รายงานวิจัยฉบับสมบูรณ์

โครงการ การโคลนยีนและการเสดงออกของยีนควิเนต ดีไฮโดรจิเนส จากเชื้อ *Gluconobacter* เพื่อการผลิตสารชิคิเมต

โดย

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย

สารบัญ

Abstract

The quinate dehydrogenase (QDH) from the acetic acid bacteria, *Gluconobacter oxydans* IFO3244, exhibits high affinity towards quinate suggesting its applicable role in metabolic engineering of bacteria for shikimate production. The gene encoding QDH was cloned and sequenced. The nucleotide sequence analysis revealed a full-length *qdh* gene of 2,475 bp encoding a protein of 824 amino acids. The *qdh* gene has an unusual TTG translation initiation codon. The conserved regions and a signature sequence for quinoprotein family were clearly observed. The phylogenetic analysis demonstrated the relatedness of QDH to other bacterial quinate/shikimate dehydrogenases with highest similarity (56%) towards that of *Acinetobacter calcoaceticus* ADP1 and partial similarity (36%) of a membrane-bound glucose dehydrogenase (mGDH) of *E. coli*. The similarity hydropathy patterns between QDH and mGDH were depicted near the amino terminus. The function of the gene coding for QDH was confirmed by heterologous gene expression in a PQQ-synthesizing bacteria, *Pseudomonas putida* HK5. After the intensive investigations of gene expression conditions to suppress indigenous enzyme activity of the host cells, the recombinant *P. putida* HK5 harboring pBBR1-MCS5::*qdh* showed QDH activity (0.13 ± 0.01 μmol.min^{-1}.mg^{-1}) which was approximately 30% of that in *G. oxydans* IFO3244 wild type.

บทคัดย่อภาษาไทย

ยีนถอดรหัสให้เอนไซม์ควิโนโปรตีน ควิเนตดิไฮโดรจิเนส จากเชื้อกลูโคโนแบคเตอร์ IFO3244 เป็นเอนไซม์ที่มีความจำเพาะสูงต่อควิเนตซึ่งเป็นซับสเตรทของเอนไซม์ จึงมีศักยภาพในการใช้เพื่อทำวิศวกรรมเมแทบอลิซึมเพื่อผลิตสารชิคิเมต ยีนดังกล่าวจึงได้ถูกโคลนและหาลำดับเบสที่ถูกต้อง จากการวิเคราะห์พบว่ายีนถอดรหัสให้เอนไซม์ควิโนโปรตีน ควิเนตดิไฮโดรจิเนส นี้ประกอบด้วย 2,475 คู่เบส ซึ่งถอดรหัสให้เอนไซม์ควิโนโปรตีน ควิเนตดิไฮโดรจิเนส ที่ประกอบด้วย 824 กรดอะมิโน ยีนดังกล่าวนี้มีโคดอนเริ่มต้นคือ TTG มีบริเวณลำดับของกรดอะมิโนที่ conserve และบริเวณลำดับของกรดอะมิโนจำเพาะของเอนไซม์ในกลุ่มที่มีไพโรโลควิโนลีนควิโนนเป็นหมู่ร่วมอย่างชัดเจน ผลการวิเคราะห์ phylogenetic ชี้ให้เห็นความคล้ายคลึงของยีนนี้ในเชื้อกลูโคโนแบคเตอร์ IFO3244 และยีน quinate/shikimate dehydrogenases ในเชื้อ Acinetobacter calcoaceticus ADP1 (56%) และความคล้ายคลึงบางส่วนกับ membrane-bound glucose dehydrogenase (mGDH) ในเชื้อ *E. coli* การทดลองเพื่อยืนยันหน้าที่ของยีนดังกล่าวทำได้โดยการโคลนและแสดงออกของยีนนี้ในเชื้อ *Pseudomonas putida* HK5 พบว่าเชื้อรีคอมบิแนนท์ที่มีพลาสมิด pBBR1-MCS5::*qdh* มีเอนไซม์แอคติวิตีประมาณ 0.13 $\pm$ 0.01 μmol.min^{-1}.mg^{-1} ซึ่งคิดเป็น 30% ของเอนไซม์แอคติวิตีของเชื้อกลูโคโนแบคเตอร์ IFO3244

หน้าสรุปโครงการ (Executive Summary)

ประกอบด้วยหัวข้อ ดังต่อไปนี้

1. ชื่อโครงการ

2. ชื่อหัวหน้าโครงการ

3. สาขาวิชาที่ทำการวิจัย

4. งบประมาณทั้งโครงการ

5. ระยะเวลาดำเนินงาน

6 ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

7. วัตถุประสงค์

8. ระเบียบวิธีวิจัย

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1. ชื่อโครงการ (ภาษาไทย) การโคลนยีนและการเสดงออกของยีนควิเนต ดีไฮโดรจิเนสจากเชื้อ *Gluconobacter* เพื่อการผลิตสารชิคิเมต

(ภาษาอังกฤษ) Cloning and Expression of Quinate dehydrogenase gene from *Gluconobacter* strain for shikimate production

2. ชื่อหัวหน้าโครงการ

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3. สาขาวิชาที่ทำการวิจัย ชีวเคมี

5. ระยะเวลาดำเนินงาน

The project can be briefly divided into 4 phases including (in order): (1) Cloning of qdh gene from *Gluconobacter oxydans* IFO3244 and sequence analysis; (2) Construction of a *qdh* disruptant; (3) Transformation and examination of the suitable expression system in *G. oxydans* IFO3244; and (4) Investigate the suitable conditions for qdh gene expression in *G. oxydans* IFO3244

6 ปัญหาที่ทำการวิจัย และความสำคัญของปัญหา

Shikimate pathway is known to be an important pathway for the biosynthesis of aromatic amino acids (tyrosine, phenylalanine and tryptophan), in plants and microorganisms as well as several important industrial and pharmaceutical substances including antibiotics, for example rifamycin, ansamycin, candicidin, nocardicin, etc. Recently, shikimate has been found to be an important intermediate for the synthesis of an antiviral infection drug named Tamiflu. Shikimate is one of the key intermediates in the shikimate pathway and is also one of the major branch-points to the production of various industrial important end products. According to the metabolic pathways for shikimate biosynthesis, shikimate is derived remotely from glucose, an initial substrate. In order to yield shikimate and, subsequently other intermediates in the shikimate pathway, two different metabolic pathways have to merge together, i.e. phospho-enolpyruvate derived from glycolytic pathway and erythrose-4-phosphate originated from pentose phosphate pathway. Therefore, it is difficult to control and genetically improve the production of shikimate as well as other intermediates in the shikimate pathway from these biosynthesis routes. Attempts for metabolic engineering of microorganism, i.e. *E.coli* strain, for shikimate production have been carried out and still in progress. However, there are some major drawbacks in that metabolic engineering routes. Therefore, effective method for the production of shikimate as well as the intermediates in the shikimate pathway is necessary and yet remained to be developed.

A novel and effective method for enzymatic production of shikimate and other intermediates in the shikimate pathway has been proposed and recently demonstrated by the oxidative fermentation of acetic acid bacteria using quinate as the starting substrate. The rationale of this enzymatic method is based on that quinate metabolic pathway and shikimate pathway share two common intermediates, 3-dehydroquinate (DQ) and 3-

dehydroshikimate (DS), which are precursors for shikimate. In addition, quinate can be found and extracted from barks and leaves of cinchona, a local plant in South Asian countries. Therefore, it is encouraging to use quinate as the starting substrate for shikimate production. With quinate metabolic route, the first enzyme involved in a uni-directional one-step oxidation of quinate to DQ is the membrane-bound quinate dehydrogenase (QDH). DQ is then found to accumulate in the culture medium and further oxidized to yield DS, then shikimate, respectively. While the oxidative products from the quinate oxidation of acetic acid bacteria can be collected, the oxidative intermediates are not accumulated in other bacteria such as *Acinetobacter* and *Pseudomonas*, and, in fact, they are rapidly catabolized through protocatechuate pathway. Therefore, acetic acid bacteria would be usefully exploited to produce the intermediates in the shikimate pathway.

