2003 (DhaK) and NBRC3255_3084 (DerK), have been annotated as DHA kinases in the NBRC 3255 draft genome. Because the double deletion derivative for *dhaK* and *derK* showed ATP-dependent DHA kinase activity similar to that of the wild type, we attempted to purify DHA kinase from Δ*dhaK* Δ*derK* cells to identify the gene for DHA kinase. The identified gene was *NBRC3255_0651*, of which the product was annotated as glycerol kinase (GlpK). Mutant strains with several combinations of deletions for the *dhaK*, *derK*, and *glpK* genes were constructed. The single deletion strain Δ*glpK* showed approximately 10% of wild-type activity and grew slower on glycerol than the wild type. The double deletion strain Δ*derK* Δ*glpK* and the triple deletion strain Δ*dhaK* Δ*derK* Δ*glpK* showed DHA kinase activity less than a detection limit and did not grow on glycerol. In addition, although Δ*derK* Δ*glpK* consumed a small amount of DHA in the late phase of growth, Δ*dhaK* Δ*derK* Δ*glpK* did not show DHA consumption on glucose–glycerol medium. The transformants of the Δ*dhaK* Δ*derK* Δ*glpK* strain that expresses one of the genes from plasmids showed DHA kinase activity. We concluded that all three DHA kinases, DhaK, DerK, and GlpK, are involved in DHA metabolism of *G. thailandicus*.

Key points
• *Dihydroxyacetone (DHA) is produced but degraded by Gluconobacter thailandicus.*
• *Phosphorylation rather than reduction is the first committed step in DHA metabolism.*
• *Three kinases are involved in DHA metabolism with the different properties.*

Keywords Acetic acid bacteria · *Gluconobacter* · Dihydroxyacetone · Glycerol · Kinase

Naoya Kataoka and Kaori Hirata contributed equally to this work.

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Introduction

Acetic acid bacteria have a unique ability to oxidize a wide variety of alcohols including polyols such as glycerol to the corresponding ketones and organic acids (Matsushita et al. 1994). The conversion rate of the oxidative biotransformation is quite high, particularly at high concentrations of starting materials. Importantly, these microorganisms hardly consume the oxidized products such as dihydroxyacetone. Thus, incomplete oxidation is referred to as oxidative fermentation and some of them are applied in the industrial production processes such as L-sorbose from D-sorbitol as well as dihydroxyacetone from glycerol.

Glycerol is regiospecifically oxidized by membrane-bound glycerol dehydrogenase (GLDH) of *Gluconobacter* spp. not only to dihydroxyacetone (DHA), a suntan agent, but also to glyceric acid by membrane-bound alcohol dehydrogenase (ADH) (Habe et al. 2009a, b). Thus, the bacterial strain lacking ADH is beneficial to DHA production (Habe et al. 2010). We attempted to produce DHA using *Gluconobacter thailandicus* strain NBRC3255 (formerly *Gluconobacter suboxydans* var. α IFO3255), which naturally possesses a functionally critical single nucleotide polymorphism in the gene for ADH, resulting in the loss of ADH function (Charoenyingcharoen et al. 2015; Matsushita et al. 1991). However, the NBRC3255 strain degraded DHA, even though glycerol was effectively oxidized to DHA (Adachi et al. 2008). Therefore, minimizing DHA degradation by NBRC3255 is needed for the efficient production of DHA from glycerol. In this study, we attempted to elucidate the metabolic pathway of DHA in *G. thailandicus* NBRC3255, since the pathways for DHA metabolism in *Gluconobacter* spp. remain largely unknown.

G. thailandicus NBRC3255 oxidizes glycerol to DHA, which is then degraded presumably by intracellular metabolism. Since the ΔsldA derivative that is devoid of GLDH consumes glycerol, the NBRC3255 strain possesses a metabolic pathway on glycerol (Adachi et al. 2008). Based on the NBRC3255 draft genome (Matsutani et al. 2013), we predicted a metabolic pathway for glycerol and DHA, as shown in Fig. 1. Like *Gluconobacter oxydans* (Krajewski et al. 2010), a final metabolite is acetic acid using pyruvate decarboxylase, since the NBRC3255 strain lacks the genes for succinyl CoA synthetase and succinate dehydrogenase (Matsutani et al. 2013). Because no genes are related to the phosphotransferase system in the NBRC3255 genome (Matsutani et al. 2013; Prust et al. 2005), glycerol and DHA are likely incorporated into the cell without phosphorylation. We anticipate two reactions as the first committed step for DHA metabolism: reducing DHA to glycerol and phosphorylating it to dihydroxyacetone phosphate (DHAP) (Fig. 1). Although it is theoretically possible to consider the isomerization of DHA to glyceraldehyde as the third route (Fig. 1) (Adachi et al. 2008), we examined DHA reductase and DHA kinase at the beginning of this study.
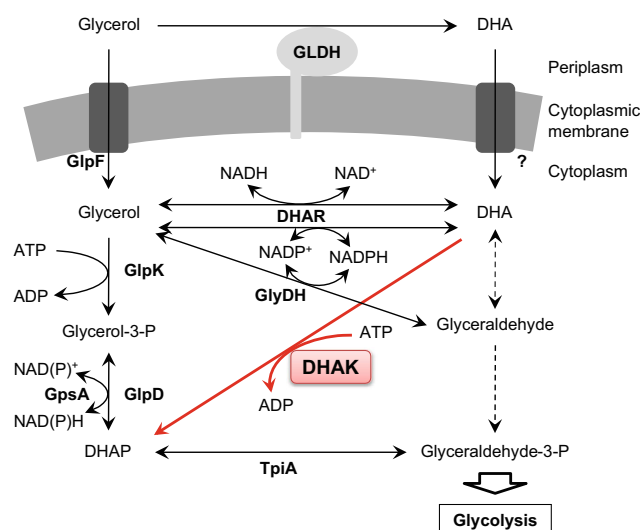

