Graduate School of Sciences and Technology for Innovation

Yamaguchi University

Physiological role and catalytic mechanism of membrane-bound aldehyde dehydrogenase in acetic acid fermentation by *Gluconacetobacter* sp.

（*Gluconacetobacter* 属酢酸菌が行う酢酸発酵における膜結合型アルデヒド脱水素酵素の生理学的役割と触媒機構）

A DISSERTATION

Submitted by

Roni Miah

in Partial Fulfillment of the Requirement for the Degree of

DOCTOR OF LIFE SCIENCE

March 2022

DECLARATION

I hereby declare that the performed experiment reported in this research project were undersigned. I also declare the appropriate acknowledgment to the source of information of other workers results as reference.

March, 2022 Yamaguchi University

Author

Roni Miah

PREFACE

Our laboratory of Applied microbiology has a very long history on acetic acid bacteria research. From that perspective, this dissertation contributed to the understanding of the role, structure and molecular mechanism of membrane bound aldehyde dehydrogenase (ALDH) in acetic acid fermentation process of *Gluconacetobacter* sp.. It discusses the essential role of ALDH in acetic acid fermentation and ensure its prosthetic group (Chapter 1) by reverse genetic study that was a long debating topic between the researchers group and Chapter 2 delineate the molecular mechanism (intramolecular electron transport) of ALDH upon acetaldehyde oxidation through classical biochemical and genetic engineering study in *Ga. diazotrophicus* PAL5.

The first study was performed to understand the role of membrane bound ALDH in acetic acid fermentation and detects its prosthetic group by reverse genetic study because biochemical analysis might be influenced by contamination with other molecules. The experimental results clarify that there are three membrane bound ALDH enzymes (AldFGH, AldSLC and AdhAB) in *Ga. diazotrophicus* PAL5 where AldFGH is the major player among them and have an essential role in efficient acetic acid fermentation and most importantly dependent on a form of molybdopterin species but not pyrroloquinoline quinone.

The second study was performed for understanding the intramolecular electron transport in AldFGH complex through classical biochemical approach and genetic engineering for functional analysis of each subunit. The intact complex of AldFGH having the ubiquinone reduction activity upon acetaldehyde oxidation was purified successfully and genetically dissected AldFGH complex helped to assess function at the subunit level and to determine the mechanism of the enzyme complex.

This dissertation is organized in three main sections. The first section is general introduction whereby I described fundamental understanding about acetic acid bacteria specially *Gluconacetobacter diazotrophicus* PAL5 and expressed rationale of carrying out this research. The two following sections are corresponding with two studies respectively.

I believe this research would be interesting for all readers who are interested in bacteriology, biochemistry, genetics as well as molecular biology.

ACKNOWLEDGEMENTS

This research was conducted in the Laboratory of Applied Microbiology, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan since I was a research student until nearly finishing my PhD course between September 2018 and March 2022.

First and foremost, praise and thanks to the Almighty Allah, for His showers of blessing and giving me the strength in every step of life and throughout my work to complete the research successfully. One of the joys of completion is to look over the journey past and remember all the peoples who have supported me along this long but fulfilling road.

I pay esteem and heartfelt thanks to my mentor Prof. Dr. Toshiharu Yakushi who supervized me throughout my PhD student life with his scholastic guidance, endless inspiration, constructive criticism, endearing company, never failing interest and a great deal of patience were invaluable for this works. I acknowledge that my background knowledge in research was very poor but fortunately his kind supervision helped me to acquire improvement. He guided me to acquire knowledge on technique regarding to biochemistry, microbiology, molecular biology as well as making a good presentation, writing a scientific paper and also many of life lessons, throughout his supervision. It is my great honor to be his student. He is patience, hard working and kindly tought me since my research student life in Yamaguchi University. After three and a half years being his student, I feel confident to access to wherever I will work in the future. Once again, I would like to say thank you from the bottom of my heart for all of things that you did during my PhD student life.

I would like to express my special thanks to Prof. Dr. Kazunobu Matsushita who is a kind hearted, enthusiastic, expertise in acetic acid bacteria research and during my study, his advanced comments greatly contributed to my research.

I am deeply indebted to Prof. Dr. Osao Adachi who behaved me like a friend and provided careful suggesstions to found out an easy way when I had trouble in my experiments. His warmly care helped me to overcome all difficult day of missing my home and family.

I am deeply thankful to Dr. Naoya Kataoka who patiently gave good suggesstions during my molecular study and helped in some HPLC experiments. During my study, his comments and advices contributed to make my research much better.

I am deeply grateful to Prof. Dr. Mamoru Yamada and Dr. Tomoyuki Kosaka for all advanced comments and helpful advices for my research.

I would like to express my sincere thanks to Dr. Minenosuke Matsutani and Dr. Nami Matsumoto who responses for excellent bioinformatic analysis support. Moreover, they always kindly help and teach me whenever I faced problems.

I am deeply indebted to Ms. Miyaji, Ms. Okamoto, Ms. Takeshita. They helped to prepare all of my documents not only in school but also in my japan living time. My life in Japan was smooth, wonderful and all troubles were solved through their professional work.

I would like to acknowledge all Obi laboratory members for their kind assistance in solving numerous problems during the research work. Specially, I am thankful to Takeru Murate, my tutor who helped me for the first time to start experiment in laboratory. We have a happy moment together and thank you for all things.

I am deeply thankful to the Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) of Japan for financial support and giving me an opportunity to study and research in Yamaguchi University during my PhD's life.

I am deeply indebted to Yamaguchi University's staffs and teachers for their kind support and dedicated teaching to me. One of wonderful university that I had an opportunity to study.

I am deeply grateful to all of my Bangladeshi and international friends in Yamaguchi University who always supported me, gave advices and helped me during my student life.

Alhamdulillah (praise to the God-Allah), I would like to express the deepest gratitude to my parents, wife (Sunjida Tabassum) and all of my family members who tirelessly supported me and encouraged me to overcome difficulties to obtain the highest achievements throughout my study journey.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AAB	Acetic acid bacteria
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
PQQ	Pyrroloquinoline quinone
Km	Kanamycin
Tc	Tetracycline
Ap	Ampicillin
Gm	Gentamycin
Suc	Sucrose
MCD	Molybdopterin cytosine dinucleotide
Q1	2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4 benzoquinone
Q_2	2,3-dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone
FC^{3-}	Ferricyanide
DM	*n*-dodecyl-β-D maltoside
KPB	Potassium phosphate buffer
DCIP	Dichlorophenolindophenol
PMS	Phenazinemethosulfate
rpm	Round per minute

CHAPTER ONE

GENERAL INTRODUCTION

1.1. Acetic acid bacteria

Acetic acid bacteria (AAB), first described as "vinegar bacteria" by Louis Pasteur over 150 years ago, are an important and diverse group of bacteria involved in the production of fermented foods and beverages. The species of AAB are well known to have a high capability to oxidize alcohols, aldehydes, sugars or sugar alcohols in the presence of oxygen. As a result of these oxidative activities, the corresponding oxidation products accumulate in the culture medium. These unique oxidative fermentation of AAB is carried out by membrane-bound dehydrogenases located in the outer surface of cytoplasmic membrane or in the periplasmic space. The electrons generated by the action of these dehydrogenases are transferred to ubiquinone in the membrane. The reducing equivalents are then further transferred to the terminal ubiquinol oxidase in the cytoplasmic membranes. Thus, the organisms generate bioenergy through the dehydrogenases enzyme activities (without exception). AAB are classified in 17 genera, of which many species have been reported in the genera *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, and *Komagataeibacter*. Other genera are *Acidomonas*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia*, *Ameyamaea*, *Endobacter*, *Nguyeni- bacter*, *Swingsia*, *Neokomagataea* and *Gammaproteobacteria* (Adachi et al., 2007 and Matsushita et al., 1994, 2016).

1.2. Acetic acid fermentation

Acetic acid fermentation is the oxidation of ethanol by acetic acid bacteria. This is a process involving successive oxidation reactions catalyzed by membrane-bound enzymes: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Matsushita et al., 1994). ADH bound to cytoplasmic membrane surfaces, catalyzes the first reaction of acetic acid fermentation by oxidizing ethanol to acetaldehyde, and this is coupled with the reduction of ubiquinone in the membrane. The resultant acetaldehyde is further oxidized to acetic acid by the membrane-bound ALDH. The electrons generated by the action of these ADH and ALDH are transferred to ubiquinone in the membrane. The reducing equivalents are then further transferred to the terminal ubiquinol oxidase in the cytoplasmic membranes and thus the organisms generate bioenergy.

1.3. Alcohol dehydrogenase

Alcohol dehydrogenase (ADH) catalyzes the oxidation of ethanol to acetaldehyde, coupled with the reduction of ubiquinone in the cytoplasmic membrane in acetic acid fermentation (Yakushi et al., 2010). ADHs of different species of acetic acid bacteria differ in subunit composition. ADHs of *Acetobacter* spp. and *Gluconobacter* spp. are composed of three subunits—AdhA, AdhB, and AdhS, whereas those of *Komagataeibacter* spp. and *Gluconacetobacter* spp. are composed of two subunits—AdhA and AdhB (Yakushi et al., 2010). The AdhA and AdhB subunits are a quinohemoprotein subunit containing pyrroloquinoline quinone (PQQ) and one *c*-type cytochrome center as the prosthetic groups, and a *c*-type cytochrome subunit containing three hemes, respectively (Yakushi et al., 2010; Matsushita et al., 1996). AdhB is the site of ubiquinone reduction (Matsushita et al., 1996). AdhS has no characteristic cofactors but the several reports suggest the function as a molecular chaperone that assists maturation of the AdhA subunit (Kondo et al., 1995; Masud et al., 2010).

1.4. Aldehyde dehydrogenase

Aldehyde dehydrogenase (ALDH) catalyzes the oxidation of acetaldehyde to produce acetic acid. Physiological role and catalytic mechanism of ALDH in acetic acid fermentation have not been studied for a long time. Recently, we reported that there are two molecular species of ALDH, AldFGH and AldSLC in both *Acetobacter pasteurianus* strain SKU1108 (Yakushi et al., 2018) and *Gluconacetobacter diazotrophicus* strain PAL5 (Miah et al., 2021). Amino acid identities between AldH and AldL, AldF and AldC, and AldG and AldS of the PAL5 strain are 24%, 32%, and 59%, respectively. AldFGH is physiologically important for acetic acid fermentation (Yakushi et al., 2018; Miah et al., 2021). According to the predicted amino acid sequences, the AldH protein is an 80-kDa molybdopterin-containing subunit that is the site of oxidation of the substrate (Huber et al.,1996); AldF is a 45-kDa c-type cytochrome containing three hemes; and AldG is a 17-kDa protein possessing two binding motifs for [2Fe–2S] clusters (Thurner et al., 1997).

1.5. Ubiquinol oxidase

The respiratory chain of acetic acid bacteria branches into the cytochrome bo_3 ubiquinol oxidase and a cyanide-insensitive bypass oxidase at the ubiquinol site (Yakushi et al., 2010). During acetic acid fermentation process generated reducing equivalents are transferred to ubiquinone which is, in turn, re-oxidized by ubiquinol oxidase,

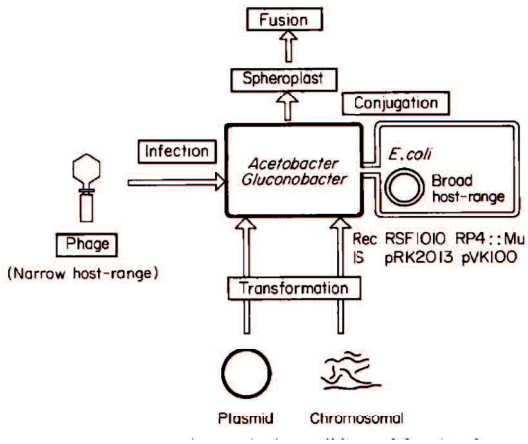
reducing oxygen to water and produced a proton motive force necessary for energy production through a membrane-bound ATPase in the cytoplasmic membranes and thus the organisms generate bioenergy.

1.6. *Gluconacetobacter diazotrophicus* PAL5

Gluconacetobacter diazotrophicus (formerly *Acetobacter diazotrophicus*) is a strict aerobe and N_2-fixing endophyte originally isolated from sugarcane roots and stems (Cavalcante and Dobereiner 1988; Gillis et al. 1989; Yamada et al. 1997). This microorganism is a member of the acetic acid bacteria group and produces acetic acid from ethanol via two-step oxidation catalyzed by the membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the periplasmic space (Gómez-Manzo et al. 2010; Gómez-Manzo et al. 2008; Matsushita et al. 1994). An interesting property of acetic acid fermentation by *Ga. diazotrophicus* PAL5, which has been proposed in terms of ADH function, is that ADH oxidizes not only ethanol but also acetaldehyde. Gómez-Manzo et al. (2015) reported that ADH produces acetic acid from ethanol on its own in vitro, without the accumulation of acetaldehyde. In that case, even the *Ga. diazotrophicus* PAL5 mutant strain that is devoid of ALDH can produce acetic acid without the accumulation of acetaldehyde, unlike *A. pasteurianus* SKU1108 (Yakushi et al. 2018). Gómez- Manzo et al. (2010) also reported that the prosthetic group of ALDH of *Ga. diazotrophicus* strain PAL5 is pyrroloquinoline quinone (PQQ) through biochemical study but these data were inconsistent with one of our our previous genetic study (Takemura et al. 1994).

1.7. Genetic modification of acetic acid bacteria

Gene manipulation techniques, which may be used for *Acetobacter* and the related genus *Gluconobacter,* are summarized in the following image.



(Fukaya et al., 1992)

In most of the genetic modification systems of aceic acid bacteria, broad host-range vectors which are available among gram-negative bacteria are transferred to recipient by conjugation or electroporation from *Escherichia coli*. In the host-vector system a plasmid vector carrying genetic marker allows gene disruption or replacement by integration of a foreign gene into the chromosome through the insertional inactivation occurring as a result of homologous recombination between the chromosome DNA of acetic acid bacteria and the cloned gene of interest. Markerless gene disruption is considered as major experimental system used in reverse genetics studies to understand the molecular mechanisms underlying microbial physiology. Kostner et al., 2013 developed a *codA*-based marker-less deletion system for *Gluconobacter oxydans* by using the *codAB* genes of *E. coli* for the counter selection of a mutant strain with 5-fluorouracil and suggested its application to a wide variety of acetic acid bacteria, such as *Acetobacter pasteurianus* LMG1513 and *Ga.diazotrophicus* DSM5601 (synonym of PAL5). Schäfer et al. (Schäfer et al. 1994) equipped the mobilizable derivative pK18mob carrying the *sacB* gene, which confers sucrose sensitivity to gram-negative bacteria for the counter selection of a mutant strain in the presence of high concentrations (10.0%) of sucrose, which was reported applicable to the construction of gene deletion mutant in *Gluconobacter oxydans*, a species of acetic acid bacteria (Krajewski et al. 2010).

1.8. Objective

We aimed to develope a markerless gene disruption method for *Ga. diazotrophicus* PAL5, to produce a wide variety of multiple gene deletion mutant strains to understand whether ADH itself, without any contribution from ALDH, is sufficient for acetic acid fermentation in the PAL5 strain (Gómez-Manzo et al. 2015), to investigate whether ALDH is dependent on PQQ (Gómez-Manzo et al. 2010) or molybdopterin (Thurner et al. 1997). We also aimed to investigate the molecular mechanism (intramolecular electron transport) of ALDH upon acetaldehyde oxidation through classical biochemical and genetic engineering study in *Ga. diazotrophicus* PAL5.

CHAPTER TWO

Major aldehyde dehydrogenase AldFGH of *Gluconacetobacter diazotrophicus* is independent of pyrroloquinoline quinone but dependent on molybdopterin for acetic acid fermentation

ABSTRACT

Acetic acid fermentation involves the oxidation of ethanol to acetic acid via acetaldehyde as the intermediate and is catalyzed by the membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) of acetic acid bacteria. Although ADH depends on pyrroloquinoline quinone (PQQ), the prosthetic group associated with ALDH remains a matter of debate.

This study aimed to address the dependency of ALDH of *Gluconacetobacter diazotrophicus* strain PAL5 on PQQ and the physiological role of ALDH in acetic acid fermentation. We constructed deletion mutant strains for both the ALDH gene clusters of PAL5, *aldFGH* and *aldSLC*. In addition, the *adhAB* operon for ADH was eliminated, since it shows ALDH activity. The triple- deletion derivative Δ*aldFGH* Δ*aldSLC* Δ*adhAB* failed to show ALDH activity, which suggested that ALDH activity in PAL5 is derived from these three enzyme complexes. Since the single-gene cluster deletion derivative Δ*aldFGH* lost most ALDH activity, and accumulated much higher acetaldehyde than wild type under acetic acid fermentation conditions, we concluded that AldFGH functions as the major ALDH in PAL5. Furthermore, deletion of the PQQ biosynthesis gene cluster (*pqqABCDE*) abolished ADH activity completely, but did not affect ALDH activity. Instead, the molybdopterin biosynthesis gene deletion derivatives lost ALDH activity. Thus, we concluded that the AldFGH and AldSLC complexes of *Ga. diazotrophicus* PAL5 require a form of molybdopterin but not PQQ for ALDH activity.

2.1. INTRODUCTION

Gluconacetobacter diazotrophicus (formerly *Acetobacter diazotrophicus*) is a strict aerobe and an N_2-fixing endophyte originally isolated from sugarcane roots and stems (Cavalcante and Dobereiner 1988; Gillis et al. 1989; Yamada et al. 1997). This microorganism is a member of the acetic acid bacteria group and produces acetic acid from ethanol via two-step oxidation catalyzed by the membrane-bound alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in the periplasmic space (Gómez-Manzo et al. 2010; Gómez-Manzo et al. 2008; Matsushita et al. 1994). ADH bound to cytoplasmic membrane surfaces catalyzes the first reaction of acetic acid fermentation that oxidizes ethanol to acetaldehyde, and this is coupled with the reduction of ubiquinone in the membrane (Yakushi and Matsushita 2010). The resultant acetaldehyde is further oxidized to acetic acid by the membrane-bound ALDH, which is also coupled with ubiquinone reduction (Adachi and Yakushi 2016). The reducing equivalents of the reduced form of ubiquinone are linked to the respiratory chain in order to reduce molecular oxygen to water. ALDH is the key enzyme involved in acetic acid fermentation, since the accumulation of acetaldehyde due to the loss of ALDH represses the cell growth of *Acetobacter pasteurianus* SKU1108 (Yakushi et al. 2018).

Pyrroloquinoline quinone (PQQ) is the prosthetic group of ADH for ethanol oxidation, whereas whether PQQ is associated with ALDH for acetaldehyde oxidation remains a matter of debate. To the best of our knowledge, this was first reported by our group in 1981, where extracts from the purified ALDH of *Gluconobacter oxydans* (formerly *Gluconobacter suboxydans*) that were treated with 90% methanol had activated the apo-form of glucose dehydrogenase, and this form is dependent on exogenous PQQ (Ameyama et al. 1981; Matsushita et al. 1994). However, a genetic study revisited this issue, in which a mutant strain of *Acetobacter sp*. BPR2001 that had failed to produce PQQ retained ALDH activity but lost ADH activity. Therefore, the study had concluded that the prosthetic group of ALDH is not PQQ (Takemura et al. 1994). Later, Thurner et al. (1997) had determined the nucleotide sequence of the *aldFGH* genes that encode for ALDH and suggested that the ALDH prosthetic group is molybdopterin cytidine dinucleotide (MCD). This was suggested based on the homology in the amino acid sequence with other molybdopterin-dependent enzymes, such as isoquinoline oxidoreductase and xanthine dehydrogenase (Thurner et al. 1997). Nevertheless, a biochemical study reported that the prosthetic group of ALDH of *Gluconacetobacter diazotrophicus* strain PAL5 (also referred to as PA15 elsewhere) is PQQ (Gómez Manzo et al. 2010). The authors showed the presence of PQQ in the purified ALDH that was missing the smallest subunit from *Ga. diazotrophicus* by performing electron paramagnetic resonance spectroscopy to detect semiquinone radical of PQQ. Furthermore, a peak that corresponded to authentic PQQ was also detected by HPLC from the purified ALDH extract.

Since these data were inconsistent with our previous study (Takemura et al. 1994), we attempted to examine this issue using a reverse genetic approach to identify the ALDH prosthetic group.

In addition to the *aldFGH* gene cluster, we found that *aldSLC* is an additional gene cluster for ALDH in the genome of *Acetobacter pasteurianus* strain SKU1108, a species of acetic acid bacteria (Yakushi et al. 2018). The *Ga. diazotrophicus* PAL5 genome possesses homologous gene clusters to *aldFGH* and *aldSLC*, and the amino acid sequence identity between AldHs (the catalytic subunit of the AldFGH complex) of *A. pasteurianus* strain SKU1108 and of the PAL5 strain is 68%, and that between AldLs (the catalytic subunit of the AldSLC complex) of the two bacterial strains is 61% (Giongo et al. 2010). Therefore, the physiological significance of the two ALDHs in acetic acid fermentation was also investigated in this study.

Another interesting property of acetic acid fermentation by *Ga. diazotrophicus* PAL5, which has been proposed in terms of ADH function, is that ADH oxidizes not only ethanol but also acetaldehyde. Gómez-Manzo et al. (2015) reported that ADH produces acetic acid from ethanol on its own in vitro, without the accumulation of acetaldehyde. In that case, even the *Ga. diazotrophicus* PAL5 mutant strain that is devoid of ALDH can produce acetic acid without the accumulation of acetaldehyde, unlike *A. pasteurianus* SKU1108 (Yakushi et al. 2018).

Thus, we have three unanswered issues regarding the mechanisms of ALDH and ADH of *Ga. diazotrophicus* PAL5. To address these issues, we developed a markerless gene disruption method for *Ga. diazotrophicus* PAL5, to produce a wide variety of multiple gene deletion mutant strains. In the present study, we targeted the *aldFGH* and *aldSLC* gene clusters, the *adhAB* operon codes for ADH, the *pqqABCDE* gene cluster for PQQ biosynthesis, and *moaA* and *moeA* for two molybdopterin biosynthesis genes (Leimkühler et al. 1999; Rieder et al. 1998). We observed that the aldFGH gene products are the major ALDH for acetic acid fermentation in this microorganism, which is dependent on molybdopterin but independent of PQQ.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Acetaldehyde and pyrroloquinoline quinone (PQQ) were purchased from Fluka (St. Louis, MO, USA) and Wako (Osaka, Japan), respectively. Yeast extract and Hipolypepton were obtained from Oriental Yeast (Tokyo, Japan) and Nihon Pharmaceutical (Tokyo, Japan), respectively. Restriction endonucleases were obtained from Toyobo (Osaka, Japan). All other materials were purchased from an analytical grade commercial source.

