Expression and Characterization of a Class III Alcohol Dehydrogenase Gene from *Gluconobacter frateurii* in the Presence of Methanol during Glyceric Acid Production from Glycerol

Shun Sato¹, Naoki Morita², Dai Kitamoto¹ and Hiroshi Habe¹*

¹ Research Institute for Innovation in Sustainable Chemistry, National Institute of Advanced Industrial Science and Technology (AIST) (Tsukuba Central 5-2, 1-1-1 Higashi, Tsukuba, Ibaraki 305-8565, JAPAN)
² Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST) (2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo 062-8517, JAPAN)

Abstract: Some acetic acid bacteria have been shown to produce large amounts of glyceric acid (GA) from glycerol, which is a by-product of biodiesel fuel (BDF) production. Previously, a *Gluconobacter* strain was found that produced decreased amounts of GA from glycerol in the presence of methanol, a major ingredient of raw glycerol derived from the BDF industry. Thus, a comparative transcriptome analysis of *Gluconobacter frateurii* NBRC103465 was performed to investigate changes in gene expression during GA production from glycerol in the presence of methanol. Cells grown with methanol showed upregulated expression of a class III alcohol dehydrogenase homolog (*adhC*<sub>Gf</sub>) and decreased GA production. *adhC*<sub>Gf</sub> was cloned and expressed heterologously in *Escherichia coli*, and the presence of an additional protein with an approximate molecular mass of 39 kDa in the cytosol of the recombinant *E. coli* cells was identified by SDS-PAGE. Activity measurements of the cytosol revealed that the translational product of *adhC*<sub>Gf</sub> exhibited formaldehyde dehydrogenase activity in the presence of nicotinamide adenine dinucleotide and glutathione. *Gluconobacter frateurii* cells grown in 1% methanol-containing glycerol were found to have fivefold higher formaldehyde dehydrogenase activity than cells grown without methanol, suggesting that *adhC*<sub>Gf</sub> in *G. frateurii* cells functions in the dissimilation of methanol-derived formaldehyde.

Key words: acetic acid bacteria, *adhC*, formaldehyde dehydrogenase, glyceric acid, *Gluconobacter frateurii*, methanol

1 Introduction

Biodiesel fuel (BDF) is a major renewable energy source and alternative to petroleum diesel. Its production is based on an ester exchange reaction between triacylglycerol and methanol under alkaline conditions, resulting in the formation of a crude glycerol waste (raw glycerol) containing methanol and alkali catalysts. Due to the considerable increase in BDF production worldwide over the last few years, surplus raw glycerol derived from BDF production has become an alternative feedstock for the production of chemicals and fuels¹. The quality of raw glycerol is rather low; large quantities of methanol, water, and alkali catalysts are present in the raw glycerol solution, and expensive refining processes to obtain the required quality of glycerol are necessary. Thus, most raw glycerol is wasted or used for thermal recovery rather than used as a feedstock for chemical production². As a result, technical developments for the use of raw glycerol as a feedstock for the production of value-added chemicals without refining are desired. Accordingly, attention should be paid to methanol, which is a major ingredient of raw glycerol.

Glyceric acid (GA) is a glycerol derivative found in some plants³,⁴ with a variety of reported biological activities⁵−⁷. We previously developed an oxidative method by fermentation for producing GA from glycerol using acetic acid bacteria⁸,⁹, and we demonstrated the application of GA for developing value-added chemicals such as surfactants¹⁰, bioplastics¹¹, and biologically active compounds¹². By optimizing our fermentation conditions and strains, the GA productivity reached 136 g/L after 6 days of fed-batch fer-
mentation using *Glucanobacter frateurii* NBRC103465. In another report, we investigated the feasibility of using raw glycerol derived from the BDF and oleochemical industries in GA production by *Glucanobacter* sp. NBRC3259\(^\text{[2]}\). Unfortunately, this strain produced little GA from raw glycerol containing methanol. We concluded that methanol was the major reason for the decrease in GA production from raw glycerol. Thus, investigating the correlations between methanol and GA production in acetic acid bacteria is of great importance. However, no studies have examined of methanol. This is the first report on the identification and functional analysis of an newly identified class III alcohol dehydrogenase in bacteria, and a plausible mechanism for methanol dissimilation in *G. frateurii* NBRC103465, the best GA producer, in fed-batch fermentation using a synthetic alkaline solution prepared according to the raw glycerol composition derived from BDF production (glycerol, 66 wt%; methanol, 30 wt%; NaOH, 1 M)\(^\text{[3]}\).

