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Identification of Genes Essential for the Biogenesis of Quinohemoprotein Amine Dehydrogenase

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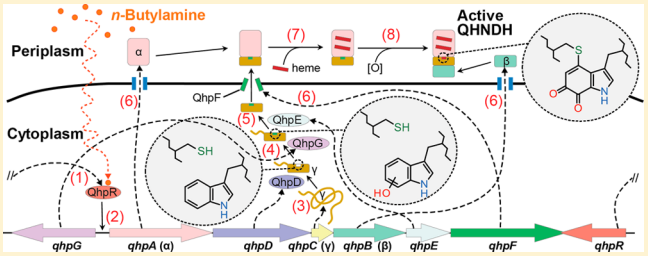
Supporting Information

ABSTRACT: The structural genes encoding quinohemoprotein amine dehydrogenase (QHNDH) in Gram-negative bacteria constitute a polycistronic operon together with several nearby genes, which are collectively termed "*qhp*". We previously showed that the *qhpD* gene, which lies between *qhpA* and *qhpC* (encoding the α and γ subunits of QHNDH, respectively), and the *qhpE* gene, which follows *qhpB* (encoding the β subunit), both encode enzymes specifically involved in the posttranslational modification of the γ subunit and are hence essential for QHNDH biogenesis in *Paracoccus denitrificans* [Ono, K., et al. (2006) *J. Biol. Chem. 281*, 13672−13684; Nakai, T., et al. (2012) *J. Biol. Chem. 287*, 6530−6538]. Here we further demonstrate that the *qhpF* gene, which follows *qhpE*, and the *qhpG* and *qhpR* genes, peripherally located in the complementary strand, are also indispensable for QHNDH biogenesis. The *qhpF* gene encodes an efflux ABC transporter, which probably translocates the γ subunit into the periplasm in a process coupled with hydrolysis of ATP. The *qhpG* gene encodes a putative FAD-dependent monooxygenase, which is required for the generation of the quinone cofactor in the γ subunit. Finally, the *qhpR* gene encodes an AraC family transcriptional regulator, which activates expression of the *qhp* operon in response to the addition of *n*-butylamine to the culture medium. Database analysis of the *qhp* genes reveals that they are very widely distributed, not only in many Gram-negative species but also in a few Gram-positive bacteria.



Quinohemoprotein amine dehydrogenase (QHNDH) is inducibly formed in the periplasm of several Gram-negative bacteria, including *Pseudomonas putida*[1−3] and *Paracoccus denitrificans*.[4] The enzyme catalyzes oxidative deamination of various aliphatic primary amines so that they can be assimilated as energy, carbon, and nitrogen sources. Our previous studies showed that QHNDH is composed of three nonidentical subunits, designated α, β, and γ on the basis of their molecular sizes (see Figure S1 of the Supporting Information).[5−7] The α subunit, the largest (∼60 kDa), has a four-domain structure with two heme *c* groups contained in the N-terminal diheme cytochrome *c*-like domain. The β subunit (∼37 kDa) has a seven-bladed β propeller structure that is well-conserved across quinoproteins. The γ subunit, the smallest of the three (∼9 kDa), is buried inside the α subunit; it has a particularly unusual structure consisting mostly of featureless coils with a covalently bound quinone cofactor, cysteine tryptophylquinone (CTQ), derived from Trp and Cys residues, and three intrapeptidyl thioether cross-links formed between Cys and Glu or Asp residues. These structural features of the γ subunit clearly indicate that it must undergo multiple posttranslational modifications before it can form an active QHNDH complex with the α and β subunits.

The structural genes encoding the three QHNDH subunits constitute an operon harboring six apparent open reading frames (ORFs) that are transcribed in a coordinated manner upon addition of amines to the culture medium, although the promoter region has not yet been identified (Figure 1A). The subunits constituting QHNDH are encoded by *ORF1* (α subunit), *ORF4* (β subunit), and *ORF3* (γ subunit). Of the other genes in the operon, *ORF2* encodes an [Fe-S] cluster- and *S*-adenosylmethionine (SAM)-binding protein, a member of the "radical SAM superfamily",[8] and *ORF5* encodes a protein of approximately 22.5 kDa belonging to subfamily S8A of peptidase family S8 (the subtilisin family) with the conserved Asp/His/Ser catalytic triad characteristic of this subfamily. We have previously shown that the *ORF2* gene product in *P. denitrificans* plays an essential role in the posttranslational modification of the γ subunit, probably by participating in intrapeptidyl thioether cross-link formation via an [Fe-S] cluster- and SAM-dependent mechanism.[9] More recently, we demonstrated that the subtilisin-like serine protease encoded by *ORF5* is also essential for QNHDH biogenesis, acting as a