Fig. 1 Potential metabolic pathway for glycerol and DHA in *Gluconobacter thailandicus* NBRC3255. The metabolic pathways for glycerol and DHA were constructed from Adachi et al. (2008) and from the genome information of *G. oxydans* ATCC621H and *G. thailandicus* NBRC3255. Dashed arrows indicate the pathways where the responsible enzymes are missing in the genome data. The gene products corresponding to the enzymes shown here are as follows. Glycerol uptake facilitator (GlpF), NBRC3255_0655; PQQ-dependent glycerol dehydrogenase (GLDH), NBRC3255_0026-27 or NBRC3255_0238-39; NADH-dependent DHA reductase (NADH-DHAR), NBRC3255_0712; NADPH-dependent DHA reductase (NADPH-DHAR), NBRC3255_1149; glycerol kinase (GlpK), NBRC3255_0654; NAD(P)$^+$-dependent glycerol-3-phosphate dehydrogenase (GpsA), NBRC3255_0877; acceptor-dependent glycerol-3-phosphate dehydrogenase (GlpD), NBRC3255_0656; NADP$^+$-dependent glycerol dehydrogenase (GlyDH), NBRC3255_1138; DHA kinase (DHAK), NBRC3255_2012 and NBRC3255_3096, triosephosphate isomerase (Tpi), NBRC3255_0730, NBRC3255_0831, NBRC3255_0667, or NBRC3255_3012. The homologous proteins in *G. oxydans* strain ATCC621H are listed in Table S1

Materials and methods

Materials

Dihydroxyacetone was obtained from MP Biomedicals (Santa Ana, CA). Yeast extract and polypeptone were obtained from Oriental Yeast (Osaka, Japan) and Nihon Pharmaceutical (Tokyo, Japan), respectively. 5-Fluorocytosine was purchased from Fluorochem (Glossop, UK). Restriction endonucleases and modification enzymes for genetic engineering were purchased from Toyobo (Osaka, Japan). All other materials were purchased from a commercial source of analytical grade.

Bacterial strains and cultivation

The *G. thailandicus* strains used in this study are listed in Table 1. *G. thailandicus* strain NBRC3255 was obtained from the NITE Biological Resource Center (NBRC, https://www.nite.go.jp/nbrc/). *G. thailandicus* cells were cultivated in ΔP (10 g yeast extract, 10 g polypeptone, 5 g glucose, and 20 g

Table 1 Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Source or reference
Gluconobacter thailandicus		
NBRC3255	Wild type	NBRC
KAO-1	NBRC3255 Δ*dhaK*	This study
KAO-2	NBRC3255 Δ*derK*	This study
KAO-3	NBRC3255 Δ*dhaK* Δ*derK*	This study
KAO-4	NBRC3255 Δ*glpK*	This study
KAO-5	NBRC3255 Δ*dhaK* Δ*glpK*	This study
KAO-6	NBRC3255 Δ*derK* Δ*glpK*	This study
KAO-7	NBRC3255 Δ*dhaK* Δ*derK* Δ*glpK*	This study
Plasmid		
pRK2013	Plasmid for conjugal plasmid transfer, Km^R	Figurski and Helinski 1979
pT7Blue	Cloning vector, T7 promoter, Ap^R	Novagen
pK18mobGII	Suicide vector, *mob gusA*, Km^R	Katzen et al. 1999
pKOS6b	Suicide vector, *mob codBA*, Km^R	Kostner et al. 2013
pBBR1MCS-4	Broad-host-range plasmid, *mob*, *lacZ* promoter, Ap^R	Kovach et al. 1995
pEK12	pK18mobGII, a 1.6-kb fragment of the Δ*dhaK* allele	This study
pEK15	pK18mobGII, a 1.3-kb fragment of the Δ*derK* allele	This study
pKH4	pKOS6b, a 1.3-kb fragment of the Δ*derK* allele	This study
pKH7	pT7Blue, a 2.7-kb fragment of *glpK*	This study
pKH10	pKOS6b, a 1.4-kb fragment of the Δ*glpK* allele	This study
pKH18	pBBR1MCS-4, a 2.7-kb fragment of *glpK*	This study
pKH24	pBBR1MCS-4, a 2.1-kb fragment of *derK*	This study
pEK20	pBBR1MCS-4, a 1.8-kb fragment of *dhaK*	This study

dhaK, *NBRC3255_2003*; *derK*, *NBRC3255_3084*; *glpK*, *NBRC3255_0651*

glycerol per liter), YPGD (5 g yeast extract, 5 g polypeptone, 5 g glycerol, and 5 g glucose per liter), YPG (5 g yeast extract, 5 g polypeptone, and 10 g glycerol per liter), or YPDHA (5 g yeast extract, 5 g polypeptone, and 5 g DHA per liter) medium. A stock solution of DHA at 40% (w/v) was freshly prepared and sterilized by filtration (0.22 μm). The cells were pre-cultivated in 2 mL of ΔP medium in a tube at 30 °C with shaking at 200 rpm. For the main cultivation, 1 mL of the pre-culture was transferred into a 100-mL medium in a 500-mL Erlenmeyer flask, and the cells were cultivated at 30 °C with shaking at 200 rpm. For *G. thailandicus*, kanamycin and ampicillin were used at final concentrations of 100 and 50 μg mL^{-1}, respectively.

