RESEARCH LETTER

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

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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 (α_2 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 $\alpha_2\beta_2$ 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.

Pseudomonas putida HK5, an alcohol-utilizing bacterium, produces three different quinoprotein ADHs: one Type I (ADH-I) and two Type II (ADH-IIB and ADH-IIG), depending on the growth substrate provided (Toyama *et al.*, 1995). ADH-I is produced in cells grown on ethanol, 1-propanol or 1,2-propanediol. ADH-IIB is expressed in cells grown on 1-butanol as well as alcohols with longer carbon chain, while ADH-IIG is produced on 1,2-propanediol or glycerol. The multiplicity of quinoprotein ADH expression in one microorganism is unique, although examples for two enzymes have been stated: a MDH and a Type III ADH in *Acetobacter* (*Acidomonas*) *methanolicus* (Matsushita *et al.*, 1992), a Type I ADH and a Type II ADH from *Pseudomonas butanovora* (Vangnai *et al.*, 2002) and two Type I ADHs in the genome sequence from *P. putida* KT2440 (Vrionis *et al.*, 2002).

In *P. putida* HK5, the substrate specificity of the three enzymes has been investigated previously and it is well related to the inducing alcohols, although ADH-IIG reacts with 1-butanol, a noninducing substance, better than 1,2-propanediol (Toyama *et al.*, 1995). It is interesting to examine how the expression of the three enzymes is distinguished and regulated at the molecular level. Cloning and molecular analysis of genes encoding ADH-IIB (Toyama *et al.*, 2003) and ADH-IIG (Toyama *et al.*, 2005) were performed previously. In this work, molecular cloning of the ADH-I gene, *qedA*, and ten adjacent genes were carried out. Gene insertion mutagenesis was then performed on the selected genes in the cluster, and the effect of the mutation on expression of the three quinoprotein ADHs was then analyzed.

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% $(NH_4)_2SO_4$, 0.2% Na_2HPO_4, 0.1% KH_2PO_4, 0.02% $MgSO_4 \cdot 7H_2O$ 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. *Escherichia coli* strains with plasmids were cultured in LB medium with 50 μg ampicillin mL^{-1}. For HK5 mutant selection, 50 μg kanamycin mL^{-1} and 200 μg piperacillin mL^{-1} were used. The plasmids constructed and used in this study are listed in supplementary Table S1.

Amplification of part of the ADH I gene

Genomic DNA of HK5 was prepared according to the protocols described by Sambrook *et al.* (1989). Primers 415F and 1680R (supplementary Table S2) were designed by Clone Manager (Scientific and Educational Software), from the conserved regions of quinoprotein ADH of KT2440 (Vrionis *et al.*, 2002) and *Pseudomonas aeruginosa* (Keitel *et al.*, 2000) and butanol dehydrogenase of *P. butanovora* (Vangnai *et al.*, 2002). PCR was performed according to the manufacturer's protocol using a Ready To Go/PCR Beads kit (GE Healthcare, UK) with genomic DNA as a template. The PCR reaction conditions were as follows: 30 cycles of 30 s at 94 °C, 30 s at 57 °C and 1 min at 72 °C. The PCR product was then cloned into pGEM-T Easy vector (Promega), resulting in plasmid pWP1265. *Escherichia coli* DH5α strain was used as a host for electrotransformation.

Inverse PCR and gene walking

Pseudomonas putida HK5 genomic DNA was digested with the appropriate restriction enzyme (SalI or XhoI). Then, the digested DNA was self-ligated with T4 ligase (Fermentas) at 16 °C, overnight. The inverse PCR primers invL279 and invR407 were designed and PCR was performed using *Ex Taq* DNA polymerase (Takara, Japan) and the self-ligated DNA as a template. The PCR reaction conditions were as follows: 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 4.5 min at 72 °C, in the presence of 5% (v/v) dimethylsulfoxide. The inverse PCR products of *c.* 5 and 4.5 kbp were cloned into the pGEM-T Easy vector, resulting in plasmids pWP5000 and pWP4500, respectively. To obtain the DNA fragment containing *agmR* and further downstream, primers W3047 and invR3296 were used, resulting in plasmid pWPAgmF.