### 2.2 Microarray analysis

In order to investigate changes of gene expression profiles induced by the presence of methanol during the cultivation, DNA microarray analysis was performed. Total RNA was prepared from the cells cultured for 12 or 24 h in a 5-L jar-fermenter under the condition as described in the former section. In this cultivation, methanol was added to the culture broth along with the synthetic alkaline solution. As a control, the culture fed with a methanol-free version of the synthetic solution (glycerol, 66 wt%; NaOH, 1 M) was performed in parallel. Cells taken from the jar culture were treated with RNAProtect Bacteria Reagent (Qiagen, Hilden, Germany).

To design microarray probes, the draft genomic sequence of *G. frateurii* NBRC103465 was first analyzed (Genarist Inc., Yokohama, Japan; the method and genomic data from the sequence analysis are to be reported in a more specialized journal). The DNA microarray was then prepared on 12×135 K slides (Roche Diagnostics K. K., Tokyo, Japan). A set of five probes corresponding to each ORF was designed in one area comprising 135 K probes. RNA extraction, cDNA preparation, Cy3 labeling, hybridization, and quantification of the scanned slide images were performed by Hokkaido System Science Ltd. (Sapporo, Japan). Genes showing a fold change of >1.5 or <0.67 were identified as upregulated or downregulated, respectively. The presented values for each gene were based on six independent experiments.

### 2.3 Analysis of the culture broth

The glycerol, GA, dihydroxyacetone (DHA), and methanol concentrations in the culture broth were determined using high-performance liquid chromatography, as described previously\(^\text{[2]}\). Briefly, samples were prepared at the appropriate dilution from culture broth by centrifugation and membrane filtration (0.45 μm). A 20-μL portion of the sample was applied to a SUGAR SC1011 column (Showa Denko, Tokyo, Japan) maintained at 80°C with double-distilled water as the mobile phase at 1 mL/min for glycerol and DHA analysis, and to a SUGAR SH1011 column (Showa Denko) maintained at 60°C with 5 mM H₂SO₄ as the mobile phase at 1 mL/min for GA and methanol analysis. The effluents were monitored with a refractive index detector.

Bacterial growth was evaluated by optical density measurements at 600 nm using a V-530 UV/VIS spectrophotom-
eter (Jasco Corp., Tokyo, Japan).

2.4 Gene cloning and heterologous expression of \textit{adhC}\textsubscript{Gf}

An \textit{adhC} homolog was amplified by polymerase chain reaction (PCR) using genomic DNA from \textit{G. frateurii} NBRC103465 as template. The primers used to amplify the coding sequences were as follows: forward direction, 5′-GTTTTTTTGGCTAGCAGGGAATTCAAAATGAAATCACGTCGGCTGGC-3′, and reverse direction, 5′-CAGCTCTAGGGATCCTCAGTGGAGTTTTTTGGGCTAGCAGGAGGAATTCAAAATGAAATC-3′. The underlined sequences indicate the EcoRI site in the N-terminal primer and BamHI site in the C-terminal primer. Using the same restriction sites, the amplified DNA fragments were cloned into pBBad22T\textsuperscript{14} linearized by EcoRI, yielding the expression plasmid pBADHC. The cloned DNA sequence was confirmed to be identical to that of the genomic DNA.

An amino acid sequence alignment between the deduced sequence of \textit{adhC}\textsubscript{Gf} and other class III alcohol dehydrogenases was performed on the DDBJ server with the ClustalW algorithm (ver. 2.1).

Recombinant \textit{E. coli} DH5\textalpha harboring pBADHC or pBBad22T\textsuperscript{-} (negative control) was cultivated at 30°C for 20 h. LB medium plus 0.1 wt% l-arabinose was used to induce the expression of \textit{adhC} under \textit{pBAD} promoter regulation. After cultivation, cells collected by centrifugation were suspended in chilled 0.1 M sodium-phosphate buffer (pH 8.0), followed by sonication to prepare a crude lysate. The lysate was centrifuged, and the supernatant was used for enzyme assays and determination of the protein concentration.

2.5 Enzyme assays

The protein concentration in each cell lysate supernatant was determined by the Bradford method using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% gels for separation; the protein bands were visualized by staining with Coomassie brilliant blue.