2.2.2. Bacterial strains, plasmids, and growth conditions

The *Ga. diazotrophicus* strains and plasmids used in this study are listed in Table 1. *Gluconacetobacter diazotrophicus* strain PAL5 was obtained from ATCC (American Type Culture Collection) as the ATCC49037 strain. The PAL5 strain and its derivatives were used in this study. The suicide vector pK18mobSacB was provided by Dr. Alfred Pühler (Universität Bielefeld) and the vector was used for the purpose of markerless gene deletion. The *Ga. diazotrophicus* cells were routinely grown on DYGS medium (2 g glucose, 1.5 g Hipolypepton, 2 g yeast extract, 0.5 g KH2PO4, 0.5 g MgSO4·7H2O, 1.5 g sodium L-glutamate monohydrate per liter, pH 6). For acetic acid fermentation, *Ga. diazotrophicus* cells were aerobically pre-cultivated on YPGD (5 g yeast extract, 5 g Hipolypepton, 5 g glycerol, 5 g glucose per liter) at 30 °C overnight. Then, 1 mL pre-culture was transferred into 100 mL of YPG medium (5 g yeast extract, 5 g Hipolypepton, 5 g glycerol, per liter) in a 500 mL Erlenmeyer flask, in which ethanol was supplemented at 20 mL L^{-1} . Acetic acid fermentation was carried out aerobically at 30 °C on a rotary shaker at 180 rpm. Kanamycin was used at a final concentration of 200 μg mL^{-1}. PQQ was sterilized by filtering through a 0.22-μm pore filter and used at a final concentration of 1 μM, when necessary.

Escherichia coli strains DH5α (Hanahan 1983) and HB101 (Boyer and Roulland-Dussoix 1969) were used for strain construction and triparental mating. The *E. coli* cells were grown on modified Luria-Bertani medium (5 g yeast extract, 10 g Hipolypepton, 5 g NaCl per liter, adjusted pH to 7 with NaOH). Kanamycin was used at a final concentration of 50μg/mL.

2.2.3. Plasmid construction

We constructed plasmids to eliminate four gene clusters, *adhAB* (Gdia_0262-0263) codes for the large and cytochrome subunits of PQQ-dependent ADH (Tamaki et al. 1991), *aldFGH* (Gdia_3086-3088) codes for three subunits of the membrane-bound ALDH (Thurner et al. 1997), *aldSLC* (Gdia_0014-0016) codes for three subunits of another membrane-bound ALDH (Yakushi et al. 2018), and *pqqABCDE* (Gdia_1982-1978) codes for the components of PQQ biosynthesis (Goosen et al. 1989), and two other genes, *moaA* (Gdia_0124) (Rieder et al. 1998) and *moeA* (Gdia_0146) (Leimkühler et al. 1999), code for molybdopterin biosynthesis components. The synthetic oligonucleotides used in this study are listed in Supplementary Table 2.S1. Genomic DNA of *Ga. diazotrophicus* PAL5 was isolated by the method of Marmur (Marmur 1961) with some modifications (Kawai et al. 2013).

For Δ*aldFGH* allele, a 5.8-kb DNA fragment containing the *aldFGH* gene cluster and its 5′ and 3′ flanking regions were amplified using Herculase II fusion DNA polymerase (Agilent Technologies, CA, USA), the genomic DNA of PAL5, and the D-aldF-Hin(+) and D-aldH-Xba(−) pair of oligonucleotides. The PCR product was digested with *EcoRI* and *XbaI* to yield a 1.0-kb DNA fragment that contained the 3′ flanking region. A 1.0-kb DNA fragment containing the 5′ flanking region was amplified with the D-aldF-Hin(+) and D- aldF-5-RI(−)

oligonucleotide pair and digested with *HindIII* and *EcoRI*. The two DNA fragments were inserted into the *HindIII* and *XbaI* sites of pKOS6b (Kostner et al., 2013) to yield pRM5. The 2.0 kb *HindIII* and *XbaI* DNA fragment containing the pMR5 Δ*aldFGH* allele was transferred to the corresponding site of pK18mobsacB (Schäfer et al., 1994) to yield pMRA. The suicide plasmids carrying Δ*aldSLC*, Δ*adhAB*, and Δ*pqqABCDE* were constructed using methods similar to those used to generate the pMRA (Fig. 2.S1).

The plasmids for Δ*moaA* and Δ*moeA* alleles were con- structed using a different method. Two PCR reactions were performed with the D-moaA-Hin(+) and D-moaA-fsn(−) pair of oligonucleotides for the 5′ flanking region of *moaA* and with the D-moaA-fsn(+) and D-moeA-Xba(−) pair of oligonucleotides for the 3′ flanking region. The two PCR products were connected and amplified by PCR, and the second PCR product was digested with *HindIII* and *XbaI* and inserted into the corresponding pK18mobsacB sites to yield pMRE. The plasmid for the Δ*moeA* allele was constructed using a similar procedure. All nucleotide sequences of the PCR cloning were confirmed by cycle sequencing techniques according to the manufacturer's instructions (Applied Biosystems, Waltham, MA, USA).

2.2.4. *Ga. diazotrophicus* transformation

The *Ga. diazotrophicus* PAL5 strain was transformed with pK18mobsacB derivatives via a triparental mating method using the *E. coli* HB101 strain harboring pRK2013 as the helper strain (Figurski and Helinski, 1979). Gentamycin (50 μg mL^{-1}) was added to the media to eliminate *E. coli* growth. Colonies of gentamycin- and kanamycin-resistant (200 μg mL^{-1}) conjugants were screened twice on DYGS agar for pK18mobSacB derivatives. Finally, the transconjugants were screened in liquid DYGS medium containing 50 μg mL^{-1} kanamycin.

2.2.5. Construction of deletion mutant strains

The *Ga.diazotrophicus* PAL5 strain was transformed with pMRA, pMRB, pMRC, pMRD, pMRE, and pMRF as described above, and the transformants were referred to as the first recombinants. The first recombinant cells were grown on DYGS without kanamycin at 30°C overnight and inoculated on DYGS + 10% sucrose agar after appropriate serial dilutions. Sucrose-resistant colonies were screened again on the same agar medium to examine for kanamycin sensitivity. Finally, the genomic DNA of Suc^R and Km^S strains was isolated and confirmed either the wild-type or deletion allele of *aldFGH, aldSLC, adhAB*, *pqqABCDE, moaA*, and *moeA*. One of the strains with the deletion allele was used as the gene deletion strain. By repeating the gene deletion procedures described above, we constructed double and triple deletion mutant strains.

2.2.6. Determination of acidity and acetaldehyde concentration

The cultures were withdrawn at the time indicated and centrifuged at 10,000 × g for 5 min to remove the cells. The acidity of the culture broth was determined by alkali titration with

NaOH, using phenolphthalein as the pH indicator, and the acidity was expressed as a percentage (w/v) equivalent to acetic acid. Acetaldehyde concentration was determined by an enzymatic method as described previously (Adachi et al., 1980), with the membrane fraction of our recombinant *A. pasteurianus* strain mNS3 (Δ*adhAB* Δ*aldSLC*) that harbored the pTK10 (*aldFGH*$^{+}$) plasmid, in which the genomic *adhAB* and *aldSLC* genes had been deleted but plasmid-born AldFGH were overproduced (SN et al., unpublished). The membrane fraction did not oxidize glucose, glycerol, or ethanol under our experimental setup (data not shown). An acetaldehyde calibration curve was prepared up to a concentration of 2.5mM. The detection limit of our determination system was 5 mM acetaldehyde in the medium.

2.2.7. Membrane preparation

Ga. diazotrophicus PAL5 and its derivative strains were pre-cultivated in a tube containing 2- mL YPGD medium at 30°C for 24 h, and 1-mL pre-culture was transferred to 100 mL YPG medium in a 500-mL flask and incubated at 30°C for 24 h. The cells were collected by centrifugation at 9,000 × g for 10 min at 4°C, washed with ice-cold 10 mM MES buffer containing 1 mM $CaCl_2$ (pH 6.0), and re-suspended in the same buffer supplemented with 0.5 mM phenylmethanesulfonyl fluoride (PMSF). The cell suspension was passed through a French pressure cell press (American Instrument Company, Silver Spring, MD, USA) at 1100 kg cm^{-2} twice. After the removal of intact cells and cell debris at 9,000 × g for 10 min at 4°C, the supernatant was further centrifuged at 100,000 × g for 1 h at 4°C. The precipitate was suspended in ice-chilled 10 mM MES buffer with 1 mM $CaCl_2$ (pH 6.0), which was used as the membrane fraction.

2.2.8. Enzyme assays

Ethanol or acetaldehyde:ferricyanide oxidoreductase activity was determined by the ferric dupanol assay at 25°C using 100 mM ethanol or acetaldehyde, respectively, as described previously (Adachi et al. 1980). McIlvain buffer (mixture of 100 mM citric acid and 200 mM Na_2HPO_4) was used, with a pH 5.0 for ADH and pH 4.0 for ALDH. Glucose dehydrogenase activity of *Ga. diazotrophicus* was determined spectrophotometrically at 522 nm using phenazine methosulfate (PMS) and dichlorophenol indophenol (DCPIP) as electron acceptors (Matsushita and Ameyama, 1982). The reaction mixture contained 0.12 mM DCPIP, 0.2 mM PMS, and 20 mM D-glucose in a total volume of 1 mL of 50 mM potassium phosphate buffer (pH 6.5). A molecular absorption coefficient of 8.6×10^3 was used for DCPIP (Armstrong, 1964). One unit of the enzyme was defined as the amount of enzyme that oxidizes 1 μmol of substrate per min. The protein content was determined by the modified Lowry method, and bovine serum albumin was used as the standard (Dulley and Grieve, 1975).

2.3. RESULTS

2.3.1. Marker-free gene deletion for *Ga. diazotrophicus* PAL5

Kostner et al., 2013 developed a *codA*-based marker-less deletion system for *Gluconobacter oxydans* by using the *codAB* genes of *E. coli* for the counter selection of a mutant strain with 5-fluorouracil and suggested its application to a wide variety of acetic acid bacteria, such as *Acetobacter pasteurianus* LMG1513 and *Ga. diazotrophicus* DSM5601 (synonym of PAL5). In this study, we first attempted to construct a *Ga. diazotrophicus* PAL5 gene deletion mutant strain by using the *codA* system (Kostner et al., 2013). We constructed the plasmid pMR5, a derivative of the pKOS6b suicide vector that carries the Δ*aldFGH* allele.

While screening the PAL5 strain transconjugants with the pKOS6b derivative pMR5 with 200 μg mL^{-1} kanamycin, we eliminated *E. coli* growth by adding acetic acid to the medium at a final concentration of 0.1% (w/v). However, we found that the wild-type PAL5 strain had kanamycin resistance up to 250 μg mL^{-1} in the presence of 0.1% (w/v) acetic acid (data not shown); thus, we failed to screen out the transconjugants. We next used gentamycin at a final concentration of 50 μg ml^{-1} in the media to eliminate *E. coli*. It was noted that the transconjugants on the agar medium that contained kanamycin and gentamycin possessed the Δ*aldFGH* allele (data not shown), which allowed us to proceed to the second recombination step.

We failed to screen the second recombinant, since the first recombinant (Km^R) strain was insensitive to 5-fluorocytosine (data not shown). Therefore, we attempted another counter selection method using the *sacB* system. Schäfer et al. (Schäfer et al. 1994) equipped the mobilizable derivative pK18mob carrying the *sacB* gene, which confers sucrose sensitivity to gram-negative bacteria for the counter selection of a mutant strain in the presence of high concentrations (10%) of sucrose, which was reported applicable to the construction of gene deletion mutant in *Gluconobacter oxydans*, a species of acetic acid bacteria (Krajewski et al. 2010). We constructed the plasmid pMRA, a derivative of the pK18mobsacB suicide vector that carries the Δ*aldFGH* allele. While screening the PAL5 strain transconjugants with the pK18mobsacB derivative pMRA with 200μg mL^{-1} kanamycin, we eliminated *E. coli* growth by adding gentamycin to the medium at a final concentration of 50 μg mL^{-1}. The first recombinants constructed with the pK18mobsacB derivative pMRA carrying the Δ*aldFGH* allele were unable to grow on DYGS agar that contained 10% sucrose, although the wild-type strain successfully grew on the same agar medium. More than 50 colonies with sucrose resistance (Suc^R) were appeared, and then we picked up 32 colonies to examine the kanamycin sensitivity. We obtained 11 colonies of Suc^R and kanamycin-sensitive (Km^S). The genome DNA were isolated from the 11 Suc^R Km^S recombinants and subjected to an ex- amination of the *aldFGH* alleles by PCR, either wild type or deletion. Finally, we isolated four markerless Δ*aldFGH* recombinants of *Ga. diazotrophicus* PAL5, while other seven recombinants possessed the wild-

type allele. Then, we combined several gene deletions to construct double and triple deletion mutant strains (Table 2.1).

2.3.2. AldFGH is the major ALDH in *Ga. diazotrophicus* strain PAL5

We hypothesized that the ALDH activity that is relevant to acetic acid fermentation of the PAL5 strain was derived from three enzyme complexes: AdhAB, AldFGH, and AldSLC. Therefore, we constructed the gene cluster deletion mutant derivatives of the PAL5 strain Δ*aldFGH* Δ*aldSLC,* Δ*aldFGH* Δ*adhAB,* Δ*aldSLC* Δ*adhAB,* and Δ*aldFGH* Δ*aldSLC* Δ*adhAB*, which possess combinations of gene-cluster deletions as well as each single gene cluster deletion mutant (Table 2.1). No ALDH activity was detected in the membranes of the triple mutant strain Δ*aldFGH* Δ*aldSLC* Δ*adhAB*, while the other three double mutants showed different degrees of ALDH activity (Fig. 2.1). The Δ*aldSLC* Δ*adhAB* double deletion derivative that contains AldFGH as the sole ALDH showed similar ALDH activity to wild type, whereas the other two double deletion derivatives, Δ*aldFGH* Δ*adhAB* that contains AldSLC alone as the ALDH, and Δ*aldFGH* Δ*aldSLC* that contains AdhAB as the sole ALDH, showed low ALDH activity that was approximately 20% that of wild type (Fig. 2.1). These findings suggest that all three enzyme complexes are responsible for ALDH activity in the PAL5 strain.

To understand the effect of *aldFGH* and *aldSLC* gene deletion on acetic acid fermentation with *Ga.diazotrophicus* PAL5, we cultivated single and double gene cluster deletion derivatives under acetic acid fermentation conditions (Figs. 2.2 and 2.S2). All the gene deletion mutant strains grew as well as wild type in the presence and absence of ethanol, as shown in Fig. 2.2a and Fig. 2.S2, respectively. Under acetic acid fermentation conditions, the Δ*aldSLC* strain produced acetic acid similarly to the wild-type strain, which produced an acidity of 1.6% (w/v). This indicates a small contribution of AldSLC in acetic acid fermentation. On the other hand, the Δ*aldFGH* and Δ*aldFGH* Δ*aldSLC* strains produced less than one-half of the acidity produced with the wild-type strain, since the deletion mutants produced the acidity of 0.7% (w/v). All of the derivatives of *Ga. diazotrophicus* strain PAL5 including the wild type failed to produce any titratable acids in the absence of ethanol (data not shown).

2.3.3. Acetaldehyde accumulates during acetic acid fermentation with the Δ*aldFGH* strain

Because less acetic acid production with Δ*aldFGH* and Δ*aldFGH* Δ*aldSLC* derivatives implied acetaldehyde accumulation, we examined the level of acetaldehyde in the culture medium under acetic acid fermentation conditions. Since the difference in acidity was marginal between Δ*aldFGH* and Δ*aldFGH* Δ*aldSLC*, we anticipated that AldFGH plays a crucial role in acetic acid fermentation. Therefore, we examined the acetaldehyde level of the culture with the Δ*aldFGH* derivative (Fig. 2.2b). At 32 h of cultivation, Δ*aldFGH* accumulated acetaldehyde at a maximum concentration of approximately 140 mM, which declined immediately, probably

due to evaporation (Yakushi et al., 2018) and oxidation to acetic acid (Fig. 2.2c). In contrast, wild type produced a low amount of approximately 20 mM acetaldehyde, and this suggests that AldFGH produces acetic acid at a high rate from acetaldehyde, which is produced from ethanol by ADH.

2.3.4. AldFGH and AldSLC are independent of PQQ

We constructed an auxotrophic derivative of the *Ga. diazotrophicus* PAL5 strain for PQQ by deleting the *pqqABCDE* gene cluster (Goosen et al., 1989). The resultant mutant Δ*pqqABCDE* strain, which we refer to as ΔPQQ hereafter, failed to show ADH activity in the membranes of cells grown in ordinary glycerol medium, but recovered ADH activity in cells grown in medium supplemented with 1 μM PQQ (Fig. 2.S3). In contrast, the ΔPQQ strain showed similar levels of ALDH activity to the wild-type strain, regardless of whether PQQ was supplemented to the culture medium (Fig. 2.S3). These results indicated that ALDH in the PAL5 strain is independent of PQQ.

To examine which ALDH complex, AldFGH or AldSLC, is independent of PQQ, we constructed Δ*aldFGH* Δ*adhAB* ΔPQQ and Δ*aldSLC* Δ*adhAB* ΔPQQ strains, respectively. The elimination of PQQ biosynthesis led to the complete loss of membrane-bound glucose dehydrogenase activity (GDH), which is known to be PQQ-dependent (Fig. 2.3a). The Δ*aldSLC* Δ*adhAB* ΔPQQ strain that theoretically possesses only AldFGH as the sole ALDH showed ALDH activity similar to the reference strain Δ*aldSLC* Δ*adhAB* (Fig. 2.3b). A similar result was obtained for Δ*aldFGH* Δ*adhAB*. Taken together, we conclude that AldFGH and AldSLC were both independent of PQQ.

2.3.5. Molybdopterin is crucial for ALDH activity

Thurner *et al*. (1997) suggested that AldFGH of *Komagataeibacter europaeus* (formerly *Acetobacter europaeus*) is an enzyme that uses molybdopterin cytosine dinucleotide as a cofactor. To examine this idea, we constructed gene deletion strains for *moaA* and *moeA* genes, the product of which is required for the synthesis of cyclic pyranopterin monophosphate, an important molybdopterin biosynthesis intermediate (Rieder et al., 1998), and that is needed for molybdenum insertion into mlybdopterin (Leimkühler et al., 1999). The Δ*moaA* and Δ*moeA* strains lost most of the ALDH activity but showed similar ADH activity to the parental strain (Fig. 2.4). It can be reasonably speculated that the remaining ALDH activity in the molybdopterin mutants corresponds to the ALDH activity derived from ADH (Fig. 2.1), we eliminated the *adh* operon in these mutant strains. Deletion of the *adhAB* genes in these mutant strains defective in the molybdopterin biosynthesis lost ALDH activity completely, while GDH activity was not affected by the deletion of the genes for molybdopterin (Fig. 2.4c,d). These results indicate that ALDH in *Ga. diazotrophicus* strain PAL5 is dependent on molybdopterin as the prosthetic group.

2.4. DISCUSSION

We examined three issues concerning the mechanism of ALDH and ADH in acetic acid fermentation with *Ga. diazotrophicus* PAL5. The first is whether ADH itself, without any contribution from ALDH, is sufficient for acetic acid fermentation in the PAL5 strain (Gómez-Manzo et al. 2015). Second, we investigated which molecular species of the two ALDHs (Yakushi et al. 2018) were functionally crucial for acetic acid fermentation in the PAL5 strain. Finally, we investigated whether ALDH is dependent on PQQ (Gómez-Manzo et al. 2010) or molybdopterin (Thurner et al. 1997).

We showed that ADH indeed oxidizes acetaldehyde (Fig. 2.1), which is consistent with previous studies which reported that purified ADH oxidizes acetaldehyde in vitro (Gómez-Manzo et al. 2008; Kanchanarach et al. 2010). That is also the case in *Gluconobacter* ADH, i.e., reverse genetic studies evealed that ADH oxidizes not only a wide variety of primary alcohols but also a wide variety of aldehydes (Mientus et al. 2017, Peters et al. 2013). Furthermore, the Δ*aldFGH* Δ*aldSLC* strain that possesses only ADH but not ALDH produced acetic acid from ethanol, even though the acidity levels were much lower than those of the wild type (Fig. 2.2). These results indicate that ADH is a bifunctional enzyme that oxidizes ethanol and acetaldehyde and may imply that ADH is sufficient for acetic acid fermentation, as suggested by Gómez-Manzo et al. (Gómez-Manzo et al. 2015). However, much higher levels of acetaldehyde were observed in Δ*aldFGH* than in wild type (Fig. 2.2), which suggests that acetaldehyde oxidation is a rate-limiting step and that impaired acetaldehyde oxidation limits the acetic acid production by the Δ*aldFGH* strain. Thus, we conclude that ALDH, particularly AldFGH, is essential for efficient acetic acid fermentation.

We suggest that AldFGH is the major ALDH in acetic acid fermentation by the PAL5 strain, as determined from our reverse genetic study. Acetic acid fermentation was severely impacted in the *aldFGH* strain, whereas the *aldSLC* strain showed ALDH activity and fermentation ability similar to that of wild type. The *A. pasteurianus* SKU1108 strain possesses at least two ALDH gene clusters: *aldFGH* and *aldSLC*. AldFGH is the major ALDH, because the *aldL* gene mRNA levels are reduced under acetic acid fermentation conditions (Yakushi et al. 2018), and this indicates that *aldFGH* is functionally crucial in acetic acid fermentation in this microorganism. *Komagataeibacter europaeus* (formerly *Gluconacetobacter europaeus*), an industrial acetic acid bacterium that produces high concentrations of acetic acid, possesses only the *aldFGH* gene cluster in the draft genome (Andres-Barrao et al. 2011). Altogether, it is conceivable that the *aldFGH* gene cluster is widely distributed in acetic acid bacteria for acetic acid fermentation.

In this study, we showed that both AldFGH and AldSLC were independent of PQQ. Instead, molybdopterin, presumably a form of molybdopterin cytosine dinucleotide (Thurner et al. 1997), might be the ALDH prosthetic group in the PAL5 strain. Since ADH is abundant in the membranes and also shows some ALDH activity, it may perturb its purification, and therefore, the biochemical analysis of ALDH and may lead to incorrect conclusions in some cases. The present study constructed the Δ*adhAB* Δ*aldSLC* Δ*PQQ* and Δ*adhAB* Δ*aldFGH* Δ*PQQ* strains, which showed similar ALDH activity to their prototrophic reference strains as for PQQ. Thus, these two strains are useful for biochemical analysis, including purification of AldFGH and AldSLC, respectively. Biochemical studies with purified proteins are a prerequisite for the identification of the prosthetic group of AldFGH and AldSLC. We are currently attempting to purify AldFGH for biochemical characterization.

