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Analysis of the promoter activities of the genes encoding three quinoprotein alcohol dehydrogenases in *Pseudomonas putida* HK5

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The transcriptional regulation of three distinct alcohol oxidation systems, alcohol dehydrogenase (ADH)-I, ADH-IIB and ADH-IIG, in *Pseudomonas putida* HK5 was investigated under various induction conditions. The promoter activities of the genes involved in alcohol oxidation were determined using a transcriptional *lacZ* fusion promoter-probe vector. Ethanol was the best inducer for the divergent promoters of *qedA* and *qedC*, encoding ADH-I and a cytochrome *c*, respectively. Primary and secondary C3 and C4 alcohols and butyraldehyde specifically induced the divergent promoters of *qbdBA* and *aldA*, encoding ADH-IIB and an NAD-dependent aldehyde dehydrogenase, respectively. The *qgdA* promoter of ADH-IIG responded well to (*S*)-(+)-1,2-propanediol induction. In addition, the roles of genes encoding the response regulators *exaE* and *agmR*, located downstream of *qedA,* were inferred from the properties of *exaE*- or *agmR*-disrupted mutants and gene complementation tests. The gene products of both *exaE* and *agmR* were strictly necessary for *qedA* transcription. The mutation and complementation studies also suggested a role for AgmR, but not ExaE, in the transcriptional regulation of *qbdBA* (ADH-IIB) and *qgdA* (AGH-IIG). A hypothetical scheme describing a regulatory network, which directs expression of the three distinct alcohol oxidation systems in *P. putida* HK5, was derived.

INTRODUCTION

Alcohol dehydrogenases (ADHs) whose reaction is independent of NAD(P) have been found in many aerobic bacteria. Most of these enzymes contain pyrroloquinoline quinone (PQQ) as a prosthetic group and are termed quinoprotein ADHs (qADHs). Methanol dehydrogenase, found in methylotrophic bacteria, was the first quinoprotein shown to have a prosthetic PQQ (Anthony, 1982). Although considerable research has been carried out on the biochemistry and physiology associated with the qADHs (Anthony & Williams, 2003; Chen *et al.*, 2002; Matsushita *et al.*, 1999; Toyama *et al.*, 2004), much less is known about the transcriptional regulation of these enzymes. Insights into the complexity of the transcriptional regulation of qADHs have been obtained from studies of *Pseudomonas aeruginosa* (Gliese *et al.*, 2004), *Pseudomonas butanovora* (Vangnai *et al.*, 2002), *Pseudomonas putida* (Promden *et al.*, 2008) and *Methylobacterium extorquens*, in which at least 28 genes have been shown to be involved in the oxidation of methanol to formaldehyde (Lidstrom *et al.*, 1994; Springer *et al.*, 1995). In *P. aeruginosa*, the transcription of a quinoprotein ethanol dehydrogenase (qEDH) promoter, namely the *exaA* promoter, is regulated by a two-component system: a histidine sensor kinase (ExaD), which is presumably located in the cytoplasm, and a response regulator (ExaE). The AgmR response regulator has been shown to control transcription of a regulon consisting of the three operons *exaBC*, *exaDE* and *pqqABCDE*, the gene products of which are essential for ethanol oxidation (Gliese *et al.*, 2004; Schobert & Görisch, 2001).

P. putida HK5 was originally isolated as a 1-octanol utilizer. When grown on different alcohols, *P. putida* HK5 expresses three distinct soluble ADHs (ADH-I, ADH-IIB and ADH-IIG), each of which contains a PQQ prosthetic group. ADH-I and ADH-IIB are formed in cells either

Abbreviations: ADH, alcohol dehydrogenase; PQQ, pyrroloquinoline quinone; qADH, quinoprotein ADH; qEDH, quinoprotein ethanol dehydrogenase.

grown on or induced with short-chain-length alcohols, while the induction of ADH-IIG is mainly restricted to 1,2-propanediol (Toyama *et al.*, 1995). *P. putida* HK5 is one of only two organisms reported so far that expresses more than one type of PQQ-ADH in response to exposure to alcohols (Vangnai *et al.*, 2002). Therefore, it is interesting to examine how the expression of the three enzymes is distinguished and regulated at a molecular level. The cloning and molecular analysis of the three PQQ-ADH genes, encoding ADH-I, ADH-IIB and ADH-IIG, was performed previously (Promden *et al.*, 2008; Toyama *et al.*, 2003, 2005). The aim of the present study was to analyse the promoter activities under various induction conditions in *P. putida* HK5, using a transcriptional *lacZ* fusion promoter-probe vector as a reporter, and to investigate the role of a two-component system that controls the three ADH clusters.

METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are listed in Table 1. *Escherichia coli* was cultivated at 37 °C in Luria–Bertani (LB) medium. *P. putida* HK5 wild-type and mutant strains were cultivated at 30 °C, either in LB medium or in basal medium (Promden *et al.*, 2008). Alcohols used as inducers were added to a final concentration of 0.5 % (v/v) (equivalent to 50–85 mM, depending on the alcohol used), while aldehydes were used at 0.1 % (v/v) (10–18 mM). Antibiotics were added to the following final concentrations: ampicillin, 50 μg ml^{-1}; piperacillin, 200 μg ml^{-1}; tetracycline, 25 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

DNA manipulations and construction of plasmids. Routine recombinant DNA work was performed according to the protocols described by Sambrook *et al.* (1989). DNA plasmids used in this study are listed in Table 1, and their principal constructs are shown schematically in Fig. 1. The promoter-probe vector pQF50 (Farinha & Kropinski, 1990), harbouring the reporter *lacZ* and the test gene, was constructed to study the transcriptional regulation with the upstream region of the gene of interest. The upstream region of each gene was obtained by PCR amplification based on the nucleotide sequences available in the GenBank database, i.e. accession numbers AB333783, AB091400 and AB204833 for the ADH-I, ADH-IIB and ADH-IIG gene clusters, respectively (Table 1, Fig. 1). The gene complementation study was carried out by cloning a target gene including its promoter into the pCM62 broad-host-range plasmid (Marx & Lidstrom, 2001). Transformation of the plasmids pQF50, pCM62 and their derivatives into *P. putida* HK5 was performed as described by Choi *et al.* (2006). DNA sequencing was done using an ABI PRISM 310 (PE Biosystems).

