Disruption of quinoprotein ethanol dehydrogenase gene and adjacent genes in Pseudomonas putida HK5

Worrawat Promden¹, Alisa S. Vangnai¹, Piamsook Pongsawasdi¹, Osao Adachi², Kazunobu Matsushita² & Hirohide Toyama²,³

¹Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand; ²Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Yamaguchi, Japan; and ³Applied Molecular Bioscience, Graduate School of Medicine, Yamaguchi University, Yamaguchi, Japan

Abstract

Pseudomonas putida HK5 produces three different quinoprotein alcohol dehydrogenases: ADH-I, ADH-IIB and ADH-IIG. Gene organization of qeda, the gene for ADH-I, and other 10 genes in the cluster was related to the genome sequences of five other Pseudomonas strains. Insertion mutations in either qeda, exaE or agmR eliminated ADH-I activity, although the mutants were still able to grow on ethanol but more slowly than the wild-type strain. Mutant analysis demonstrated the requirement of agmR and exaE in ADH-I expression, and the tentative involvement of agmR, but not exaE, in the induction of ADH-IIB and ADH-IIG activities.

Introduction

Many aerobic bacteria are able to grow on alcohols as the sole carbon and energy sources, and some of them produce quinoprotein alcohol dehydrogenases (ADH) containing pyrroloquinoline quinone (PQQ) as the prosthetic group (Matsushita et al., 2002; Toyama et al., 2004). The most well-examined examples are quinoprotein methanol dehydrogenases (MDH), which are produced in bacteria able to grow on methanol, requiring a complex system with more than 20 genes to produce an active enzyme (Goodwin & Anthony, 1998). Other quinoprotein ADHs can be classified into three types, according to their molecular properties, catalytic properties and localization (Goodwin & Anthony, 1998; Toyama et al., 2004). Type I ADH is a soluble, dimeric protein of identical subunits (α₂ structure) having a PQQ and a calcium ion in each active center (Keitel et al., 2000). Type I ADH has molecular properties similar to MDH, although it has additional small subunits with an unknown function, comprising an α₂β₂ structure. Type II ADH is a soluble, monomeric quinohemoprotein, having a PQQ-containing catalytic domain and an additional cytochrome c domain (Toyama et al., 2004). Type III ADH is a quinohemoprotein–cytochrome complex with three non-identical subunits, and attached on the cytoplasmic membrane of acetic acid bacteria. Quinoprotein ADHs function by linking to an intracellular respiratory chain at the outer surface of bacterial cells through different electron mediators: cytochromes c for Type I ADH and MDH, cytochromes c or azurin for Type II ADH, and ubiquinone for Type III ADH (Toyama et al., 2004). PQQ is tightly bound to quinoprotein ADH and is not released during the reaction process; therefore, quinoprotein ADHs do not require external addition of coenzyme.
**Materials and methods**

**Chemicals**

All chemicals were of analytical grade. Yeast extract was a kind gift from the Oriental Yeast Co. Ltd, Japan.

**Bacterial strains, media and growth conditions**

The strains used in this study are listed in supplementary Table S1. For *P. putida* HK5 and its mutants, preculture cells were grown overnight in Luria–Bertani (LB) medium at 30 °C. Then, a 1-mL aliquot of preculture cells was washed and inoculated to 100-mL basal medium (0.2% (NH₄)₂SO₄, 0.2% Na₂HPO₄, 0.1% KH₂PO₄, 0.02% MgSO₄·7H₂O and 0.05% yeast extract in tap water at pH 7.0, modified from (Toyama et al., 1995), containing the appropriate alcohol at the final concentration of 0.5% (v/v). The cell growth at 30 °C was monitored as turbidity using a Klett–Summerson colorimeter. The concentration of 0.5% (v/v). The cell growth at 30 °C was monitored as turbidity using a Klett–Summerson colorimeter. The concentration of 0.5% (v/v). The cell growth at 30 °C was monitored as turbidity using a Klett–Summerson colorimeter.