2.5. CONCLUSION

We have developed a markerless gene disruption method for *Ga. diazotrophicus* PAL5, to produce a wide variety of multiple gene deletion mutant strains. In this study, we deleted the *aldFGH* and *aldSLC* gene clusters, the *adhAB* operon codes for ADH, the *pqqABCDE* gene cluster for PQQ biosynthesis, and *moaA* and *moeA* for two molybdopterin biosynthesis genes. We observed that the ALDH activity in PAL5 strain is derived from three enzyme complex; *aldFGH*, *aldSLC* and *adhAB* where *aldFGH* gene products are the major ALDH and plays the essential role for efficient acetic acid fermentation in this microorganism and most importantly, which is dependent on a form of molybdopterin but independent of PQQ.

2.6. ACKNOWLEDGEMENTS

We are grateful to the Japanese Government (Monbukagakusho: MEXT) Scholarships that supported me to work in this study. We thank Oriental Yeast (Tokyo, Japan) and Toyobo (Osaka, Japan) for gifting us with yeast extract and restriction endonucleases, respectively. We are grateful to Tomoyuki Kosaka and Mamoru Yamada for their invaluable suggestions. We would like to thank Dr. Osao Adachi, Applied Microbiology laboratory, Yamaguchi university and Dr. Hirohide Toyama, Bioscience and Biotechnology, University of the Ryukyus for encouraging our ALDH work.

TABLES AND FIGURES IN CHAPTER TWO

Table 2.1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Description	Source or reference
Bacterial strains		
Gluconacetobacter diazotrophicus		
ATCC49037	Wild type, synonym of PA1 5 (PAL5)	ATCC, (Yamada et al. 1997)
MR2	PAL5 Δ*aldFGH*	This study
MR5	PAL5 Δ*aldSLC*	This study
MR8	PAL5 Δ*adhAB*	This study
MR11	PAL5 Δ*pqqABCDE*	This study
MR12	PAL5 Δ*aldFGH* Δ*aldSLC*	This study
MR13	PAL5 Δ*aldFGH* Δ*adhAB*	This study
MR14	PAL5 Δ*aldSLC* Δ*adhAB*	This study
MR15	PAL5 Δ*aldFGH* Δ*aldSLC* Δ*adhAB*	This study
MR16	PAL5 Δ*aldFGH* Δ*adhAB* Δ*pqqABCDE*	This study
MR17	PAL5 Δ*aldSLC* Δ*adhAB* Δ*pqqABCDE*	This study
MR20	PAL5 Δ*moaA*	This study
MR23	PAL5 Δ*moeA*	This study
MR24	PAL5 Δ*moaA* Δ*adhAB*	This study
MR25	PAL5 Δ*moeA* Δ*adhAB*	This study
Acetobacter pasteurianus		
SKU1108	Wild type, synonym of NBRC101655	(Saeki et al. 1997)
mNS3	SKU1108 Δ*adhAB* Δ*aldSLC*	SN et al., unpublished
Plasmids		
pRK2013	Plasmid mediates plasmid transfer, Km^R	(Figurski and Helinski 1979)
pK18mobsacB	A suicide vector, *mob*, *sacB*, Km^R	(Schäfer et al. 1994)
pCM62	A broad host range plasmid, *mob lacZ*α, Tc^R	(Marx and Lidstrom 2001)

pTK10	pCM62, the 5.4-kb DNA fragment containing the *aldFGH* genes of SKU1108	(Yakushi et al. 2018)
pMRA	pK18mobsacB, the 2.0-kb fragment containing the Δ*aldFGH* allele	This study
pMRB	pK18mobsacB, the 1.8-kb fragment containing the Δ*aldSLC* allele	This study
pMRC	pK18mobsacB, the 1.8-kb fragment containing the Δ*adhAB* allele	This study
pMRD	pK18mobsacB, the 2.0-kb fragment containing the Δ*pqqABCDE* allele	This study
pMRE	pK18mobsacB, the 1.8-kb fragment containing the Δ*moaA* allele	This study
pMRF	pK18mobsacB, the 1.8-kb fragment containing the Δ*moeA* allele	This study

Table 2.S1. Oligonucleotides used in this study.

Name	Sequence (5' → 3') [a]	Objective [b]
PAL5-D-aldF-Hin(+)	aagcttcgctatgacgggc	Δ*aldFGH*
PAL5-D-aldF-5-RI(-)	gaattcgatgctggcggtgtcg	Δ*aldFGH*
PAL5-D-aldH-Xba(-)	tctagataggggatggcgtcg	Δ*aldFGH*
PAL5-D-aldS-Xba(+)	tctagagggcgaggaatacgacgc	Δ*aldSLC*
PAL5-D-aldC-3-Sal(+)	gtcgacctggcgaactacacgatc	Δ*aldSLC*
PAL5-D-aldC-Hin(-)	aagcttcccgcaccgattcgaacg	Δ*aldSLC*
PAL5-adhA-Hin(+)	aagcttcatcggggacgtgc	Δ*adhAB*
PAL5-D-adhB-RI(+)	gaattcgcggacgtggtcaacttc	Δ*adhAB*
PAL5-adhB-Xba(-)	tctagaatttcgccgaaccatacg	Δ*adhAB*
PAL5-pqqA-Hin(+)	aagcttgatgatcgtgatggtggtc	Δ*pqqABCDE*
PAL5-D-pqqA-Pvu(-)	cagctgaacctcggtgatcttg	Δ*pqqABCDE*
PAL5-pqqA-Xba(-)	tctagaacgtgacgctgttcac	Δ*pqqABCDE*
Pal5-D-moaA-Hin(+)	aagcttcgacgaaatcacccggg	Δ*moaA*
Pal5-D-moaA-fsn(-)	ggtcggtggccccttcatggcggtccgttaccgagattc	Δ*moaA*
al5-D-moaA-fsn(+)	gaatctcggtaacggaccgccatgaaggggccaccgacc	Δ*moaA*
Pal5-D-moaA-Xba(-)	tctagacctatcgcttcaccgcc	Δ*moaA*
Pal5-D-moeA-Hin(+)	aagcttcggctggaacgtcatgc	Δ*moeA*
Pal5-D-moeA-fsn(-)	catggcgaaaacgatggccccgtccggggtgacgac	Δ*moeA*
Pal5-D-moeA-fsn(+)	gtcgtcaccccggacggggccatcgttttcgccatg	Δ*moeA*
Pal5-D-moeA-Xba(-)	tctagaagggcaggtgaatcgc	Δ*moeA*

[a] Recognition sites for the restriction endonucleases (HindIII, EcoRI, XbaI, SalI, PvuII) are underlined.

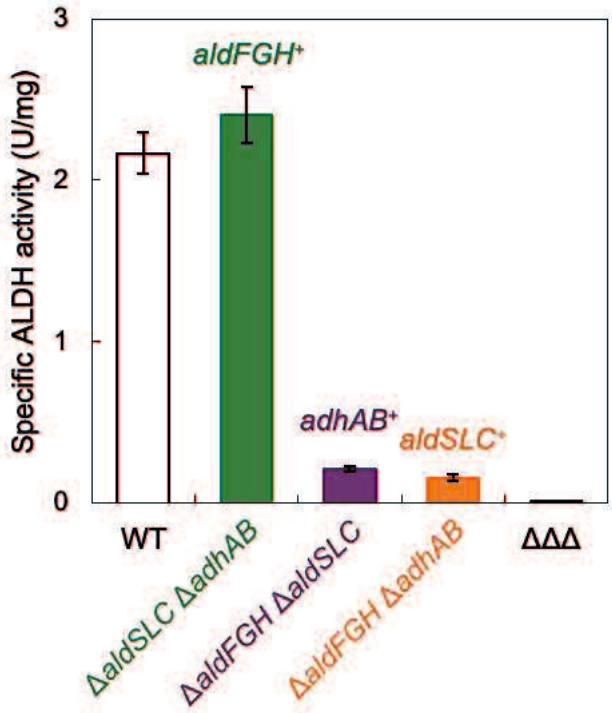


Figure 2.1. AldFGH is the major ALDH in the PAL5 strain. Specific ALDH activities were measured in the membranes of the wild-type (white, WT), Δ*aldSLC* Δ*adhAB* (green), Δ*aldFGH* Δ*aldSLC* (purple), Δ*aldFGH* Δ*adhAB* (orange), and Δ*aldFGH* Δ*aldSLC* Δ*adhAB* (gray, ΔΔΔ) strains by ferricyanide reductase assay at pH 4.0.

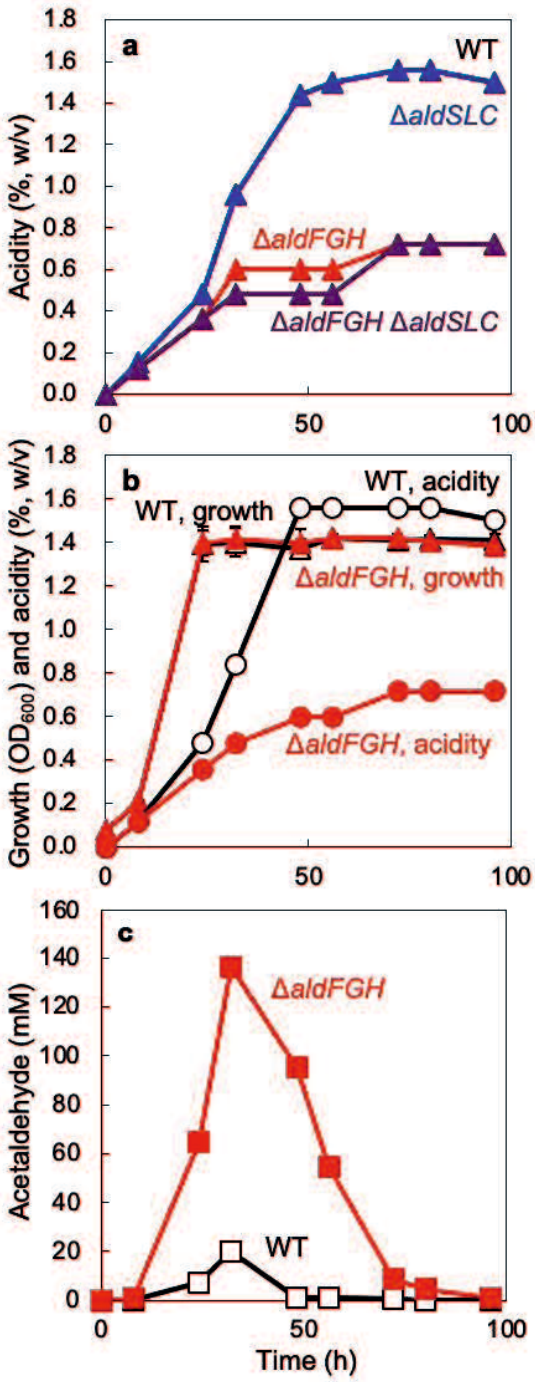


Figure 2.2. AldFGH is physiologically relevant to acetic acid fermentation. (A) The wild-type (white, WT), Δ*aldFGH* (red), Δ*aldSLC* (blue), and Δ*aldFGH* Δ*aldSLC* (purple) cells were pre-cultivated in YPGD and then in 100 mL YPG medium supplemented with ethanol at 20 mL/L at 30 °C on a rotary shaker at 180 rpm. The cultures were withdrawn at the time indicated, and the growth (OD_{600}, shown in Fig. S2) and acidity [% (w/v)] of the medium were determined. The data of wild-type are overlayed with those of Δ*aldSLC*. (B, C) Triplicate experiments for the wild-type (white, WT) and Δ*aldFGH* (red) strains. The growth (OD_{600}, triangle in panel B), acidity [% (w/v), circle in panel B], and acetaldehyde concentration (mM, square in panel C)

of the medium were determined. The growth data of wild-type are overlayed with those of Δ*aldFGH.*

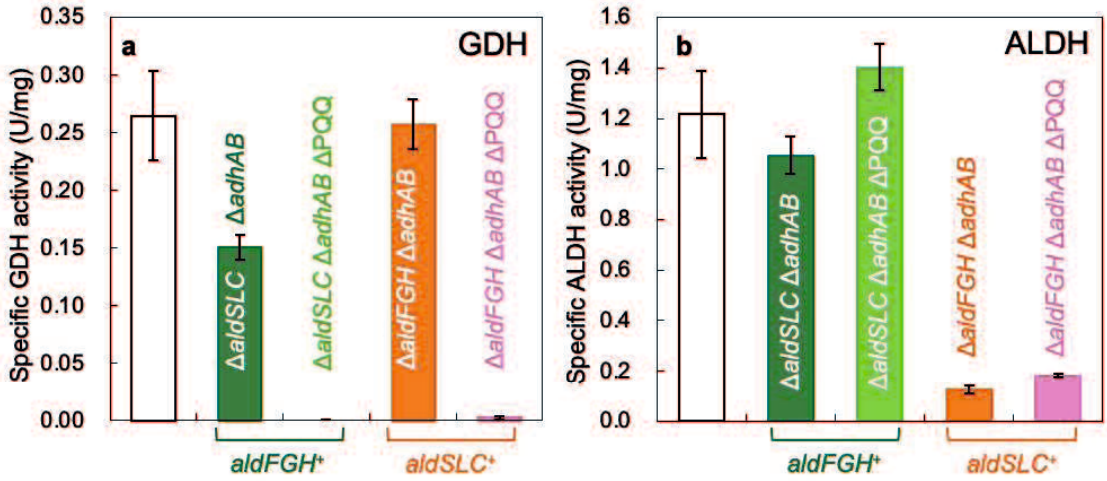


Figure 2.3. AldFGH and AldSLC aldehyde dehydrogenases are independent of PQQ. Specific GDH (A) and ALDH (B) activities were measured in the membranes of the wild-type (white, WT), Δ*aldSLC* Δ*adhAB* (green), Δ*aldSLC* Δ*adhAB* Δ*pqqABCDE* (lime green), Δ*aldFGH* Δ*adhAB* (orange), and Δ*aldFGH* Δ*adhAB* Δ*pqqABCDE* (pink) strains. GDH and ALDH were measured by the PMS-DCPIP assay at pH 6.5 and by the ferricyanide reductase assay at pH 4.0, respectively.

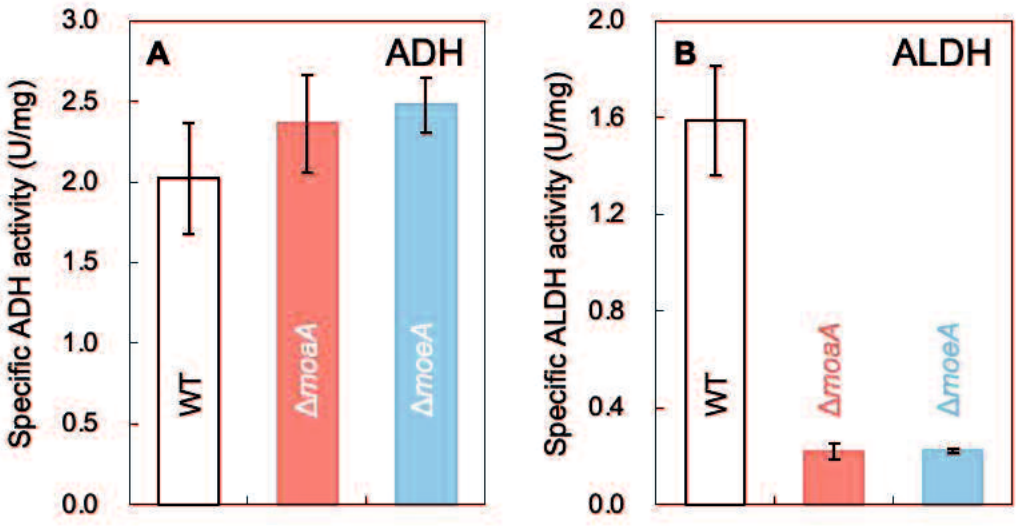


Figure 2.4. ALDH is dependent on molybdopterin. Specific ADH (A), ALDH (B and D), and GDH (C) activities were measured in the membranes of the wild-type (white, WT), Δ*moaA* (salmon pink), Δ*moeA* (sky blue), Δ*adhAB* (gray), Δ*moaA* Δ*adhAB* (red) and Δ*moeA* Δ*adhAB* (blue) strains. ADH and ALDH were determined by the ferricyanide reductase assay at pH 5.0 and pH 4.0, respectively**.** GDH activity was measured by the PMS-DCPIP assay at pH 6.5.

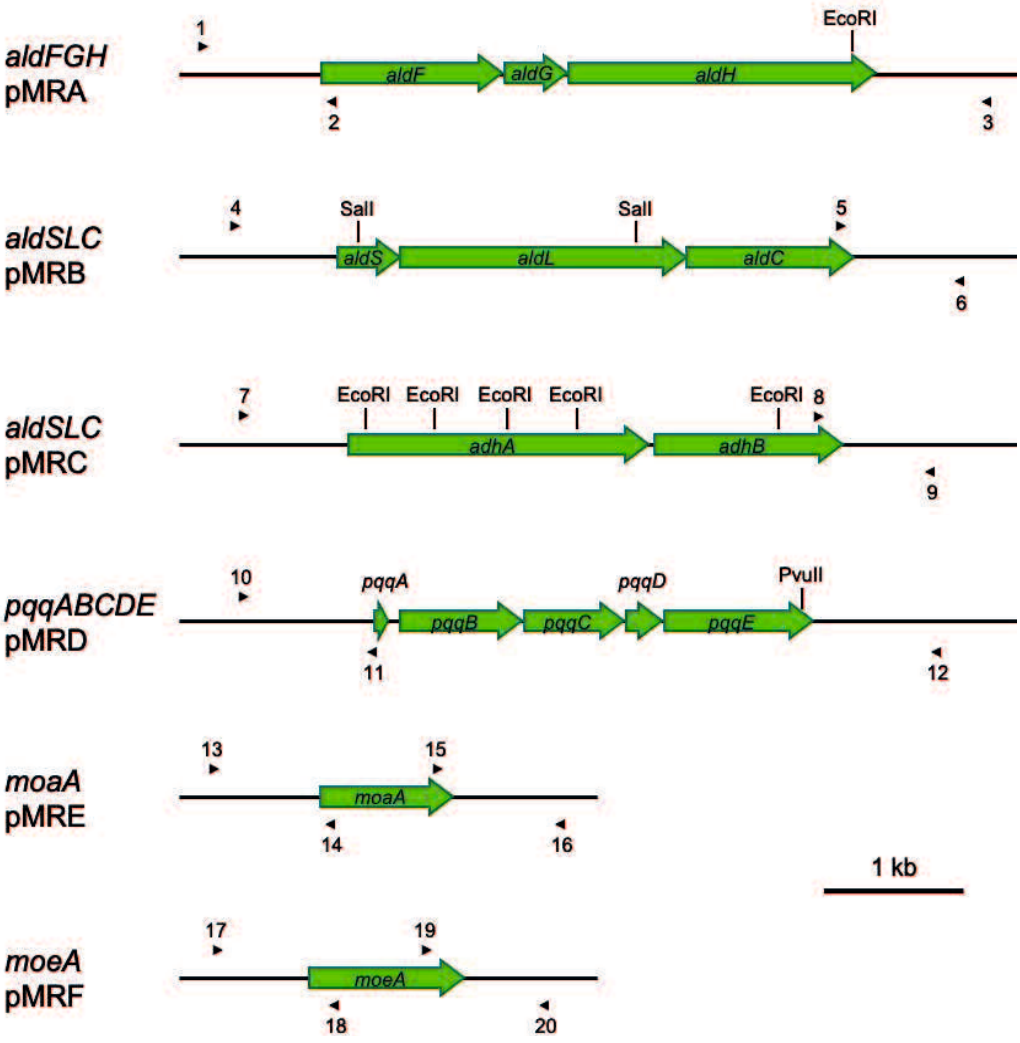


Figure 2.S1. Construction of plasmids for gene deletion in this study. The green arrows indicate the genes of interest. Arrow heads with numbers indicate primers for plasmid construction. The numbers correspond to those in Table 2.S1.

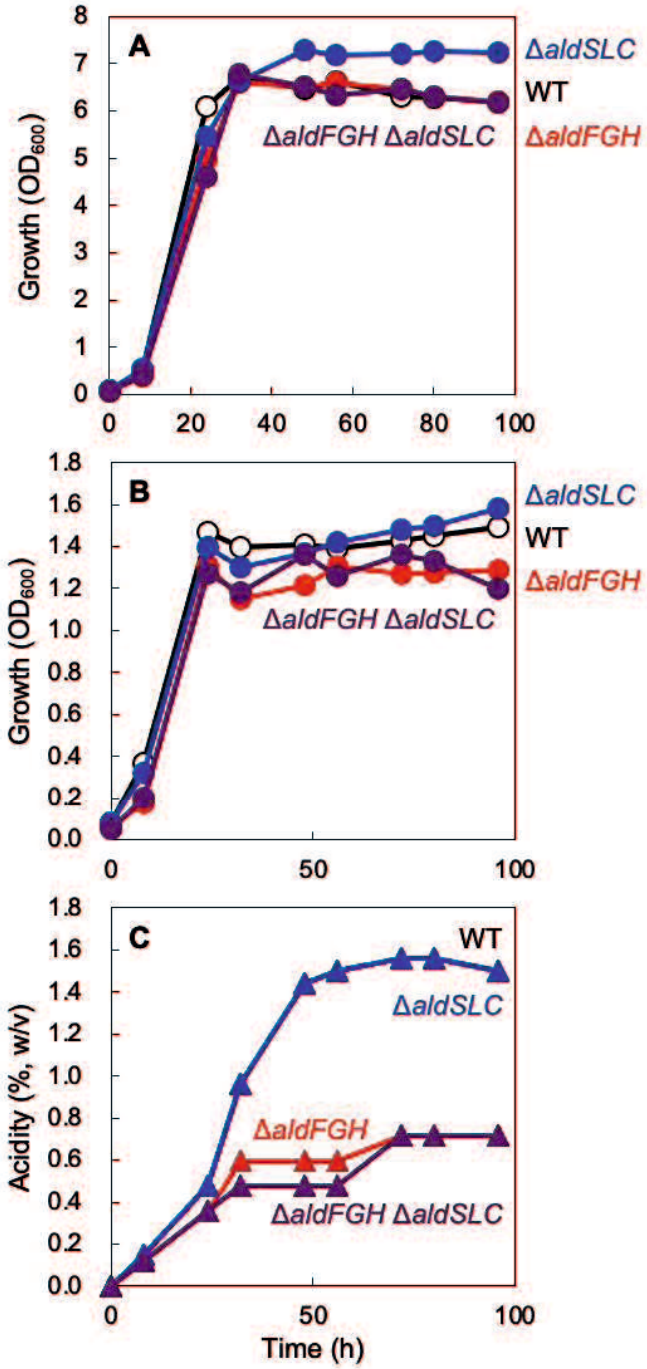


Figure 2.S2. Growth of *Gluconacetobacter diazotrophicus* derivatives on medium with and without ethanol. The wild-type (white, WT), Δ*aldFGH* (red), Δ*aldSLC* (blue), and Δ*aldFGH* Δ*aldSLC* (purple) cells were pre-cultivated in YPGD, and then, in 100 mL YPG medium supplemented with ethanol at 20 mL/L (B and C) or without ethanol (A) at 30 °C on a rotary shaker at 180 rpm. The cultures were withdrawn at the time indicated, the growth (OD600, A and B) and acidity [% (w/v), C] of the medium were determined. Panel C is the same as Fig. 2A. No acidity was detected under the conditions without ethanol (data not shown).