Received: December 4, 2013
Revised: January 16, 2014
Published: January 17, 2014

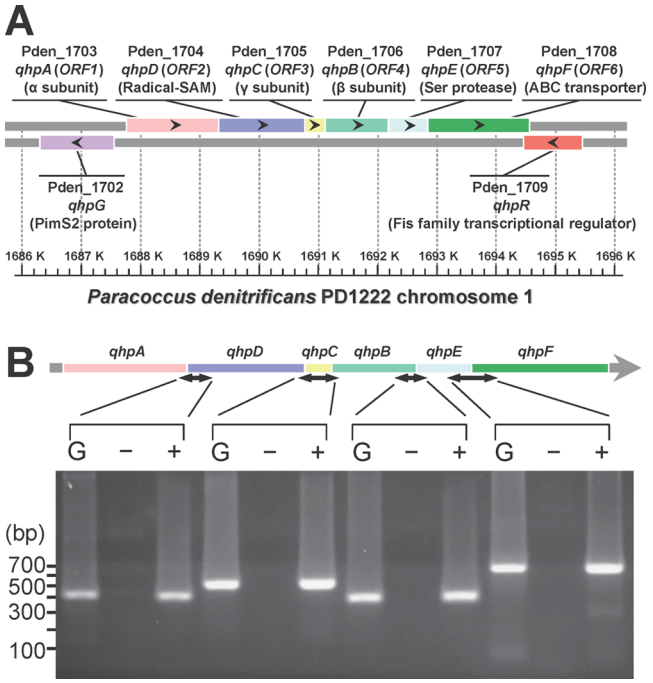


Figure 1. Structure of the *qhp* operon and reverse transcription polymerase chain reaction (PCR) analysis. (A) Arrangement of the *qhp* genes (Pden_1702−Pden_1709) in *P. denitrificans* Pd1222 chromosome 1. Protein names initially annotated by the genome project for each *qhp* gene product are given in parentheses. (B) Reverse transcription and colony PCR detection of transcripts from the *qhp* operon. Reverse transcription PCR products from cDNA prepared with (+) or without (−) reverse transcriptase are shown beside colony PCR products amplified from the corresponding genomic regions (G). The amplified regions are indicated with double-headed arrows.

processing protease that cleaves off the γ subunit leader peptide with negligible catalytic turnover.[10]

In the years since QHNDH protein structures[5−7] and other studies on the enzyme[9,10] were first published, genome sequences have been released for numerous bacteria, and analysis of these sequences reveals that the genes encoding QHNDH subunits, and those in the surrounding regions, are highly conserved in a large number of bacteria, mostly Gram-negative organisms belonging to the *Proteobacteria* (Table 1). In addition to the *ORF1−5* genes mentioned above, we observed a high degree of conservation of three additional ORFs found in the *P. denitrificans* genome, although the arrangement and orientation of these genes are less conserved than those of the *ORF1−4* genes (Table 1).

In this paper, instead of identifying genes by the name "*ORF*" together with a number that would be inapplicable for other bacterial genomes with different arrangements of the genes, we have renamed the operon containing the QHNDH subunit genes and associated genes "*qhp*" (Figure 1A), a name that has been used occasionally for annotations of QHNDH homologues identified in bacterial genome projects.[11] We demonstrate, using gene disruption and plasmid complementation, that the products of the three newly identified, highly conserved genes are indispensable for the biogenesis of QHNDH and that each plays a distinct role. In addition, we report that the *qhp* genes are very widely distributed, particularly in Gram-negative bacteria.

MATERIALS AND METHODS

Materials, Bacterial Strains, and Culture Conditions. Plasmid pUC4K, containing a kanamycin resistance (K_m^r) gene, was obtained from the National Institute of Genetics (Mishima, Japan). Suicide vector pGRPd1,[12] *P. denitrificans* wild-type strain Pd1222, and *Escherichia coli* strain S17-1[13] were kindly provided by R. J. van Spanning (Vrije Universiteit, Amsterdam, The Netherlands). *E. coli* strains DH5α, S17-1, and C41(DE3) were used for plasmid preparation, diparental mating, and protein expression, respectively. *E. coli* was grown aerobically at 37 °C in Luria broth (LB) medium [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl] supplemented with appropriate antibiotics when necessary. *P. denitrificans* was grown aerobically at 30 °C in LB medium or a minimal mineral medium containing 6.0 mg/mL Na_2HPO_4, 3.0 mg/mL KH_2PO_4, 0.5 mg/mL NaCl, 1.0 mg/mL NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 73 μM Na_2MoO_4, 1.6 μM $CuSO_4$, 20 μM ammonium Fe(III) citrate, and 0.5% (w/v) *n*-butylamine hydrochloride, with or without 20 mM choline chloride, which supports bacterial growth as an alternative carbon source.[14−16] Final concentrations of antibiotics added to the culture medium were 50 μg/mL ampicillin, 50 μg/mL kanamycin (K_m), 20 μg/mL rifampicin, 10 μg/mL (for *E. coli*) or 1 μg/mL (for *P. denitrificans*) tetracycline, and 50 μg/mL streptomycin.

Reverse Transcription Polymerase Chain Reaction (PCR). To detect polycistronic transcripts from the *qhp* operon, total RNA was purified, using a NucleoSpin RNA (Macherey-Nagel) column, from a cell extract of *P. denitrificans* Pd1222 cultivated to mid-log phase in the minimal mineral medium containing 0.5% (w/v) *n*-butylamine hydrochloride and 20 mM choline chloride. Genomic DNA contaminating the eluted RNA solution was removed by DNase digestion. Reverse transcription was performed with a SuperScript III first-strand synthesis system (Life Technologies) using the isolated total RNA as a template and primers specific for the coding regions of *qhpDBEF* genes (Q2R, Q4R, Q6R, and Q8R) (Table S1 of the Supporting Information). The manufacturer's protocol for transcripts with high GC content was used for the cDNA synthesis. Controls without reverse transcriptase were included in all experiments. Regions spanning two or three *qhp* genes in the polycistronic transcripts were then amplified by PCR using the reverse transcription products as a template and KOD-Plus-NEO DNA polymerase (Toyobo) in a reaction mixture containing 10% (v/v) dimethyl sulfoxide; the program consisted of 35 cycles of denaturation at 98 °C (10 s), annealing at 62 °C (30 s), and polymerase reaction at 68 °C (40 s). The Q1F/Q2R, Q3F/Q4R, Q5F/Q6R, and Q7R/Q8R primer pairs (Table S1 of the Supporting Information) were used to amplify the *qhpA-qhpD*, *qhpD-qhpC-qhpB*, *qhpB-qhpE*, and *qhpE-qhpF* regions, respectively (see Figure 1B). The same reverse transcription PCR primers were used to directly amplify chromosomal DNA from single colonies of *P. denitrificans* Pd1222 as a positive control. Products of reverse transcription and colony PCR were analyzed on a 2% (w/v) agarose gel and detected with SYBR Safe DNA gel stain (Life Technologies).