RT-PCR procedure. Total RNA was isolated from *P. putida* HK5 cells grown to late-exponential phase on ethanol or 1-butanol (Chuang *et al.*, 1993). The RT-PCR kit (mRNA Selective PCR kit, AMV) was obtained from TaKaRa, and used according to the manufacturer's instructions. The primers used for RT-PCR were designed based on the intergenic region of the two adjacent genes of interest to generate a PCR product of approximately 230–500 bp. A negative control, for the exclusion of contaminating genomic DNA amplification, was performed in each case using *Taq* polymerase without any reverse transcriptase.

β*-Galactosidase assay.** *P. putida* HK5 containing pQF50-*lacZ* derivatives was grown overnight in 5 ml LB medium containing 200 μg piperacillin ml^{-1} at 30 °C on a rotary shaker at 200 r.p.m. Cell cultures (1 ml) were collected by centrifugation at 15 000 ***g for 5 min, washed with basal medium, and resuspended in an equal volume of basal medium. The cell suspension was then diluted fivefold in basal medium containing piperacillin and the desired alcohol (at 0.5 %, v/v, final concentration) and/or 20 mM glucose. After shaking the culture for 6 h at 30 °C, the *β*-galactosidase activity was determined with cells treated with chloroform, according to the procedure of Miller (1992).

Determination of ADH activity and protein assay. Cells from 100 ml culture of OD_{600} 0.8–1.0 were harvested, washed once with ice-cold saline, and resuspended in 3 ml 50 mM Tris/HCl buffer (pH 8.0). The suspension was passed through a French pressure cell at 16 000 p.s.i. (110 400 kPa) at 4 °C. The cell debris was removed by centrifugation at 15 000 ***g*** for 20 min, and the resulting supernatant was obtained as a crude fraction. Phenazine methosulfate (PMS) reductase activity was measured for ADH-I activity, and ferricyanide reductase activity was used for ADH-IIB and ADH-IIG activity assays, as described previously (Toyama *et al.*, 1995). The rate of reduction of electron acceptor observed with substrate was subtracted from that obtained without substrate. Protein content was estimated by a modified method of Lowry (Dulley & Grieve, 1975) with serial dilutions of BSA (Sigma) as a standard.

RESULTS AND DISCUSSION

ADH-I gene cluster: promoter activities of structural genes, *qedA*(*B*) and *qedC*

Genes encoding ADH-I (*qedA*) and cytochrome *c* (*qedC*) are adjacent, but divergently transcribed. This cytochrome *c* homologue is reported to be an essential component of the ethanol oxidation system in *P. aeruginosa* (Schobert & Görisch, 1999). The upstream region of *qedA* of *P. putida* HK5 contains the putative *E. coli* σ^{70} promoter consensus sequence (Harley & Reynolds, 1987), i.e. −35[TTCCCG] and −10[TATCTG]. This promoter region of *P. putida* HK5 also exhibits high similarity to the putative promoter sequence of the qEDHs of *P. putida* KT2440 and *Pseudomonas fluorescens* Pf5.

Promoter-probe vectors, i.e. pQW-ADHI and pQW-CYT (Fig. 1a), were constructed and used to monitor the promoter activities of *qedA* and *qedC* genes, respectively. In response to various alcohol inducers, both promoters exhibited similar transcriptional patterns, though to different magnitudes. Significant promoter activities were observed towards primary and secondary short-chain-length alcohols (C2–C4), diols and glycerol. The highest promoter activity was obtained with ethanol induction, while the activity diminished upon induction with longer-chain-length alcohols (C5–C8) (Fig. 2a, b). Previous results, based upon the relative activities of ADH-I towards different substrates, indicated that C2–C8 primary alcohols were good substrates for the enzyme (Toyama *et al.*, 1995). Nonetheless, our present work suggested that longer-chain-length alcohols act as repressors for the *qedA* and *qedC* promoters. In contrast, though the secondary alcohols, diols and glycerol have been reported to be poor substrates for ADH-I (Toyama *et al.*, 1995), significant promoter

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant trait(s)	Source or reference
Strains		
E. coli DH5α	r^-m^+ *recA1 lacZYA* Φ80 d*lac*Δ (*lacZ*)*M15*	Bethesda Research Laboratories
P. putida HK5	Wild-type	Toyama *et al.* (1995)
qedA::Km^r	HK5 derivative, Km^r cassette with opposite orientation of *qedA* gene	Promden *et al.* (2008)
exaE::Km^r	HK5 derivative, Km^r cassette with opposite orientation of *exaE* gene	Promden *et al.* (2008)
agmR::Km^r	HK5 derivative, Km^r cassette with opposite orientation of *agmR* gene	Promden *et al.* (2008)
Plasmids		
pQF50	Ap^r; *lacZ* promoter-probe vector	Farinha & Kropinski (1990)
pQF50 derivative promoter-probe vectors (Ap^r): ADH-I cluster (corresponding to base position of nucleotide sequence accession no. AB333783)		
pQW-CYT	414 bp of *qedC* upstream region (4279–3866)	This study
pQW-ADHI	354 bp of *qedA* upstream region (3787–4140)	This study
pQW-PEN	528 bp of *qedB* upstream region (5916–6443)	This study
pQW-EXAE	530 bp of *exaE* upstream region (7935–7406)	This study
pQW-AGMR	400 bp of *agmR* upstream region (7838–8237)	This study
pQF50 derivative promoter-probe vectors (Ap^r): ADH-IIB cluster (corresponding to base position of nucleotide sequence accession no. AB091400)		
pQW-ALDA	524 bp of *aldA* upstream region (1096–573)	This study
pQW-ADHIIB	427 bp of *qbdB* upstream region (493–919)	This study
pQF50 derivative promoter-probe vectors (Ap^r): ADH-IIG cluster (corresponding to base position of nucleotide sequence accession no. AB204833)		
pQW-ORFG1	561 bp of *orf1* upstream region (1079–521)	This study
pQW-ADHIIG	466 bp of *qgdA* upstream region (444–909)	This study
pCM62	Tc^r; broad-host-range plasmid	Marx & Lidstrom (2001)
pCM62 derivative, complementation plasmids (Tc^r): *qedA, exaE and agmR* (corresponding to base position of nucleotide sequence accession no. AB333783)		
pCM-ADHI	2224 bp of *qedA* gene (3974–6197), ADH-I expressed under control of *qedA* promoter	This study
pCM-ADHIZ	2077 bp of *qedA* gene (4121–6197), ADH-I expressed under control of *lacZ* promoter	This study
pCM-EXAE	1524 bp of *exaE* gene (8140–6617), ExaE expressed under control of *exaE* promoter	This study
pCM-EXAEZ	712 bp of *exaE* gene (7475–6764), ExaE expressed under control of *lacZ* promoter	This study
pCM-AGMRZ	706 bp of *agmR* gene (8129–8833), AgmR expressed under control of *lacZ* promoter	This study