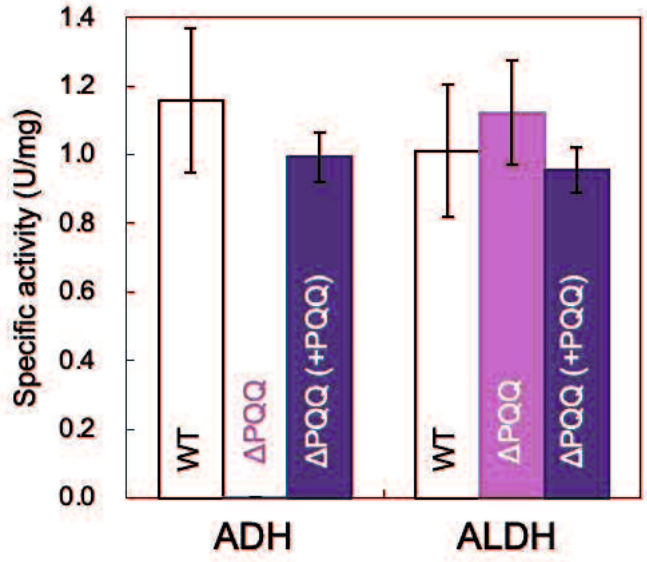


Figure 2.S3. ALDH activity is independent of PQQ. Specific ADH and ALDH activities in the membranes were measured using the ferricyanide reductase assay at pH 5.0 (ADH) and pH 4.0 (ALDH) for the wild-type (white, WT) and Δ*pqqABCDE* (magenta) strains grown on ordinary YPG medium, and for the Δ*pqqABCDE* strain grown on YPG medium supplemented with 1 μM PQQ (deep purple).

CHAPTER THREE

Dissection and reconstitution provide insights into electron transport in the membrane-bound aldehyde dehydrogenase complex of *Gluconacetobacter diazotrophicus*

ABSTRACT

Acetic acid bacteria catalyze the two-step oxidation of ethanol to acetic acid using the membrane-bound enzymes pyrroloquinoline quinone-dependent alcohol dehydrogenase and molybdopterin-dependent aldehyde dehydrogenase (ALDH). Although the reducing equivalents from the substrate are transferred to ubiquinone in the membrane, intramolecular electron transport in ALDH is not understood. Here, we purified the AldFGH complex, the membrane-bound ALDH that is physiologically relevant to acetic acid fermentation in *Gluconacetobacter diazotrophicus* strain PAL5. The purified AldFGH complex showed acetaldehyde:ubiquinone (Q_2) reductase activity. *C*-type cytochromes of the AldFGH complex (in the AldF subunit) were reduced by acetaldehyde. Then, we genetically dissected the AldFGH complex into AldGH and AldF units and reconstituted them. The AldGH subcomplex showed acetaldehyde:ferricyanide reductase activity, but not Q_2 reductase activity. The ALDH activity of AldGH was not found in membranes but in the soluble fraction of the recombinant strain, suggesting that the AldF subunit is responsible for membrane binding of the AldFGH complex. AldFGH complex reconstituted from the AldGH subcomplex and AldF showed Q_2 reductase activity. Absorption spectra of the purified AldGH subcomplex suggested the presence of an [Fe–S] cluster, which can be reduced by acetaldehyde. We propose a model in which electrons from the substrate are abstracted by a molybdopterin in the AldH subunit and transferred to [Fe–S] cluster(s) in the AldG subunit, followed by electron transport to *c*-type cytochrome centers in the AldF subunit, which is the site of ubiquinone reduction in the membrane.

3.1. INTRODUCTION

Acetic acid fermentation is the oxidation of ethanol by acetic acid bacteria. This is a process involving successive oxidation reactions catalyzed by membrane-bound enzymes: alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Matsushita et al., 1994). ADH catalyzes the oxidization of ethanol to acetaldehyde, coupled with the reduction of ubiquinone in the cytoplasmic membrane (Yakushi et al., 2010). ADHs of different species of acetic acid bacteria differ in subunit composition. ADHs of *Acetobacter* spp. and *Gluconobacter* spp. are composed of three subunits—AdhA, AdhB, and AdhS, whereas those of *Komagataeibacter* spp. and *Gluconacetobacter* spp. are composed of two subunits—AdhA and AdhB (Yakushi et al., 2010). The AdhA and AdhB subunits are a quinohemoprotein subunit containing pyrroloquinoline quinone (PQQ) and one *c*-type cytochrome center as the prosthetic groups, and a *c*-type cytochrome subunit containing three hemes, respectively (Yakushi et al., 2010; Matsushita et al., 1996). AdhB is the site of ubiquinone reduction (Matsushita et al., 1996). AdhS has no characteristic cofactors but the several reports suggest the function as a molecular chaperone that assists maturation of the AdhA subunit (Kondo et al., 1995; Masud et al., 2010).

The functions of the membrane-bound ALDH of acetic acid bacteria, which catalyzes hydroxylation and oxidation of acetaldehyde to produce acetic acid, have not been studied for a long time. In both *Acetobacter pasteurianus* strain SKU1108 (Yakushi et al., 2018) and *Gluconacetobacter diazotrophicus* strain PAL5 (Miah et al., 2021), there are two molecular species of ALDH, AldFGH and AldSLC. Amino acid identities between AldH and AldL, AldF and AldC, and AldG and AldS of the PAL5 strain are 24%, 32%, and 59%, respectively. AldFGH is physiologically important for acetic acid fermentation (Yakushi et al., 2018; Miah et al., 2021). However, the presence of multiple enzymes with ALDH activity complicates purification of AldFGH. According to the predicted amino acid sequences, the AldH protein is an 80-kDa molybdopterin-containing subunit that is the site of oxidation of the substrate (Huber et al.,1996); AldF is a 45-kDa *c*-type cytochrome containing three hemes; and AldG is a 17-kDa protein possessing two binding motifs for [2Fe–2S] clusters (Thurner et al., 1997).

ALDH activity of PQQ-dependent ADH might perturb purification of ALDH (Kanchanarach et al., 2010; Gómez-Manzo et al., 2008), which is presumably one of the reasons that the prosthetic group of ALDH was a matter of debate for a long time: either PQQ or molybdopterin (Thurner et al., 1997; Ameyama et al., 1981; Takemura et al., 1994; Gómez-Manzo et al., 2010). Recently we confirmed that PQQ is not involved in ALDH, but a form of molybdopterin is required by this enzyme (Miah et al., 2021). This work used a triple deletion strain, *Ga. diazotrophicus* PAL5 Δ*aldSLC* Δ*adhAB* Δ*PQQ*, called strain MR17, which lacks the genes encoding AldSLC and ADH and has AldFGH as the sole ALDH (Miah et al., 2021);

in this strain, the genes for biosynthesis of PQQ (*pqqABCDE*) were also deleted to enable identification of the prosthetic group of AldFGH.

In the present study, we purified the AldFGH complex from *Ga. diazotrophicus* strain while maintaining the important functions, that is, ubiquinone reduction ability upon acetaldehyde oxidation, and reduction of ALDH with acetaldehyde. Ubiquinone reductase activity is relevant to the *in vivo* function of the enzyme, but only a few studies have considered this activity (Gómez-Manzo et al., 2010) and purification of ALDH having ubiquinone reductase activity has not yet been reported. Reduction of the purified ALDH with the substrate is also functionally critical (Gómez-Manzo et al., 2010) because AldFGH is predicted to possess *c*-type cytochrome centers and an [Fe–S] cluster. We genetically dissected the AldFGH complex into an AldGH subcomplex and the AldF subunit because the presence of cytochromes in AldF would mask the absorption of the [Fe–S] cluster of the AldG subunit and inhibit spectral analysis. By dissection and reconstitution of the AldFGH complex, we propose a model for intramolecular electron transport upon acetaldehyde oxidation coupled with ubiquinone reduction.

3.2. RESULTS

3.2.1. Stabilizing agent for ALDH of *Ga. diazotrophicus*

Before starting the purification of the AldFGH complex, we experimented with purification of the AldGH subcomplex (see section "The AldF subunit is responsible for membrane binding of the AldFGH complex," below). The AldGH subcomplex rapidly lost its ferricyanide reductase activity. We determined whether substrate could repress the inactivation of the AldGH subcomplex. Because aldehydes are volatile, we tried aldehydes with relatively high boiling points, namely propionaldehyde and butyraldehyde. The K_M values for ferricyanide reductase activity of the AldGH subcomplex for acetaldehyde, propionaldehyde, and butyraldehyde were determined to be 0.48, 0.68, and 3.2 mM, respectively (Fig.3.S1). We examined 2 mM butyraldehyde as a stabilizing agent for the AldGH subcomplex over 4 d at 4°C, as well as 50 mM benzaldehyde, which we previously used as a stabilizing agent for the membrane-bound ALDH of *Gluconobacter* sp. (Adachi et al., 1980). Butyraldehyde maintained the activity of the AldGH subcomplex, but benzaldehyde did not (Fig. 3.S2). Thus, 2 mM butyraldehyde was included in all the buffers used in the purification procedures in this study.

3.2.2. Properties of the AldFGH complex

We purified the AldFGH complex from *Ga. diazotrophicus* strain MR17 (PAL5 Δ*adhAB*Δ*aldSLC* ΔPQQ; see the Materials and Methods section), which lacks the genes encoding ADH, AldSLC, and PQQ biosynthesis. Strain MR17 produces AldFGH as the sole

molecular species that exhibits ALDH activity in the membrane (Miah et al., 2021). A summary of the purification is shown in Table 3.1. The final recovery of activity was approximately 20% with specific acetaldehyde:ferricyanide (FC^{3-}) reductase activity of 350 U (mg protein)$^{-1}$ and acetaldehyde:ubiquinone-2 (Q_2) reductase activity of 20 U (mg protein)$^{-1}$. The Q_2 reductase activity was approximately 5% of the FC^{3-} reductase activity in each purification step (Table 1), indicating that the purification procedures in this study maintained the physiologically relevant ubiquinone reductase activity.

In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in denaturing conditions, our final enzyme preparation showed the presence of three main bands (Fig. 3.1A). High amounts of protein (33 μg) were loaded on the SDS-PAGE consisted of high concentrations of acrylamide to see the small subunit AldG. However, the large subunit AldH migrated as a lager molecular species than the expected size (80 kDa) in the gel. We anticipated high amount of detergent may perturb the migration of large protein in SDS-PAGE, because the detergent in the enzyme preparation was concentrated by ultrafiltration. Therefore, lower amounts of the ALDH (2 μg) were loaded on SDS-PAGE consisted of lower acrylamide concentration in Fig.3. 1B. The large subunit AldH migrated as having expected molecular size. Taken together, we concluded that the enzyme preparation contains three subunits of 80, 45, and 17 kDa, which correspond to the expected molecular masses ofAldH, AldF, and AldG, respectively. Moreover, the *N*-terminal amino acid sequence of the 17-kDa band was TTFRL, which corresponded to the expected sequence for AldG (Giongo et al., 2010). Heme-staining based on heme-dependent peroxidase activity showed that the 45-kDa band contained heme covalently associated with the protein, namely heme *c* (Fig. 3.1C).

Visible spectroscopy of the as-purified AldFGH complex showed an absorption peak at 410 nm, but no obvious peaks around 520 or 550 nm (black line in Fig. 3.2). However, when the AldFGH complex was incubated with the substrate acetaldehyde at a final concentration of 10 mM, absorption peaks were observed at 417, 522, and 550nm, which correspond to the γ, β, and α absorption bands of a typical *c*-type cytochrome, respectively (red line in Fig. 3.2). We suggest that the as-purified AldFGH complex is mostly in the oxidized form, even though we added butyraldehyde to all the buffers used for the purification. Addition of acetaldehyde reduced the AldFGH complex. Importantly, addition of sodium dithionite after addition of acetaldehyde did not change the absorption spectrum much (dashed-blue line in Fig. 3.2, compared with the red line), suggesting that almost all cytochromes in the purified AldFGH complex were reduced in the presence of substrate. The absorption spectrum also showed shoulders at around 530 and 556 nm (inset in Fig. 3.2), similar to previous reports that suggested the presence of a *b*-type cytochrome (Thurner et al., 1997, Gómez-Manzo et al., 2010).

Similar to previous reports by Thurner *et al.* (1997) and Gómez-Manzo *et al.* (2010), the absorption spectrum of the purified AldFGH complex had a shoulder at 556 nm (inset in Fig. 3.2). Because previous reports suggested the presence of heme *b*, we attempted to detect it in our purified AldFGH complex. The reduced *minus* oxidized difference spectrum of the pyridine hemochromogen failed to detect heme *b*, showing only one peak (absorption maximum at 550 nm), consistent with heme *c* (Fig. S3*)*. Moreover, we attempted to extract hemes by acid–acetone treatment (Puustinen et al., 1991). Heme *c* cannot be extracted from protein by this method because the heme is covalently bound to the polypeptide chain, but noncovalently bound hemes such as hemes *a* and *b* are extracted. However, we did not detect any heme in the acetone-soluble fraction extracted from AldFGH. For comparison, hemes *a* and *b* were extracted by acid–acetone treatment from partially purified cytochrome ba_3 ubiquinol oxidase (Matsushita et al.,1990), which was derived from subfractions of the AldFGH purification. The acetone was evaporated and the pyridine hemochromes of the extracted hemes were examined (Fig. 3.S3). We conclude that the AldFGH complex does not contain heme *b*.

For the determination of heme *c*, a reduced *minus* oxidized difference spectrum was recorded for the pyridine hemochromogen of the AldFGH complex (Fig. 3.S3). The heme *c* content in the AldFGH complex was calculated to be 4.4 μM with 0.30 mg protein mL^{-1}. Assuming the molecular mass of the AldFGH complex is 142 kDa, the heme *c* content would be 2.1 mol per mol of AldFGH complex. However, even though the nearest integer was 2, we suggest that the AldFGH complex possesses three hemes *c*, because impurities in the enzyme preparation may lower the experimentally observed value. The value of three is also consistent with that reported in prior work (Gómez-Manzo et al., 2010), and with the number of heme *c* binding motifs (Cys–Xxx–Xxx–Cys–His) in AldF (Thurner et al., 1997).

3.2.3. The AldF subunit is responsible for membrane binding of the AldFGH complex

We examined the membrane association of the AldFGH complex. The ALDH activity of *Ga. diazotrophicus* strain MR17 was mostly detected in the membranes: 77% of the total activity was found in the membrane fractions (Fig. 3.3). The remaining ALDH activity (in the crude soluble fraction) was not a soluble form of the enzyme: we tried to isolate ALDH from the crude soluble fraction by column chromatography, but the elution profile of the ALDH activity was different from that of the solubilized AldFGH complex or the AldGH subcomplex. ALDH activity in the crude soluble fraction is presumably derived from small membrane fragments that were not sedimented by ultracentrifugation.

In the quadruple deletion strain *Ga. diazotrophicus* MR25 (Δ*adhAB* Δ*aldSLC* ΔPQQ Δ*aldF*), almost all the ALDH activity was detected in the crude soluble fraction; only 1% of the total activity was detected in the membrane fraction. These results suggest that AldF is responsible for membrane binding of the AldFGH complex.

3.2.4. Heterologous expression of *Ga. diazotrophicus* AldFGH

We genetically dissected the AldFGH complex using heterologous expression in *A. pasteurianus* strain mNS4 (SKU1108 Δ*adhAB* Δ*aldSLC* Δ*aldFGH*; see the Materials and Methods section), from which the genes encoding AldFGH, AldSLC, and ADH have been deleted. The membranes of *A. pasteurianus* strain mNS4 show negligible levels of ALDH activity at pH 4: less than 0.01U (mg protein)$^{-1}$ (S.N. et al., unpublished). As described in the Materials and Methods section, we constructed plasmids carrying several combinations of the *aldFGH* genes of *Ga. diazotrophicus* strain PAL5 and expressed them in *A. pasteurianus* mNS4.

The membranes of the recombinant *Acetobacter* strain harboring the plasmid encoding *Ga. diazotrophicus aldFGH* showed acetaldehyde:ferricyanide reductase activity (see section "Reconstitution of the AldFGH complex," below). The crude soluble fraction of the recombinant *Acetobacter* strain harboring the plasmid encoding *aldGH* also showed this activity (Fig. 3.S4), suggesting that the AldGH subcomplex can function as a dehydrogenase even without the AldF subunit. However, cells harboring the *aldH*-encoding plasmid did not show any activity (Fig. 3.S4), suggesting a functionally critical role of AldG.

The membranes of recombinant *Acetobacter* expressing the *Ga. diazotrophicus* AldFGH complex reduced ubiquinone-1 [45 mU (mg protein)$^{-1}$ at pH 4.0] and consumed dissolved oxygen [56 mU (mg protein)$^{-1}$ at pH 5.0], while the cell-free extract of recombinant *Acetobacter* expressing AldGH failed to reduce ubiquinone or molecular oxygen.

3.2.5. Properties of the AldGH subcomplex

To obtain clues about the function of the [Fe–S] cluster in the AldG subunit, we attempted to purify the *Ga. diazotrophicus* AldGH subcomplex from recombinant *A. pasteurianus* mNS4 as described in Materials and Methods. We finally obtained 1.8 mg of the AldGH subcomplex with specific FC^{3-} reductase activity of 100 U (mg protein)$^{-1}$ (Table 3.2). In SDS-PAGE, the final preparation of the AldGH subcomplex showed two main bands having molecular masses of approximately 80 and 17 kDa (Fig. 3.S5). We attempted to purify the AldGH subcomplex further by Superdex 200 gel filtration chromatography. The elution profile of the two bands corresponded with that of FC^{3-} reductase activity. The data suggest that the 80-kDa and 17-kDa bands are AldH and AldG, respectively (Fig. 3.S5). Furthermore, the five amino acids at the *N*-terminus of the 17-kDa band were as expected for AldG.

Absorption spectra of the purified AldGH subcomplex suggested the presence of an [Fe–S] cluster (Fig. 3.4). We performed spectrophotometric analysis of the AldGH subcomplex in fraction number 19 from the gel filtration column chromatography because the fewest impurities were observed in this fraction in SDS-PAGE analysis (Figs. 3.4 and 3.S5). The overall absorption spectrum of the as-purified AldGH subcomplex was that of typical protein with an absorption peak at around 280 nm (Fig. 3.4B), but a small shoulder was also observed

at around 480 nm (inset of Fig. 3.4B). We anticipated that the [Fe–S] cluster of the purified AldGH subcomplex would be present in a reduced form because our purification system contained butyraldehyde. Therefore, we added potassium ferricyanide (final concentration 0.5 mM) to oxidize the subcomplex. The shoulder at around 480 nm showed increased absorbance, and a new shoulder appeared at around 580 nm (blue line in inset of Fig. 3.4B). Finally, we added acetaldehyde (final concentration 1 mM) to reduce the AldGH subcomplex. The two shoulders were now close to those in the original "as-purified" spectrum (compare the red and black lines in the inset of Fig. 3.4B). These results suggest that the AldGH subcomplex contains an [Fe–S] cluster that is involved in intramolecular electron transport for the catalytic reaction of the enzyme. It also suggests that the AldGH subcomplex purified in this study was in a reduced form.

3.2.6. Reconstitution of the AldFGH complex

We constructed a recombinant *A. pasteurianus* mNS4 strain harboring the plasmid carrying the *Ga. diazotrophicus* aldF gene. Because we suggest (see above) that the AldF subunit is responsible for the membrane binding of the AldFGH complex, we prepared the membranes of the recombinant strain (hereafter referred to as "AldF membrane") for experiments to try to reconstitute the AldFGH complex using the AldGH subcomplex. The AldF membranes showed low ALDH activity of less than 0.01 U (mg protein)$^{-1}$ at pH 4. When crude soluble fraction containing *Ga. diazotrophicus* AldGH was mixed with AldF membranes, the acetaldehyde: FC^{3-} oxidoreductase activity at pH 4.0 was significantly elevated in an AldF-dependent manner (Fig.3. S6). These results suggest that the AldF membrane contains active molecules that enhance the activity of AldGH, and that genetically dissected Ald subunits can be reconstituted to form the AldFGH complex.

We tried to reconstitute acetaldehyde:ubiquinone-2 oxidoreductase activity with the crude soluble fraction containing AldGH and the AldF membranes. However, because high protein concentrations due to impurities perturb the enzyme assay, we did not detect Q_2 reductase activity. Therefore, we partially purified AldF from detergent [Mydol 10 (decyl glucoside)]-solubilized membranes by column chromatography to reconstitute the AldFGH complex with the purified AldGH subcomplex. When the purified AldGH with specific activity of 120 U (mg protein)$^{-1}$ at pH 5.0 was mixed with variable amounts of the partially purified AldF, the reconstituted ALDH showed a saturation curve of FC^{3-}reductase activity at pH 4.0 (Fig.3.5A). The FC^{3-}reductase activity of the AldGH subcomplex at pH 4.0 was activated in an AldF-dependent manner and AldF:AldGH ratio 920 (in protein amount) showed a saturated activation in this experiment. The pH-dependence profile of FC^{3-}reductase activity of the reconstituted ALDH with an AldF:AldGH ratio of 920 (Fig.3.5B) was different from that of

the purified AldGH subcomplex (Fig.3.5C), but similar to that of the purified AldFGH complex (Fig.3.4D).

We detected acetaldehyde-dependent Q_2 reductase activity with 27 U(mg AldGH)$^{-1}$ by reconstitution of AldGH with the partially purified AldF with AldF:AldGH ratio 920 (Fig.3.5E). The Q_2 reductase activity was detected with neither the purified AldGH subcomplex alone nor the partially purified AldF subunit (Table 3.S1). For the AldFGH complex purified from *Ga. diazotrophicus* MR17, the Q_2 reductase activity was approximately 5% of the FC^{3-}reductase activity (Table 3.1). By considering FC^{3-}reductase activity of the reconstituted ALDH with an AldF:AldGH ratio of 920 is approximately 1000 U (mg AldGH)$^{-1}$, Q_2 reductase activity with 50 U (mg AldGH)$^{-1}$ can be expected for the reconstituted ALDH. Taken together, our data show that the AldF subunit enhances catalysis with FC^{3-}as the electron acceptor, presumably pulling the electrons from the AldGH subcomplex and passing them to FC^{3-}. More importantly, the results clearly indicate that AldF is the site of ubiquinone reduction.

