MOLECULAR STUDIES ON TRIMETHYLAMINOBUTANOL DEHYDROGENASE AND TRIMETHYLAMINOBUTYRALDEHYDE DEHYDROGENASE PRODUCED BY *PSEUDOMONAS* SP. 13CM

(*Pseudomonas* sp. 13CM のトリメチルアミノブタノール脱水素酵素
及び
トリメチルアミノブチルアルデヒド脱水素酵素の分子的研究)

MD. REZAUL BARI

2013

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A Dissertation

By

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DEDICATION

I would like to dedicate this humble thesis to

My beloved wife Rumana Yeasmin
and
my sweet daughter Rihana Bari Mimnoon

For their generous love and support

CONTENTS

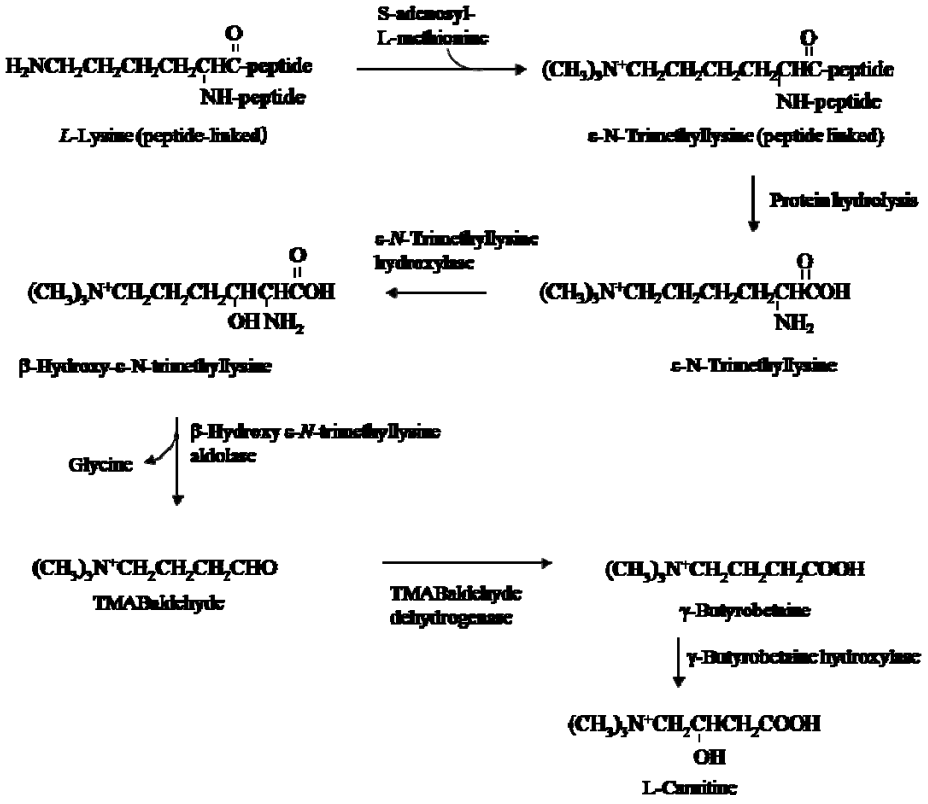
LIST OF ABBREVIATIONS

DMABaldehyde	= 4-dimethylaminobutyraldehyde
DTT	= Dithiothreitol
EDTA	= Ethylene diamine tetra acetic acid
IPTG	= Isopropyle thio β-D-galactoside
kDa	= Kilo dalton
MALDI-TOF MS	= Matrix assisted laser desorption/ionization-time of flight mass spectrometry
NBT	= Nitro blue tetrazolium
OD_{660}	= Optical density at 600 nm
ORF	= Open reading frame
PMS	= Phenazine methosulfate
QACs	= Quaternary ammonium compounds
SDS–PAGE	= Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TMABaldehyde DH	= 4-*N*-trimethylaminobutyraldehyde dehydrogenase
TMABaldehyde	= 4-*N*-trimethylaminobutyraldehyde
TMA-Butanol DH	= 4-*N*-trimethylamino-1-butanol dehydrogenase
TMA-Butanol	= 4-*N*-trimethylamino-1-butanol
X-gal	= 5-bromo-4-chloro-indolyl-β-D-galactopyranoside

INTRODUCTION

Naturally occurring quaternary ammonium compounds (QACs) are widely distributed in biosphere including well-known representatives' choline, glycine betaine, and carnitine which have different biological functions. Choline is widely distributed in foods mainly in the form of phosphatidylcholine (Anthoni *et al*., 1991). The unavoidable role played by choline includes; constitutes of phospholipids in cell membranes, methyl group metabolism, cholinergic neurotransmission, transmembrane signaling and lipid cholesterol transport and metabolism (Zeisel and Blusztajn, 1994). The microorganisms using the choline as a sole source of carbon and nitrogen and their respective degradation pathway have been well studied (Abe *et al*., 1990; Boch *et al*., 1994, Ikuta *et al*., 1977; Mori *et al*., 1980, 1992, 2002; Nagasawa *et al*., 1976). In recent past, the microorganisms, degrading homocholine as the only source of carbon and nitrogen have been isolated in the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan and proposed a possible degradation pathway of homocholine (Mohamed Ahmed *et al*., 2009a, 2009b, 2010). Glycine betaine is an osmoprotectant in plants and microorganisms. Much attention has been paid to the osmoprotective role of glycine betaine in a number of diverse microbial systems (Annamalai and Venkitanaraynan, 2009; Smith *et al*., 1988; Roberts, 2005). Carnitine is a highly polar zwitterionic quaternary amine carboxylic acid has attracted increasing attention because of its association with fatty acid metabolism, enables β-oxidation of the long-chain fatty acids in mitochondria (Mc Garry and Brown 1997; Ramsay *et al*. 2001). Carnitine is present in some prokaryotes and all eukaryotes (Ramsay *et al*., 2001). In prokaryotic cells it serves either as a nutrient (Kleber, 1997), or as an osmoprotectant (Jung *et al*., 1990; Robert *et al*., 2000). However, in eukaryotic cells, it serves exclusively as a carrier of acyl moieties through various subcellular compartments (Ramsay and Arduini, 1993). Furthermore, it involves in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to

CO_2 and H_2O in Krebs cycle (Jakobs and Wanders, 1995; Verhoeven *et al*., 1998). Bacteria are able to metabolize carnitine under aerobic or anaerobic conditions. Under aerobic conditions it is oxidized to 3-dehydrocarnitine by carnitine dehydrogenase which has been purified and characterized from different bacterial populations (Aurich *et al*., 1968; Goulas, 1988; Mori *et al*., 1988; Mori *et al*., 1994; Hanschmann *et al*., 1994; Hanschmann and Kleber, 1997; Setyahadi *et al*., 1997; Arima *et al*., 2010).



Scheme 1 Pathway of carnitine biosynthesis in mammals (Rebouche, 1991)

In the L-carnitine biosynthetic pathway, trimethyllysine is first hydroxylated at β-position by ε-trimethyllysine hydroxylase in a reaction requiring α-ketoglutarate, oxygen, ascorbic acid, and iron as cofactors, to yield β-hydroxytrimethyllysine (Hulse *et al*.,

1978; Sachan and Hoppel, 1980; Vaz *et al*., 2001). Aldolytic cleavage of β-hydroxytrimethyllysine yields 4-*N*-trimethylaminobutyraldehyde (TMABaldehyde) and glycine, a reaction catalyzed by β-hydroxytrimethyllysine aldolase. Subsequently, TMABaldehyde is oxidized by NAD^+ dependent 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde DH) to yield γ-butyrobetaine. Finally, the resulting γ-butyrobetaine is hydroxylated at the β-position by γ-butyrobetaine hydroxylase, yielding L-carnitine (Bremer, 1983; Rebouche and Engel, 1980; Englard, 1979) (Scheme 1). Among the enzymes, TMABaldehyde DH has been purified to homogeneity from bovine liver (Hulse and Henderson, 1980) and from rat liver (Vaz *et al*., 2000). Recently, Tylichova *et al*. (2010) characterize two isoforms of plant aminoaldehyde dehydrogenase from *Pisum sativum* (PsAMADH1 and PsAMADH2) with broad specificity; can oxidize TMABaldehyde into carnitine precursor γ-butyrobetaine. Crystal structures of these carnitine biosynthetic enzymes from plant have been solved and elucidated the structural clues to the substrate specificity by site-directed mutagenesis (Tylichova *et al*., 2010; Kopency *et al*., 2011). Kaufman and Broquist (1977) demonstrated that TMABaldehyde is an intermediate in the L-carnitine biosynthesis of *Neurospora crassa* using isotope labeling experiments. Rebouche and Engel (1980) showed that TMABaldehyde DH activity was highest in the cytosolic fraction of human liver and kidney, but very low in brain, heart, and muscle homogenates. In contrast to the many enzymatic studies of TMABaldehyde DH in mammals, investigations of microbial TMABaldehyde DH are few. Recently, one microorganism from soil have been isolated and identified as *Pseudomonas* sp. 13CM (Hassan *et al*., 2007), preserved in the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan. 4-*N*-trimethylamino-1-butanol dehydrogenase (TMA-Butanol DH) and TMABaldehyde DH have been purified to apparent homogeneity from *Pseudomonas* sp. 13CM. Amino terminal sequences analysis and some properties of these enzymes have been described previously (Hassan *et al*., 2007, 2008).

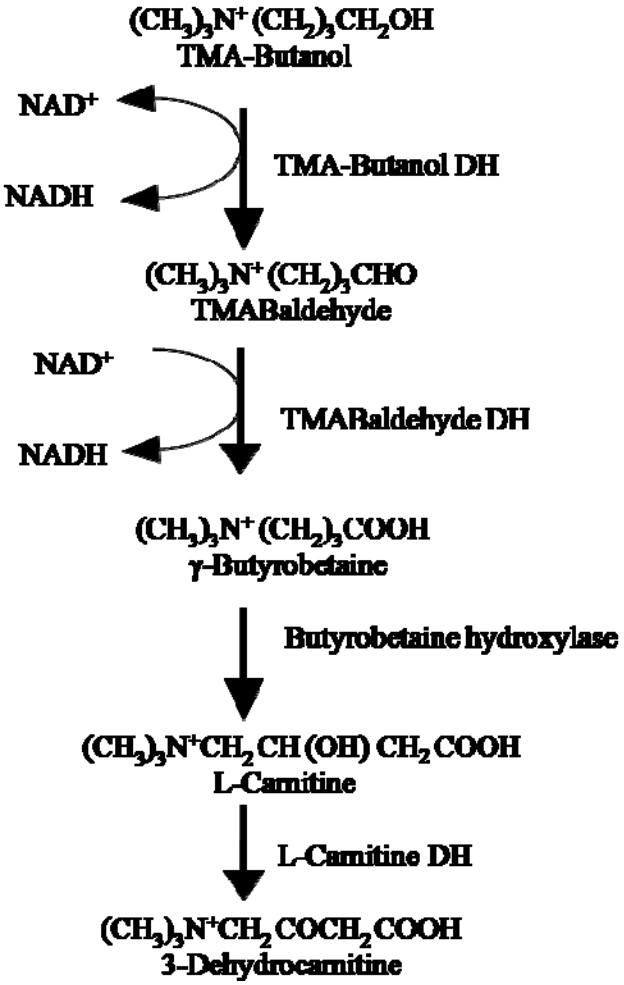
Choline	$(CH_3)_3N^+CH_2CH_2OH$
Glycine betaine	$(CH_3)_3N^+CH_2COOH$
L-Carnitine	$(CH_3)_3N^+CH_2CH(OH)CH_2COOH$
4-*N*-Trimethylamino-1-butanol (TMA-Butanol)	$(CH_3)_3N^+CH_2CH_2CH_2CH_2OH$
4-*N*-Trimethylaminobutyraldehyde (TMABaldehyde)	$(CH_3)_3N^+CH_2CH_2CH_2CHO$

Fig. 1 Structural features of five quaternary ammonium compounds

A number of brief reports have appeared which describe L-carnitine metabolism in bacteria under aerobic and anaerobic conditions. The oxidation of L-carnitine is catalyzed by NAD^+ dependent L-carnitine dehydrogenase (EC.1.1.1.108) under aerobic conditions. Enterobacteriaceae such as *Escherichia coli*, *Salmonella typhimurium*, and *Proteus vulgaris* do not assimilate the carbon and nitrogen skeleton, able to convert L-carnitine via crotonobetaine to gamma-butyrobetaine in the presence of carbon and nitrogen sources under anaerobic conditions (Seim *et al*., 1982).

To evaluate the structure and function of QACs degrading enzymes, the previous efforts were concentrated with isolation of *Pseudomonas* sp. 13CM and characterization of the enzymes TMA-Butanol DH and TMABaldehyde DH which degrades the 4-*N*-trimethylamino-1-butanol (TMA-Butanol), considerable structural resemblance to choline as a theme (Fig. 1) (Hassan *et al*., 2007, 2008). These studies were specifically addressed TMA-Butanol DH and TMABaldehyde DH from *Pseudomonas* sp. 13CM that can use TMA-Butanol as a sole source of carbon and nitrogen. The isolated enzyme TMA-Butanol DH converts TMA-Butanol into TMABaldehyde (Hassan *et al*., 2007).

Further, an enzyme TMABaldehyde DH purified from the same bacteria *Pseudomonas* sp. 13CM, oxidize TMABaldehyde to yield carnitine precursor γ-butyrobetaine (Hassan *et al*., 2008). Consistent with these observations, postulated a complete pathway of TMA-Butanol degradation (Scheme 2).



Scheme 2 Proposed pathway for 4-*N*-trimethylamino-1-butanol degradation (Hassan, 2008)

Considering the above facts, the author prompted to study the *Pseudomonas* sp. 13CM genes encoding the enzymes TMA-Butanol DH and TMABaldehyde DH. To isolate the genes shotgun cloning technique has been carried out and obtained a new enzyme, designated as TMABaldehyde DH II (Chapter 1).

To get *Pseudomonas* sp. 13CM TMA-Butanol DH gene and TMABaldehyde DH I gene, general cloning technique has been applied using the primers designed from the N-terminal amino acid sequences of the proteins and from the conserved sequences in closely-related species (Chapter 2).

TMABaldehyde DH I has been structurally closer to *Pseudomonas putida* GB-1 aldehyde dehydrogenase (B0KJD3), where as TMABaldehyde DH II has been closer to *P. putida* KT2440 putative betaine aldehyde dehydrogenase (Q88PZ0). Both of TMABaldehyde DHs furthermore show the sequence identity to *E. coli* K-12 YdcW betaine aldehyde dehydrogenase (P77674) with a percentage around 35–40%. The natures of these enzymes were also having been found to be different in substrate binding and affinity. Site-directed mutagenesis of TMABaldehyde DHs from *Pseudomonas* sp. 13CM and analyzed the resulting mutated proteins has been carried out in order to assess the ability of the corresponding enzymes to utilize the substrates which will be described in Chapter 3.

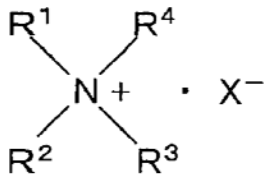
REVIEW OF LITERATURE

The view of this section is to provide selective review of the literatures of the previous researches relevant to the present study. A remarkable number of studies have been carried out all over the world including Japan on different aspect of quaternary ammonium compounds (QACs) degrading enzymes. The literature related to the research work has been briefly reviewed under the following sub-headings.

QUATERNARY AMMONIUM COMPOUNDS

Quaternary ammonium compounds (QACs) are a type of ionic compound that can be regarded as derived from ammonium compounds by replacing the hydrogen atoms with organic groups. In a like manner, QACs are molecule with at least one hydrophobic long alkyl chain attached to a positively charged nitrogen atom. In short, QACs or cationic detergents are synthetic derivatives of ammonium chloride (Arena *et al*., 1964).

General formula:



Where, R_{1}-$_{4}$ represent(s) alkyl or aryl substituents and X represents a halogen, such as bromide, iodide, or chloride (Dreisbach and Robertson, 1987; Budavari, 1996).

QACs are prepared by alkylation of tertiary amines, in a process called quaternization (Smith and March, 2001). Typically one of the alkyl groups on the amine is larger than the others (Kosswig, 2002).

A typical synthesis is for benzalkonium chloride from a long-chain alkyldimethylamine and benzyl chloride:

$$CH_3(CH_2)_nN(CH_3)_2 + ClCH_2C_6H_5 \rightarrow CH_3(CH_2)_nN(CH_3)_2CH_2C_6H_5]^+Cl^-$$

Naturally occurring QACs constitute a class of metabolites with more than 100 reported examples including well-known representatives such as choline, glycine betaine, and L-carnitine which have different biological functions, for example, adaptation of organisms to environmental stress and transportation of chemical groups in many metabolic processes (Anthoni *et al*., 1991). QACs have a wide-range of commercial and consumer uses such as detergents, antistatics, wetting and softening agents, biocides, germicides, deodorizers, wood preservatives and emulsifiers. In percentage, this utilization translates into mostly fabric softeners (66%), coated clays (16%), and biocides (8%) (Cross, 1994). After use, the residual product is discharged to sewage treatment plants or surface waters and finally to coastal waters. Released into the environment, the biodegradability of QACs is limited by their antimicrobial activity (van Ginkel, 1991; Nishiyama *et al*., 1995). Each of the QACs has its own chemical and anti-microbiological characteristics. As biocides QACs are very active against Gram-negative and Gram-positive bacteria and some viruses, fungi and protozoa (Lopez, 1986; Macdonnel and Russell, 1999). However, some microorganisms are able to demonstrate intrinsic resistance through inactivation of the biocide, and this will assist in the removal of such agents from the environment. As a result, some strains of *Pseudomonas* from the environment, including *Pseudomonas* sp. and *Pseudomonas* fluorescens, have high resistance to QACs and are thought to be responsible for biodegradation of QACs in activated sludge (van Ginkel *et al*., 1992; Nishihara *et al*., 2000).

CHOLINE

Choline is a quaternary amine which occurs in the phospholipid phosphatidylcholine and the neurotransmitter acetylcholine, and is an important methyl donor in intermediary metabolism. It is an essential nutrient that is widely distributed in foods, mainly in the form of phosphatidylcholine. Organ meats, eggs, soybeans, nuts, fish, and broccoli are particularly good sources (Howe *et al*., 2004). The only source of choline other than diet is from *de novo* biosynthesis of phosphatidylcholine from phosphatidylethanolamine (Zeisel, 1990). Choline is required to make the phospholipids phosphatidylcholine, lysophosphatidylcholine, choline plasmalogen, and sphingomyelin-essential components of all cell membranes. It is a precursor for the biosynthesis of the neurotransmitter acetylcholine, and in biological methylation reactions. Choline phospholipids in cell membranes play a role in generating various second messengers during signal transduction (Zeisel, 1990; Zeisel and Blusztajn, 1994). Choline can be acetylated, phosphorylated, and oxidized. Only a small fraction of dietary choline is acetylated, catalyzed by choline acetyltransferase (EC.2.3.1.6) (Zeisel, 1990; Zeisel and Blusztajn, 1994). This enzyme is highly concentrated in the terminals of cholinergic neurons, but also present in such non-nervous tissue as the placenta. Phosphorylation of choline is the first step in the major pathway for phosphotidylcholine synthesis, catalyzed by choline kinase (EC.2.7.1.32), using Mg^{2+} and ATP. This enzyme is widely distributed in mammalian tissues, including the liver, brain, kidney, and lung. Choline deficiency results in liver dysfunction, fatty liver, decreased in growth, infertility, Alzheimer's disease, abnormal kidney function, decreased red blood cell synthesis, high blood pressure, and liver cancer (Zeisel, 1990; Zeisel and Canty, 1993; Zeisel and Blusztajn, 1994). The microorganisms using the choline as a sole source of carbon and nitrogen and their respective degradation pathway have been well studied (Abe *et al*., 1990; Boch *et al*., 1994, Ikuta *et al*., 1977; Mori *et al*., 1980, 1992, 2002; Nagasawa *et al*., 1976a).

HOMOCHOLINE

Homocholine (3-*N*-trimethylamino-1-propanol) resembles choline in many aspects of cholinergic metabolism (Boksa and Collier, 1980). It is an analogue of choline; the amino alcohol group is lengthened by one CH_2-group. After it is transported into the rat brain synaptosome, it is acetylated and released as acetylhomocholine from a superior cervical ganglion and minces of mouse forebraine by a calcium-dependent process during depolarization (Collier *et al*., 1977; Carroll and Aspry, 1980). Homocholine, however, differs dramatically from choline in a very important aspect, not acetylated by solubilized cholineacetyltransferase, but acetylated with intact rat brain synaptosomes (Collier *et al*., 1977). This phenomenon may happen due to homocholine is acetylated with choline acetyltransferase that is associated with vesicular and/or neuronal membranes (Carroll and Aspry, 1980). Nelson *et al*. (1980) assumed that the extracellular precursors of choline and homocholine may be directly accumulated by a crude vesicular fraction of mouse forebrain, independently of the cytoplasm, and may be utilized to replace the loss in acetylcholine. Additionally, they have been suggested that the extracellular products acetylcholine and acetylhomocholine may not be capable of replacing acetylcholine lost from a crude vesicular fraction. Moreover, acetylhomocholine is released from brain slices both spontaneously and in response to simulation *via* mechanism similar to those that released acetylcholine, but some differences in specificity of the acetylcholine storage and/or release process might be present (Nelson *et al*., 1980). Previous studies on acetylhomocholine synthesis and release have partly classified these false transmitter criteria by showing that homocholine is acetylated in tissues containing cholinergic nerve terminals (Collier *et al*., 1977), that acetylhomocholine is released by a calcium-dependent mechanisms during nerve stimulation, and that acetylehomocholine has actions qualitatively similar to but quantitatively different from those of acetylcholine on both muscarinic and nicotinic receptors (Hunt and Renshaw, 1934; Curtis and Ryall, 1966; Barrass *et al*., 1970). In pharmacology, choline analogues such as homocholine, methylcholine, and ethylcholine are used to study the chemical specificity of the various processes involved in neurotransmission and in particular it has been used to investigate the sub cellular

origin of the transmitter released by stimulation. From the choline like structure, one would expect that homocholine is degraded similarly and a rate comparable to that of choline. Recently, the microorganisms, degrading homocholine as the only source of carbon and nitrogen have been isolated as well as proposed a possible degradation pathway (Mohamed Ahmed *et al*., 2009a, 2009b, 2010).

GLYCINEBETAINE

Glycinebetaine is found in microorganisms, plants, and animals, and is a significant component of many foods, including wheat, shellfish, spinach, and sugar beets (Craig, 2004). Glycine betaine is compatible osmolyte that increases the water retention of cells, replaces inorganic salts, and protect intracellulars enzymes against osmotically induced or temperature-induced inactivation. In human, glycine betaine is catabolized *via* a series of enzyme reactions that occure mainly in the mitochondria of liver and kidney cells. Glycinebetaine also acts as a catabolic source of methyl groups *via* transmethylation for use in many biochemical pathways. The formations of methionine from homocysteine can occur either *via* glycinebetaine or 5-methyltetrahydrofolate. The conversion of homocysteine to methionine is important to conserve methionine, detoxify homocysteine, and produce S-adenosylmethionine. Glycine betaine is synthesized by two step oxidation of choline or by methylation reaction from glycine. Two *N*-methyltransferases genes from halotolerant cyanobacterium *Aphanothece halophytica* have been isolated (Waditee *et al*., 2003).

CARNITINE

Carnitine is a naturally occurring dipolar amino acid like compound, widely distributed in food from animal sources but limited in plant sources (Kendler, 1986), present in some prokaryotes and all eukaryotes (Ramsay *et al*., 2001). This highly polar zwitterionic quaternary ammonium compound was discovered in meat, and was

extracted by Russian scientists Gulewitsch and Krimberg *i*n 1905. The term carnitine is derived from the Latin term *carnis* means meat. Carnitine is chemically known as β-hydroxy-γ-trimethyl ammonium butyrate or 4-*N*-trimethyl-3-hydroxy butyrate and it is a betaine derivative, structurally resembles choline. In 1957, Fraenkel and Friedmann identified an essential factor required for the growth of the meal worm *Tenebrio molitor* named as vitamin BT means biological B complex group of vitamins for the Tenebrio meal worm. The same was later on resolved as carnitine (Williams, 1994). Carnitine biosynthesis takes place in living cells of liver and kidney. The precursors required for synthesis are essential amino acid lysine, methionine (Steiber *et al*., 2004) and a cofactor L-ascorbic acid (Rebouche, 1991; Dunn *et al*., 1984). Therefore, it is not an essential dietary component due to *in vivo* synthesis. Carnitine exists as two types of stereoisomers, amongst them, L-carnitine is naturally occurring and found to be biologically active, where as D-carnitine is biologically inactive and does not occur naturally (Liedtke *et al*., 1982). However, D-carnitine used to deplete tissue L-carnitine level partially (Paulson and Shug, 1981). Generally, the term carnitine comprises number of different compounds such as L-carnitine, Propionyl-L-carnitine and acetyl-L-carnitine (Rebouche, 1990). In prokaryotic cells, carnitine serves either as a nutrient, such as a carbon and nitrogen source (Kleber, 1997), or as an osmoprotectant (Jung *et al*., 1990; Robert *et al*., 2000). However, in eukaryotic cells, it serves exclusively as a carrier of acyl moieties through various subcellular compartments (Ramsay and Arduini, 1993). It has an important role in the transport of activated long-chain fatty acids across the inner mitochondrial membrane (Bieber, 1988; McGarry and Brown, 1997). Furthermore, it is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle (Jakobs and Wanders 1995; Verhoeven *et al*., 1998). Data from animal studies showed that L-carnitine is synthesized in the liver but stored in skeletal (Mitchell, 1978).

ROLE OF CARNITINE

Metabolically, carnitine plays an important role in energy generation by fatty acid oxidation in the mitochondrial matrix (Olson, 1966). It participated in the transport of activated fatty acid (acyl CoA) from cytoplasm across inner mitochondrial membrane through carnitine transport proteins termed as "carnitine shuttle". Generally, fatty acid containing more than fourteen carbon atoms needs carnitine transport; they constitute either dietary fatty acids or fatty acids released from adipose tissues in response to hormone sensitive triacylglycerol lipase action (David *et al*., 2005). There are four Carnitine Palmitoyl Transferase (CPT) isoforms found, they are CPT1a in liver, CPT1b in muscle and other tissues, CPT1c in brain and testis and CPT II (Price *et al*., 2002). The two isoenzymes form of CPT are CPT-I and CPT-II, CPT-I localized in inner side of the outer mitochondrial membrane that catalyse esterification of acyl coA and carnitine in to acyl-carnitine complex (Murthy *et al*., 1987; Fiona *et al*., 1997; van der Leij *et al*., 1999).

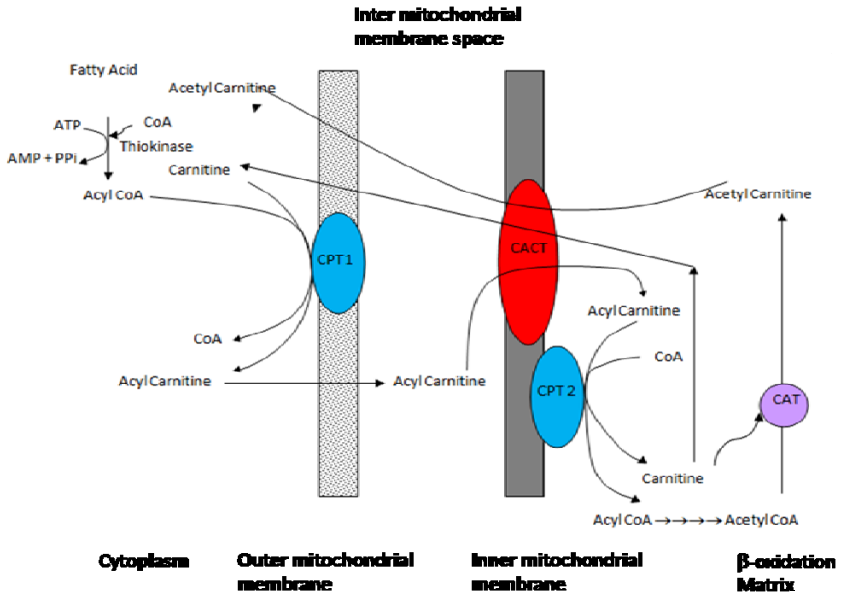


Fig. 2 Role of L-carnitine in transport of fatty acid in to matrix (Dayanand *et al*., 2011)

CPT-I, Carnitine Palmitoyl Transeferase -I; CPT-II, Carnitine Palmitoyl Transeferase-II; CACT, Carnitine Acyl Carnitine Translocase; CAT, Carnitine Acetyl Transferase.

Acyl-carnitine complex transported across inner mitochondrial membrane through specific transporter, Carnitine acyl Carnitine Translocase (CACT) (Dayanand *et al*., 2011). The inner side of mitochondrial membrane, CPT-II is located; it breaks the acyl bond of acyl-carnitine releases fatty acid. Simultaneously, coenzyme A recycling takes place and carnitine enters for transport of another molecule of fatty acid. The transported acyl CoA from cytoplasm undergoes β-oxidation for rapid energy release (Fig. 2). Acetylcarnitine formed by acetyl CoA carboxylase transported into cytoplasm where it serves as precursor for fatty acid or acetyl-choline synthesis. The carnitine uptake defect occurs due to the defect in cellular carnitine transporter (CT) that impairs the mechanism of fatty acid transport in to mitochondria for oxidation.

CARNITINE HOMEOSTASIS

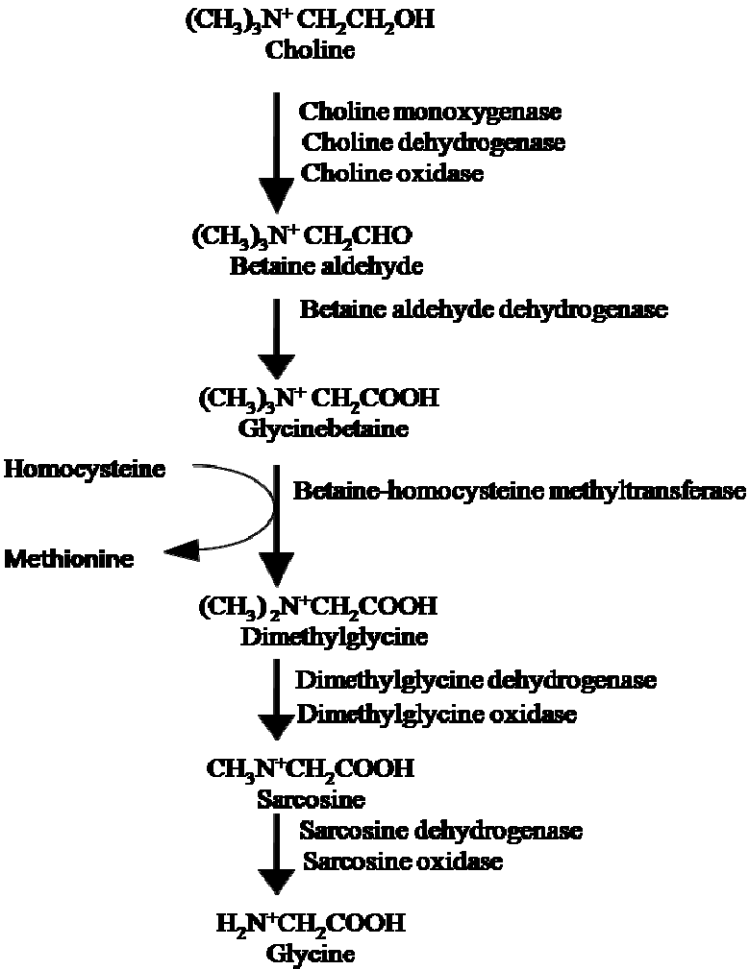
Carnitine homeostasis is maintained by endogenous synthesis, absorption from dietary foods, and tubular reabsorption by the kidney. Vegetarians obtain little carnitine in their diet, therefore, the carnitine biosynthesis is highly significant in them in comparison to omnivorous humans due to their major source of carnitine is meat (Rebouche, 1992). However, in omnivorous humans, carnitine is obtained about 75% from the diet and 25% from the *de novo* synthesis (Tein *et al*., 1996). Carnitine, a branched non-essential amino acid, is synthesized from the essential amino acids lysine and methionine. Ascorbic acid, ferrous iron, pyroxidine and niacin are also necessary cofactors (Williams, 1994) and deficiencies of any of these can lead to carnitine deficiency. In mammals, certain proteins like actin, myosin, calmodulin, cytochrome-c and histones contains L-lysine amino acid residues, which during their post translational modifications event undergoes transmethylation reactions (Husazar, 1975). This reaction is catalyzed by specific methyl transferases using active methionine i.e. adesonosyl methionine as methyl group donor (Palik and Kim, 1971). The product produced in this reaction is protein bound trimethyllysine residues (which is the initial metabolite in carnitine biosynthesis) and adenosyl homocysteine. Subsequently, trimethyllysine

hydroxylated and formed β-hydroxytrimethyllysine and then TMABaldehyde. Next, TMABaldehyde oxidized by TMABaldehyde DH to form γ-butyrobetaine (Hulse and Henderson, 1980; Sachan and Hoppel, 1980; Vaz *et al*., 2001). The purification and characterization of TMABaldehyde DH from rat liver cytosol and the identification of corresponding rat cDNA was reported. The translated coding sequence of TMABaldehyde DH cDNA is highly homologous with that of the previously reported human aldehyde dehydrogenase 9 enzyme (EC.1.2.1.19) (Vaz *et al*., 2000). Most of the tissues are able to produce this product efficiently. But, the conversion of γ-butyrobetaine into L-carnitine only takes place in humans particularly in liver, kidney, brain and testes. Therefore only liver and kidney can transport carnitine to the blood. Furthermore, the enzyme butyrobetaine dehydrogenase is a dioxygenase catalyses, the stereo-specific hydroxylation of γ-butyrobetaine to L-carnitine and oxidative decarboxylation of 2-oxoglutarate into succinate and carbon dioxide (Bremer 1983; Rebouche and Engel 1980; Englard 1979). The enzyme γ-butyrobetaine dehydrogenase activity is age dependent, its activity low at birth, increases to adult values during puberty, however in kidney activity is unaltered in birth (Olson and Rebouche, 1987).

Free L-carnitine, absorbed from dietary intake or synthesized in liver and kidney, reaches the blood stream and the extracellular fluid. Its transport within cells of various tissues is limited by their respective uptake capacities (Angelini *et al*., 1987). Plasma concentration of free carnitine is in dynamic balance with acylcarnitines with the acyl to free carnitine ration of $\leq$ 0.4 being considered normal (Bellinghieri *et al*., 2003). Acetylcarnitine esters are formed intracellularly during regular metabolic activity. Long chain acetylcarnitine esters transport fatty acyl moieties into the mitochondria. Short and medium-chain acetyl esters, formed in the mitochondria and peroxisomes, participate in the removal of organic acids (Rebouche and Seim, 1998). Acetyl-L-carnitine is the principal acylcarnitine ester (Rebouche and Seim, 1998). Acetyl-L-carnitine participates in both anabolic and catabolic pathways in cellular metabolism (Rebouche and Seim, 1998).

CHOLINE OXIDATION

A major use for choline is *via* irreversible oxidation forming glycine betaine, an important methyl donor. Once glycinebetaine is formed, it cannot be reduced to reformed choline; however, it can donate a methyl group to homocysteine, thereby producing dimethylglycine and methionine. Dimethylglycine is converted to sarcosine and then glycine, producing an l-fragment (Zeisel, 1990).



Scheme 3 Choline degradation pathway

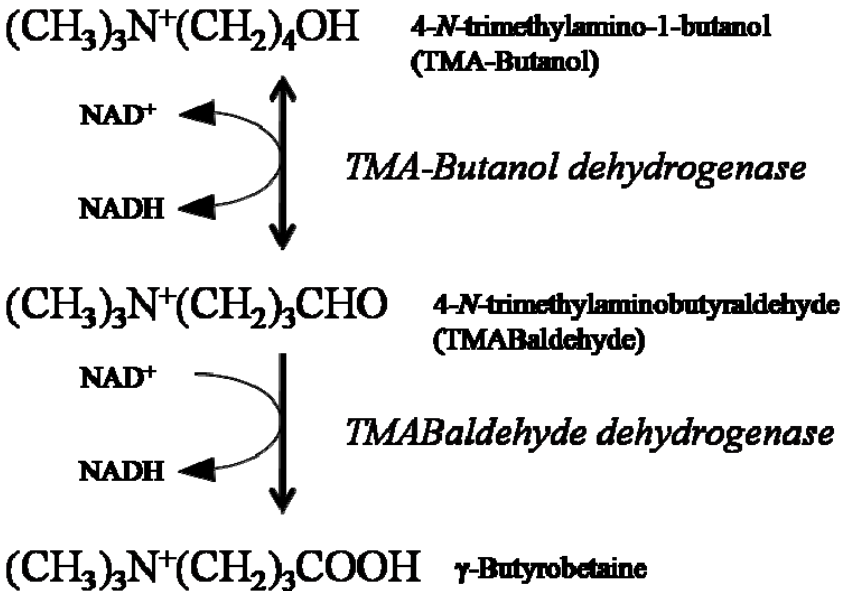
In microorganisms, plants and animals, glycinebetaine is synthesized from choline by two reactios: (1) choline to betaine aldehyde and (2) betaine aldehyde to glycinebetaine. In plants such as spinach (Rathinasabapathi *et al*., 1997), sugar beet and amaranth (Russel *et al*., 1998), the first step reaction is catalyzed by choline monoxygenase (EC.1.14.15.7), a ferredoxin-dependent soluble Rieske-type protein, whereas in animals and microorganisms, it is catalyzed by either membrane-bound choline dehydrogenase (EC.1.1.99.1) (Nagasawa *et al*., 1976a; Landfald and Strom, 1986; Russel and Scopes, 1994; Tsuge *et al*., 1980) or soluble flavoprotein, choline oxidase (EC.1.1.3.17) (Ikuta *et al*., 1977; Yamada *et al*., 1979; Rozwadowski *et al*., 1991). Choline monooxygenase is in fact unique to plants. Unlike other plant oxygenases, choline monooxygenase purified from spinach leaves are shown to be soluble and insensitive to carbon monoxide and it appears not to be a P450-type enzyme (Rathinasabapathi *et al*., 1997). Choline oxidase catalyzes the four-electron oxidation of choline to glycinebetaine *via* betaine aldehyde as intermediate (Ikuta *et al*., 1977). Based on amino acid sequence comparisons, the enzyme can be grouped in the glucose-methanol-choline flavine-dependent (GMC) oxidoreductase enzyme superfamily, which comprises enzymes like glucose oxidase, cholesterol oxidase, or cellubiose dehydrogenase, that utilizes FAD as cofactor for catalysis and non-activated alcohols as substrate. The highly GC rich *codA* gene encoding for choline oxidase was cloned from genomic DNA of *Arthrobacter globiformis* and expressed to high yield in *Escherichia coli* (Fan *et al*., 2004). The resulting recombinant enzyme was highly purified and showed to be a dimer of identical subunits containing covalently bound FAD.