Gene Disruption. To facilitate construction of the plasmids used for gene disruption, a DNA fragment containing a multiple cloning site (MCS) obtained by BssHII digestion of pBluescript II SK+ (Stratagene) was ligated into pGRPd1, which had been linearized by digestion with EcoRI and HindIII and blunt-ended using Klenow fragment, to form pGRPd1-MCS. Subsequently, DNA fragments of ∼1100 bp, containing

dx.doi.org/10.1021/bi401625m | *Biochemistry* 2014, 53, 895−907

Table 1. Bacterial Distribution of *qhp* and Associated Genes Identified by BLAST Searching

bacterial species[a]	no. of hits	gene order[b]
Alphaproteobacteria (16 species)	34	
Caenispirillum salinarum (strain AK 4)	2	*GADCBEF*
Citreicella sp. (strain SE45)	2	*GADCBEFR*
Labrenzia aggregata (strain IAM 12614)	2	*RGADCBFER*
Magnetospirillum sp. (strain SO-1)	1	*FEBCDA*
Meganema perideroedes	1	*RFEBCDAG*
Novosphingobium aromaticivorans (strain DSM 12444)	3	*ADCBGFE*
Novosphingobium nitrogenifigens (strain DSM 19370)	2	*ADCBGFE*
P. denitrificans (strain Pd1222)	7	*GADCBEFR*
Paracoccus sp. (strain TRP/N5)	2	*GADCBEFR*
Polymorphum gilvum (strain SL003B-26A1)	3	*EFBCDAGR*
Phaeobacter gallaeciensis	1	*RGADCBFE*
Rhodobacterales bacterium (strain Y4I)	2	*RGADCBFER*
Sphingobium ummariense (strain RL-3)	1	*ADCBGFER*
Sphingobium xenophagum	1	*EFGBCDA*
Sphingobium yanoikuyae (strain ATCC 51230)	3	*ADCBGFE*
unidentified α-proteobacterium LLX12A	1	*EFGBCDA*
Betaproteobacteria (11 species)	46	
Aromatoleum aromaticum (strain EbN1)	6	*G−FBCDAR—ADCBFE*
Azoarcus sp. (strain BH72/KH32C)	6	*RFEGADCB*
Burkholderia cepacia (strain GG4)	5	*BCDA−RFEG*
Burkholderia sp. (strain TJI49)	2	*ADCB−F*
Methyloversatilis universalis (strain FAM5)	2	*RADCBFE−G*
Pseudogulbenkiania sp. (strain NH8B)	3	*RFEG−ADCB−R*
Pseudogulbenkiania ferrooxidans (strain 2002)	3	*RFEG−ADCB−R*
Thauera aminoaromatica (strain S2)	2	*BCDARG—RADCBERR*
Thauera linaloolentis (strain DSM 12138)	4	*ADCB—BCDARG*
Thauera phenylacetica (strain B4P)	3	*RF−REBCDA*
Thauera sp. (strain 27/28/63/MZ1T)	10	*FEGRADCB−RR−BCDA*
Gammaproteobacteria (20 species)	111	
Amphritea japonica	2	*RADCBFEG−ADCB−R*
Halomonas sp. (strain KM-1)	1	*G—EFBCDA*
Marinobacterium stanieri	2	*R−BCDA—GEFBCDA—R*
Neptuniibacter caesariensis	4	*ADCBFEG−ADCB−R*
Pseudomonas aeruginosa (strain WC55/BWHPSA028)	3	*BCDA−GEFR—CB*
Pseudomonas alcaligenes (strain OT 69)	1	*BCDA—GEF−R*
Pseudomonas chlororaphis (strain O6)	2	*BCDA—GEFR*
Pseudomonas chlororaphis ssp. *aureofaciens* (strain 30-84)	1	*RFEG—ADCB*
Pseudomonas denitrificans (strain ATCC 13867)	2	*ADCB−RFEG*
Pseudomonas entomophila (strain L48)	5	*FG−RADCBE*
Pseudomonas fluorescens (strain Pf0-1)	8	*RFEG—ADCB*
Pseudomonas monteilii	1	*BCDA−GEFR*
Pseudomonas plecoglossicida (strain NB2011)	1	*BCDA—GEFR*
Pseudomonas protegens (strain Pf-5/CHA0)	4	*ADCB−R—RFEG*
Pseudomonas pseudoalcaligenes (strain KF707)	2	*RFEG−ADCB*
Ps. putida (strain KT2440/S16/HB3267/S11/BIRD-1/LF54/H8234/W619/GB-1/NBRC 14164/F1/ND6/DOT-T1E/LS46/TRO1/CSV86)	42	*RFEG−ADCB*
Pseudomonas resinovorans (strain NBRC 106553)	5	*R−BCDA—GEFR*
Pseudomonas sp. [strain EGD-AK9/GM84/GM21/GM17/GM18/GM41(2012)/M1/GM60/GM67/GM33/GM49/GM48/G5(2012)/GM55/GM74/GM78/GM25]	22	*RFEG−ADCB*
Pseudomonas thermotolerans	2	*BCDA—GEF−R—EBCDA*
Thiothrix disciformis	1	*RGADCBFE*
Deltaproteobacteria (two species)	5	
Desulfobacula toluolica (strain Tol2)	3	*EF—BC−DA*
Geopsychrobacter electrodiphilus	2	*ADCB—FE*
Epsilonproteobacteria (two species)	11	