3.3. DISCUSSION

We purified the AldFGH complex from strain MR17 of *Ga. diazotrophicus*, which lacks the genes encoding AldSLC and ADH to avoid contaminating ALDH activity, and the genes for PQQ biosynthesis because PQQ is not involved in AldFGH function (Miah et al., 2021). Gómez-Manzo *et al.* (Gómez-Manzo, 2010) purified ALDH from *Ga. diazotrophicus* strain PAL5. Even though the wild-type strain has the *aldFGH*, *aldSLC*, and *adhAB* gene clusters, the purified ALDH consisted of two subunits and the large and middle subunits of purified ALDH were identified as the *aldH* and *aldF* gene products, respectively, by determining the amino acid sequences (Gómez-Manzo et al., 2010). Our enzyme preparation contained a 17-kDa band, the *N*-terminal amino acid sequence of which was identical to that of AldG, which possesses two [2Fe–2S] cluster-binding motifs (Giongo et al., 2010). The previous report did not mention the presence of this small subunit (Gómez-Manzo et al., 2010). The AldFGH complex purified in the present study showed acetaldehyde:Q_2 reductase activity, whereas this activity was not detected for purified ALDH in the previous report (Gómez-Manzo et al., 2010). More importantly, the previous report failed to reduce the cytochromes of the enzyme with the substrate. However, almost all cytochromes of the purified AldFGH complex were reduced when using acetaldehyde as the substrate in the present study (Fig. 3.2). We suggest that the detergent used in the purification may be an important factor to maintain the intrinsic properties of the AldFGH complex, although we did not try to purify the AldFGH complex with Triton X-100 as used in the previous report (Gómez-Manzo et al., 2010). We summarize the comparison between the previous study and our present study in Table 3.3.

We genetically dissected the AldFGH complex of *Ga. diazotrophicus* to understand the function of each subunit and the catalytic mechanism of the complex. The results shown in Fig.3. 3 clearly indicate that even in the absence of the AldF subunit, the AldG and AldH proteins show acetaldehyde:ferricyanide oxidoreductase activity and Fig.3.4 indicates that the two proteins work as an AldGH subcomplex. As shown in Fig. 3.3, AldF is responsible for the membrane binding of the complex. The acetaldehyde:ferricyanide oxidoreductase activity of the AldFGH complex (Fig. 3.5D) and the AldGH subcomplex (Fig. 3.5C) as a function of pH were completely different from each other. Upon reconstitution with AldF, the pH-dependence of the AldGH subcomplex changed to be similar to that of the AldFGH complex (Fig. 3.5B). Furthermore, the reconstituted ALDH complex showed acetaldehyde:Q2 oxidoreductase activity, although the AldGH subcomplex alone did not (Fig.3.5E). Taken together, it is reasonable to conclude that we prepared an active AldF subunit and AldGH subcomplex and functionally reconstituted an AldFGH complex.

Several discrepancies in the properties of purified ALDHs are reported by different research groups (Adachi et al., 1980; Fukaya et al., 1989; Gómez-Manzo et al., 2010). Fukaya *et al*. (1989) reported purification and characterization of the ALDH of *Komagataeibacter polyoxogenes* (formerly *Acetobacter polyoxogenes*) strain NBI1028. They reported that the ALDH possessed no cytochromes, but the absorption spectrum showed an absorption shoulder at around 480 nm similar to the AldGH subcomplex purified in the present study. However, they did not mention the presence of an [Fe–S] cluster in the purified enzyme. The optimum pH for the ferricyanide reductase activity of *K. polyoxogenes* ALDH was also similar to that of the AldGH subcomplex in the present work. A summary of the comparison of the ALDHs is shown in Table 3.4. We suggest that the ALDH purified by Fukaya *et al*. was the AldGH subcomplex of *K. polyoxogenes*.

We observed reduction of an [Fe–S] cluster by acetaldehyde in the purified AldGH subcomplex (Fig. 3.4). These results strongly support the idea that the [Fe–S] cluster is involved in catalysis by the AldFGH complex. Experiments in Fig. 5 reconstituted electron transfer from the AldGH subcomplex to the AldF subunit where the Q_2 molecule finally accepts the electrons. The intramolecular electron transport pathway in the AldFGH complex is proposed in Fig. 3.6A. A molybdopterin cofactor in the AldH subunit is the site of oxidation of acetaldehyde. Electrons from molybdopterin are transported to the [Fe–S] (presumably [2Fe–2S]) cluster in the AldG subunit, and then to hemes *c* in the AldF subunit, which is responsible for the membrane binding and the ubiquinone reduction.

The reconstitution experiment in this study may have reproduced the final step of the assembly of the AldFGH complex *in vivo*. In this study, we constructed two recombinant *Acetobacter* strains that respectively produced the AldGH subcomplex and the AldF subunit to

reconstitute the AldFGH complex *in vitro*. All the Ald proteins are synthesized in the cytoplasm (Fig. 3.6B). The AldG and AldH precursors are expected to be folded and assembled with cofactors molybdopterin and the [Fe–S] cluster in the cytoplasm. The precursors of these proteins do not possess a signal peptide for the Sec protein translocator system, but the AldH precursor possesses a signal peptide for the twin arginine protein translocator (TAT) for secretion of folded, cofactor-bound proteins to the periplasm (Palmer et al., 2020). The folded and assembled AldG would associate with AldH to hitchhike to the periplasm. The AldF precursor would be secreted to the periplasm by the Sec system, and assembled with the *c* hemes in the periplasm (Kranz et al., 2009). The matured AldF would associate with the cytoplasmic membrane, but its binding mode to the membrane remains unknown, because AldF apparently does not have a transmembrane segment for membrane anchoring. Finally, the AldGH subcomplex and the AldF subunit would meet in the cytoplasmic membrane to complete the AldFGH complex assembly, as we reconstituted the complex *in vitro*.

Determination of heme *c* showed 2.1 mols of heme *c* per mol of the AldFGH complex. However, we suggest there are likely to be 3 mols of heme *c* per mol of the AldFGH complex, even though the nearest integer was 2 because our enzyme preparation contained protein impurities that can lower the experimentally observed value. The value three corresponds with there being three heme *c*-binding CXXCH motifs in AldF (Thurner et al., 1997) and the experimental results in a prior report (Gómez-Manzo et al., 2010). Although we failed to detect heme *b* in the purified AldFGH complex (Fig. 3.S3), the absorption spectrum showed a shoulder at 556 nm (Fig. 3.2) similar to previous reports (Gómez-Manzo et al., 2010). The AldF, AldG, and AldH proteins do not contain the amino acid sequence for heme *b* binding suggested by Li *et al.* (2011). Because the absorption peak of the α-band of *c*-type cytochromes is variable (Yamanaka et al., 1992), we suggest that one of the three hemes *c* is responsible for the absorption at 556 nm. Intriguingly, the as-purified AldFGH complex in the present work was oxidized even though 2 mM butyraldehyde was included in the buffers used in the purification steps (Fig. 3.2), but the AldGH subcomplex was reduced (Fig. 3.5), hinting that the AldFGH complex can be oxidized by molecular oxygen in an AldF-dependent manner. Such oxygen reduction ability of the AldFGH complex will be addressed in the near future.

3.4. MATERIALS AND METHODS

3.4.1. Materials

Acetaldehyde (99.5%) and n-dodecyl-β-D-maltoside were purchased from Fluka (St. Louis, MO, USA) and Dojindo (Kumamoto, Japan), respectively. Yeast extract and Hipolypepton were from Oriental Yeast (Tokyo, Japan) and Nihon Pharmaceutical (Tokyo,

Japan), respectively. Mydol 10 (decyl glucoside) was from Kao (Tokyo, Japan). All other materials were purchased from commercial sources and were of analytical grade.

3.4.2. Bacterial strains, plasmids, and growth conditions

The *Ga. diazotrophicus* and *A. pasteurianus* strains and plasmids used in this study are listed in Table 3.5. *Ga. diazotrophicus* strain PAL5 (ATCC 49037) was obtained from the American Type Culture Collection (Manassas, VA). Suicide vector pK18mobSacB was provided by Dr. Alfred Pühler, Universität Bielefeld, Bielefeld, Germany, and was used for markerless gene deletion. *A. pasteurianus* strain mNS4 (Δ*adhAB*Δ*aldSLC*Δ*aldFGH*) was used as a host for heterologous expression of the *Ga. diazotrophicus aldFGH* genes. The mNS4 strain shows only negligible levels of ALDH activity [less than 0.01 U (mg membrane protein) $^{-1}$] at pH 4, but does have ALDH activity with FC^{3-} at pH 6 [0.02-0.03 U (mg membrane protein) $^{-1}$; S.N. et al., unpublished].

Ga. diazotrophicus cells were grown at 30°C in DYGS medium (2.0 g glucose, 1.5 g Hipolypepton, 2.0 g yeast extract, 0.5 g KH2PO4, 0.5 g MgSO4·7H2O, and 1.5 g sodium L-glutamate monohydrate per liter, pH 6.0); YPGD medium (5 g yeast extract, 5 g Hipolypepton, 5 g glycerol, and 5 g glucose per liter); or YPG medium (5 g yeast extract, 5 g Hipolypepton, and 10 g glycerol, per liter). Kanamycin was used at a final concentration of 200 μg/mL. *A. pasteurianus* cells were cultivated at 30°C in YPGD medium or YPGDP medium (5 g yeast extract, 5 g Hipolypepton, and 10 g glycerol, per liter, supplemented with 50 mM potassium - phosphate, pH 6.5). Tetracycline was used at a final concentration of 50 μg/mL.

The *Escherichia coli* strains DH5α (Hanahan et al., 1983) and HB101 (Boyer et al., 1969) were used for strain construction and triparental mating, respectively. *E. coli* cells were grown in modified Luria-Bertani medium (10 g Hipolypepton, 5 g yeast extract, and 5 g NaCl per liter, adjusted pH to 7 with NaOH). Ampicillin, kanamycin, and tetracycline were used at final concentrations of 50 μg mL^{-1}, 50 μg mL^{-1}, and 10 μg ml^{-1}, respectively.

3.4.3. Construction of plasmids

The outline of the construction of plasmids used in this study is shown in Fig. 3.S7. Genomic DNA of *Ga. diazotrophicus* strain PAL5 was isolated by the method of Marmur (Marmur et al., 1961) with some modifications (Kawai et al.,2013). The *aldFGH* genes (*Gdia_3086-3088*) were amplified by PCR with Herculase II fusion DNA polymerase (Stratagene, CA, USA), PAL5 genomic DNA, and several pairs of oligonucleotides (Table 3.S2). For elimination of the *aldF* gene from the genome of *Ga. diazotrophicus* PAL5, the pair of oligonucleotides Pal5-D-aldF-Hin(+) and Pal5-D-aldF-5-RI(−) was used to amplify the 1.0-kb DNA fragment containing the 5′-flanking region, and the primer pair Pal5-D-aldF-3-RI(+) and Pal5-D-aldF-Xba(−) was used to amplify the 1.0-kb 3′-flanking region. The two resulting DNA fragments were treated with *Hin*dIII and *Eco*RI and *Eco*RI and

*Xba*I, respectively, and inserted in the *Hin*dIII and *Xba*I sites of K18mobsacB (Schäfer et al., 1994) to yield pMRG (Fig. 3.S7, Δ*aldF*).

For construction of *aldFGH*-expression plasmid pTM16, the 4.4-kb DNA fragment containing *aldFGH* including the putative promoter region was amplified using genomic DNA of *Ga. diazotrophicus* PAL5 as the template and oligonucleotide pair Pal5-aldpro-RI(+) and Pal5-ex-aldH-Xba(−), and inserted into plasmid pT7Blue (Novagen) to yield pTM15. The 4.4-kb DNA fragment obtained by *Xba*I treatment of pTM15 was inserted into the corresponding site of pCM62 (Marx et al., 2001) to yield pTM16 (Fig. 3.S7, *aldFGH*). pTM8, pTM14, and pTM10 for expression of *aldGH*, *aldH*, and *aldF*, respectively, were constructed using similar procedures (outlined in Fig. 3.S7), and the oligonucleotides used are shown in Table 3.S2.

3.4.4. Transformation of acetic acid bacteria

Ga. diazotrophicus and *A. pasteurianus* were transformed with plasmids via a triparental mating method using *E. coli* HB101 harboring pRK2013 as the helper strain (Figurski et al., 1979), as described previously (Yakushi et al., 2018).

3.4.5. Construction of Δ*aldF* deletion strain

Ga. diazotrophicus MR17, a Δ*aldSLC* Δ*adhAB* ΔPQQ derivative of strain PAL5, was transformed with suicide plasmid pMRG (Δ*aldF*) to construct the quadruple deletion mutant MR25 (Δ*aldSLC* Δ*adhAB* ΔPQQ Δ*aldF*), as described previously (Miah et al., 2021).

3.4.6. Preparation of membranes and crude soluble fractions

Ga. diazotrophicus strains were precultivated in 100 mL YPGD medium at 30°C for 24h, and 5 mL of the preculture was transferred to 500 mL YPG medium in a 3-L flask and incubated at 30°C for 24 h. Recombinant *A. pasteurianus* strains were precultivated in 100 mL YPGDP medium containing 50 μg mL^{-1} tetracycline at 30°C for 48 h, and 5 mL of the preculture was transferred to 500 mL of the same medium in a 3-L flask and incubated at 30°C for 48 h. The cells were collected by centrifugation at 8,000 × *g* for 10 min at 4°C, washed with ice-chilled 10 mM K^+-MES (2-morpholinoethanesulfonic acid; pH 6.0), and resuspended in the same buffer supplemented with 0.5 mM phenylmethanesulfonyl fluoride. The cell suspension was passed through a French pressure cell press (American Instrument Company, Silver Spring, MD) at 1100 kg cm^{-2}, twice. After removal of intact cells and cell debris by centrifugation at 8,000 × *g* for 10 min at 4°C, the supernatant was further centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was used as the crude soluble fraction, and the precipitate was suspended in ice-chilled 10 mM K^+-MES (pH 6.0) and used as the membrane fraction.

3.4.7. Purification of the AldFGH complex

The membranes of *Ga. diazotrophicus* strain MR17 were solubilized with 1.0% (w/v) *n*-dodecyl-β-D maltoside (DDM) in 10 mM Na^+-acetate (pH 5.0) with protein concentration 10

mg mL^{-1}. After incubation at 4°C for 1 h with gentle stirring, the membrane suspension was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was applied to a CM-Toyopearl column (Tosoh, Tokyo, Japan). After washing the column with 10 mM Na^+-acetate (pH 5.0) containing 0.02% (w/v) DDM and 2 mM butyraldehyde, ALDH was eluted with a linear NaCl gradient from 0 to 0.5 M. The acetaldehyde:FC^{3-} reductase activity in the eluate fractions was determined.

Active fractions from the CM-Toyopearl column chromatography were pooled and K^+-phosphate (pH 6.0) was added to a final concentration of 10 mM, followed by pH adjustment to 6.0 with potassium hydroxide, and the solution was applied to a ceramic hydroxyapatite column (Bio-Rad, Hercules, CA). After washing the column with 10 mM K^+-phosphate (pH 6.0) containing 0.02% (w/v) DDM and 2 mM butyraldehyde, ALDH was eluted by a stepwise increase in phosphate concentration: 50, 100, 200, 300, 400, and 500 mM. The acetaldehyde:FC^{3-} reductase activity in the eluate fractions was determined. Active fractions from the ceramic hydroxyapatite column eluted with 500 mM K^+-phosphate (pH 6.0) containing 0.02% (w/v) DDM and 2 mM butyraldehyde were pooled and concentrated by ultrafiltration with Amicon Ultra centrifugal filters (50 k; Merck, Darmstadt, Germany) and dialyzed into 10 mM K^+-phosphate (pH 6.0) containing 0.02% (w/v) DDM and 2 mM butyraldehyde at 4°C.

The dialysate was applied to a DEAE-Toyopearl column (Tosoh). ALDH was eluted with a linear NaCl gradient from 0 to 0.5 M, after washing the column with 10 mM K^+-phosphate (pH 6.0) containing 0.02% (w/v) DDM and 2 mM butyraldehyde. The active fractions were pooled, concentrated as described above, and stored at 4°C.

3.4.8. Purification of the AldGH subcomplex

The crude soluble fraction of the recombinant *A. pasteurianus* mNS4 strain harboring pTM8 (*aldGH*$^+$) was applied to a DEAE-Toyopearl column. After washing the column with 10 mM K^+-MES (pH 6.0) containing 2 mM butyraldehyde, ALDH was eluted with a linear NaCl gradient from 0 to 0.3 M. The acetaldehyde:FC^{3-} reductase activity in the eluate fractions was determined.

Active fractions from the DEAE-Toyopearl column chromatography were pooled and solid ammonium sulfate was added to 25% saturation, followed by application to a Phenyl-Toyopearl column (Tosoh). After washing the column with 10 mM K^+-MES (pH 6.0) containing 2 mM butyraldehyde and ammonium sulfate (25% saturation), ALDH was eluted with a linear ammonium sulfate gradient from 25% to 0% saturation. Active fractions from Phenyl-Toyopearl column chromatography were pooled and dialyzed into 10 mM K^+-phosphate (pH 6.0) containing 2 mM butyraldehyde at 4°C.

The dialysate was applied to a ceramic hydroxyapatite column. ALDH was eluted with a stepwise increase in phosphate concentration: 10, 20, 50, 100, and 1000 mM. Most activity was recovered in the 100 mM K^+-phosphate fraction. Active fractions from the ceramic hydroxyapatite column were pooled and concentrated ultrafiltration as described above, and stored at 4°C.

3.4.9. Partial purification of the AldF subunit

The membranes of the recombinant *A. pasteurianus* mNS4 strain harboring pTM14 (*aldF*$^+$) were solubilized with 1.0% (w/v) Mydol 10 (decyl glucoside) in 10 mM Na^+-acetate (pH 5.0) at a protein concentration of 10 mg mL^{-1}. After incubation at 4°C for 1 h with gentle mixing, the membrane suspension was centrifuged at 100,000 × *g* for 1 h at 4°C. The supernatant was applied to a CM-Toyopearl column. After washing the column with 10 mM Na^+-acetate (pH 5.0) containing 0.2% (w/v) Mydol 10, AldF was eluted with a linear NaCl gradient from 0 to 0.2M. AldF in the eluate fractions was detected by activation ability of the AldGH subcomplex (see Figs. 3.4 and 3.S5). The acetaldehyde:FC^{3-}oxidoreductase activity of the AldGH subcomplex at pH 4.0 is low, but it is enhanced by AldF. Thus, we detected AldF by examining the ability of eluate from the chromatography column to activate AldGH. Active fractions were pooled and dialyzed into 10 mM K^+-phosphate (pH 6.0) containing 0.2% (w/v) Mydol 10 at 4°C.

The dialysate was applied to a ceramic hydroxyapatite column. ALDH was eluted with a stepwise increase in phosphate concentration: 100, 200, 500, and 1000 mM. AldGH-activation activity was recovered in the 200 and 500 mM K^+-phosphate fractions. Active fractions were pooled and dialyzed into 10 mM Na^+-acetate (pH 5.0) containing 0.2% (w/v) Mydol 10, followed by application to a small CM-Toyopearl column to concentrate the AldF. The activity was eluted with 10 mM Na^+-acetate (pH 5.0) containing 0.2% (w/v) Mydol 10 and 0.2 M NaCl.

3.4.10. Reconstitution of the AldFGH complex

The purified AldGH subcomplex and the partially purified AldF subunit were mixed in 10 mM Na+-acetate (pH 5.0) containing 0.2% (w/v) Mydol 10 and incubated at 25°C for 10 min

3.4.11. Enzyme assays

Acetaldehyde:ferricyanide oxidoreductase activity was determined by the ferric-Dupanol assay at 25°C using 100 mM acetaldehyde, as described previously (Adachi et al., 1980). McIlvaine buffer (a mixture of 100 mM citric acid and 200 mM Na_2HPO_4) was used at pH 4.0 to 7.0. Acetaldehyde:ubiquinone-1[Q_1,2,3-dimethoxy-5-methyl-6-(3-methyl-2-butenyl)-1,4 benzoquinone] and acetaldehyde:ubiquinone-2 (Q_2, 2,3-dimethoxy-5-geranyl-6-methyl-1,4-benzoquinone) oxidoreductase activities were determined at 25°C by monitoring the decrease

in absorbance at 275 nm in McIlvaine buffer (pH 4.0) containing either 50 μM Q_1 or 20 μM Q_2, 4 mM NaN_3, membranes or enzyme solution, and 100 mM acetaldehyde. The molecular absorption coefficient used was ε_{275} = 12.25 mM^{-1} cm^{-1} (Redfearn et al.,1967). One unit of enzyme was defined as the amount that oxidized 1 μmol of substrate per min. Oxygen consumption activity was measured by a Clark-type oxygen electrode (YSI model 5300; Yellow Spring Instrument, Yellow Springs, OH, USA) at 25 °C. The electrode was calibrated by using air-saturated McIlvaine buffer (pH 5.0), assuming the concentration of molecular oxygen to be 249 μM (Mitchell et al., 1979). Sodium dithionite was used for calibration to reduce molecular oxygen completely. The reaction mixture contained membranes, McIlvaine buffer (pH 5.0) and 100 mM acetaldehyde. One unit was defined as 1 μmol of half a molecular oxygen (equivalent to oxygen atom) consumed per minute. Protein content was determined by the modified Lowry method, and bovine serum albumin was used as the standard (Dulley et al., 1975).

3.4.12. Absorption spectra

Absorption spectra of the purified AldFGH complex and AldGH subcomplex were recorded at 25°C using a UV-1900i spectrophotometer (Shimadzu, Kyoto, Japan) and 1-cm light path cuvettes.

3.4.13. Determination of hemes in the AldFGH complex

For quantification of heme, the purified AldFGH complex, pyridine, and NaOH were mixed at final concentrations of 0.30 mg mL^{-1}, 20% (v/v), and 0.5 M, respectively. The sample was reduced with a few grains of sodium dithionite or oxidized with a few grains of potassium ferricyanide. Reduced-*minus*-oxidized difference absorption spectra were recorded at 300–700 nm. Heme *c* content was determined using the extinction coefficient 23.97 mM^{-1} cm^{-1} for the absorbance difference reduced *minus* oxidized at 550 nm *minus* 535 nm (Berry et al., 1987).