In the second step, the oxidation of betaine aldehyde is catalyzed by a soluble, $NAD(P)^+$ dependent betaine aldehyde dehydrogenase (EC.1.2.1.8) which has been purified to homogeneity from spinach (Arakawa *et al*., 1987), amaranth (Valenzuela-Soto *et al*., 1994), *Pseudomonas aeruginosa* (Nagasawa *et al*., 1976), *E. coli* (Falkenberg and Strom, 1990), *Xanthomonas translucens* (Mori *et al*., 1992), *A. globiformis* (Mori *et al*., 2002), and *Cylindrocarpon didymium* (Mori *et al*., 1980). Chern and Pietruszko (1995) reported that the human aldehyde dehydrogenase E3 isozyme is a betaine aldehyde dehydrogenase.

Betaine-homocysteine methyltransferase is an enzyme calyzing methyl transfer reaction from glycinebetaine to homocysteine, forming dimethylglycine and methionine, respectively. Betaine-homocysteine methyltransferase (EC.1.2.1.8) has been purified to homogeneity from rat (Lee *et al*., 1992), pig (Garrow, 1996), human (Skiba *et al*., 1982), and cyanobacteria (Waditee and Incharoensakdi, 2001). It is a zinc metalloenzyme consisting of a hexamer of 45 kDa subunits and is active in the livers and kidneys of humans and pigs, but only in the livers of rats (Mckeever *et al*., 1991; Millian and Garrow, 1998). Following the betaine-homocysteine methyltransferase reaction, the last two reactions of choline oxidation convert dimethylglycine to glycine *via* sarcosine as intermediate. The oxidative demethylation of dimethylglycine to form sarcosine is catalyzed by dimethylglycine oxidase (Mori *et al*., 1980) or membrane bound dimethylglycine dehydrogenase (Porter *et al*., 1985). Subsequently, the formation of glycine from sarcosine is catalyzed by the mitochondrial enzyme sarcosine dehydrogenase (Porter *et al*., 1985) or sarcosine oxidase (Frisell, 1971; Mori *et al*., 1980).

4-*N*-TRIMETHYLAMINO-1-BUTANOL DEGRADATION

4-*N*-trimethylamino-1-butanol (TMA-Butanol) is an analogue of choline, in which the amino alcohol group is lengthened by two CH_2-groups, structurally resemblance to choline, as a theme. In the screening for TMA-Butanol degrading microorganisms from soil, many bacterial strains were isolated. Among them, *Pseudomonas* sp. 13CM was found to degrade TMA-Butanol with very first reaction and showed as a high and stable NAD^+-dependent alcohol dehydrogenase activity, selected as a TMA-Butanol degrading strain (Hassan, 2008). In *Pseudomonas* sp. 13CM, it was observed that TMA-Butanol was oxidized to TMABaldehyde by a NAD^+-dependent TMA-Butanol dehydrogenase (Scheme 4). Further, an enzyme TMABaldehyde was also isolated from the same bacteria, oxidized TMA-Butanol to yield γ-bytyrobetaine by a NAD^+-dependent reaction (Hassan *et al*., 2007, 2008).



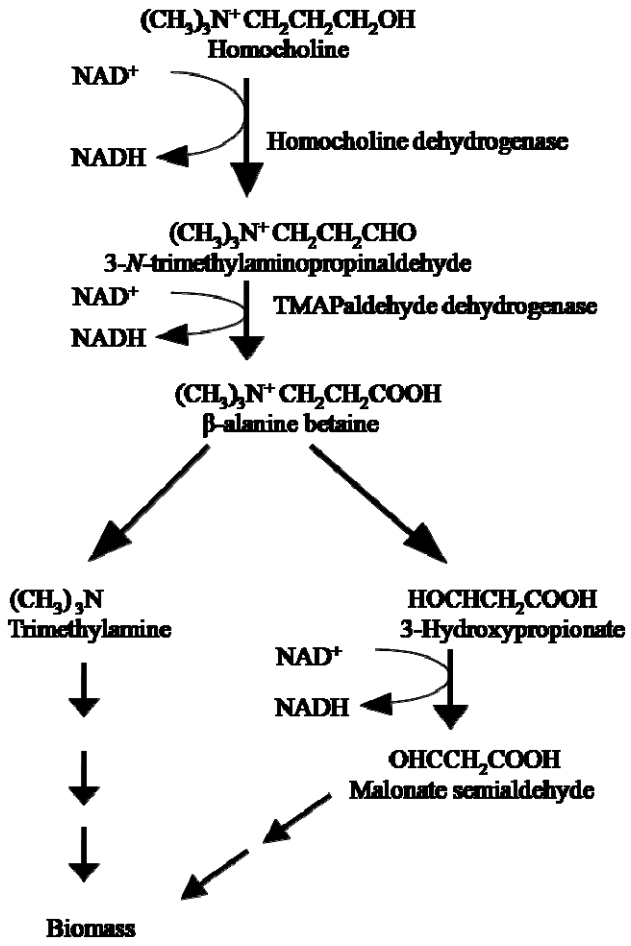
Scheme 4 4-*N*-trimethylamino-1-butanol (TMA-Butanol) degradation pathway in *Pseudomonas* sp. 13CM (Hassan, 2008)

Kinetic studies on substrates and substrate analogues of both enzymes showed that positively charged trimethylammonium or trimethylammonium groups of the substrates have critical effect on the catalytic activity of the enzymes (Hassan, 2008). Similarly, Gadda *et al*., (2004) reported that the trimethyl ammonium moiety is critically important for the binding of ligands at the active site of choline oxidase.

HOMOCHOLINE DEGRADATION

Very recently a study has been carried out designed the microbial degradation of homocholine (Mohamed Ahmed, 2010). Investigation of relevant properties, proposed a degradation pathway of homocholine in *Pseudomonas* sp. strain A9. First of all, homocholine is oxidized to trimethylaminopropionaldehyde (TMAPaldehyde) by a NAD^+-dependent homocholine dehydrogenase, and consequently, TMAPaldehyde oxidized to β-alanine betaine by a NAD^+-dependent aldehyde dehydrogenase. After that,

cleavage of β-alanine betaine C-N bond yielded trimethylamine and 3-hydroxypropionate (C-3 moiety). 3-Hydroxypropionate was further oxidized to malonate semi-aldehyde by a NAD^+-dependent hydroxypropionate dehydrogenase (Scheme 5).



Scheme 5 Homocholine degradation pathway (proposed) in *Pseudomonas* sp. strain A9 (Mohamed Ahmed, 2010)

BACTERIAL CARNITINE METABOLISM

Various species of bacteria are able to grow aerobically on L-carnitine as sole source of carbon and nitrogen, namely *Pseudomonas* and *Agrobacterium* species. The catabolic step is the oxidation of the β-hydroxy group of L-carnitine with concomitant formation of 3-Dehydrocarnitine by NAD^+ dependent L-carnitine dehydrogenase (EC.1.1.1.108) (Aurich *et al*., 1968; Goulas, 1988; Mori *et al*., 1988; 1994). 3-dehydro carnitine, formed by the L-carnitine dehydrogenase could be degraded to glycinebetaine by the addition of NAD^+, ATP, and CoA (Lindstedt *et al*., 1967). Various *Agrobacterium* species also can grow on the D-carnitine medium as the sole source of carbon and nitrogen, NAD^+ dependent D-carnitine dehydrogenase (EC.1.1.1.254) has been purified and biochemically characterized (Hanschmann *et al*., 1994; Setyahadi *et al*., 1997). *Acinetobacter calcoaceticus* 69/V is able to metabolize L-carnitine, L-acylcarnitines, and γ-butyrobetaine as sole carbon sources (Kleber *et al*., 1977). D-carnitine is metabolized only if an additional carbon source like L-carnitine is present in the incubation mixture of if the bacteriaare pre-incubated with L- or DL-carnitine but no growth was observed with D-carnitine as sole source of carbon (Kleber *et al*., 1977). The utilization of these compounds and the growth of the organisms are correlated with the stoichiometric formation of trimethylamine. In addition Miura-Fraboni and Englard (1983) showed that D-carnitine effectively supports the growth of *A. calcoaceticus*, and that utilization of carnitine resulted in stoichiometric formation of trimethylamine and equivalent loss of the carboxyl-labelled carbon backbone from the growth medium. Certainly, D-carnitine does not induce the initial enzyme of carnitine degradation in *A. calcoaceticus* 69/V.

CHAPTER 1

GENE CLONING AND BIOCHEMICAL CHARACTERIZATION OF 4-*N*-TRIMETHYLAMINOBUTYRALDEHYDE DEHYDROGENASE II FROM *PSEUDOMONAS* SP. 13CM

1.1 INTRODUCTION

A microorganism isolated from soil, identified as *Pseudomonas* sp. 13CM, growing on 4-*N*-trimethylamino-1-butanol (TMA-Butanol) as sole source of carbon and nitrogen and the enzyme 4-*N*-trimethylamino-1-butanol dehydrogenase (TMA-Butanol DH) purified to apparent homogeneity from it. The isolated enzyme converted TMA-Butanol (considerable structural resemblance to choline as a theme) into trimethylamino butyraldehyde (TMABaldehyde) (Hassan *et al*., 2007). Furthermore, another enzyme 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde DH) was isolated from the same organism, oxidized TMABaldehyde to yield γ-butyrobetaine (Hassan *et al*., 2008). Consistent with these observations, postulated a pathway of TMA-Butanol degradation in *Pseudomonas* sp. 13CM. Substrate specificity, the equilibrium for the reactions, and several properties of the TMA-Butanol degrading enzymes (TMA-Butanol DH and TMABaldehyde DH) have been described (Hassan *et al*., 2007, 2008).

From another point of view, TMABaldehyde DH is responsible for the reaction of the biosynthetic route of L-carnitine (Hulse *et al*., 1978) that serves either as a nutrient (Kleber, 1997) or as an osmoprotectant (Jung *et al*., 1990; Robert *et al*., 2000). The enzyme, TMABaldehyde DH has been also purified to homogeneity from bovine liver (Hulse and Henderson, 1980), and from rat liver (Vaz *et al*., 2000). Much attention has been paid on the study of TMABaldehyde DH from mammals. However, until recently, little is known about the bacterial TMABaldehyde DH as well as TMA-Butanol DH. To our knowledge, no study to date has had complete genome sequences encoding the enzymes TMABaldehyde DH and TMA-Butanol DH. To clarify the function and role of

Pseudomonas sp. 13CM TMABaldehyde DH and TMA-Butanol DH, it is necessary to obtain enough amount of the protein.

Considering the above scenario, we emphasizes the importance of these genes and attempted to sequence the genes encoding TMABaldehyde DH and TMA-Butanol DH using the bacterial strain *Pseudomonas* sp. 13CM as a gene donor. To isolate the genes of these TMA-Butanol degrading enzymes from *Pseudomonas* 13CM, we applied shotgun cloning technique using the activity staining assay for screening the recombinants. By the investigation, a new type of enzyme that catalyzes the dehydrogenation reaction toward TMABaldehyde was obtained, the primary structure of which is clearly different from the previously obtained *Pseudomonas* sp. 13CM TMABaldehyde DH (Hassan *et al*., 2008). We renamed the obtained second enzyme as "TMABaldehyde DH II". In this chapter, expression cloning i.e., the genomic DNA digested with *Bam*HI restriction enzyme, incorporated on a multiple cloning site of the vector and active clones produced by *E. coli* DH5α and characterization of the recombinant enzyme are described. Additionally, properties of the purified recombinant TMABaldehyde DH II are also compared with the native TMABaldehyde DH I (Hassan *et al*., 2008), as well as, with those of TMABaldehyde DH which was purified from rat liver and beef liver.

1.2 MATERIALS AND METHODS

1.2.1 Materials

1.2.1.1 Reference organism

Reference organism *Pseudomonas* sp. 13CM (Hassan, 2008) collected from the repository of the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan, used as the donor strain in this study.

1.2.1.2 Competent cells and plasmids

Competent cells of *E. coli* DH5α [F^-, Φ80d*lacZ*ΔM15, Δ (*lacZYA*-argF) U169, *deo*R, *recA*1, *endA*1, *hsdR*17 (r_k^-, m_k^+), *phoA*, *supE*44, λ^-, *thi*-1, *gyrA*96, *relA*1] purchased from Takara Bio Inc. (Shiga, Japan), used to prepare the recombinant. *E. coli* JM109 and *E. coli* BL21 (DE3) obtained from Takara Co. Ltd. (Shiga, Japan) and Novagen Inc. (Madison, WI, USA), respectively. The plasmid vector pUC18 was collected from Takara Bio Inc. (Shiga, Japan) and Nippon Gene (Tokyo, Japan) (Fig. 1.1). The vector pET24b (+) was a product of Novagen Inc. (Madison, WI, USA) used for the high level expression of the recombinant (Fig. 1.2).

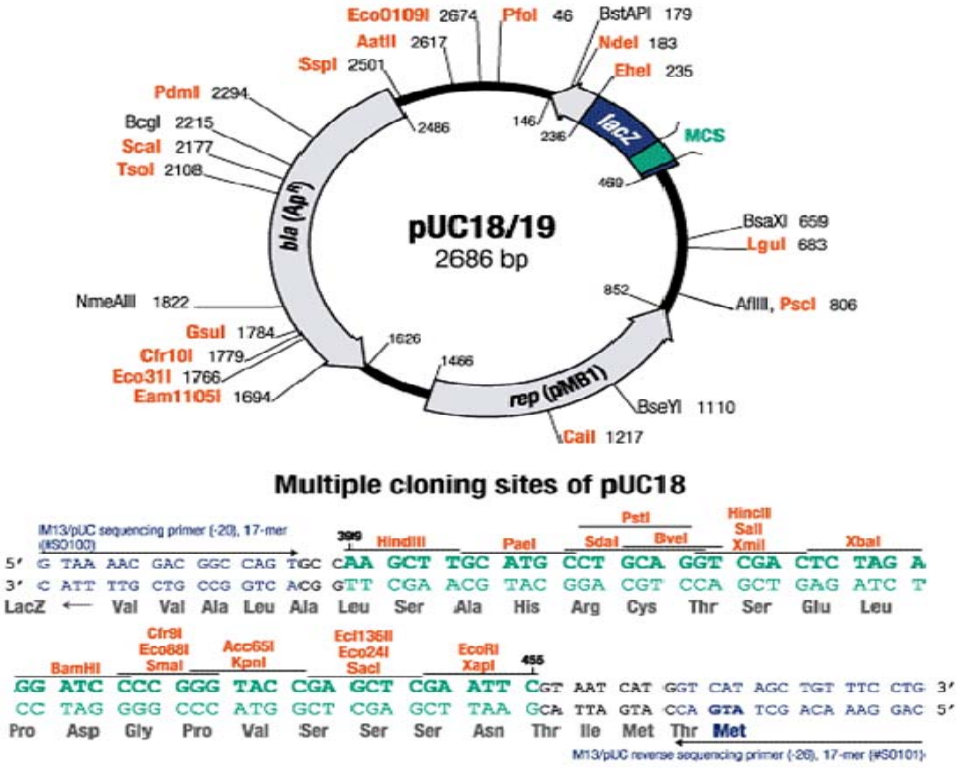


Fig. 1.1 pUC18 vector map with multiple cloning sites (adapted from Thermo Scientific)

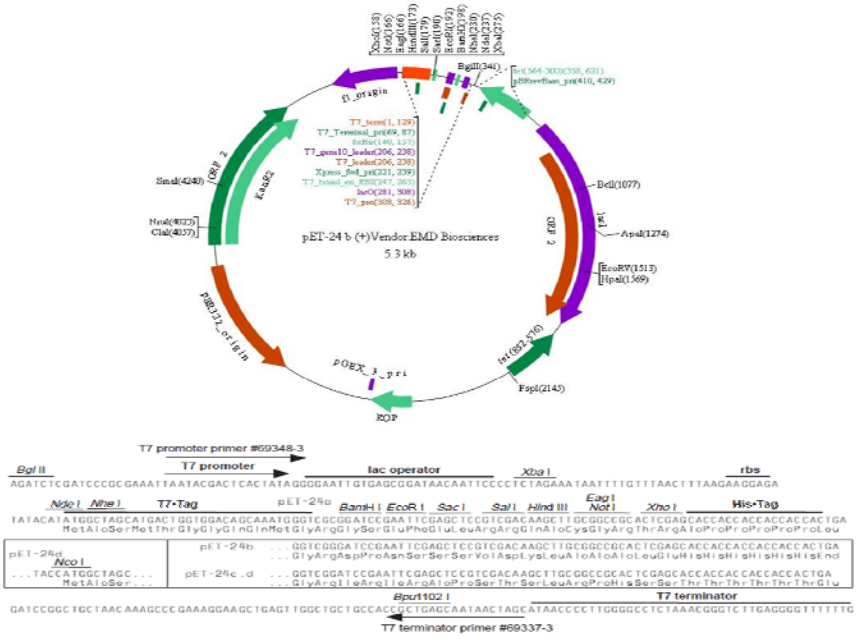


Fig. 1.2 Structure of vector pET24b (+) (adapted from EMD Bioscience)

1.2.1.3 Culture media

The isolates of *Pseudomonas* sp. 13CM was grown on meat extract agar medium (Table 1.1). An isolated colony was cultivated in TMA-Butanol medium for 3 to 6 days at 30 ºC in a reciprocal shaker (Table 1.2). The cells from transformation were grown on LB medium (Table 1.3) supplemented with relevant antibiotics.

Table 1.1 Culture medium for *Pseudomonas* sp. 13 CM

Meat extract	10.0	g
Glucose	5.0	g
Peptone	5.0	g
NaCl	1.0	g
Total volume	1000	ml

pH was adjusted to 7.0 with 1.0 M NaOH or 1.0 M HCl

Table 1.2 Basal culture medium for *Pseudomonas* sp. 13CM

TMA-Butanol iodide	5.0	g
KH_2PO_4	3.0	g
K_2HPO_4	3.0	g
$MgSO_4$. $7H_2O$	0.5	g
Yeast extract	0.5	g
Total volume	1000	ml

pH was adjusted to 7.3 with 1.0 M NaOH or 1.0 M HCl

Table 1.3 Luria-Bertani (LB) medium

Bacto tryptone	10.0	g
Bacto yeast extract	5.0	g
NaCl	10.0	g
(Agar	15.0	g)
Total volume	1000	ml

pH was adjusted to 7.0 with 1.0 M NaOH or 1.0 M HCl

1.2.1.4 Cofactors and chemicals for substrates

The cofactors NAD^+ and $NADP^+$ were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). 4-Dimethylamino-1-butanol (DMA-Butanol), 4-aminobutyraldehyde dimethylacetal, 3-dimethylamino-1-propanol (DMA-Propanol), 5-chloro-1-pentanol, and 6-dimethylamino-1-hexanol (DMA-Hexanol) were purchased from Tokyo Kasei (Tokyo, Japan).

1.2.1.5 PCR reagents

PCR primers (Table 1.4) were synthesized at Operon Biotechnologies (Tokyo, Japan) as degenerate primers corresponding to the partial sequences in the fragments. DNA ligation kit, M13 primer M4 (5′-GTTTTCCCAGTCACGAC-3′), and M13 primer RV(d(CAGGA-AACAGCTATGAC) were from Takara Bio Inc. (Shiga, Japan).

Table 1.4 PCR primers used for sequencing

Primers	Direction	Sequences (5′-3′)
F1	Forward	CGA GCT GGC CGA GCA GCT
F2	Forward	CCT GGT GGC CAC CGT CTG T
F3	Forward	CCA CCC AGG TCA GGC TGG
F4	Forward	CGC CAC ACC CAC GGC AAA G
F5	Forward	GCA CAG GCG CGG TCA GTT C
R1	Reverse	CGG CGT TTT CGG CGA TCG T
R2	Reverse	CCG AAC TGC CGG TGC TGG A
R3	Reverse	GCT GAC GAG CCG GCG TGA A

Restriction enzymes and kits for genetic manipulation were from New England Biolabs (Massachusetts, USA), Takara Bio Inc. (Shiga, Japan), Toyobo (Osaka, Japan) and Nippon Gene (Tokyo, Japan). Agarose SeaKem GTG was used in preparation of deletion mutants collected from BMA (Rockland, ME, USA). Loading quick λ/*Sty*I and loading quick λ/*Hind*III were purchased from Toyobo, Japan. Psychrophilic bacterium alkaline phosphatase (PAP) was from Biodynamics, Japan. MagExtractor-Genome kit and MagExtractor-Plasmid kit were from Toyobo (Osaka, Japan). BigDye Terminator Cycle Sequencing Ready Reaction kit ver 3.1 was from Applied Biosystems (Foster City, CA). The dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, rT*th* pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), magnesium chloride, and buffer were premixed into a single tube of Ready Reaction Mix and were ready to use.

1.2.1.6 Culture, purification, and polyacrylamide gel electrophoresis

Agar and yeast extract were from Difco (Lawrence, USA). Carbenicillin and kanamycin were collected from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Phenyl-Toyopearl 650 M was from Tosoh Corporation (Tokyo, Japan). SDS–PAGE was performed using the mini slab size 5–20% gradient polyacrylamide gels purchased from Atto (Tokyo, Japan) and the enzyme samples were prepared for loading by mixing with an equal volume of 2x EzApply sample buffer (Atto, Tokyo, Japan). TSK-gel G3000SW (0.78 × 30 cm) column was from Tosoh Corp. (Tokyo, Japan). Standard protein kit for gel filtration was from BIO-RAD (Hercules, CA, USA). Color Plus prestained protein marker for SDS–PAGE was from New England Biolabs (Massachusetts, USA).

1.2.1.7 Other reagents

Proteinase K, ribonuclease A, and lysozyme were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). All other chemicals and materials were of the highest purity grade, used without further processing.

1.2.2 Methods

1.2.2.1 General methods

Mupid-2X submarine electrophoresis system apparatus (Advance Co. Ltd., Japan) were used for agarose gel electrophoresis. General weight measurements were made using IB-200H electronic balance (Shimadzu corp. Kyoto, Japan) and smaller quantities measurements for the preparation of standards were performed using Mettler AE240 analytical balance (Mettler, Toledo, AG, Switzerland). Cell growth was measured using the Novaspec II from Amersham Pharmachia Biotech (Piscataway, NJ, USA). TOMY Ultrasonic Disruptor (UD-200) was used for disruption of cells. For measurement of enzyme activity, Shimadzu UV-2100S was used. Gilson HPLC System was used for gel

filtration with the Unipoint^{TM} HPLC system control software was used for data analysis. Pharmachia LKB GradiFrac, Pump P-1, Optical Unit UV-1, Control UV-1, Valve IV-7, and Valve PSV-50 from Pharmachia Biotech were used for chromatography apparatus. Micro Tube PumpMP-3 from EYELA, Tokyo Rikakikai Co. Ltd. was used for buffer circulation, and Consta Power 3500 was used for power supply purchased from Atto Corp. (Tokyo, Japan). Products derived from PCR were purified by using Centri-Sep spin columns from Applied Biosystems (Foster city, CA) and MagExtractor-Genome kit from Toyobo (Osaka, Japan). Magical Trapper from Toyobo (Osaka, Japan) was used during preparation of genomic DNA, plasmids DNA, and purification of PCR products. For measurement of purity of plasmid DNA, UVmini-1240 UV-Visible spectrophotometer from Shimadzu Corp. (Kyoto, Japan) was used. pH was determined using an Horiba F-22 pH meter (Horiba Ltd, Kyoto, Japan). Cultures and extracts were centrifuged using Hitachi Himac Compact Centrifuges RX II Series CF16RXII (rotor: 36, T16A31; 44, T15A36; 46, T15A36; 24, T9A31; 50, T5SS31) and Hitachi Himac CT15E (Hitachi Koki Company Ltd., Tokyo, Japan). Amicon Ultra-15 centrifugal filter devices collected from Millipore Corporation (Massachussetts, USA) were used for ultrafiltration. Slide-A-Lyzer Dialysis Products from Pierce (Rockford, Il, USA) were used for dialysis of enzyme samples. Inoculated media were incubated either at 37 ºC in MIR-262 (Sanyo Incubator, Tokyo, Japan) or with recipro shaker NR-1; at 25 ºC using a NR-300 double shaker (Taitec Corporation, Tokyo, Japan) or at 30 ºC and low temperature in LTI-60ISD EYALA low temperature incubator equipped with NR-30 double shaker (EYALA, Tokyo, Japan). Incubation of plates occurred in Taitec M-260F temperature controlled incubate box. Media were autoclaved at 121 ºC for 20 minutes using BS-235 high pressure steam sterilizer (Tomy Seiko Co., Ltd., Tokyo, Japan). Filter sterilization of solutions was carried out using 0.22 μm disposable filters MILLEX-GA (Millipore, Molshiem, France). Samples were vortexes using a Vortex Genie 2 (Scientific Industries, Inc., USA). Thermal Cycler Dice with model TP-600 or TP-100 were used for PCR reactions (Takara Bio Inc., Shiga, Japan).

1.2.2.2 Chemical synthesis

TMABaldehyde iodide was prepared from 4-aminobutyraldehyde. Two equivalents of methyl iodide (20 mmol) were added to a solution of 4-aminobutyraldehyde dimethylacetal (10 mmol) in 10 ml methanol in the presence of three equivalents of potassium carbonate (30 mmol) as a proton sponge. The mixture was refluxed for 5 hour at 60 ºC. Next, the mixture was evaporated after cooling at room temperature and added water to dissolve the residue. After extraction with dichloromethane (100 ml x 4 times), the combined organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated in *vacuo* to give 4-dimethylaminobutyraldehyde dimethylacetal. To a solution of 4-dimethylaminobutyraldehyde dimethylacetal (21 mmol) in ethylacetate (80 ml) was added five equivalents of methyl iodide (105 mmol). The mixture was stirred for 2 hours at roomtemperature and concentrated in *vacuo* to give 4-*N*-trimethylaminobutyraldehyde dimethylacetal iodide. Then the preparation was subjected to hydrolyze by 0.1 M of HCl overnight at room temperature to produce TMABaldehyde iodide as a 30 mM solution.

1.2.2.3 Bacterial strains, plasmids, and culture conditions

Pseudomonas sp. 13CM strain, isolated from soil, was used as the TMABaldehyde DH gene donor. Original isolates was grown in the medium containing 1.0% meat extract, 0.5% glucose, 0.5% of peptone, 0.1% of NaCl (Table 1.1) at pH 7.0 with reciprocal shaking at 30 ºC for overnight in reciprocal shaker at 125 strokes.min^{-1}, used for the preparation of genome DNA, used for isolation of genomic DNA. The plasmid pUC18 (Takara Bio Inc., Japan) was used for shotgun cloning and pUC18 (Nippon Gene, Japan) was used for construction of deletion mutants from recombinants (Fig. 1.1). Plasmid pET24b (+) was used for the expression of the recombinant (Fig. 1.2). *E. coli* DH5α and *E. coli* BL21 (DE3) were used, respectively, as a host strain for general cloning procedures and high level of recombinant expression. For identification of

native enzyme activity during cultivation stages, *Pseudomonas* sp. 13CM was grown on TMA-Butanol medium, as described by Hassan *et al*., (2007).

E. coli DH5α, *E. coli* JM109, and *E. coli* BL21 (DE3) carrying the recombinant plasmids were grown in Luria-Bertani (LB) medium consisting 1.0% peptone, 0.5% yeast extract, and 1.0% NaCl, at pH 7.0 (Table 1.2), supplemented with kanamycin (50 $\mu g.ml^{-1}$ of medium) and 0.3 mM isopropyle-β-D-thiogalactopyranoside (IPTG).

1.2.2.4 DNA isolations and manipulations

Chromosomal DNA was isolated from *Pseudomonas* sp. 13CM as described by Saito and Miura (Saito and Miura, 1963) after pretreatment with lysozyme, RNase and proteinase K. Small scale plasmid purified from *E. coli* using the MagExtractor-Plasmid kit (Toyobo, Japan). DNA fragments were recovered from agarose gels using a MagExtractor-Genome kit (Toyobo, Japan).

1.2.2.5 Enzyme assay

The catalytic activity of TMABaldehyde DH was determined at 30 ºC by monitoring the increase in absorbance at 340 nm which is the characteristic absorption wavelength of NADH and using an extinction coefficient of 6,200 $M^{-1}.cm^{-1}$. The standard reaction mixture (1.5 ml) contained 3.0 mM of NAD^+, 0.8 mM TMABaldehyde iodide, and an appropriate amount of the enzyme in 150.0 mM glycine-NaOH buffer (pH 9.5) (Table 1.5). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute under the assay conditions. The specific activity was defined as units of enzyme activity per milligram of protein.

Table 1.5 Assay system of TMABaldehyde DH

Glycine-NaoH buffer (pH 9.5, 450 mM)	0.50	ml
TMABaldehyde (30 mM)	0.04	ml
NAD^+ (60 mM)	0.05	ml
Enzyme solution	0.2~0.05	ml
Distilled water	0.86~0.71	ml
Total volume	1.5	ml

1.2.2.6 Protein measurement

The protein concentration was determined using the Lowry method (1951) with bovine serum albumin as the standard (Fig. 1.3) or by the absorbance at 280 nm, where an $E_{1cm}^{1\%}$ value of 10.0 was used. Specific activity was defined as units of enzyme activity per mg protein.

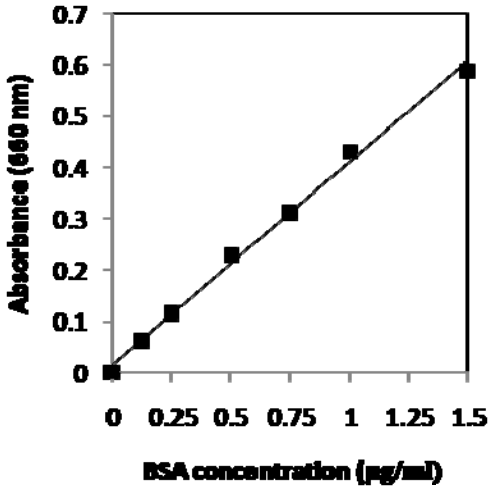


Fig. 1.3 Bovine serum albumin standard curve according to the Lowry method

1.2.2.7 Shotgun-cloning

The prepared genomic DNA from *Pseudomonas* sp. 13CM was partially digested by *BamH*I, ligated with *Bam*HI-digested plasmid pUC18. Next, *E. coli* DH5α was transformed with the ligated plasmid. Briefly, *E. coli* DH5α-competent cells (200 µl) were mixed with ligated plasmid (3 µl) in 1.5 ml tubes, by tapping the container of the mixture lightly and incubated the tube on ice for 30 min. The transformation was carried out with heat shock method (incubated the microtubes into waterbath at 42 ºC for 45 second). Tubes were backed on ice for 2 min to reduce the damage to the *E. coli* cells. Next, 0.5 ml of Super Optimal Broth medium (SOC medium) (Table 1.6) were added into each tube and again incubated at 37 ºC for 1 hour.

Table 1.6 Recipes for SOC medium

Bactotryptone	20	g
Bacto yeast extract	5	g
NaCl	0.585	g
KCl	0.186	g
$MgCl_2$ (1 M)	10	ml
$MgSO_4$ (1 M)	10	ml
Glucose (2 M)	10	ml
Total volume	1000	ml

The mixture (300 µl) was then spread on a pre-warmed (37 ºC) agar plate with LB medium containing 50 $\mu g.ml^{-1}$ carbenicillin, 0.3 mM IPTG and 40 $\mu g.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside and incubated at 37 ºC for 16 hours. Distinct colonies appeared on the plate. To screen the transformants, colonies (six hundred) were replicated onto a filter paper and treated with 2 ml of lysing solution, 100 mM potassium phosphate buffer (pH 8.0) containing 0.5% lysozyme and 10 mM EDTA

and incubated at 37 ºC for 30 min; assayed the TMA-Butanol DH and TMABaldehyde DH activities using TMA-Butanol and TMABaldehyde, respectively as substrates, with the activity-staining assay method (Raetz, 1975). Briefly, the filter paper with lysis cells was sunk in a solution supplemented with energy generating system inhibitor (NaN_3, 10 mM; NaF, 10 mM; Na_2AsO_4, 1 mM) for 5 min. Next, the filter paper was subjected to froze and thaw three times to inactivate the energy-generating system. After dry, the filter paper was then sunk in a solution prepared for activity staining (Table 1.7). The reductions of the nitroblue tetrazolium (NBT) lead to the formation of the purple color, indicator of the enzymatic activity. Positive clones by the activity staining assay were subjected to subsequent enzymatic activity assay by the spectrophotometric method.

Table 1.7 Reaction mixture for activity staining

Glycine-NaOH buffer (pH 9.5)	100	mM
1-methoxy phenazine methosulfate	64	μM
NBT	0.24	mM
NAD^+	1	mM
TMABaldehyde or TMA-Butanol	1	mM
Total volume	2	ml

An insert fragment of pAN1 was then sequenced using the primers shown in Table 1.4 and several different deletion mutants of pAN1 were constructed. The deduced amino acid sequences of the Open Reading Frames (ORFs) were compared with other similar polypeptides.

1.2.2.8 Construction of the vector for over-expression

Based on the sequence of TMABaldehyde DH II, the sense primer, AN1_aldeF: 5′-AAT CCG CAT ATG ACC ACT TCC CAT-3′ and antisense primer, AN1_aldeR: 5′-GCT TAC CCG GCG GTT CGA AGG GGC C-3′ was designed for construction of pET24b (+) vector. Both of the primers contained restriction sites (underlined) respectively, *Nde*I site and *Hind*III site for directional cloning into the multiple cloning sites. PCR was performed using these two primers and a DNA fragment containing the gene encoding TMABaldehyde DH II (*tmabadhII*) was amplified. PCR products were digested with the appropriate restriction enzymes, alongside the relevant vector, gel purified and then ligated at 2:1 (insert: vector) ratio using T4 DNA ligase (Fig. 1.4). Resulting ligation mixture was then transformed into the *E. coli* BL21 (DE3) cells for small-scale protein expression tests (Sambrook *et al*., 1989). A suitable control was set constructing the transformant with vector pET24b (+) only.

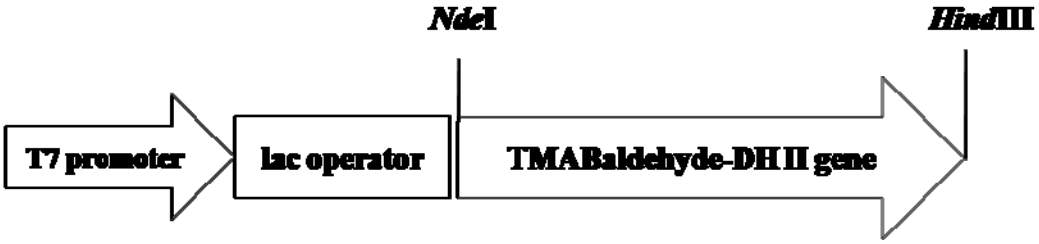


Fig. 1.4 Schematic diagram of pET24b-*tmabadhII*

1.2.2.9 Expression of TMABaldehyde DH II in E. coli BL21 (DE3)

The recombinant strain of *E. coli* BL21 (DE3) harboring pET24b ligated with TMABaldehyde DH II gene was subjected to grow in 75 ml of LB broth medium with 50 $\mu g.ml^{-1}$ kanamycin at 25 ºC until the optical density OD_{660} reached to 0.5–0.6. Expression was induced by adding IPTG to the medium with a final concentration of 0.3

mM and cultivation was continued for additional 16 hours. Cells were harvested through centrifugation at 14,000 x g for 20 min at 4 ºC and washed twice with a 0.85% potassium chloride solution. Cell pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. The mixture was sonicated (TOMY Ultrasonic Disruptor, UD-200, Japan) and clarified by centrifugation at 14,000 x g for 20 min at 4 ºC. The highly expressed recombinant protein was detected in crude extracts of *E. coli* BL21 (DE3) by SDS–PAGE on a 5–20% linear polyacrylamide gradient gel under denaturing conditions (Laemmli, 1970). Gels were stained with Coomasie brilliant blue R-250. The Color Plus prestained protein marker consisting *E. coli* MBP-truncated-β-galatosidase (80.0 kDa), *E. coli* MBP-CBD (58 kDa), rabbit muscle aldolase (46.0 kDa), *E. coli* triosephosphate isomerase (30.0 kDa), soybean trypsin inhibitor (23.0 kDa), and chicken egg white lysozyme (17.0 kDa) were used as standard proteins.

1.2.2.10 Purification of recombinant TMABaldehyde DH II

Recombinant TMABaldehyde DH II was purified by hydrophobic chromatography using Phenyl-Toyopearl spin column under native conditions with the crude cell-free extracts. All operations were conducted at 4 °C. Potassium phosphate buffer (50 mM, pH 7.5) containing 1.0 mM of DTT was used throughout the purification procedure unless otherwise stated. Briefly, cell pellet was resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and disrupted at 4 ºC by sonic treatment and centrifuged. To the cell-free extract, same volume of 0.5 M of ammonium sulfate solution in the 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT was added. The resulting solution was loaded onto a Phenyl-Toyopearl spin column equilibrated with the same buffer which contained 0.25 M of ammonium sulfate and desalted through repeated concentration or dilution against low salt buffer (50 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT). An identical procedure was successfully used to overexpress and purify the recombinant TMABaldehyde DH. The homogeneity of purified proteins was confirmed using SDS gel electrophoresis

(Laemmli, 1970) on a 5–20% linear polyacrylamide gradient gel under denaturing conditions and stained with Coomassie brilliant blue (CBB) R-250.