Escherichia coli strains DH5α (Hanahan 1983) and HB101 (Boyer and Roulland-Dussoix 1969) were used for plasmid construction and triparental mating, respectively. *E. coli* cells were cultivated in modified Luria–Bertani (LB) medium (Sambrook and Russel 2001), which consists of 5 g of yeast extract, 10 g of polypeptone, and 5 g of NaCl per liter, adjusted pH to 7 with NaOH. For *E. coli*, kanamycin and ampicillin were used at final concentrations of 50 μg mL^{-1}.

Construction of plasmids

The plasmids used in this study are listed in Table 1. Herculase DNA polymerase (Stratagene, Santa Clara, CA,

USA) was used for PCR. The DNA primers used in this study are listed in Table S2. The nucleotide sequence of the PCR products was confirmed by Sanger sequencing after DNA cloning. Genomic DNA used as the parental DNA for PCR was isolated from *G. thailandicus* strain NBRC3255 as described by Marmur (1961), with some modifications (Kawai et al. 2013). To construct plasmids for the deletion of *dhaK* (*NBRC3255-2003*), a ca. 2.8-kb DNA fragment containing the 5′ and 3′ flanking regions of *dhaK* was amplified with a pair of the primers, namely ΔGTH2012-5-Pst(+) and ΔGTH2012-3-Bam(−). The DNA fragment was digested with *Pst*I, *Bam*HI, and *Sph*I to obtain two DNA fragments ca. 0.8-kb 5′ region and ca. 0.8-kb 3′ region to be inserted into the *Pst*I and *Bam*HI sites of pK18mobGII, yielding pEK12. To construct plasmids for the deletion of *derK* (*NBRC3255-3084*), a ca. 2.3-kb DNA fragment containing the 5′ and 3′ flanking regions of *derK* was amplified with a pair of primers, namely ΔGTH3096-5-Sal(+) and ΔGTH3096-3-Bam(−). The DNA fragment was digested with *Sal*I, *Bam*HI, and *Nco*I to obtain two DNA fragments, a ca. 0.7-kb 5′ region and ca. 0.6-kb 3′ region, to be inserted into the *Sal*I and *Bam*HI sites of pK18mobGII, yielding pEK15. pEK15 was treated with *Sal*I and *Sma*I to obtain the ca. 1.3 kb DNA fragment containing the Δ*derK* allele, which was then inserted into the corresponding site of pKOS6b to yield pKH4.

glpK (*NBRC3255_0651*) was amplified with a pair of primers, ΔglpK-5-Hin(+) and ΔglpK-3-Xba(−), and inserted into the *Eco*RV site of pT7Blue (Novagen) to yield pKH7. The pKH7 plasmid was treated with *Hin*dIII and *Eco*RV to obtain a ca. 0.7-kb DNA fragment containing the 5′ flanking region of *glpK*. Then, the 3′ flanking region of *glpK* was obtained by PCR with a pair of primers, ΔglpK-5-RV(+) and ΔglpK-3-Xba(−), and the PCR product was treated with *Eco*RV and *Xba*I. The 5′ and 3′ regions of *glpK* were inserted into the *Hin*dIII and *Xba*I sites of pKOS6b to yield pKH10.

The pKH7 plasmid carrying *glpK* (*NBRC3255_0651*) was treated with *Sma*I and *Sal*I to obtain a ca. 2.7-kb DNA fragment containing *glpK*. It was then inserted into the corresponding site of pBBR1MCS-4 to yield pKH18. To construct the plasmid for expression of *derK* (*NBRC3255_3084*), a 2.1-kb DNA fragment was obtained with a pair of primers, namely GTH3096-5-Kpn(+) and GTH3096-3-Sal(−). The DNA fragment was treated with *Kpn*I and *Sal*I to be inserted into the corresponding site of pBBR1MCS-4 to yield pKH24. For the *dhaK* (*NBRC3255_2003*) expression, the plasmid pEK20 was constructed by a similar procedure with a pair of primers, GTH2012-5-Kpn(+) and GTH2012-3-Sal(−).

Construction of gene deletion mutants

G. thailandicus strain NBRC3255 was transformed with plasmids via a triparental mating method, as described previously (Kawai et al. 2013). In the case of pK18mobGII derivatives, the first recombinants with kanamycin-resistant (Km^R) and β-glucuronidase-positive (GUS^+) phenotypes were screened to conduct second recombination procedures. Non-blue colonies were screened on ΔP agar in the presence of 20 μg mL^{-1} 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (cyclohexylammonium salt). Thus, β-glucuronidase-negative (GUS^-) and kanamycin-sensitive (Km^S) second recombinants were isolated, and their target alleles were confirmed by PCR. In the case of pKOS6b derivatives, the first recombinants with Km^R and 5-fluorocytocine-sensitive ($5\text{-}FC^S$) phenotypes were screened to conduct second recombination procedures. FC-resistant ($5\text{-}FC^R$) colonies were screened on ΔP agar in the presence of 60 μg mL^{-1} 5-FC. Thus, $5\text{-}FC^R$ and Km^S second recombinants were isolated, and their target alleles were confirmed by PCR. Second recombinants with only the allele deletion were obtained and referred to as the deletion mutant strains.