GD-FALDH activity was measured at 25°C using a spectrophotometer (model 1700; Shimadzu, Otsu, Japan) to follow the absorbance changes in the reaction mixture at 340 nm due to NADH formation, as reported previously\textsuperscript{15}. One unit of activity was defined as the amount of protein resulting in the formation of 1 \textmu mol of NADH in 1 min. NADH formation was estimated based on a molar absorption efficiency of 6220 M\textsuperscript{-1} cm\textsuperscript{-1}.

2.6 Sequence accession number

The sequence data for \textit{adhC}\textsubscript{Gf} and other genes found in the transcriptome analysis have been deposited in the DDBJ database. The accession codes are shown in Table 2.

3 Results and discussion

3.1 Transcriptome analysis of \textit{G. frateurii} cultivated by fed-batch fermentation with methanol supplementation

Our previous report revealed that the presence of methanol in raw glycerol used for GA fermentation by \textit{Gluconobacter} sp. NBRC3259 decreased GA production\textsuperscript{33}. To understand the changes in gene expression involved in the decrease in GA production caused by methanol, a comparative transcriptome analysis of \textit{G. frateurii} during GA production from glycerol with or without methanol supplementation was performed. Fed-batch fermentation in a 5-L jar fermenter was conducted using a synthetic solution containing 66% glycerol and 30% methanol in 1 M NaOH as a feeding solution, which was modeled after raw glycerol\textsuperscript{33}. Culture broth was taken from the jar after 12 or 24 h of cultivation for product analysis and total RNA preparation. As a control, the same fermentation process using a methanol-free version of the feeding solution (66% glycerol in 1 M NaOH) was performed. As shown in Table 1, the cell growth profiles were almost the same regardless of the presence of methanol between the 12- and 24-h cultures. In contrast, the concentrations of GA and DHA, a by-product of GA fermentation, in the cultures began to change between 12 and 24 h of cultivation. The methanol concentrations accumulated in the culture broth after 12 and 24 h were 2.9 and 12.4 g/L, respectively. We roughly estimated the amount of methanol fed to the culture broth based on the consumption of the synthetic alkaline solution during the cultivation: 5.4 and 15.0 g/L of methanol for 12 and 24 h, respectively. During the culture period from 12 to 24 h, most of the supplemented methanol (9.6 g/L) still remained in the culture broth (9.5 g/L), although the strain grew well at this period as judged by OD\textsubscript{600} values (Table 1). This suggests a poor ability of \textit{G. frateurii} to assimilate methanol. A little difference between the amounts of methanol supplemented to and residual in the culture broth (2.5 and 2.6 g/L for 12 and 24 h, respectively) may also arise from evaporation of methanol from the culture broth.

cDNAs were prepared from the extracted RNA harvested from cells cultured for 12 or 24 h, and transcriptome analyses were performed using DNA microarrays. Table 2 summarizes the upregulated genes (i.e., those showing a fold change of >1.5) in \textit{G. frateurii} cells cultured for 12 h with methanol supplementation. Several genes related to amino acid metabolism (\textit{gloA}, \textit{metE}, and \textit{lysA}), inorganic ion transport and metabolism (\textit{cirA} and \textit{fepC}), coenzyme metabolism (\textit{cohN}), and energy metabolism (\textit{adhC}), were upregulated. In cells cultured for 24 h, over 140 genes were upregulated including ribosomal proteins and chaperones, probably as a stress response to the presence of methanol in the culture broth (data not shown). In contrast, no significantly downregulated genes (i.e., showing a fold change of <0.67) were found in cells cultured for 12 or 24 h.
Moreover, no genes related to GA or DHA production were found. This suggests that methanol altered the product selectivity without significantly influencing the expression of genes encoding membrane-bound alcohol dehydrogenase and membrane-bound glycerol dehydrogenase, which are related to GA or DHA production, in G. frateurii.

An adhC homolog encoding a putative class III alcohol dehydrogenase was upregulated in cells cultured for 12 h (1.99 fold; Table 2). Because this gene product may be involved in methanol dissimilation by oxidizing formaldehyde, we searched for changes in the expression levels of a series of genes involved in methanol dissimilation; however, no candidate genes other than the adhC homolog were identified.