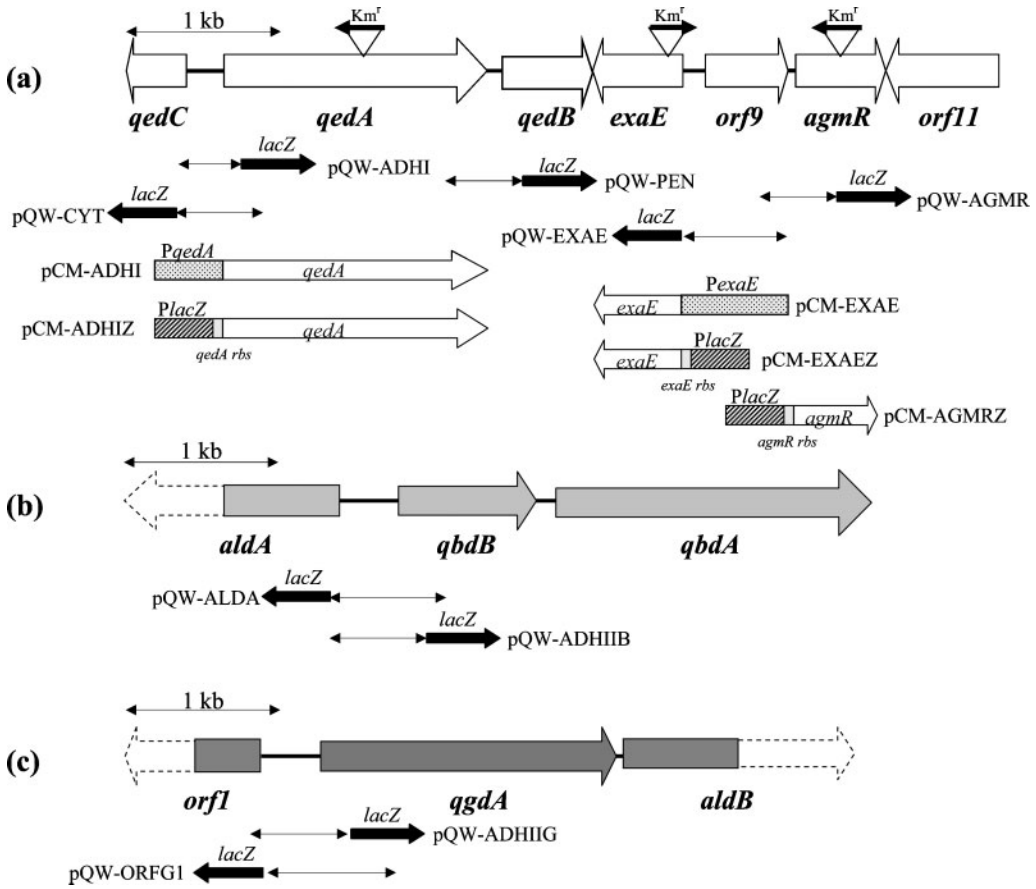

Fig. 1. Organization of the qADH genes within the (a) ADH-I, (b) ADH-IIB and (c) ADH-IIG clusters of *P. putida* HK5, and plasmid construction. The arrows at the insertion sites indicate the transcriptional direction of the inserted kanamycin-resistance genes. For construction of the *lacZ* promoter-probe vectors (pQW plasmids), PCR fragments of promoter region (divergent arrows) were cloned into pQF50. Black arrows indicate the *lacZ* gene. pCM plasmids were constructed by cloning PCR fragments into pCM62. Stippled boxes indicate the promoter regions of *qedA* (P*qedA*) and exaE (P*exaE*); striped boxes indicate the *lacZ* promoter region (P*lacZ*) of pCM62. The consensus sequence-predicted positions of the ribosome-binding sites (*rbs*) are shown.

activities of *qedA* and *qedC* with these inducers were observed (Fig. 2a, b). These results suggested that short-chain-length alcohols (C2–C4) act as signal molecules for a specific regulator that controls transcription of *qedA* and *qedC*. Although the similar transcriptional patterns of the two promoters under alcohol induction suggested that *qedA* and *qedC* might be under the control of the same regulator, the expression level of *qedC* was higher than that of *qedA* under the same conditions. This directly contrasts with an earlier study of *P. aeruginosa*, in which the promoter activity of *exaB* (cytochrome c_{550}) was reported to be significantly lower than that of *exaA* (qEDH) (Schobert & Görisch, 2001). The high expression level of *qedC* was anticipated, because *P. putida* HK5 has three types of qADHs, each of which requires cytochrome *c* for electron translocation during alcohol oxidation. In the present work, *qedA* and *qedC* promoters were found to likely be affected by a catabolite repression control by glucose, as the combination of glucose with ethanol led to a low induction of the *qedA* and *qedC* promoter activities (Fig. 2a, b). Lactate, acetate and tricarboxylic acid cycle intermediates such as citrate and succinate were also found to act as catabolite repressors of *qedA* (data not shown).