dx.doi.org/10.1021/bi401625m | *Biochemistry* 2014, 53, 895−907

Table 1. continued

bacterial species[a]	no. of hits	gene order[b]
Arcobacter butzleri (strain RM4018/ED-1/7h1h/JV22)	8	*R**ADCB**FEG*
Arcobacter sp. (strain L)	3	*R**ADCB**FEG*
Bacilli (four species)	6	
Aneurinibacillus aneurinilyticus (strain ATCC 12856)	1	*EF*–*BDACR*
Bacillus azotoformans (strain LMG 9581)	2	*EF*–*BDACR*
Brevibacillus sp. (strain phR)	1	*EF*–*BDACR*
Geobacillus thermoglucosidans (strain TNO-09.020)	2	*R**CADB*—*FE*

[a]Listed in alphabetical order within each bacterial class. Unidentified species (sp.) in the same genus, and subspecies, are counted as one species in the total number of species shown in parentheses in the top row for each class. The strain names in parentheses are shown if they are given in the genome database. [b]Using the *qhpGADCBEFR* genes of *P. denitrificans* Pd1222 as references, homologous genes in each bacterial species were searched for, and those identified are shown with alphabetical characters (when no homologues are identified for the *qhpGEFR* genes but nearby genes with predicted functions close to them are found, they are indicated). Genes identified in the complementary strand in the genome database are underlined. When two genes are separated by a gap of several hundred base pairs or interrupted by another ORF, a dash (−) is inserted. When two genes are separated by >1000 bp or two or more ORFs, a long dash (—) is inserted. Strongly conserved gene order (***ADCB***), and its reverse encoded in the complementary strand (***BCDA***), are shown with boldface letters. Within a given bacterial species, different gene orders may be found in different strains; the gene order shown is representative of the species.

5′- and 3′-terminal regions of each target gene, were amplified by PCR using a pair of primers designed to hybridize with 1000 bp sequences outside the gene and 100 bp sequences within it (see Figure S2 of the Supporting Information) based on the nucleotide sequence of *P. denitrificans* Pd1222 chromosome 1 (GenBank accession no. CP000489), and to contain a specific restriction site at the 5′-terminus (Table S1 of the Supporting Information). PCR was performed in a reaction mixture containing one pair of forward and reverse primers, 10% (v/v) dimethyl sulfoxide, and genomic DNA from *P. denitrificans* Pd1222 as a template, with a program consisting of 30 cycles of denaturation at 98 °C (10 s), annealing at 61 °C (30 s), and polymerase reaction at 68 °C (80 s). The PCR products were purified using a FastGene Gel/PCR extraction kit (Nippon Genetics), digested with a restriction enzyme(s), inserted into the MCS of pBluescript II SK+ vector, and sequenced to confirm the presence of the introduced restriction site and the absence of undesirable mutations. The pBluescript II SK+ derivatives thus obtained and the pUC4K vector were digested with appropriate restriction enzymes to yield 5′- and 3′-fragments of the target gene and the K_m^r gene, which were then inserted into the MCS of pGRPd1-MCS in the order 5′-fragment, K_m^r gene, and 3′-fragment, to produce the final plasmids used for gene disruption (pGRPd1-QhpN::K_m^r, where N = F, R, or G) (Figure S2 of the Supporting Information).

The *qhpFRG* genes of *P. denitrificans* were disrupted by homologous recombination essentially as described previously.[9] Briefly, the donor *E. coli* S17-1 cells carrying pGRPd1-QhpN::K_m^r (N = F, R, or G) were conjugated with the recipient Pd1222 cells,[9,12] and *qhp* gene-disrupted mutants of Pd1222 were selected from K_m^r and streptomycin-sensitive colonies. Gene disruption was confirmed by colony PCR using primers designed to amplify a DNA fragment covering each *qhp* gene interrupted by the K_m^r gene (Table S1 of the Supporting Information). Finally, mutants of Pd1222 carrying *qhpN*::K_m^r (N = F, R, and G) genes in the genome, designated PdΔqhpF, PdΔqhpR, and PdΔqhpG, respectively, were obtained and used without removing the K_m^r gene.