In order to develop the production route of shikimate and other intermediates in the shikimate pathway, quinate dehydrogenase (QDH), the first enzyme accessing to the metabolic pathway from quinate has been characterized (8). Among acetic acid bacteria, QDH activity was reported in *Gluconobacter melanogenus* IFO3294, *Gluconobacter oxydans* IFO3292, with the highest QDH activity found in *Gluconobacter oxydans* IFO3244. Therefore, the expression conditions to improve QDH activity in *Gluconobacter oxydans* IFO3244 has been studied. Interestingly, the information obtained illustrated that the induction of quinate dehydrogenase in each strain of *Gluconobacter* is different depending on types of carbon source and type of the inducer used. In addition, it has been shown that either quinate, shikimate or protocatechuate could be a good inducer for quinate dehydrogenase. According to the previous study of the purification and characterization of QDH from *Gluconobacter oxydans* IFO3244, QDH from this strain exhibits higher affinity and higher specific activity towards quinate than those of QDH purified from *Acinetobacter* sp. strain SA1. These results indicate that QDH from *Gluconobacter oxydans* IFO3244 is the most appropriate enzyme in order to drive the quinate oxidation leading to shikimate production. Since the purified QDH exhibits excellent and desirable enzyme properties in term of high specific activity as well as high affinity towards quinate and low affinity towards shikimate, it is an appropriate enzyme for the conversion of quinate to dehydroquinate leading to shikimate pathway. Therefore, in order to further study for metabolic engineering of *Gluconobacter oxydans* IFO3244 for shikimate production, gene coding for quinate dehydrogenase (*qdh* gene) will be cloned and expression.

The significance of this research project is that the sequence analysis and comparison of qdh gene cloned from *Gluconobacter oxydans* IFO3244 will reveal the differences between *qdh* gene sequence from this organism when compared to others in terms of substrate-recognition site and specifically binding region. The information obtained will be useful if the protein engineering will be attempted to improve the enzyme properties. In addition, this *qdh* gene will be cloned into the suitable expression vector for *Gluconobacter* to significantly increase the conversion of quinate to dehydroquinate in the quinate oxidation which is the first step leading to shikimate production. In addition, the result of this research will illustrate the cloning and expression of a gene coding for a membrane-bound PQQ-dependent enzyme which will also contribute to and strengthen the knowledge in the research field of protein-derived cofactor and quinone-related proteins. As the knowledge of gene encoding the periplasmic pyrroloquinoline quinone-containing proteins has been extensively studied in the recent years, the research results of this project will be a gateway for further understanding of gene-coding for a membrane-bound PQQ-containing dehydrogenases as well as the expression in acetic acid bacteria.

7. วัตถุประสงค์

7.1 To determine inducer(s) and the expression conditions for quinate dehydrogenase (QDH) activity in *Gluconobacter oxydans* IFO3244

Quinate dehydrogenase (QDH) is the first enzyme entering to the quinate utilization pathway, which leads to the biosynthesis of shikimate, and consequently the biosynthesis of other important intermediates in the shikimate pathway. Therefore, the suitable expression conditions of QDH activity in *Gluconobacter oxydans* IFO3244, carrying the highest QDH activity detected so far (Adachi et al. 2003a), will be examined. The suitable and specific inducer(s) for QDH activity will be identified for further development and/or scaling-up of the production of shikimate.

7.2 To establish the solubilization protocols to remove the contaminated enzymes (such as a PQQ-containing alcohol dehydrogenase and a PQQ-containing glucose dehydrogenase) having structural similarities to QDH and to facilitate the purification of quinate dehydrogenase.

The purification of QDH from *Gluconobacter* strain have been attempted since the importance has been recognized. However, all attempts were failed due to high

contamination of other structural-related and/or property-resembling proteins usually found in *Gluconobacter* strains, for example a membrane-bound glucose dehydrogenase and alcohol dehydrogenase. The solubilization protocol will be established prior to the purification of QDH of *Gluconobacter oxydans* IFO3244 in order to facilitate the removal of contaminated proteins. The solubilization protocol developed from this research project will be useful as a solubilization guideline in order to obtain other membrane-bound dehydrogenases in *Gluconobacter* strains, which have not yet been characterized (Deppenmeier et al. 2002).

7.3 To purify of QDH and to characterize QDH properties involving quinate oxidation in *Gluconobacter oxydans* IFO3244.

According to the previous reports, quinoprotein quinate dehydrogenase (QDH) in acetic acid bacteria (*Gluconobacter* strains) leads to the production of 3-dehydroquinate (DQ) by oxidative fermentation and, subsequently the production of shikimate which can be further develop to an industrial scale (Adachi *et al*. Japanese Patent Application 2001, 267257). The development of shikimate biosynthesis using quinate as a substrate will also lead to the revival of cinchona agriculture in Asian countries.

Therefore, to develop the production route of shikimate through quinate oxidation, it is necessary to understand the suitable working conditions for quinate dehydrogenase. QDH of *Gluconobacter oxydans* IFO3244 is the most promising useful enzyme due to its highest activity. Therefore, the optimum oxidizing conditions including the kinetics information of the enzyme from this organism must be fully understood. Accordingly, the purification of QDH is necessary. A purified QDH will be further characterized to understand its biochemical characteristics, for example, the activation and optimum working conditions as well as its stability.

The biochemical characteristic and kinetics information acquired from this PQQ-containing enzyme will also be academically useful in the research field of protein-derived cofactor and quinone-related proteins.

8. ระเบียบวิธีวิจัย

8.1 Cloning and sequence analysis of qdh gene from Gluconobacter oxydans IFO3244.

Gene encoding quinate dehydrogenase has been previously reported from *Acinetobacter calcoaceticus* (1) and *Xanthomonas campestris* (3). However, the enzyme

reported in *Acinetobacter* strain exhibits low affinity towards quinate while having high affinity towards shikimate. Thus, the information of the sequence differences responsible for these distinguish properties would be important information.

Experimental approach: Quinate dehydrogenase from *G. oxydans* IFO3244 is a PQQ-dependent enzyme which has been purified and characterized (8). In order to clone qdh gene, the alignment of gene sequences encoding qdh gene from other organisms will be performed. Since in *Gluconobacter* strain, there are several PQQ-containing enzymes, therefore, in order to design the specific primers for PCR amplification of qdh gene and to avoid mispriming to other genes encoding PQQ-enzymes, the primers will be designed based on not only the conserved regions, but also based on the deduced N-terminal amino acid sequence obtained from the analysis of the purified enzyme. The PCR product will then be subcloned into pGEM-T Easy vector (Promega, USA). The PCR product will be then used as a probe for Southern hybridization. Gene walking as well as inverse PCR technique will be attempted in order to obtain the full sequence of *qdh* gene. The sequence analysis and comparison will be performed thoroughly.

8.2 *qdh* Gene disruption to confirm a sole involvement the acquiring gene in quinate oxidation in *G. oxydans* IFO3244

Experimental approach: A gene disruption by antibiotic cassette insertion will be performed to generate a double-crossover Gluconobacter disruptant. QDH activity will be examined from the membrane fraction of the disruptant compared to that of the wildtype strain.

8.3 Subcloning of *qdh* gene into the expression vector and determination of the heterologous gene expression

Experimental approach: Genes encoding QDH which has been individually cloned into pGEM-T Easy vector will be then subcloned into the expression vector; tentatively pUC119 (Takara Bio Inc., Japan). The expression level of *qdh* gene in the expression vector will then be determined.