Downstream of *qedA* lies a pentapeptide repeated sequence, namely qedB (Promden *et al.*, 2008). The homologue of qedB was also observed downstream of type I ADH genes in *P. aeruginosa* ATCC 17933 (gene PA1981). Although it has been presumed that this possible ORF is co-transcribed with the *exaA* gene of *P. aeruginosa* (Görisch, 2003), the RT-PCR results from the present work showed that in ethanol-grown cells, *qedA* and qedB were not stably transcribed as a single transcript. Moreover, neither a σ^{54}- nor a σ^{70}-promoter consensus sequence could be found in the qedAB intergenic region, and no promoter activity was attained with the qedAB intergenic

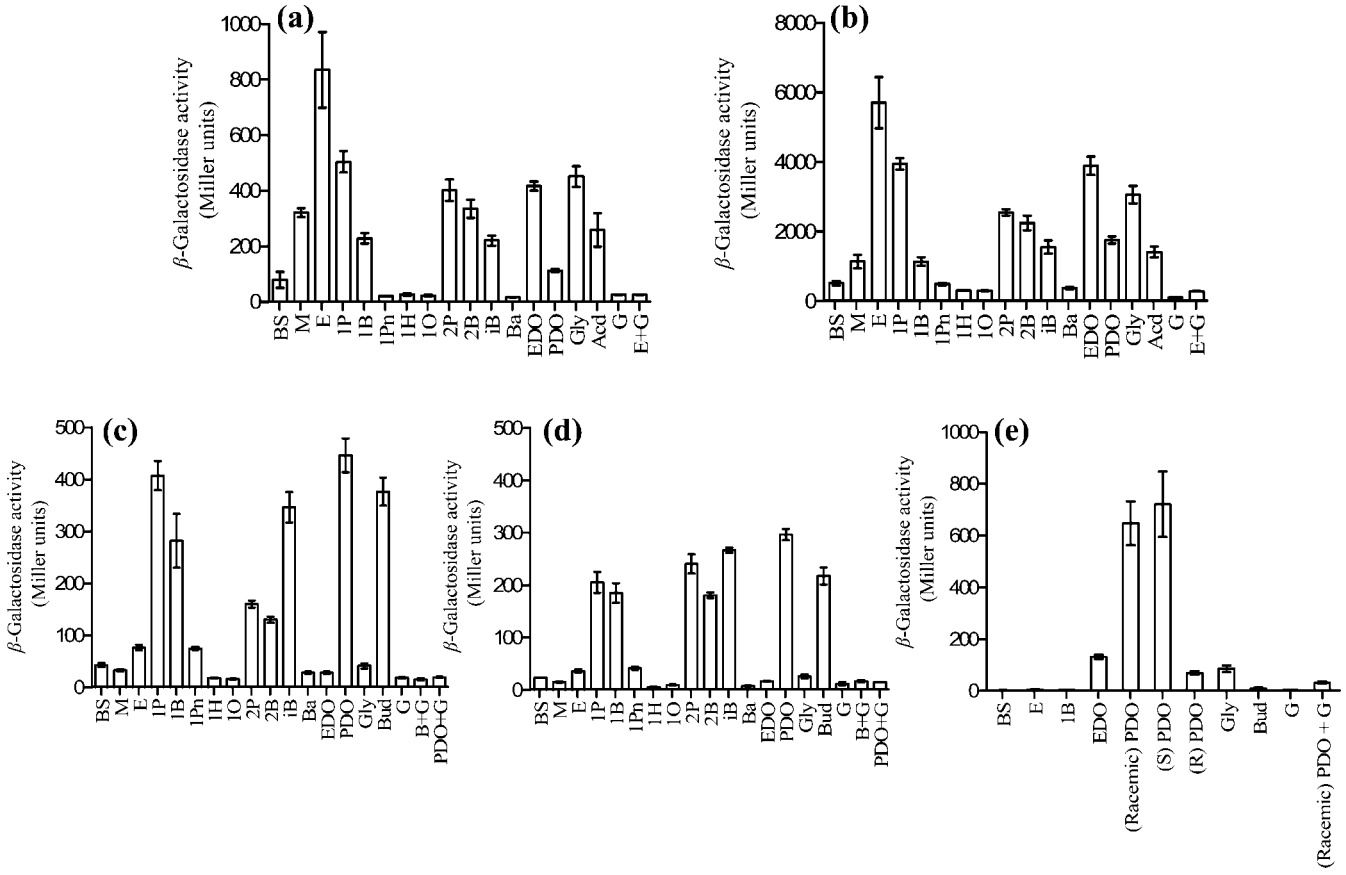

Fig. 2. Promoter activities of (a) *qedA*; pQW-ADHI, (b) *qedC*; pQW-CYT, (c) *aldA*; pQW-ALDA, (d) *qbdBA*; pQW-ADHIIB, and (e) *qgdA*; pQW-ADHIIG in *P. putida* HK5 after induction by various substrates (0.5 %, v/v, alcohols, 0.1 %, v/v, aldehyde or 20 mM glucose). Data are shown as the mean ± SD and are derived from three independent experiments. Abbreviations: BS, basal medium only; M, methanol; E, ethanol; 1P, 1-propanol; 1B, 1-butanol; 1Pn, 1-pentanol; 1H, 1-heptanol; 1O, 1-octanol; 2P, 2-propanol; 2B, 2-butanol; iB, *iso*-butanol; Ba, benzyl alcohol; EDO, ethanediol; PDO, 1,2-propanediol; Gly, glycerol; Acd, acetaldehyde; Bud, butyraldehyde; G, glucose.

region-containing construct (pQW-PEN) under the conditions tested (data not shown). Thus, according to these results, qedB may not be a true ORF.