1.2.2.11 Characterization of TMABaldehyde DH II

The molecular mass of the enzyme was determined using SDS–PAGE and gel filtration on a column (0.78 × 30 cm) TSK-gel G3000SW (Tosoh Corp. Tokyo) equilibrated with 50 mM of potassium phosphate buffer (pH 7.5) containing 1.0 mM of DTT and 150 mM potassium chloride, at a flow rate of 0.5 $ml.min^{-1}$. The isoelectric focusing was done with minor modification, as described by Hassan *et al*., (2008). Native PAGE was performed using 10% gels at pH 8.8 and that gels were run at 4 ºC. The protein was stained with CBB or checked for enzyme activity at room temperature. The reaction mixture for activity staining for the native gels contained 150 mM of glycine-NaOH buffer (pH 9.5), 64 µM of 1-methoxy phenazine methosulfate, 0.24 mM of NBT, 3 mM of NAD^+ and 0.8 mM of TMABaldehyde iodide (Table 1.8).

Table 1.8 Reaction mixture for activity staining

Glycine-NaOH buffer (pH 9.5, 450 mM)	6.67	ml
TMABaldehyde iodide (30 mM)	0.53	ml
NAD^+ (60 mM)	1.00	ml
1-methoxy phenazine methosulfate (6.4 mM)	0.20	ml
Nitroblue tetrazolium (NTB) (2.4 mM)	2.00	ml
Total volume	20	ml

1.2.2.12 Molecular mass by mass spectrometry and N-terminal sequencing

The purified sample of TMABaldehyde DH II was assayed for molecular mass using MALDI-TOF mass spectrometry and analyses the mass by Bruker Daltonics flex Analysis. The N-terminal sequencing was performed by Edman's degradation method using Shimadzu PPSQ-31A Protein Sequencer.

1.2.2.13 Accession number

The nucleotide sequence of the *Pseudomonas* sp. 13CM gene cluster containing the TMABaldehyde DH II gene has been deposited in the DDBJ database under the accession number AB713749.

1.3 RESULTS

1.3.1 Cloning and sequencing of TMABaldehyde DH II gene

E. coli DH5α was transformed with ligated plasmid (*Bam*HI digested pUC18 and *Pseudomonas* sp. 13CM genome DNA) and distinct colonies were appeared on the plate. Six hundred transformants were subjected to check the enzyme activity based on the activity staining procedure. All of them were found to be inert against TMA-Butanol as substrate. However, two clones, namely *E. coli* DH5α/pAN1 and *E. coli* DH5α/pAN2 were found to active with TMABaldehyde as substrate. Plasmids isolated from the positive transformants were designated as pAN1 and pAN2. Restriction enzyme digestion of plasmids showed that both plasmids contained an approximately 7 kbp insert DNA fragment.

However, *E. coli* DH5α harboring pAN1 was selected as a potential strain for production of TMABaldehyde DH by virtue of its activity. Next, by evaluating the electrophoretic patterns of pAN1 plasmid with single and double restriction digestions, obtained the internal restriction map that contained *Pst*I, *Sma*I, *Kpn*I, *Sac*I, *Sph*I, and *Hind*III sites (Fig. 1.5). To check the activity, recombinant *E. coli* were grown on LB medium containing carbenicillin and IPTG for 16 hours at 25 ºC and enzyme assay was carried out as described in the Materials and methods.

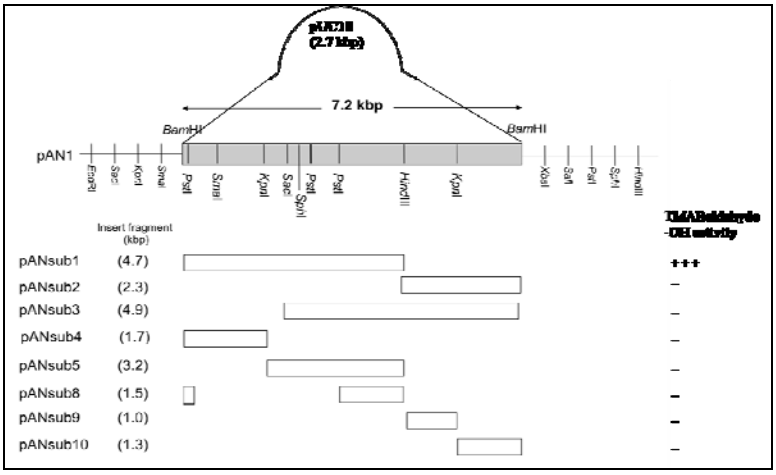


Fig. 1.5 Physical map of pAN1 and its derivatives

The localization of the gene of enzyme precisely within the 7.2 kbp insert fragments of pAN1 was determined by various fragments which subcloned in pUC18. The plasmid pAN1 digested with *Hind*III and DNA fragment containing about 4.75 kbp was self-ligated. The plasmid, pANsub1was obtained by transformation with *E. coli* DH5α. Next, digestion with the *Hind*III and fragment 2.3 kbp was obtained and ligated to plasmid vector pUC18 to obtain recombinant pANsub2, and transformed with *E. coli* DH5α. Likewise, the recombinants pANsub3, pANsub4, were constructed, respectively, with the restriction enzymes *Sac*I, and *Kpn*I; self ligated and vector ligated. Moreover, the recombinant pANsub1 and pANsub2 were digested with *Kpn*I, self ligated to construct the plasmid pANsub5 and pANsub9, respectively, and transformed into *E. coli*

DH5α to prepare the recombinants. The plasmid pANsub1 also digested with *Pst*I, and DNA fragment containing pANsub1-*Pst*I was self ligated to produce plasmid pANsub8. The plasmid pANsub2 was double digested with *Bam*HI and *Hind*III, ligated into plasmid vector pUC18 and transformed into *E. coli* DH5α competent cells to obtain recombinant pANsub10. All transformants were checked for the enzyme activity using TMABaldehyde iodide as substrate. The activity was only found with the crude extract prepared with *E. coli* DH5α harboring pANsub1 (Fig. 1.5). The inert attitude of the transformant pANsub4 which was constructed by *Kpn*I denoted the possibility of significance of the terminal area of pANsub1.

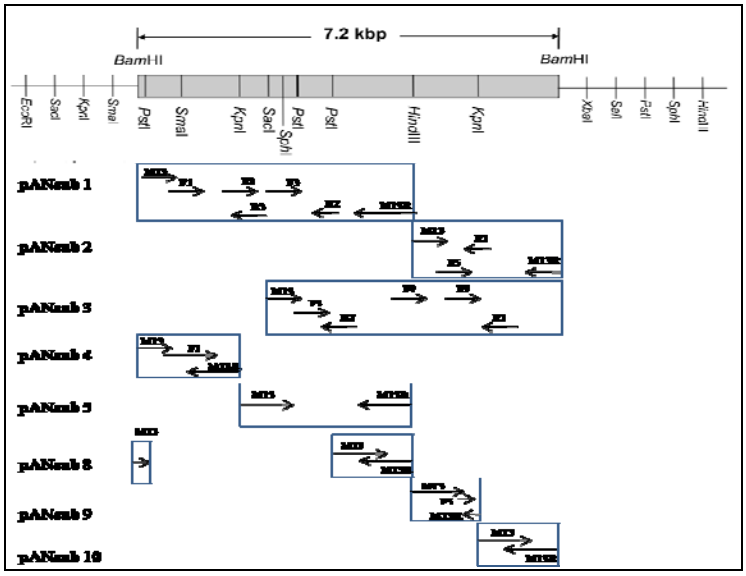


Fig. 1.6 Sequencing strategy for TMABaldehyde DH II gene

The thin arrow below restriction map shows the direction and length of each sequence analyzed. F, stands for forward; whereas R, stands for reverse primer.

The nucleotides of the 7.2 kbp DNA fragment containing TMABaldehyde DH gene of pAN1 were sequenced by the strategy shown in Figure 1.6. A complete nucleotide sequence from pAN1 which consist about 7.0 kbp fragment of the *Pseudomonas* sp. 13CM was shown in Figure 1.7.

```
        10         20         30         40         50         60         70
ggatccccag tgcggcggtc ggcctgtgct tcgtcaacct gccggggcaa ttcgtcggcc cgctcggcga
        80         90        100        110        120        130        140
cctggccggg ggcatcgacc tgagcctgcc ggtcaccctg ggcctggccg gcgcgctgta cctgctgctg
       150        160        170        180        190        200        210
ctcaacctgt tccctgaacc tgctggcgtg tatggcccca atggcccgcg ctgggtgcgc tgcaaaagca
       220        230        240        250        260        270        280
cccggcacat gcccgtgacc actgccgaaa tggcctgact ggaatccgac catgaccact tcccattaca
                                                    →ORF1 start
       290        300        310        320        330        340        350
tcgctggccg ctggctcgaa ggccaaggca gcgactgcat taccgtcaac gacccagcac tgggtgtgcc
       360        370        380        390        400        410        420
cttcgccgag ctgacggccg ccagcgtcgc ccaggtcgac caggccgtag ctgcggcccg cgatgccctg
       430        440        450        460        470        480        490
ccgacctgga aagccctcag cgccaagacc cgcgcggcgt ttctgcgtgg ctttgccgag caacttggcc
       500        510        520        530        540        550        560
agcgccgcga aacgctgctg gccctgcaga tgcgcaacaa cggcaagccg cgccatgagg ccgaaatcga
       570        580        590        600        610        620        630
cctggacgac gccatcgcca ccttcgccta ctacgccgag ctggccgagc agctacctga aaagaaccgt
       640        650        660        670        680        690        700
gaagtgcccc tggccgcccc cgggttcacc gcccgcacac gcctggagcc ggtgggcgtg gtcggcctga
       710        720        730        740        750        760        770
tcgtgccgtg gaacttcccg ctggtgacca gcgcctggaa gctcgccccg gccttggctg ccggctgcac
       780        790        800        810        820        830        840
cgtggtgctc aagccctcgg aagtcacccc actgatcgag caggcctacg gccagatcgc cgacaccctg
       850        860        870        880        890        900        910
ggcctgccgg ccggcgtgct gaacatcgtc aacggcaagg ccgaaaccgg cgcggccctg agcagccaca
       920        930        940        950        960        970        980
acggcctgga caagctgtcc ttcaccggca gcaacagcgt cggcagccag gtgatgcgca gcgcggcggc
       990       1000       1010       1020       1030       1040       1050
gcagtgccgg ccggtgaccc tggagctggg gggcaagtcg gcgatcgtgg tgttcgatga ctgcgacgtg
      1060       1070       1080       1090       1100       1110       1120
gaccaggcgg tggagtggat cgtcgccggc atcacctgga atgccgggca gatgtgctcg gccacctcgc
      1130       1140       1150       1160       1170       1180       1190
gcctgctggt gcaggacggc attgccgatg cgctgctgcc acgcctgcag caggcgctgg aaaaactgcg
      1200       1210       1220       1230       1240       1250       1260
ggtgggcaac ccgctgagcg aagaagtgga catggggccg ctgaccagcc aggcgcaatg gctgaaagtg
      1270       1280       1290       1300       1310       1320       1330
gccagctact tcgccactgc ccgcgaagaa ggcctgcagt gcctggccgg cggcaaggcg ctggaccgcg
      1340       1350       1360       1370       1380       1390       1400
acggctggtt cgtcagcccg acgctgtatg ccgatgtgcc caaggaaagc cgcctgtgga ccgaggaaat
      1410       1420       1430       1440       1450       1460       1470
tttcggcccg gtgctgtgcg cacgccgttt cgccagcgaa caacaggcca ttgccgaagc caacgacagc
      1480       1490       1500       1510       1520       1530       1540
cgctttggcc tggtggccac cgtctgttca gcggacctgg tgcgcgccga gcgcgtggcc gatgcgctgg
      1550       1560       1570       1580       1590       1600       1610
aagtcggcca tgtgtggatc aactcggtgc aggcggtgtt cgtcgaaaca tcgtggggcg gtaccaaggg
      1620       1630       1640       1650       1660       1670       1680
cagcgggatc gggcgtgagc tcgggccttg ggggttgtcc gggtacctgt cggtgaagca tgtgacgcgc
      1690       1700       1710       1720       1730       1740       1750
tgcctgggct gacgaccgcg aatgggccgc caagcggccc cggtttcacg ccggctcgtc agccggccgt
           ORF1 stop                                  ORF2 stop
      1760       1770       1780       1790       1800       1810       1820
gccccttcct gcaccagcac catgctctgc ggcgaagaca gggcaatgcc ctcatcgcgc aactggccaa
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Fig. 1.7 Complete nucleotide sequence from pAN1 prepared with *E. coli* DH5α

Fig. 1.7 (continue)

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      1830       1840       1850       1860       1870       1880       1890
ggatggtgaa cagcaagtcg ctgcgcgccc ccgacacctg ccgcggcccc gccacataac cactcacgcc
      1900       1910       1920       1930       1940       1950       1960
aatcaccatg ccgttgctgg tcaggtcctt gaaggtcacc gacggcgacg gcgcatcgag caccgcctcg
      1970       1980       1990       2000       2010       2020       2030
tgttcctggt aggccttgag caacacctcg cgcacgcggt tggcatcggt ttccagcggc aaggtcaggg
      2040       2050       2060       2070       2080       2090       2100
tgatgccgac cacgcccagg gcattgccca tggtcacgtt gcgcacgttc tgcgaaatga actgcgagtt
      2110       2120       2130       2140       2150       2160       2170
gggcacgatc accgtggagc ggtcggacat ctggatctcg gtggcgcgca cgttgatccg ccgaatgtca
      2180       2190       2200       2210       2220       2230       2240
ccctcgacgc cagccaggct cacccagtcg cccactttga ccgggcgttc ggtgagcagg atcaggccgg
      2250       2260       2270       2280       2290       2300       2310
aaatgaagtt ctgcacgatc tgctgcaagc cgaaaccgat accgaccgac agcgcactga ccacccaggt
      2320       2330       2340       2350       2360       2370       2380
caggctggtg aggttgatgc gcagggtcga catcaccagc atggccagga acaggaagcc caggtaaccc
      2390       2400       2410       2420       2430       2440       2450
accagggtca ccagcgaggc gcgcatgccg gcgtccatgt cggtttccgg cagcaagcgc tcgctcagcc
      2460       2470       2480       2490       2500       2510       2520
agcgcttgag cacgcgaatg ccgaacaggc cgccgaagaa gatcgccatg gccagcagga tgtcctgggg
      2530       2540       2550       2560       2570       2580       2590
cacgatgttc aggttgccga gcagcttgcc accggtgccg tcccagtcgc ccaggctcag cagcagctcg
      2600       2610       2620       2630       2640       2650       2660
ctggggctgg tgcccgaggg catcagcgcc agcaacacgg cgaggaacaa cagcacggtg cggccgatac
      2670       2680       2690       2700       2710       2720       2730
cggtgagtat ggtgctggcc tgcgcctggt ggcgcggcgc caggcccaag gtcgcagcca gcgccaggcc
      2740       2750       2760       2770       2780       2790       2800
accgggctgg cggggcgaga gcagggtttc gcagaggtcg ctgaggaagg tggtcaacag gtaggcgcag
      2810       2820       2830       2840       2850       2860       2870
gtggccacca cgctgatcca cagcagcttg gcagtgagga agtaggccag ggtcaggtag ccggtcaaca
      2880       2890       2900       2910       2920       2930       2940
gcgccagcac gatcagcccg acccatacca cgatcacgaa cgggatcaac ccggccaggc cggtggggcg
      2950       2960       2970       2980       2990       3000       3010
ctccagcgcg tgcttgcgca gggtacggcg gtagcgcatc agcgccatcg agaaaatcac cgccaccacc
      3020       3030       3040       3050       3060       3070       3080
agcgcggtca ggccgttggt cgccacggtg agcgccaggc tggtgccgat cacgctgttg atgcgctcca
      3090       3100       3110       3120       3130       3140       3150
tggtcagcat caccatcagc gccagggcca gcagcttggg gaaccagccc agggcgtggg ccacttcgtc
      3160       3170       3180       3190       3200       3210       3220
ggcgatcggc ggcaggcgcc aggacgggcg ctgcagcatc agcatggcgc ggcccaggcc ggaaatgaag
      3230       3240       3250       3260       3270       3280       3290
gcgctgaaca ccaccagggt gaggaagtgg ttggtcaggc tggcaatgtc ggagcccagt tcggagctgc
      3300       3310       3320       3330       3340       3350       3360
tttccaggcc ccagcgcagc aacgacacgg agcctgcgat ggtgcccagg gttgccaggc tcacgctcag
      3370       3380       3390       3400       3410       3420       3430
ggccagggca ctgcggcgca ggcggccctc gggcagccag cggatcatcg catcggccag caagcgttcg
      3440       3450       3460       3470       3480       3490       3500
agcacccggc gtaccagggt ccagaccagg atcgccgcca ccaggctggc aatgaagaac caacggtgct
      3510       3520       3530       3540       3550       3560       3570
cggcactgaa ggcactggcc acggcatcgg ccgcctcgcc gcgcaggtcg cgcaggcgca tcacgtcttc
      3580       3590       3600       3610       3620       3630       3640
gtcggtgggg cggatcagcg attgccagaa ggccggggtc agcgggctgg tggcgcggct ggtgacctgc
      3650       3660       3670       3680       3690       3700       3710
gagttgaact ggctgcggcg caggttgacg atctgcgtgg acaggtcgcg ggccgactgg gtcagcttgg
     3720       3730       3740       3750       3760       3770       3780
cggcctgctc ctgctcggcg agcaccgcct tcttctcggc ttcgagctgg ttgcgctgct cggtgaggct
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Fig. 1.7 Complete nucleotide sequence from pAN1 prepared with *E. coli* DH5α

Fig. 1.7 (continue)

```
      3790       3800       3810       3820       3830       3840       3850
gcgggcttcg tccggttgca ccgggccgat gaccttgagc ttgtcgtcca gcttctccac atcggccgtg
      3860       3870       3880       3890       3900       3910       3920
cgcaaggcgc tcagcgcatc ggcctggcgc tggacctgca cagcgcccag gcgcagctgc aggagcaggt
      3930       3940       3950       3960       3970       3980       3990
cgtcgttggc attgctggtc acgccctggc ggatctggtc gagccgttcg ctcaactgct ccaggctggc
      4000       4010       4020       4030       4040       4050       4060
gctttcatcc agcaccggca gttcggcagc ggccaggctg gacaccggcg tagccagtgc cgaccaggcc
      4070       4080       4090       4100       4110       4120       4130
gggctggcaa acgccagcat cagggtcatg acacccaggt aaaaacaggc aaggctggaa cgcctcatcg
                                                                 ORF2 start←
      4140       4150       4160       4170       4180       4190       4200
aatgttcctc tttacatttc gatcaggcgg tggattctac gcggcttgtg gcgatccgaa gagtgccgtt
      4210       4220       4230       4240       4250       4260       4270
tctcgcaaca atcctgcgcc attcgcttgc ggcgcaagaa agtgtcctgt tcccttcagt ctgccactgc
                                                    ORF3 stop
      4280       4290       4300       4310       4320       4330       4340
ccgcagcttg cccactcggc ggtacgactg ccgggcaaag cacacgaaca gcccggccat gatcgccagc
      4350       4360       4370       4380       4390       4400       4410
gccatcggca acccatgcgg cgccacatcc atggccgcac cactgaccaa cggcccgatc aggctgccga
      4420       4430       4440       4450       4460       4470       4480
cgccccacag caagccgacg ctggcattgg cggtgaccag gtcctgcccc ttgaagcgct gcccgatcaa
      4490       4500       4510       4520       4530       4540       4550
caccagcgcc agggtataga tgccccccgc caccgcgccc agcaccacca gcagcggcca cagcagccag
      4560       4570       4580       4590       4600       4610       4620
gtcatgttca gcagccaagg caaggcaata ccgatggcca tcgccaccag cccgcatacc aggtgcagac
      4630       4640       4650       4660       4670       4680       4690
cggtgcgctc gacccggtcg gccagccagc ccaacggcag ctggaagacc atgtcaccgg cgaacaccac
      4700       4710       4720       4730       4740       4750       4760
ggtcaccatc agcgccgcca cacccacggc aaagccatgg ctggtggcat acaccggcag cagcgacagc
      4770       4780       4790       4800       4810       4820       4830
accacggcat cgaagaacga gaagaacagc accgccacgc acagcgctgg cgccacccgg aagaaaccgg
      4840       4850       4860       4870       4880       4890       4900
ccaggccgaa gctttttcg ccttcctcgc catgctcgac atggtcgttg ggcacggtga acaggatgca
      4910       4920       4930       4940       4950       4960       4970
cagcagggcc aggccgtagc acagggtcac cacaccggtg atccacgggc tgtcggcccc gagcagcgcc
      4980       4990       5000       5010       5020       5030       5040
agcaatgccg ggcccagtac ctggaagccg gtgaagctgg tggcatacag ggccatgatc ttgccgcggt
      5050       5060       5070       5080       5090       5100       5110
tatggtccgg gcacagttcg ttgacccagg actcgccgag gatgatggcg atgcccatgc cgatgcccag
      5120       5130       5140       5150       5160       5170       5180
ccccaggcgc agcacggcca gcagccagag cgaatcgaat gccgactcca gcagcgcgat gctgacggtg
      5190       5200       5210       5220       5230       5240       5250
cacaggctga agcacagcag gtagatggtg cgccgggtca gcaggcggca gcaggcgtcg accatgaacg
      5260       5270       5280       5290       5300       5310       5320
ccgagagcat catgcccgcg gcggggatgg ccgagatgat gccgatttgc agggtgccgg caccggcttc
      5330       5340       5350       5360       5370       5380       5390
gtgcaggcgc aacgacacca gcggcaggct ggctcccaaa ctgaagccga cgaccgagac ggcgaacaga
      5400       5410       5420       5430       5440       5450       5460
aggcccgcca gcaaacgcat gttcatgtac cactccaagc aaaaccgaaa agacgtgcag atacagacgc
               ORF3 start←
      5470       5480       5490       5500       5510       5520       5530
ccacgcgcac accgggaagg cggacgacgc acaggcgcgg tcagttcagg gcaaggtgtg gcgagaaggt
```

Fig. 1.7 Complete nucleotide sequence from pAN1 prepared with *E. coli* DH5α

Fig. 1.7 (continue)

```
      5540       5550       5560       5570       5580       5590       5600
gaaggcaaac agcacgctgc ggcgacgctt gagcaccgtg tcgctcggcc acgcgcgacg catggcctgg
      5610       5620       5630       5640       5650       5660       5670
agggcaggct ggcgggcatg ggggaggctt tgggtacggc gcattgctgt gggactacgc aggtgtgtgg
      5680       5690       5700       5710       5720       5730       5740
aaagaggcgg cactctaggc taagcctggt tgaatcgtca attgctgggg gccgctttgc ggccccaacc
      5750       5760       5770       5780       5790       5800       5810
atttcagcgc ttgccggtat tgggcagaaa caccgccagc aagccaaaca acggcaagaa cgagcacagc
ORF4 stop
      5820       5830       5840       5850       5860       5870       5880
ccatatacat actcgatacc ccgcaaatcc gccaggtacc ccagcagcgc cgcgccaatc ccgccgaagc
      5890       5900       5910       5920       5930       5940       5950
caaacattaa cccgaagaag atcccggcaa tcatgcccac gctgcccggc accagctcct gcgcatacac
      5960       5970       5980       5990       6000       6010       6020
cacgatcgcc gaaaacgccg acgccaggat aaagccgatc accacgctga gcacggtggt ccagaacagg
      6030       6040       6050       6060       6070       6080       6090
tcggcatagg gcaacgccag ggtgaacggc gccacgccga ggatcgagaa ccagatcacc gccttgcgcc
      6100       6110       6120       6130       6140       6150       6160
cgatacggtc accgatgggc ccgccgaaga acgtaccggc agccaccgcg cccaggaaca ggaacaggtg
      6170       6180       6190       6200       6210       6220       6230
cagctgcgaa ctggccaccg acaggccgaa cttctcgatc aggtagaagg tgaagtagct ggtgaagctg
      6240       6250       6260       6270       6280       6290       6300
gccatgtaga agtacttgga gaacaccagc aggcccagca cgatcaaggc agcgatcacc cgctggcgcg
      6310       6320       6330       6340       6350       6360       6370
aaatgccatg ggtggcctgc acggccttgc gcgccttggc ctggttgagg tgttccttgt accagcggcg
      6380       6390       6400       6410       6420       6430       6440
cagcatcagg gtcacggcga agaacaacag cccggccacg ccgaaccagg ccacgtgggt ctgcccgaac
      6450       6460       6470       6480       6490       6500       6510
gggatgacga tggccgctgc cagcagtggg ccgaaggcgg agccggcgtt gccaccgacc tggaaggtcg
      6520       6530       6540       6550       6560       6570       6580
actgggccag gccgaagcgc ccgcccgagg ccaggcgcgc gatccgcgac gtctccgggt ggaacgtcga
      6590       6600       6610       6620       6630       6640       6650
cgagccgata cccaccagtg ccgaagccag gagaatcatc gggaagctgc cgacaaaggc cagcatgacg
      6660       6670       6680       6690       6700       6710       6720
atgcccacca gggtgcacag ggtgccgagc ggcagcaggt tcggcgccgg ccggcggtcg gtgaagaagc
      6730       6740       6750       6760       6770       6780       6790
cgacccaggg ctgcagcagg gaagcggtga cctggaaggt cagggtaatc aggccgatct gggcgaagct
      6800       6810       6820       6830       6840       6850       6860
caggtcgtag ttgaccttga gcatcgggta gatcgccggc agcaccgact ggataaggtc gttgatcagg
      6870       6880       6890       6900       6910       6920       6930
tgcgccaagg cgcagaacgt gatgatacgc atcaccagcg ggttggcttg gctcgccgaa ccggtgctgg
      6940       6950       6960       6970       6980       6990       7000
cggagggggt gctgctgatg gccatgggct tgagttccgt ccgcaggttt gggatcc
              ORF4 start←
```

Fig. 1.7 Complete nucleotide sequence from pAN1 prepared with *E. coli* DH5α

Positions and directions ORFs are indicated with blue and lacZ with green. Restriction sites are underlined: *Bam*HI (GGATCC), *Sal*I (GTCGAC), *Pst*I (CTGCAG), *Sma*I (CCCGGG), *Kpn*I (GGTACC), and *Sac*I (GAGCTC).

Sequence analysis indicated that the DNA fragment of 6,987 bp included four complete ORFs (Fig. 1.8). Among them, the ORF1 comprising 1,431 nucleotides starting with ATG (Met) and end with TGA. ORF2 (2,400 bp), ORF3 (1,160 bp), and ORF4 (1,210 bp), respectively, were identified downstream to the ORF1 but in the opposite direction on the complementary strand.

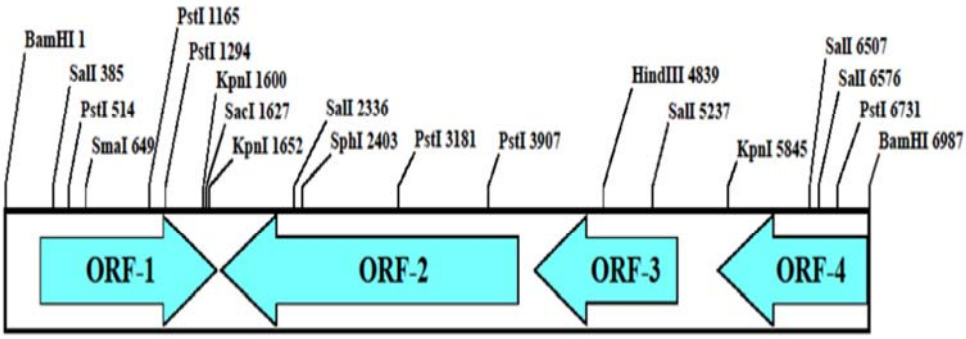

Fig. 1.8 Physical and genetic map of 6,987 bp DNA fragment containing the cluster gene in *Pseudomonas* sp. 13CM chromosome

The arrows shown denote the open reading frames and the direction of transcription.

The translated ORF1 has 92% positional identity with the putative betaine aldehyde dehydrogenase of *P. putida* KT2440 (Q88PZ0). Of the other ORFs, the encoded proteins of ORF2, ORF3, and ORF4 were, respectively, similar to the putative membrane protein (Q88PZ1), major facilitator family transporter (Q88PZ6), and the putative MFS efflux transporter (Q88PZ7). The amino acid sequence of 476 amino acids deduced from the nucleotide sequence of ORF1 is shown in Figure 1.9.

```
GGATCCCCAGTGCGGCGGTCGGCCTGTGCTTCGTCAACCTGCCGGGGCAATTCGTCGGCC     60
CGCTCGGCGACCTGGCCGGGGGCATCGACCTGAGCCTGCCGGTCACCCTGGGCCTGGCCG    120
GCGCGCTGTACCTGCTGCTGCTCAACCTGTTCCCTGAACCTGCTGGCGTGTATGGCCCCA    180
ATGGCCCGCGCTGGGTGCGCTGCAAAAGCACCCGGCACATGCCCGTGACCACTGCCGAAA    240
TGGCCTGACTGGAATCCGACCATGACCACTTCCCATTACATCGCTGGCCGCTGGCTCGAA    300
                      M  T  T  S  H  Y  I  A  G  R  W  L  E      13
GGCCAAGGCAGCGACTGCATTACCGTCAACGACCCAGCACTGGGTGTGCCCTTCGCCGAG    360
 G  Q  G  S  D  C  I  T  V  N  D  P  A  L  G  V  P  F  A  E      33
CTGACGGCCGCCAGCGTCGCCCAGGTCGACCAGGCCGTAGCTGCGGCCCGCGATGCCCTG    420
 L  T  A  A  S  V  A  Q  V  D  Q  A  V  A  A  A  R  D  A  L      53
CCGACCTGGAAAGCCCTCAGCGCCAAGACCCGCGCGGCGTTTCTGCGTGGCTTTGCCGAG    480
 P  T  W  K  A  L  S  A  K  T  R  A  A  F  L  R  G  F  A  E      73
CAACTTGGCCAGCGCCGCGAAACGCTGCTGGCCCTGCAGATGCGCAACAACGGCAAGCCG    540
 Q  L  G  Q  R  R  E  T  L  L  A  L  Q  M  R  N  N  G  K  P      93
CGCCATGAGGCCGAAATCGACCTGGACGACGCCATCGCCACCTTCGCCTACTACGCCGAG    600
 R  H  E  A  E  I  D  L  D  D  A  I  A  T  F  A  Y  Y  A  E     113
CTGGCCGAGCAGCTACCTGAAAAGAACCGTGAAGTGCCCCTGGCCGCCCCCGGGTTCACC    660
 L  A  E  Q  L  P  E  K  N  R  E  V  P  L  A  A  P  G  F  T     133
GCCCGCACACGCCTGGAGCCGGTGGGCGTGGTCGGCCTGATCGTGCCGTGGAACTTCCCG    720
 A  R  T  R  L  E  P  V  G  V  V  G  L  I  V  P  W  N  F  P     153
CTGGTGACCAGCGCCTGGAAGCTCGCCCCGGCCTTGGCTGCCGGCTGCACCGTGGTGCTC    780
 L  V  T  S  A  W  K  L  A  P  A  L  A  A  G  C  T  V  V  L     173
AAGCCCTCGGAAGTCACCCCACTGATCGAGCAGGCCTACGGCCAGATCGCCGACACCCTG    840
 K  P  S  E  V  T  P  L  I  E  Q  A  Y  G  Q  I  A  D  T  L     193
GGCCTGCCGGCCGGCGTGCTGAACATCGTCAACGGCAAGGCCGAAACCGGCGCGGCCCTG    900
 G  L  P  A  G  V  L  N  I  V  N  G  K  A  E  T  G  A  A  L     213
AGCAGCCACAACGGCCTGGACAAGCTGTCCTTCACCGGCAGCAACAGCGTCGGCAGCCAG    960
 S  S  H  N  G  L  D  K  L  S  F  T  G  S  N  S  V  G  S  Q     233
GTGATGCGCAGCGCGGCGGCGCAGTGCCGGCCGGTGACCCTGGAGCTGGGGGGCAAGTCG   1020
 V  M  R  S  A  A  A  Q  C  R  P  V  T  L  E  L  G  G  K  S     253
GCGATCGTGGTGTTCGATGACTGCGACGTGGACCAGGCGGTGGAGTGGATCGTCGCCGGC   1080
 A  I  V  V  F  D  D  C  D  V  D  Q  A  V  E  W  I  V  A  G     273
ATCACCTGGAATGCCGGGCAGATGTGCTCGGCCACCTCGCGCCTGCTGGTGCAGGACGGC   1140
 I  T  W  N  A  G  Q  M  C  S  A  T  S  R  L  L  V  Q  D  G     293
ATTGCCGATGCGCTGCTGCCACGCCTGCAGCAGGCGCTGGAAAAACTGCGGGTGGGCAAC   1200
 I  A  D  A  L  L  P  R  L  Q  Q  A  L  E  K  L  R  V  G  N     313
CCGCTGAGCGAAGAAGTGGACATGGGGCCGCTGACCAGCCAGGCGCAATGGCTGAAAGTG   1260
 P  L  S  E  E  V  D  M  G  P  L  T  S  Q  A  Q  W  L  K  V     333
GCCAGCTACTTCGCCACTGCCCGCGAAGAAGGCCTGCAGTGCCTGGCCGGCGGCAAGGCG   1320
 A  S  Y  F  A  T  A  R  E  E  G  L  Q  C  L  A  G  G  K  A     353
CTGGACCGCGACGGCTGGTTCGTCAGCCCGACGCTGTATGCCGATGTGCCCAAGGAAAGC   1380
 L  D  R  D  G  W  F  V  S  P  T  L  Y  A  D  V  P  K  E  S     373
CGCCTGTGGACCGAGGAAATTTTCGGCCCGGTGCTGTGCGCACGCCGTTTCGCCAGCGAA   1440
 R  L  W  T  E  E  I  F  G  P  V  L  C  A  R  R  F  A  S  E     393
CAACAGGCCATTGCCGAAGCCAACGACAGCCGCTTTGGCCTGGTGGCCACCGTCTGTTCA   1500
 Q  Q  A  I  A  E  A  N  D  S  R  F  G  L  V  A  T  V  C  S     413
GCGGACCTGGTGCGCGCCGAGCGCGTGGCCGATGCGCTGGAAGTCGGCCATGTGTGGATC   1560
 A  D  L  V  R  A  E  R  V  A  D  A  L  E  V  G  H  V  W  I     433
AACTCGGTGCAGGCGGTGTTCGTCGAAACATCGTGGGGCGGTACCAAGGGCAGCGGGATC   1620
 N  S  V  Q  A  V  F  V  E  T  S  W  G  G  T  K  G  S  G  I     453
GGGCGTGAGCTCGGGCCTTGGGGGTTGTCCGGGTACCTGTCGGTGAAGCATGTGACGCGC   1680
 G  R  E  L  G  P  W  G  L  S  G  Y  L  S  V  K  H  V  T  R     473
```

Fig. 1.9 Nucleotide and deduced amino acid sequences of TMABaldehyde DH II

Fig. 1.9 (continue)

```
TGCCTGGGCTGACGACCGCGAATGGGCCGCCAAGCGGCCCCGGTT                    1725
 C  L  G  *                                                       476
```

Fig. 1.9 Nucleotide and deduced amino acid sequences of TMABaldehyde DH II

The numbers to the right of the sequence indicate the nucleotide and amino acid positions. The deduced amino acid sequence is shown under the nucleotide sequence. The initiation codon (ATG) is in bold. Asterisk denotes the translational stop codon (TGA). A possible Shine–Dalgarno sequence is shaded at seven nucleotides upstream of the start codon. Restriction enzyme sequences are underlined: *Bam*HI (GGATCC), *Sal*I (GTCGAC), *Pst*I (CTCCAG), *Sma*I (CCCGGG), *Kpn*I (GGTACC), and *Sac*I (GAGCTC). Sequence data are deposited in the DDBJ database under the accession number of AB713749.

The primary structure of protein encoded in ORF1 closely resembled putative betaine aldehyde dehydrogenases from the *Pseudomonas* genus and shared a high amino acid similarity (30–45%) to *E. coli* γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883), cod betaine aldehyde dehydrogenase (P56533), spinach betaine aldehyde dehydrogenase (M31480), barley betaine aldehyde dehydrogenase type 1 (AB063179), barley betaine aldehyde dehydrogenase type 2 (AB063178) (Table 1.9, Fig. 1.10). The deduced primary structure was differed from the previously reported N-terminus of the TMABaldehyde DH of *Pseudomonas* sp. 13CM (Hassan *et al*., 2008). Based on its sequence and other properties, we proposed to name the new protein as "TMABaldehyde DH II" which is encoded by the ORF1.

Table 1.9 Comparison of TMABaldehyde DH II (ORF1) with other protein databases

Origin	Locus ID	Protein	Alignment length	Identity (%)
P. putida KT2440	Q88PZ0	Putative betaine ald. dehydrogenase	476	92
Barley	AB063179	Betaine ald. dehydrogenase type 1	505	45
Spinach	M31480	Betaine ald. dehydrogenase	497	45
Barley	AB063178	Betaine ald. dehydrogenase type 2	503	43
E. coli	P23883	γ-Glutamyl-γ-aminobutyraldehyde dehydrogenase (PuuC)	495	38
E. coli	M77739	Betaine ald. dehydrogenase (*bet*B)	483	37
Cod	P56533	Betaine ald. dehydrogenase	500	37
E. coli	P77674	Betaine ald. dehydrogenase (YdcW)	474	37
R. norvegicus	AF170918	TMABaldehyde DH	494	35

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DH II    ----------------MTTSHYIAGRWVEGQG-SDCITVNDPALGVPFAELTAASVAQV 42
```