**DNA sequencing and sequence analysis**

Plasmids for sequencing were prepared using the QIAprep Spin Miniprep Kit (Qiagen, Germany). All DNA fragments were subjected to DNA sequencing using ABI PRISM 310 (PE Biosystems). The nucleotide sequences were assembled by SEQUENCER (Gene Codes Cooperation) and analyzed using GENETYX-MAC (Software Development Co. Ltd). Homology search analysis and gene alignment were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL W (http://www.ebi.ac.uk/clustalw). The sequence described in this work has been deposited with the DNA Data Bank of Japan (DDBJ) under accession number AB333783.
Construction of *P. putida* HK5 mutants

Each desired region was amplified by PCR from the strain HK5 genomic DNA using the primers listed in supplementary Table S2. PCR products were ligated into the pGEM-T Easy vector and then subcloned into pUC118 or pUC119 in the case of orf3, orf4 and orf9. The plasmids were digested with appropriate restriction enzyme, blunt-ended and ligated with the 0.9-kb fragment of a nonpolar kanamycin-resistant cassette (Yoshida *et al.*, 2003), resulting in the plasmids listed in supplementary Table S1. Transformation of the plasmid into HK5 was performed as described (Choi *et al.*, 2006). A potential site-directed double-crossover mutant with a Km phenotype was selected for the loss of piperacillin resistance and was confirmed using PCR analysis with specific primers.

Native-polyacrylamide gel electrophoresis (PAGE) and activity staining

Cell-free extracts obtained from different growth or induction conditions were loaded onto a native-PAGE. After electrophoresis, the gel was stained for quinoprotein ADH activity in a reaction mixture containing phenazine methosulfate, nitro-blue tetrazolium and a mixture of alcohol substrates (1 mM ethanol, 10 mM 1-butanol and 10 mM 1,2-propanediol) (Toyama *et al.*, 1995).

Results and discussion

Sequence analysis of *qedA* and its flanking region

A DNA segment of c. 10 kbp, including *qedA*, a gene coding for ADH-I, was obtained using a combination of PCR techniques. Ten complete and two incomplete ORFs were acquired (Fig. 1a and Table 1).

The amino acid sequence deduced from the 34th to 47th residues of QedA shows a complete match to the N-terminal amino acid sequence of the purified ADH-I determined by Edman sequencing (data not shown: Toyama *et al.*, 1995). QedA is highly homologous to QedH from *P. putida* KT2440 (Vrionis *et al.*, 2002), ExaA from *P. aeruginosa* ATCC17933, which is identical to the enzyme from *P. aeruginosa* PAO1 (Keitel *et al.*, 2000), and BOH from *P. butanovora* (Vangnai *et al.*, 2002), with 93%, 86% and 66% identities, respectively. In addition to *qedA*, HK5 also
contains genes for ADH-IIB (qbdA) and ADH-IIG (qgdA). The Qeda exhibits 38% and 36% identity to the PQQ domains of Qbda and Qgda, respectively. The identities among three quinoprotein ADHs in HK5 are, however, comparatively lower than that between ADH-I and other putative Type I ADH reported in the genome of KT2440 and P. aeruginosa F1, where a 53% identity was obtained. This implies that ADH-IIB and ADH-IIG are not derived from gene duplication of qeda in HK5, alternatively, by lateral gene transfer from other microorganisms.

A possible ORF, namely qedB, exists in the same transcriptional direction and adjacent downstream of qeda, with an intergenic region of only 102 nucleotides. QedB has a putative sec-dependent signal sequence with 17 amino acids. The homologs of QedB are also observed downstream of the Type I ADH genes in other Pseudomonas species (Fig. 1b, see below), for instance PP2673 and PA1981 in the genome sequences of P. putida KT2440 and P. aeruginosa PAO1, respectively.

Upstream of qeda, an ORF having 145 amino acids is transcribed divergently. This ORF is designated as qedC, having a heme c-binding motif (CAACH) and a sec-dependent signal sequence 25 amino acids in length. It is highly homologous to PP2675 of KT2440 and PA1983 (exaD) of PAO1 with 70% and 68% identity, respectively (Table 1). This cytochrome c is essential for ethanol oxidation in P. aeruginosa (Schober & Görisch, 1999).

The gene for ExaE, showing similarity to the LuxR DNA-binding response regulator, is found downstream of qedaB. In P. aeruginosa, exaE appeared with exaD, the gene for a sensor kinase (Schober & Görisch, 2001); however, there is no exaD-like gene found in the genomic region sequenced in HK5.