8.4 Determination of suitable expression vector for *qdh* gene in *Gluconobacter* and determination of the gene expression in *G. oxydans* IFO3244

Experimental approach: Three expression vectors, which have been previously developed for Gram negative bacteria include pSA19 (developed for *Acetobacter* strain;

unpublished), pSG8 (7), and pCM62 (4), will be examined. The expression level of qdh gene on each of these vectors will be tested and compared when transformed in to *Gluconobacter* host strain.

เนื้อหางานวิจัย

Cloning and Expression of Quinate dehydrogenase gene from *Gluconobacter* strain for shikimate production

1. Introduction

Shikimic acid and intermediates in the shikimate pathway can be utilized as a building block for the production of aromatic amino acids, various fine chemicals (Bongaerts et al. 2001) and pharmaceutical compounds such as the anti-influenza drug GS-4104 (Kim et al. 1997; Rohloff et al. 1998). Shikimic acid can be synthesized from glucose using genetically modified strains of *E.coli* (Yi et al. 2003). Nonetheless, the biosynthesis pathways in *E.coli* are complicated and required rigorously controlled fermentation conditions in order to minimize by-product formation (Knop et al. 2001).

Alternatively, shikimic acid and other intermediates in the pathway can be produced via quinate oxidation pathway using acetic acid bacteria, *Gluconobacter* strain (Adachi et al. 2003b). Quinate is an abundant natural product which can be oxidized by *Pseudomonas* sp. and *Acinetobacter* sp. and subsequently converted to shikimate via dehydroshikimate. The first enzymatic step in the pathway is the oxidation of quinate which is catalyzed by quinate dehydrogenase (QDH). QDH observed in Gram negative bacteria are generally known to act on either quinate or shikimate, therefore it has been named as quinate/shikimate dehydrogenase. While QDH is an NAD-dependent QDH in fungi (Ahmed and Giles 1969), *Aerobacter aerogenes* (Mitsuhashi and Davis 1954), and *Pseudomonas aeruginosa* (Ingledew and Tai 1972), it is an NAD(P)-independent QDH (EC 1.1.99.25) in *Acinetobacter calcoaceticus* (Tresguerres et al. 1970), and acetic acid bacteria (Whiting and Coggins 1967). Purification and characterization of a membrane-bound QDH from *A. calcoaceticus* SA1 (Adachi et al. 2003b) and *Gluconobacter oxydans* IFO3244 (Vangnai et al. 2004) confirmed the involvement of pyrroloquinoline quinone (PQQ) as an enzyme cofactor. It also revealed that QDH of *A. calcoaceticus* SA1 exhibited similar substrate affinity towards quinate and shikimate having an apparent K_m of 0.20 and 0.26 mM, respectively. On the other hand, QDH from *G. oxydans* IFO3244 showed higher substrate affinity towards quinate than shikimate having an apparent K_m of 0.19 and 29.7 mM, respectively, suggesting that the enzyme from *G. oxydans* IFO3244 is more applicable for a metabolic engineering of bacteria for shikimate production. Therefore, it is important to perform further genetic investigation of genes involved in

quinate oxidation pathway, *i.e*. genes coding for quinate dehydrogenase, dehydroquinate dehydratase and shikimate dehydrogenase in *G. oxydans* IFO3244.

The present paper reports the molecular cloning and characterization of the gene encoding quinate dehydrogenase from *G. oxydans* IFO3244. The primary structure of the deduced amino acids was analyzed and compared with known QDH sequences. Heterologous expression of QDH was carried out to illustrate the completeness of gene sequence and its function.

2. Materials and methods

Bacterial strains, Plasmids, and Cultivation - The bacterial strains and plasmids used in this study are listed in Table 1. The following media were used to cultivate *E. coli* (37^oC) (Hanahan 1983) and *P. putida* HK5 (30^oC) cells, *i.e.* LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl (w/v), pH 7.2), the basal medium (0.2% $NaNO_3$, 0.2% $(NH_4)SO_4$, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4.7H_2O$, and 0.05% yeast extract (w/v), pH 7.0) (Toyama et al. 1995). Growth of *Gluconobacter* was according to Vangnai et al. (2004) (Vangnai et al. 2004). As an inducer, quinate (0.2%, w/v) was provided in the medium. In the presence of plasmid, an appropriate antibiotic was added to the medium to the final concentration of 50 $\mu g.ml^{-1}$, *e.g.* ampicillin (Ap), and gentamycin (Gm).

DNA isolation, Cloning and DNA sequencing - Extraction of *G. oxydans* IFO3244 genomic DNA and routine molecular techniques were performed as outlined by Sambrook and Russell (Sambrook and Russell 2001). Plasmids were prepared using the QIAprep Spin Miniprep kit (Qiagen, Oslo, Norway). DNA fragments were purified from agarose gels with the QIAquick Gel Extraction kit (Qiagen, Oslo, Norway). Restriction enzymes were purchased from NBI Fermentas (Vilnius, Lithuania) and TOYOBO (Japan). DNA sequence analyses were performed by Macrogen Inc. (Seoul, Korea).

PCR amplification, Southern hybridization, Inverse PCR and Gene walking - *G. oxydans* IFO3244 genomic DNA was used as a template for PCR amplification of *qdh*. PCR primers were shown in Table 2. For Southern analysis, the genomic DNA was digested for 10 h with a restriction enzyme, *i.e. Eco*RI, *Pst*I, *Hind*III, *Bam*HI, *Xho*I, *Kpn*I, *Sal*I, *Nar*I, and *Sac*I. The hydrolyzed DNA was separated by agarose gel electrophoresis and transferred onto a Hybond N^+ membrane (Amersham BioSciences) by capillary

blotting and was fixed by UV cross-link (Sambrook and Russell 2001). A 1,083-bp hybridization probe was PCR-amplified using the primers 231QF and 592QR. The probe labeling, hybridization, washes and detection were carried out using the ECL direct nucleotide labeling system (Amersham BioSciences) according to the recommendations of the manufacture. Subsequently, PCR primers for inverse PCR were designed from the known sequence of *G. oxydans* IFO3244 *qdh* gene fragments. Inverse PCR and gene walking were then performed. DNA sequences were manipulated and assembled using Sequencher (Gene Codes Corporation) to obtain a full length of *qdh* gene.

Analysis of DNA and protein sequence – Sequence similarity search and alignment were performed using BLAST at the National Center for Biotechnology Information (NCBI) and ClustalW2 (the European Bioinformatics Institute) (Thompson et al. 1994), respectively. The analysis of protein domain and phylogenetic tree construction were carried out using TreeDomViewer (Alako et al. 2006). The analysis of protein transmembrane segments was conducted using the Dense Alignment Surface (DAS) method (Cserzo et al. 1997).

Construction of pBBR1-MCS5 vector harboring qdh gene - *qdh* gene including the putative ribosome-binding site was amplified from *G. oxydans* IFO3244 chromosomal DNA using PCR primers tagged with *Sal* I restriction site (QDH-N1-*Sal* I) and *Bgl* II restriction site (QDH-C-*Bgl* II) (Table 1). The 2,536-bp PCR product was cloned into a cloning vector pGEM-T Easy (Promega). The DNA fragment was then hydrolyzed with *Sal* I and *Bgl* II and cloned into the broad-host range vector pBBR1-MCS5 (Kovach et al. 1995) where the *qdh* gene was arranged co-linear to and downstream of the *lacZ* promoter. The recombinant plasmid was transformed into *E. coli* DH5α. The recombinant colonies were selected on LB-XGal plate supplemented with 50 $\mu g.ml^{-1}$ gentamicin (Gm). The recombinant plasmid containing *qdh* gene was named pBBR1-MCS5::*qdh*. The presence of *qdh* gene was confirmed by restriction mapping and DNA sequencing. The recombinant plasmid, pBBR1-MCS5::*qdh*, was then transformed into *P. putida* HK5 by electroporation.