Alternatively, hemes in the purified AldFGH complex (1.0 mg protein) or partially purified cytochrome ba_3 ubiquinol oxidase (1.0 mg protein), which was obtained by anion-exchange column chromatography of subfractions in the purification of the AldFGH complex, were separated into protein-bound and free forms by treatment with acid–acetone [65% (v/v) acetone and 35 mM HCl]. The extracted materials were dissolved in 50% acetonitrile as described by Puustinen and Wikstrom (Puustinen et al., 1991), followed by application of the above procedure for quantification of hemes.

3.4.14. SDS-PAGE

SDS-PAGE was performed as described previously (Laemmli et al., 1970), followed by staining with Coomassie Brilliant Blue R-250, or for heme-dependent peroxidase activity using tetramethylbenzidine (Thomas et al., 1976). Broad-range (250–10 kDa) Precision Plus Protein Dual Color Standards (Bio-Rad) were used as molecular size standards.

For determination of *N*-terminal amino acid sequences, proteins in a gel were electrophoretically transferred onto a polyvinylidene difluoride membrane (Merck) in 10 mM Na^+-CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid; pH 11.0) containing 20% (v/v) methanol. The membrane was stained with Coomassie Brilliant Blue R-250. *N*-terminal amino acid sequencing was performed by automated Edman degradation (PPSQ-33A, Shimadzu, Kyoto, Japan).

3.5. CONCLUSIONS

Two membrane-bound enzymes of acetic acid bacteria—pyrroloquinoline quinone-dependent alcohol dehydrogenase and molybdopterin-dependent aldehyde dehydrogenase (ALDH)—are responsible for vinegar production. Upon oxidation of acetaldehyde, ALDH reduces ubiquinone in the cytoplasmic membrane. ALDH is an enzyme complex of three subunits. Here, we tried to understand how ALDH works by using a classical biochemical approach and genetic engineering to dissect the enzyme complex into soluble and membrane-bound parts. The soluble part had limited activity *in vitro*, and did not reduce ubiquinone. However, enzyme complex reconstituted from the soluble and membrane-bound parts showed ubiquinone reduction activity. The proposed working model of ALDH provides a better understanding of how the enzyme works in the vinegar fermentation process.

3.6. ACKNOWLEDGEMENTS

We are grateful to the Japanese Government (Monbukagakusho: MEXT) Scholarships for supporting the work of RM. We thank Oriental Yeast (Tokyo, Japan), Kao (Tokyo, Japan), and Toyobo (Osaka, Japan) for gifting us yeast extract, Mydol 10 (decyl glucoside), and restriction endonucleases, respectively. We thank Rio Izumi for her skillful technical assistance. We are grateful to Tomoyuki Kosaka and Mamoru Yamada for invaluable suggestions. We thank Osao Adachi and Hirohide Toyama for continuously encouraging our work on ALDH. This work was supported by KAKENHI grant number 23580115.

TABLES AND FIGURES IN CHAPTER THREE

Table 3.1. Summary of the purification of *Gluconacetobacter diazotrophicus* AldFGH complex from the triple deletion strain MR17 (PAL5 Δ*aldSLC* Δ*adhAB*ΔPQQ).

Purification step	Total protein (mg)	Specific activity (U/mg)		Total activity (U)		Yield (%)	
		Ferricyanide reductase	Q2 reductase	Ferricyanide reductase	Q2 reductase	Ferricyanide reductase	Q2 reductase
Membranes	4,900	2.5	0.12	12,000	580	100	100
Solubilized	860	12	0.55	9,900	460	83	82
CM-Toyopearl	250	36	1.6	8,300	400	69	68
Hydroxyapatite	8.9	310	15	3,800	170	31	30
DEAE-Toyopearl	5.7	350	20	2,500	120	21	21

The activity was assayed in the standard conditions described in Materials and Method

Table 3.2. Summary of purification of the AldGH subcomplex from recombinant *Acetobacter pasteurianus* mNS4[a].

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)
Crude soluble fraction	1,000	0.64	660	100
DEAE-Toyopearl	95	6.1	580	88
Phenyl-Toyopearl	-	-	280	42
Hydroxyapatite	1.8	100	180	28

[a]Acetaldehyde:ferricyanide reductase activity was measured at pH 5.0.

[b]Accurate protein content was not determined because of the presence of ammonium sulfate that may affect the Lowry method.

Table 3.3. Summary of properties of ALDH complexes purified from *Gluconacetobacter diazotrophicus* strain PAL5.

Properties	Gomez-Manzo et al. 2010	This study
Sp. FC^{3-} reductase activity (U/mg)	800	300
Optimum pH for FC^{3-} reductase activity	3.5	4.0
Ubiquinone reductase	no	yes
Small subunit	no	yes
Heme staining	Middle subunit	Middle subunit
Heme C	3 Hemes C	3 Hemes C[a]
Heme B	1 Heme B	no
Reduction of cytochromes with the substrate	no	yes

[a]Determination of heme *c* showed 2.1 mols per mol of the AldFGH complex. However, we suggest that there are 3 mols of heme *c* per mol of the AldFGH complex, even though the nearest integer was 2, because impurities in the enzyme preparation may lower the experimentally observed value.

Table 3.4. Summary of properties of the ALDH complexes from *Komagataeibacter polyoxogenes* strain NBI1028 and the AldGH subcomplex of *Gluconacetobacter diazotrophicus* strain PAL5.[a]

Properties	Fukaya et al. 1989	This study
Sp. FC^{3-} reductase activity (U/mg)[b]	800	100
Optimum pH for FC^{3-} reductase activity	7.0	7.0[c]
Ubiquinone reductase	n.i.[d]	no
Subunits	Large and small subunits	Large and small subunits
Cytochromes	no	no
Absorption shoulder (nm)	~ 480	~ 480
Absorption changes upon redox	n.i.	yes

[a]*Komagataeibacter polyoxogenes* strain NBI1028 was formerly referred to as *Acetobacter polyoxogenes*.

[b]AldGH activity was routinely determined at pH 5.0 in this study, but at pH 6.0 in the study by Fukaya *et al*. The ferricyanide reductase activity at pH 5.0 would be half of that at pH 6.0.

[c]The ferricyanide reductase activity at pH 8.0 was higher than that at pH 7.0, but weak chemical reduction of ferricyanide by acetaldehyde occurred. We tentatively conclude that pH 7.0 is optimal for the ferricyanide reductase activity of the AldGH subcomplex.

[d]N.i., no information.

Table 3.5. Bacterial strains and plasmids used in this study.[a]

Strains and plasmids	Description	Source or reference
Bacterial strains		
Gluconacetobacter diazotrophicus		
ATCC49037	Wild type, synonym of PAL5	ATCC, (Yamada *et al.*, 1997)
MR17	PAL5 Δ*aldSLC* Δ*adhAB* Δ*pqqABCDE*	(Miah *et al.*, 2021)
MR25	PAL5 Δ*aldSLC* Δ*adhAB* Δ*pqqABCDE* Δ*aldF*	This study
Acetobacter pasteurianus		
SKU1108	Wild type, synonym of NBRC101655	(Saeki et al., 1997)
mNS4	SKU1108 Δ*adhAB* Δ*aldSLC* Δ*aldFGH*	SN et al., unpublished
Plasmids		
pT7Blue	Cloning vector, Ap^R	Novagen
pRK2013	Plasmid mediates plasmid transfer, Km^R	(Figurski et al., 1979)
pK18mobsacB	A suicide vector, *mob*, *sacB*, Km^R	(Schäfer et al., 1994)
pCM62	A broad host range plasmid, *mob lacZ*α, Tc^R	(Marx et al., 2001)
pMRG	pK18mobsacB, the 2-kb fragment containing the Δ*aldF* allele	This study
pTM8	pCM62 *aldGH*	This study
pTM10	pCM62 *aldH*	This study
pTM14	pCM62 *aldF* with putative *aldFGH* promoter in the opposite orientation to the *lac* promoter	This study
pTM15	pT7Blue *aldFGH* with putative *aldFGH* promoter	This study
pTM16	pCM62 *aldFGH* with putative *aldFGH* promoter in the opposite orientation to the *lac* promoter	This study

[a] Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R, tetracycline resistance.

Table 3. S1. Q_2 reductase activity of the reconstituted AldFGH complex.

Sample	Protein amount in the assay mix (ng)		Q_2 reductase activity in the assay mix (mU)
	AldGH	AldF	
Purified AldGH subcomplex	96	-	n.d.[a]
Partially purified AldF	-	88×10^3	n.d.
Reconstituted	96	88×10^3	2.6 ± 0.4

Table 3.S2. Oligonucleotides used in this study.

Name	Sequence (5' to 3')	Objective
Pal5-ex-aldF-Hin(+)	aagcttttcccgtccgcaggac	
Pal5-aldF-SEQ4000(+)	ccttcccgttcgaccatttc	
Pal5-aldF-SEQ4500(+)	tctatctggccgacctggac	
Pal5-ex-aldG-BamHin(+)	ggatccaagcttgagatgacagacggagcc	Expression of *aldGH*
Pal5-ex-aldH-Hin(+)	aagcttcgcgacgccatcaagaag	Expression of *aldH*
Pal5-ex-aldG-Kpn(-)	ggtacctacccattggacggttcc	
Pal5-aldH-SEQ6100(+)	ggtcgaggggatggtctatg	
Pal5-aldH-SEQ6600(+)	aggacaagatcgtcctgcgc	
Pal5-aldH-SEQ7600(+)	acgctatcttcaacgccgtc	
Pal5-ex-aldH-Xba(-)	tctagattcaggttgatgcggtgccg	Expression of *aldFGH*, *aldGH*, and *aldH*
Pal5-aldpro-RI(+)	gaattcatcgtgacggccgatgg	Expression of *aldFGH* and *aldF*
Pal5-ex-aldF-Xba(-)	tctagatgtcggggctccgtctg	Expression of *aldF*
Pal5-D-aldF-Hin(+)	aagcttcgctatgacgggc	Construction of Δ*aldF*
Pal5-D-aldF-5-RI(-)	gaattcgatgctggcggtgtcg	Construction of Δ*aldF*
Pal5-D-aldF-3-RI(+)	gaattctaccattccctgaccg	Construction of Δ*aldF*
Pal5-D-aldF-Xba(-)	catctagaccgaccagcttc	Construction of Δ*aldF*

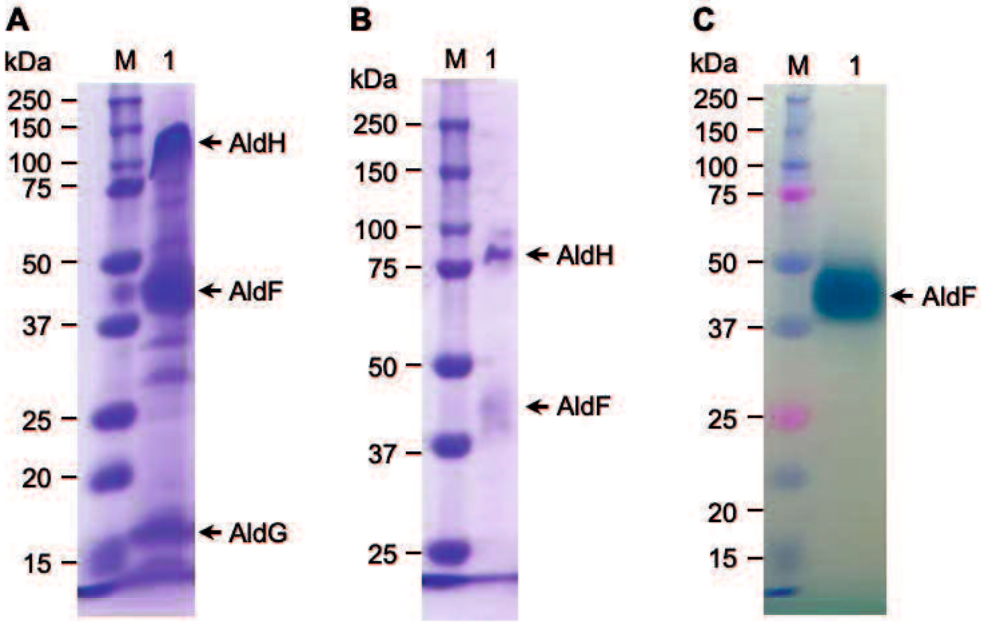


Figure 3.1. SDS-PAGE of the purified AldFGH complex of *Gluconacetobacter diazotrophicus* strain MR17 (PAL5 Δ*aldSLC* Δ*adhAB* ΔPQQ). A. Thirty-three micrograms of purified protein separated by SDS-PAGE [10% (w/v) acrylamide] followed by staining with Coomassie Brilliant Blue R-250. B. Similar to panel A, but 2 µg of purified protein and 7.0% (w/v) acrylamide. C. Twenty-two micrograms of purified protein, and staining for heme-dependent peroxidase activity using tetramethylbenzidine.

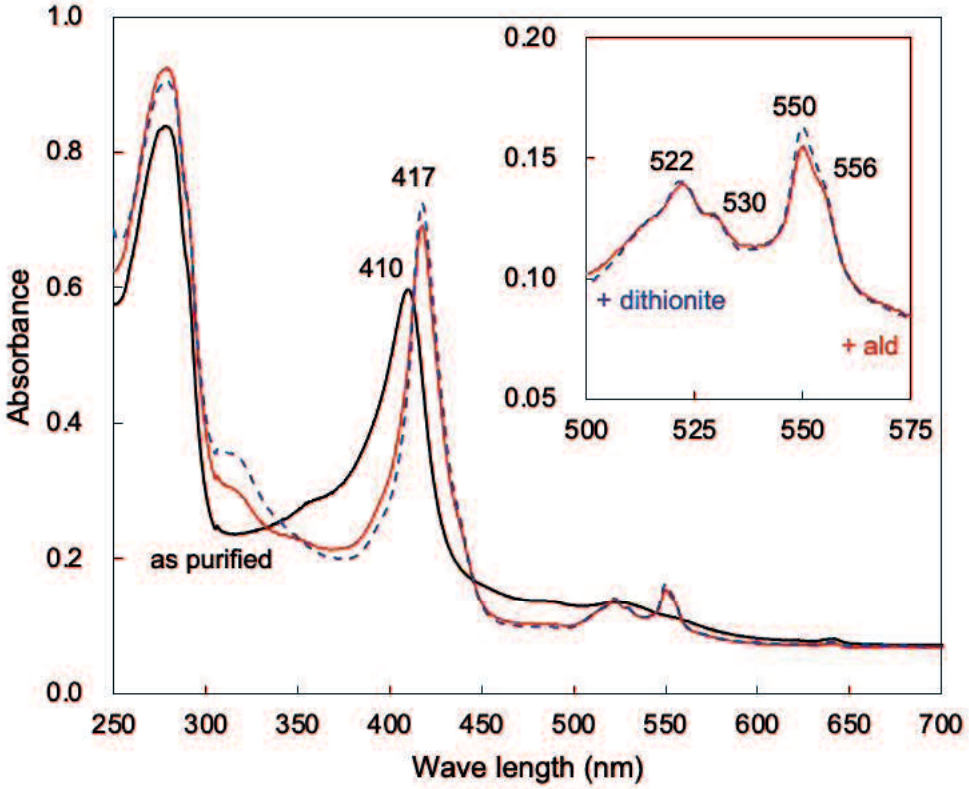


Figure 3.2. Absorption spectra of purified AldFGH complex from *Ga. diazotrophicus* strain MR17. Absorption spectra from 250 to 700 nm with enzyme concentration 0.7 mg protein mL^{-1} in 10 mM K^+-phosphate buffer (pH 6.0). The solid black line is the absorption spectrum of the "as purified" AldFGH complex. The AldFGH complex was reduced with 10 mM acetaldehyde (solid-red line), then, a few grains of sodium dithionite were added to the protein solution to chemically reduce the AldFGH complex (dashed-blue line). Inset: Enlarged absorption spectra of acetaldehyde-reduced and dithionite-reduced AldFGH complex from 500 to 575 nm.

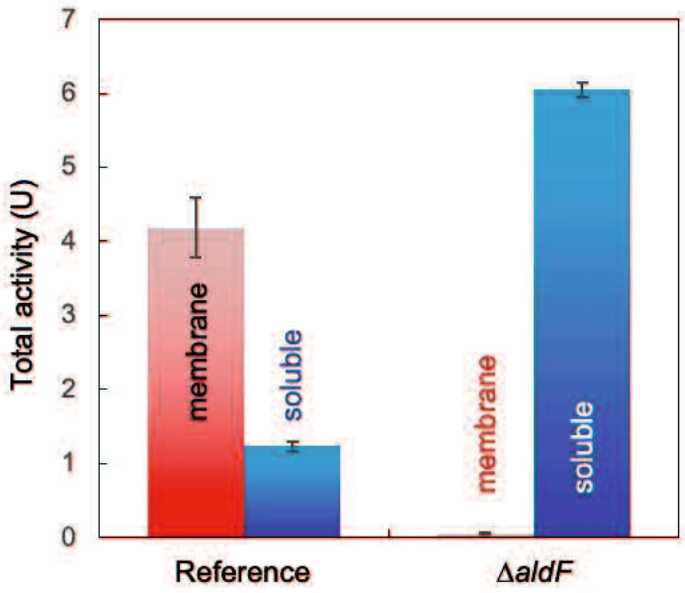


Figure 3.3. AldF subunit is responsible for membrane binding of the AldFGH complex. *Ga. diazotrophicus* strain MR17, referred to as the Reference strain, and strain MR25 (MR17 Δ*aldF*), referred to as the Δ*aldF* strain, were cultivated in 100-mL YPG medium. The membranes (red) and the crude soluble fraction (blue) were separated by ultracentrifugation. Acetaldehyde:ferricyanide oxidoreductase activity was determined at pH 6.0. Mean values and standard deviations from triplicate assays are shown.

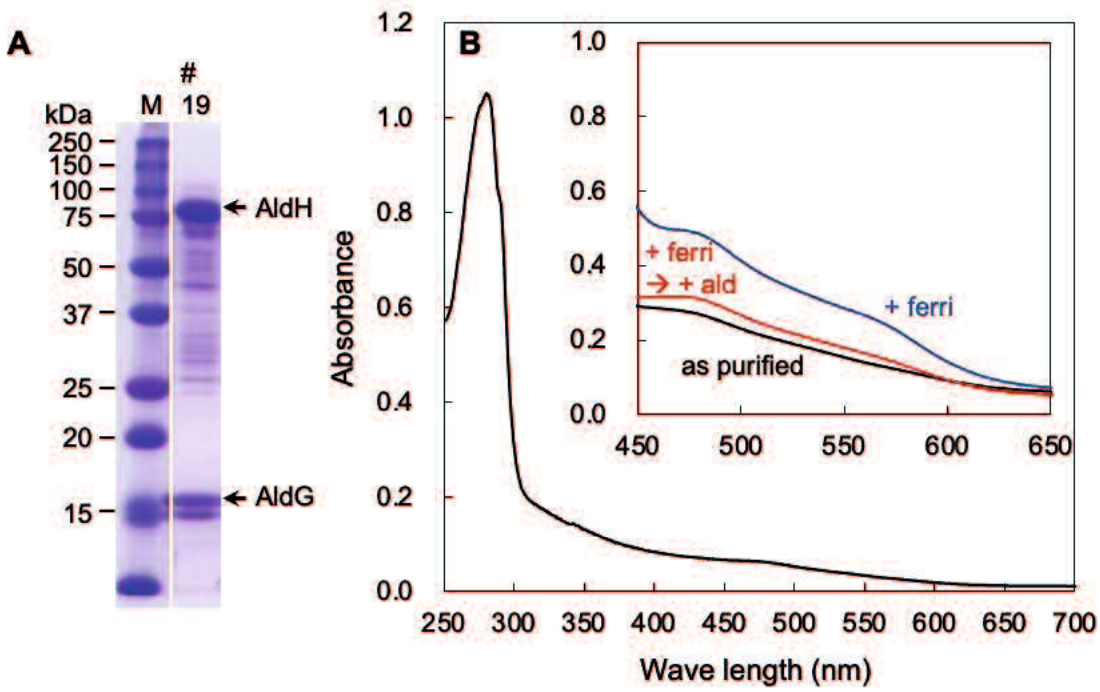


Figure 3.4. [Fe–S] cluster of the AldGH subcomplex is reduced by acetaldehyde. A. SDS-PAGE of fraction number 19 from Superdex 200 gel filtration chromatography of the AldGH subcomplex. Thirty micrograms of protein were separated by SDS-PAGE, followed by staining with Coomassie Brilliant Blue R-250. B. Absorption spectrum from 250 to 700 nm of the "as purified" fraction number 19 (0.55 mg protein mL^{-1}). Inset: Absorption spectrum from 450 to 650 nm of "as purified" fraction number 19 (2.2 mg protein mL^{-1}) (black line). Potassium ferricyanide was added to a final concentration of 0.5 mM (blue line). Then, acetaldehyde was added to a final concentration of 1 mM (red line).