Determination of glycerol and DHA

The glycerol and DHA concentrations in the media were analyzed using a high-performance liquid chromatography (HPLC) system equipped with a refractive index (RI) detector. Glycerol and DHA were quantified with a Pb^{2+} cation-exchange column (SP0810 (SUGAR), 8.0 mm I.D. × 300 mm L; Shodex, Showa Denko KK, Kawasaki, Japan) at 80 °C using H_2O as the mobile phase at a flow rate of 0.5 mL min^{-1}. The retention times for glycerol and DHA were 28.5 and 30.9 min, respectively.

Preparation of the soluble fraction

Gluconobacter cells were cultivated on YPG or YPGD medium until the late exponential phase. After cultivation, the cells were collected by centrifugation at 9000×*g* for 10 min at 4 °C. The collected cells were suspended in 50 mM K$^+$-phosphate (pH 7.0) and disrupted using a French pressure cell press at 1100 kg cm^{-2}. The cell debris was removed by centrifugation at 9000×*g* for 10 min at 4 °C. The resulting supernatant was centrifuged at 100,000×*g* for 1 h at 4 °C to separate the membranes. The supernatant was used as the soluble fraction for measuring enzyme activity.

Enzyme assays

DHA kinase activity was measured using a coupled method by reducing dihydroxyacetone phosphate with glycerol-3-phosphate dehydrogenase (G3PDH) (Sigma-Aldrich, St. Louis, MO), as described previously (Siebold et al. 2003). The assay mixture consisted of 100 mM Tris-HCl (pH 6.5 or 7.0, as specified), 25 mM DHA, 5 mM $MgCl_2$, 2.5 mM ATP, 0.16 mM NADH, 2.5 U mL^{-1} G3PDH, 1 mM KCN, 7 mM NaN_3, and the appropriate amount of enzyme. The activity was determined by the rate of decrease in the absorbance at 340 nm with a molecular coefficient of 6.22 mM^{-1} cm^{-1}. The glycerol kinase activity was measured in a similar way (Deutscher and Sauerwald 1986). The assay mixture consisted of 100 mM K_2CO_3-$NaHCO_3$ (pH 10), 2 mM glycerol, 4 mM $MgCl_2$, 1 mM ATP, 3 mM NAD$^+$, 2.5 U mL^{-1} G3PDH, and an appropriate amount of enzyme. The activity was measured by the rate of increase in absorbance at 340 nm by the oxidation of glycerol-3-phosphate with G3PDH.

NADH-dependent DHA reductase activity was measured by the rate of decrease in absorbance at 340 nm concomitant with the oxidation of NADH. The assay mixture consisted of 50 mM Tris-HCl (pH 7.5), 7 mM NaN_3, 1 mM KCN, 0.125 mM NADH, 10 mM DHA, and an appropriate amount of enzyme, as described previously (Adachi et al. 2008). NADPH-dependent DHA reductase activity was measured by the rate of decrease in absorbance at 340 nm with a molecular coefficient of 6.22 mM^{-1} cm^{-1}. The assay mixture consisted of 50 mM Tris-HCl (pH 7.5), 0.125 mM NADPH, 10 mM DHA, and an appropriate amount of enzyme, as described previously (Adachi et al. 2008). One unit of enzyme activity was defined as the amount of enzyme catalyzing the phosphorylation or reduction of 1 μmol of substrate per min under the assay conditions.

The protein content was determined by a modified Lowry method with bovine serum albumin as the standard protein (Dulley and Grieve 1975).

Partial purification of DHA kinase

The buffer used for the purification of DHA kinase was 50 mM K^+-phosphate (pH 8.0) containing 2 mM EDTA and 3 mM 2-mercaptoethanol. KAO-3 ($\Delta dhaK$ $\Delta derK$) cells were grown on YPG medium for 36 h. The resulting soluble fraction was applied to a DEAE-Toyopearl column that had been equilibrated with the buffer. The enzyme was eluted from the column by increasing the NaCl concentration to 0.1 M. The active fractions from the DEAE-Toyopearl column were pooled and ammonium sulfate was added to the enzyme solution to 30% saturation. Then, it was centrifuged at 10,000×g for 10 min at 4 °C. The supernatant was applied to a Butyl-Toyopearl column that had been equilibrated with buffer containing 30% ammonium sulfate. The enzyme was eluted from the column by decreasing the ammonium sulfate concentration to 10% saturation. The active fractions were pooled and concentrated using a centrifugal filter unit (Amicon Ultra 50 k; Millipore, Burlington MA). The concentrated enzyme solution was applied to a Sephacryl S-200 column that had been equilibrated with the buffer. The purity of the active fractions was examined by SDS-PAGE (Laemmli 1970). The N-terminal amino acid sequence of the band that shows an elution profile similar to that of the enzyme activity in the column chromatography was determined using a Procise 494 HT Protein Sequencing System (Applied Biosystems, Foster City, CA), as described previously (Kataoka et al. 2015).