Heterologous expression of qdh gene in P. putida HK5 - *P. putida* HK5 harboring pBBR1-MCS5::*qdh* was cultured with orbital agitation (250 rpm) for 18 h, at

30°C, in the presence of 50 μg.ml^{-1} gentamicin in several types of medium, *i.e.* LB medium or basal medium supplemented either with 0.2% (w/v) quinate, 80 mM glucose, 80 mM fructose, or 80 mM citrate. Cell growth was interval determined using a spectrophotometer (DU800, Beckman Coulter) at 600 nm. The cells were harvested by centrifugation at 8,000 rpm for 10 min, washed and resuspended with 50 mM potassium phosphate buffer pH 7.0. After cells were disrupted using a French pressure cell, and the cell debris was removed by low-speed centrifugation, cell-free extract was used for enzyme activity assay.

Enzyme assay and Protein determination - QDH activity was determined with quinate (5 mM) as a substrate using phenazine methosulfate (PMS) and 2,6-dichlorophenol-indophenol (DCIP) as the electron acceptors (Vangnai et al. 2004). Mcllvaine buffer (pH 6.0 and pH 8.0) were used. One unit of enzyme activity is defined as the amount of enzyme catalyzing the oxidation of 1.0 μmol of substrate per min under indicated conditions. Protein concentration was measured by a modified Lowry method using bovine serum albumin as the standard protein (Dully and Grieve 1975).

3. Results and Discussion

Molecular Cloning and Sequence Analysis of the Quinate Dehydrogenase gene of G. oxydans IFO3244 – Several sets of primers were designed based on the conserved amino acid residues of gene encoding bacterial quinate dehydrogenase to amplify *qdh* gene from *G. oxydans* IFO3244. The 1,083-bp PCR product was successfully obtained when the primers 231QF and 592QR (**Fig. 1**) were used. The 1,083-bp *qdh* gene fragment was then employed as a probe for Southern hybridization in which it bound specifically to the partially digested *G. oxydans* IFO3244 genomic DNA. *Sac*I-digestion gave two DNA fragments with amenable size of approximately 4.2 kb and 4.8 kb. These SacI-digested DNA fragments were gel-extracted, re-ligated and used for inverse PCR. Two sets of primers, *i.e.* QSacI1_L1 and QSacI1_R1; QSacI2_L1 and QSacI2_R1 (**Table 1**), were used to amplify the 4.2 kb and 4.8 kb DNA fragments. After the inverse PCR, several gene walking steps and DNA fragment assembly were carried out, a full length of *qdh* gene of 2,475 bp was acquired. For further investigation, the *qdh* gene was obtained by PCR amplification from a genomic DNA of *G. oxydans* IFO3244 using a proof-reading DNA Taq polymerase enzyme and the primers (TF160 and TR3034). The complete

sequence of *qdh* gene of *G. oxydans* IFO3244 was submitted to GenBank under the accession number **EU371510**. A promoter was not clearly shown upstream of the gene, but putative sequences, *i.e.* -35 (TTCATA) and -10 (TCTCATTCT), were proposed at 173 and 153 bp upstream of the translation initiation codon of *qdh* gene, respectively. The putative Shine Dalgarno sequence of AGGAGA was observed at 12 bp upstream of an unusual TTG translation initiation codon. Although, this translation initiation codon is not common, it is not unprecedented as it has also been found in *Bacillus* sp. (Kato and Asano 2003; Zhang et al. 2006).

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Description	Source
Strains		
G. oxydans IFO3244	Wild-type strain	IFO[a]
G. suboxydans IFO12528	Wild-type strain	IFO[a]
P. putida HK5	Wild-type strain	(*15*)
E.coli DH5α	F− *endA1 hsdR*17 *recA*1 *supE*44 *thi-1* ψ80d*lacZ*ΔM15	(*14*)
Plasmids		
pGEM-T Easy	*E. coli* cloning vector; Amp^r	Promega
pSA19	*Gluconobacter* cloning vector; Amp^r	(*35*)
pSG8	*Gluconobacter* cloning vector; Amp^r	(*36*)
pCM62	Hybrid of pUC19 and pCM51 (originated from pTJS75, a small IncP plasmid)	(*37*)
pBBR1-MCS5	Broad-host-range cloning vector; Gm^r	(*20*)
pBBR1-MCS5::*qdh*	pBBR1-MCS5 subcloned with 2,536-bp *Sal*I-*Bgl*II restriction fragment of *qdh* gene	This study