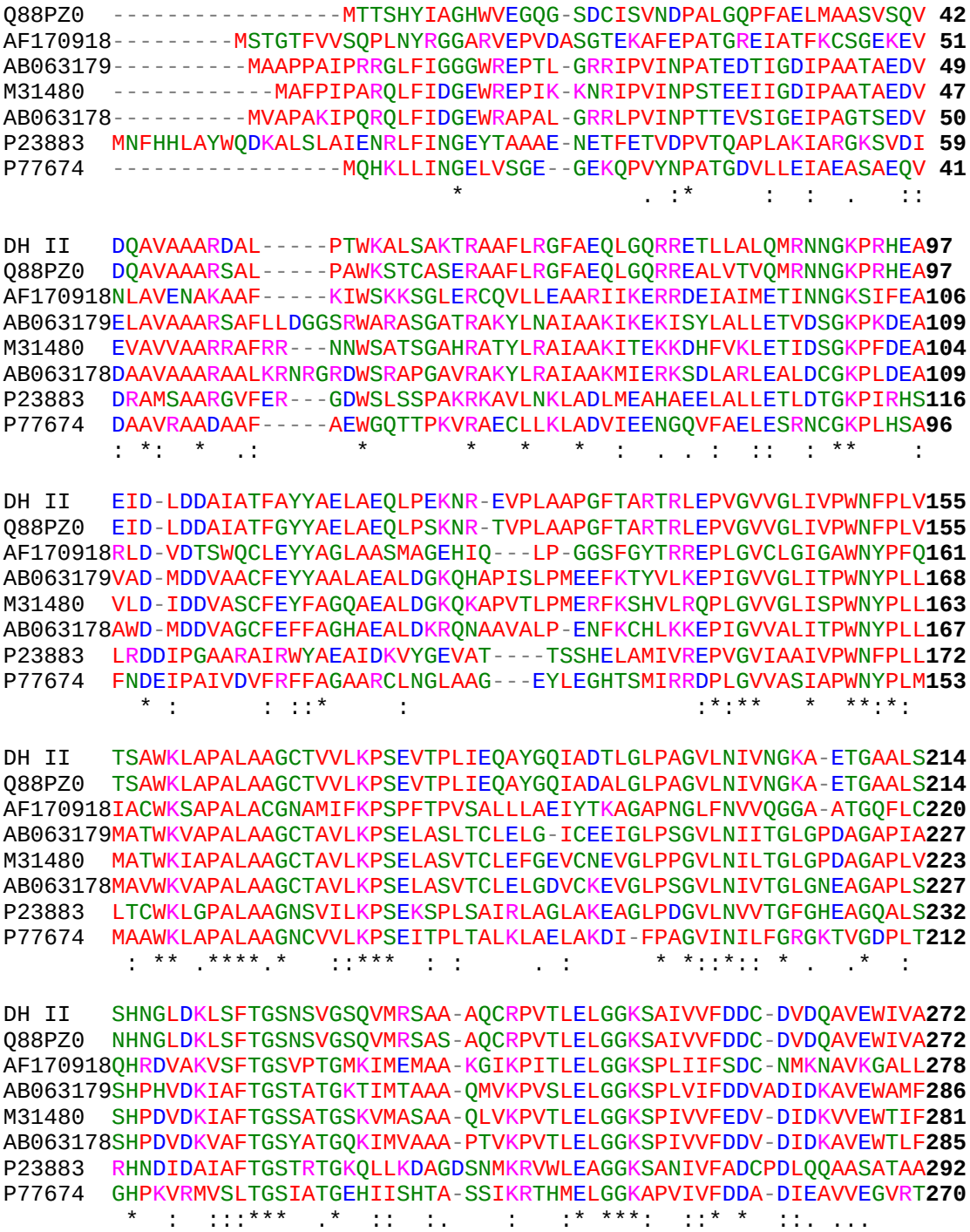

Fig. 1.10 Amino acid sequences of TMABaldehyde DH II and similar polypeptides

Fig. 1.10 (continue)

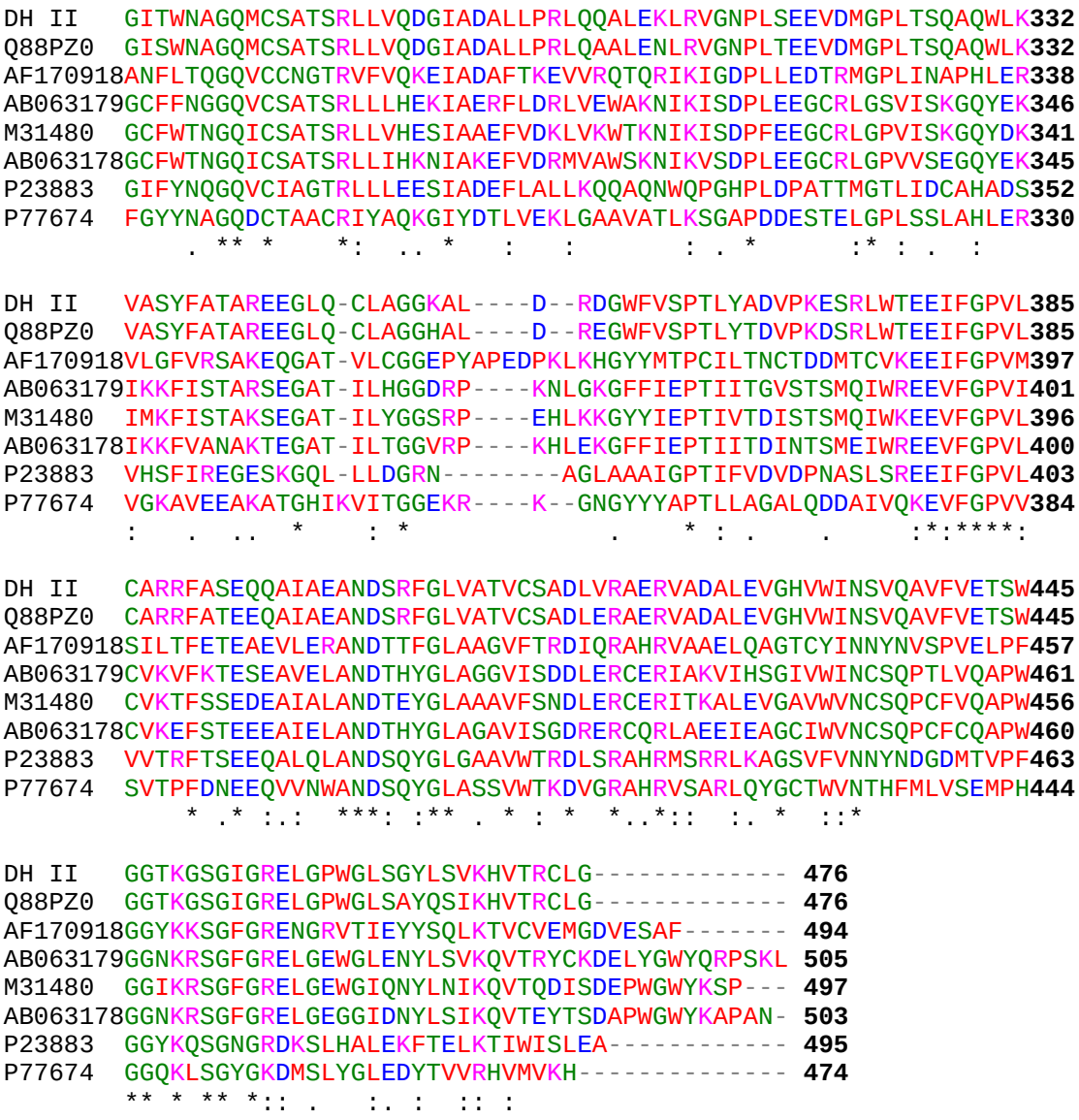

Fig. 1.10 Amino acid sequences of TMABaldehyde DH II and similar polypeptides

The sequences are from *Pseudomonas* sp. 13CM TMABaldehyde DHII (AB713749, top line), *Pseudomonas putida* KT2440 putative betaine aldehyde dehydrogenase (Q88PZ0), rat TMABaldehyde DH (AF170918), barley betaine aldehyde dehydrogenase type 1 (AB063179), spinach betaine aldehyde dehydrogenase (M31480), barley betaine aldehyde dehydrogenase type 2 (AB063178), *E. coli* γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883), and *E. coli* betaine aldehyde dehydrogenase (YdcW) (P77674). Numerals show quantities of amino acid residues, starting from the initial codon. "*", residues that are identical in all sequences in the alignment; ":", conserved substitutions; ".", semi-conserved substituitions; "-", no corresponding amino acid.

1.3.2 Expression of TMABaldehyde DH II in *E. coli* BL21 (DE3)

To over-express the TMABaldehyde DH II, two primers were designed for construction of pET24b (+) plasmid (described in materials and methods). The recombinant *E. coli-tmabadh* was cultured in LB medium and induced with optimal concentration of IPTG. The TMABaldehyde DH II gene was expressed from the bacterophage T7 polymerase strong promoter of pET24b (+) expression vector in *E. coli* BL21 (DE3). The cell-free soluble extracts of *E. coli-tmabadh* revealed the presence of a protein having an approximate molecular weight of 52 kDa on SDS−PAGE (Fig. 1.11, 1.12) which was not observed in the cell-free extract of host *E. coli* BL21 (DE3). The activity measurement for cell-free soluble extract also exhibited 16.2 unit.mg^{-1} protein at 0.3 mM IPTG induction conditions with TMABaldehyde as a substrate and NAD^+ as a cofactor.

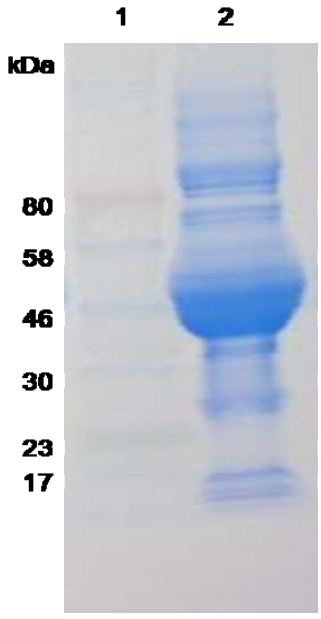

Fig. 1.11 SDS–PAGE analysis of cell-free TMABaldehyde DH II expressed in *E. coli* BL21 (DE3) on a 5–20% linear polyacrylamide gradient gel

Induction was carried out for 16 hrs under the experimental conditions described in the text. Lane 1, Color Plus prestained protein marker (New England Biolabs Inc., Massachusetts, USA); lane 2, cell free TMABaldehyde DH II.

1.3.3 Purification and polyacrylamide gel electrophoresis of TMABaldehyde DH II

The purification steps and results are presented in Table 2.5. Single purification step including hydrophobic chromatography on Phenyl-Toyopearl caused 2.1-fold purification with a yield of 33% and a specific enzyme activity of 33.6 unit.mg^{-1} protein in the final enzyme solution.

Table 1.10 Purification of recombinant TMABaldehyde DH II

Purification step	Total activity (U)	Total protein (mg)	Sp. activity (U.mg^{-1})	Purification (fold)	Yield (%)
Cell-free extract	4347	269	16.2	1.0	100
Phenyl-Toyopearl	1422	43	33.6	2.1	33

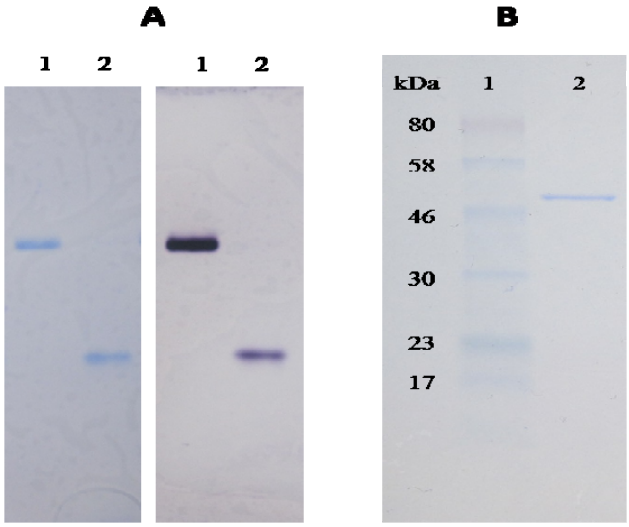

Fig. 1.12 Native PAGE and SDS–PAGE analysis of purified recombinant TMABaldehyde DHs

Native PAGE (A) of the purified enzymes [1, TMABaldehyde DH I; 2, TMABaldehyde DH II]. Gels were stained for protein with Coomassie blue (left) and for enzyme activity with TMABaldehyde (right). SDS gel electrophoresis (B) of the purified TMABaldehyde DH II on a 5–20% linear polyacrylamide gradient gel under denaturing conditions and stained for protein with Coomassie blue (predicted MW~52 kDa). *E. coli* MBP-truncated-β-galatosidase (80.0 kDa), *E. coli* MBP-CBD (58 kDa), rabbit muscle aldolase (46.0 kDa), *E. coli* triosephosphate isomerase (30.0 kDa), soybean trypsin inhibitor (23.0 kDa), and chicken egg white lysozyme (17.0 kDa) were used as standard proteins. Lane 1, Color Plus prestained protein marker (New England Biolabs Inc., Massachusetts); lane 2, purified enzyme TMABaldehyde DH II.

The successfully over-expressed purified soluble proteins were analyzed by polyacrylamide gel electrophoresis (Fig. 1.12). According to the native PAGE results of the purified enzyme, the single band of enzyme activity staining accorded with the main stained protein band (Fig. 1.12a). SDS–PAGE revealed one band at a molecular mass of 52 kDa (Fig. 1.12b) which was coincided with those of molecular mass about 51 kDa measured by MALDI-TOF MS as shown in figure 1.13.

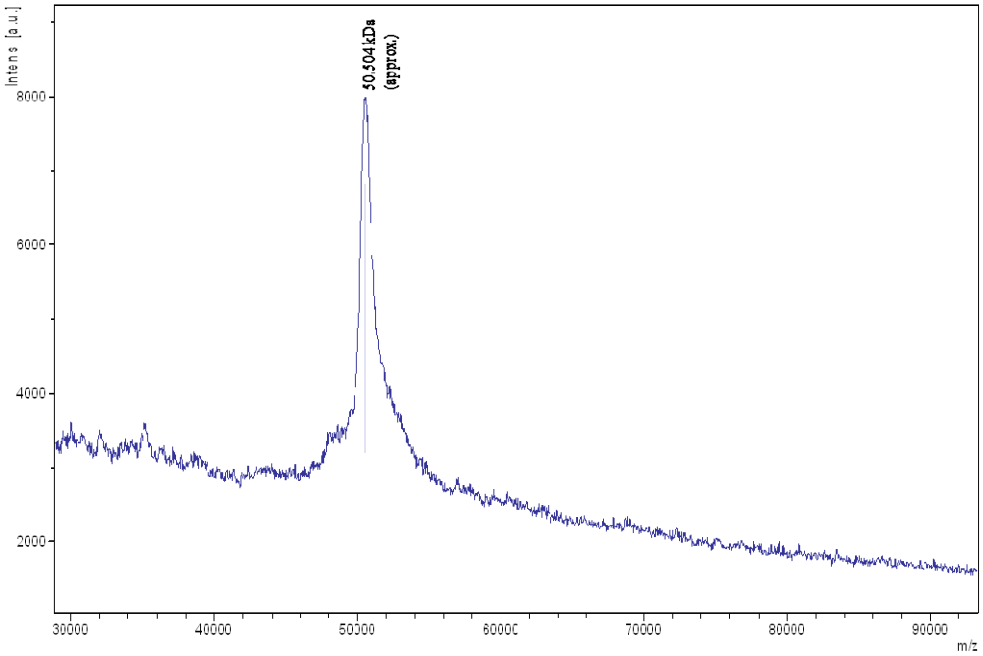

Fig. 1.13 MALDI-TOF MS analysis of TMABaldehyde DH II for molecular mass. The matrix-solution consists of 20 mg ml^{-1} 3, 5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid)

The molecular mass of the enzyme was estimated at about 150 kDa using gel filtration (Fig. 1.14), indicating that the native enzyme appeared to be a trimer with identical 52 kDa subunits. The purified enzyme was subjected to isoelectric focusing and showed a pI value of 4.5.

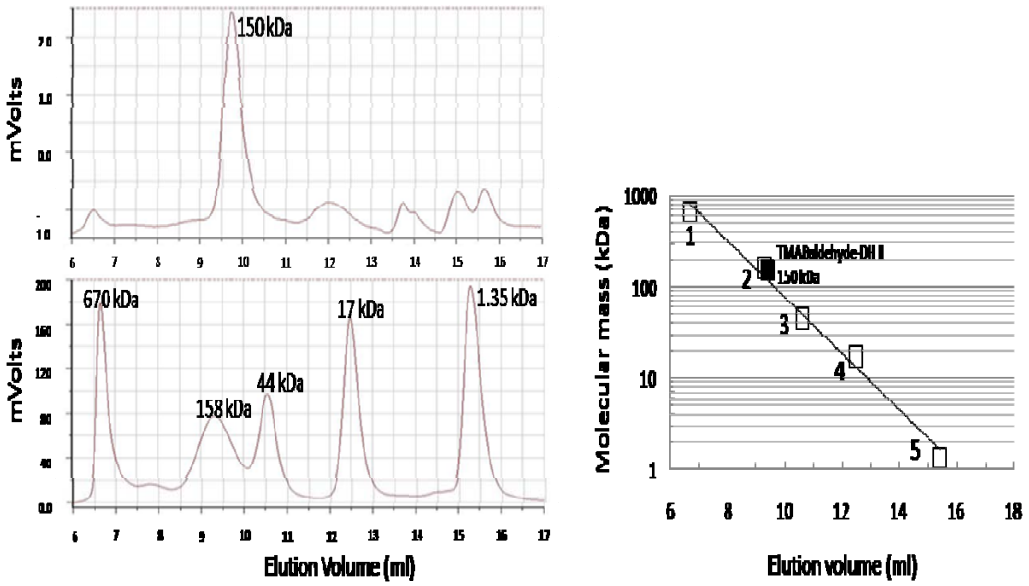

Fig. 1.14 Estimation of molecular mass of TMABaldehyde DH II by gel filtration on TSK-Gel G3000SW column in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and 150 mM potassium chloride, at a flow rate of 0.5 ml.min^{-1}

Approximate 51 µg of protein was loaded. Molecular mass standards were the following: 1, thyroglobulin (bovine, 670 kDa); 2, gamma-globulin (bovine, 158 kDa); ovalbumin (chicken, 44 kDa); myoglobin (horse, 17 kDa); vitamin B12 (1.35 kDa). TMABaldehyde DH II eluted at an apparent size of 150 kDa, based on the calibration.

To check the appearance of new TMABaldehyde DH in *Pseudomonas* sp. 13CM, the wild type enzymes from *Pseudomonas* sp. 13CM of several cultivation phases and recombinant enzyme were subjected to electrophoresis and checked for enzyme activity (data not shown). Each of the wild type enzymes shares the same characteristics, which was migrated at the same position on the gel. In contrast, TMABaldehyde DH II activity was detected at a different position on the gel. These results indicated that the electrical property of TMABaldehyde DH II differs with the previously reported TMABaldehyde DH I. The N-terminal 5 amino acid residues were sequenced as TTSHY.

1.3.4 Effect of temperature and pH

The residual activity of the enzyme was measured after heat treatment at various temperatures for 30 min in 50 mM of potassium phosphate buffer (pH 7.5) containing 1.0 mM of DTT. It was stable up to 25 °C. Incubation for 30 min at 30 ºC and above led to rapid inactivation of the enzyme (Fig. 1.15). The optimal temperature was observed at 40 °C (Fig. 1.15).

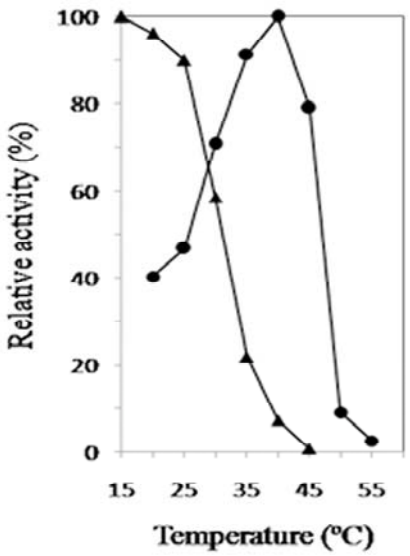

Fig. 1.15 Effect of temperature on TMABaldehyde DH II activity and stability

Thermostability (▲): Enzyme solution in 50 mM of potassium phosphate buffer, pH 7.5, containing 1.0 mM of DTT was incubated for 30 min at each temperature. Enzyme activity was assayed as described in Materials and methods. Optimal temperature (●): Enzyme activity was assayed as described in Materials and Methods, except that the reaction temperature was varied.

The influence of the pH on the enzyme activity was studied in the range 6.5–10.5 using different buffers. The optimum pH was 9.5 (Fig. 1.16a). The pH stability of the enzyme was measured after pre-incubation for 15 min at 30 °C using various buffers. The enzyme was stable between pH 8.0 and 9.0. It was inactivated completely at pH 10.0 (Fig. 1.16b).

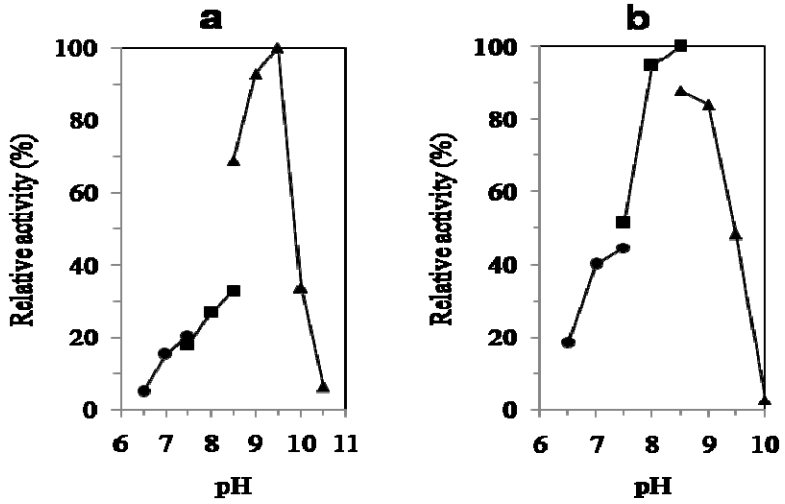

Fig. 1.16 Effects of pH on TMABaldehyde DH activity and stability

Enzyme activity was assayed under standard assay conditions (a), except that the following buffers were used at a final concentration of 150 mM in the incubation mixture: potassium phosphate buffer (●), Tris-HCl buffer (■), and glycine-NaOH buffer (▲). The enzyme was incubated for 15 min at 30 °C (b) in the following buffers which were used at a final concentration of 150 mM in the incubation mixture: potassium phosphate buffer (●), Tris-HCl buffer (■), and glycine-NaOH buffer (▲).

1.3.5 Effects of metal ions on activity

The effects of various metal ions and other reagents on the enzyme activity were examined. The enzyme was inhibited not only by heavy metal ions such as Hg^{2+} and Cu^{2+}, but also by sulfhydryl agents such as *p*-chloromercuribenzoate, 5,5′-dithiobis-(2-nitrobenzoic acid), and iodoacetoamide (data not shown). K^{+}, Zn^{2+}, Li^{2+}, Ba^{2+}, Co^{2+}, and semicarbazide caused 10–30% inhibition. Rb^{+}, Na^{+}, Mg^{2+}, Ca^{2+}, and chelating agents such as potassium fluoride, sodium azide, 2,2′-dipyridyl, and EDTA had no effect on the enzyme activity. At high concentrations of TMABaldehyde, substrate inhibition was not observed. From the above facts, we consider that the sulfhydral group is important in the enzyme activity, as are other aldehyde dehydrogenases.

1.3.6 Substrate specificity and Michaelis constants

To gain insight into the substrate specificity of the TMABaldehyde DH II, various aminoaldehydes as well as aliphatic aldehydes were used in the enzymatic assay. TMABaldehyde DH II oxidized mainly TMABaldehyde and DMABaldehyde. 4-aminobutyraldehyde and other aliphatic aldehydes such as acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde, and pivaleraldehyde were slightly oxidized; whereas betaine aldehyde, trimethylacetaldehyde, and 3-methylbutyraldehyde were not dehydrogenated. TMABaldehyde DH II did not react with $NADP^+$, and was very specific for NAD^+ (Table 1.11). The K_m values for TMABaldehyde, DMABaldehyde, and NAD^+ were calculated respectively as 0.31 mM, 0.62 mM, and 1.16 mM (Fig. 1.17).

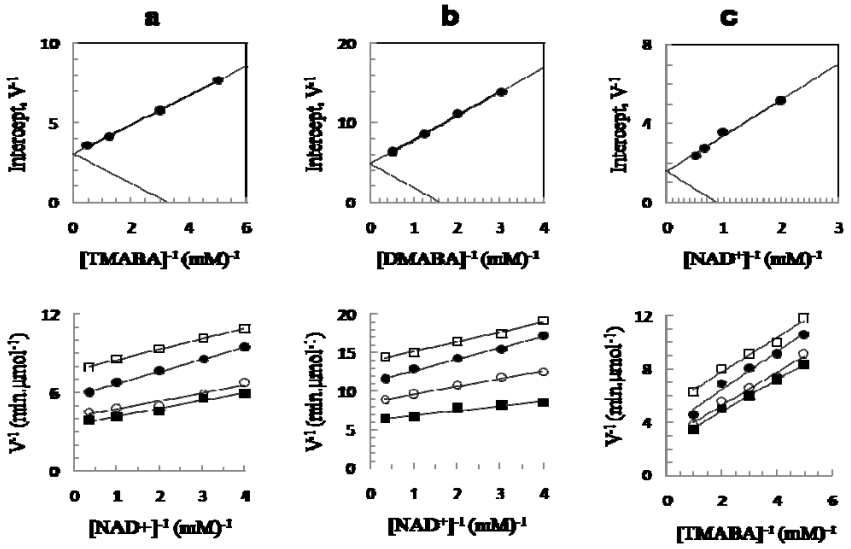

Fig. 1.17 Double-reciprocal plots of initial velocity of the TMABaldehyde DH II reaction against substrate concentration (lower panels). Upper figures show the secondary plots of the intercept against the reciprocal concentration of the invariant substrate

The enzyme reaction was conducted as described in Materials and Methods. a, NAD^+ as a variable substrate at various fixed concentrations of TMABaldehyde (TMABA): 0.2 mM (□), 0.33 mM (●), 0.8 mM (○) and 2.0 mM (■). b, NAD^+ as a variable substrate at various fixed concentrations of DMABaldehyde (DMABA): 0.33 mM (□), 0.5 mM (●), 0.8 mM (○) and 2.0 mM (■). c, TMABA as a variable substrate at various fixed concentrations of NAD^+: 0.5 mM (□), 1.0 mM (●), 1.5 mM (○) and 2.0 mM (■).

1.4 DISCUSSION

TMABaldehyde DH gene from *Pseudomonas* sp. 13CM was cloned and expressed in recombinant *E. coli*, so far never been reported before from a microorganism. The N-terminal end of TMABaldehyde DH II translated sequence (Fig. 1.9) is not identical to the N-terminal sequence of a purified TMABaldehyde DH I (Hassan *et al*., 2008). The present preparation shows the maximum identity with *Pseudomonas* spp. putative betaine aldehyde dehydrogenases, rat TMABaldehyde DH, and bovine mitochondrial aldehyde dehydrogenase (Table 1.9; Fig. 1.10). However, it oxidizes TMABaldehyde and DMABaldehyde, inert to betaine aldehyde (Table 1.11). TMABaldehyde dehydrogenase from *Bos taurus* and *Rattus norvegicus* can oxidize several kinds of aliphatic aldehydes. The enzyme from *B. taurus* can oxidize acetaldehyde, propionaldehyde, butyraldehyde, and benzaldehyde with very low relative activity. *R. norvegicus* TMABaldehyde dehydrogenase catalyzes the dehydrogenation of 4-aminobutyraldehyde, betaine aldehyde, aliphatic aldehydes in the C2-C8 range, and longer aldehydes such as hexadecanal and octadecanal. Both NAD^+ and $NADP^+$ can be used as coenzyme in *R. norvegicus*, but enzymes of *B. taurus*, *Pseudomonas* sp. 13CM, and TMABaldehyde DH II are highly specific for NAD^+ (Hulse and Henderson, 1980; Vaz *et al*., 2000) (Table 1.11). The gene organization of TMABaldehyde DH II showed that, ORF 2, 3, and 4 were positioned at downstream to the ORF1 but oriented in the opposite directionon the complementary strand which is similar to the gene organization of *P. putida* KT2440 (Q88PZ0). It is particularly interesting that γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883), which catalyzes an oxidation of γ-glutamyl-γ-aminobutyraldehyde to γ-glutamyl-γ-aminobutyrate in the putrescine utilization pathway in *E. coli* K-12 also showed 38% identity to ORF1 (Fig. 1.10). To our knowledge, γ-glutamyl-γ-aminobutyraldehyde, which has a glutamate group, binds with aminobutyraldehyde group instead of trimethylammonium group as for TMABaldehyde, but has never been reported to be oxidized by any TMABaldehyde DH.

To over-express the new enzyme, the gene encoding TMABaldehyde DH II from *Pseudomonas* sp. 13CM into pET24b (+) was cloned which placed protein expression

under control of a strong promoter (T7) that is inducible with IPTG. Over-expression of the recombinant protein was achieved with *E. coli* BL21 (DE3) cells. Our results thus far show that the TMABaldehyde DH II from *Pseudomonas* sp. 13CM was highly expressed using the *E. coli* BL21 (DE3) as the host organism (Fig. 1.11).

The cell-free extract prepared by *E. coli-tmabadh* using *Pseudomonas* sp. 13CM as a gene donor was subjected to purify with hydrophobic chromatography on a Phenyl-Toyopearl. Since the sulfahydryl-reducing agents were found to protect the enzyme activity, DTT was included throughout the isolation procedure. The specific activity 33.6 $U.mg^{-1}$ in the present preparation denoted that it has been successfully expressed and almost three times higher than the wild type TMABaldehyde DH from *Pseudomonas* sp. 13CM (Hassan *et al*. 2008). It was also higher than those of the wild type TMABaldehyde DH I from *Pseudomonas* sp. 13CM, as well as from *B. taurus* and *R. norvegicus* (Table 1.11). The molecular weight of TMABaldehyde DH II was calculated as 50,739 based on the amino acid sequence of ORF1 and confirmed by SDS−PAGE and MALDI-TOF MS analyses (Fig. 1.12, 1.13) and showed the pI value of 4.5; differed from those of TMABaldehyde DH I, which had a sub-unit molecular mass of 50 kDa with an isoelectric point of 5.5. Comparison of the properties of TMABaldehyde DH II with other TMABaldehyde DHs from *Pseudomonas* sp. 13CM, bovine and rat liver also revealed some similarities and dissimilarities. All reported TMABaldehyde DHs have a subunit molecular mass of 55,000 Da where as present preparation with 52,000 Da (Table 1.11). Betaine aldehyde dehydrogenase of human liver (Chern and Pietruszko, 1995), *Cylindrocarpon didymum* M-1 (Mori *et al*., 1980), *Escherichia coli* (Falkenberg and Strom, 1990), *Xanthomonas translucens* (Mori *et al*., 1992) and *Arthrobacter globiformis* (Mori *et al*., 2002) is a homotetramer with 50–55 kDa subunits. However, betaine aldehyde dehydrogenase from *Bacillus subtilis* (Boch *et al*., 1997), *Pseudomonas aeruginosa* (Nagasawa *et al*., 1976; Velasco-Gracia *et al*., 1999) and plants (Arakawa *et al*., 1987; Livingstone *et al*., 2003; Valenzuela-Soto and Munoz-Clares, 1994; Weretilnyk and Hanson, 1990) is a homodimer of 61–63 kDa.

Table 1.11 Specific activities, molecular mass, and K_m values among TMABaldehyde DHs

Origin	*Pseudomonas* sp. 13CM produced by *E. coli* BL21 (DE3)	*Pseudomonas* sp. 13CM*	*B. taurus***	*R. norvegicus****
Specific activity ($U.mg^{-1}$)	33.6	12.4	5.1	0.77
Molecular mass (kDa)				
Native	150	160	160	*NT*
Subunit	52	50	55	55
*K*m value (μM)				
4-*N*-TMABaldehyde	310	7.4	4.2	1.4
4-DMABaldehyde	620	51.0	*NT*	*NT*
4-Aminobutyraldehyde	+	–	*NT*	24.0
Betaine aldehyde	–	–	*NT*	123.0
Acetaldehyde	+	–	+	102.0
Propinaldehyde	+	–	+	16.0
Butyraldehyde	+	–	+	13.0
Pentanal	*NT*	*NT*	–	10.0
Hexanal	*NT*	*NT*	–	9.0
Heptanal	*NT*	*NT*	–	7.0
Octanal	*NT*	*NT*	–	6.0
Hexadecanal	*NT*	*NT*	–	8.8
Trimethylacetaldehyde	–	–	–	*NT*
3-Methylbutyraldehyde	–	–	–	*NT*
Benzaldehyde	*NT*	*NT*	+	*NT*
Isovaleraldehyde	–	*NT*	*NT*	*NT*
Pivaleraldehyde	–	*NT*	*NT*	*NT*
NAD^+	1160	125.0	+	28.0
$NADP^+$	–	–	–	1600

*Hassan *et al*. (2008); **Hulse and Henderson (1980); ***Vaz *et al*. (2000);

+, oxidized; –, inert; *NT*, not tested

The recombinant enzyme appears to be stable at 30 ºC, but became unstable at a temperature above 35 ºC (Fig. 1.15), most likely due to thermal denaturation. The optimum pH of 9.5 is lower than the TMABaldehyde DH I with the pH optimum of 10.0 but comparable to that of TMABaldehyde DH of *B. taurus* (pH 9.5–9.8) (Fig. 1.16) (Hulse and Henderson, 1980). The alkaline environment preference of the enzyme activity agrees well with the other reported TMABaldehyde DH (Hassan *et al*., 2008; Hulse and Henderson 1980; Vaz FM *et al*., 2000).

The K_m value of 0.31 mM for TMABaldehyde (Fig. 1.17) is higher than the K_m value for TMABaldehyde DHs from *Pseudomonas* sp. 13CM (Hassan *et al*., 2008), *B. taurus* (Hulse and Henderson, 1980) and *R. norvegicus* (Vaz *et al*., 2000), 7.4 μM, 4.2 μM and 1.4 μM, respectively. Similar to other TMABaldehyde DHs from *Pseudomonas* sp. 13CM (Hassan *et al*., 2008), *B. taurus* (Hulse and Henderson, 1980), and *R. norvegicus* (Vaz *et al*., 2000), TMABaldehyde DH II also showed the lowest K_m value for TMABaldehyde compared with other substrates.

1.5 CONCLUSIONS

This study was conducted to isolate the *Pseudomonas* sp. 13CM genes by shotgun cloning using the activity staining assay method. Screening of TMABaldehyde oxidation activity in the recombinant by activity staining assay and spectrophotometer assays showed that NAD^+ dependent TMABaldehyde DH is dominant in the prepared recombinants. Considering the properties of TMABaldehyde DH which was discovered initially in *Pseudomonas* sp. 13CM, the results of this work clearly indicate that the recombinant prepared by shotgun cloning using the competent cells *E. coli* DH5α is a new type of TMABaldehyde DH. The obtained second enzyme here we renamed as "TMABaldehyde DH II". The culture supernatant of the wild strain *Pseudomonas* sp. 13CM contains dominantly TMABaldehyde DH I, and thus far it has been difficult to detect TMABaldehyde DH II during the purification process from the culture supernatant because of its extremely low content. Under such circumstances, the molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the discovery of TMABaldehyde DH II followed by production of a large quantity of the enzyme in a recombinant strain *E. coli* DH5α/pAN1. At this point, the physiological roles of TMABaldehyde DH II in *Pseudomonas* sp. 13CM remain unclear. Therefore, it deserves more studies with the plans to investigate its physiological roles. In addition, experiments designed, engineering of the gene of TMABaldehyde DH II are in progress to modify the enzyme functions such as thermal stability and enzyme-cofactor interactions.

CHAPTER 2

GENES ENCODING 4-*N*-TRIMETHYLAMINO-1-BUTANOL DEHYDROGENASE AND 4-*N*-TRIMETHYLAMINOBUTYRALDEHYDE DEHYDROGENASE FROM *PSEUDOMONAS* SP. 13CM

2.1 INTRODUCTION

4-*N*-trimethylamino-1-butanol dehydrogenase (TMA-Butanol DH) and 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde DH) from *Pseudomonas* sp. 13CM were purified and characterized (Hassan *et al*., 2007; 2008). However, until recently, little is known about the structure of bacterial TMABaldehyde DH as well as TMA-Butanol DH. To our knowledge, no study to date has had complete genome sequences encoding the enzymes TMABaldehyde DH and TMA-Butanol DH. Only the N-terminal amino acid sequences are available for these enzymes.

To isolate the genes of these 4-*N*-trimethylamino-1-butanol (TMA-Butanol) degrading enzymes, shotgun cloning technique has been carried out using the bacterial strain *Pseudomonas* sp. 13CM as a gene donor. The resultant recombinants have been screened by the activity staining assay method. However, from the investigation, a new type of enzyme that catalyzes the dehydrogenation reaction toward TMABaldehyde was obtained, the primary structure of which is clearly different from the previously obtained *Pseudomonas* sp. 13CM TMABaldehyde DH (Hassan *et al*., 2008). We renamed the obtained second enzyme as "TMABaldehyde DH II" (Chapter 1).

The N-terminal amino acid sequences of TMA-Butanol DH and TMABaldehyde DH of *Pseudomonas* sp. 13CM has been reported by Hassan *et al*. (2007, 2008). To get both of the genome sequences of *Pseudomonas* sp. 13CM TMA-Butanol DH and TMABaldehyde DH I, we further attempted to apply general cloning technique, i.e.,

gene amplification of *Pseudomonas* sp. 13CM by PCR using the primers designed from the N-terminal amino acid sequences of the proteins and from the conserved sequences in closely-related species. It was observed that, the resulting amino acid sequences deduced from the nucleotide sequence conserved the identical residues of N-terminal sequences.

In this chapter, the design of an oligonucleotidic probe, sequences of the genes encoding the enzymes TMABaldehyde DH I and TMA-Butanol DH from *Pseudomonas* sp. 13CM by PCR are described. The chapter also covers the construction of recombinant TMA-Butanol DH and TMABaldehyde DH I, over-expressed in *Escherichia coli*. Moreover, enzymological properties of over-expressed TMABaldehyde DH I comparing with the characteristics of the native enzyme have been well described.

2.2 MATERIALS AND METHODS

2.2.1 Materials

2.2.1.1 Reference organism

Reference organism *Pseudomonas* sp. 13CM (Hassan, 2008) collected from the repository of the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan, used as the donor strain for the TMABaldehyde DH I and TMA-Butanol DH gene and subsequent experiments in this study.

2.2.1.2 Competent cells and plasmids

Competent cells of *E. coli* JM109 and *E. coli* BL21 (DE3) obtained from Takara Co. Ltd. (Shiga, Japan) and Novagen Inc. (Madison, WI, USA), respectively. The plasmid vector pET24b (+) was a product of Novagen Inc. (Madison, WI, USA).