The protein of the 10th orf showed high identity to AgmR from KT2440 and PAO1 (Table 1). The significance of agmR was also shown with ADH activity for the utilization of ethanol and 1,2-propanediol in KT2440 (Vrionis et al., 2002) and PAO1 (Gliese et al., 2004).

**Comparison of gene organization including qeda among Pseudomonas species**

Up-to-date, genome information of 10 Pseudomonas strains is available in the database. Five of them possess qeda homologs (Fig. 1b), whereas the other five from *Pseudomonas entomophila* L48, *Pseudomonas fluorescens* PIO-1, *Pseudomonas syringae* B728a, *P. syringae* phaseolicola 1448A and *P. syringae* pv. tomato DC3000 (Genbank accession nos. CT573326, CP000094, CP000075, CP000058 and AE016873, respectively) do not, although they contain genes for quinoprotein dehydrogenase with membrane-spanning sequences like quinoprotein glucose dehydrogenase (Yamada et al., 2003). None of the 10 genomes contains Type II ADH.

A comparison of ORFs within qeda cluster with the five *Pseudomonas* genomes (KT2440, F1, Pf-5, PAO1 and UCBPP-PA14: Genbank accession nos. AE015451, CP000712, CP000076, AE004091 and CP000438, respectively) illustrated that although the overall organization of ORFs is different in the strain HK5, it is nearly identical to the genomic regions among KT2440, F1 and Pf-5 whereas PAO1 and UCBPP-PA14 are less similar to others (Fig. 1b).

A set of qedaB and qedaC appears in all six sequences in the same organization (Fig. 1b). A gene sequence corresponding to a NAD-dependent aldehyde dehydrogenase was found downstream of qedaC in PAO1 and UCBPP-PA14 (Schober & Görisch, 1999), while a putative corresponding gene was detected in KT2440, F1 and Pf-5 nearby but in a different location. However, in this study, such a gene was not detected around the investigated region in HK5.

### Table 1. Genes found around qeda in the genome of *Pseudomonas putida* HK5

<table>
<thead>
<tr>
<th>Gene</th>
<th>Size (amino acid)</th>
<th>Function</th>
<th>Predicted location</th>
<th>PP homolog(^a)</th>
<th>PA homolog(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>orf1(^c)</td>
<td>165</td>
<td>Enolyl-CoA hydrogenase/isomerase family protein</td>
<td>C</td>
<td>PP3491 (74%)</td>
<td>PA0744 (29%)</td>
</tr>
<tr>
<td>acdA</td>
<td>384</td>
<td>Acyl-CoA dehydrogenase</td>
<td>C</td>
<td>PP3492 (90%)</td>
<td>PA2552 (58%)</td>
</tr>
<tr>
<td>orf3</td>
<td>309</td>
<td>Hydrolase, putative</td>
<td>C</td>
<td>PP2678 (67%)</td>
<td>PA1415 (26%)</td>
</tr>
<tr>
<td>orf4</td>
<td>251</td>
<td>Hypothetical protein</td>
<td>P or OM</td>
<td>PP2677 (73%)</td>
<td>None</td>
</tr>
<tr>
<td>qedC</td>
<td>145</td>
<td>Cytochrome c</td>
<td>P</td>
<td>PP2675 (70%)</td>
<td>PA1983 (68%)</td>
</tr>
<tr>
<td>qedA</td>
<td>623</td>
<td>Quinoprotein ethanol dehydrogenase</td>
<td>P</td>
<td>PP2674 (93%)</td>
<td>PA1982 (86%)</td>
</tr>
<tr>
<td>qedB</td>
<td>216</td>
<td>Pentapeptide-repeat family protein</td>
<td>OM or P</td>
<td>PP2673 (72%)</td>
<td>PA1981 (64%)</td>
</tr>
<tr>
<td>exaE</td>
<td>214</td>
<td>DNA-binding response regulator, LuxR family</td>
<td>C</td>
<td>PP2672 (71%)</td>
<td>PA1980 (64%)</td>
</tr>
<tr>
<td>orf9</td>
<td>178</td>
<td>Hypothetical protein</td>
<td>C</td>
<td>PP2666 (63%)</td>
<td>None</td>
</tr>
<tr>
<td>agmR</td>
<td>221</td>
<td>DNA-binding response regulator</td>
<td>C</td>
<td>PP2665 (86%)</td>
<td>PA1978 (90%)</td>
</tr>
<tr>
<td>orf11</td>
<td>267</td>
<td>Hypothetical protein</td>
<td>C</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>aroF (^d)</td>
<td>202</td>
<td>3-deoxy-7-phosphoheptulonate synthase</td>
<td>C</td>
<td>PP3080 (77%)</td>
<td>PA2943 (73%)</td>
</tr>
</tbody>
</table>

\(^a\)Homologs found in the genome sequences of *P. putida* KT2440 and *P. aeruginosa* PAO1, respectively.