ADH-IIB gene cluster: C3/C4 alcohols and butyraldehyde as the inducers of ADH and ADH-IIB expression

The quinohaemoprotein ADH-IIB gene cluster of *P. putida* HK5 consists of the ADH gene (*aldA*), an ORF of unknown function (*qbdB*), and the ADH-IIB structural gene (*qbdA*) (Fig. 1b) (Toyama *et al.*, 2003). The RT-PCR result suggested that the *qbdBA* genes were co-transcribed from a promoter upstream of *qbdB* (data not shown). A σ^{54}-consensus sequence, with the conserved sequence (underlined) centred around −24 and −12, respectively (Barrios *et al.*, 1999), was found upstream of *aldA* [TGGCACAA]GGG[TTGCT] and *qbdBA* [TGGCACGA]AGC[CTGCT]. The promoter-probe vectors pQW-ALDA and pQW-ADHIIB were constructed (Fig. 1b), and the transcriptional expression levels in response to various alcohol substrates were evaluated. The results, summarized in Fig. 2(c, d), suggest that glucose acted as a catabolite repressor of the *aldA* and *qbdBA* promoters. The promoter activities of *aldA* and *qbdBA* demonstrated a similar induction pattern in response to primary and secondary C3 and C4 alcohols as well as butyraldehyde induction (Fig. 2c, d). Since the *qedA* and *qbdBA* promoters were differentially induced by alcohols that differed in their chain lengths, their activities may be either controlled by different regulators or influenced by different regulatory factors. Nonetheless, it cannot be excluded that these regulators have broad specificity to other short-chain alcohols, resulting in the concurrent expression of ADH-I and ADH-IIB (Promden *et al.*, 2008; Toyama *et al.*, 1995).

ADH-IIG gene cluster: (*S*)-(+)-1,2-propanediol is a specific inducer

The ADH-IIG gene cluster consists of *qgdA*, a structural gene of quinohaemoprotein ADH-IIG, followed by the

aldB gene, encoding an NAD-aldehyde dehydrogenase in the same direction, and *orf1*, encoding a hypothetical protein oriented in the opposite direction and located upstream of *qgdA* (Toyama *et al.*, 2005). The activities of the divergent *qgdA* and *orf1* promoters were investigated when induced with alcohols and aldehydes. The *lacZ* promoter-probe vector of *orf1*, i.e. pQW-ORFG1 (Fig. 1c), showed a low constitutive activity with all substrates tested, suggesting that *orf1* might not be directly involved in alcohol utilization in *P. putida* HK5 (data not shown). Since the σ^{54}-dependent promoter consensus sequence, [TGGCATGG]CGG[TTGCG], was found at the upstream region of the *qgdA* gene, the *qgdA* promoter-probe vector, pQW-ADHIIG (Fig. 1c), was constructed. The highest promoter activity of pQW-ADHIIG was observed after growth on (*S*)-(+)-1,2-propanediol. Ethanediol, glycerol and (*R*)-(−)-1,2-propanediol were weaker inducers, whereas ethanol, 1-butanol and butyraldehyde were ineffective inducers (Fig. 2e). It should be noted that (*S*)-(+)-1,2-propanediol not only is a strong inducer for the *qgdA* promoter, but is also the preferred substrate of ADH-IIG with a higher substrate specificity (K_m=0.055 mM) than that of (*R*)-(−)-1,2-propanediol (K_m=3.32 mM) (Toyama *et al.*, 2005). Interestingly, even though 1-butanol was a substrate of ADH-IIG (K_m=0.043 mM) (Toyama *et al.*, 1995, 2005), it was not found to be an effective signal molecule for the *qgdA* promoter. These results suggest that the *qgdA* promoter is specifically regulated by (*S*)-(+)-1,2-propanediol and that different regulators control the activities of the *qedA*, *qbdB* and *qgdA* promoters.

Roles of regulatory genes, *exaE* and *agmR*, for alcohol oxidation in *P. putida* HK5

Downstream of *qedA* (ADH-I structural gene) lie *exaE* and *agmR*, two regulatory genes that encode likely DNA-binding response regulators, and *orf9*, which encodes a hypothetical protein similar to that in *P. putida* KT2440. The RT-PCR results suggested that *orf9* and *agmR* did not form an operon (data not shown). In fact, no *orf9* transcriptional product was detected under ethanol induction. Although the role of *orf9* has not yet been conclusively determined, a previous report has shown that disruption of the *orf9* sequence adversely affects the expression of ADH-I (Promden *et al.*, 2008).

An earlier study of the genetic regulation of qADH in *P. aeruginosa* ATCC 17933 revealed a two-component regulatory system composed of the sensor kinase ExaD and the DNA binding response regulatory protein ExaE (Schobert & Görisch, 2001). However, in *P. putida* HK5, the homologous gene corresponding to *exaD* could not be observed within the 10 kb ADH-I cluster gene fragment (Promden *et al.*, 2008). Nevertheless, the activity of the *exaE* promoter was investigated in this study using the promoter-probe vector pQW-EXAE, which was constructed with the putative σ^{70} promoter region −35[TCGACA] and −10[AGTAGT], upstream of the *exaE* gene (Fig. 1a). The results revealed that the *exaE* promoter was transcribed to an approximately similar level with all alcohols and the aldehyde tested, but that this expression level was relatively low (Fig. 3). In addition, it was found that the *exaE* promoter was likely to be affected by catabolite repression control by glucose, as the combination of glucose with ethanol led to a low activity of the *exaE* promoter (Fig. 3). To investigate the promoter activity of *agmR*, the plasmid pQW-AGMR was used (Fig. 1a). The results are consistent with the notion that the *agmR* promoter is constitutively transcribed (Fig. 3).