Construction of Plasmids for Gene Complementation. For plasmid complementation of disrupted genes in the genome of *P. denitrificans* strain Pd1222, DNA fragments carrying the *qhpN* (N = F, R, or G) genes were amplified by PCR using a sense primer containing an NdeI site at the 5′-terminus and an antisense primer containing a BamHI site at the 3′-terminus (Table S1 of the Supporting Information), with Pd1222 genomic DNA as a template, and cloned into the pGEM-T Easy vector (Promega). The constructs were sequenced to confirm the presence of the introduced restriction sites and the absence of undesirable mutations. Each of the *qhpFRG* genes obtained by NdeI and BamHI digestion was cloned into either pRK-P_{A800} (for *qhpF* and *qhpG*) or pRK-P_{R600C} and pRK-P_{weak} (for *qhpR*). Plasmid pRK-P_{A800} is derived from a broad-host-range, multicopy plasmid, pRK415-1,[17] harboring a tetracycline-resistant gene for plasmid maintenance and the *n*-butylamine inducible promoter located within ∼800 bp 5′ of the *qhpA* (*ORF1*) gene of *P. denitrificans* (previously designated Pbau).[9] Plasmids pRK-P_{R600C} and pRK-P_{weak} are also derived from pRK415-1 and contain the original promoter located within ∼600 bp 5′ of the *qhpR* gene of *P. denitrificans* (in the complementary strand) and a weak constitutive promoter contained in pET-11a, respectively; for construction of these plasmids, see Promoter Assay. The plasmids thus constructed were designated pRK-P_{A800}-QhpF, pRK-P_{A800}-QhpG, pRK-P_{R600C}-QhpR, and pRK-P_{weak}-QhpR (Figure S2 of the Supporting Information). Transformation of the gene-disrupted mutant strains PdΔqhpF, PdΔqhpG, and PdΔqhpR with these plasmids was conducted by diparental mating using *E. coli* S17-1 as donor cells or by electroporation.

Site-Specific Mutagenesis. Two conserved residues (Asp500 and Glu501) in the presumed ATP-binding domain (ABD) of QhpF [identified as an efflux ATP-binding cassette (ABC) transporter as described later (see Figures S3A and S4 of the Supporting Information)] were mutated, either individually or simultaneously, into Asn and Gln residues, respectively, by PCR-based site-directed mutagenesis using primers listed in Table S1 of the Supporting Information. Codon replacements were confirmed by sequencing the entire coding regions to eliminate PCR-derived errors, if any. The resulting plasmids, which were designated pRK-P_{A800}-$QhpF^{D500N}$, pRK-P_{A800}-$QhpF^{E501Q}$, and pRK-P_{A800}-$QhpF^{D500N/E501Q}$, were used to transform the *qhpF* gene-disrupted mutant strain PdΔqhpF by diparental mating as described above.

Promoter Assay. Promoter activity of the *qhp* operon was measured by the *β*-galactosidase assay method[18] using *o*-nitrophenyl *β*-D-galactopyranoside (ONPG) as a substrate. To construct plasmids carrying a promoter region fused to the *lacZ* gene, the *lacZ* gene was amplified from the genome of *E. coli* BL21(DE3) by colony PCR using primers containing NdeI and BamHI restriction sites (Table S1 of the Supporting Information). The PCR product was partially digested with NdeI (maintaining the NdeI site in *lacZ*) and BamHI and inserted into the NdeI and BamHI site of vector pET-11a. After elimination of the NdeI and EcoRI sites in the resulting plasmid (at nucleotides 2971 and 3019, respectively, in *lacZ*) by site-directed mutagenesis, the NdeI−HindIII fragment containing the *lacZ* gene and T7 terminator was inserted into pRK-P_{A800}-ORF3 (a modified pKO30,[9] from which the intrinsic NdeI site of pRK415-1 had been eliminated by site-directed mutagenesis), replacing the *qhpC* (*ORF3*) gene, to yield pRK-P_{A800}-lacZ (Figure S2 of the Supporting Information). Other plasmids (pRK-P_{A200}-lacZ, pRK-P_{G200C}-lacZ, pRK-P_{R600C}-lacZ, and pRK-P_{weak}-lacZ) for promoter assays were similarly constructed by replacing the promoter region (the NdeI−EcoRI fragment) with other fragments (suffix "C" denotes the 5′ to 3′ direction of the complementary strand). Nucleotide sequences of the inserted promoter regions are given, with explanatory comments, in Table S2 of the Supporting Information. P_{weak}, which was used as a negative control, is derived from the T7 promoter contained in pET-11a, which is expected to behave as a weak constitutive promoter without activating gene expression in Pd1222 cells carrying no T7 RNA polymerase gene. All the plasmids thus constructed were transformed into the wild-type Pd1222 and mutant PdΔqhpR strains by diparental mating as described above. Promoter activities were determined by ONPG assays and expressed as Miller units,[18] calculated using the formula $1000 \times A_{420}/(t \times V \times OD_{600})$, where A_{420}, t, V, and OD_{600} are the absorbance at 420 nm of *o*-nitrophenol generated from ONPG, the reaction time in minutes, the reaction volume in milliliters, and the cell density measured at 600 nm, respectively.

Database Analysis. Homology searching was performed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) against the database of nonredundant protein sequences (protein BLAST) and the Position-Specific Iterated BLAST (PSI-BLAST) or Domain Enhanced Lookup Time Accelerated BLAST (DELTA-BLAST) algorithm, available at http://blast.ncbi.nlm.nih.gov/Blast.cgi. To search for functionally distinct homologues of QhpFRG proteins (those not directly related to QHNDH), protein BLAST was conducted for each of the QhpFRG proteins with the database specified as Protein Data Bank (PDB) and/or UniProtKB/Swiss-Prot. When a homologue with a known three-dimensional structure was found, a structure-based search was then conducted with this homologue as a query sequence using the Dali (distant matrix alignment) server.[19] Protein motifs were searched for in the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database (http://www.genome.jp/kegg/ko.html), and annotation of each gene was assigned according to the most common annotation in the Gene Function Identification Tool (GFIT) table obtained from the KO database search. Prediction of promoters, terminators, ribosomal binding sites, and transcription factor binding sites (TFBSs) in prokaryotes was conducted with a web-based regulon mining system [Prediction of Prokaryote Promoter Elements and Regulons (PePPER)].[20]