Phylogenetic tree construction

To estimate the phylogenetic relationship of DHA kinase homologs, phylogenetic analysis was performed using amino acid sequences. Ten amino acid sequences of DHA kinase homologs from acetic acid bacteria, *C. freundii*, *Mycobacterium smegmatis*, and *E. coli* were retrieved from the public database (see Fig. 2). The sequences were aligned using MUSCLE v3.8.31 (Edgar 2004), and well-aligned regions were extracted using Gblocks 0.91b (Talavera and Castresana 2007). The maximum likelihood phylogenetic tree of the DHA kinases of acetic acid bacteria, *C. freundii*, *Mycobacterium*, and *E. coli* was constructed using RAxML version 8.2.2 (Stamatakis 2014) using the PROTGAMMAWAG model with 1000 rapid bootstrap. The tree was visualized using MEGA 5 (Tamura et al. 2011).
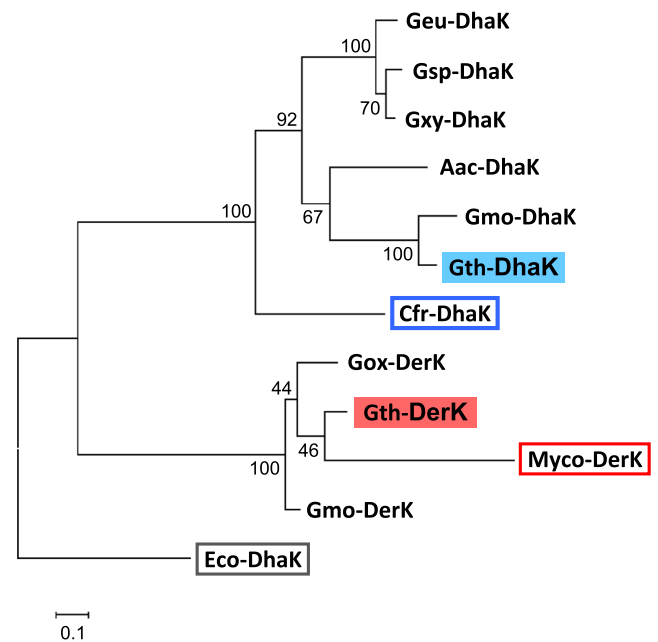

Fig. 2 Maximum likelihood phylogenetic trees of DHA kinases of acetic acid bacteria, *Citrobacter freundii*, *Mycobacterium smegmatis*, and *Escherichia coli*. Amino acid sequences were mined from public databases with the following protein IDs (organism and UniProtKB accession number, RefSeq ID, or locus tag): Gth-DhaK (*G. thailandicus* NBRC3255, NBRC3255_2003), Gth-DerK (*G. thailandicus* NBRC3255, NBRC3255_3084), Gox-DerK (*G. oxydans* 621H, GOX2222), Gmo-DhaK (*Gluconobacter morbifer* G707, GMO_08860), Gmo-DerK (*G. morbifer* G707, GMO_24250), Gxy-DhaK (*Ga. xylinus* NBRC3288, GLX_05270), Gsp-DhaK (*Gluconacetobacter* sp. SXCC-1, SXCC_04742), Geu-DhaK (*Gluconacetobacter europaeus* LMG18890, WP_019086540.1), Aac-DhaK (*Acetobacter aceti* NBRC14818, EMQ_0169), Cfr-DhaK (*C. freundii* DSM30040, P45510), Msm-DerK (*M. smegmatis*, LI99_16265), and Eco-DhaK (*E. coli*, b1200)

Results

ATP-dependent DHA phosphorylation in *G. thailandicus* NBRC3255

There are two potential pathways (reducing and phosphorylating DHA) as the first committed step for DHA metabolism (Fig. 1). There are two types of DHA kinases: ATP-dependent DHA kinase of homodimer of single subunits (Siebold et al. 2003) and phosphoenolpyruvate (PEP)-dependent DHA kinase of three subunits (DhaK, DhaL, and DhaM) (Gutknecht et al. 2001). The two kinases are homologous to each other and have been proposed to function under a similar catalytic mechanism, even though they use different phosphate donors (Bachler et al. 2005; Shi et al. 2011). ATP-dependent DHA kinase activity was much higher than NADH- and NADPH-dependent DHA reductase activities (Table 2). We failed to detect DHA kinase activity when ATP was replaced with PEP. Interestingly, the DHA kinase activity ceased in the late stationary phase (Fig. S1).

Table 2 Enzyme activity in the soluble fraction of *G. thailandicus* strain NBRC3255 and its derivatives

Bacterial strain	Enzyme	Activity (mU mg^{-1})
Wild type	DHA reductase (NADH)	20 ± 3
	DHA reductase (NADPH)	< 1
	DHA kinase	450 ± 60
	Glycerol kinase	64 ± 3
Δ*glpK*	DHA kinase	30 ± 7
	Glycerol kinase	n.d.
Δ*dhaK* Δ*derK*	DHA kinase	590 ± 10
Δ*dhaK* Δ*glpK*	DHA kinase	56 ± 6
Δ*derK* Δ*glpK*	DHA kinase	n.d.
Δ*dhaK* Δ*derK* Δ*glpK*	DHA kinase	n.d.

The cells were cultivated in YPGD medium until the late exponential growth phase. The soluble fraction was prepared by cell disruption on a French press followed by ultracentrifugation for removal of cell membranes

n.d. not detected (less than a detection limit of 0.5 mU mg^{-1})

Two putative DHA kinases annotated in the draft genome study

According to a draft genome study on *G. thailandicus* NBRC3255, two genes for putative DHA kinase were found: *NBRC3255_2003* and *NBRC3525_3084* (Matsutani et al. 2013). Homologous proteins were retrieved by BLAST with UniProt (Swiss-Prot) as a target. The DHA kinase of *Citrobacter freundii* (Siebold et al. 2003) and D-erythrulose kinase of *Mycobacterium smegmatis* (Huang et al. 2015) were the first hits with a high amino acid identity for NBRC3255_2003 and NBRC3255_3084, respectively. In addition, a phylogenetic analysis of these four gene products, the DHAK homologs of other acetic acid bacteria and the DhaK subunit of *E. coli* PEP-dependent DHA kinase, suggested that NBRC3255_2003 and NBRC3255_3084 are phylogenetically distinguished from each other (Fig. 2). Thus, hereafter, NBRC3255_2003 and NBRC3255_3084 are referred to as DhaK and DerK.