### 3.2 Functional analysis of adhC<sub>fr</sub>

Transcriptome analysis revealed the upregulation of adhC<sub>fr</sub>, which encodes a putative class III alcohol dehydrogenase capable of formaldehyde oxidation in the presence of NAD and glutathione. In the enzymatic reaction shown in Fig. 1, formaldehyde reacts with glutathione spontaneously to form S-hydroxymethylglutathione. Next, class III alcohol dehydrogenase catalyzes oxidation of the primary hydroxy group, resulting in the formation of S-formylglutathione. This class of enzyme has been well characterized in humans<sup>16</sup>, rats<sup>17</sup>, yeasts<sup>18</sup>, and bacteria such as E. coli<sup>15</sup> and is known to be involved in formaldehyde dissimilation. However, no homologous enzymes from acetic acid bacteria have been characterized. To investigate the function of

### Table 2

<table>
<thead>
<tr>
<th>Accession code</th>
<th>Gene</th>
<th>Gene products</th>
<th>Fold changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB778925</td>
<td>gloA</td>
<td>Putative glyoxalase</td>
<td>2.65 ± 0.91</td>
</tr>
<tr>
<td>AB778926</td>
<td></td>
<td>Hypotectila protein GOX2205</td>
<td>2.47 ± 0.83</td>
</tr>
<tr>
<td>AB778927</td>
<td></td>
<td>Predicted metal-binding protein</td>
<td>2.43 ± 0.84</td>
</tr>
<tr>
<td>AB778928</td>
<td>metE</td>
<td>5-Methyltetrahydropteroylglutamate – homocysteine S-methyltransferase</td>
<td>2.39 ± 0.93</td>
</tr>
<tr>
<td>AB7789929</td>
<td></td>
<td>Putative GTPase (G3E family)</td>
<td>2.31 ± 0.76</td>
</tr>
<tr>
<td>AB778930</td>
<td>gloA</td>
<td>Putative glyoxalase</td>
<td>2.26 ± 0.78</td>
</tr>
<tr>
<td>AB778931</td>
<td>cirA</td>
<td>Outer membrane receptor proteins, mostly Fe transport</td>
<td>1.99 ± 0.60</td>
</tr>
<tr>
<td>AB735534</td>
<td>adhC</td>
<td>Zn-dependent alcohol dehydrogenase, class III</td>
<td>1.99 ± 0.50</td>
</tr>
<tr>
<td>AB778932</td>
<td></td>
<td>Hypothetical protein</td>
<td>1.88 ± 0.58</td>
</tr>
<tr>
<td>AB778933</td>
<td>gloA</td>
<td>Putative glyoxalase</td>
<td>1.87 ± 0.66</td>
</tr>
<tr>
<td>AB778934</td>
<td>gloB</td>
<td>Zn-dependent hydrolase, including glyoxylases</td>
<td>1.82 ± 0.64</td>
</tr>
<tr>
<td>AB778935</td>
<td>lysA</td>
<td>Ornithine decarboxylase</td>
<td>1.66 ± 0.70</td>
</tr>
<tr>
<td>AB778936</td>
<td>fepC</td>
<td>ABC-type cobalamin/Fe&lt;sup&gt;3+&lt;/sup&gt;-siderophores transport systems, ATPase components</td>
<td>1.62 ± 0.63</td>
</tr>
<tr>
<td>AB778937</td>
<td></td>
<td>Hypothetical protein</td>
<td>1.57 ± 0.35</td>
</tr>
<tr>
<td>AB778938</td>
<td>cobN</td>
<td>Cobalamin biosynthesis protein CobN and related Mg-chelatases</td>
<td>1.54 ± 0.38</td>
</tr>
</tbody>
</table>