[a] Institute for Fermentation, Osaka, Japan

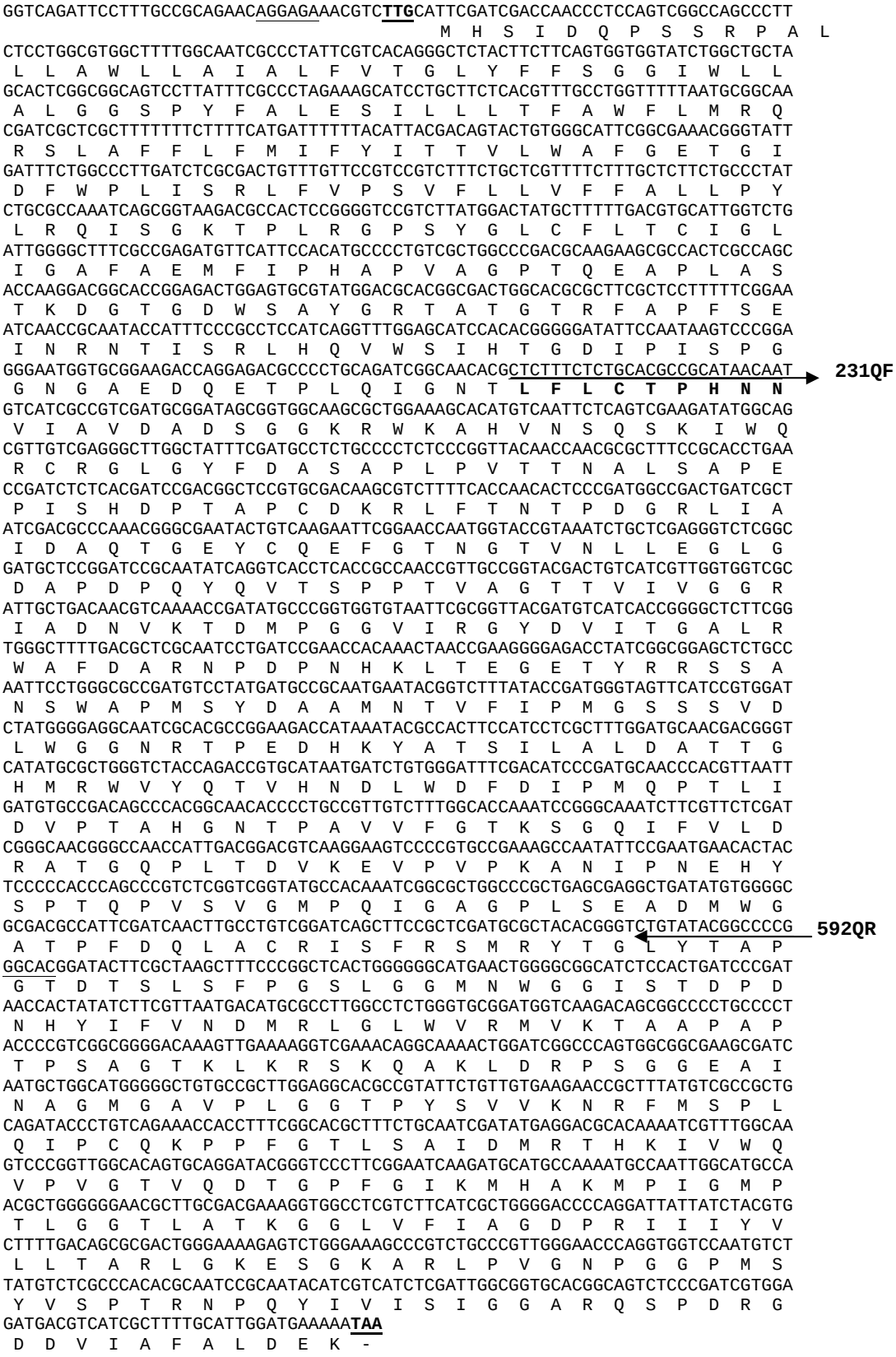


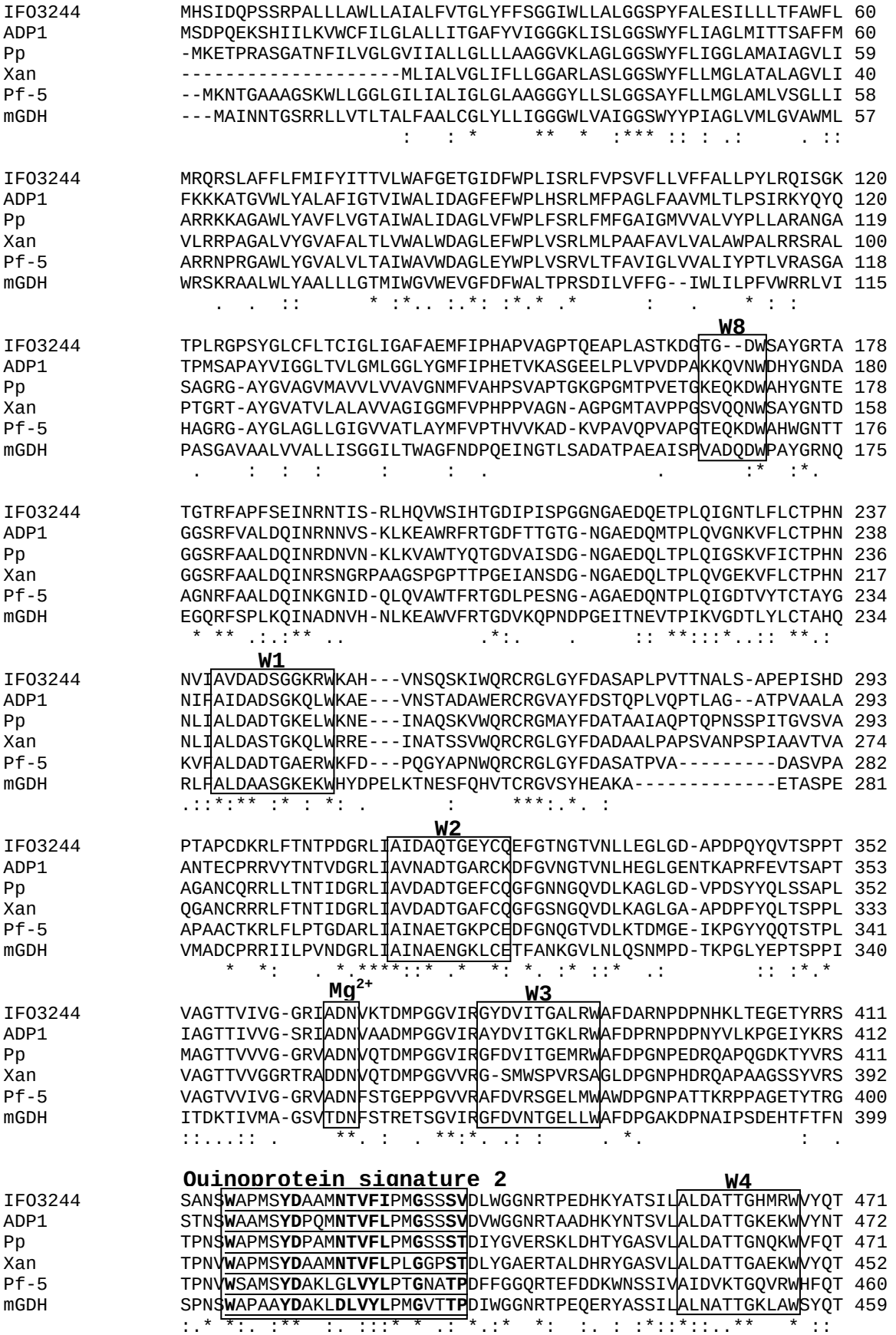
Fig. 1. **Nucleotide sequence of *Gluconobacter oxydans* IFO3244 quinate dehydrogenase and the deduced amino acid sequence of the encoded protein.** The initiation and stop codons of the gene are bolded and underlined. The putative ribosome binding site (AGGAGA) is underlined upstream of the translation starting codons. The location and sequences of the two PCR primers

(231QF and 592QR) successfully used to initially amplify the 1,083-*qdh* gene fragment are indicated *via* arrows.

The analysis of the deduced amino acid sequence of *qdh* gene (824 aa) revealed a protein molecular mass of 88.8 kDa (Gasteiger et al. 2003). This calculated molecular mass agrees with the molecular mass of the purified QDH (86 kDa) prior to proteolysis (Elsemore and Ornston 1994; Adachi et al. 2003b; Vangnai et al. 2004). The deduced amino acid residues at N-terminus (LH**SI**DQP**SS**R**PAL**) depicted partial similarity (bolded and underlined) to that of the purified enzyme previously reported (GS**SI**GEV**SSPA**G**L**) (Vangnai et al. 2004). Subsequently, the deduced amino acid sequence of *qdh* gene of *G. oxydans* IFO3244 was aligned to other quinate/shikimate dehydrogenase sequences using ClustalW2 program (Thompson et al. 1994) (**Fig. 2**). Eight significant sequence motifs, called "W-motifs" (W1-W8) found in a superbarrel structure having eight sets of four anti-parallel β-sheets of PQQ-containing quinoproteins (Toyama et al. 2004), were observed in all 6 sequences including a membrane-bound glucose dehydrogenase (mGDH) of *E. coli* (Fig.2). A putative Mg^{2+}-binding site was also noticed at the amino acid residues 365-367 (ADN) because a Mg^{2+} ion is known to be an activator for the enzyme (Vangnai et al. 2004). The QDH sequence of *G. oxydans* IFO3244 exhibited the highest similarity (55-56%) to those of *A. calcoaceticus* ADP1, *X. campestris* and *P. putida* KT2440, respectively. The analysis of protein domain using TreeDomViewer (Alako et al. 2006), a web-based tool adopting an evolutionary perspective of phylogenetic tree description with PHYLIP format (Felsenstein 2002) and a protein domain prediction, InterProScan (Quevillon et al. 2005), as well as PROSITE, a database of protein families and domains (Hulo et al. 2007), illustrated bacterial quinoprotein dehydrogenase signature 2, (W-x(4)-[YF]-D-x(3)-[DN]-[LIVMFYT]-[LIVMFY](3)-x(2)-G-x(2)-[STAG]-[PVT]) (Duine and Jongejan 1989; Gallop et al. 1989; Inoue et al. 1989; Cleton-Jansen et al. 1991), at the amino acid residues 416-437 of the QDH. The phylogenetic analysis (**Fig. 3**) also confirmed a close relatedness of QDH of *G. oxydans* IFO3244 to that of *A. calcoaceticus* ADP1. The QDH sequence analysis showed a comparatively fair similarity (36%) towards a membrane-bound glucose dehydrogenase (mGDH) of *E.coli*. This result is in agreement with a previous report (Yamada et al. 2003) in which the evolutional relation between QDH and mGDH was speculated. Moreover, the analysis of transmembrane segments using the Dense Alignment Surface (DAS) method (Cserzo et al. 1997) indicated the similarity hydropathy patterns between mGDH (Yamada et al. 2003) and QDH (**Fig. 4**) in

which five membrane-spanning regions were predicted near the amino terminus (Elsemore and Ornston 1994).