We constructed the three NBRC3255 derivatives Δ*dhaK*, Δ*derK*, and Δ*dhaK* Δ*derK* by eliminating the genes, as described in the "Materials and methods" section. However, all of the constructed strains showed DHAK activities and DHA consumption similar to that of the wild type (Fig. S2). We tentatively concluded that *dhaK* and *derK* are not greatly involved in DHA metabolism in the NBRC3255 strain, and other ATP-dependent DHAK may be involved. Thus, we attempted to identify the enzymes responsible for DHAK activity and DHA consumption.

Identification of DHAK in the Δ*dhaK* Δ*derK* strain

DHAK was partially purified from the soluble fraction of the Δ*dhaK* Δ*derK* double mutant strain to determine the N-terminal amino acid sequence. We attempted to determine the five amino acid sequences of the approximately 60-kDa band that showed elution behaviors similar to that of DHAK. The N-terminal amino acid sequence obtained was Thr-Gln-Lys-Asp-Xxx (Xxx is Cys or modified amino acid), corresponding to (Met)-Thr-Gln-Lys-Asp-Cys of the predicted N-terminal sequence of the *NBRC3255_0651* gene product, annotated as glycerol kinase (GlpK). The partially purified DHAK preparation was able to phosphorylate not only DHA but also glycerol. The specific DHAK and glycerol kinase activities of the partially purified enzyme were 1.0 and 0.15 U mg^{-1}, respectively.

We eliminated *NBRC3255_0651* to construct the Δ*glpK* strain. The DHA kinase activity of the Δ*glpK* strain was decreased to less than 10% of that of the wild type (Table 2). Thus, we concluded that GlpK accounts for a major part of DHAK activity in *G. thailandicus*. In addition, we anticipated that the remaining DHAK activity in the Δ*glpK* strain may be due to the action of *dhaK* or *derK* products. Therefore, we constructed *G. thailandicus* NBRC3255 derivatives with all the combinations of the deletions in the three genes, *glpK*, *dhaK*, or *derK*, to determine the involvement of DhaK and DerK in DHA metabolism under the Δ*glpK* background.

Deletion mutant strains for *dhaK*, *derK*, and *glpK*

Although the Δ*glpK* strain showed a marked decrease in DHAK activity (Table 2), it consumed DHA as the wild type with a short delay (Fig. 3). The late start of DHA consumption is likely due to it having a slower growth than the wild type. The combination of Δ*dhaK* and Δ*glpK* decreased the growth of the single Δ*glpK* mutant on glycerol, while the double mutant strain grew slowly on glycerol and DHA (Fig. 3c, d). Consistent with the growth behavior, the Δ*dhaK* Δ*glpK* strain showed considerable DHAK activity (Table 2). On the other hand, the Δ*derK* Δ*glpK* double mutant strain showed DHAK activity less than a detection limit of 0.5 mU mg^{-1} (Table 2) and was unable to grow in a medium containing glycerol or DHA alone (Fig. 3c, d). The triple deletion mutant Δ*dhaK* Δ*derK* Δ*glpK* strain showed similar results as the Δ*derK* Δ*glpK* strain in terms of its DHAK activity and growth on glycerol or DHA. Our findings suggest that GlpK plays a major role in DHA metabolism and that DerK supports the function of GlpK. By contrast, since the role of DhaK in DHA metabolism has been unclear, we attempted to find a clue for it through the following experiments.

Because the Δ*dhaK* Δ*derK* Δ*glpK* and Δ*derK* Δ*glpK* strains cannot grow on glycerol or DHA as sole carbon sources, we cultivated the two strains on medium containing glucose and glycerol to monitor DHA metabolism. The single deletion mutants for *glpK* and *derK* showed growth and DHA consumption similar to those on glycerol as the sole carbon source (Fig. S3). The Δ*dhaK* Δ*derK* Δ*glpK* and Δ*derK*

Fig. 3 Growth, pH, and metabolite profile of the wild-type and deletion mutant strains on glycerol and DHA. Each strain was pre-cultivated on ΔP medium overnight. The pre-culture was inoculated into YPG (a–c) and YPDHA (d) at a 1% ratio. The OD$_{600}$ (solid lines in a, c, and d) and pH (dashed lines in a) values, and glycerol (open symbols in b) and DHA (closed symbols in b) concentrations in the medium were measured. The mean values and standard deviations were calculated from the triplicate cultivations in a–c. Wild type, black; ΔglpK, blue; ΔdhaK ΔglpK, orange; ΔderK ΔglpK, purple; ΔdhaK ΔderK, magenta; ΔdhaK ΔderK ΔglpK, red