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 SEQUENCHER (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/clistalw). 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^r 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, nitroblue 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

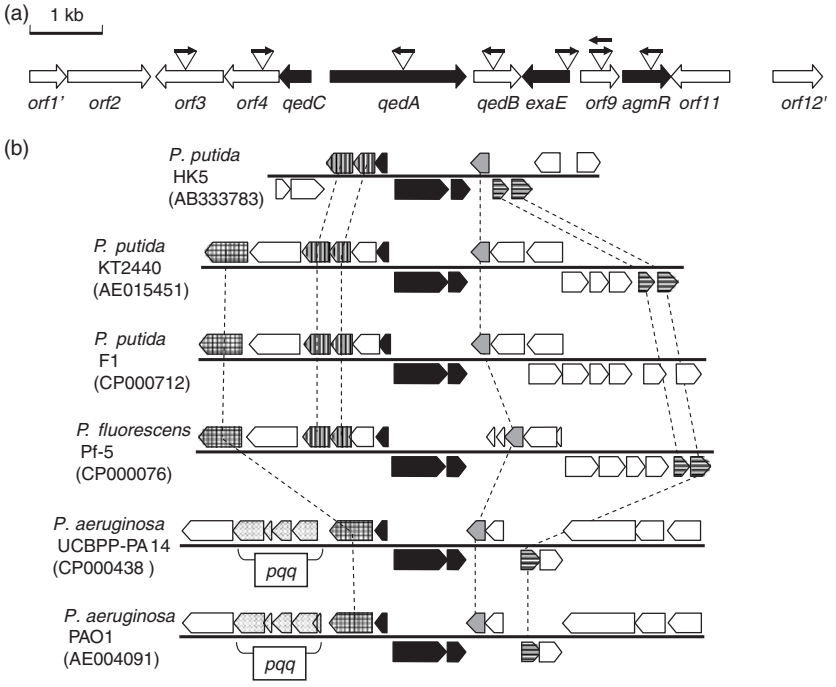

Fig. 1. (a) Gene organization of *qedA* and related genes obtained in this study. The sites used for construction of the knockout mutants are also shown. The arrow of the insertion site indicates the transcriptional direction of the kanamycin-resistance gene inserted. Black arrows indicate the genes that are shown to be essential for ADH-I activity and ethanol oxidation. (b) Comparison of the genetic organization of *qedA* and neighboring genes in diverse *Pseudomonas* species. Related genes are connected by broken lines. Black block arrows indicate *qedC*, *qedA* and *qedB* (from left to right). Within the upstream region of *qedA* (from left to right): the cross-hatched block bar indicates the gene of NAD-dependent aldehyde dehydrogenase; the dotted light-gray block arrows indicate genes for PQQ biosynthesis; the block arrows with vertical stripes are *orf3* and *orf4*, respectively. The downstream region of *qedA* (from left to right) consists of the dark gray block arrow indicates *exaE*, and the block arrows with horizontal stripes indicate *orf9*, followed by *agmR*.

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

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

*,†Homologs found in the genome sequences of *P. putida* KT2440 and *P. aeruginosa* PAO1, respectively.

‡Incomplete deduced amino acid sequence.

C, cytoplasm; P, periplasm; OM, outer membrane.

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. putida* 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 adjacently 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 (*exaB*) of PAO1 with 70% and 68% identity, respectively (Table 1). This cytochrome *c* is essential for ethanol oxidation in *P. aeruginosa* (Schobert & 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 (Schobert & 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* PfO-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 *qedC* 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 *qedC* in PAO1 and UCBPP-PA14 (Schobert & 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.