Expression of the quinate dehydrogenase gene of G. oxydans IFO3244 and Enzyme activity assay – In order to illustrate whether the gene cloned from an organism is complete and functional, there are several techniques available. Homologous or heterologous expression of the gene is one of the alternative means to confirm its function. To demonstrate the function of *qdh* gene cloned from *G. oxydans* IFO3244, the gene was cloned into several expression vectors, *i.e.* an expression vector suitable for acetic acid bacteria, pSA19 (Tonouchi et al. 1994), an expression vector suitable for *Gluconobacter*, pSG8 (Tonouchi et al. 2003), and a versatile broad-host range vector for Gram negative bacteria, pCM62 (Marx and Lidstrom 2001). The expression vector with *qdh* gene insertion was transformed back into the original host, *G. oxydans* IFO3244. However, after several transformation procedures were intensively carried out, all attempts were not accomplished.


IFO3244 MHSIDQPSSRPALLLAWLLAIALFVTGLYFFSGGIWLLALGGSPYFALESILLLTFAWFL 60
ADP1 MSDPQEKSHIILKVWCFILGLALLITGAFYVIGGGKLISLGGSWYFLIAGLMITTSAFFM 60
Pp -MKETPRASGATNFILVGLGVIIALLGLLLAAGGVKLAGLGGSWYFLIGGLAMAIAGVLI 59
Xan ----------MLIALVGLIFLLGGARLASLGGSWYFLLMGLATALAGVLI 40
Pf-5 --MKNTGAAAGSKWLLGGLGILIALIGLGLAAGGGYLLSLGGSAYFLLMGLAMLVSGLLI 58
mGDH ---MAINNTGSRRLLVTLTALFAALCGLYLLIGGGWLVAIGGSWYYPIAGLVMLGVAWML 57
MRQRSLAFFLFMIFYITTVLWAFGETGIDFWPLISRLFVPSVFLLVFFALLPYLRQISGK 120
FKKKATGVWLYALAFIGTVIWALIDAGFEFWPLHSRLMFPAGLFAAVMLTLPSIRKYQYQ 120
ARRKKAGAWLYAVFLVGTAIWALIDAGLVFWPLFSRLFMFGAIGMVVALVYPLLARANGA 119
VLRRPAGALVYGVAFALTLVWALWDAGLEFWPLVSRLMLPAAFAVLVALAWPALRRSRAL 100
ARRNPRGAWLYGVALVLTAIWAVWDAGLEYWPLVSRVLTFAVIGLVVALIYPTLVRASGA 118
WRSKRAALWLYAALLLGTMIWGVWEVGFDFWALTPRSDILVFFG--IWLILPFVWRRLVI 115
TPLRGPSYGLCFLTCIGLIGAFAEMFIPHAPVAGPTQEAPLASTKDGTG--DWSAYGRTA 178
TPMSAPAYVIGGLTVLGMLGGLYGMFIPHETVKASGEELPLVPVDPAKKQVNWDHYGNDA 180
SAGRG-AYGVAGVMAVVLVVAVGNMFVAHPSVAPTGKGPGMTPVETGKEQKDWAHYGNTE 178
PTGRT-AYGVATVLALAVVAGIGGMFVPHPPVAGN-AGPGMTAVPPGSVQQNWSAYGNTD 158
HAGRG-AYGLAGLLGIGVVATLAYMFVPTHVVKAD-KVPAVQPVAPGTEQKDWAHWGNTT 176
PASGAVAALVVALLISGGILTWAGFNDPQEINGTLSADATPAEAISPVADQDWPAYGRNQ 175
TGTRFAPFSEINRNTIS-RLHQVWSIHTGDIPISPGGNGAEDQETPLQIGNTLFLCTPHN 237
GGSRFVALDQINRNNVS-KLKEAWRFRTGDFTTGTG-NGAEDQMTPLQVGNKVFLCTPHN 238
GGSRFAALDQINRDNVN-KLKVAWTYQTGDVAISDG-NGAEDQLTPLQIGSKVFICTPHN 236
GGSRFAALDQINRSNGRPAAGSPGPTTPGEIANSDG-NGAEDQLTPLQVGEKVFLCTPHN 217
AGNRFAALDQINKGNID-QLQVAWTFRTGDLPESNG-AGAEDQNTPLQIGDTVYTCTAYG 234
EGQRFSPLKQINADNVH-NLKEAWVFRTGDVKQPNDPGEITNEVTPIKVGDTLYLCTAHQ 234
NVIAVDADSGGKRWKAH---VNSQSKIWQRCRGLGYFDASAPLPVTTNALS-APEPISHD 293
NIFAIDADSGKQLWKAE---VNSTADAWERCRGVAYFDSTQPLVQPTLAG--ATPVAALA 293
NLIALDADTGKELWKNE---INAQSKVWQRCRGMAYFDATAAIAQPTQPNSSPITGVSVA 293
NLIALDASTGKQLWRRE---INATSSVWQRCRGLGYFDADAALPAPSVANPSPIAAVTVA 274
KVFALDADTGAERWKFD---PQGYAPNWQRCRGLGYFDASATPVA---------DASVPA 282
RLFALDAASGKEKWHYDPELKTNESFQHVTCRGVSYHEAKA------------ETASPE 281
PTAPCDKRLFTNTPDGRLIAIDAQTGEYCQEFGTNGTVNLLEGLGD-APDPQYQVTSPPT 352
ANTECPRRVYTNTVDGRLIAVNADTGARCKDFGVNGTVNLHEGLGENTKAPRFEVTSAPT 353
AGANCQRRLLTNTIDGRLIAVDADTGEFCQGFGNNGQVDLKAGLGD-VPDSYYQLSSAPL 352
QGANCRRRLFTNTIDGRLIAVDADTGAFCQGFGSNGQVDLKAGLGA-APDPFYQLTSPPL 333
APAACTKRLFLPTGDARLIAINAETGKPCEDFGNQGTVDLKTDMGE-IKPGYYQQTSTPL 341
VMADCPRRIILPVNDGRLIAINAENGKLCETFANKGVLNLQSNMPD-TKPGLYEPTSPPI 340
VAGTTVIVG-GRIADNVKTDMPGGVIRGYDVITGALRWAFDARNPDPNHKLTEGETYRRS 411
IAGTTIVVG-SRIADNVAADMPGGVIRAYDVITGKLRWAFDPRNPDPNYVLKPGEIYKRS 412
MAGTTVVVG-GRVADNVQTDMPGGVIRGFDVITGEMRWAFDPGNPEDRQAPQGDKTYVRS 411
VAGTTVVGGRTRADDNVQTDMPGGVVRG-SMWSPVRSAGLDPGNPHDRQAPAAGSSYVRS 392
VAGTVVIVG-GRVADNFSTGEPPGVVRAFDVRSGELMWAWDPGNPATTKRPPAGETYTRG 400
ITDKTIVMA-GSVTDNFSTRETSGVIRGFDVNTGELLWAFDPGAKDPNAIPSDEHTFTFN 399
SANSWAPMSYDAAMNTVFIPMGSSSVDLWGGNRTPEDHKYATSILALDATTGHMRWVYQT 471
STNSWAAMSYDPQMNTVFLPMGSSSVDVWGGNRTAADHKYNTSVLALDATTGKEKWVYNT 472
TPNSWAPMSYDPAMNTVFLPMGSSSTDIYGVERSKLDHTYGASVLALDATTGNQKWVFQT 471
TPNVWAPMSYDAAMNTVFLPLGGPSTDLYGAERTALDHRYGASVLALDATTGAEKWVYQT 452
TPNVWSAMSYDAKLGLVYLPTGNATPDFFGGQRTEFDDKWNSSIVAIDVKTGQVRWHFQT 460
SPNSWAPAAYDAKLDLVYLPMGVTTPDIWGGNRTPEQERYASSILALNATTGKLAWSYQT 459
Mg2+
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W8
Quinoprotein signature 2