### Table 1

<table>
<thead>
<tr>
<th>Cultivation time (h)</th>
<th>OD600</th>
<th>Glycerol (g/L)</th>
<th>Glyceric acid (g/L)</th>
<th>Dihydroxyacetone (g/L)</th>
<th>Methanol (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With methanol supplementation</td>
<td></td>
<td>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>0.17 ± 0.04</td>
<td>164 ± 23</td>
<td>ND&lt;sup&gt;e&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>2.15 ± 0.44</td>
<td>183 ± 13</td>
<td>2.3 ± 0.3</td>
<td>7.7 ± 0.6</td>
<td>2.9 ± 0.4</td>
</tr>
<tr>
<td>24</td>
<td>5.66 ± 0.44</td>
<td>168 ± 13</td>
<td>4.5 ± 0.3</td>
<td>27.9 ± 1.9</td>
<td>12.4 ± 2.5</td>
</tr>
<tr>
<td>Without methanol supplementation</td>
<td></td>
<td>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>0.15 ± 0.03</td>
<td>167 ± 27</td>
<td>ND</td>
<td>ND</td>
<td>−&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>2.21 ± 0.20</td>
<td>187 ± 15</td>
<td>3.2 ± 0.4</td>
<td>8.0 ± 0.9</td>
<td>−</td>
</tr>
<tr>
<td>24</td>
<td>5.45 ± 0.31</td>
<td>215 ± 14</td>
<td>18.5 ± 1.6</td>
<td>18.5 ± 1.5</td>
<td>−</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cells were cultivated in a 5L-jar fermenter containing 2.5 L of glycerol medium. Data were averages and standard deviations from six independent experiments. <sup>b</sup>ND, not deteted. <sup>c</sup>−, not tested.
adhlC, a sequence alignment was performed with ClustalW using the deduced amino acid sequence of adhlC and that of known class III alcohol dehydrogenases from E. coli, Saccharomyces cerevisiae, rats, and humans. As shown in Fig. 2, the ligands to the catalytic Zn at Cys-40, His-62, and Cys-169, and to the non-catalytic Zn at Cys-92, -95, -98, and -106 in ADHC are strictly conserved in all class III alcohol dehydrogenases. The residues for substrate contact points at Asp-51, Tyr-88, and Arg-110 in ADHC are also conserved. These results suggest that the translated product of adhlC in G. frateurii is an enzyme capable of catalyzing formaldehyde dehydrogenation in the presence of NAD and glutathione. Note that residue 111 in ADHC (Glu) is less conserved, except in E. coli FrmA, although this position was also considered to be important for constructing the substrate-binding pocket in class III alcohol dehydrogenases. At this position, human ADH3 and rat ADH5 contain valine, S. cerevisiae SFA1 and rice FDH1 contain alanine, and Arabidopsis thaliana FDH1 contains serine, suggesting variation in the amino acid at this position in class III alcohol dehydrogenases.

Next, cloned adhlC was expressed heterologously in recombinant E. coli cells, and the supernatant after centrifugation of a crude lysate prepared by sonication was subjected to enzyme assays. As shown in Fig. 3A, SDS-PAGE of the soluble fraction of cells harboring adhlC showed a 39-kDa band not present in cells harboring the control vector. The molecular mass calculated from the deduced amino acid sequence of adhlC was 39.1 kDa, indicating successful expression of adhlC in E. coli. In addition, the GD-FALDH activity of the soluble fraction from cells harboring adhlC showed tenfold higher GD-FALDH activity than control cells (Fig. 3B). This result indicates that the cloned adhlC gene encodes a class III alcohol dehydrogenase that functions in formaldehyde oxidation in a NAD- and glutathione-dependent manner.

Class III alcohol dehydrogenase from E. coli and some methylotrophic yeasts was previously reported to show higher Km values toward the substrate S-hydroxymethylglutathione compared to that from the non-methylotrophic yeast S. cerevisiae and mammalian cells for adaptation to

![Diagram of formaldehyde dehydrogenation catalyzed by class III alcohol dehydrogenase](image)

**Fig. 1** Dehydrogenation of formaldehyde catalyzed by class III alcohol dehydrogenase in the presence of glutathione and NAD.

**Fig. 2** Amino acid sequence alignment of the deduced amino acid sequence of adhlC (Gf-ADHC) and class III alcohol dehydrogenases from E. coli (Ec-FrmA), S. cerevisiae (Sc-SFA1), rats (Rat-ADH5), and humans (Human-ADH3). The GenBank accession numbers are P25437 for E. coli, P32771 for S. cerevisiae, P12711 for rats, and M30471 for humans. Critical amino acids that are part of the substrate or coenzyme binding domain are shown in bold. Conserved amino acid residues across the sequences are indicated by *.
the higher formaldehyde concentrations present in their habitats\textsuperscript{53}. Considering the high sequence identity (86\%) between ADHC\textsubscript{Gf} and \textit{E. coli} FrmA, ADHC\textsubscript{Gf} might show low affinity toward \textit{S}-hydroxymethylglutathione and \textit{G. frateurii} could be comparable in terms of its tolerance to formaldehyde with \textit{E. coli}.