Involvement of *exaE* and *agmR* in ADH-I and cytochrome *c* expression

To investigate the influence of *exaE* and *agmR* on ADH-I expression, the ADH-I-defective mutants were tested for complementation with plasmids harbouring individually the *qedA*, *exaE* or *agmR* genes. Two plasmids harbouring the *qedA* gene were constructed (Table 1) for the complementation study, i.e. pCM-ADHI and pCM-ADHIZ, in which *qedA* was under the control of the *qedA* promoter (P*qedA*) and the *lacZ* promoter (P*lacZ*), respectively. Each plasmid was transformed into *P. putida* HK5 wild-type and mutant strains. The transformants were then grown on ethanol and ADH-I activity was determined (Table 2). Introduction of pCM-ADHI into HK5 WT (wild-type) cells increased the observed ADH-I activity by 2.5-fold, while it fully restored the activity in the *qedA*::Km^r mutant cells (Table 2). The complementation results for pCM-ADHIZ in either wild-type or mutant cells indicated that *qedA* was expressed at a low level under the control of the *lacZ* promoter. Previous work has demonstrated that the insertion of a kanamycin-resistance cassette

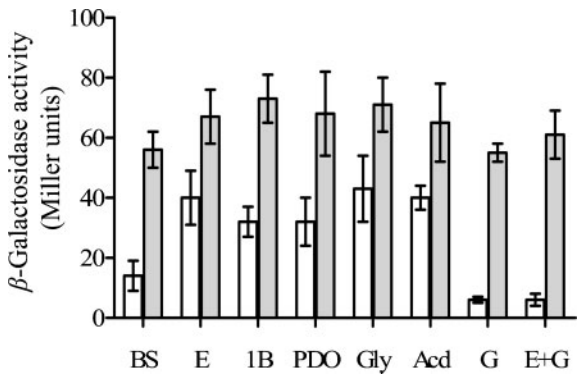

Fig. 3. Promoter activities of *exaE*; pQW-EXAE (white bars) and *agmR*; pQW-AGMR (grey bars) in *P. putida* HK5 cells after induction by various substrates (0.5 %, v/v, alcohols, 0.1 %, v/v, aldehyde or 20 mM glucose). Data are shown as the mean ± SD and are derived from three independent experiments. Abbreviations: BS, basal medium only; E, ethanol; 1B, 1-butanol; PDO, 1,2-propanediol; Gly, glycerol; Acd, acetaldehyde; G, glucose.

Table 2. Specific activities of three ADHs from *P. putida* HK5 wild-type, mutant and complemented mutant strains

Data shown are mean ± SD, and are derived from at least three independent replicates. WT, wild-type.

Strain	ADH specific activities [U (mg protein)$^{-1}$]		
	ADH-I*	ADH-IIB†	ADH-IIG‡
HK5 WT	1.50 ± 0.12	0.76 ± 0.08	0.21 ± 0.01
HK5 WT/pCM-ADHI	3.97 ± 0.38		
HK5 WT/pCM-ADHIZ	1.46 ± 0.02		
qedA::Km^r	0	0.76 ± 0.14	0.20 ± 0.01
qedA::Km^r/pCM-ADHI	1.45 ± 0.20		
qedA::Km^r/pCM-ADHIZ	0.17 ± 0.01		
exaE::Km^r	0	0.85 ± 0.05	0.22 ± 0.01
exaE::Km^r/pCM-ADHI	0.17 ± 0.08		
exaE::Km^r/pCM-ADHIZ	0.21 ± 0.07		
exaE::Km^r/pCM-EXAE	1.33 ± 0.09		
exaE::Km^r/pCM-EXAEZ	1.22 ± 0.06		
exaE::Km^r/pCM-AGMRZ	0		
agmR::Km^r	0	0.47 ± 0.07	0.14 ± 0.01
agmR::Km^r/pCM-ADHI	0		
agmR::Km^r/pCM-ADHIZ	0.10 ± 0.01		
agmR::Km^r/pCM-EXAE	0		
agmR::Km^r/pCM-EXAEZ	0		
agmR::Km^r/pCM-AGMRZ	1.40 ± 0.15	0.70 ± 0.06	0.21 ± 0.01

*ADH-I, †ADH-IIB and ‡ADH-IIG activities were obtained from ethanol-, butanol- and 1,2-propanediol-grown cells, respectively.

(Km^r) in *qedA*, *exaE* and *agmR* genes completely eliminated ADH-I activity (Promden *et al.*, 2008). Complementation with either pCM-ADHI or pCM-ADHIZ, but not pCM-AGMRZ, in *exaE*::Km^r could only partially restore the ADH-I activity, while complementation with pCM-EXAE (under P*exaE* control) and pCM-EXAEZ (under P*lacZ* control) resulted in ~80–90 % ADH-I activity relative to that seen in wild-type cells (Table 2). This result indicates a likely important role for ExaE as a regulatory protein involved in P*qedA* transcriptional activity. Thus, the potential role of AgmR as another regulatory protein was also examined. In the presence of ExaE alone, in *agmR*::Km^r/pCM-EXAEZ, *qedA* expression was not detected. Complementation of *agmR* using the *agmR*::Km^r/pCM-AGMRZ mutant resulted in a 95 % restoration of ADH-I activity relative to wild-type cells, indicating that the presence of both ExaE and AgmR is necessary for the transcriptional regulation of P*qedA*. Moreover, the *lacZ* promoter-probe vector harbouring the *qedA* promoter, pQW-ADH-I (Fig. 1a), was examined in the wild-type, *exaE*::Km^r and *agmR*::Km^r mutant cells with ethanol induction (Fig. 4b). The promoter activity of the *qedA* construct in wild-type cells was 835 ± 136 Miller units compared with no detectable activity in *exaE*::Km^r and *agmR*::Km^r mutant strains, supporting the hypothesis that both ExaE and AgmR are necessary for the transcription of P*qedA*.