Other Methods. Preparation of periplasmic and cytoplasmic fractions of *P. denitrificans* Pd1222 cells, QHNDH activity and protein assays, sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), Western blotting, and quinone staining were performed as described previously.[9,10]

RESULTS AND DISCUSSION

Renaming of the QHNDH Operon and Identification of Other Conserved Genes. As a result of cloning and sequencing the genes encoding QHNDH in *Ps. putida* and *P. denitrificans*,[5,6] we found that the structural genes encoding the QHNDH subunits constitute an operon that we would have preferred to name "*bau*" because of the involvement of the enzyme in butylamine (or benzylamine) utilization by the bacterium. However, *bau* had already been used for the genes involved in the acinetobactin-mediated process of iron acquisition in *Acinetobacter baumannii*.[21] In our subsequent studies of the roles of the genes in the QHNDH operon,[9,10] we therefore employed the terminology "*ORF*" with a number to specify each gene in the operon. However, because the numbering system cannot be applied to homologous genes from other bacteria in which the gene order is different, here we propose that the operon be renamed "*qhp*", which has been used occasionally for annotations of QHNDH homologue genes (it is probably an abbreviation of quinohemoprotein).[11] Accordingly, the genes encoding the *α*, *β*, and *γ* subunits of QHNDH are here renamed *qhpA* (*ORF1*), *qhpB* (*ORF4*), and *qhpC* (*ORF3*), respectively (Figure 1A). The product of *qhpD* (*ORF2*) is the radical SAM protein that is needed for the formation of intrapeptidyl thioether cross-links in the *γ* subunit,[9] although it was also called "*qhpX*" as a putative SAM radical-dependent activating subunit of QHNDH. The product of *qhpE* (*ORF5*) is a subtilisin-like serine protease that cleaves the leader peptide of the *γ* subunit in a nearly disposable manner.[10] Both *qhpD* and *qhpE* are thus essential for QHNDH biogenesis. Another name, "*pea*", has also been given to some of the QHNDH subunit genes and associated genes in bacterial genome databases, based on their involvement in the utilization of 2-phenylethylamine[22] [*peaA* for *qhpA* (*α* subunit), *peaB* (encoding a putative QHNDH modification protein) for *qhpD*, *peaC* for *qhpC* (*γ* subunit), and *peaD* for *qhpB* (*β* subunit)], but *qhp* may be preferable to *pea* because of the enzyme's ability to degrade not only 2-phenylethylamine but also other amines, including *n*-butylamine and benzylamine.

Besides *qhpD* and *qhpE*, we observed that surrounding the *qhp* genes of *P. denitrificans* there are three additional ORFs[10] that are also highly conserved in numerous bacteria, as described below (Table 1). The gene termed *qhpF* (*ORF6*) that follows *qhpE* encodes a protein identified as an efflux ABC transporter, on the basis of the results of the BLAST search and the most common annotation in the GFIT table obtained from the KO database. Other frequent annotations for the *qhpF* gene product QhpF are ABC-type multidrug transporter and lipid A export permease. The *qhpF* gene is also occasionally called *peaH* because it encodes a 2-phenylethylamine uptake protein, PeaH. In the protein sequence resulting from translation of *qhpF*, motifs such as ABC transporter transmembrane and ATP-binding regions are identifiable (Figure S3A of the Supporting Information).

The two genes named *qhpR* and *qhpG* are located 3′ of *qhpF* and 5′ of *qhpA*, respectively, in the complementary strand of

the genome in *P. denitrificans* strain Pd1222 (Figure 1A). On the basis of the BLAST search results and annotation in the GFIT table, *qhpR* encodes an AraC family transcriptional regulator (hence the name *qhpR*) with helix−turn−helix DNA-binding motifs in the C-terminal half (Figure S3B of the Supporting Information), and *qhpG* encodes an FAD-dependent monooxygenase with an N-terminal FAD-binding motif (originally annotated as a PimS2 protein) (Figure S3C of the Supporting Information). The *qhpR* and *qhpG* genes have also been named *peaR* and *peaF* on the grounds that they encode a Fis family transcriptional regulator, PeaR, and a 2-phenylethylamine degradation protein, PeaF, respectively.[22] The *qhp* genes of this locus in chromosome 1 of *P. denitrificans* Pd1222 are arranged in the order *GADCBEFR* (Figure 1A), and the *qhpADCBEF* and *qhpG* genes are transcribed individually under the common control of QhpR, as described below.

Approximately 1200 bp 3′ downstream of *qhpF*, another enzyme, annotated as betaine aldehyde dehydrogenase, is encoded in the *P. denitrificans* Pd1222 genome (accession no. Pden_1710); it belongs to an aldehyde dehydrogenase superfamily, and BLAST searching shows it to be highly conserved among bacteria possessing the *qhp* genes (not shown). Because one of the reaction products of QHNDH is an aldehyde that is further utilized as a carbon source, it is not surprising that the aldehyde dehydrogenase gene is conserved along with the *qhp* genes among those bacteria that assimilate amines as energy and carbon sources. Although the aldehyde dehydrogenase gene appears to be regulated in a manner similar to that of the *qhp* genes by QhpR, as described later, it is presumably not directly relevant to QHNDH biogenesis and is therefore not considered further in this paper.