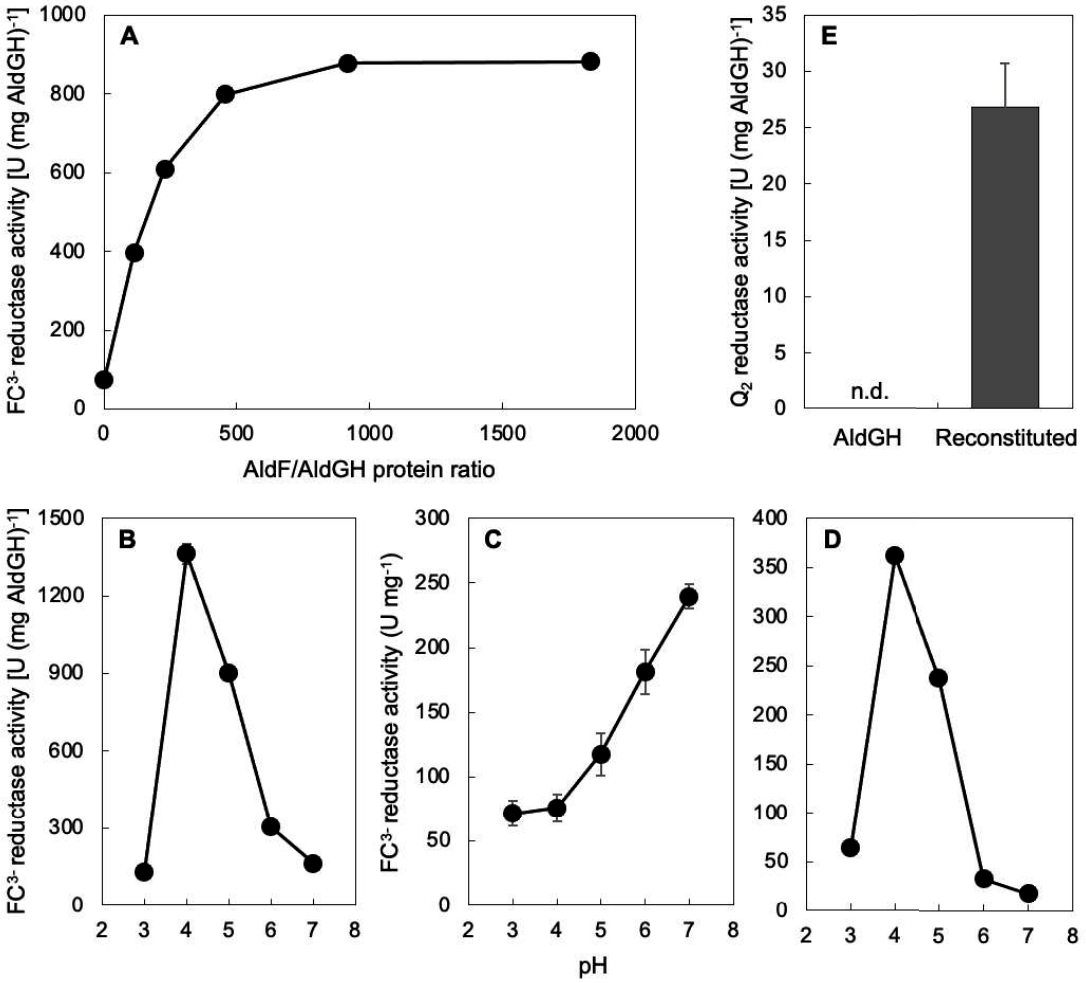


Figure 3.5. Dissection of the AldFGH complex and reconstitution of the AldGH subcomplex with AldF. (A) Saturation curve of the ferricyanide (FC^{3-}) reductase activity as a function of a protein ratio in milligrams of partially purified AldF to the purified AldGH subcomplex. The mixture of the AldGH subcomplex and AldF was incubated at 25°C for 10 min to accelerate reconstitution. Next, the acetaldehyde: FC^{3-} oxidoreductase activity of the mixture was assayed at pH 4.0. (B) Specific FC^{3-} reductase activity of the reconstituted AldFGH complex (protein ratio of 920) as a function of pH. (C) Specific FC^{3-} reductase activity of the purified AldFG subcomplex as a function of pH for comparison with the data in panel B. (D) Specific FC^{3-} reductase activity of the purified AldFG subcomplex as a function of pH for comparison with the data in panel B. (E) Q2 reductase activity of the purified AldGH subcomplex (AldGH) and the reconstituted AldFGH complex (protein ratio of 920, Reconstituted) at pH 4.0. Mean values and standard deviations from triplicate assays are shown as the specific activity to the protein amount of AldGH (A to C and E) or AldFGH (D). n.d., not detected.

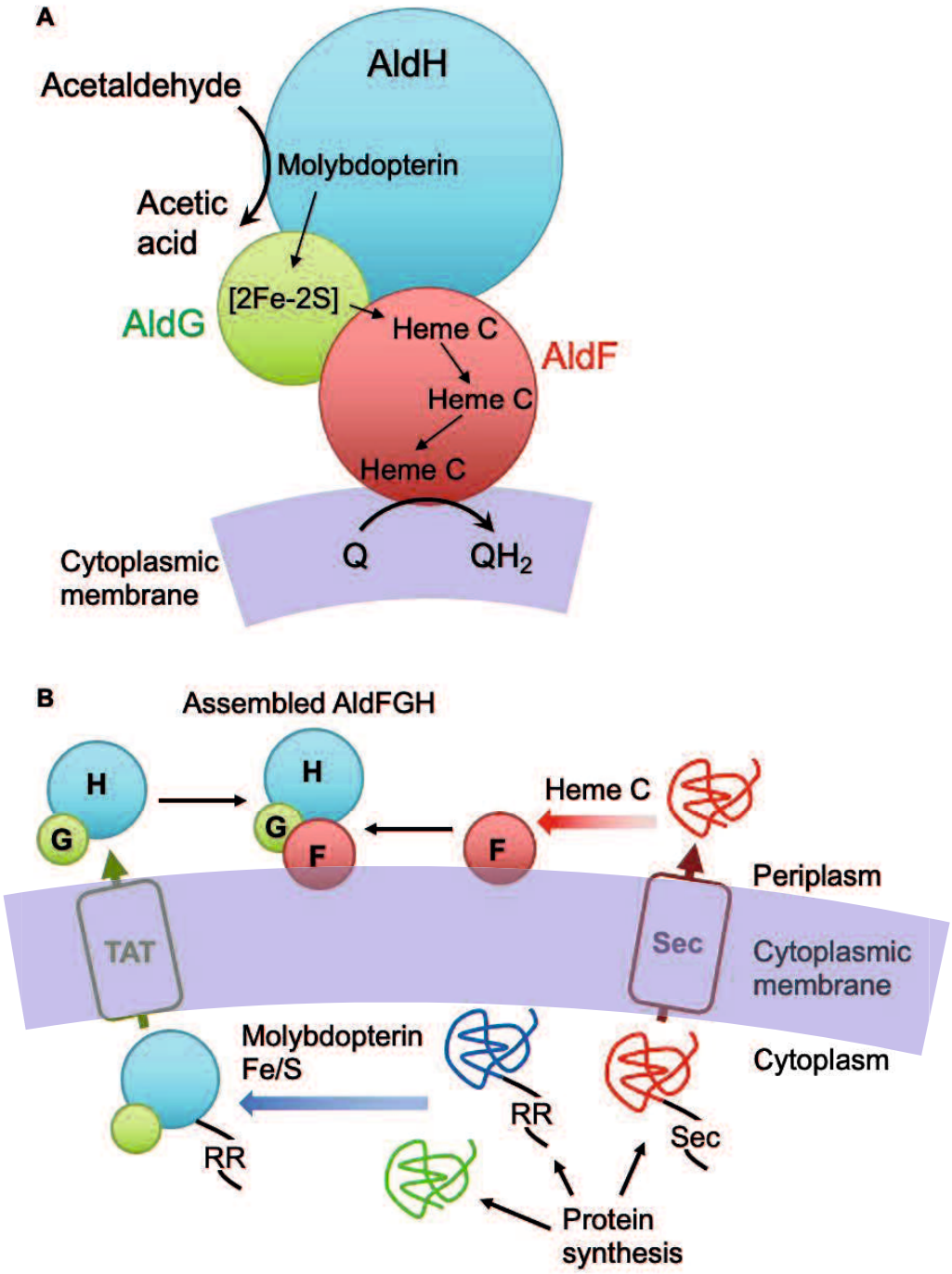


Figure 3.6. Models of intramolecular electron transport (A) and molecular assembly (B) of the AldFGH complex of *Ga. diazotrophicus*. A. A molybdopterin cofactor in the AldH subunit is the site of acetaldehyde oxidation. Electrons from molybdopterin are transferred to the [Fe–S] cluster in the AldG subunit, presumably the [2Fe–2S] cluster suggested previously (7), and then they are transferred to *c*-type cytochrome centers in the AldF subunit, which is responsible for the membrane binding of the complex and ubiquinone reduction. B. All the Ald subunits are synthesized in the cytoplasm. AldH and AldG are folded and

assembled with a molybdopterin cofactor and an [Fe–S] cluster, respectively, followed by complex formation of AldG and AldH in the cytoplasm. The folded, assembled, and complexed AldG and AldH precursor is translocated to the periplasm via the TAT ("twin arginine transport") protein translocator. The AldF precursor is translocated to the periplasm via the Sec translocator without protein folding or assembly with any cofactors. After translocation, the hemes *c* are covalently attached to the polypeptide, the AldF subunit is folded, and the protein associates with the cytoplasmic membrane. Finally, the AldGH subcomplex and the AldF subunit meet at the cytoplasmic membrane to form the AldFGH complex. TAT, the TAT protein translocator; Sec, the Sec protein translocator.

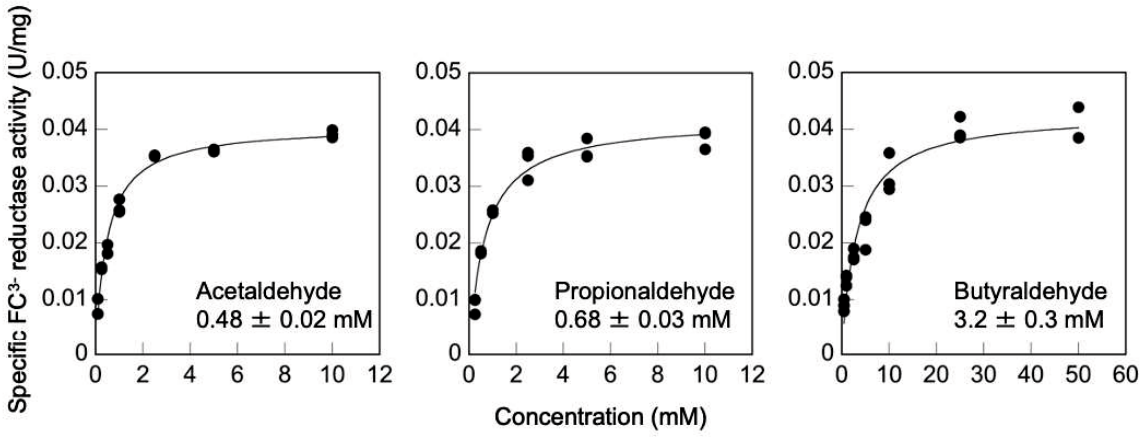


Figure 3.S1. Determination of K_M values of AldGH for short-chain aldehydes. Aldehyde:ferricyanide (FC^{3-}) reductase activity at pH 6.0 of the crude soluble fraction of *Gluconacetobacter diazotrophicus* PAL5 quadruple deletion derivative (Δ*aldF* Δ*aldSLC* Δ*adhAB* ΔPQQ) was assayed with deferent concentrations of acetaldehyde, propionaldehyde, and butyraldehyde. The V_{max} and K_M values were determined on KaleidaGraph v4.5 (Synergy Software, Reading, PA, USA).

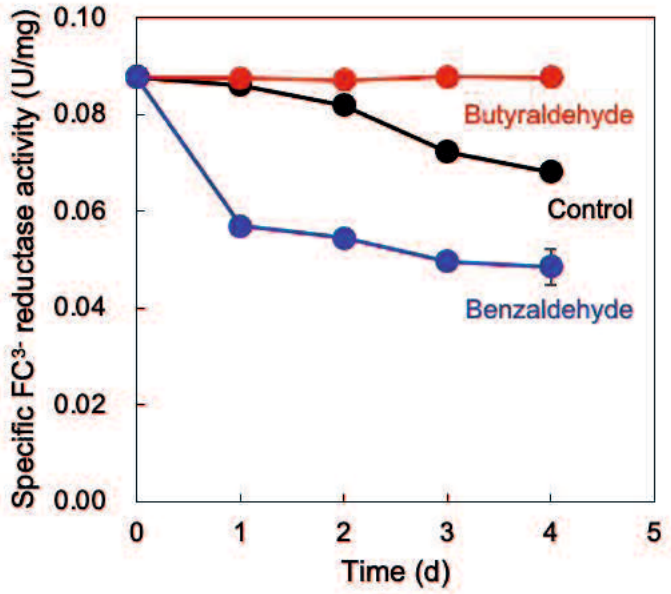


Figure 3.S2. Effect of butyraldehyde on stability of AldGH. The crude soluble fraction of *Gluconacetobacter diazotrophicus* PAL5 quadruple deletion derivative (Δ*aldF* Δ*aldSLC* Δ*adhAB* ΔPQQ) was kept at 4°C for four days in the presence of 2 mM butyraldehyde (red line) or 50 mM benzaldehyde (blue line; Adachi et al., 1980) or in the absence of aldehydes (black line). Aldehyde:ferricyanide (FC^{3-}) reductase activity at pH 6.0 was assayed once a day. Mean value and standard deviation from triplicate enzyme assay are shown.

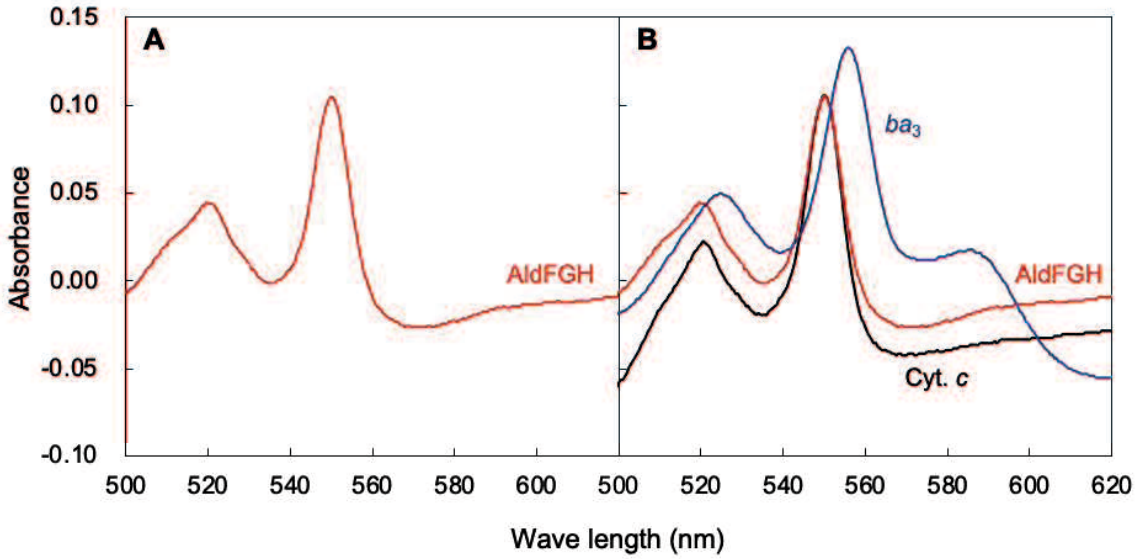


Figure 3.S3. Reduced minus oxidized difference spectrum of pyridine hemochrome of the AldFGH complex. The purified AldFGH complex of 0.30 mg protein mL^{-1} were treated with pyridine and NaOH. Reduced minus oxidized difference spectrum of the treated sample was recoded (red trace in A and B). The heme C contents in the AldFGH complex were calculated at 2.1 mol heme C (mol AldFGH complex)$^{-1}$, if considered the molecular mass is 142 kDa. For comparison, horse heart cytochrome *c* of 60 µg protein mL^{-1} were used for determination of heme C (black trace in B). The heme C contents in cytochrome *c* were calculated at 1.0 mol heme C (mol cytochrome *c*)$^{-1}$, if considered the molecular mass is 12 kDa. For another comparison, hemes B and A were extracted from partially purified cytochrome ba_3 ubiquinol oxidase. Reduced minus oxidized difference spectrum of the extracted hemes was recoded (blue trace in B). By comparison of the three spectra, it is plausibly concluded that the purified AldFGH complex does not contain heme B.

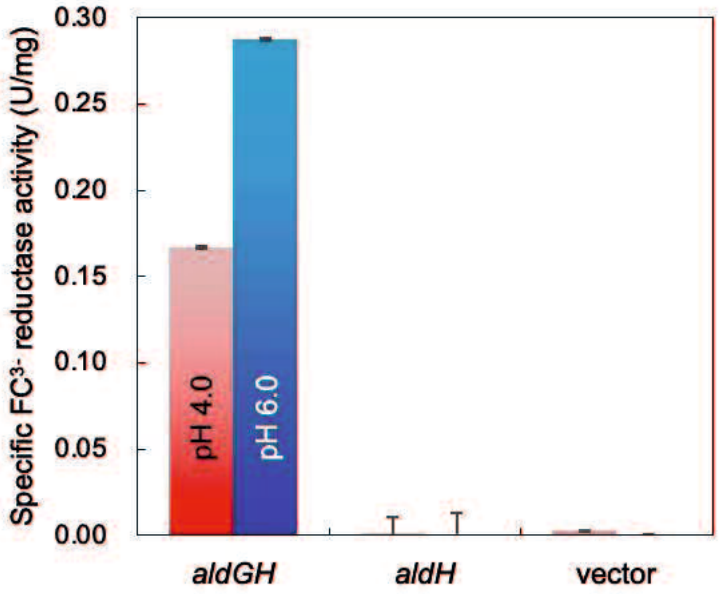


Figure 3.S4. The *aldG* gene is essential for ALDH activity. *A. pasteurianus* mNS4 strain (Δ*adhAB* Δ*aldFGH* Δ*aldSLC*) harboring pTM8 (*aldGH*$^{+}$), pTM10 (*aldH*$^{+}$), and pCM62 (vector control) were cultivated in the YPGD medium containing 50 μg mL^{-1} tetracycline at 30°C overnight. Acetaldehyde:ferricyanide (FC^{3-}) oxidoreductase activity of the crude soluble fraction was measured at pH 4.0 and 6.0.

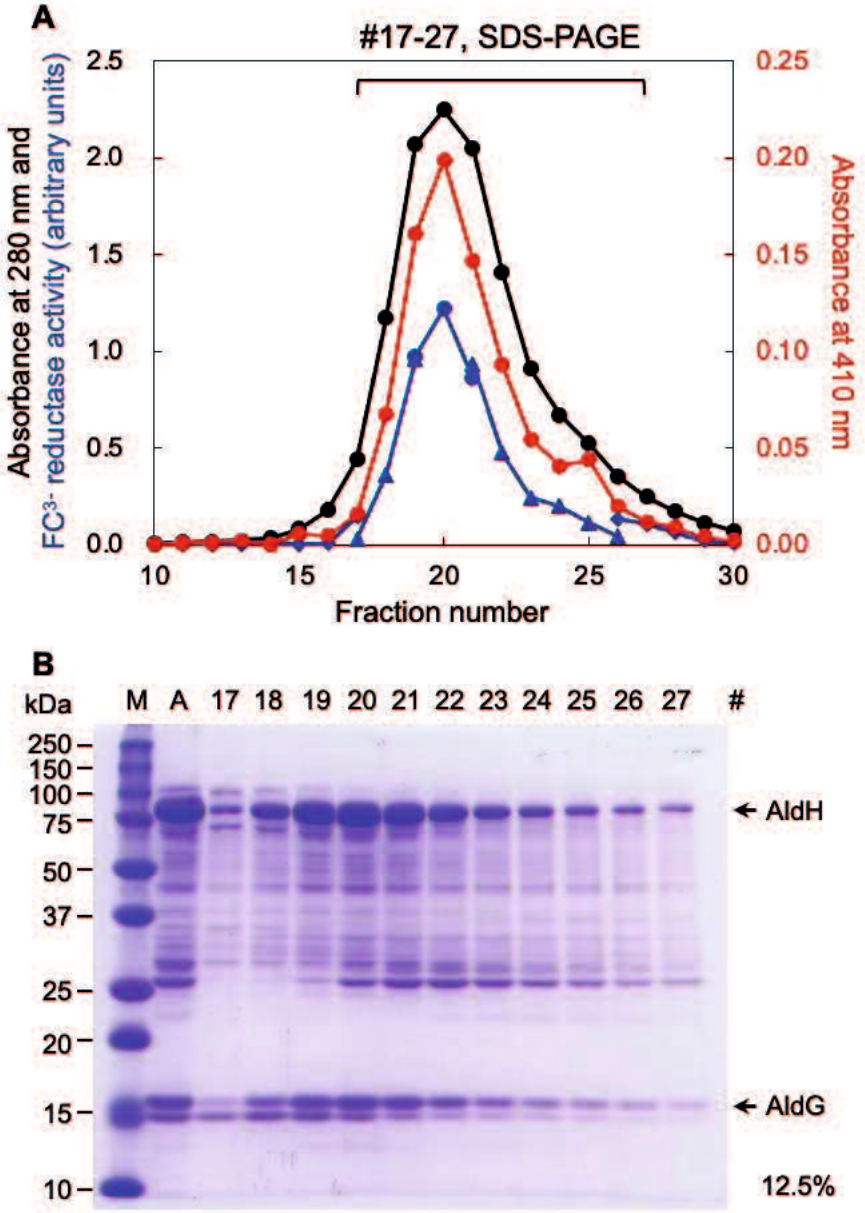


Figure 3.S5. Superdex 200 gel filtration column chromatography of AldGH. The purified AldGH subcomplex (0.19 mg mL^{-1}) was concentrated with AmiconUltra (50 k). Two hundred microliter of the concentrated AldGH (8.9 mg protein mL^{-1}) was applied on Superdex 200 (10/300) and eluted with 50 mM K^{+}-phosphate (pH 6.0) containing 2 mM butyraldehyde. A. Acetaldehyde:ferricyanide (FC^{3-}) reductase activity at pH 5.0 (blue), absorbances at 280 nm (black) and 410 nm (red) are shown. B. Six microliter of fraction number 17 to 27 was applied on SDS-PAGE (12.5% acrylamide) and stained with Coomassie brilliant blue R-250. The molecular mass standard and the concentrated purified AldGH subcomplex prior to gel filtration (11 µg protein) were loaded to the lanes M and A, respectively.

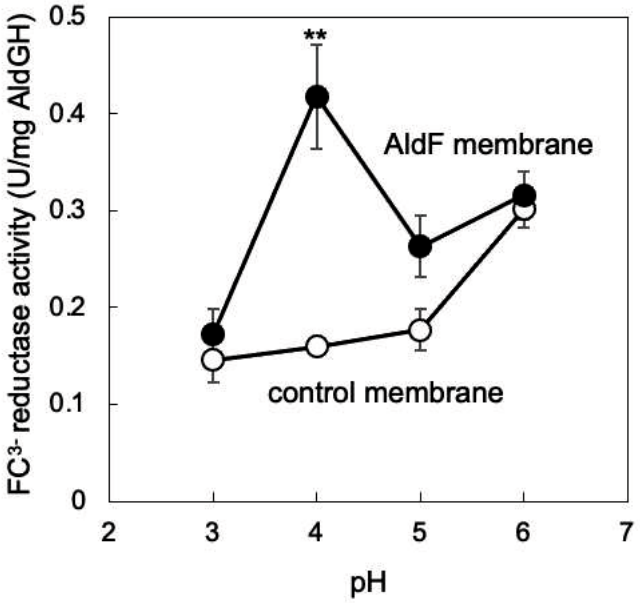


Figure 3.S6. Effect of AldF on ferricyanide reductase activity of AldGH. The crude soluble fraction of *Acetobacter pasteurianus* mNS4 triple deletion derivative (Δ*adhAB* Δ*aldSLC* Δ*aldFGH*) harboring pTM8 (*aldGH*$^{+}$) was incubated with the membrane fraction of the mNS4 strain harboring either pCM62 (vector control) or pTM14 (*aldF*$^{+}$). One volume of the soluble fraction of AldGH (7.2 mg protein mL^{-1}) and one volume of the membrane suspension (13 mg protein mL^{-1} for control membrane; 16 mg protein mL^{-1} for AldF membrane) were mixed and incubated fat 30°C for 10 min. Aldehyde:ferricyanide (FC^{3-}) reductase activity at pH 3.0 to 6.0 was assayed. The specific activity was calculated to the soluble fraction containing AldGH. Mean value and standard deviation from triplicate enzyme assay are shown. Asterisks indicate *P* values between the AldF membrane and the control membrane using Student's *t* test. *, $P < 0.05$; **, $P < 0.01$.