\(^b\)Incomplete deduced amino acid sequence.

C, cytoplasm; P, periplasm; OM, outer membrane.
exaE appears downstream of qedAB in the opposite transcriptional direction in all six genomes. However, a corresponding sensor kinase exaD was not observed in this region in HK5.

A response regulator gene, agmR, is located close to qedA in five genomes, except strain F1, where the agmR homolog exists far from qedA (data not shown). In strains PAO1 and UCBPP-PA14, a gene encoding a protein with 10 transmembrane helices is placed downstream of agmR with only a 10 bp intergenic region, suggesting that they may form an operon. However, the disruption of the gene did not abolish the growth on ethanol, 1,2-propanediol and 1-butanol (Gliese et al., 2004). The corresponding gene is not found in HK5. Nonetheless, orf9 encoding a putative periplasmic protein was found 45 bp upstream of agmR, and a similar set of the genes was also found in KT2440, F1, and Pf-5 (Fig. 1b).

**Mutation analysis of qedA, exaE, orf3, orf4 and qedB**

Disruption of qedA severely reduced the growth rate on ethanol in liquid medium (Fig. 2), although the cell yield of the qedA::Km mutant after a 60-h cultivation was comparable to that of the wild type at 24-h growth. Even though the qedA::Km mutant could grow slowly on ethanol, no ADH-I activity was detected (Fig. 3a). Nonetheless, ethanol-grown wild type and qedA::Km mutant expressed low ADH-IIB activity, showing similar band intensity on native-PAGE (Fig. 3a). Furthermore, the qedA gene disruption did not adversely influence cell growth on 1-butanol and 1,2-propanediol (Fig. 2) and ADH-IIB and ADH-IIG activities (Fig. 3b and c). Growth and ADH-I activity of the mutant were complemented by the plasmid with qedA (data not shown).

ExaE in *P. aeruginosa* is a regulator protein working together with ExaD, which is thought to be sensing a certain molecule in the cytoplasm (Schobert & Görisch, 2001). In HK5, exaE disruption caused a delayed cell growth on ethanol similar to that of qedA disruption (Fig. 2). Growth on glycerol was also slightly delayed (~10 h slower) compared with that of wild type (data not shown). Disruption of the exaE gene resulted in the total disappearance of

---

**Fig. 2.** Growth of *Pseudomonas putida* HK5 and mutant strains on different alcohols. Cells were cultured on 0.5% ethanol (a), 1-butanol (b) or 1,2-propanediol (c). Wildtype (●), qedA::Km (●), exaE::Km (×), agmR::Km (▲), orf9::KmF (□), orf9::KmR (■).

**Fig. 3.** Native-PAGE with enzyme activity staining for ADH-I, ADH-IIB and ADH-IIG. The cell-free extracts (20 μg protein) from the cells grown on ethanol (a), 1-butanol (b) and 1,2-propanediol (c) were loaded on a native PAGE and activity stained with the alcohols. Arrows indicate the position of the activity bands of ADH-I, ADH-IIB and ADH-IIG, from top to bottom. Lane 1, cell-free extract from the wild-type cells (a positive control); Lanes 2–9, cell-free extract from mutants: qedA::Km, exaE::Km, agmR::Km, qedB::Km, orf3::Km, orf4::Km, orf9::KmF and orf9::KmR, respectively.
ADH-I activity (Fig. 3a), while normal induction of ADH-IIB and ADH-IIG were still observed (Fig. 3b and c). The plasmid with exaE complemented both growth and ADH-I activity in trans (data not shown). These results indicate that exaE is essential for the expression of ADH-I activity but for neither ADH-IIB nor ADH-IIG.