To investigate the involvement of *exaE* and *agmR* in regulating the expression of genes under the cytochrome *c* promoter (P*qedC*), the *lacZ* promoter-probe vector harbouring the *qedC* promoter, pQW-CYT (Fig. 1a), was constructed and its expression level was examined in wild-type, and *exaE*::Km^r and *agmR*::Km^r mutant strains, under induction with ethanol (Fig. 4a). The promoter activity of *qedC* in wild-type cells (5704 ± 740 Miller units) was significantly reduced in the *exaE*::Km^r mutant (708 ± 262 Miller units) and completely abolished (no detectable activity) in the *agmR*::Km^r strain. The complementation of *exaE* (pCM-EXAE) and *agmR* (pCM-AGMRZ) in the presence of pQW-CYT could partially recover *qedC* promoter activity by ~30 % (1725 ± 80 Miller units) and ~16 % (955 ± 100 Miller units), respectively, relative to that seen in the wild-type cells (data not shown). These results are consistent with the notion that both ExaE and AgmR are necessary for the transcription of not only P*qedA* but also P*qedC*. The regulation of ethanol oxidation by ExaE and AgmR regulators in *P. putida* HK5 is partly distinct from that in *P. aeruginosa*. In *P. aeruginosa*, ExaDE regulators regulate only the expression of ethanol dehydrogenase, but not that of cytochrome c_{550} (Görisch, 2003; Schobert & Görisch, 2001). In the present work, it was shown that the AgmR regulator governed the activity of the *exaE* promoter. When the *agmR* gene was disrupted, the *exaE* promoter activity was almost completely eliminated (Fig. 4c), suggesting that AgmR is a general regulator of quinoprotein ethanol oxidation in *P. putida* HK5, similar to the situation in *P. aeruginosa* ATCC 17933 (Gliese *et al.*, 2004).

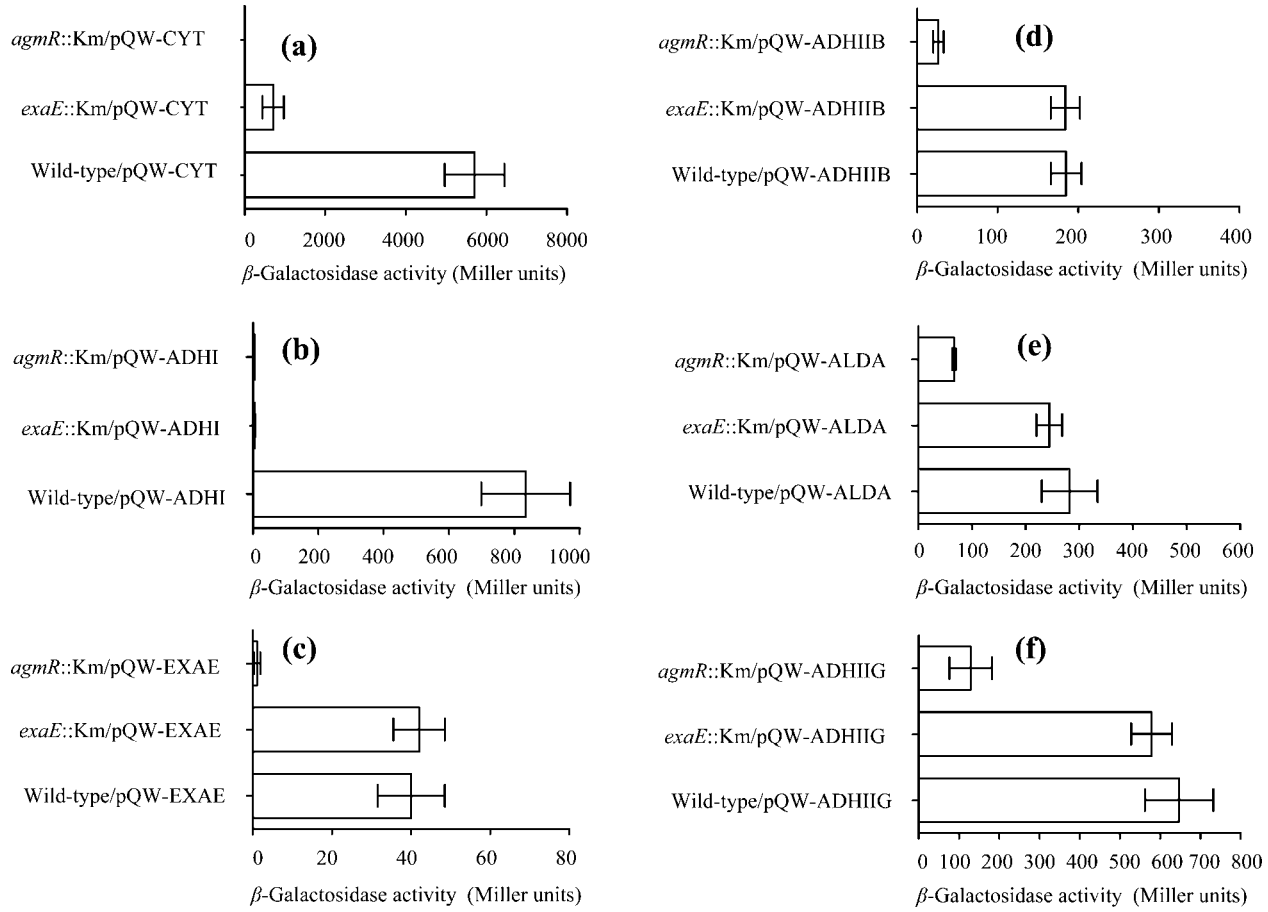

Fig. 4. Promoter activities of (a) *qedC*; pQW-CYT, (b) *qedA*; pQW-ADHI, (c) *exaE*; pQW-EXAE, (d) *qbdBA*; pQW-ADHIIB, (e) *aldA*; pQW-ALDA, and (f) *qgdA*; pQW-ADH-IIG in HK5 wild-type and *exaE*::Km and *agmR*::Km mutant *P. putida* strains when induced with 0.5 % (v/v) ethanol (a–c), butanol (d–e) or 1,2-propanediol (f). Data are shown as the mean ± SD and are derived from three independent experiments

Involvement of *agmR*, but not *exaE*, in ADH-IIB and ADH-IIG expression

The disruption of *agmR* completely abolished the ADH-I activity, while the activities of ADH-IIB and ADH-IIG were reduced by 38 and 33 %, respectively (Table 2). Complementation of the *agmR* gene using pCM-AGMRZ fully restored the ADH-IIB and ADH-IIG activities, and neither enzyme activity was affected when the *exaE* gene was disrupted.