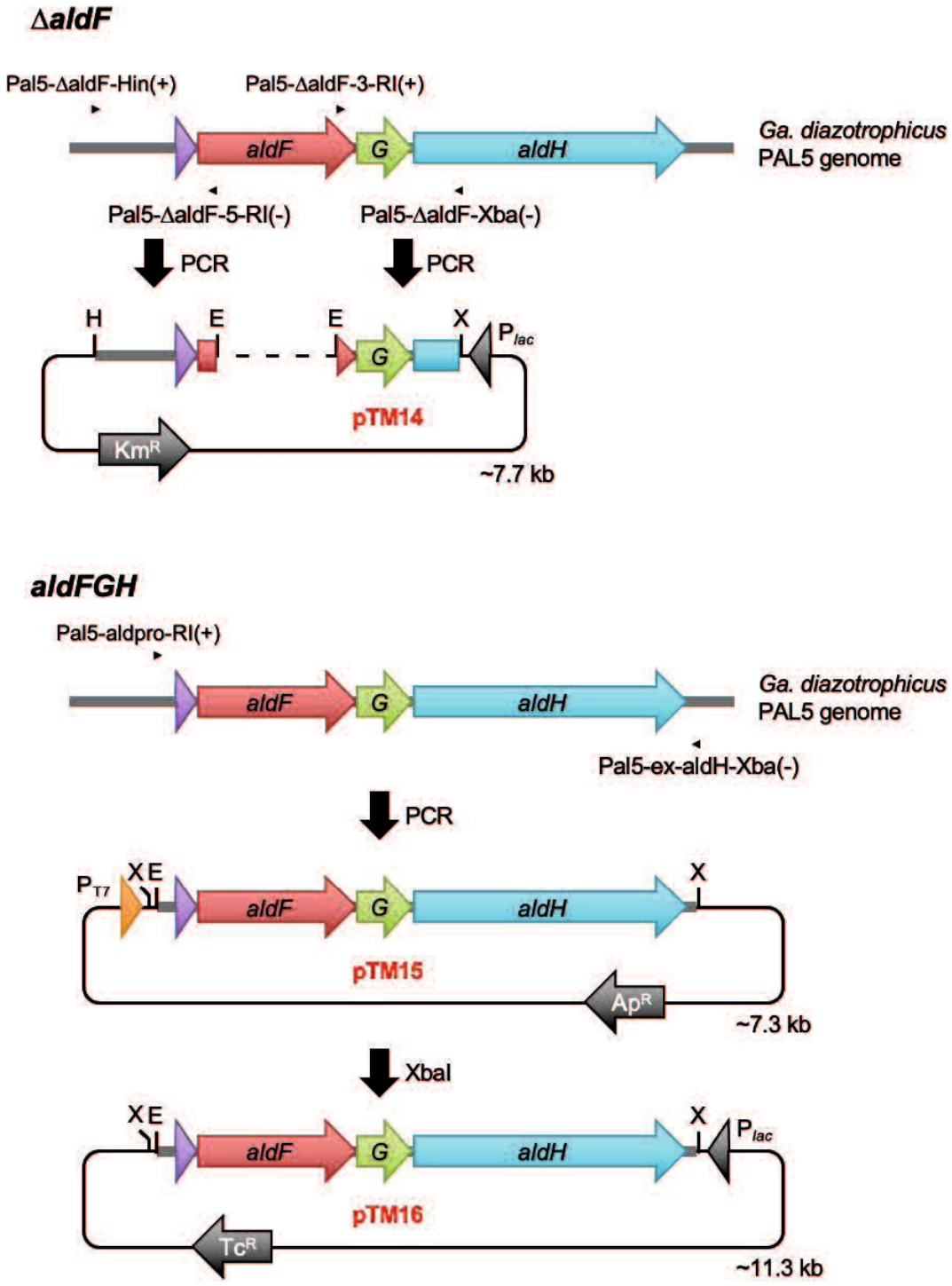

ΔaldF
Pal5-ΔaldF-Hin(+)
Pal5-ΔaldF-3-RI(+)
aldF
G
aldH
Ga. diazotrophicus
PAL5 genome
Pal5-ΔaldF-5-RI(-)
Pal5-ΔaldF-Xba(-)
PCR
PCR
H
E
E
X
Plac
G
pTM14
KmR
~7.7 kb
aldFGH
Pal5-aldpro-RI(+)
aldF
G
aldH
Ga. diazotrophicus
PAL5 genome
Pal5-ex-aldH-Xba(-)
PCR
PT7
X E
X
aldF
G
aldH
pTM15
ApR
~7.3 kb
XbaI
X E
X
Plac
aldF
G
aldH
pTM16
TcR
~11.3 kb

Figure 3. S7.

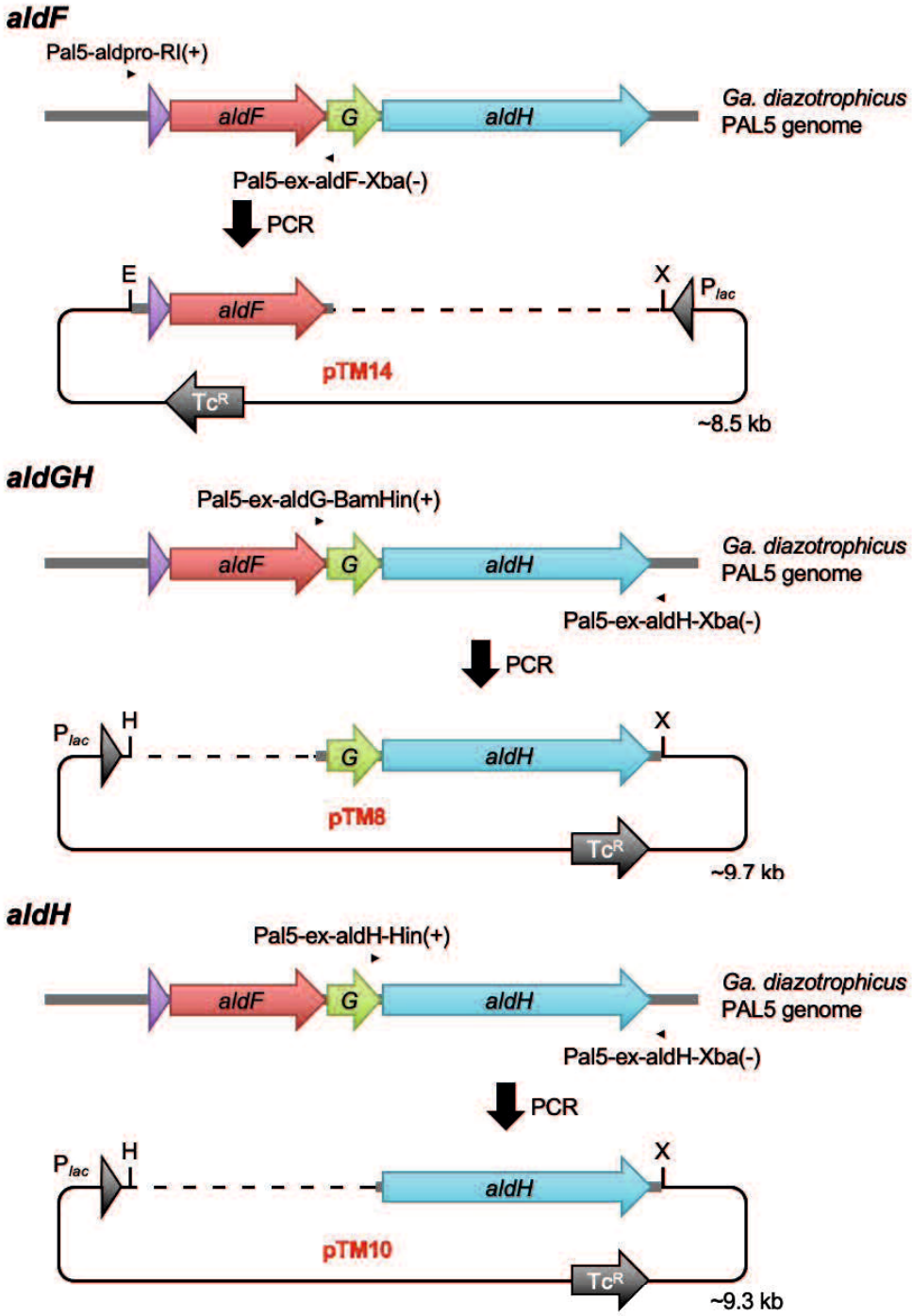


Figure 3.S7. Construction of plasmids used in this study. All of the DNA fragments used in this study were prepared by PCR using genomic DNA of *Gluconacetobacter diazotrophicus* PAL5. The positions where each DNA primer binds to the genomic are shown by black arrow heads with identification of the primer name. H, HindIII; X, XbaI; E, EcoRI; purple arrowhead, putative promoter region for *aldFGH*; red arrow, *aldF*; green arrow, *aldG*; blue arrow, *aldH*; gray arrowheads, *lac* promoter; orange arrowhead, T7 promoter; gray arrow, antibiotics resistance gene; Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Tc^R, tetracycline resistance; dashed line, no DNA.

REFERENCES

Adachi, O., Tayama, K., Shinagawa, E., Matsushita, K., Ameyama, M. 1980. Purification and characterization of membrane-bound aldehyde dehydrogenase from *Gluconobacter suboxydans*. Agric Biol Chem 44: 503-15.

Adachi, O., Yoshitaka, A., Hirohide, T., Matsushita, K. 2007. Biooxidation with PQQ - and FAD - Dependent Dehydrogenases. Modern. Bioox.: Enz., React. and Appl. 6:1- 41.

Adachi, O., Yakushi, T. 2016. Membrane-bound dehydrogenases of acetic acid bacteria. *in* Acetic Acid Bacteria: Ecology and Physiology (Matsushita, K., Toyama, H., Tonouchi, N., Okamoto-Kainuma, A. eds.), Springer Japan, Tokyo. pp 273-97.

Ameyama, M., Matsushita, K., Ohno, Y., Shinagawa, E., Adachi, O. 1981. Existence of a novel prosthetic group, PQQ, in membrane-bound, electron transport chain-linked, primary dehydrogenases of oxidative bacteria. FEBS Lett 130: 179-83.

Andres-Barrao, C., Falquet, L., Calderon-Copete, S.P., Descombes, P., Ortega Perez, R., Barja, F. 2011. Genome sequences of the high-acetic acid-resistant bacteria *Gluconacetobacter europaeus* LMG 18890^{T} and *G. europaeus* LMG 18494 (reference strains), *G. europaeus* 5P3, and *Gluconacetobacter oboediens* 174Bp2 (isolated from vinegar). J Bacteriol 193: 2670-671.

Armstrong, J.M. 1964. The molar extinction coefficient of 2,6-dichlorophenol indophenol. Biochim Biophys Acta 86: 194-97.

Berry EA, Trumpower BL. 1987. Simultaneous determination of hemes *a*, *b*, and *c* from pyridine hemochrome spectra. Anal Biochem 161:1-15.

Boyer, H.W., Roulland-Dussoix, D. 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41: 459-72.

Cavalcante, V.A., Dobereiner, J. 1988. A new acid-tolerant nitrogen-fixing bacterium associated with sugarcane. Plant and Soil 108: 23-31.

Dulley, J.R., Grieve, P.A. 1975. A simple technique for eliminating interference by detergents in the Lowry method of protein determination. Anal Biochem 64: 136-41.

Figurski, D.H., Helinski, D.R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc Natl Acad Sci U S A. 76:1648-52.

Fukaya M, Tayama K, Okumura H, Kawamura Y, Beppu T. 1989. Purification and characterization of membrane-bound aldehyde dehydrogenase from *Acetobacter polyoxogenes* sp. nov. Appl Microbiol Biotechnol 32:176-80.

Fukaya, M., Park, Y.S., and Toda, K. 2008. Improvement of acetic acid fermentation by molecular breeding and process development. J. Appl. Microbiol 73. 447 - 54.

Gillis, M., Kersters, K., Hoste, B., Janssens, D., Kroppenstedt, R.M., Stephan, M.P., Tetxeira, K.R.S., Dobereiner, J., Ley, J. 1989. *Acetobacter diazotrophicus* sp. nov, a nitrogen-fixing acetic-acid bacterium associated with sugarcane. Int J Syst Bacteriol 39: 361-64.

Giongo, A., Tyler, H.L., Zipperer, U.N., Triplett, E.W. 2010. Two genome sequences of the same bacterial strain, *Gluconacetobacter diazotrophicus* PAl 5, suggest a new standard in genome sequence submission. Stand Genomic Sci 2:309-17.

Gómez-Manzo, S., Chavez-Pacheco, J.L., Contreras-Zentella, M., Sosa-Torres, M.E., Arreguín-Espinosa, R., Pérez de la Mora, M., Membrillo-Hernández, J., Escamilla, J.E. 2010. Molecular and catalytic properties of the aldehyde dehydrogenase of *Gluconacetobacter diazotrophicus*, a quinoheme protein containing pyrroloquinoline quinone, cytochrome *b*, and cytochrome *c*. J Bacteriol 192: 5718-24.

Gómez-Manzo, S., Contreras-Zentella, M., González-Valdez, A., Sosa-Torres, M., Arreguín-Espinoza, R., Escamilla-Marván, E. 2008. The PQQ-alcohol dehydrogenase of *Gluconacetobacter diazotrophicus*. Int J Food Microbiol 125: 71-78.

Gómez-Manzo, S., Escamilla, J.E., González-Valdez, A., López-Velázquez, G., Vanoye-Carlo, A., Marcial-Quino, J., de la Mora-de la Mora, I., Garcia-Torres, I., Enríquez-Flores, S., Contreras-Zentella, M.L., Arreguín-Espinosa, R., Kroneck, P.M., Sosa-Torres, M.E. 2015. The oxidative fermentation of ethanol in *Gluconacetobacter diazotrophicus* is a two-step pathway catalyzed by a single enzyme: alcohol-aldehyde dehydrogenase (ADHa). Int J Mol Sci 16:1293-1311.

Goosen, N., Horsman, H.P., Huinen, R.G., van de Putte, P. 1989. *Acinetobacter calcoaceticus* genes involved in biosynthesis of the coenzyme pyrrolo-quinoline-quinone: nucleotide sequence and expression in *Escherichia coli* K-12. J Bacteriol 171: 447-55.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166: 557-80.

Huber R, Hof P, Duarte RO, Moura JJ, Moura I, Liu MY, LeGall J, Hille R, Archer M, Romão MJ. 1996. A structure-based catalytic mechanism for the xanthine oxidase family of molybdenum enzymes. Proc Natl Acad Sci U S A 93:8846-51.

Kanchanarach, W., Theeragool, G., Yakushi, T., Toyama, H., Adachi, O., Matsushita, K. 2010. Characterization of thermotolerant *Acetobacter pasteurianus* strains and their quinoprotein alcohol dehydrogenases. Appl Microbiol Biotechnol 85: 741-51.

Kawai, S., Goda-Tsutsumi, M., Yakushi, T., Kano, K., Matsushita, K. 2013. Heterologous overexpression and characterization of a flavoprotein-cytochrome *c* complex fructose dehydrogenase of *Gluconobacter japonicus* NBRC3260. Appl Environ Microbiol 79: 1654-60.

Kondo, K., Beppu, T., Horinouchi, S. 1995. Cloning, sequencing, and characterization of the gene encoding the smallest subunit of the three-component membrane-bound alcohol dehydrogenase from *Acetobacter pasteurianus*. J Bacteriol 177: 5048–5055.

Kostner, D., Peters, B., Mientus, M., Liebl, W., Ehrenreich, A. 2013. Importance of *codB* for new *codA*-based markerless gene deletion in *Gluconobacter* strains. Appl Microbiol Biotechnol 97: 8341-49.

Kranz RG, Richard-Fogal C, Taylor JS, Frawley ER. 2009. Cytochrome *c* biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. Microbiol Mol Biol Rev 73:510-28, Table of Contents.

Krajewski V, Simic P, Mouncey NJ, Bringer S, Sahm H, Bott M. 2010. Metabolic engineering of *Gluconobacter oxydans* for improved growth rate and growth yield on glucose by elimination of gluconate formation. Appl Environ Microbiol 76:4369–76.

Leimkühler, S., Angermüller, S., Schwarz, G., Mendel, R.R., Klipp, W. 1999. Activity of the molybdopterin-containing xanthine dehydrogenase of *Rhodobacter capsulatus* can be restored by high molybdenum concentrations in a *moeA* mutant defective in molybdenum cofactor biosynthesis. J Bacteriol 181: 5930-39.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5.

Li T, Bonkovsky HL, Guo JT. 2011. Structural analysis of heme proteins: implications for design and prediction. BMC Struct Biol 11:13.

Marmur J. 1961. A procedure for the isolation of deoxyribonucleic acid from micro-organisms. J Mol Biol 3:208-18.

Masud, U., Matsushita, K., Theeragool, G. 2010. Cloning and functional analysis of *adhS* gene encoding quinoprotein alcohol dehydrogenase subunit III from *Acetobacter pasteurianus* SKU1108. Int J Food Microbiol 138:39–49

Marx, C.J., Lidstrom, M.E. 2001. Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. Microbiology 147: 2065-75.

Matsushita, K., Ameyama, M. 1982. D-Glucose dehydrogenase from *Pseudomonas fluorescens*, membrane-bound. Methods Enzymol 89: 149-54.

Matsushita K, Shinagawa E, Adachi O, Ameyama M. 1990. Cytochrome a1 of acetobacter aceti is a cytochrome ba functioning as ubiquinol oxidase. Proc Natl Acad Sci U S A 87:9863-7.

Matsushita, K., Toyama, H., Adachi, O. 1994. Respiratory chains and bioenergetics of acetic acid bacteria. in Advances in Microbial Physiology (Rose, A.H., Tempest, D.W. eds.), Academic Press, London. pp 247-301.

Matsushita K, Yakushi T, Toyama H, Shinagawa E, Adachi O. 1996. Function of multiple heme *c* moieties in intramolecular electron transport and ubiquinone reduction in the quinohemoprotein alcohol dehydrogenase-cytochrome *c* complex of *Gluconobacter suboxydans*. J Biol Chem 271:4850-7.

Miah R, Nina S, Murate T, Kataoka N, Matsutani M, Matsushita K, Yakushi T. 2021. Major aldehyde dehydrogenase AldFGH of *Gluconacetobacter diazotrophicus* is independent of pyrroloquinoline quinone but dependent on molybdopterin for acetic acid fermentation. Appl Microbiol Biotechnol 105:2341-50.

Mitchell, S. F. and Burns C. W. 1979. Oxygen consumption in the epilimnia and hypolimnia of two eutrophic, warm-monomictic lakes. N. Z. J. Mar. Freshwater Res.13: 427-441.

Palmer T, Stansfeld PJ. 2020. Targeting of proteins to the twin-arginine translocation pathway. Mol Microbiol 113:861-71.

Puustinen A, Wikström M. 1991. The heme groups of cytochrome *o* from *Escherichia coli*. Proc Natl Acad Sci U S A 88:6122-6.

Redfearn ER. 1967. Isolation and determination of ubiquinone. Methods in Enzymology 10:381-84.

Rieder, C., Eisenreich, W., O'Brien, J., Richter, G., Götze, E., Boyle, P., Blanchard, S., Bacher, A., Simon, H. (1998) Rearrangement reactions in the biosynthesis of molybdopterin - An NMR study with multiply $^{13}C/^{15}N$ labelled precursors. *Eur J Biochem* 255, 24-36.

Saeki, A., Theeragool, G., Matsushita, K., Toyama, H., Lotong, N., Adachi, O. 1997. Development of thermotolerant acetic acid bacteria useful for vinegar fermentation at higher temperatures. Biosci Biotechnol Biochem 61: 138-45.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., Pühler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene 145: 69-73.

Takemura, H., Tsuchida, T., Yoshinaga, F., Matsushita, K., Adachi, O. 1994. Prosthetic group of aldehyde dehydrogenase in acetic acid bacteria not pyrroloquinoline quinone. Biosci Biotechnol Biochem 58: 2082-83.

Tamaki, T., Fukaya, M., Takemura, H., Tayama, K., Okumura, H., Kawamura, Y., Nishiyama, M., Horinouchi, S., Beppu, T. 1991. Cloning and sequencing of the gene cluster encoding two subunits of membrane-bound alcohol dehydrogenase from *Acetobacter polyoxogenes*. Biochim Biophys Acta 1088: 292-300.

Thomas PE, Ryan D, Levin W. 1976. An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. Anal Biochem 75:168-76.

Thurner, C., Vela, C., Thony-Meyer, L., Meile, L., Teuber, M. 1997. Biochemical and genetic characterization of the acetaldehyde dehydrogenase complex from *Acetobacter europaeus*. Arch Microbiol 168: 81-91.

Yakushi, T., Fukunari, S., Kodama, T., Matsutani, M., Nina, S., Kataoka, N., Theeragool, G., Matsushita, K. 2018. Role of a membrane-bound aldehyde dehydrogenase complex AldFGH in acetic acid fermentation with *Acetobacter pasteurianus* SKU1108. Appl Microbiol Biotechnol 102: 4549-61.

Yakushi, T., Matsushita, K. 2010. Alcohol dehydrogenase of acetic acid bacteria: structure, mode of action, and applications in biotechnology. Appl Microbiol Biotechnol 86: 1257-65.

Yamada, Y., Hoshino, K., Ishikawa, T. 1997. The phylogeny of acetic acid bacteria based on the partial sequences of 16S ribosomal RNA: the elevation of the subgenus *Gluconoacetobacter* to the generic level. Biosci Biotechnol Biochem 61: 1244-51.

Yamanaka T. 1992. The Biochemistry of Bacterial Cytochromes Springer Verlag, Tokyo.

LIST OF PUBLICATIONS

Chapter 2

Miah R, Nina S, Murate T, Kataoka N, Matsutani M, Matsushita K, Yakushi T. 2021. Major aldehyde dehydrogenase AldFGH of *Gluconacetobacter diazotrophicus* is independent of pyrroloquinoline quinone but dependent on molybdopterin for acetic acid fermentation. Applied Microbiology and Biotechnology 105:2341-50.

Chapter 3

Miah R, Nina S, Murate T, Kataoka N, Matsutani M, Ano Y, Matsushita K and Yakushi T. 2022. Dissection and reconstitution provide insights into electron transport in the membrane-bound aldehyde dehydrogenase complex of *Gluconacetobacter diazotrophicus*. Journal of Bacteriology (Accepted) doi:10.1128/jb.00558-21.