***qhpADCBEF* Genes Constitute a Hexacistronic Operon.** Reverse transcription PCR analysis using primers specific for coding regions of *qhpADCBEF* genes was conducted to examine whether the *qhpADCBEF* genes constitute a hexacistronic operon. As shown in Figure 1B, when regions spanning two (*qhpA-qhpD*, *qhpB-qhpE*, and *qhpE-qhpF*) or three (*qhpD-qhpC-qhpB*) genes were amplified from the cDNA of the transcripts, their sizes corresponded closely to those amplified from the genomic DNA by colony PCR. Moreover, nucleotide sequence analysis by PePPER indicated that at a position 137 bp 3′ of the *qhpF* gene, a 10 bp inverted repeat (IR) ($\Delta G = -4.1$ kcal/mol) is followed by a stretch of 15 AT-rich nucleotides (*vide infra*), suggesting that this region can form a rho-independent transcriptional terminator.[23] At ∼200 bp 5′ of *qhpA*, where the transcriptional regulator QhpR presumably binds, there is also an IR that may function as an AraC-like transcriptional promoter. Collectively, these observations strongly suggest that the *qhpADCBEF* genes are transcribed continuously as a hexacistronic operon.

Effect of Gene Disruption on Bacterial Growth and QHNDH Activity. To investigate the roles of the peripheral genes newly identified in the *qhp* operon and to determine whether they are essential for the production of QHNDH, we disrupted each of them by homologous recombination. The gene-disrupted *P. denitrificans* mutant strains PdΔqhpF, PdΔqhpR, and PdΔqhpG were cultivated in minimal mineral medium containing *n*-butylamine as the sole carbon and energy source. In marked contrast with the rate of growth of the wild-type strain Pd1222 in this medium, none of the mutant strains grew well unless 20 mM choline chloride, which supports *n*-butylamine-independent growth of *P. denitrificans*,[14−16] was added (Figure 2A). After cultivation for 36 h in the *n*-butylamine-containing medium, the wild-type Pd1222 cells showed high QHNDH activity, whereas those of PdΔqhpF, PdΔqhpR, and PdΔqhpG exhibited no QHNDH activity even when their growth was supported by the addition of choline (Figure 3). These results suggest that, in addition to the structural genes encoding QHNDH subunits (*qhpABC*) and

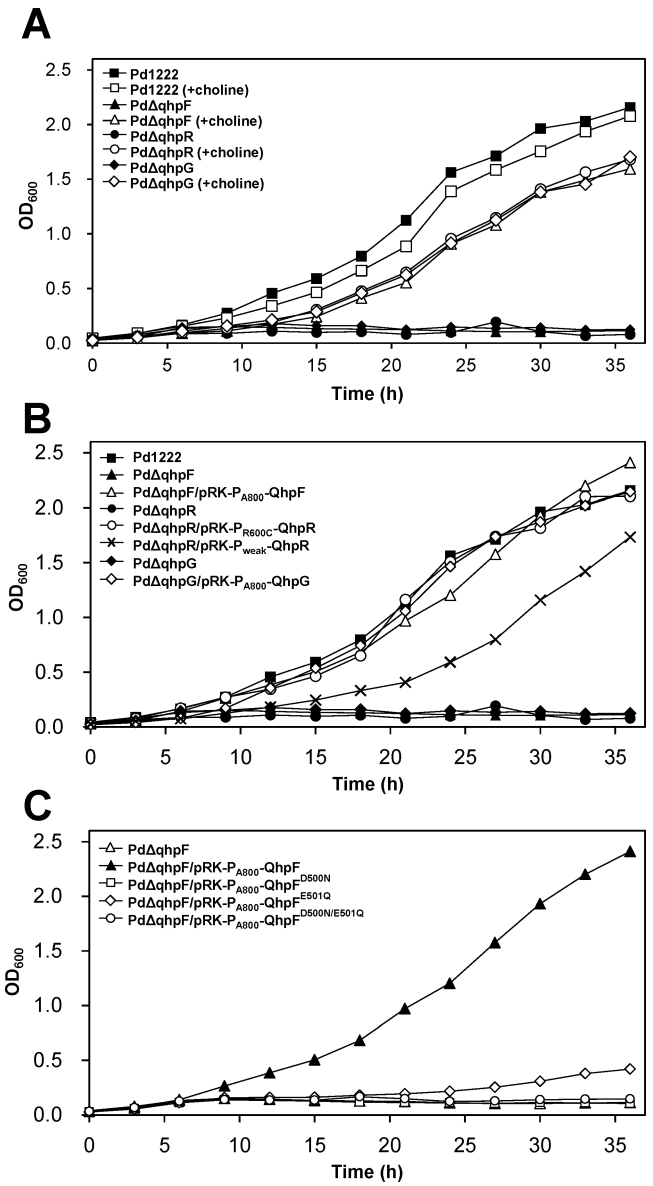


Figure 2. Growth of wild-type and *qhp* gene-disrupted mutant cells of *P. denitrificans* Pd1222. Each bacterial strain was grown in minimal medium supplemented with *n*-butylamine. Cell densities measured using optical density at 600 nm were plotted against culture time. (A) Growth of wild-type Pd1222 in the absence (■) or presence (□) of choline, PdΔqhpF in the absence (▲) or presence (△) of choline, PdΔqhpR in the absence (●) or presence (○) of choline, and PdΔqhpG in the absence (◆) or presence (◇) of choline. (B) Growth of wild-type Pd1222 (■), PdΔqhpF alone (▲), or PdΔqhpF transformed with pRK-P_{A800}-QhpF (△), PdΔqhpR alone (●) or PdΔqhpR transformed with pRK-P_{R600C}-QhpR (○) or pRK-P_{weak}-QhpR (×), and PdΔqhpG alone (◆) or PdΔqhpG transformed with pRK-P_{A800}-QhpG (◇). (C) Growth of PdΔqhpF alone (△) or PdΔqhpF transformed with pRK-P_{A800}-QhpF (▲), pRK-P_{A800}-$QhpF^{D500N}$ (□), pRK-P_{A800}-$QhpF^{E501Q}$ (◇), or pRK-P_{A800}-$QhpF^{D500N/E501Q}$ (○).