Gene-disrupted mutants for qedB, orf3 and orf4 were constructed. All mutants grew on ethanol and other growth substrates like the wild type did. They also expressed activities for ADH-I, ADH-IIB and ADH-IIG comparable to the wild-type cells, indicating that these genes are dispensable for growth on ethanol and expression of quinoprotein ADH activities (Fig. 3).

The construction of a mutant harboring a qedC-disrupted gene was also attempted, but failed due to unknown reasons.

**Involvement of agmR in the expression of ADH-I, ADH-IIB and ADH-IIG**

In *P. aeruginosa*, AgmR is the essential regulator for exaA expression, through the expression of exaDE (Gliese et al., 2004). Similarly, AgmR was demonstrated to be important for qedA expression in strain HK5 because disruption of agmR not only markedly delayed cell growth on ethanol, but also abolished ADH-I activity (Figs 2 and 3a). Both growth and ADH-I activity were complemented by the plasmid with agmR (data not shown). Unlike the exaE-knockout mutant in which the gene disruption affected only ADH-I activity but not ADH-IIB, the agmR-knockout mutant grew slowly not only on ethanol but also on 1-butanol, and it showed significantly decreased ADH-IIB activity as well (Figs 2 and 3b). The loss of ADH-I expression in both the exaE::Km and the agmR::Km mutants suggested the roles of both genes for expression of ADH-IIB. However, ADH-IIB expression was adversely affected in the agmR::Km mutant, but not in the exaE::Km mutant, indicating that AgmR also regulates expression of ADH-IIB but not through ExaE. The disruption of agmR also affected cell growth on 1,2-propanediol (Fig. 2). ADH-IIB activity was still detected in the agmR-knockout mutant grown on 1,2-propanediol, although the activity was lower than that in the wild-type cells (Fig. 3c). These results indicated that AgmR may be involved in the ADH-IIB expression, but not solely essential.

As mentioned above, orf9 might be organized in an operon with agmR. Two orf9-disrupted mutants were constructed: the orf9::KmF and the orf9::KmR strains, in which the kanamycin-resistant gene was inserted in the same and opposite orientations, respectively, to the direction of orf9 transcription (Fig. 1a). As shown in Figs 2 and 3, the orf9::KmR strain had a growth rate on ethanol comparable to that of the wild-type strain, but lower ADH-I activity. On the contrary, the orf9::KmF strain showed a long lag phase for growth and no ADH-I activity on ethanol. However, at the stationary phase of growth, ADH-I activity was detected (data not shown). Both mutants grew slower on 1-butanol and 1,2-propanediol than the wild-type strain (Fig. 2). While ADH-IIB in mutants grown on 1-butanol seemed to be normally induced to a level comparable to the wild-type strain (Fig. 3b), the expression of ADH-IIG in 1,2-propanediol-grown mutants was weaker than that of the wild-type strain (Fig. 3c). The disruption of orf9 affected the expression of both ADH-I and ADH-IIG; however, the role of orf9 is not yet conclusive.

According to the results, it is suggested that AgmR governs expression of both ADH-I and ADH-IIB, whereas ExaE does regulate the expression of ADH-I, but not ADH-IIB. AgmR potentially controls the expression of ExaE, a phenomenon that has been described previously in *P. aeruginosa* (Gliese et al., 2004). Expression of ADH-IIG is partially affected by AgmR, but it may be mainly controlled by unknown regulator protein. Further analysis is required to understand how HK5 regulates the expression of three quinoprotein ADHs in response to different alcohols.

**Acknowledgements**

This work was supported by the Thailand Research Fund (TRF) under The Royal Golden Jubilee PhD Program (grant No. PHD/0030/2548).

**References**


Schobert M & Görisch H (1999) Cytochrome c\textsubscript{550} is an essential component of the quinoprotein ethanol oxidation system in *Pseudomonas aeruginosa*: cloning and sequencing of the genes encoding cytochrome c\textsubscript{550} and an adjacent acetaldehyde dehydrogenase. *Microbiology* 145: (Part 2): 471–481.


**Supplementary material**

The following supplementary material is available for this article:

**Table S1.** Bacterial strains and plasmids used in this study.

**Table S2.** Oligonucleotide primers used in this study.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.01060.x (This link will take you to the article abstract.)

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.