derivatives maintained much higher concentrations of DHA than the wild type. Interestingly, the triple deletion strain accumulated higher DHA than the double deletion strain (Fig.
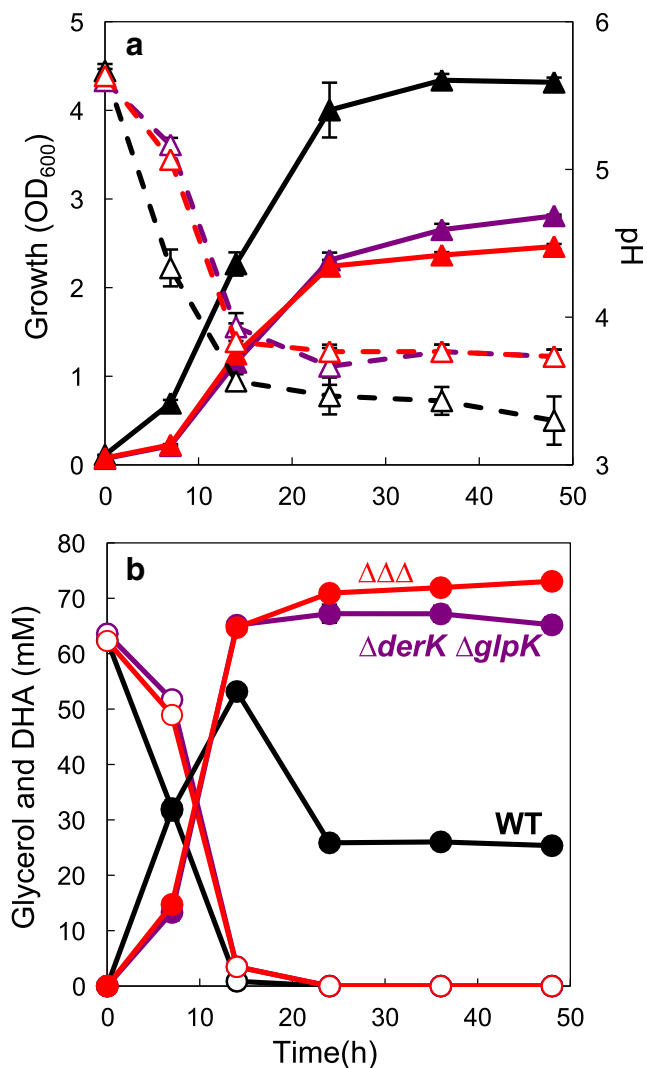
Fig. 4 Growth, pH, and metabolite profile of the wild-type and multiple gene deletion strains on YPGD medium. The wild-type, ΔderK ΔglpK, and ΔdhaK ΔderK ΔglpK cells were pre-cultivated overnight on ΔP medium. Then, 1 mL of pre-culture was inoculated into 100-mL YPGD medium. The OD$_{600}$ (solid lines in a) and pH values (dashed lines in a) and glycerol (open symbols in b) and DHA (closed symbols in b) concentrations in the medium were measured. The mean values and standard deviations were calculated from triplicate cultivations. Wild type (WT), black; ΔderK ΔglpK, purple; ΔdhaK ΔderK ΔglpK (ΔΔΔ), red

ΔglpK strains grew much worse on glucose–glycerol medium than the wild type, and the difference in pH change might reflect the difference in growth. The triple deletion strain showed worse growth than the double deletion strain, particularly in the late phase of growth (Fig. 4a). The two deletion

4b), which is consistent with the growth behavior (Fig. 4a). The double deletion strain assimilated a small amount of DHA and used it for cell growth, while the triple deletion strain did not. Since we failed to detect DHA kinase activity in the cell extract of the $\Delta derK \ \Delta glpK$ strain (Table 2), a small enzyme activity less than a detection limit might be involved in the DHA consumption. These results suggest that DhaK plays a role in DHA metabolism in the late phase of growth, although no roles of $dhaK$ in DHA metabolism were found when the deletion derivatives were cultivated in glycerol medium.

Complementation of the triple gene deletion strain with DHAKs expressed from plasmids

We attempted to express three DHAKs to complement the growth defect of the triple deletion strain on glycerol. All transformants harboring the plasmids for DHAKs grew on glycerol, although the strain with $dhaK$ was slower than the other transformants (Fig. 5a). Importantly, cell extracts of these strains showed DHA kinase activity (Fig. 5b). Taken altogether, we suggest that all three DHAKs are involved in glycerol and DHA metabolism as the catalyst of ATP-dependent phosphorylation of DHA, albeit to different degrees.

Discussion

The *G. thailandicus* strain NBRC3255 regiospecifically oxidizes glycerol to produce DHA, but degrades DHA completely. In this study, we investigated DHA metabolism in *G. thailandicus* using reverse genetics and a biochemical approach. It was found that the three ATP-dependent DHA-phosphorylating enzymes are involved in its metabolism, indicating that the phosphorylation is a metabolically crucial step in the DHA degradation in *G. thailandicus* strain NBRC3255. It is also plausibly suggested that an upstream step of the DHA phosphorylation, i.e., DHA transport can be a committed step in the DHA metabolism. GlpK plays a central role in DHA metabolism because only the $\Delta glpK$ strain of the three single gene deletion derivatives delayed growth on glycerol and DHA consumption (Fig. 3), that is, the $\Delta dhaK$ and $\Delta derK$ strains grew as the wild type (Fig. S2). The combination of gene deletions of DHAK genes suggests that DerK is a secondarily important enzyme and that DhaK is involved in DHA metabolism only in the late phase of growth (Fig. 4). The triple gene deletion strain constructed in this study accumulated the highest levels of DHA with a ca. 100% yield when the cells were grown on glucose and glycerol.