In addition, the *lacZ* promoter-probe constructs harbouring the *qbdBA*, *aldA* and *qgdA* promoters (Fig. 1b, c) were examined in the wild-type, and *exaE*::Km^r and *agmR*::Km^r mutant strains, under induction with butanol (for *qbdBA* and *aldA* promoter-probe vectors) or 1,2-propanediol (for the *qgdA* promoter-probe vector) (Fig. 4d–f). The promoter activities of *qbdBA* (pQW-ADHIIB) and *aldA* (pQW-ALDA) in the wild-type and *exaE*::Km^r mutant strains were not significantly different (Fig. 4d, e). For *qgdA* (pQW-ADHIIG), the promoter activities were higher. Nevertheless, for all the three promoters, the activities were significantly reduced in *agmR*::Km^r mutant strains. These results suggest that the AgmR, but not the ExaE, regulator plays a major role in the regulation of ADH-IIB and ADH-IIG expression. The findings agree well with results from a previous report on total ADH activities demonstrated by native PAGE with *in situ* enzyme activity staining (Promden *et al.*, 2008).

Hypothetical scheme of the regulatory network controlling the alcohol oxidation system of *P. putida* HK5

P. putida HK5 expresses three distinct qADHs, depending on the type of alcohol presented as the signal molecule. The regulatory network scheme controlling the transcriptional expression of these three ADH genes and alcohol oxidation systems in *P. putida* HK5 was derived from the properties of *exaE*- and *agmR*-disrupted mutants and the promoter activities of each ADH gene cluster examined under various growth and induction conditions (Fig. 5). The results indicated that AgmR is a primary, although not sole,

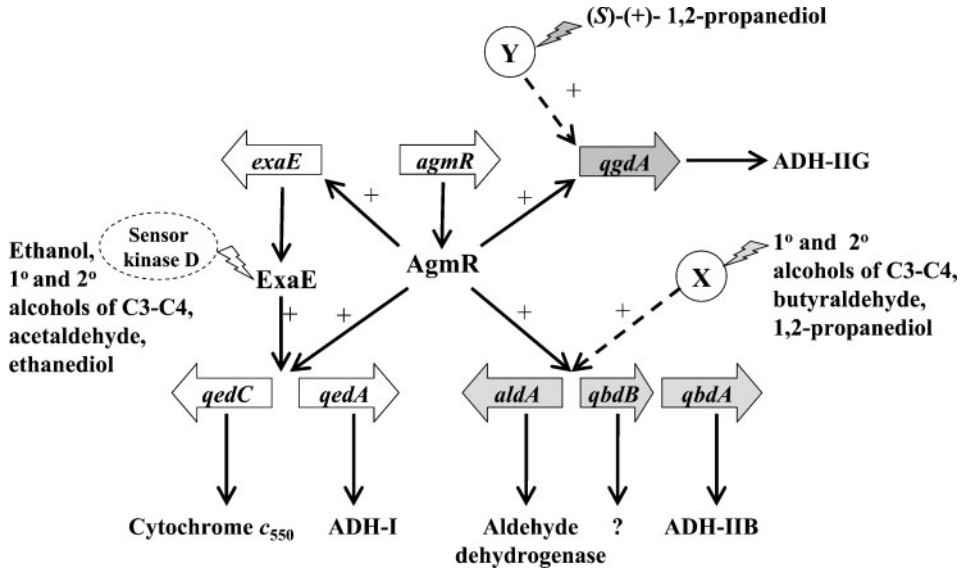

Fig. 5. Hypothetical scheme of the regulatory network controlling transcription of the quinoprotein alcohol oxidation systems in *P. putida* HK5. The scheme is derived from results obtained with *exaE* and *agmR* regulatory mutants, promoter activities and complementation studies. Genes are indicated by letters in arrows, whilst the hypothetical regulatory genes proposed for ADH-IIB and ADH-IIG are indicated by X and Y, respectively.

regulator involved in all three alcohol oxidation systems. The product of *agmR* also regulates the transcription of *exaE*. ExaE subsequently influences the transcription of *qedA* (for ADH-I expression), but ExaE cannot direct the transcription of *qedA* in the absence of AgmR. Both AgmR and ExaE are therefore annotated as response regulators, for which corresponding sensor kinases are generally required. Since a sensor kinase homologue of the AgmR regulator is not currently known (Gliese *et al.*, 2004), and the *exaD* gene encoding the histidine sensor kinase of ExaE (Schobert & Görisch, 2001) could not be detected in *P. putida* HK5 cloned fragments, there may be other sensing regulatory factor(s) which respond to ethanol, and the primary and secondary C3 and C4 alcohols involved. As for the transcription of *qbdBA* (ADH-IIB) and *qgdA* (ADH-IIG), AgmR played a partial role in governing their transcription. In this case, AgmR may be differentially controlled by other promoter(s), if any. In addition, we cannot rule out that besides AgmR and ExaE, other regulator(s) or effector(s) could be involved. For instance, the involvement of a protein X (Fig. 5), which responds to the primary and secondary C3- and C4-chain-length alcohols, butyraldehyde and 1,2-propanediol, in directing *qbdBA* transcription, and a protein Y (Fig. 5), which senses the stereospecific (*S*)-(+)-1,2-propanediol for controlling *qgdA* transcription, could be proposed.

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