2.2.1.3 Cofactors and chemicals for substrates

The cofactors NAD^+ and $NADP^+$ were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). 4-Dimethylamino-1-butanol (DMA-Butanol), 4-aminobutyraldehyde dimethylacetal, 3-dimethylamino-1-propanol (DMA-Propanol), 5-chloro-1-pentanol, and 6-dimethylamino-1-hexanol (DMA-Hexanol) were purchased from Tokyo Kasei (Tokyo, Japan).

2.2.1.4 Culture and polyacrylamide gel electrophoresis

Kanamycin, proteinase K, and ribonuclease-A were collected from Wako Pure Chemical Industries (Osaka, Japan). Agar and yeast extract were from Difco (Lawrence, USA). Phenyl-Toyopearl was purchased from Tosoh Corporation (Tokyo, Japan). SDS–PAGE was performed using the mini slab size 5–20% gradient polyacrylamide gels purchased from Atto (Tokyo, Japan) and samples were prepared by mixing with an equal volume of 2x EzApply sample buffer (Atto, Tokyo, Japan). Standard protein kit for gel filtration supplied from BIO-RAD (Hercules, CA, USA). Color Plus prestained protein marker for SDS–PAGE was from New England Biolabs (Massachusetts, USA).

2.2.1.5 PCR reagents

PCR primers were synthesized at Operon Biotechnologies (Tokyo, Japan). Restriction enzymes and kits for genetic manipulation were from New England Biolabs (Massachusetts, USA), Takara Bio Inc. (Shiga, Japan), Toyobo (Osaka, Japan) and Nippon Gene (Tokyo, Japan). Agarose SeaKem GTG was from BMA (Rockland, ME, USA). Loading quick λ/*Sty*I and loading quick λ/*Hind*III were collected from Toyobo (Osaka, Japan). NucleospinExtract II and Nucleospin Plasmid were from Macherey Nagel GmbH & Co. KG (Germany). MagExtractor-Genome kit, MagExtractor-PCR & Gel clean up kit, and MagExtractor-Plasmid kit were from Toyobo (Osaka, Japan).

BigDye Terminator Cycle Sequencing Ready Reaction kit ver 3.1 was from Applied Biosystems (Foster City, CA). The dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, rT*th* pyrophosphatase (a component in AmpliTaq DNA Polymerase, FS), magnesium chloride, and buffer were mixed into a single tube of Ready Reaction Mix and were ready to use.

2.2.1.6 Other reagents

All other chemicals and materials were of the highest purity grade from Wako Pure Chemical Industries (Osaka, Japan), and Applied Biosystems (Foster City, USA), used in this study without further processing.

2.2.2 Methods

2.2.2.1 General methods

General weight measurements were made using IB-200H electronic balance (Shimadzu corp. Kyoto, Japan) and smaller quantities measurements for the preparation of standards were made using Mettler AE240 analytical balance (Mettler, Toledo, AG, Switzerland). Mupid-2X submarine electrophoresis system apparatus (Advance Co. Ltd., Japan) was used for agarose gel electrophoresis. Cell growth was measured using the Novaspec II from Amersham Pharmachia Biotech (Piscataway, NJ, USA). TOMY Ultrasonic Disruptor (UD-200) was used for disruption of cells. For measurement of enzyme activity, Shimadzu UV-2100S spectrophotometer was used. Gilson HPLC System was used for gel filtration and the Unipoint^{TM} HPLC system control software was used for data analysis. Products derived from PCR were purified by using Centri-Sep spin columns from Applied Biosystems (Foster city, CA). For measurement of purity of plasmid DNA, UVmini-1240 UV-Visible spectrophotometer from Shimadzu Corp. (Kyoto, Japan) was used. pH was determined using an Horiba F-22 pH meter

(Horiba Ltd., Kyoto, Japan). Cultures and extracts were centrifuged using Hitachi Himac Compact Centrifuges RX II Series CF16RXII (rotor: 36, T16A31; 44, T15A36; 46, T15A36; and 24, T9A31) and Hitachi Himac CT15E (Hitachi Koki Company Ltd., Tokyo, Japan). Inoculated media were incubated either in at 37 ºC with recipro shaker NR-1 or at 25 ºC using a NR-300 double shaker (Taitec Corporation, Tokyo, Japan) or at 30 ºC and low temperature in LTI-60ISD EYALA low temperature incubator equipped with NR-30 double shaker (EYALA, Tokyo, Japan). Incubation of plates occurred in Taitec M-260F temperature controlled incubate box. Media were autoclaved at 121 ºC for 20 minutes using BS-235 high pressure steam sterilizer (Tomy Seiko Co., Ltd., Tokyo, Japan). Filter sterilization of solutions was carried out using 0.22 µm disposable filters MILLEX-GA (Millipore, Molshiem, France). Samples were vortexes using a Vortex Genie 2 (Scientific Industries, Inc., USA). PCR thermal Cycler Dice model TP 600 of Takara was used for PCR reactions (Takara Bio Inc., Shiga, Japan). Magical Trapper from Toyobo (Osaka, Japan) was used during preparation of genomic DNA, plasmids DNA, and purification of PCR products.

2.2.2.2 Chemical synthesis

TMA-Butanol iodide, TMABaldehyde iodide, 5-dimethylamino-1-pentanol (DMA-Pentanol), 3-*N*-trimethylamino-1-propanol (TMA-Propanol) iodide, 5-*N*-trimethyl-amino-1-pentanol (TMA-Pentanol) iodide, and 6-*N*-trimethylamino-1-hexanol (TMA-Hexanol) iodide were prepared according to the method of Hassan (2008).

TMA-Butanol iodide was prepared using DMA-Butanol (100 mmol) solution in diethyl ether and dropwise adding the methyl iodide (160 mmol) into it at room temperature. The prepared mixture was filtered after proper mixing (stirring for 1 hour). Then the precipitate was dried in vacuum desiccators for overnight, and afforded white powder. TMA-Butanol iodide was stored in light resistant desiccators. The preparation was used without further purification for enzyme assay.

TMABaldehyde iodide was prepared from 4-aminobutyraldehyde dimethylacetal according to the method described previously in Chapter 1.

DMA-Pentanol was prepared with the mixing of 5-chloro-1-pentanol (8.6 mmol) and dimethylamine (0.2 mol) at room temperature for overnight. The pH of solution was adjusted to a pH range of 10 to 12 with the addition of potassium carbonate. Then the mixture was extracted with diethyl ether. The organic layer was dried over anhydrous magnesium sulfate, filtered, and evaporated to produce colorless oil as DMA-Pentanol.

TMA-Propanol, TMA-Pentanol, and TMA-Hexanol were prepared by the dropwise adding of methyl iodide (160 mmol) to a solution of 100 mmol diethyl ether solution of DMA-Propanol, DMA-Pentanol, and DMA-Hexanol, respectively. After stirring for 1 hour at room temperature the mixtures were filtered. The precipitates were dried in individual vacuum desiccators for overnight, produced a white powder.

2.2.2.3 Bacterial strains, plasmids, and culture conditions

Pseudomonas sp. 13CM strain was isolated from soil isolates that can use TMA-Butanol iodide as the sole source of carbon and nitrogen, maintained in the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan, used as the donor strain for the TMA-Butanol DH and TMABaldehyde DH gene (Hassan *et al*., 2007). Original isolates was grown in the medium containing 1.0% meat extract, 0.5% glucose, 0.5% of peptone, 0.1% of NaCl (Table 1.1) at pH 7.0 with the conditions described in Chapter 1. Plasmid pET24b (+) was used for the general cloning and for the expression (Fig. 1.2).

Competent cells *E. coli* JM109 was used as the host strain for general cloning procedures, obtained from Takara Co. Ltd. (Shiga, Japan). *E. coli* BL21 (DE3) cells were used as the host strain for gene expression, purchased from Novagen Inc. (Madison, WI, USA). *E. coli* cells were grown in Luria-Bertani (LB) medium according to the method described in Chapter 1.

2.2.2.4 DNA isolations and manipulations

Chromosomal DNA was prepared from *Pseudomonas* sp. 13CM as described by Saito and Miura (Saito and Miura, 1963) after pretreatment with lysozyme, RNase and proteinase K. Small scale plasmid purified from *E. coli* using the MagExtractor Plasmid (Toyobo, Japan) and Nucleospin Plasmid kit (Macherey Nagel GmbH Co. KG, Germany). DNA fragments were recovered from agarose gels using a MagExtractor Genome (Toyobo, Japan) and NucleospinExtract II (Macherey Nagel GmbH Co. KG, Germany).

2.2.2.5 PCR for the core region of the Pseudomonas sp. 13CM gene

The N-terminus MIDNLSPLSRQS and PQLRDAAYWRAQS from the *Pseudomonas* sp. 13CM, encodes respectively, TMA-Butanol DH and TMABaldehyde DH I (Hassan *et al*., 2007, 2008), obtained by Edman degradative sequencing (Edman and Begg, 1967).

The primers aldeNF, aldeR, alcoNF, alcoNR, and alcoR (Table 2.1) were designed on the basis of N-terminal amino acid sequences of TMABaldehyde DH I and TMA-Butanol DH from *Pseudomonas* sp. 13CM determined as described previously and the multiply aligned sequences across the conserved regions of proteins.

Table 2.1 PCR primer used for sequencing of the *Pseudomonas* sp. 13CM TMABaldehyde DH I and TMA-Butanol DH genes and construction of expression vector

Primer	Direction	Sequence (5´-3´)	Tm (ºC)
aldeNF	Forward	CCSCARCTSAGRGAYGCSGCSTAYTGG	68.1
aldeR	Reverse	AGSACSGGSCCGAAGATYTCYTC	61.4
alcoNF	Forward	ATGATYGAYAAYCTSTCSCCSCT	56.5
alcoNR	Reverse	AGSGGSGASAGRTTRTCRATCA	54.5
alcoR	Reverse	TCSGCSCCSGTSCCSGCSGTSGT	69.3
alde2F	Forward	CCTTCTGGTCAGCCGACCTCGG	75.0
alde2R	Reverse	GTCCACGTCAGGCTCACGGC	72.8
alco3F	Forward	CCTGGTGGCCCGGCAGAC	73.8
alco3R	Reverse	TGCCACTCGCGGAACGTCG	75.3
[a]13CM_aldeF	Forward	GAGGGGATGCATATGCCGCAACTCAG	64.3
[b]13CM_aldeR	Reverse	ACAGGGGGGAAAGCTTGTCAATCACG	62.9
[a]13CM_alcoF	Forward	GAAGGTGCACTCATATGATTGACAACC	59.6
[b]13CM_alcoR	Reverse	CATCAGCGCCCTTCGAAGCTGGACCGA	65.7

[a]*Nde*I site underlined; [b]*Hind*III site underlined (primers for construction of expression vectors)

The coding region was amplified using standard PCR method. Using the different combination of the synthesized primers, PCR amplification followed by DNA sequencing of the PCR product yielded various sizes of DNA. The PCR reaction was done for 30 cycles with 50 ng *Pseudomonas* sp. 13CM genomic DNA. Each cycle was run at 95 ºC for 15 sec, 50 ºC for 30 sec, and 72 ºC for 100 sec. PCR products were sequenced using the dideoxy chain termination method (Sanger *et al*., 1977). The sequencing mixture and PCR conditions are shown in Table 2.2, 2.3 and 2.4.

Table 2.2 Sequencing PCR reaction solution 1

Template DNA	2.0	µl
T7 univ primer(5 pmol/µl)	1.0	µl
Big dye terminator	1.0	µl
5x sequencing buffer	1.5	µl
Milli Q water	4.5	µl
Total volume	10.0	µl

Table 2.3 Sequencing PCR reaction solution 2

Template DNA	2.0	µl
T7 terminator primer(5 pmol/µl)	1.0	µl
Big dye terminator	1.0	µl
5x sequencing buffer	1.5	µl
Milli Q water	4.5	µl
Total volume	10.0	µl

Table 2.4 Sequencing PCR reaction condition

Denaturation	95 ºC	20 sec
Denaturation	95 ºC	10 sec
Annealing	50 ºC	15 sec
Elongation	60 ºC	150 sec

2.2.2.6 Inverse PCR for DNA sequences flanking the core region

To obtain additional part of the DNA sequences of the *Pseudomonas sp*. 13CM genes, inverse PCR approach was applied effectively. On the basis of the nucleotide sequences of the core region the "inside-out" primers alde2F, alde2R, alco3F and alco3R (Table 2.1) were designed based on inverted repeats border sequences (Triglia *et al*., 1988, Ochman *et al*., 1988, Silverman *et al*., 1989). Next, using the obtained DNA

sequences, a restriction enzyme map of the known sequence was produced with the program GENETYX ver.10. The restriction enzymes *Nco*I (New England Biolabs, USA), *Pst*I (Takara, Japan), *Sac*I and *Sal*I (Nippon Gene, Japan) were chosen for digestion of 4 aliquot of genomic DNA (1.0 µg) from *Pseudomonas sp*. 13CM in a volume of 40 µl. After restriction digestion with the incubation at 37 ºC, purification and then gel-clean up were performed according to the directions supplied with the MagExtractor-PCR and Gel-clean up kit (Toyobo, Japan). Intramolecular ligations of the restriction fragments were performed using T4 DNA ligase, DNA Ligation kit Ver. 2.1 (Takara, Shiga, Japan) for overnight at 16 ºC in a total volume of 20 µl. The ligation products were amplified using primer sets designed from the partial DNA fragment of the *Pseudomonas* sp. 13CM gene. Using the resulting DNA circles as templates, the above mentioned primers, the inverse PCR was performed to determine the nucleotide sequence of the regions upstream and downstream of the TMABaldehyde DH I and TMA-Butanol DH gene (Triglia *et al*., 1988, Ochman *et al*., 1988, Silverman *et al*., 1989).

The PCR reaction was done for 30 cycles (15 sec at 94 ºC, 30 sec at 60 ºC, and 100 sec at 72 ºC). Then, the PCR products were cleaned with NucleoSpin Extract II kit (Macherey-Nagel, Gmbh & Co. KG, Germany) and directly sequenced using the dideoxy chain termination method (Sanger *et al*., 1977), giving the remaining sequences of the gene. Assembly of the overlapping sequence fragments allowed reconstituting two Open Reading Frames (ORFs), and coding for amino acid residue polypeptide.

2.2.2.7 DNA sequence analysis

The entire sequence described, was determined on both strands. Analysis of the sequence was carried out using the Sequence Scanner Software v1.0 (Applied Biosystems, USA). Protein sequences were compared with the data-banks using the BLAST similarity search program from NCBI (Altschul *et al*., 1990).

2.2.2.8 Accession numbers

The entire nucleotide sequence data encoding the TMABaldehyde DH I and TMA-Butanol DH genes have been deposited in the DDBJ database under the accession numbers AB741624 and AB741625, respectively.

2.2.2.9 Construction of expression vector for TMABaldehyde DH I

The T7 promoter over-expression system of *E. coli* was used for recombinant TMABaldehyde DH I production. A DNA fragment containing the structural gene encoding TMABaldehyde DH (1488 bp) of *Pseudomonas* sp. 13CM (DDBJ: AB741624) was amplified by PCR using the sense primer 13CM_aldeF 5′-GAG GGG ATG CAT ATG CCG CAA CTC AG-3′ with *Nde*I site (underlined) and the antisense primer 13CM_aldeR 5′-ACA GGG GGG AAA GCT TGT CAA TCA CG-3′ with *Hind*III site (underlined) (Table 2.1) and thereby incorporating the 5′-*Nde*I and 3′-*Hind*III restriction sites with the target of directional cloning into the multiple cloning site of pET24b (+) (Novagen, Madison, USA). The PCR was performed by 1 cycle of 2 min at 94 ºC, 35 cycles with 40 ng of *Pseudomonas* sp. 13CM genomic DNA. Each cycle was run at 94 ºC for 15 sec, 50 ºC for 30 sec, and 72 ºC for 180 sec. The PCR products containing the structural gene of *tmabadh* were purified by NucleospinExtract II kit, digested with the appropriate restriction enzymes, alongside the relevant vectors; gel purified and then ligated into the equivalent sites of the pET24b (+) downstream of the T7 Lac promoter and upstream of His_6 tag using T4 DNA ligase to produce a plasmid pET24-*tmabadh* for protein overproduction (Fig. 2.1).

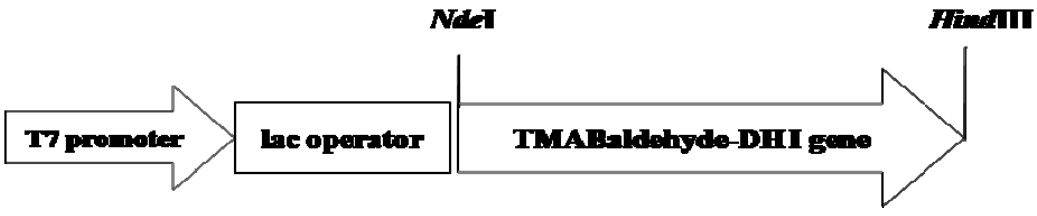

Fig. 2.1 Schematic diagram of pET24b-*tmabadh*

Resulting recombinant plasmid was then transformed into the competent *E. coli* JM109 cells and the mixture was plated on LB agar plates containing 50 μg.ml^{-1} of kanamycin. Positive clones were grown in LB medium, and colonies were screened for recombinant. For this, growing the recombinant of LB broth, plasmid DNA sample was extracted using standard plasmid DNA isolation techniques. The pET24-*tmabadh* was verified by performing restriction site and DNA sequence analysis. A plasmid harboring the *tmabadh* gene inserted downstream of the T7 promoter was selected (pET24b-TMABaldehyde DH). This recombinant plasmid was then heat-shock transformed into expression host *E. coli* strain BL21 (DE3) competent cell for small-scale protein expression tests (Sambrook *et al*., 1989).

2.2.2.10 Construction of expression vector for TMA-Butanol DH

The 5′ and 3′ terminal DNA sequences of the TMA-Butanol DH gene (*tmabdh*) of *Pseudomonas* sp. 13CM (DDBJ: AB741625) were used to design the following primers: upstream, alco_F, 5′-GAA GGT GCA CTC ATA TGA TTG ACA ACC-3′ and downstream, alco_R, 5′-CAT CAG CGC CCT TCG AAG CTG GAC CGA-3′ (underlined nucleotides indicate *Nde*I and *Hind*III sites, respectively). These primers were used to amplify the coding region of *tmabdh* gene (1182 bp) from genomic DNA of *Pseudomonas* sp. 13CM by PCR. The amplified PCR fragment was then purified by NucleospinExtract II kit, digested with the appropriate restriction enzymes, alongside the relevant vectors; ligated into the pET24b (+) vector and transformed into *E. coli* JM109 (Fig. 2.2). The resulting plasmid, pET24b-*tmabdh*, was then verified by restriction digestion and sequence analysis. The positive recombinant was then transformed into *E. coli* BL21 (DE3) cells to express the small scale protein.

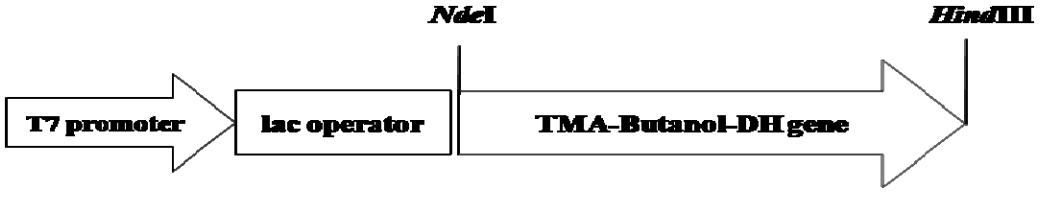

Fig. 2.2 Schematic diagram of pET24b-*tmabdh*

2.2.2.11 Expression of TMABaldehyde DH I and TMA-Butanol DH in E. coli

The recombinant *E. coli* clone hosting desired gene encoding TMABaldehyde DH I and TMA-Butanol DH, cultivated at 37 ºC for overnight in 6 ml lysogeny broth medium containing 50 $\mu g.ml^{-1}$ kanamycin, in a shaking incubator with 125 $strokes.min^{-1}$. An aliquot (0.75 ml) of the culture was used to inoculate 75 ml of the same medium. Culture was then incubated at 25 ºC in a reciprocal shaker (125 $strokes.min^{-1}$) until a cell density (optical density at 660 nm) of 0.5–0.7 was reached. Flasks containing the cultures were supplemented with isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.3 mM and the culture was continuously incubated at 25 ºC for a further 16 hour to over-express the recombinants.

2.2.2.12 Purification of recombinant TMABaldehyde DH I

Purification of recombinant TMABaldehyde DH was performed by hydrophobic chromatography using Phenyl-Toyopearl spin column. Over-expressed cells were harvested by centrifugation and pellets and washed twice with 0.85% KCl solution, resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. The mixture was sonicated (TOMY Ultrasonic Disruptor, UD-200, Japan) and crude cell lysate was prepared according to the method in Chapter 1. To the cell free extract, same volume of 0.5 M of ammonium sulfate solution in the 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT was added. The resulting solution was loaded onto a Phenyl-Toyopearl spin column equilibrated with the same buffer which contained 0.25 M of ammonium sulfate and desalted through repeated concentration or dilution against low salt buffer (50 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT). The purity of the recombinant protein was verified using SDS-PAGE as described in Chapter 1.

2.2.2.13 Enzyme activity assay for TMABaldehyde DH I and TMA-Butanol DH

The routine assay of recombinant TMABaldehyde DH I and TMA-Butanol DH was performed at 30 ºC by monitoring the increase in absorbance at 340 nm. The reaction mixture (1.5 ml) in a cuvette contained 150 mM glycine-NaOH buffer (pH 9.5), 3.0 mM of NAD^+ and an appropriate amount of the enzyme. The enzyme reaction was initiated by the addition of the TMABaldehyde iodide for TMABaldehyde DH I at a final concentration of 0.8 mM (Table 2.5) and TMA-Butanol iodide for TMA-Butanol DH at a final concentration of 4.5 μM (Table 2.6).

Table 2.5 Assay system of TMABaldehyde DH I

Glycine-NaoH buffer (pH 9.5, 450 mM)	0.50	ml
TMABaldehyde (30 mM)	0.04	ml
NAD^+ (60 mM)	0.05	ml
Enzyme solution	0.2~0.05	ml
Distilled water	0.86~0.71	ml
Total volume	1.5	ml

Table 2.6 Assay system of TMA-Butanol DH

Glycine-NaoH buffer (pH 9.5, 450 mM)	0.50	ml
TMA-Butanol (90 mM)	0.05	ml
NAD^+ (60 mM)	0.05	ml
Enzyme solution	0.20~0.05	ml
Distilled water	0.85~0.70	ml
Total volume	1.5	ml

1.2.2.14 Polyacrylamide gel electrophoresis of TMABaldehyde DH I

Native PAGE was performed using 10% gels at pH 8.8 and that gels were run at 4 ºC. The protein was stained with Coomasie brilliant blue R-250 or checked for enzyme activity at room temperature. For the activity staining, immediately after electrophoresis, gels were incubated at room temperature for 15 min and placed in the reaction mixture which contained 150 mM of glycine-NaOH buffer (pH 9.5), 64 μM of 1-methoxy phenazine methosulfate, 0.24 mM of nitroblue tetrazolium (NBT), 0.12 mM of TMABaldehyde, and 3 mM of NAD^+ (Table 1.7).

SDS–PAGE was performed following the method of Laemmli (1970) at room temperature using the mini slab size 5–20% gradient polyacrylamide gels purchased from Atto (Tokyo, Japan). The purified enzyme samples were prepared for SDS–PAGE by mixing with an equal volume of 2x EzApply sample buffer (Atto, Tokyo, Japan) and boiled for 5 min before use. Gels were stained with Coomasie brilliant blue R-250. The Color Plus prestained protein marker consisting the standards proteins are described in Chapter 1.

1.2.2.15 Measurement of molecular mass of recombinant TMABaldehyde DH I

The molecular mass of purified enzyme was estimated by SDS–PAGE and gel filtration on a TSK-gel G3000SW column (0.78 × 30 cm, Tosoh Corp., Tokyo, Japan) equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT at a flow rate of 0.5 $ml.min^{-1}$. Approximate 42 μg of protein was loaded. The fraction (0.5 ml each) was quantified by absorption 280 nm and by assaying the enzymatic activity.

1.2.2.16 Kinetic assay of recombinant TMABaldehyde DH I

Apparent K_m for TMABaldehyde, DMABaldehyde and NAD^+ of the recombinant TMABaldehyde DH were obtained from the initial-rate measurements under the

conditions in which one reactant was varied, typically at 12 different levels, over a concentration range of 5–10 times the apparent K_m, and the other reactant was present at a constant and saturating concentration. The experimental data were evaluated using Lineweaver-Burk plot. It was considered that the total concentration (mg protein.ml^{-1}) of purified protein was fixed.

1.2.2.17 Analytical methods

Codons were selected from Codon Usage Database (Nakamura *et al*., 2000) (http://www.kazusa.or.jp/codon/). The nucleotide and amino acid database searching was done using BLAST (Altschul *et al*., 1990). Multiple sequence alignments were obtained with the ClustalW program (Thompson *et al*., 1994) at GenomeNet (http://www.genome.ad.jp/).

2.3 RESULTS

2.3.1. Genes encoding TMABaldehyde DH I and TMA-Butanol DH

The N-terminal amino acid sequences of TMABaldehyde DH I and TMA-Butanol DH of *Pseudomonas* sp. 13CM had the similarity with, respectively, aldehyde dehydrogenase (B0KJD3) and iron containing alcohol dehydrogenase (B0KJD2) of *Pseudomonas putida* GB-1. Therefore, we assumed that both DHs obtained were the homologous with the putative enzymes of *Pseudomonas putida* GB-1.

To obtain the genes encoding TMABaldehyde DH I and TMA-Butanol DH, several attempts were made using PCR technique. Primers were synthesized based on the N-terminal sequences of *Pseudomonas* sp. 13CM TMABaldehyde DH I and TMA-Butanol DH and highly conserved region found in aldehyde and alcohol dehydrogenases as described in materials and methods. The primers aldeNF and aldeR amplified the DNA fragment of about 1.2 kbp, aldeNF and alcoNR amplified about 1.5 kbp and aldeNF and alcoR amplified about 2.0 kbp of fragments (Fig. 2.3).

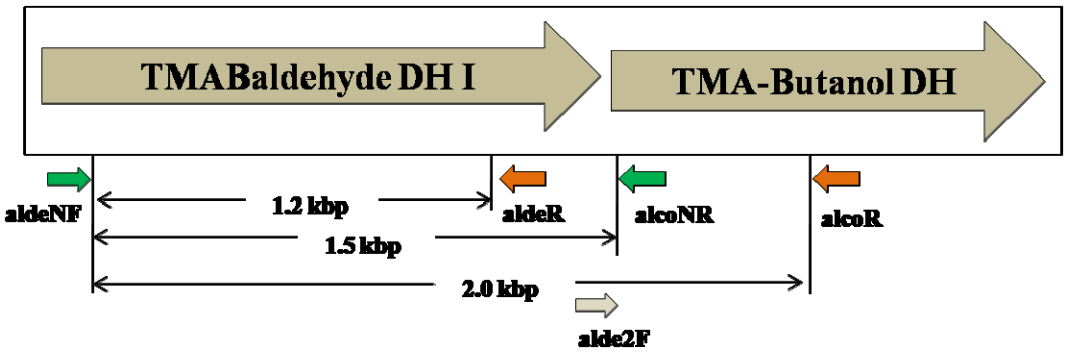

Fig. 2.3 Schematic representation of the gene of *Pseudomonas* sp. 13CM and amplified products with the various combination of primers set

To walk from the known region to an unknown region in the genomic DNA, inverse PCR-based method was used. Information on the regions flanking this sequence was obtained using the primers especially designed for inverse PCR (described in materials and methods). The nucleotide sequences of the 5′- and 3′- flanking regions and the core region were connected. Using this inverse PCR technique, we obtained gene containing two Open Reading Frames (ORFs) coding for a 496 and 394 amino acids polypeptide chain for TMABaldehyde DH I and TMA-Butanol DH, resepectively (Fig. 2.4).

The deduced primary structure from ORF1 and ORF2 were completely matched with the N-terminus from *Pseudomonas* sp. 13CM, respectively, TMABaldehyde DH I and TMA-Butanol DH, analyzed previously by Edman degradation (Hassan *et al*., 2007, 2008) and showed a calculated molecular weight 53.02 and 42.08 kDa, respectively. The deduced amino acid sequences from *Pseudomonas* sp. 13CM were compared with other protein sequences. Database analysis revealed that the primary structure of protein encoded in ORF1 and ORF2 showed high identity with aldehyde dehydrogenase (Table 2.7, Fig. 2.5) and iron containing alcohol dehydrogenase of *Pseudomonas putida* GB-1 (Table 2.8, Fig. 2.6). ORF1 (TMABaldehyde DH I) which encodes 496 amino acids also shares identity with betaine aldehyde dehydrgenase of *Pseudomonas aeruginosa*, TMABaldehyde DH of Rat (Fig. 1.6). Interestingly, γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883) which is responsible for oxidation of γ-glutamyl-γ-aminobutyraldehyde into γ-glutamyl-γ-aminobutyrate in putrescene utilization pathway of *E. coli* K-12 (Gamma-glutamyl-gamma-aminobutyraldehyde + NAD^+ + H_2O = gamma-glutamyl-gamma-aminobutyrate + NADH) also showed a considerable positional identity to TMABaldehyde DH I of *Pseudomonas* sp. 13CM (Table 2.7, Fig. 2.5). Additionally, the primary structure of protein encoded TMABaldehyde DH I showed similarity with aldehyde dehydrogenases from other *Pseudomonas* genus and shared a high amino acid similarity with 55–60% (Table 2.7).

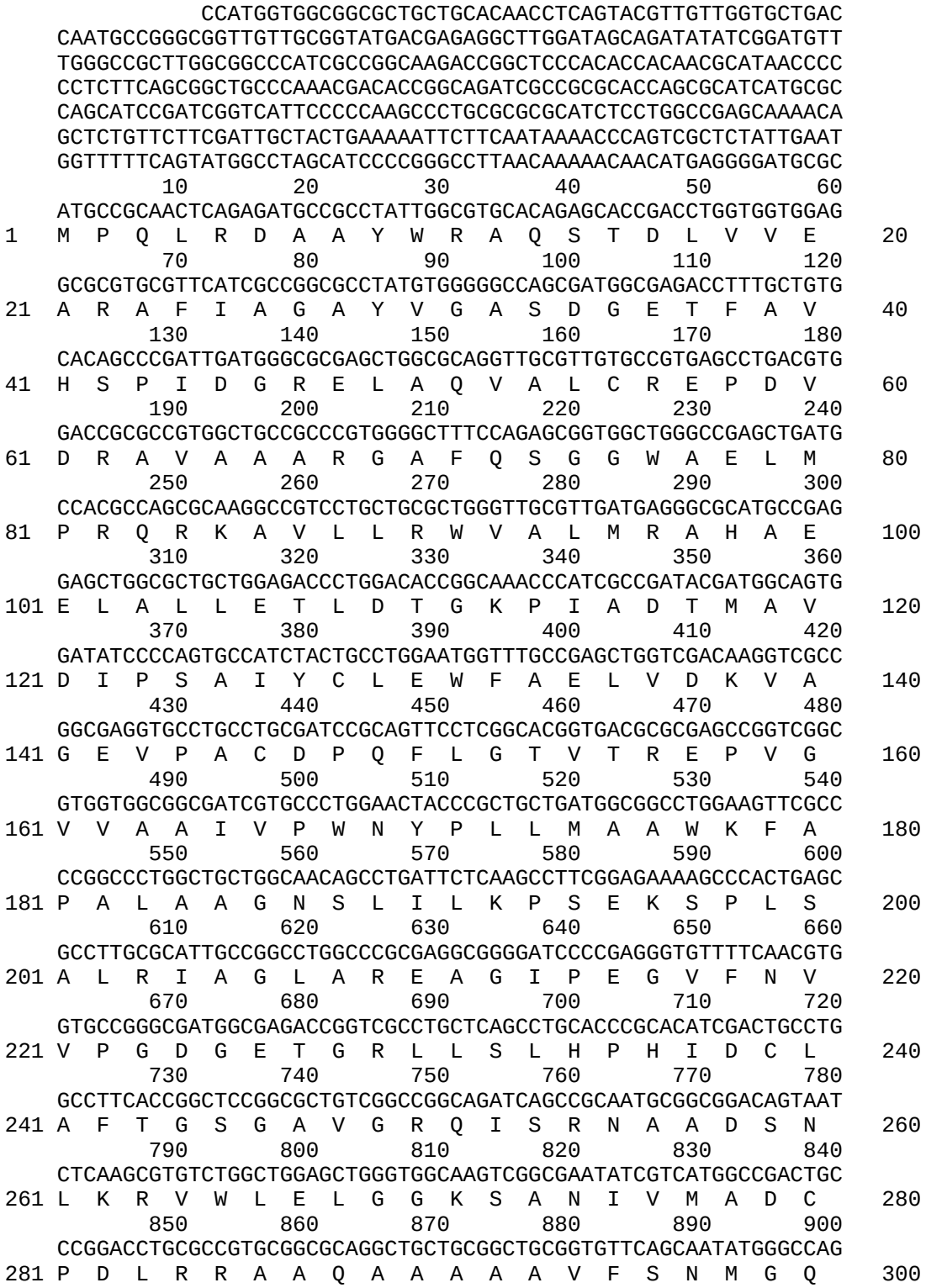

Fig. 2.4 Nucleotide and deduced amino acid sequences of the TMABaldehyde DH I and TMA-Butanol DH genes

Fig. 2.4 (continue)

```
          910       920       930       940       950       960
    GTGTGCAGCGCAGGTTCGCGCCTGCTGGTGCAGCGATCCATCGCCGAGGCTTTCATCGCC
301 V  C  S  A  G  S  R  L  L  V  Q  R  S  I  A  E  A  F  I  A    320
          970       980       990      1000      1010      1020
    GAACTGCTGGAGGCCGCGCGCGCCTATCTGCCTGGCGACCCGCTGGACCCGGCCAGCGTC
321 E  L  L  E  A  A  R  A  Y  L  P  G  D  P  L  D  P  A  S  V    340
         1030      1040      1050      1060      1070      1080
    ACCGGCAGCCTGATCGATGATGCCCAGTACCAGCGTGTGCGCCACTACATCGAGCTGGGC
341 T  G  S  L  I  D  D  A  Q  Y  Q  R  V  R  H  Y  I  E  L  G    360
         1090      1100      1110      1120      1130      1140
    AAGCGTGAAGCACGCCTGCTGCTGGGCGGCGAGGGCTGCGCGGTGGTCCCCGGCGGGCAC
361 K  R  E  A  R  L  L  L  G  G  E  G  C  A  V  V  P  G  G  H    380
         1150      1160      1170      1180      1190      1200
    TATCTGCAACCGACCATCTTTGCCACCGACGCCACCTCGCGCATCGCGTGTGAAGAGGTG
381 Y  L  Q  P  T  I  F  A  T  D  A  T  S  R  I  A  C  E  E  V    400
         1210      1220      1230      1240      1250      1260
    TTCGGGCCGGTGCTGAGCGTCATCGAATTCGATGGGCTGGACGACGCCATCGCGCTCGCC
401 F  G  P  V  L  S  V  I  E  F  D  G  L  D  D  A  I  A  L  A    420
         1270      1280      1290      1300      1310      1320
    AATGCCAGCGAATACGGCCTGGCGGCGGCCTTCTGGTCAGCCGACCTCGGCGCCATCCGC
421 N  A  S  E  Y  G  L  A  A  A  F  W  S  A  D  L  G  A  I  R    440
         1330      1340      1350      1360      1370      1380
    CGTGCCTCGCGACGCCTGCGTGCCGGCACCGTGTGGGTGAACTGCTACGACGAGCTGCTC
441 R  A  S  R  R  L  R  A  G  T  V  W  V  N  C  Y  D  E  L  L    460
         1390      1400      1410      1420      1430      1440
    GACATGAACTTCCCGTTCGGCGGCTTCAAGGAGTCCGGCAATGGCCGCGACAACTCCGTG
461 D  M  N  F  P  F  G  G  F  K  E  S  G  N  G  R  D  N  S  V    480
         1450      1460      1470      1480      1490      1500
    CACGCCCTGGACAAGTACAGCGAACTCAAGTCGACCATCATCCGTTGCTGAAGGTGCACT
481 H  A  L  D  K  Y  S  E  L  K  S  T  I  I  R  C  *             496
         1510      1520      1530      1540      1550      1560
    ATCGTGATTGACAACCTTTCCCCCCTGTCGCGGCAGAGCTGGAGCCTGCCACTTCCCATC
1      M  I  D  N  L  S  P  L  S  R  Q  S  W  S  L  P  L  P  I    19
         1570      1580      1590      1600      1610      1620
    GAGTACGGCCCCGGCGCGCGGACCGCGCTGGTGGATTTGTGCAAGCGCCATGGCATTTCC
20  E  Y  G  P  G  A  R  T  A  L  V  D  L  C  K  R  H  G  I  S    39
         1630      1640      1650      1660      1670      1680
    CGGCCGCTGATCGTGACCGACCAGGGCTCGCTGCGCCTACCCTTCGTGGCCGAGCTGCAG
40  R  P  L  I  V  T  D  Q  G  S  L  R  L  P  F  V  A  E  L  Q    59
         1690      1700      1710      1720      1730      1740
    GCCCTGCTGAACGAGGCGGGCCTGGCCTGCGGCCTGTTCGGTGAGATCGAGCCCAACCCC
60  A  L  L  N  E  A  G  L  A  C  G  L  F  G  E  I  E  P  N  P    79
         1750      1760      1770      1780      1790      1800
    AGTGACCTCGCGGTGCTCAAAGGCGCTGCGACGTTCCGCGAGTGGCACGCCGACGGTATC
80  S  D  L  A  V  L  K  G  A  A  T  F  R  E  W  H  A  D  G  I    99
         1810      1820      1830      1840      1850      1860
    ATCGCCCTCGGTGGCGGTAGCGGCCTGGACGGCGGCAAGGCCGTGGCCCTGGTGGCCCGG
100 I  A  L  G  G  G  S  G  L  D  G  G  K  A  V  A  L  V  A  R    119
         1870      1880      1890      1900      1910      1920
    CAGACACGCCTGCCGTTGTGGGCGTTCGATTTCGACAAGCCAGTTCCCGAAGGCTTGGTC
120 Q  T  R  L  P  L  W  A  F  D  F  D  K  P  V  P  E  G  L  V    139
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Fig. 2.4 Nucleotide and amino acid sequences of TMABaldehyde DH I and TMA-Butanol DH

Fig. 2.4 (continue)

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          1930      1940      1950      1960      1970      1980
    GCCAGCGATTTCCCGCCGGTGATCTGCATCCCGACCACGGCCGGCACCGGTGCCGAGACC
140 A  S  D  F  P  P  V  I  C  I  P  T  T  A  G  T  G  A  E  T     159
          1990      2000      2010      2020      2030      2040
    GAGAGCACGGCAATGCTCACCGACAGCGAGCGCGCTATCAAAGGTTGCGTCTGGCATCCG
160 E  S  T  A  M  L  T  D  S  E  R  A  I  K  G  C  V  W  H  P     179
          2050      2060      2070      2080      2090      2100
    CTGGCGCGCCCCCAGGCGGTGATCCTCGACCCCGAACTGACCCTCAGCCTGCCGGCACAC
180 L  A  R  P  Q  A  V  I  L  D  P  E  L  T  L  S  L  P  A  H     199
          2110      2120      2130      2140      2150      2160
    CTGACCGCCTGGACCGGCCTGGATGCGGTAATCCATGCCCTGGAAGCCTACTTTGTGCCG
200 L  T  A  W  T  G  L  D  A  V  I  H  A  L  E  A  Y  F  V  P     219
          2170      2180      2190      2200      2210      2220
    ACCTTCAATCCGCTCTGCGACGGTGCGGCGCTGCAGGCACTGGAGCTGCTCTGGGGTTCA
220 T  F  N  P  L  C  D  G  A  A  L  Q  A  L  E  L  L  W  G  S     239
          2230      2240      2250      2260      2270      2280
    CTTGAGCAAGCGGTGGAGCAGGGCGACGACCTTGAAGCACGCGCACGCATGCTGATCGGT
240 L  E  Q  A  V  E  Q  G  D  D  L  E  A  R  A  R  M  L  I  G     259
          2290      2300      2310      2320      2330      2340
    TCGTGCCTGGCGGGCGTGGCTTTTCTCAAAGGGCTTGGCCTGGTCCATGCGCTCAGCCAC
260 S  C  L  A  G  V  A  F  L  K  G  L  G  L  V  H  A  L  S  H     279
          2350      2360      2370      2380      2390      2400
    ATGGTCGGCGCCACCTACAACAGCCACCATGGTTTGACCAACGCGATGATCCTGCCCAGG
280 M  V  G  A  T  Y  N  S  H  H  G  L  T  N  A  M  I  L  P  R     299
          2410      2420      2430      2440      2450      2460
    GTGCTGCGCTTCAACCGCGCGGCCATCGAGCCGCGGCTGGGGCCGGTGTGCCGCGCCATG
300 V  L  R  F  N  R  A  A  I  E  P  R  L  G  P  V  C  R  A  M     319
          2470      2480      2490      2500      2510      2520
    GGCCTGGCGGGCGAAGACTTCGAGAGCTTCGAGCAGGCGATCTGTGCGCGCCTGGACCGC
320 G  L  A  G  E  D  F  E  S  F  E  Q  A  I  C  A  R  L  D  R     339
          2530      2540      2550      2560      2570      2580
    CTTGGCATCCCGGTTTCCCTGGCCTCGCTGGGCCTGCGCCGCGACGATATTCCAGCCATT
340 L  G  I  P  V  S  L  A  S  L  G  L  R  R  D  D  I  P  A  I     359
          2590      2600      2610      2620      2630      2640
    GCGCGCAAGGCCATGGGCGATCCGGCGCGCCAGACCAACCCACGCCCCAGCAGTTTCGAA
360 A  R  K  A  M  G  D  P  A  R  Q  T  N  P  R  P  S  S  F  E     379
          2650      2660      2670      2680      2670      2680
    GACCTGCAAACGCTGCTGCATCTGGCCCTGGAAGAGGAACGGGCGTAGTCGCGGGAAGCG
380 D  L  Q  T  L  L  H  L  A  L  E  E  E  R  A  *                 394
          2690      2700      2710
    ACGACCTTGCTGTGCTCACAACAAAACAAATAAAGGTAC
```

Fig. 2.4 Nucleotide and deduced amino acid sequences of the TMABaldehyde DH I and TMA-Butanol DH genes

The numbers to the right of the sequence stands for nucleotide and amino acid positions. The deduced amino acid sequenced is shown under the nucleotide sequence. Asterisks represents the translational stop codon. A possible ribosome binding site (RBS) is boxed with red line. Goldberg-Hoggness box in the promoter region is not identified within this nucleotides. The sequence data are deposited in the DDBJ database under the accession numbers AB741624 and AB741625, respectively.