### 3.3 Involvement of \textit{adhC\textsubscript{Gf}} in \textit{G. frateurii} methanol dissimilation

We then evaluated GD-FALDH activity in the soluble fraction of \textit{G. frateurii} grown in 170 g/L glycerol with methanol. The soluble fraction of cells grown for 24 h with 1 vol\% methanol showed fivefold higher GD-FALDH activity than cells grown with 0 or 0.5 vol\% methanol (Fig. 4), although protein bands corresponding to ADHC\textsubscript{Gf} were not identified by SDS-PAGE in soluble fractions from \textit{G. frateurii} cells grown with 1 vol\% methanol. This indicates that 1 vol\% methanol induced GD-FALDH activity in \textit{G. frateurii} cells and that this enzyme is involved in the dissimilation of methanol-derived formaldehyde. A previous study reported that GD-FALDH activity in non-methylotrophic \textit{E. coli} was induced by exogenous formaldehyde but not methanol\textsuperscript{52}. Therefore, formaldehyde formation may be necessary to induce GD-FALDH activity in \textit{G. frateurii}.

Considering the weak activity of membrane-bound alcohol dehydrogenase from \textit{Gluconobacter oxydans} toward methanol\textsuperscript{54}, other enzymes homologous to intracellular alcohol dehydrogenase and catalase, some of which were found in the draft genome sequence (data not shown), might also contribute to formaldehyde formation. Our transcriptome analysis, however, indicated that their expression levels in \textit{G. frateurii} cells grown in the presence of methanol did not change significantly. Note that GD-FALDH was reported to be involved in DHA dissimilation in \textit{S. cerevisiae}\textsuperscript{23}. Considering that no significant differences in DHA concentration were observed between our 12-h cultures by jar fermentation regardless of methanol supplementation (Table 1), the expression of \textit{adhC} in \textit{G. frateurii} would be induced not by DHA, but methanol-derived formaldehyde, and could contribute to DHA production in methanol-supplemented culture.

Because GD-FALDH activity was observed in \textit{G. frateurii} cells grown in glycerol with methanol (Fig. 4), \textit{S}-formylglutathione, the product of the reaction catalyzed by GD-FALDH (Fig. 1), may be an intermediate of methanol dissimilation in \textit{G. frateurii}. In this plausible metabolic pathway, intracellular formaldehyde, which is converted from methanol by intracellular alcohol dehydrogenase or catalase, is spontaneously trapped by glutathione to form \textit{S}-hydroxymethylglutathione. This compound undergoes dehydrogenation by GD-FALDH to form \textit{S}-formylglutathione, which is then hydrolyzed to formic acid and glutathione by \textit{S}-formylglutathione hydrolase or formate dehydrogenase, the latter of which was present in the draft genome sequence as a homologous gene. Although formate dehydrogenase is known to catalyze the formation of carbon dioxide from formate, some formate dehydrogenases from methanol-utilizing yeasts exhibit a preference toward the hydrolysis of \textit{S}-formylglutathione\textsuperscript{21}. Formic acid is further converted to carbon dioxide or methylation reactants, including 10-formyltetrahydrofolate, which acts as a formyl donor in nucleic acid and protein biosynthesis. Expression of the genes encoding these enzymes, other than GD-
FALDH, was not altered significantly in the presence of methanol. Therefore, engineering of this plausible pathway for methanol dissimilation by disruption or recombination of *adhC* and other candidate genes will enable *G. frateurii* to produce GA efficiently in the presence of methanol. Further investigation on the genomic sequence of *G. frateurii*, which is currently underway, will also be necessary to study how methanol dissimilation by *G. frateurii* can be enhanced to increase GA production from raw glycerol.

4 Conclusions

Our comparative transcriptome analysis revealed the up-regulated expression of an *adhC* homolog in *G. frateurii* cells exposed to methanol during GA fermentation. SDS-PAGE and enzymatic activity assays of the soluble fraction from recombinant *E. coli* harboring *adhC* revealed that *adhC* encodes a GD-FALDH. In addition, GD-FALDH activity was confirmed in *G. frateurii* cells grown in the presence of methanol. No significant changes in the expression levels of genes related to GA and DHA production were detected. Since *adhC* promoted the dissimilation of formaldehyde derived from methanol in *G. frateurii*, enhanced *adhC* function in recombinant *G. frateurii* and other acetic acid bacteria may enable the use of impure raw glycerol for the production of glycerol-derived chemicals, including GA.

Acknowledgments

The authors thank Mikie Daigo for her technical assistance. This work was financially supported in part by the Japan-U.S. Cooperation Project for Research and Standardization of Clean Energy Technologies from the Ministry of Economy, Trade, and Industry of Japan.

References