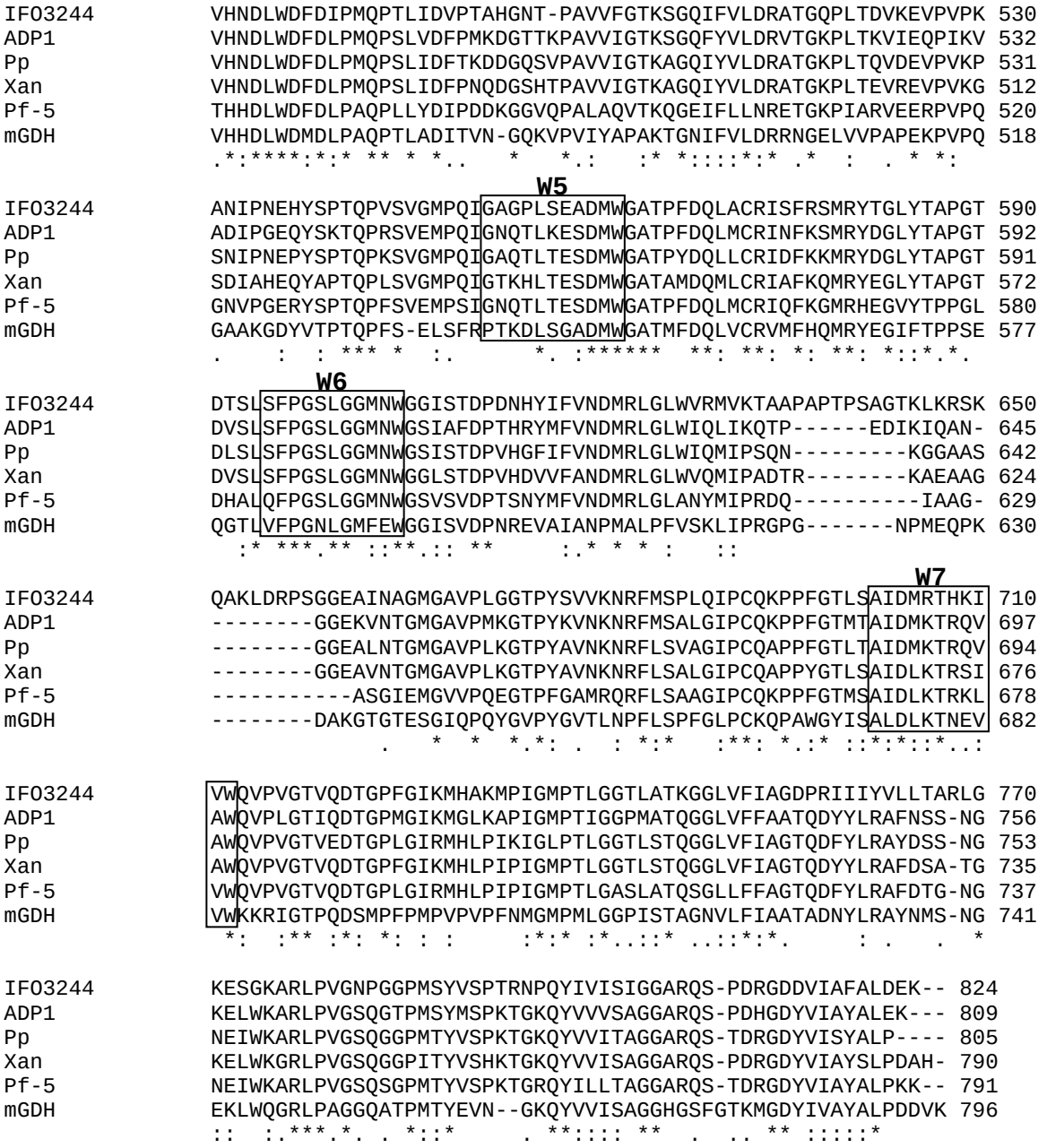

Fig. 2. Alignment of the amino acid sequence of *G. oxydans* IFO3244 QDH with other bacterial quinate/shikimate dehydrogenases. Gene sequences were from *G. oxydans* IFO3244 (IFO3244; GenBank accession no. EU371510), *Acinetobacter* sp. ADP1 (ADP1; GenBank accession no. AAC37161), *P. putida* KT2440 (Pp; GenBank accession no. NP_745706), *Xanthomonas campestris* (Xan; GenBank accession no. AAD38453), *P. fluorescens* Pf-5 (Pf-5; GenBank accession no. YP_262726) and a membrane-bound glucose dehydrogenase of *E. coli* K12 (mGDH; GenBank accession no. NP_414666). W-motifs (W1 to W8), a putative Mg^{2+}-binding site, and a quinoprotein signature sequence 2 are indicated in labeled boxes. Consensus symbols are indicated as " * " (identical residue), " : " (conserved residue with substitution), and " . " (semi-conserved residue with substitution).

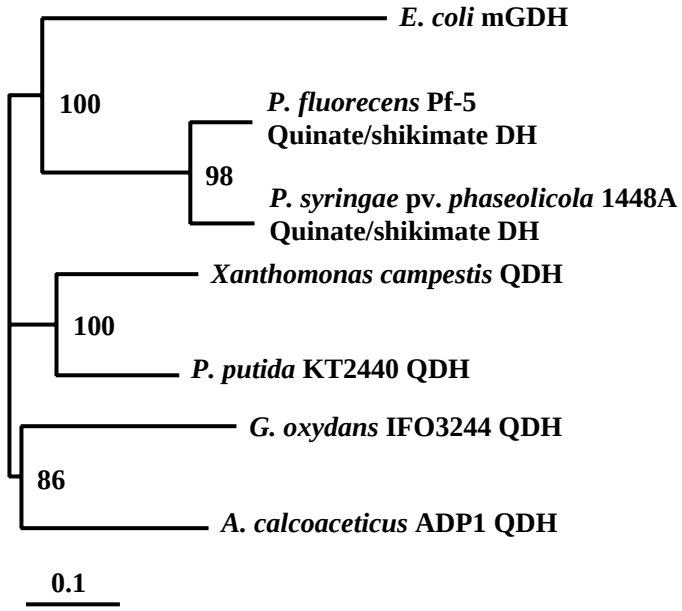

Fig. 3. Phylogenetic relationship among quinate dehydrogenases from various bacteria. The tree topology was inferred by using TreeDomViewer (*17*). The scale bar represents 0.1 amino acid substitution per site. The values adjacent to a node indicate the percentage of 1000 bootstrap trees that contain the node. (*G. oxydans* IFO3244 QDH, GenBank accession no. EU371510; Quinate/shikimate dehydrogenase from *Acinetobacter* sp. ADP1, GenBank accession no. AAC37161; *P. putida* KT2440 , GenBank accession no. NP_745706; *Xanthomonas campestris*, GenBank accession no. AAD38453; *P. fluorescens* Pf-5, GenBank accession no. YP_262726; and a membrane-bound glucose dehydrogenase of *E. coli* K12, GenBank accession no. NP_414666).

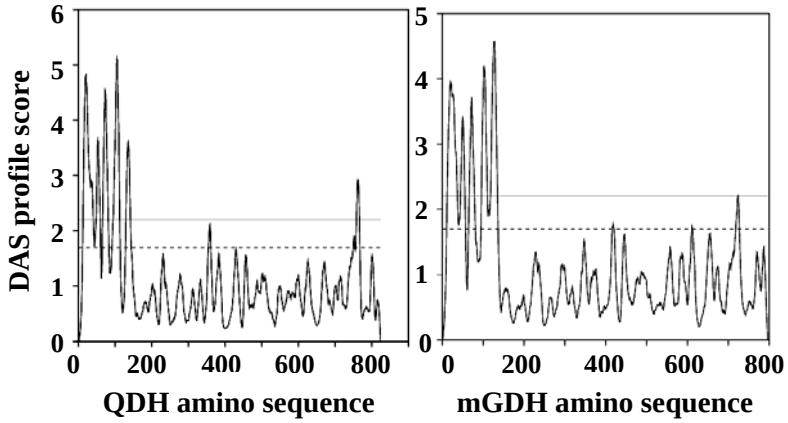

Fig. 4. Putative five transmembrane helices of QDH of *G .oxydans* IFO3244 (824 aa) and of mGDH of *E.coli* (796 aa). The prediction was carried out using the Dense Alignment Surface (DAS) method and was reported as DAS profile score, i.e. loose-cutoff scoring matrix (dashed line) and strict-cutoff scoring matrix (grey line).