Cod betaine aldehyde dehydrogenase (PA5373), spinach betaine aldehyde dehydrogenase (M31480), barley betaine aldehyde dehydrogenase type 1 (AB063179), barley betaine aldehyde dehydrogenase type 2 (AB063178), and *E. coli* betaine aldehyde dehydrogenase (M77739, P77674) also showed about 35–43% structural similarity with *Pseudomonas* sp. 13CM TMABaldehyde DH I polypeptide (Table 2.7). The amino acid sequence of TMABaldehyde DH I also displayed a consistent identity with *Pseudomonas* sp. 13CM TMABaldehyde DH II (39%) and *R. norvegicus* TMABaldehyde DH (36%).

Table 2.7 Compare of *Pseudomonas* sp. 13CM TMABaldehyde DH I with other protein

Origin	Locus ID	Protein	Alignment length	Identity (%)
P. putida GB-1	B0KJD3	Aldehyde dehydrogenase	496	94
P. stutzeri A1501	PST_0046	Aldehyde dehydrogenase family	497	57
P. entomophila	PSEEN5424	Aldehyde dehydrogenase (Aldh)	497	56
P. putida KT2440	PP_5278	Aldehyde dehydrogenase family protein	497	55
E. coli	P23883	γ-glutamyl-γ-amino-butyraldehyde dehydrogenase	495	53
Cod	PA5373	Betaine aldehyde dehydrogenase	490	44
E. coli	M77739	Betaine aldehyde dehydrogenase (BetB)	483	41
P. sp. 13CM	AB713749	TMABaldehyde DH II	476	39
Barley	AB063179	Betaine aldehyde dehydrogenase 1	483	39
Barley	AB063178	Betaine aldehyde dehydrogenase 2	482	38
Rat	AF170918	TMABaldehyde dehydrogenase	494	36
E. coli	P77674	Betaine aldehyde dehydrogenase (YdcW)	494	35
Spinach	M31480	Betaine aldehyde dehydrogenase	500	35

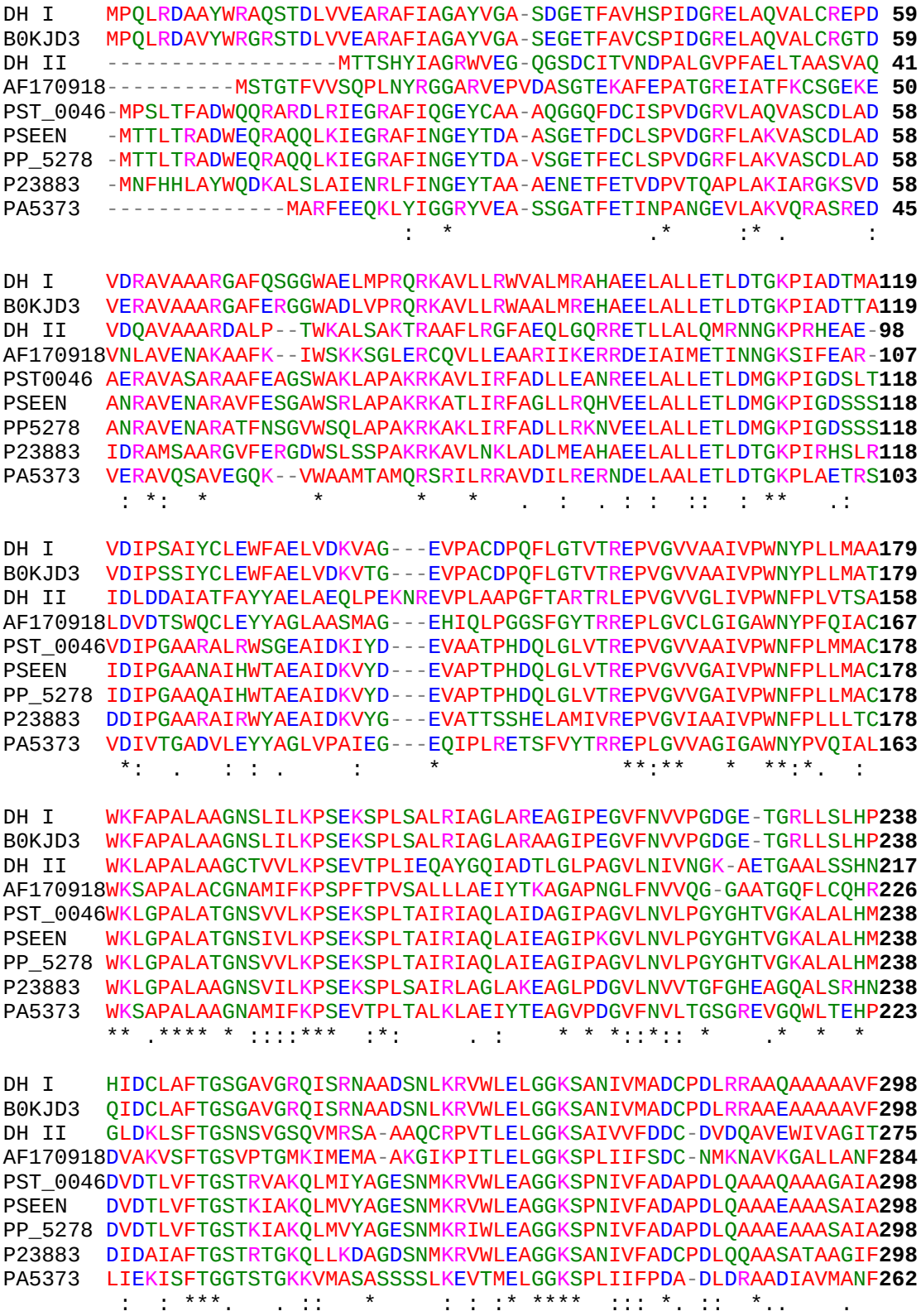

Fig. 2.5 Amino acid sequences of TMABaldehyde DH I and similar polypeptides

Fig. 2.5 (continue)

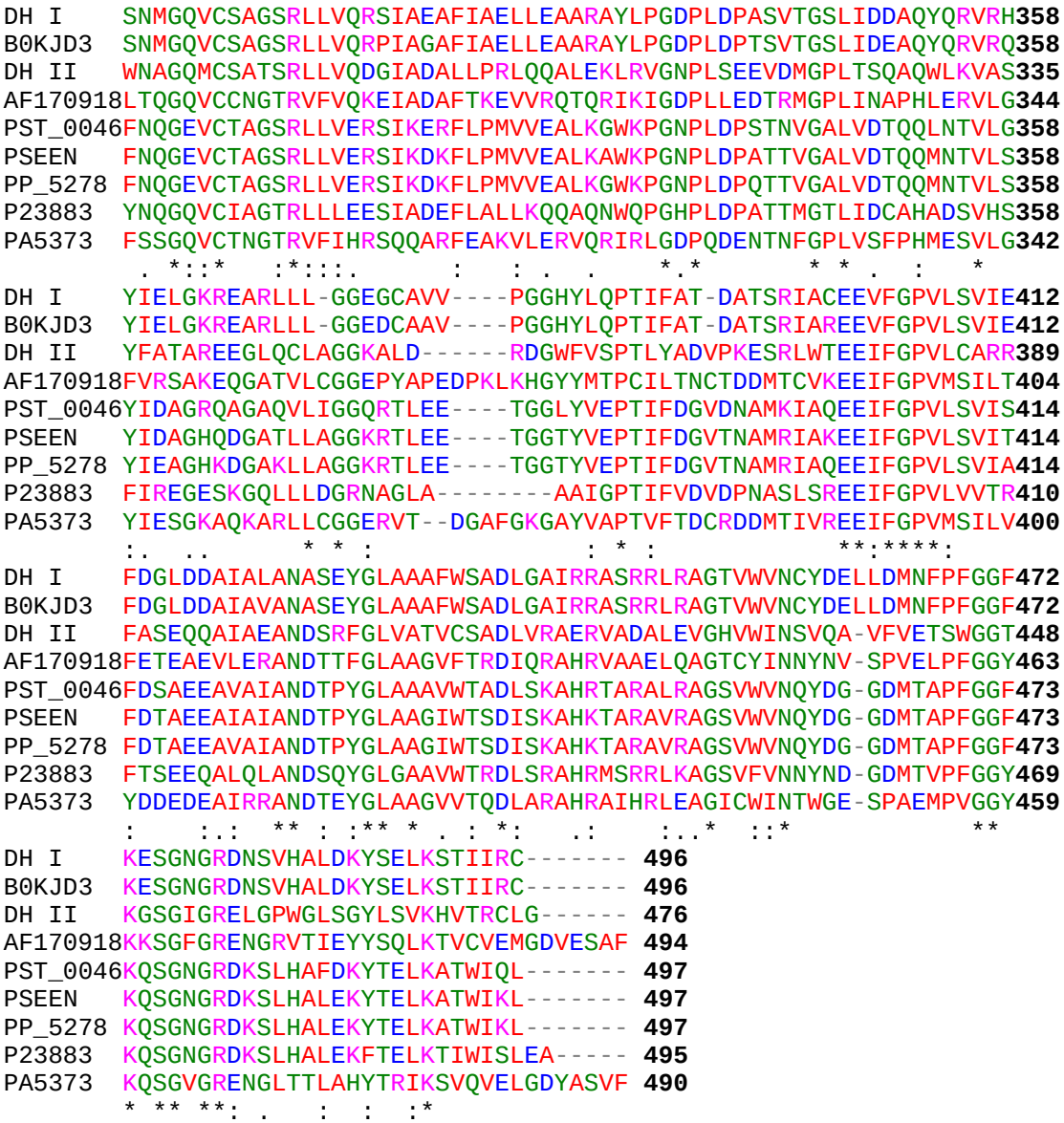

Fig. 2.5 Amino acid sequences of TMABaldehyde DH I and similar polypeptides

The sequences are from *Pseudomonas* sp. 13CM TMABaldehyde DH I (top line, AB741624), *Pseudomonas putida* GB1 aldehyde dehydrogenase (B0KJD3), *Pseudomonas* sp. 13CM TMABaldehyde DH II (AB713749), rat TMABaldehyde DH (AF170918), *Pseudomonas stutzeri* A1501 aldehyde dehydrogenase family (PST_0046), *Pseudomonas entomophila* L48 (PSEEN5424) aldehyde dehydrogenase (Aldh), *Pseudomonas putida* KT2440 aldehyde dehydrogenase family protein (PP_5278), *E. coli* K-12 γ-glutamyl-γ-aminobutyraldehyde dehydrogenase (P23883), and *Pseudomonas aeruginosa* betaine aldehyde dehydrogenase (PA5373). Numerals show quantities of amino acid residues, starting from the initial codon. "*", residues that are identical in all sequences in the alignment; ":", conserved substitutions; ".", semi-conserved substituitions; "-", no corresponding amino acid.

The other hand, the deduced amino acid sequence of *Pseudomonas* sp. 13CM TMA-Butanol DH showed very high identity (87%) to that of *P. putida* GB-1 (B0KJD2) iron-containing alcohol dehydrogenase (Table 2.8, Fig. 1.7). It was also exhibited 30–40% similarity with alcohol dehydrogenase of *Pelagibacter* sp. HTCC7211 (B6BT37), *Pseudovibrio* sp. FO-BEG1 (G8PVZ2), *Silicibacter* sp. (C9D0Z7), *Rhizobium leguminosarum* bv. *trifolii* WSM2304 (B5ZWN1), *Sinorhizobium meliloti* (M77739), *Mesorhizobium* sp. BNC1 (Q11H64), and *Sinorhizobium medicae* WSM419 (A6UBD1) (Table 2.8, Fig.2.6).

Table 2.8 Comparison between *Pseudomonas* sp. 13CM TMA-Butanol DH with other protein

Origin	Locus ID	Protein	Alignment length	Identity (%)
P. putida GB-1	B0KJD2	Iron cont. alcohol dehydrogenase	394	87
Pelagibacter sp.	B6BT37	Probable alcohol dehydrogenase	396	39
Pseudovibrio sp.	G8PVZ2	Iron cont. alcohol dehydrogenase	387	36
Silicobacter sp.	C9D0Z7	Iron cont. alcohol dehydrogenase	381	36
R. leguminosarum	B5ZWN1	Iron cont. alcohol dehydrogenase	383	36
S. meliloti	M77739	Iron cont. alcohol dehydrogenase	381	35
Mesorhizo. sp.	Q11H64	Iron cont. alcohol dehydrogenase	381	35
S. medicae	A6UBD1	Iron cont. alcohol dehydrogenase	381	34

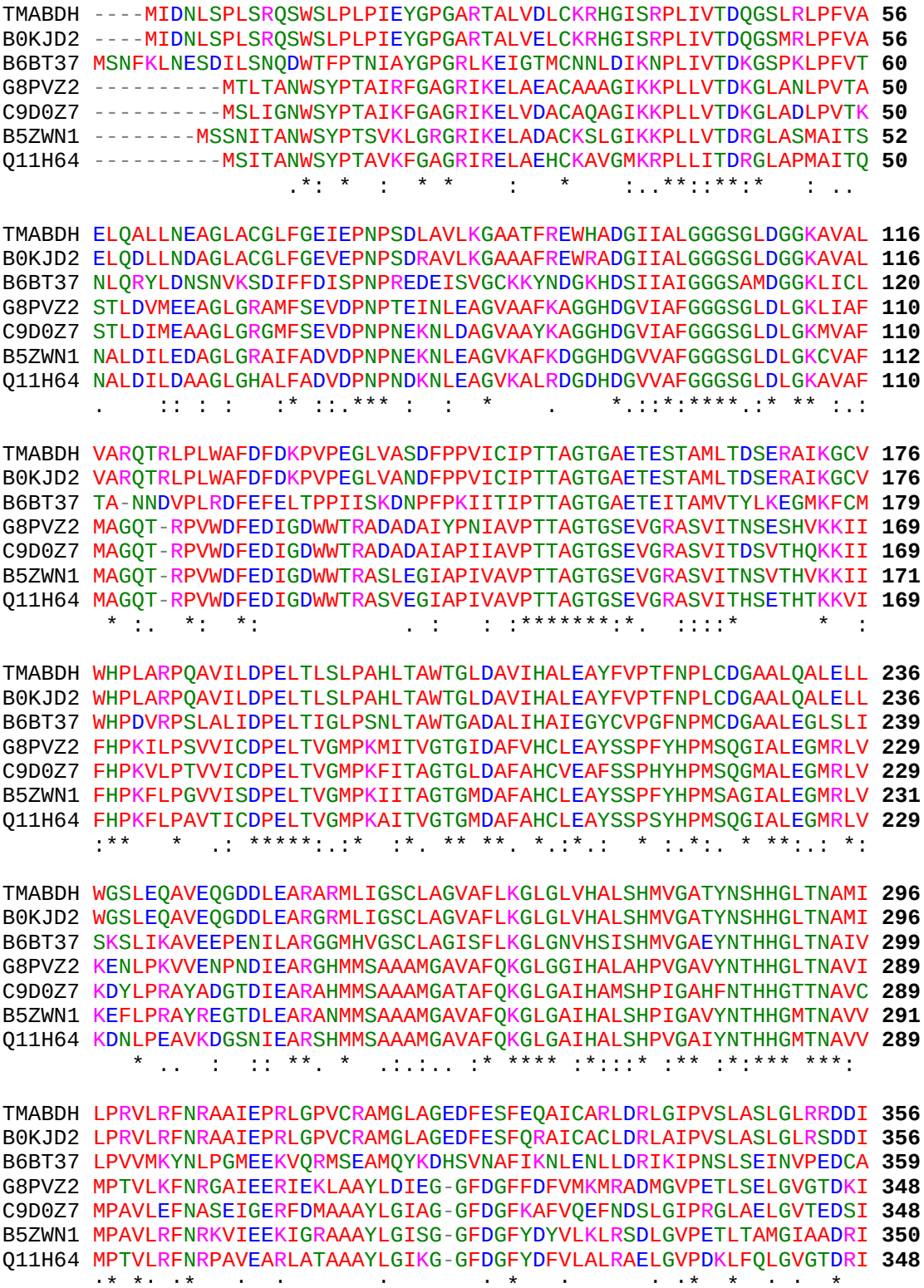

Fig. 2.6 Amino acid sequences of TMA-Butanol DH and similar polypeptides

Fig. 2.6 (continue)

```
TMABDH PAIARKAMGDPARQTNPRPSSFEDLQTLLHLALEEERA- 394
B0KJD2 PAIARKAMGDPARQTNPRPSSLEDLEALLHQALEEKRA- 394
B6BT37 ERIAEKAMKDQAYATNPKVASLAEMKEMVLQSIKKAR-- 396
G8PVZ2 DVLAEEAIKDPSAGGNPVELTLEAAKALLIEAIGEQVSA 387
C9D0Z7 PELVKGAIIDPSCGGNPVKLTEENLTQLFKAAL------ 381
B5ZWN1 DELSAMAIEDPSAGGNPVAMTLENTKALFRDCF------ 383
Q11H64 DEMAAMAIVDPTAGGNPVELTLEAAKSLYAECI------ 381
          :   *: * :   **   :      :   .:
```

Fig. 2.6 Amino acid sequences of TMA-Butanol DH and similar polypeptides

The sequences are from *Pseudomonas* sp. 13CM TMA-Butanol DH (top line, AB741625), *Pseudomonas putida* GB-1 putative iron containing alcohol dehydrogenase (B0KJD2), *Pelagibacter* sp. probable alcohol dehydrogenase (B6BT37), *Pseudovibrio* sp. iron containing alcohol dehydrogenase (G8PVZ2), *Silicobacter* sp. iron containing alcohol dehydrogenase (C9D0Z7), *Rhizobium leguminosarum* iron containing alcohol dehydrogenase (B5ZWN1) and *Mesorhizobium* sp. iron containing alcohol dehydrogenase (Q11H64). Numerals show quantities of amino acid residues, starting from the initial codon. "*", residues that are identical in all sequences in the alignment; ":", conserved substitutions; ".", semi-conserved substituitions; "-", no corresponding amino acid.

2.3.2 Over-expression of TMABaldehyde DH I and TMA-Butanol DH

To clarify the function and role of *Pseudomonas* sp. 13CM TMABaldehyde DH and TMA-Butanol DH, we have constructed the expression vector for production of TMABaldehyde DH I and TMA-Butanol DH as described in materials and methods section. Upon induction with isopropyl-β-D-thiogalactopyranoside, recombinant TMABaldehyde DH I and TMA-Butanol DH were expressed at 25 ºC. Comparing the activity with native enzymes, the specific activity of the crude cell-free extract of recombinant TMABaldehyde DH I and TMA-Butanol DH were shown in Table 2.9.

Table 2.9 Recombinant TMABaldehyde DH I and TMA-Butanol DH (cell-free) activity

Enzyme	Total activity (U)	Total protein (mg)	Sp. activity ($U.mg^{-1}$)	Productivity ($U.ml^{-1}$)
TMABaldehyde DH I (native)	305	349	0.9	0.3
TMABaldehyde DH I (recom.)	2199	377	5.8	112.8
TMA-Butanol DH (native)	138	1443	0.10	0.9
TMA-Butanol DH (recom.)	283	82	3.45	1.9

2.3.3 Purification of recombinant TMABaldehyde DH I

The expressed TMABaldehyde DH I was purified by hydrophobic chromatography using Phenyl-Toyopearl spin column giving a preparation with a specific activity at 31.0 unit.mg^{-1} (Table 2.10). The purified recombinant TMABaldehyde DH I appeared as single protein band on native–PAGE and the enzyme activity was detected at the same position on the gel, additionally, moved as a single protein band with SDS–PAGE at around 50 kDa (Fig. 2.7). The molecular mass of the native enzyme was estimated by gel filtration on a TSK-Gel G3000SW column. The enzyme was eluted at a molecular mass of about 160 kDa (Fig. 2.8).

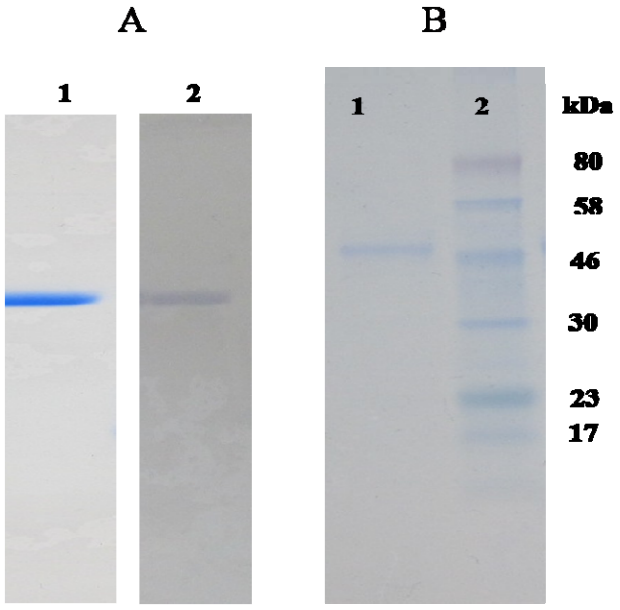

Fig. 2.7 Native PAGE and SDS−PAGE of purified recombinant *Pseudomonas* sp. 13CM TMABaldehyde DH I

A, Native PAGE: Gels were stained for protein with CBB (1) and enzyme activity with the TMABaldehyde (2). B, SDS-PAGE: Lane 1, purified recombinant TMABaldehyde DH I; lane 2, molecular weight markers (color plus prestained protein marker, New England Biolabs).

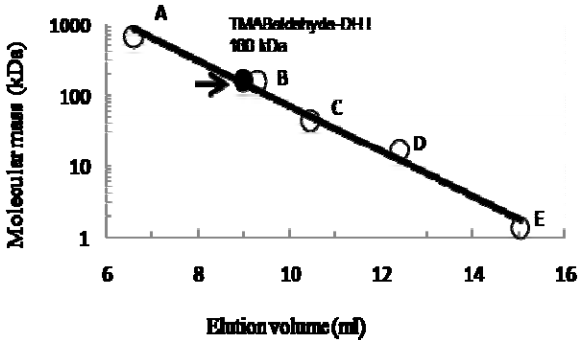

Fig. 2.8 Molecular weight determination of recombinant TMABaldehyde DH I

The molecular mass of recombinant TMABaldehyde DH I was estimated by HPLC from a semilogarithmic plot of molecular mass against retention time with each standard protein: A, bovine thyroglobulin; B, bovine γ-globulin; C, chicken ovalbumin; D, horse myoglobin; E, vitamin B12.

2.3.4 Effect of temperature and pH on recombinant TMABaldehyde DH I activity

The effect of temperature on recombinant TMABaldehyde DH I activity was investigated from 20 ºC to 50 ºC at pH 9.5. The maximum activity for this preparation was found at 40 ºC. The enzyme appears to be stable at 30 ºC, but became very unstable at a temperature above 35 ºC (data not shown). The pH effect was studied in the range of 6.0 to 10.5 at 30 ºC using different buffers. The maximum activity was obtained at pH 9.5. The pH stability of the enzyme was measured after pre-incubation for 15 min at 30 ºC using various buffers. The enzyme was stable between pH 8.0 and 9.0. It was completely inactivated at pH 10.0 (data not shown).

2.3.5 Substrate specificity and kinetic assay of recombinant TMABaldehyde DH I

To determine the impacts of the recombinant TMABaldehyde DH I on dehydrogenase activity, various aminoaldehydes as well as aliphatic aldehydes were chosen as substrate in the enzymatic assay. The present preparation of TMABaldehyde DH I oxidized mainly TMABaldehyde and DMABaldehyde. In addition, it was found to

be slightly active against 4-aminobutyraldehyde, betaine aldehyde, acetaldehyde, propionaldehyde, butyraldehyde, isovaleraldehyde and pivaleraldehyde (Table 2.10) but was inactive against alcoholic substrates, DMA-Pentanol, TMA-Butanol, TMA-Pentanol, and TMA-Hexanol.

Table 2.10 The enzyme activities of purified recombinant TMABaldehyde DH I

Substrate (Aldehydes)	Sp. activity ($U.mg^{-1}$)	K_m (mM)
TMABaldehyde	31.0	0.12
DMABaldehyde	43.08	0.07
4-Aminobutyraldehyde	0.70	*ND*
Betainealdehyde	0.43	*ND*
Acetaldehyde	0.27	*ND*
Propionaldehyde	0.63	*ND*
Butyraldehyde	0.59	*ND*
Isovaleraldehyde	0.08	*ND*
Pivaleraldehyde	0.04	*ND*
$^{*}NAD^{+}$	TMABaldehyde	0.15

"*ND*" stands for not detected; *considered as substrate with co-substrate TMABaldehyde for K_m

The purified recombinant TMABaldehyde DH I was found to be very specific for NAD^{+} and did not react with $NADP^{+}$. The author was attempted here to measure the K_m values for TMABaldehyde iodide, DMABaldehyde iodide and NAD^{+}. The apparent K_m values for TMABaldehyde iodide, DMABaldehyde iodide and NAD^{+} were calculated to be 0.12 mM, 0.07 mM, and 0.15 mM, respectively (Table 2.10).

2.4 DISCUSSION

One way to gain a better understanding of the molecular properties of enzymes is to sequence the genes. The production of recombinant versions of TMABaldehyde DH I and TMA-Butanol DH in *E. coli* is highly desirable due to simpler culture conditions, produce a more pure product with less processing time, less variation in lot-to-lot, sufficient level of production with desired need, and minimize the cost of production. Recombinant form of any enzyme is also obviously essential for any biotechnological application, and it would also be extremely useful for further characterization studies, such as determining three-dimentional structures and structure/function studies using mutagenesis as easier to manipulate at the genetic level. An enzymatic reduction system composed of both TMABaldehyde DH I and TMA-Butanol DH for the degradation of choline like structure TMA-Butanol within the same organism, *Pseudomonas* sp. 13CM has been reported by Hassan (2008). Recently, the microorganisms, degrading homocholine as the only source of carbon and nitrogen have been isolated in the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan and proposed a possible degradation pathway (Mohamed Ahmed *et al*., 2009a, 2009b, 2010). However, the genes encoding the enzymes TMABaldehyde DH I and TMA-Butanol DH of *Pseudomonas* sp. 13CM remained unknown.

In this study, we succeed to sequence the genes encoding TMABaldehyde DH I and TMA-Butanol DH as well as construction of the recombinant TMABaldehyde DH I and TMA-Butanol DH which is the first report with bacterial genes. The specific activity of the purified recombinant TMABaldehyde DH I show 31.0 $U.mg^{-1}$ where as 12.4 $U.mg^{-1}$ in wild-type TMABaldehyde DH I isolated from cells of *Pseudomonas* sp. 13CM (Hassan *et al*., 2008). The activity of present preparation of TMABaldehyde DH I is also higher than those of TMABaldehyde DHs from *B. taurus* (Hulse and Henderson, 1980) and *R. norvegicus* (Vaz *et al*., 2000), respectively, 5.1 $U.mg^{-1}$ and 0.77 $U.mg^{-1}$. The catalytic and kinetic parameters of the native and recombinant enzyme were indistinguishable, indicating that this TMABaldehyde DH I was successfully produced in *E. coli*. This study suggests that heterologous expression in *E. coli* could potentially

be used for the large-scale production of recombinant TMABaldehyde DH I and TMA-Butanol DH for potential biotechnological applications.

The deduced amino acid sequence of *Pseudomonas* sp. 13CM TMABaldehyde DH (consisting 496 amino acid) and TMA-Butanol DH (consisting 394 amino acid), respectively, similar to those of aldehyde dehydrogenase and alcohol dehydrogenase superfamily proteins. In particular, both of the enzymes have high similarity to proteins from *P. putida* GB-1(B0KJD3, B0KJD2).

The maximum identity showed by the deduced amino acid sequence of TMA-Butanol DH with iron-containing alcohol dehydrogenases. It differs in size and metal content. The metal-free and zinc-containing alcohol dehydrogenases have a size containing approximately 250 residues and 350 residues, respectively, while typical iron-containing alcohol dehydrogenase has a larger size varying from 382 to 891 amino acids (Reid and Fewson, 1994). The 394 residues containing TMA-Butanol DH has fallen on the later group of long chain iron-containing alcohol dehydrogenase. A considerable number of study has been carried out with iron-containing alcohol dehydrogenases in mesophiles that catalyzes various alcohols such as, ethanol, 1,2-propanediol, 1,3-propanediol, and 1-butanol (Conway and Ingram, 1989; Walter *et al*., 1992; Zheng *et al*., 2004; Montella *et al*., 2005). The iron-dependence of *Pseudomonas* sp. 13CM TMA-Butanol DH (Hassan *et al*., 2007) has not been determined experimentally. In this study, uncover the link to iron dependency by the deduced amino acid sequence. Amino acid sequence identity of TMA-Butanol DH with other protein databases exhibited the high percentage similarity with iron-containing alcohol dehydrogenases which is good agreement with the findings of Hassan *et al*. (2007), the enzyme activity was strongly inhibited by a chelating agent 1,10-phenanthroline for Fe ions with a concentration of 0.1 mM. In comparison to metal-free and zinc-containing group of alcohol dehydrogenases, iron-containing enzymes are oxygen sensitive. Ying *et al*. (2009) reported that iron-containing alcohol dehydrogenase from *Thermococcus* strain ES1 lost almost 80% of their activity within 5 min exposure to the air. It was also reported that the loss of activity after exposure to the air might be due to the oxidation of

ferrous to ferric, and/or loss of ferrous ion, and replacement with other metals such as zinc (Ying *et al*., 2007). The irreversible loss of enzyme activity was also likely to be caused by the oxidation of some amino acids such as cysteine residues resulting in irreversible structure change and thus inactivation of activity (Neale *et al*., 1986).

The deduced amino acid sequences (Fig. 2.4) are in good agreement with that of the partial amino acid sequences and electrophoresis data described by Hassan *et al*. (2007, 2008). In addition, the present preparation of TMABaldehyde DH I, moved as a single protein band with SDS–PAGE at around 50 kDa (Fig. 2.7), which is in accordance with the theoretical monomeric molecular mass of the TMABaldehyde DH I 53.02 kDa. The molecular mass of the over-expressed TMABaldehyde DH I of *Pseudomonas* sp. 13CM is found to be 160 kDa by gel filtration (Fig. 2.8) suggesting that the protein existed in trimer in solution under native condition which is similar to the molecular mass of TMABaldehyde DH of *B. taurus* (Hulse and Henderson, 1980) and the same to the predicted molecular mass of TMABaldehyde DH from rat liver (Vaz *et al*., 2000).

The optimum temperature and pH were comparable to that of the native TMABaldehyde DH I from *Pseudomonas* sp. 13CM (Hassan *et al*., 2008). The alkaline environment preference of the enzyme activity agrees well with the other reported TMABaldehyde DH (Hassan *et al*., 2008; Hulse and Henderson, 1980; Vaz *et al*., 2000).

In full agreement with Hassan *et al*., (2008), the prepared TMABaldehyde DH I showed oxidative activity toward only TMABaldehyde, and no aldehyde produced by the reversible reaction. The over-expressed preparation of TMABaldehyde DH I and TMA-Butanol DH cell-free extract showed the specific activity respectively 5.8 and 3.45 $U.mg^{-1}$ those were higher than the wild-type 0.87 and 0.10 $U.mg^{-1}$ (Hassan *et al*., 2007, 2008). The purified recombinant TMABaldehyde DH I in this study displayed the specific activity 31.0 $U.mg^{-1}$ and gave a K_m value of 0.12 mM for TMABaldehyde is appeared to be higher than the K_m values for TMABaldehyde DHs from *Pseudomonas* sp. 13CM (Hassan *et al*., 2008), *B. taurus* (Hulse and Henderson, 1980), *R. norvegicus* (Vaz

et al., 2000), respectively, 7.4 μM, 4.2 μM, and 1.4 μM. Both NAD^+ and $NADP^+$ can be used as coenzyme in *R. norvegicus* (Vaz *et al*., 2000), but the enzyme of *B. taurus* (Hulse and Henderson, 1980) and the enzymes purified from *Pseudomonas* sp. 13CM were highly specific only for NAD^+.

2.5 CONCLUSIONS

This study was designed to sequence the genes encoding the enzymes TMABaldehyde DH I and TMA-Butanol DH from *Pseudomonas* sp. 13CM. Besides, preparation of recombinants expressed in *E. coli* also designed to compare the properties with native enzyme. As expected, the genes were successfully sequenced from the donor strain *Pseudomonas* sp. 13CM using the N-terminal sequences of the proteins. Molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the production of a large quantity of the enzyme in a recombinant strain. Characterization of degradative genes, may explore to evaluate the microbial populations optimal for biodegradation and bioremediation technologies. A more temperature tolerant form of TMABaldehyde DH I is highly desirable. Therefore, with the sequences, these materials and informations in hand, our further investigation will be focused on experiments designed; engineering of the gene of TMABaldehyde DHs of *Pseudomonas* sp. 13CM to modify the enzyme function such as thermal stability and enzyme-cofactor interactions.

CHAPTER 3

SITE-DIRECTED MUTAGENESES REVEAL ESSENTIAL RESIDUES FOR SUBSTRATE SPECIFICITIES OF 4-*N*-TRIMETHYLAMINOBUTYRALDEHYDE DEHYDROGENASES IN *PSEUDOMONAS* SP. 13CM

3.1 INTRODUCTION

Carnitine (3-hydroxy-4-*N*-trimethylaminobutyric acid) has attracted increasing attention because of its association with fatty acid metabolism, enables β-oxidation of the long-chain fatty acids in mitochondria (Mc Garry and Brown, 1997; Ramsay *et al*., 2001). It is present in some prokaryotes and all eukaryotes (Ramsay *et al*., 2001). In prokaryotic cells, it serves either as a nutrient (Kleber, 1997), or as an osmoprotectant (Jung *et al*., 1990; Robert *et al*., 2000). However, in eukaryotic cells, it serves exclusively as a carrier of acyl moieties through various subcellular compartments (Ramsay and Arduini, 1993). Furthermore, it involves in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle (Jakobs and Wanders, 1995; Verhoeven *et al*., 1998).