Since the triple deletion strain cannot grow on glycerol, biotransformation with the growing triple deletion cells requires an additional carbon source. Alternatively, the use of resting cells can be a solution for this issue. The broad
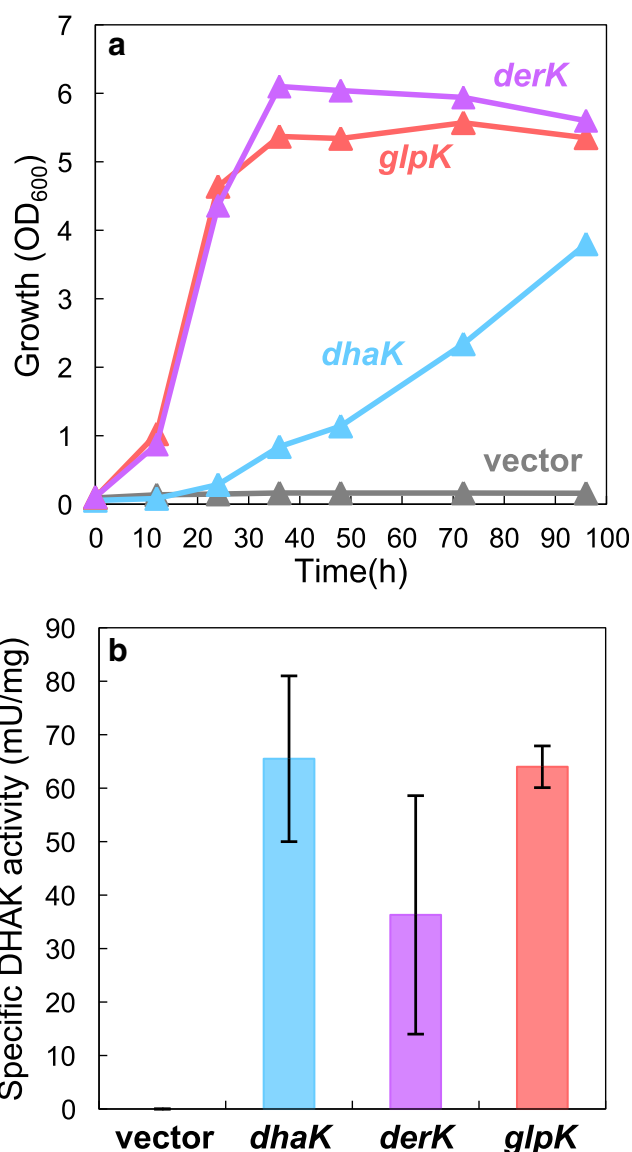
Fig. 5 The triple deletion mutant strain harboring the plasmid carrying *dhaK*, *derK*, or *glpK*. **a** Growth of the recombinant strains on the YPG medium. Each strain was pre-cultivated on ΔP medium overnight. The pre-culture was inoculated in YPG medium at a 1% (v/v) ratio. **b** DHA kinase activity in the soluble fraction of each strain was measured at pH 7.0. Mean values and standard deviations were calculated from triplicate enzyme assays. ΔΔΔ/pBBR1MCS-4 (vector), gray; ΔΔΔ/pEK20 (*dhaK*), sky blue; ΔΔΔ/pKH24 (*derK*), lilac; ΔΔΔ/pKH18 (*glpK*), salmon pink

substrate specificity of GlpK, in which the kinase phosphorylates both glycerol and DHA, would make it difficult to construct a *G. thailandicus* derivative that grows on glycerol, but accumulates high DHA. Previous studies have shown that GlpK in *E. coli* phosphorylates DHA, as well as glycerol (Hayashi and Lin 1967) and that the constitutive *glpK* derivative compensates for the growth defect of the *Klebsiella* mutant strain on DHA (Jin et al. 1982), indicating that the broad substrate specificity of the GlpK protein is widely found. To overcome this, the introduction of highly specific glycerol kinase into the triple deletion strain would help to construct

a suitable DHA producer. Some outstanding issues include the transport of DHA into the cell and the regulation of DHA metabolism. For one of the future directions, if the transport of DHA is independent of that of glycerol, identification and elimination of DHA transporter might construct a DHA producer that maintains the DHA levels. Understanding the mechanisms underlying these unaddressed processes could lead to the construction of a good strain for DHA production with *G. thailandicus*.

A construction of an inducible expression system in *G. thailandicus* might overcome the issue mentioned above. For instance, expression of a repressor protein that represses the expression of DHA kinase in the late growth phase would reduce the consumption of DHA. The recent report on the tunable, inducible expression system in *G. oxydans* (Fricke et al. 2020) encourages us to develop a similar expression system in *G. thailandicus* for the future work.

The DHA kinase purified from *G. thailandicus* NBRC 3172 (formerly *G. suboxydans* IFO 3172) does not phosphorylate glycerol (Tachiki et al. 1987), which suggests that this enzyme is different from GlpK because of its high substrate specificity. According to catalytic mechanism of *Citrobacter* DHAK (Siebold et al. 2003) and *E. coli* DhaK–DhaL complex (Shi et al. 2011), as indicated by the crystal structures, DhaK and DerK may phosphorylate DHA via a covalent enzyme-substrate intermediate: hemiaminal linkage between DHA and the crucial His residue of the enzymes. Because this covalent linkage is based on the keto group of DHA, the substrate specificity of DhaK and DerK would be narrower than that of GlpK, where glycerol is phosphorylated without a covalent intermediate (Hurley et al. 1993).

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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