Therefore, heterologous gene expression of *qdh* was performed. In this study, *E.coli* strain was not chosen as an expression host because *E. coli* strain such as *E. coli* DH5α does not synthesize PQQ which is the cofactor of QDH. Other *E.coli* strain such as *E. coli* K12 exhibits high expression of mGDH (Elias et al. 2004) which might interfere with PMS reduction-dependent enzyme activity assay. Therefore, gene expression in *E. coli* was avoided. Instead, *G. suboxydans* IFO12528 and *P. putida* HK5 were employed as the host for heterologous gene expression of *qdh* from *G. oxydans* IFO3244. Gene encoding QDH was subcloned into an expression vector, pBBR1-MCS5. The transformation of the recombinant plasmid, pBBR1-MCS5::*qdh*, into *P. putida* HK5 was successfully achieved, while, surprisingly, it was failed with *G. suboxydans* IFO12528.

The main concern of *qdh* gene expression in *P. putida* HK5 was that *P. putida* HK5 could express low level of quinate/shikimate dehydrogenase activity. Therefore, various growth substrates were investigated of how they supported cell growth and affected either expression or repression of the quinate/shikimate dehydrogenase activity. *P. putida* HK5 cells grew well in LB medium having the optical density of 1.5 within 18 hrs. The basal medium supplemented with quinate or fructose moderately supported cell growth (70% and 50% of that in LB, respectively), whereas the basal medium supplemented with glucose or citrate caused adverse effect due to significant pH change during growth of cells. The quinate/shikimate dehydrogenase activity was noticeably induced when cells were grown on the basal-quinate medium. However, *P. putida* HK5 grown on LB or the basal-fructose medium exhibited insignificant level of quinate/shikimate dehydrogenase activity, suggesting that the enzyme activity of the host cells was suppressed under these growth conditions. Further study showed that the quinate/shikimate dehydrogenase activity in *P. putida* HK5 wild-type was detected when it was assayed at pH 6.0, but it was completely diminished at pH 8.0 (**Fig. 5**). On the other hand, the activity of the purified QDH of *G. oxydans* IFO3244 remains at approximately 50% at pH 8.0, while the highest activity was obtained at pH 6.0 (Vangnai et al. 2004). Although there are reports on putative gene sequences of quinate/shikimate dehydrogenase in *Pseudomonas* sp., but there is no report on the characteristic of the enzyme. This result demonstrated that although quinate/shikimate dehydrogenases of *Pseudomonas* and *Gluconobacter* are somewhat evolutionarily related (Fig. 3), the characteristic of enzymes, *i.e.* pH dependent-enzyme activity, was different. According to this strategy, the QDH activity expressed from *P. putida* HK5 host cells and that of the recombinant plasmid, *i.e.* pBBR1-

MCS5::*qdh*, could be distinguished. Using the assay conditions indicated, QDH activity of *P. putida* HK5 harboring pBBR1-MCS5::*qdh* was 0.13±0.01 μmol.min^{-1}.mg^{-1} which was approximately 30% of that in *G. oxydans* IFO3244 wild type.

In summary, the *qdh* gene was successfully obtained from *G. oxydans* IFO3244. The sequence reported here is a complete, full-length gene. It is a functional gene which could be expressed in a PQQ-synthesizing host strain, such as *P. putida* HK5. The recombinant cells demonstrated a comparatively fair QDH activity compared to that of the wild-type strain.

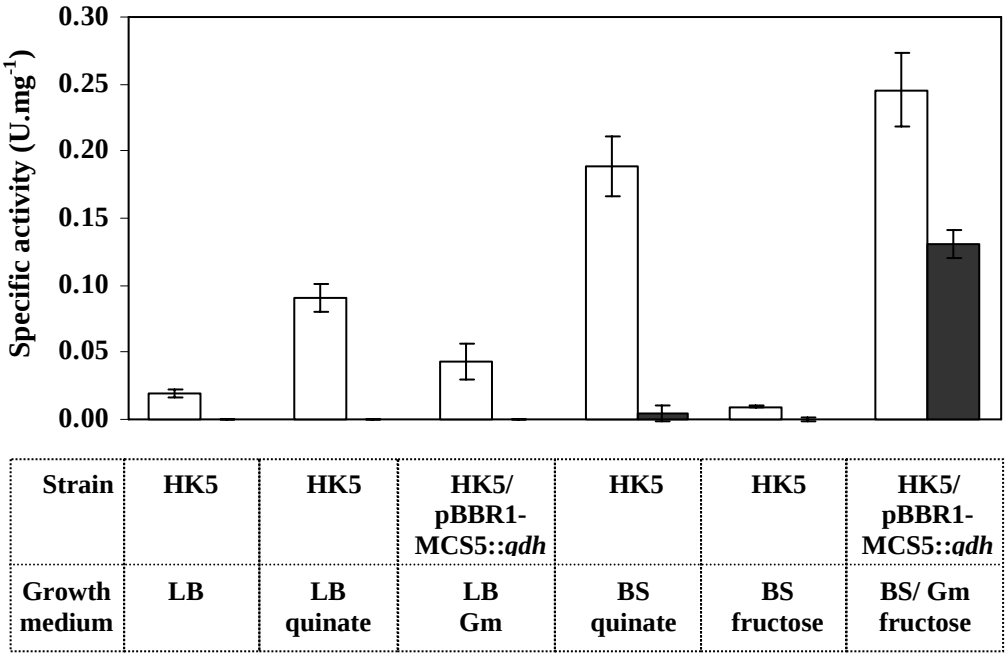

Fig. 5. Heterologous expression of *qdh* gene in *Pseudomonas putida* HK5. *P. putida* HK5 wild type and the recombinant strain, i.e. *P. putida* HK5 harboring pBBR1-MCS5::*qdh*, were grown in various types of growth medium (LB, Luria-Bertani medium; BS, basal medium) in the presence or absence of an inducer, quinate, or other carbon source, e.g. fructose. QDH activity was determined using PMS-reduction assay at two different buffering pHs. Gm (gentamicin) was provided only when the recombinant strain was used. Data are means of three individual experiments. Error bars indicate SEs.

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Output ที่ได้จากโครงการ

1. Two oral presentations related to this project were carried out:

   1.1 Chayatip Insomphun, Hirohide Toyama, and **Alisa S. Vangnai** (2007) Cloning of dehydroquinate dehydratase and shikimate dehydrogenase genes from *Gluconobacter oxydans* 621H and co-transformation in *Gluconobacter oxydans* IFO3244. University of Malaya, Kuala Lumpur, Malaysia, December 17-19. (Poster presentation)

   1.2 **Alisa S. Vangnai**, Chayathip Insomphun, Somboon Tanasupawat, Kazonubu Matsushita, Hirohide Toyama, Osao Adachi (2007) Development of Shikimate production from Quinate using Enzymes and a Metabolic-engineered Pathway in Thermotolerant Microorganism. JSPS-NRCT Concluding Joint Seminar on Development of Thermotolerant Microbial Resources and their Applications. The 33rd Congress on Science & Technology, Thailand (STT33) Science and Technology for Global Sustainability. Walailak University, Nakhon Si Thammarat, Thailand. October, 18-20. (Poster presentation)

2. The manuscript has been submitted to Bioscience, Biotechnology and Biochemistry. The previous result was rejection, but the editor encourages to re-submit the manuscript with revision as suggested by two reviewers.