Carnitine other than food sources is synthesized endogenously from trimethyllysine with a series of enzymatic reactions. Among them, 4-*N*-trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde DH) catalyzes 4-*N*-trimethylaminobutyraldehyde (TMABaldehyde) and yield γ-butyrobetaine, the final precursor of carnitine (Vaz *et al*., 2002). TMABaldehyde DH has been purified to homogeneity from beef liver (Hulse and Henderson, 1980), rat liver (Vaz *et al*., 2000) and soil microorganism *Pseudomonas* sp. 13CM (Hassan *et al*., 2008).

In contrast to the many enzymatic studies of TMABaldehyde DH in mammals, investigations of microbial TMABaldehyde DH are few. Two type of TMABaldehyde DH produced by *Pseudomonas* sp. 13CM have been purified, biochemically characterized and compared their different properties; namely TMABaldehyde DH I (Chapter 1), and TMABaldehyde DH II (Chapter 2). TMABaldehyde DH I (AB741624) is structurally closer to *Pseudomonas putida* GB-1 aldehyde dehydrogenase (B0KJD3) exhibited 94% amino acid identity, where as TMABaldehyde DH II (AB713749) is closer to *Pseudomonas putida* KT2440 putative betaine aldehyde dehydrogenase (Q88PZ0) with 92% identity. Inspite of the considerable high sequence identity (39%) between these enzymes exhibited substantial differences in their biochemical properties. Although, both of them accept NAD^+ as a cofactor but catalytic properties are different. The natures of these TMABaldehyde DHs have been also found to be different in substrate binding and affinity. It has been assumed that the protonated substrate nitrogen electro-statically interacts with highly conserved negatively charged residues in the active site of these enzymes. In fact, analyses in various mutant strains have confirmed that, indeed, this mechanism can contribute the insights of the facts.

In this study, we performed site-directed mutagenesis of TMABaldehyde DHs (TMABaldehyde DH I and TMABaldehyde DH II) from *Pseudomonas* sp. 13CM and analyzes the resulting mutated proteins in order to assess the ability of the corresponding enzymes to utilize the substrates. To explore the structural basis of the different specificities, a structural comparison among the *Pseudomonas* sp. 13CM TMABaldehyde DHs has been carried out. Based on homology models and multiply aligned sequences, assumed the map and the amino acid differences onto the structure. In this chapter we accumulate data concerning the nature of constructed mutants in order to assess the critical residues involved in the enzyme activity, stored in the substrate binding pocket

3.2 MATERIALS AND METHODS

3.2.1 Materials

3.2.1.1 Microorganism and culture

Escherichia coli JM109 was collected from Takara Bio Inc. (Shiga, Japan). *E. coli* BL21 (DE3) was obtained from Novagen Inc. (Madison, WI, USA). Transformed cells were grown on LB medium (Table 1.2) containing the relevant antibiotics.

3.2.1.2 Plasmids

The vector pET24b (+) was a product of Novagen Inc. (Madison, WI, USA). The expression plasmid pET24b (+) hosting the genes of TMABaldehyde DHs (Chapter 1 and Chapter 2) were used for the PCR templates for desired mutagenesis.

3.2.1.3 Cofactors and chemicals for substrates

The cofactors NAD^+ and $NADP^+$ were purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). 4-dimethylamino-1-butanol (DMA-Butanol), 4-aminobutyraldehyde dimethylacetal, 3-dimethylamino-1-propanol (DMA-Propanol), 5-chloro-1-pentanol, and 6-dimethylamino-1-hexanol (DMA-Hexanol) were purchased from Tokyo Kasei (Tokyo, Japan).

3.2.1.4 PCR reagents

PCR primers (Table 3.1) were synthesized at Operon Biotechnologies (Tokyo, Japan). The sequences of the pairs of oligonucleotides synthesized for the mutagenesis (Operon Biotechnologies, Tokyo, Japan) are presented in Table 3.1. *Pwo* polymerase

was from Roche Applied Science (USA). Agarose SeaKem GTG was collected from BMA (Rockland, ME, USA). Loading quick λ/*Sty*I and loading quick λ/*Hind*III were purchased from Toyobo, Japan. Nucleospin Extract II and Nucleospin Plasmid kit were from Macherey-Nagel GmbH & Co. KG (Germany). BigDye Terminator Cycle Sequencing Ready Reaction kit ver 3.1 was from Applied Biosystems (Foster City, CA). The dye terminators, deoxynucleoside triphosphates, AmpliTaq DNA Polymerase, FS, rT*th* pyrophosphatase, magnesium chloride, and buffer were premixed into a single tube of Ready Reaction Mix and were ready to use.

Table 3.1 Oligonucleotide primers used for site-directed mutagenesis

Primer Name*	**Oligonucleotide Sequence (5´-3´)****	**Tm (ºC)**	**Mutants**
DHId_IIqF+	GAACTGCTAC**cAg**GAGCTGCTCGACATGAA	65.0	D457Q
DHId_IIqR–	TTCATGTCGAGCAGCTC**cTg**GTAGCAGTTC	65.0	
DHIe_IIaF+	CTGCTACGAC**GcG**CTGCTCGACATG	66.0	E458A
DHIe_IIaR–	CATGTCGAGCAG**CgC**GTCGTAGCAG	66.0	
DHIl_IIvF+	GCTACGACGAG**gTG**CTCGACATGAA	63.0	L459V
DHIl_IIvR–	TTCATGTCGAG**CAc**CTCGTCGTAGC	63.0	
DHIdel_qavF+	GTGAACTGCTAC**cAgGcGgTG**CTCGACATGAAC	65.0	D457Q-E458A-L459V
DHIdel_qavR–	GTTCATGTCGAG**cACCgCcTg**GTAGCAGTTCAC	65.0	
DHIIq_IdF+	TCAACTCGGTG**gAc**GCGGTGTTCGTCGAAA	66.0	Q437D
DHIIq_IdR–	TTTCGACGAACACCGC**gTc**CACCGAGTTGA	66.0	
DHIIa_IeF+	ACTCGGTGCAG**GaG**GTGTTCGTCGA	65.0	A438E
DHIIa_IeR–	TCGACGAACAC**CtC**CTGCACCGAGT	65.0	
DHIIv_IlF+	TCGGTGCAGGCG**cTG**TTCGTCGAAA	65.0	V439L
DHIIv_IlR–	TTTCGACGAA**CAg**CGCCTGCACCGA	65.0	
DHIIqav_IdelF+	ATCAACTCGGTG**gAcGaGcTG**TTCGTCGAAACA	66.0	Q437D-A438E-V439L
DHIIqav_IdelR–	TGTTTCGACGAA**CAgCtCgTc**CACCGAGTTGAT	66.0	

*The sense oligonucleotides are indicated by "+" and the antisense oligonucleotides by "–".

**The mutated codons are in bold, and mismatched basepairs are in lowercase.

3.2.1.5 Culture, purification, and polyacrylamide gel electrophoresis

Agar and yeast extract were from Difco (Lawrence, USA). Ampicillin and kanamycin were collected from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Phenyl-Toyopearl was from Tosoh Corporation (Tokyo, Japan). SDS–PAGE was performed using the mini slab size 5–20% gradient polyacrylamide gels purchased from Atto (Tokyo, Japan) and the enzyme samples were prepared for loading by mixing with an equal volume of 2x EzApply sample buffer (Atto, Tokyo, Japan). Color Plus prestained protein marker for SDS–PAGE was from New England Biolabs (Massachusetts, USA).

3.2.1.6 Other reagents

All other chemicals and materials were of the highest purity grade were supplied from Wako Pure Chemical Ind. Ltd. (Osaka, Japan) and used without further processing.

3.2.2 Methods

3.2.2.1 General methods

Cell growth was measured using the Novaspec II from Amersham Pharmachia Biotech (Piscataway, NJ, USA). TOMY Ultrasonic Disruptor (UD-200) was used for disruption of cells. Mupid-2X submarine electrophoresis system apparatus (Advance Co. Ltd., Japan) were used for agarose gel electrophoresis. General weight measurements were made using IB-200H electronic balance (Shimadzu corp. Kyoto, Japan) and smaller quantities measurements for the preparation of standards were performed using Mettler AE240 analytical balance (Mettler, Toledo, AG, Switzerland). For measurement of enzyme activity, Shimadzu UV-2100S was used. Products derived from PCR were purified by using Centri-Sep spin columns from Applied Biosystems (Foster city, CA) and Nucleospin Extract II and Nucleospin Plasmid kit were used during purification of PCR products and plasmids DNA. For measurement of purity of plasmid DNA,

UVmini-1240 UV-Visible spectrophotometer from Shimadzu Corp. (Kyoto, Japan) was used. pH was determined using an Horiba F-22 pH meter (Horiba Ltd, Kyoto, Japan). Cultures and extracts were centrifuged using Hitachi Himac Compact Centrifuges RX II Series CF16RXII (rotor: 36, T16A31; 44, T15A36; 46, T15A36; and 24, T9A31) and Hitachi Himac CT15E (Hitachi Koki Company Ltd., Tokyo, Japan). Inoculated media were incubated either at 37 ºC in MIR-262 (Sanyo Incubator, Tokyo, Japan) or with recipro shaker NR-1; at 25 ºC using a NR-300 double shaker (Taitec Corporation, Tokyo, Japan). Media were autoclaved at 121 ºC for 20 minutes using BS-235 high pressure steam sterilizer (Tomy Seiko Co., Ltd., Tokyo, Japan). Samples were vortexes using a Vortex Genie 2 (Scientific Industries, Inc., USA). Thermal Cycler Dice with model TP-600 or TP-100 were used for PCR reactions (Takara Bio Inc., Shiga, Japan).

3.2.2.2 Chemical synthesis

TMA-Butanol iodide, TMABaldehyde iodide, 5-dimethylamino-1-pentanol (DMA-Pentanol), 3-*N*-trimethylamino-1-propanol (TMA-Propanol) iodide, 5-*N*-trimethyl-amino-1-pentanol (TMA-Pentanol) iodide, and 6-*N*-trimethylamino-1-hexanol (TMA-Hexanol) iodide were prepared according to the method of Hassan (2008).

TMA-Butanol iodide was prepared from 4-dimethylamino-1-butanol according to the method described in Chapter 1. TMABaldehyde iodide was prepared from 4-aminobutyraldehyde dimethylacetal according to the method described previously in Chapter 1.

3.2.2.3 Bacterial strains, plasmids, and culture conditions

E. coli JM109 and *E. coli* BL21 (DE3) were used, respectively, as a host strain for sequence confirmation and high level of recombinant expression. Plasmid pET24b (+) was used for the expression of the recombinant (Fig. 1.1). Competent cells *E. coli*,

carrying the recombinant plasmids were grown in LB medium (Table 1.2) according to the method described in Chapter 1, supplemented with 50 μg.ml^{-1} of kanamycin and 0.3 mM of isopropyle-β-D-thiogalactopyranoside (IPTG).

3.2.2.4 Enzyme assay and protein measurement

The catalytic activity of TMABaldehyde DH was determined at 30 ºC by monitoring the increase in absorbance at 340 nm which is the characteristic absorption wavelength of NADH and using an extinction coefficient of 6,200 $M^{-1}.cm^{-1}$. The standard reaction mixture (1.5 ml) contained 3.0 mM of NAD^+, 0.8 mM TMABaldehyde iodide, and an appropriate amount of the enzyme in 150.0 mM glycine-NaOH buffer (pH 9.5) (Table 1.5). One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute under the assay conditions. The specific activity was defined as units of enzyme activity per milligram of protein. Protein was measured using the Lowry method (Lowry *et al*., 1951) with BSA as the standard protein, or by absorbance at 280 nm as described in Chapter 1.

3.2.2.5 Selection of residues to construct the mutants of **TMABaldehyde DHs**

The sequences of two aldehyde dehydrogenases from *Pseudomonas* sp. 13CM were described in Chapter 1 and 2 with the accession number DDBJ: AB741624 (TMABaldehyde DH I) and DDBJ: AB713749 (TMABaldehyde DH II). Genes encoding TMABaldehyde DH I and TMABaldehyde DH II were comprised of respectively, 1488 and 1431 nucleotides, which encoded the proteins comprising of 496 and 476 amino acids, respectively. Amino acid sequences of TMABaldehyde DH I and TMABaldehyde DH II exhibited high homology with, respectively, *Pseudomonas putida* GB-1 aldehyde dehydrogenase and *Pseudomonas putida* KT2440 putative betaine aldehyde dehydrogenase. The crystal structures of TMABaldehyde DH and from these two microorganisms were not available. Therefore, the predictive three-dimentional

structural models were constructed with SWISS-MODEL server by using crystal structure of betain aldehyde dehydrogenase from *E. coli* YdcW (PDB code 1WNB) as a template (Schwede *et al*., 2003). Structure quality and minor structural conflicts were evaluated using the Discrete Optimized Protein Energy statistical potential within modeler (Shen and Sali, 2006). Target and template sequences were multiply aligned with ClustalW (Thompson *et al*., 1994) and the resulting alignment manually refined. The built model structure utilized in the work will refer to that reported in Fig. 3.1. The predictive three dimentional models of TMABaldehyde DHs revealed the similarity in structures, placement of substrate and NAD^+. Apart from the similar residues located on the identical positions, there was exist a region near the substrate binding site (less than 10Å) consisting the residues Asp, Glu, and Leu in TMABaldehyde DH I whereas Gln, Ala, and Val in TMABaldehyde DH II (will be reported in Fig. 3.2). Therefore, it was assumed that the nature of these enzymes differences in respect to the coordination of these amino acids. Additionally, multiply aligned sequences of these enzymes focused on the different amino acids in a row especially in the position of C455−F466 of TMABaldehyde DH I and S435−W445 of TMABaldehyde DH II. It was examined with the three-dimentional structures that these different residues were present in the portion of the substrate binding pocket of the enzyme. To verify the role of the residues Asp, Glu, and Leu in TMABaldehyde DH I; Gln, Ala, and Val in TMABaldehyde DH II, site-specific mutagenesis was performed.

3.2.2.6 Site-directed mutagenesis via PCR

The genes encoding TMABaldehyde DHs (TMABaldehyde DH I and TMABaldehyde DH II) in the pET24b (+) vector were cloned using two oligonucleotides containing the desired mutation. The expression plasmid pET24b (+), hosting the genes of TMABaldehyde DHs, was used as the template for construction of the mutation genes. The PCR was performed with *Pwo* DNA polymerase. The restriction enzyme *Dpn*I was directly added to the PCR reactions and the mixture was

incubated at 37 ºC for overnight. The PCR products were then gel purified following the NucleoSpinExtract II protocol and transformed into *E. coli* JM109, cultivated on the LB agar plate supplemented with 50 μg.ml^{-1} kanamycin. Plasmid DNA was purified using NucleoSpin Plasmid kit. After mutagenesis the genes were sequenced to verify the correct introduction of the mutations. To employ site-directed mutagenesis of TMABaldehyde DHs residues based on the findings obtained from the three-dimentional model of TMABaldehyde DHs and amino acid sequence alignment. To evaluate the impact of mutations on the active-site of these enzymes, mutageneses of TMABaldehyde DHs were carried out replacing the residues of one sequence with the corresponding positioned residues from another sequence.

3.2.2.7 Protein expression and purification of recombinant wild-type and mutants

The verified mutant plasmids were transformed into *E. coli* BL21 (DE3) for expression. The recombinant *E. coli* clone hosting each desired mutation gene was cultivated at 37 ºC for overnight in 6 ml lysogeny broth medium containing 50 μg.ml^{-1} kanamycin, in a shaking incubator at 125 stroke.min^{-1}. At least three independent clones of each mutant were picked. An aliquot (0.75 ml) of these cultures was used to inoculate 75 ml of the same medium. Cultures were incubated at 25 ºC, at 125 strokes.min^{-1} until a cell density (optical density at 660 nm) of 0.5–0.7 was reached. Flasks containing the cultures were supplemented with IPTG to a final concentration of 0.3 mM and the culture was continuously incubated at 25 ºC for a further 16 hours to over-express the recombinants. Cells were harvested by centrifugation and pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT. The mixture was sonicated and clarified by centrifugation at 14,000 g for 20 min at 4 ºC and washed twice with 0.85% KCl solution. Recombinant proteins were purified by hydrophobic chromatography using Phenyl-Toyopearl spin column under native conditions. For this, cell pellet were resuspended in 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT and disrupted at 4 ºC by sonic treatment and centrifuged. To the cell free

extract, same volume of 0.5 M of ammonium sulfate solution in the 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM DTT was added. The resulting solution was loaded onto a Phenyl-Toyopearl spin column equilibrated with the same buffer which contained 0.25 M of ammonium sulfate and desalted through repeated concentration or dilution against low salt buffer (50 mM potassium phosphate buffer, pH 7.5 containing 1 mM DTT). An identical procedure was successfully used to over-express and purify the TMABaldehyde DHs mutant versions, D457Q, E458A, L459V, D457Q-E458A-L459V in TMABaldehyde DH I and Q437D, A438E, V439L and Q437D-A438E-V439L in TMABaldehyde DH II. Overexpressing TMABaldehyde DHs proteins in the BL21 DE3 strain of *E. coli* in the presence (+) or absence (–) of 3 mM of IPTG and the results of purifications were monitored by SDS–PAGE under denaturing conditions as described by Laemmli (1970) using the mini slab size 5–20% gradient polyacrylamide gels (Atto, Tokyo, Japan). The concentration of protein was determined following the method of Lowry (Lowry *et al*., 1951) with bovine serum albumin as the standard or by the absorbance at 280 nm as described in Chapter 1. Specific activity was defined as units of enzyme activity per mg protein.

3.2.2.8 Determination of enzyme activities

The catalytic activities of purified TMABaldehyde DHs wild-type and mutant enzyme were determined spectrophotometrically at 30 ºC by monitoring the increase in absorbance at 340 nm. Reactions were developed in a mix solution containing 150.0 mM glycine-NaOH buffer (pH 9.5), 3.0 mM of NAD^+, and an appropriate amount of the enzyme in a final volume of 1.5 ml. The reaction was initiated by the addition of the substrates at a final concentration of 0.8 mM. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of NADH per minute under the assay conditions. A molar absorption coefficient 6,200 $M^{-1}.cm^{-1}$ for NADH was used in the calculations. The specific activity was defined as units of enzyme activity per milligram of protein.

3.3 RESULTS

3.3.1 Homology modeling

For getting more insights into the catalytic mechanism of the TMABaldehyde DHs produced by *Pseudomonas* sp. 13CM, homology modeling was performed as described in Materials and method section. In the first approach mode of SWISS-MODEL server, the three-dimentional models of TMABaldehyde DHs were automatically built (Fig. 3.1) based on the crystal structure of *E. coli* YdcW betaine aldehyde dehydrogenase (PDB code 1WNB) as a template.

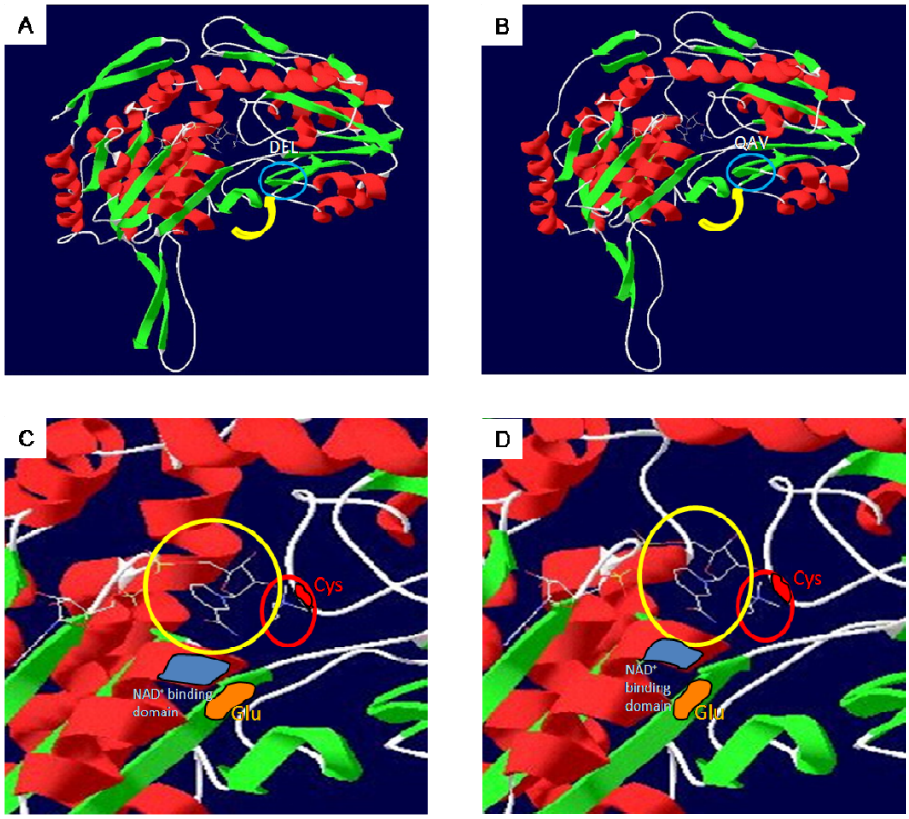

Fig. 3.1 Molecular structures of TMABaldehyde DH I and TMABaldehyde DH II

A, Overall fold of TMABaldehyde DH I, yellow arrow indicates the entrance; B, overall fold of TMABaldehyde DH II, yellow arrow indicates the entrance; C, Close-up view of the substrate binding channel of TMABaldehyde DH I; D, Close-up view of the substrate binding channel of TMABaldehyde DH II. Yellow and red cicles in C and D respectively represents NAD^+ and substrate binding sites.

A comparison of the three-dimentional structures of *E. coli* YdcW betaine aldehyde dehydrogenase as observed by Gruez *et al*. (2004) and *Pseudomonas* sp. 13CM TMABaldehyde DHs as predicted from protein modeling, indicated an overall similarity between both enzymes. Acidic and aromatic amino acid residues near the substrate (less than 10 Å) of TMABaldehyde DHs were identified (Fig. 3.2) based on the homology modeling with betaine aldehyde dehydrogenase of *E. coli* YdcW.

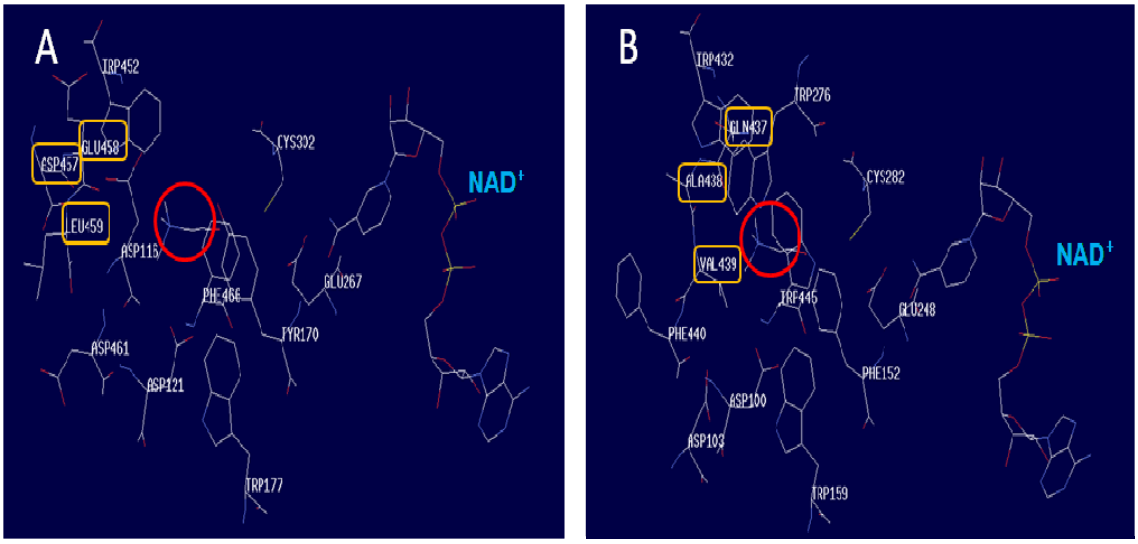

Fig. 3.2 Structure of the active sites of TMABaldehyde DHs (A, TMABaldehyde DH I; B, TMABaldehyde DH II)

Relevant residues are depicted in wireframe representation. Portion surrounded by a *red* circle represents the proposed substrate binding site of TMABaldehyde DHs. The different residues of TMABaldehyde DHs in coordination and numbers are boxed with *yellow* line.

The amino acid sequence alignment among the two TMABaldehyde DHs and the structural template from *E. coli* YdcW betaine aldehyde dehydrogenase (P77674) was shown in Fig. 3.3. Sequence identity of the target TMABaldehyde DHs and template were about 38% on average (ranging 35−40%); and 39% among each other (Fig. 3.3).

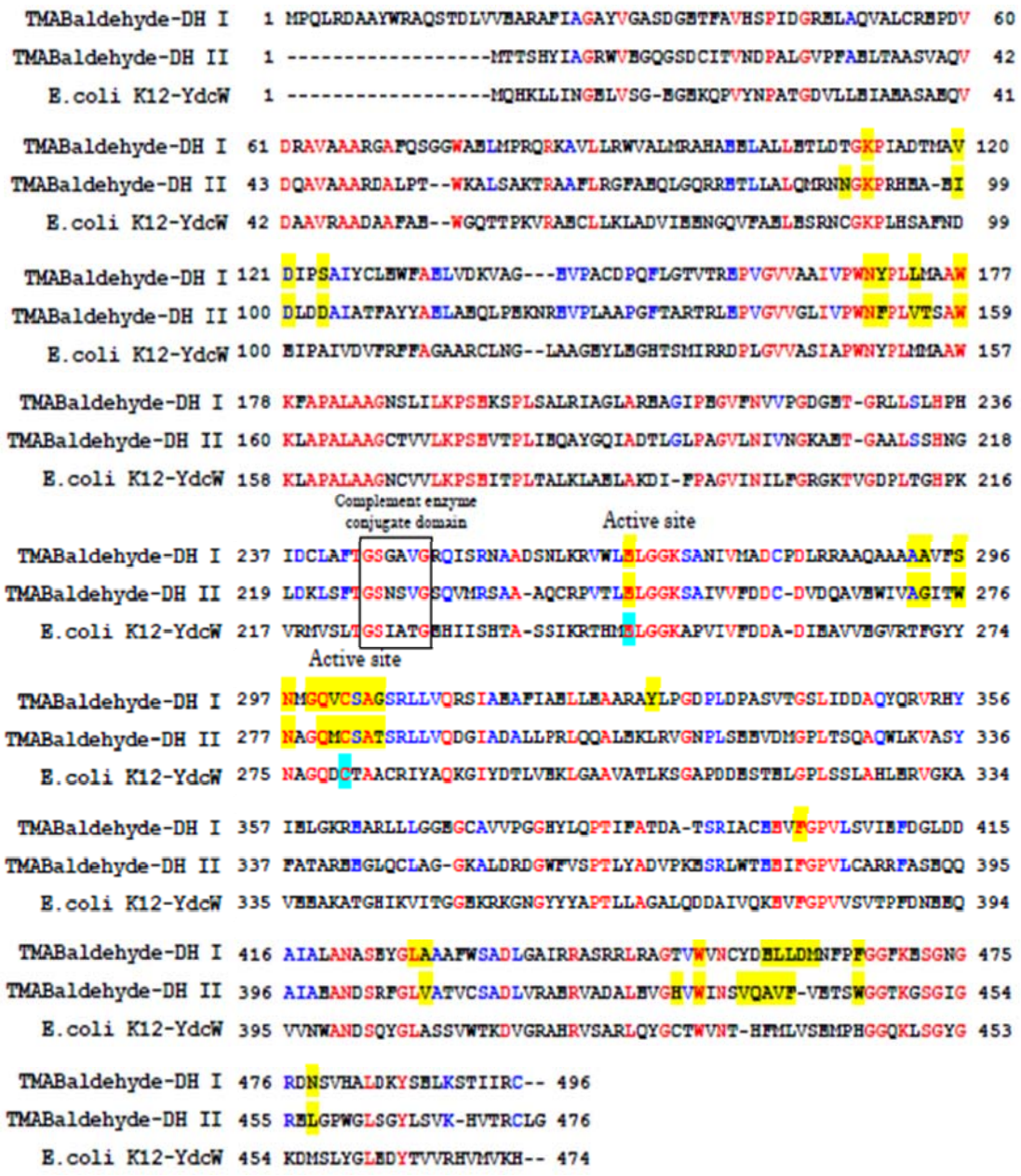

Fig. 3.3 Amino acid sequence alignment of *Pseudomonas* sp. 13CM TMABaldehyde DHs and *E. coli* K-12 YdcW betaine aldehyde dehydrogenase (PDB ID: 1WNB)

The invariant residues are indicated by *red letters*. The residues conserved in the TMABaldehyde DH I and II are indicated in *blue letters*. Homology modeling was performed using *E. coli* K-12 YdcW betaine aldehyde dehydrogenase as template and the TMABaldehyde DHs residues surrounding the substrate binding site at a less that 10 Å distances are highlighted in *yellow* with *black letters*.

3.3.2 Generation and purification of the wild-type and mutant enzymes

The figure 3.1 displayed the overall fold of the enzyme TMABaldehyde DH I (Fig. 3.1.A) and TMABaldehyde DH II (3.1.B). The predictive three-dimentional model clearly represented the structural similarity between these two enzymes along with the entrance of the substrate. The close-up view of the substrate binding channel also revealed a consistent closeness with the placement of cofactor and substrate (Fig. 3.1.C, D). Apart from the reciprocity, residues stored on these models, an area covered with the few residues clearly indicated the difference in the coordination and number (residues Asp, Glu, and Leu in TMABaldehyde DH I; Gln, Ala, and Val in TMABaldehyde DH II) (Fig. 3.2). Therefore, it was assumed that the nature of these enzymes differences in respect to the coordination of these amino acids. Additionally, an inspection with multiple sequence alignment of two TMABaldehyde DHs focused on the different amino acids in a row especially in the position of C455−F466 on TMABaldehyde DH I and S435−W445 on TMABaldehyde DH II (Fig. 3.4). Based on the homology model and alignment, clear difference in residues between TMABaldehyde DH I and II were found around the entrance to the substrate channel (the residues Asp457, Glu458, and Leu459 in TMABaldehyde DH I; and corresponding positioned residues Gln437, Ala438, and Val 439 in TMABladehyde-DH II).

Fig. 3.4 Partial amino acid sequence alignment of the catalytic domain selected for the site-directed mutagenesis study of the TMABaldehyde DH I and TMABaldehyde DH II

Residues conserved in the protein sequences are shaded black. The residues are surrounding the substrate binding site at a less that 10 Å distances are highlighted in *yellow* with *black letters*. Consistent different amino acids are boxed with *blue* line. The positions of mutated codons are indicated with arrow head (D457, E458 and L459 for TMABaldehyde DH I; Q437, A438 and V439 for TMABaldehyde DH II). Numerals show the numbers of amino acid residues, starting from the initial codons.

The predictive three-dimentional homology model and multiple sequence alignment of TMABaldehyde DHs indicated the positionally critical residues which may directly or indirectly influence the cofactor specificity for these different natured enzymes. Among them, residues Asp457, Glu458, and Leu459 in TMABaldehyde DH I; Gln437, Ala438, and Val439 in TMABaldehyde DH II (Fig. 3.4) were selected for site-specific mutagenesis to study the functional significance, if any, of these selected residues. To verify the role played by Asp, Glu, and Leu residues, codons for Asp457, Glu458, and Leu459 have been singularly modified on the TMABaldehyde DH I gene by site-specific mutagenesis in order to insert corresponding positioned three aminoacidic residues Glu437, Ala438, and Val439 from TMABaldehyde DH II (Fig. 3.4) and prepared the mutants, respectively, D457Q, E458A, and L459V. Similarly, Glu437, Ala438, and Val439 on the TMABaldehyde DH II gene have been singularly modified in order to insert corresponding amino acid from TMABaldehyde DH I (Fig. 3.4), constructed the mutants Q437D, A438E, and V439L. To study the possible cumulative effect of the mutations, next modifications consisted on the TMABaldehyde DH I with simultaneous insertion of Gln at position 457, Ala at position 458, and Val at position 459 (triple mutant D457Q-E458A-L459V); while on the TMABaldehyde DH II Asp at position 437, Glu at position 438, and Leu at position 439 (triple mutant Q437D-A438E-V439L). The native and the mutant TMABaldehyde DHs were well expressed in *E. coli* BL21 (DE3) cells under the condition as described in Materials and methods.

The expressions of TMABaldehyde DHs in recombinant *E. coli* BL21 (DE3) with the addition of IPTG, compared to absence of IPTG were shown in figure 3.5. After cell disruption and centrifugal separation, the recombinant proteins from the supernatant were purified. All of the recombinants were shown a clear single band at around 52 kDa except Q437D and Q437D-A438E-V439L (Fig. 3.6). However, for the two mutants of Q437D and Q437D-A438E-V439L in TMABaldehyde DH II, the recombinant proteins were found inactive.

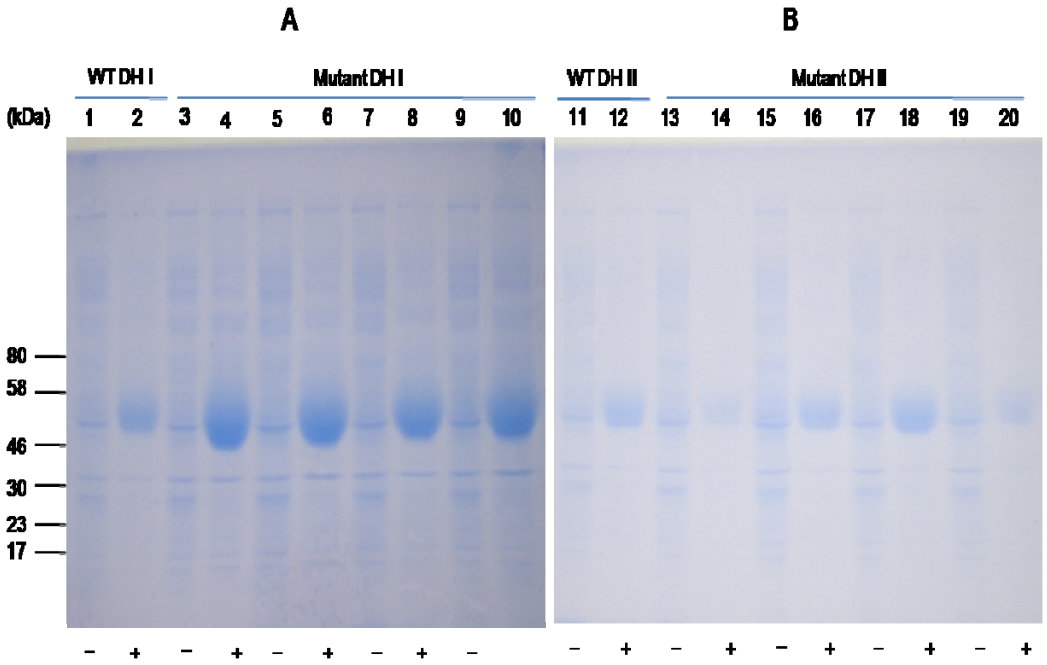

Fig. 3.5 Analysis of the wild type and mutants of the TMABaldehyde DHs separated by SDS–PAGE and stained with Coomassie Brilliant Blue dye (A, TMABaldehyde DH I; B, TMABaldehyde DH II)

Bacterial cultures overexpressing TMABaldehyde DHs proteins in the BL21 DE3 strain of *E. coli* were grown for 16 hours at 25 ºC in the presence (+) or absence (–) of 3 mM of IPTG. Lane 1 and 2, WT TMABaldehyde DH I; 3 and 4, D457Q; 5 and 6, E458A; 7 and 8, L459V; 9 and 10, D457Q-E458A-L459V; 11 and 12, WT TMABaldehyde DH II; 13 and 14, Q437D; 15 and 16, A438E; 17 and 18, V439L; 19 and 20, Q437D-A438E-V439L.

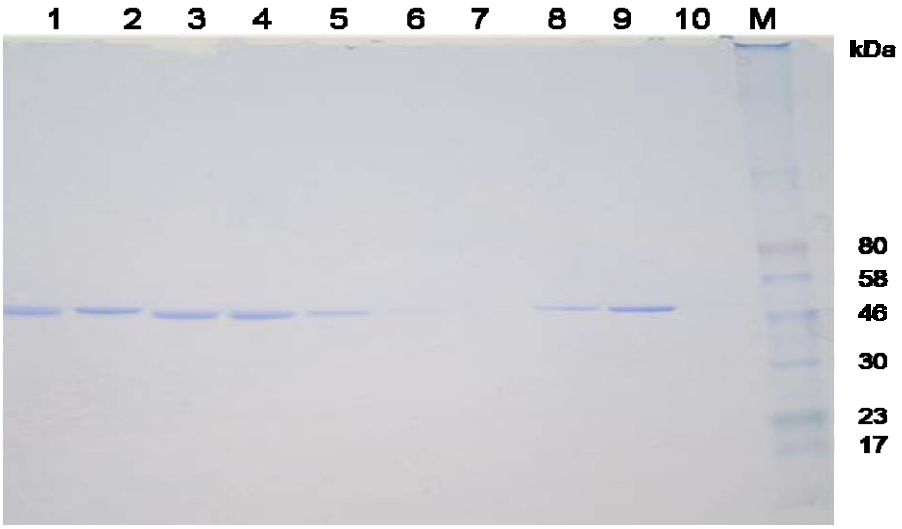

Fig. 3.6 Aliquots of purified enzyme (1.0–2.5 μg) for the wild-type and each TMABaldehyde DHs mutant were separated by 5–20% gradient gel SDS polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue dye

Lane 1, TMABaldehyde DH I; 2, TMABaldehyde DH II; 3, D457Q; 4, E458A; 5, L459V; 6, D457Q-E458A-L459V; 7, Q437D; 8, A438E; 9, V439L; 10, Q437D-A438E-V439L; M, Color Plus prestained protein marker (New England Biolabs Inc., USA).

3.3.3 Effects of mutation on TMABaldehyde DHs activitity

To determine the impacts of the mutants on dehydrogenase activity, various aminoaldehydes as well as aliphatic aldehydes were chosen as substrate in the enzymatic assay. As shown in Table 3.2 and 3.3, wild-type TMABaldehyde DH I and TMABaldehyde DH II and their mutant versions oxidized TMABaldehyde and DMABaldehyde except the mutants Q437D and triple mutant of TMABaldehyde DH II. In addition, the present preparations were found slightly active against other several chemicals with aldehyde group. Especially the activity of Q437D and triple mutant of TMABaldehyde DH II showed catalytic activity toward acetaldehyde whereas they could not oxidize TMABaldehyde and DMABaldehyde. In contrast, they were inactive for alcoholic substrates, trimethylaminobutanol and dimethylaminobutanol (data not shown). The present preparations were found to be very specific for NAD^+ and did not react with $NADP^+$ (data not shown).