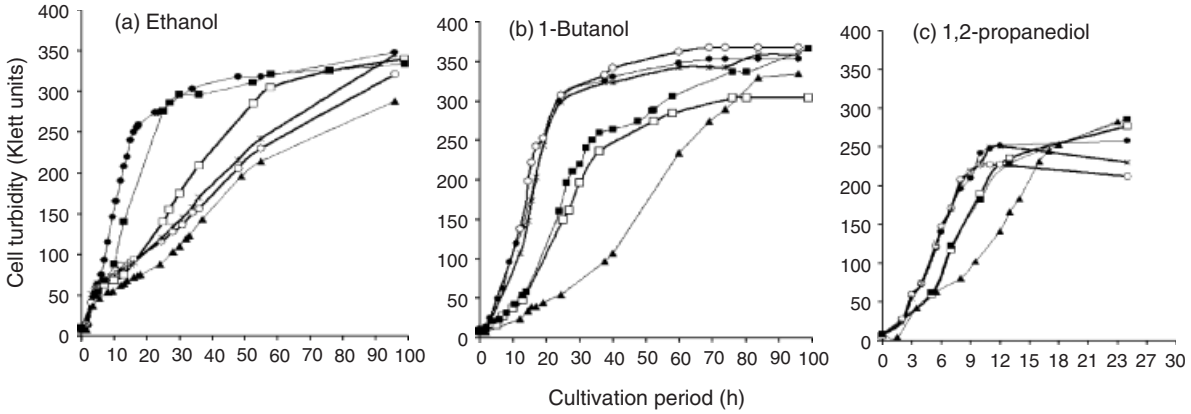

Fig. 2. Growth of *Pseudomonas putida* HK5 and mutant strains on different alcohols. Cells were cultured on 0.5% ethanol (a), 1-butanol (b) or 1,2-propanediol (c). Wildtype (●), *qedA*::Km (○), *exaE*::Km (×), *agmR*::Km (▲), *orf9*::KmF (□), *orf9*::KmR (■).

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

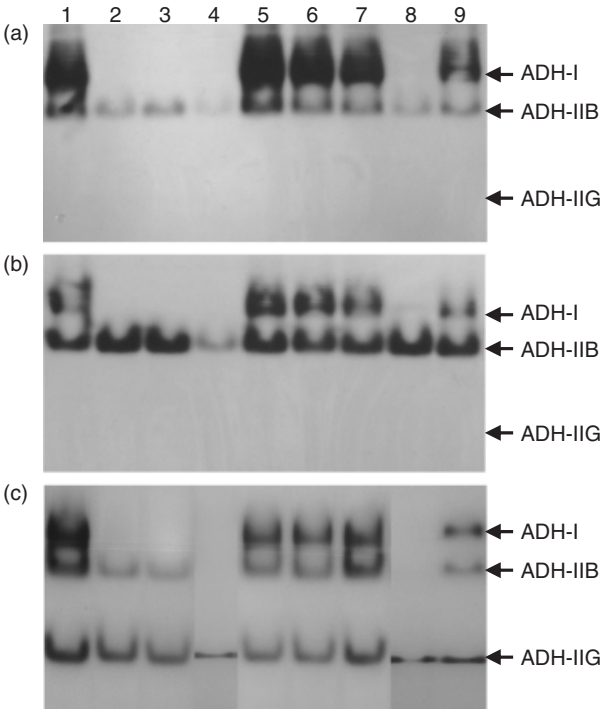

Fig. 3. Native-PAGE with enzyme activity staining for ADH-I, ADH-IIB and ADH-IIG. The cell-free extracts (20 μg protein) from the cells grown on ethanol (a), 1-butanol (b) and 1,2-propanediol (c) were loaded on a native PAGE and activity stained with the alcohols. Arrows indicate the position of the 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-I. 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-IIG 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-IIG 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.

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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.

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