Table 3.2 The specific activities of TMABaldehyde DH I and D457Q, E458A, L459V and D457Q-E458A-L459V proteins measured with 0.8 mM of substrate

Substrate	TMABaldehyde DH I	D457Q	E458A	L459V	D457Q-E458A-L459V
TMABaldehyde	31.97	6.89	21.22	6.90	0.98
DMABaldehyde	43.08	5.24	21.34	6.15	3.09
4-Aminobutyraldehyde	0.70	0.19	0.69	0.17	0.34
Betainealdehyde	0.43	*ND*	0.04	*ND*	*ND*
Acetaldehyde	0.27	0.17	0.17	0.04	0.08
Propinaldehyde	0.63	0.49	0.49	0.13	0.32
Butyraldehyde	0.59	0.75	0.58	0.09	0.38
Isovaleraldehyde	0.08	0.08	0.07	*ND*	0.06
Pivaleraldehyde	0.04	0.04	*ND*	*ND*	0.02

All values are given in unit.mg^{-1}. *ND* stands for not detectable

There were no significant changes in substrate specificity by single mutation in TMABaldehyde DH I in the ratio of oxidation for TMABaldehyde/DMABaldehyde point of view. In contrast, triple mutant of TMABaldehyde DH I showed oxidative activity toward DMABaldehyde threefold greater than that toward TMABaldehyde.

In the case of mutation on TMABaldehyde DH II, constructs Q437D and Q437D-A438E-V439L found to be inactive whereas the versions A438E and V439L maintained a little extent of activity (Table 3.3), indicating that the Gln residue of TMABaldehyde DH II is important for binding or formation of successive products. More specifically, compared to wild-type, A438E expressed about 4-times lower activity for TMABaldehyde and DMABaldehyde but maintained almost half of the activity for the oxidations of others aldehyde. The mutant V439L retained a relatively high activity for all of the substrates except pivaleraldehyde; exhibited non responsive higher activity than the wild-type for DMABaldehyde and 4-aminobutyraldehyde.

Table 3.3 The specific activities of TMABaldehyde DH II and Q437D, A438E, V439L and Q437D-A438E-V439L proteins measured with 0.8 mM of substrate

Substrate	TMABaldehyde DH II	Q437D	A438E	V439L	Q437D-A438E-V439L
TMABaldehyde	33.60	-	9.08	24.48	-
DMABaldehyde	13.81	-	3.84	20.73	-
4-Aminobutyraldehyde	0.35	-	0.21	0.76	-
Betainealdehyde	-	-	-	-	-
Acetaldehyde	0.19	-	0.09	0.16	-
Propinaldehyde	2.53	0.07	1.36	2.00	0.11
Butyraldehyde	4.36	-	4.09	3.13	-
Isovaleraldehyde	0.45	-	0.27	0.43	-
Pivaleraldehyde	0.62	-	0.32	0.20	-

All values are given in unit.mg^{-1}. "-" stands for inert

In respect to express the activity with percentage to compare with others, it may consider that, in the case of TMABaldehyde DH I, related to the wild-type mutants D457Q and D457Q-E458A-L459V showed the lower percentage of activity for TMABaldehyde, DMABaldehyde, and betaine aldehyde among the other substrates (Fig. 3.7 A). E458A mutant maintained more than 50% activity against TMABaldehyde, DMABaldehyde, and 4-aminobutyraldehyde compared with other mutant enzymes. In addition, E458A exhibited 10 times more activity against betaine aldehyde compared with any other mutant of TMABaldehyde DH I. Furthermore, the specific activity for construct L459V showed around 15% activity compare to wild-type for all substrates.

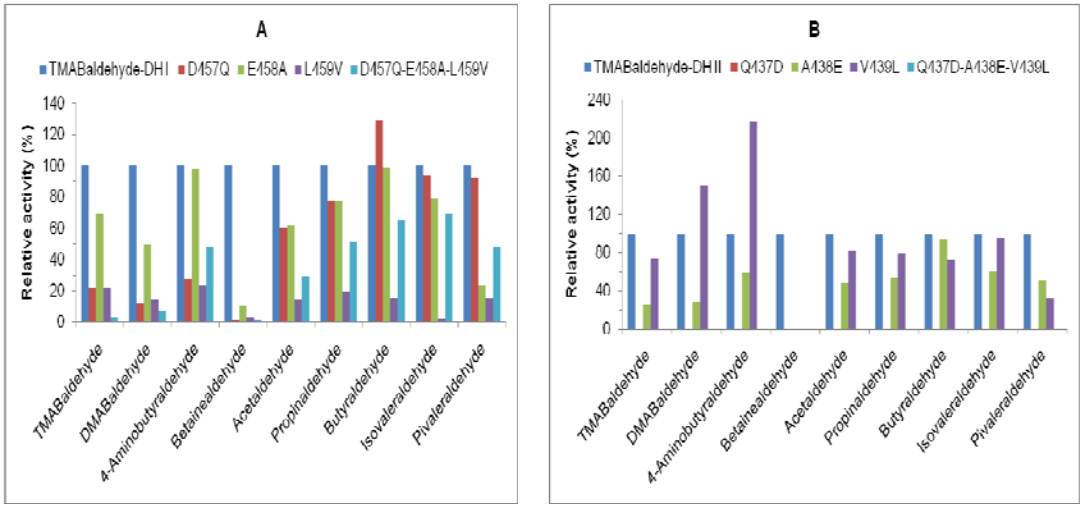

Fig. 3.7 A comparison of the substrate specificity of wild-type TMABaldehyde DHs and mutants in the aliquots of purified enzyme

The enzyme activities are shown as relative values, with wild-type activity taken as 100%

A. The substrate preference of TMABaldehyde DH I wild-type and its mutated proteins D457Q, E458A, L459V and D457Q-E458A-L459V.
B. The substrate preference of TMABaldehyde DH II wild-type and its mutated proteins D457Q, E458A, L459V and D457Q-E458A-L459V.

On the other hand, in the case of TMABaldehyde DH II, mutants Q437D and Q437D-A438E-V439L have been lost the activity. Comparing wild-type TMABaldehyde DH II, A438E showed less than 30% specific activity for TMABaldehyde and DMABaldehyde but around 50% for the others substrates (Fig. 3.7 B). Furthermore, the construct V439L retained a relatively high activity, more than 70% activity for all of the substrates except pivaleraldehyde.

3.3.4 K_m for substrates and cofactors

The products Q437D and Q437D-A438E-V439L in TMABaldehyde DH II almost abolished their activity, no kinetic parameters could be determined. As summarized in Table 3.4, comparing the K_m value 0.31 mM for TMABaldehyde in wild-type TMABaldehyde DH II, lower K_m value 0.12 mM was observed in wild-type TMABaldehyde DH I. In all mutants of TMABaldehyde DH I, the K_m values for NAD^+ of them were increased more than twice compared to the wild-type. In particular, 3.5-fold higher K_m values for NAD^+ were observed in L459V. Similarly, all TMABaldehyde DH I mutants showed K_m values for DMABaldehyde oxidation higher than wild type. In contrast, the presented data of K_m values for TMABaldehyde showed a little changes in the affinity for substrates with E458A and L458V but version D457Q displayed almost six times lowered affinity for TMABaldehyde (Table 3.4).

Table 3.4 Substrate affinities (K_m) of wild-type and mutants of TMABaldehyde DHs from *Pseudomonas* sp. 13CM

Enzyme	TMABaldehyde	DMABaldehyde	NAD^+
TMABaldehyde DH I	0.12	0.07	0.15
D457Q	0.67	0.10	0.36
E458A	0.09	0.20	0.30
L459V	0.07	0.13	0.52
D457Q-E458A-L459V	0.25	0.27	0.30
TMABaldehyde DH II	0.31	0.62	1.16
A438E	0.25	0.48	0.70
V439L	0.05	0.11	0.09

Kinetic parameters were not determined for enzyme mutants Q437D and Q437D-A438E-V439L which exhibited extremely low levels of TMABaldehyde DH activity. All given values are in mM.

On the other hand, the mutation of TMABaldehyde DH II positively affected to substrate binding affinity. Alteration of the residues Asp438 and Val439 result in A438E and V439L showed a significant lower K_m values for TMABaldehyde, DMABaldehyde, and NAD^+ with a significant improvement in the affinity (Table 3.4). Specifically,

almost two time's higher affinity was observed in A438E for NAD^+ and DMABaldehyde. A more profound effect on TMABaldehyde DH II activity was found with the mutant V439L, relative to wild-type about 13-times higher affinity for NAD^+ and almost 6-fold for TMABaldehyde and DMABaldehyde. Almost opposite scenario was observed with the mutants of TMABaldehyde DH I, except E458A and L459V for TMABaldehyde oxidation.

3.4 DISCUSSION

Many efforts were taken to evaluate the importance of the active-site residues in the aldehyde dehydrogenase superfamily (Hempel *et al*., 1993; Farre´s *et al*., 1995; Wang and Weiner 1995; Johanson *et al*., 1998; Mann and Weiner, 1999; Perez-Miller *et al*., 2003; Gruez *et al*., 2004; Kopency *et al*., 2011). The reaction of TMABaldehyde DHs, as of other aldehyde dehydrogenases (ALDH), involves a nucleophilic attack by the catalytic cysteine on the aldehyde substrate, followed by thioester formation and hydride kick to the $NAD(P)^+$ coenzyme (Wymore *et al*., 2004). The product acid is then released by hydrolysis with the precipitation of a conserved glutamate acting as an active-site base. Several studies have evaluated the importance of the active-site residues in the ALDH superfamily. The catalytic cysteine [C302 and C282 respectively in *Pseudomonas* sp. 13CM TMABaldehyde DH I and DH II] has been mutated in rat liver ALDH2 and human ALDH2 (Farre´s *et al*., 1995; Perez-Miller *et al*., 2003). Its replacement with alanine resulted in a complete loss of activity, where as its mutation to serine, as a poor nucleophile, resulted in a 10^2-10^5 fold lower dehydrogenase activity. The role of the active-site base has been studied in detail in human ALDH2 and ALDH3 (Wang and Weiner 1995; Mann and Weiner, 1999). Very recently, Tylichova *et al*., (2009) characterize two isoforms of plant aminoaldehyde dehydrogenase from *Pisum sativum* (PsAMADH1 and PsAMADH2) with broad specificity; can oxidize TMABaldehyde into carnitine precursor γ-butyrobetaine. Kaufman and Broquist (1977) demonstrated that TMABaldehyde is an intermediate in the L-carnitine biosynthesis of

Neurospora crassa using isotope labeling experiments. The structure of *E. coli* YdcW was found to be resembles that of other aldehyde dehydrogenases and using the activities experiments claimed that *E. coli* YdcW was more active on medium-chains aldehyde than on betaine aldehyde (Gruez *et al*., 2004).

In this study, the structure of TMABaldehyde DHs (*Pseudomonas* sp. 13CM) has been investigated with the site-directed mutagenesis based on their homology models using the structure of *E. coli* YdcW betaine aldehyde dehydrogenase (PDB entry 1WNB) (Gruez *et al*., 2004) whose primary sequence exhibit 38% identity with TMABaldehyde DH I and II for prediction of residues associated with substrate specificity and affinity to substrate. Apart from the structural similarity between these two TMABaldehyde DHs, differences in the residues D457−L459 on TMABaldehyde DH I and Q437−V439 on TMABaldehyde DH II were observed within 10 Å of the substrate binding site. Multiply aligned sequences also displayed a consistent length of completely different residues in a row with the position at C455−F466 on TMABaldehyde DH I and S435−W445 on TMABaldehyde DH II, assumed that the catalytic nature of these two enzymes differs with the involvement of the residues situated on the indicated area (Fig. 3.2; 3.4). It was considered that the protonated substrate nitrogen electrostatically interacts with the highly conserved negatively charged residues in the active site of TMABaldehyde DHs. To examine this assumption, the mutation of the residues Asp457, Glu458, and Leu459 on TMABaldehyde DH I; and Glu437, Ala438, and Val439 on TMABaldehyde DH II were performed by site-directed mutagenesis, significantly altered the specificity compare to wild-types.

Alteration of the residue Leu459 and subsequent construction L459V in TMABaldehyde DH I, changed the specific activity and affinity, considered that the shorter carbon chain of the side chain brought a change in substrate recognition. On the other hand, the mutant V439L in TMABaldehyde DH II with longer carbon chain showed decreased K_m values and the form of relationship between the enzyme and substrate to fit than the wild-type. It was inferred from these results that the

conformation of side chain not an important factor incases of TMABaldehyde DH II substrate affinity.

Almost similar affinity showed by the mutant E458A in TMABaldehyde DH I indicated that the negative charge of Glu did not have a major impact on the trimethylamino group. Little extent of changed affinity might be due to the space altered with the residue Ala than the change of the charge. In the case of TMABaldehyde DH II, the mutant A438E with increased side chains showed the higher substrate affinity, this was also observed with the construct V439L. It was also assumed that the possible negative charge of Glu worked on recognition of positive ammonium. However, the space of constructed enzyme might be narrowed by the substrate, not so increased the affinity.

Mutation of Asp457 in TMABaldehyde DH I with Gln led to weak activity and a significant increased K_m value (Table 3.2, 3.4) considered that this was might be due to the substitution of the negatively charged Asp with the positively charged Gln and no longer attracted the positive trimethylamino group. The evidences suggested that the Asp residue in catalytic area (Fig. 3.4) was the general base not only essential to maintain the high affinity of TMABaldehyde DH I, but also rate of its oxidation reaction. Similar findings were claimed in human aldehyde dehydrogenase 2 (ALDH2), alteration of Glu to Gln or Asp caused 10^2–10^3 fold lower the activity, where as in ALDH3, completely losts the activity (Wang and Weiner 1995; Mann and Weiner 1999). Mutation of Gln437 in TMABaldehyde DH II abolished the activity and was impossible to quantify the affinity for the substrate recognition, confirmed that Gln437 has an important role for TMABaldehyde DH II activity which is consistence with our structural model (Fig. 3.4). It was possible that this feature mainly played by changes from Gln to Asp at the three sites caused conformational disorder and it was not appeared on SDS polyacrylamide gel electrophoresis (Fig. 3.6). However, it deserves the more experiments to check the role of Gln in TMABaldehyde DH II with constructing different mutants.

Another hypothesis was taken here that the construct with replacing the three residues may change the environment for substrate binding. To examine this, we constructed the triple mutants D457Q-E458A-L459V in TMABaldehyde DH I and Q437D-A438E-V439L in TMABaldehyde DH II. The mutants D457Q-E458A-L459V displayed very low (<1% of the wild-type) enzyme activities and substrate affinity. However, the nature of the mutant Q437D-A438E-V439L was not possible to verify using this approach, abolished its activities.

3.5 CONCLUSIONS

In conclusions, the three-dimentional modeled structures of the TMABaldehyde DH I and TMABaldehyde DH II revealed several amino acid residues in their substrate binding channels that may be important for the high-affinity binding of substrates to TMABaldehyde DHs for the catalytic function of the enzyme. This work examines the consequences of mutagenic alterations of the substrate channel residues in TMABaldehyde DHs. All of the mutants versions exhibited a substantial decrease in activity along with increase of K_m displayed by TMABaldehyde DH I mutants both for substrate and cofactor. In contrast, it is noteworthy that mutant A438E and V439L of TMABaldehyde DH II exhibited kinetic properties with a significant improvement in the affinity (K_m values lower than in the wild-type), pointed the electrostatic interactions with other residues present near/in the pocket which would enhance the affinity. The data presented here are consistent with the conclusion that the carboxylate residue Asp457 and Gln437 at the entrance to the substrate channel are essential for both the activity and substrate affinity of TMABaldehyde DHs, whereas Glu458, Leu459, Ala438, and Val439 localized near surrounding the entrance, are important mainly for the catalytic rate of the enzymes. Conclusively, the selected residues especially Asp and Gln at the substrate binding site of *Pseudomonas* sp. 13CM TMABaldehyde DHs are important for binding and formation of successive products. Nevertheless, improving affinity and changing the specificity probably will require substitutions of several amino acid residues.

Structural modeling of the enzyme from *Pseudomonas* sp. 13CM and site-directed mutagenesis experiments explore understanding the role of critical residues and the mechanism of interaction between substrate and enzyme. All of the results presented here provide experimental evidence that both substrates preference and binding affinity are very complex in the *Pseudomonas* sp. 13CM TMABaldehyde DHs. However, the observed kinetic differences provide new insights into carnitine biosynthetic enzyme at the atomic level.

GENERAL CONCLUSIONS

A great number of quaternary ammonium compounds (QACs) present in the nature including well-known representatives such as choline, glycine betaine, and L-carnitine which have different biological functions; for example, adaptation of organisms to environmental stress and transportation of chemical groups in many metabolic processes. QACs have a wide-range of commercial and consumer uses such as detergents, antistatics, wetting and softening agents, biocides, germicides, deodorizers, wood preservatives and emulsifiers. In percentage, this utilization translates into mostly fabric softeners (66%), coated clays (16%), and biocides (8%). After use, the residual product is discharged to sewage treatment plants or surface waters and finally to coastal waters, raising a great concern world-wide. Released into the environment, the biodegradability of QACs is limited by their antimicrobial activity. Each of the QACs has its own chemical and anti-microbiological characteristics. However, some microorganisms are able to demonstrate intrinsic resistance through inactivation of the biocide, and this will assist in the removal of such agents from the environment including *Pseudomonas* sp.

Regarding the global importance of QACs microbial degradation and metabolism of choline, L-carnitine, D-carnitine, 4-*N*-trimethylaminobutanol (TMA-Butanol), and Homocholine have been well investigated in the Laboratory of Microbial Biotechnology, Faculty of Agriculture, Tottori University, Japan. To elucidate the structure and function of TMA-Butanol (considerable structural resemblance to choline as a theme), a microorganism from soil have been identified as *Pseudomonas* sp. 13CM, grown on TMA-Butanol, and the enzyme 4-*N*-trimethylaminobutanol dehydrogenase (TMA-Butanol DH) purified to apparent homogeneity. The isolated enzyme converts TMA-Butanol into 4-*N*-trimethylaminobutyraldehyde (TMABaldehyde). Additionally, 4-*N*-trimethylaminobutyraldehyde dehydrgenase (TMABaldehyde DH), isolated from the same organism, oxidized TMABaldehyde to yield γ-butyrobetaine. Consistent with these observations, postulated a complete pathway of TMA-Butanol degradation. Amino

terminal sequences and some properties of these two enzymes (TMA-Butanol DH and TMABaldehyde DH) have been described in previous study. From another point of view, TMABaldehyde DH mediates the reaction in carnitine biosynthetic pathway. The enzyme TMABaldehyde DH has been purified to homogeneity from beef liver and from rat liver. However, there is no report on molecular studies of these bacterial enzymes except rat TMABaldehyde DH. Moreover, the gene encoding the bacterial carnitine biosynthetic enzyme TMABaldehyde DH and TMA-Butanol DH has so far never been over-expressed. Therefore, this research was conducted to isolate the genes from *Pseudomonas* sp. 13CM, prepare and characterize the recombinants, and to identify the critical residues for the activity of TMABaldehyde DH.

In Chapter 1, the gene encoding TMABaldehyde DH from *Pseudomonas* sp. 13CM, isolated by shotgun cloning. Sequence analysis indicated that the DNA fragment of 6,987 nucleotides included four complete ORFs. The translated ORF1 has 92% positional identity with the putative betaine aldehyde dehydrogenase of *Pseudomonas putida* KT2440 (Q88PZ0). Of the other ORFs, the encoded proteins of ORF2, ORF3, and ORF4 were, respectively, similar to the putative membrane protein (Q88PZ1), major facilitator family transporter (Q88PZ6), and the putative MFS efflux transporter (Q88PZ7). The amino acid sequence of 476 amino acids deduced from the nucleotide sequence of ORF1 was inserted into an expression vector pET24b (+) and highly expressed in *Escherichia coli* BL21 (DE3) under the control of a T7 promoter. The molecular mass of the enzyme was estimated at about 150 kDa using gel filtration approach. The enzyme was found to be a trimer with identical 52 kDa subunits by SDS–PAGE, coincided to about 51 kDa by MALDI-TOF mass spectrometry analysis. The isoelectric point was found to be 4.5. Optimum pH and temperature were found respectively as pH 9.5 and 40 °C. The K_m values for TMABaldehyde, 4-dimethylaminobutyraldehyde (DMABaldehyde), and NAD^+ were respectively, 0.31 mM, 0.62 mM, and 1.16 mM. The molecular, electrical, and catalytic properties clearly differed from those of TMABaldehyde DH I, which was discovered initially in *Pseudomonas* sp. 13CM. The new type of enzyme designated as “TMABaldehyde DH II”. The results of this work clearly indicate that the TMABaldehyde DH II is a new type

of bacterial TMABaldehyde DH. The culture supernatant of the wild strain *Pseudomonas* sp. 13CM contains dominantly TMABaldehyde DH I, and thus far it has been difficult to detect TMABaldehyde DH II during the purification process from the culture supernatant, due to its extremely low content. Under such circumstances, the molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the discovery of TMABaldehyde DH II followed by production of a large quantity of the enzyme in a recombinant *E. coli* strain.

In Chapter 2, sequencing of the genes encoding TMABaldehyde DH I and TMA-Butanol DH from *Pseudomonas* sp. 13CM by general cloning technique i.e., gene amplification by PCR using the primers designed from the N-terminal sequences of the proteins and from the conserved sequences in closely-related proteins have been described. The inverse PCR was used to determine the nucleotide sequence of the regions upstream and downstream of the TMABaldehyde DH I and TMA-Butanol DH gene, designed the primers based on inverted repeats border sequences. Assembly of the overlapping sequence fragments allowed reconstituting two open reading frames, and coding for amino acid residue polypeptide. The deduced sequences have shown the complete identity with the previously reported respective N-terminus. Database analysis revealed that the primary structure of protein encoding TMABaldehyde DH I and TMA-Butanol DH have shown high similarity with aldehyde dehydrogenase (94%) and iron containing alcohol dehydrogenase (87%) of *P. putida* GB-1. The chapter also covers the preparation of recombinant TMABaldehyde DH I and TMA-Butanol DH over-expressed in *E. coli*. The enzymological properties of over-expressed recombinant TMABaldehyde DH I were also figure out in this chapter. The enzyme had a molecular mass estimated about 160 kDa by gel filtration, trimer with identical 50 kDa subunits. Optimum pH and temperature were found respectively, pH 9.5 and 50 ºC. The K_m values for TMABaldehyde, DMABaldehyde, and NAD^+ were respectively, 0.12 mM, 0.07 mM, and 0.15 mM. Characterization of purified recombinant TMABaldehyde DH I, confirmed the enzyme had essentially the same properties those of TMABaldehyde DH I which was discovered initially in *Pseudomonas* sp. 13CM. Molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the production of a large quantity of the

enzyme in a recombinant strain. The recombinant TMABaldehyde DH I from *Pseudomonas* sp. 13CM can now be obtained in large quantity necessary for further biochemical characterization and possible applications.

The study in Chapter 3 was attempted to investigate the several critical residues located in the portion of the substrate binding pocket of TMABaldehyde DHs (TMABaldehyde DH I and TMABaldehyde DH II) from *Pseudomonas* sp. 13CM, site-directed mutagenesis have been carried out. Genes encoding TMABaldehyde DH I and TMABaldehyde DH II were comprised of respectively, 1488 and 1431 nucleotides, which encoded the proteins comprising of respectively, 496 and 476 amino acids. Amino acid sequences from TMABaldehyde DH I and II exhibited high homology with respectively *P*. *putida* GB-1 putative aldehyde dehydrogenase and *P*. *putida* KT2440 putative betaine aldehyde dehydrogenase. Unfortunately, the crystal structures of TMABaldehyde DH and from these two microorganisms were not available. Therefore, a theoretical structural model was constructed by comparing the sequences of *E. coli* YdcW betain aldehyde dehydrogenase (PDB code 1WNB) whose crystal structure was available, showed 38% identity with TMABaldehyde DHs in average. Based on the homology model and alignment the residues Asp457, Glu458, and Leu459 in TMABaldehyde DH I; and corresponding positioned residues such as Gln437, Ala438, and Val 439 in TMABladehyde-DH II at the entrance to the substrate channel (less than 10 Å distances from the substrate binding site) were hypothesized to be important for the high-affinity binding of substrates to TMABaldehyde DHs for the catalytic function of the enzyme. For the investigation, the residues of TMABaldehyde DH I were replaced with that of the corresponding positioned residues of TMABaldehyde DH II and vice-versa. All of the mutants versions exhibited a substantial decrease in activity along with increase of K_m displayed by TMABaldehyde DH I mutants both for substrate and cofactor. In contrast, it is noteworthy that mutant A438E and V439L of TMABaldehyde DH II exhibited kinetic properties with a significant improvement in the affinity (K_m values lower than in the wild-type), pointed the electrostatic interactions with other residues present near/in the pocket which would enhance the affinity. The data presented here are consistent with the conclusion that the carboxylate residue Asp457 and Gln437

at the entrance to the substrate channel are essential for both the activity and substrate affinity of TMABaldehyde DHs, whereas Glu458, Leu459, Ala438, and Val439 localized near surrounding the entrance, are important mainly for the catalytic rate of the enzymes. Conclusively, the selected residues especially Asp and Gln at the substrate binding site of *Pseudomonas* sp. 13CM TMABaldehyde DHs are important for binding and formation of successive products. Nevertheless, improving affinity and changing the specificity probably will require substitutions of several amino acid residues. Structural modeling and site-directed mutagenesis experiments explore understanding the role of critical residues and the mechanism of interaction between substrate and enzyme. All of the results presented here provide experimental evidence that both substrates preference and binding affinity are very complex in the *Pseudomonas* sp. 13CM TMABaldehyde DHs. However, the observed kinetic differences provide new insights into carnitine biosynthetic enzyme at the atomic level.

Generally speaking, this study provides basic molecular information on the enzymes TMABaldehyde DH and TMA-Butanol DH involved in the microbial degradation pathway of TMA-Butanol and discussed the biochemical properties of the over-expressed TMABaldehyde DHs as well as constructed mutants. This information is important in order to explore these metabolites and enzymes in biotechnology. Further research will be focused on total refinement of these enzymes and discuss the nature by crystallizations, and their possible applications.

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ABSTRACT

The present study was aimed, molecular investigation of trimethylaminobutanol dehydrogenase (TMA-Butanol DH) and trimethylaminobutyraldehyde dehydrogenase (TMABaldehyde DH) produced by *Pseudomonas* sp. 13CM. Shotgun cloning discovered a new enzyme designated TMABaldehyde DH II has been discussed. Genes encoding the enzymes involved in TMA-Butanol DH degradation pathway have been cloned and sequenced. The recombinant enzymes were over-expressed in *Escherichia coli* and properties of recombinant TMABaldehyde DHs were investigated. Apart from the characterization of TMABaldehyde DHs, several critical residues are investigated.

The introductory part defines the background of this study and set the aim. The review of literature section provides the selective reviews relevant to this work.

In Chapter 1, the gene encoding TMABaldehyde DH from *Pseudomonas* sp. 13CM was isolated by shotgun cloning and expressed in *E. coli* DH5α. Sequence analysis indicated that the DNA fragment of 6,987 bp included four complete Open Reading Frames (ORFs). The translated ORF1 has 92% positional identity with the putative betaine aldehyde dehydrogenase of *Pseudomonas putida* KT2440 (Q88PZ0). Of the other ORFs, the encoded proteins of ORF2, ORF3, and ORF4 were, respectively, similar to the putative membrane protein (Q88PZ1), major facilitator family transporter (Q88PZ6), and the putative MFS efflux transporter (Q88PZ7). The structural gene encoding TMABaldehyde DH inserted into an expression vector pET24b (+) and transformed to *E. coli* BL21 (DE3). Over-expressed enzyme was purified to apperant homogeneity and biochemically characterized. The molecular mass of the enzyme was estimated at about 150 kDa using gel filtration. The enzyme was found to be a trimer with identical 52 kDa subunits by SDS−PAGE, and about 51 kDa by MALDI-TOF mass spectrometry analysis. The isoelectric point was found to be 4.5. Optimum pH and temperature were found, respectively, pH 9.5 and 40 °C. The K_m values for 4-*N*-trimethylaminobutyraldehyde (TMABaldehyde), 4-dimethylaminobutyraldehyde (DMABaldehyde), and NAD^+ were 0.31 mM, 0.62 mM, and 1.16 mM, respectively. It

was observed that the molecular and catalytic properties clearly differed from those of TMABaldehyde DH I, which was discovered initially in *Pseudomonas* sp. 13CM and renamed the obtained second enzyme "TMABaldehyde DH II". Discovered TMABaldehyde DH can now be obtained in large quantity necessary for further biochemical characterization and possible applications.

In Chapter 2, genes encoding TMABaldehyde DH I and TMA-Butanol DH produced by *Pseudomonas* sp. 13CM were identified. Oligonucleotidic probes were designed corresponding to the N-terminal amino acid sequences of the both enzymes and the conserved sequences in aldehyde and alcohol dehydrogenases. DNA fragment containing the core regions were obtained by PCR. To get the unknown part of the gene, inverse-PCR primers were synthesized using the known region in the genomic DNA. The sequences achieved from the inverse-PCR approach, connecting the flanking region and core region led the construct of full sequences of the genes encoding TMABaldehyde DH I and TMA-Butanol DH. The primary structures of TMABaldehyde DH I and TMA-Butanol DH showed high identities with, respectively, aldehyde dehydrogenase (94%) and iron containing alcohol dehydrogenase (87%) of *P. putida* GB-1. The section also covers the over-expression of these enzymes in *E. coli* and properties of recombinant TMABaldehyde DH I. The enzyme had a molecular mass about 160 kDa by gel filtration, trimer with identical 50 kDa subunits. Optimum pH and temperature were found, respectively, pH 9.5 and 50 ºC. The kinetic analysis showed the K_m values for TMABaldehyde, DMABaldehyde, and NAD^+ were 0.12 mM, 0.07 mM, and 0.15 mM, respectively. Characterization of purified recombinant TMABaldehyde DH I, confirmed that the enzyme had essentially the same properties to those of TMABaldehyde DH I, discovered initially in *Pseudomonas* sp. 13CM. Molecular cloning with *Pseudomonas* sp. 13CM as a gene donor led to the production of a large quantity of the enzyme in a recombinant strain.

The study in Chapter 3 was attempted to investigate the several critical residues located in the portion of the substrate binding pocket of TMABaldehyde DHs (TMABaldehyde DH I and TMABaldehyde DH II) of *Pseudomonas* sp. 13CM. Based

on the homology model and alignment, clear difference in residues of TMABaldehyde DH I and TMABaldehyde DH II were found around the entrance to the substrate channel (the residues Asp457, Glu458, and Leu459 in TMABaldehyde DH I; and corresponding positioned residues Gln437, Ala438, and Val 439 in TMABladehyde-DH II). Site-specific mutagenesis was conducted with the replacement of selected residues of one enzyme with that of the corresponding positioned residues of another, and vice-versa. Concerning the catalytic properties towards substrates, all of the mutants exhibited a substantial decrease in activity. Additionally, TMABaldehyde DH I mutants displayed a consistent increase of K_m both for substrate and cofactor. In contrast, mutant A438E and V439L of TMABaldehyde DH II exhibited kinetic properties with a significant improvement in the affinity. Because Q437D mutant of TMABaldehyde DH II exhibited extremely low level of activity, the Gln residue and its corresponding residue of *Pseudomonas* sp. 13CM TMABaldehyde DHs are important for binding and formation of successive products. The reported data represent a valuable resource to understand the relationship between structure and function of TMABaldehyde DHs.

摘 要

本研究は *Pseudomonas* sp. 13CM が生産するトリメチルアミノブタノール脱水素酵素（TMAB 脱水素酵素）、トリメチルアミノブチルアルデヒド脱水素酵素（TMAB-アルデヒド脱水素酵素）分子に関する研究である。両酵素遺伝子をクローン化し、その塩基配列を解読した。組換え型酵素を大腸菌で発現させ、組換え型酵素の性質を検討した。新たに見いだしたトリメチルアミノブチルアルデヒド脱水素酵素（トリメチルアミノブチルアルデヒド脱水素酵素 II）についても述べられている。２種類のアルデヒド脱水素酵素の性質検討に加え、両酵素の重要なアミノ酸残基についても検討されている。

序章では本研究の背景と目的が述べられている。さらに、本研究に関係のある化合物の一般的な性質が述べられている。

第１章では酵素活性を指標としたショットガンクローニング法で見出されたトリメチルアミノブチルアルデヒド脱水素酵素の全塩基配列決定と pET24b(+)を用いた大量発現及び酵素の精製と諸性質が述べられている．酵素活性を指標としたショットガンクローニング法により，約 7.2kbp の DNA 断片（pAN1）が得られた．pAN1 の全塩基配列（6,987 bp）を決定し，解析したところ，4 個の ORF が確認できた．このうち，ORF1 は Pseudomonas putida KT2440 の推定上のベタインアルデヒド脱水素酵素(Q88PZ0)と 92％の相同性を示した．その他の 3 個の ORF は「the putative membrane protein (Q88PZ1) 」，「major facilitator family transporter (Q88PZ6) 」，「the putative MFS efflux transporter (Q88PZ7) 」であった．TMAB-アルデヒド脱水素酵素遺伝子を pET24b(+)に挿入し，大腸菌 BL21(DE3)株に形質転換した．大領発現した本酵素を精製し，諸性質を検討した．本酵素の分子質量はゲル濾過法で 150kDa と算出された．SDS-PAGE 法から，本酵素はサブユニットの分子質量 52kDa の三量体であった．また，MALDI-TOF MS 法では 51kDa であった．また，本酵素の等電点は 4.5 であった．至適 pH 及び至適温度はそれぞれ 9.5 と 40℃であった．トリメチルアミノブチルアルデヒド，ジメチルアミノブチルアルデヒド及び NAD に対する Km 値はそれぞれ 0.31 mM, 0.62 mM, 1.16 mM であった．本酵素は以前に報告されていたトリメチルアミノブチルアルデヒド脱水素酵素（トリメチルアミノブチルアルデヒド脱水素酵素 I）とは分子量や基質に対する Km 値などで違いが見られた．新たに見出されたアルデヒド脱水素酵素をトリメチルアミノブチルアルデヒド脱水素酵素 II と命名した．

第２章では *Pseudomonas* sp. 13CM が生産するトリメチルアミノブタノール脱水素酵素とトリメチルアミノブチルアルデヒド脱水素酵素の両遺伝子のクローニングと塩基

配列の解読について述べられている。トリメチルアミノブタノール脱水素酵素とトリメチルアミノブチルアルデヒド脱水素酵素の N-末端アミノ酸配列とデータベース上のアルコール脱水素酵素、アルデヒド脱水素酵素に保存されているアミノ酸配列からオリゴヌクレオチドプローブを設計し，PCR 法により、DNA 断片を増幅した。さらに，インバース PCR 法により両脱水素酵素遺伝子の全長塩基配列を決定した．両酵素のアミノ酸配列のホモロジー検索を行ったところ，*Pseudomonas putida* GB-1 の推定上のアルデヒド脱水素酵素及び鉄含有アルコール脱水素酵素とそれぞれ 94%，87%のホモロジーであった．さらにこの章では両酵素の大量発現と組換え型トリメチルアミノブチルアルデヒド脱水素酵素の性質検討も述べられている．アルデヒド脱水素酵素の分子質量はゲル濾過法で 160kDa と算出され，SDS-PAGE 法から，本酵素はサブユニットの分子質量 50kDa の三量体であった．至適 pH および至適温度は pH 9.5，50℃であった．トリメチルアミノブチルアルデヒド，ジメチルアミノブチルアルデヒド及び NAD に対する Km 値はそれぞれ 0.12 mM，0.07 mM，0.15 mM であった．組換え型トリメチルアミノブチルアルデヒド脱水素酵素の性質は *Pseudomonas* sp. 13CM から精製した酵素と同じ性質を示した．

第 3 章ではトリメチルアミノブチルアルデヒド脱水素酵素 I 及び II の基質結合ポケット付近に存在する重要なアミノ酸残基について検討した．ホモロジーモデリングとアミノ酸配列の比較により，基質通路の入口付近のアミノ酸配列に違い（アルデヒド脱水素酵素 I では Asp457，Glu458，Leu459，アルデヒド脱水素酵素 II では Gln437，Ala438，Val439）が見られた．部位特異的変異法により，両酵素の 3 個のアミノ酸残基のペプチドを互いに入れ替えた変異酵素を作成した．種々の基質に対する性質を検討したところ，ほとんど全ての変異酵素はその活性が低下していた．さらに，アルデヒド脱水素酵素 I 由来の変異酵素は基質，補酵素に対する Km 値が増加していた．一方，アルデヒド脱水素酵素 II の 2 個の変異酵素（A438E， V439L）は基質に対する親和性が増大した．アルデヒド脱水素酵素 II の Q437D の変異酵素の活性が非常に低下したことから，アルデヒド脱水素酵素 II の Q437 残基とアルデヒド脱水素酵素 I の D457 残基は基質の結合と生成物の生成に重要な役割を果たしていることが示唆された．

本研究によりアルデヒド脱水素酵素の構造と機能の相関に係る重要な情報が得られた．

LIST OF PUBLICATIONS

(1) Md. Rezaul Bari, Maizom Hassan, Naoki Akai, Jiro Arima and Nobuhiro Mori, Gene Cloning and Biochemical Characterization of 4-*N*-trimethylaminobutyraldehyde Dehydrogenase II from *Pseudomonas* sp. 13CM. *World Journal of Microbiology and Biotechnology*, DOI: 10.1007/s11274-012-1224-x

This paper covers **Chapter 1** in the thesis.

(2) Md. Rezaul Bari, Naoki Akai, Jiro Arima and Nobuhiro Mori, Evaluation of Genes Encoding 4-*N*-Trimethylaminobutyraldehyde Dehydrogenase and 4-*N*-Trimethylamino-1-butanol Dehydrogenase from *Pseudomonas* sp. 13CM. *International Journal of Agriculture and Biology* (in press: 12-772/201x/00-0-000-000).

This paper partly covers **Chapter 1 and 2** in the thesis.