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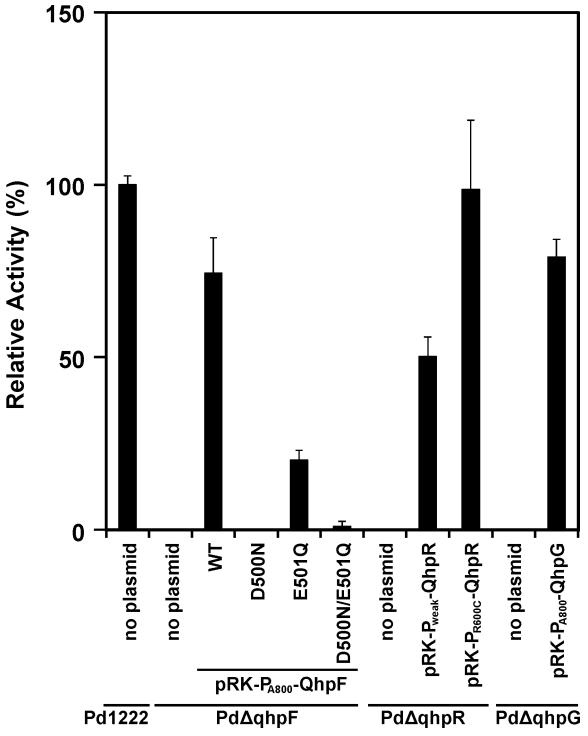

Figure 3. QHNDH activity in the periplasmic fraction of the cells. The wild-type Pd1222 and gene-disrupted mutant strains alone (no plasmid) and those transformed with the indicated plasmid carrying wild-type (WT) or mutant genes (for pRK-P_{A800}-QhpF) were cultured for 36 h in minimal mineral medium containing *n*-butylamine. To support growth of the *qhp* gene-disrupted mutant cells, 20 mM choline chloride was added to the culture medium. QHNDH activities are shown as relative values compared to that of wild-type Pd1222 cells (100%). Each bar represents the mean ± the standard error from two independent experiments.

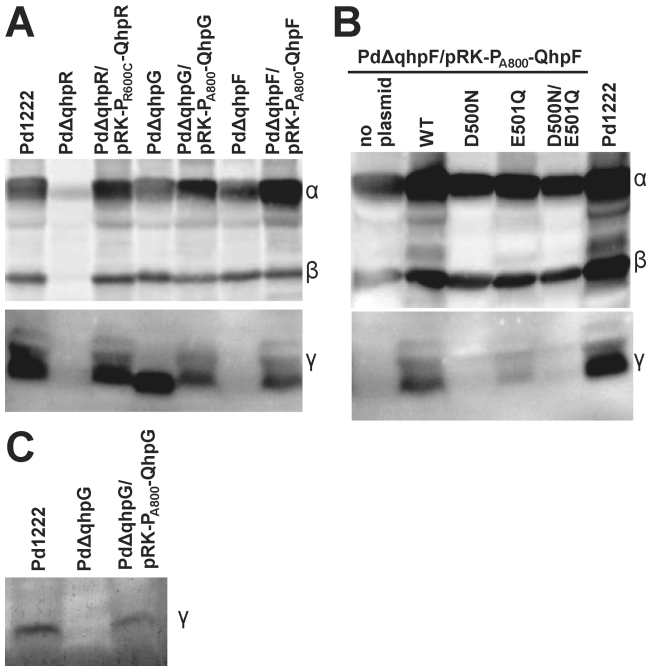

Figure 4. Detection of QHNDH subunits. (A) The α/β and γ subunits in the periplasmic fraction of Pd1222 and PdΔqhpR, PdΔqhpG, and PdΔqhpF strains with or without a plasmid, as indicated, were detected by Western blotting with an anti-QHNDH antibody (top) and an anti-γ subunit antibody (bottom), respectively. Proteins extracted from 10 mg of cells (wet weight) were loaded in each lane. Minor bands observed between the bands corresponding to the α and β subunits are probably derived from degradation of the α subunit. (B) α/β and γ subunits in the periplasmic fraction of Pd1222 and PdΔqhpF strains with or without a plasmid, as indicated, for expression of wild-type (WT) and mutant (D500N, E501Q, and D500N/E501Q) QhpF proteins. The α/β and γ subunits were detected by Western blotting with an anti-QHNDH antibody (top) and an anti-γ subunit antibody (bottom), respectively. Proteins extracted from 10 mg of cells (wet weight) were loaded in each lane. (C) QHNDH was partially purified by anion exchange column chromatography[4] from cells of Pd1222, PdΔqhpG, and PdΔqhpG transformed with pRK-P_{A800}-QhpG and was stained for the presence of a redox active quinone group after SDS−PAGE (total proteins applied in each lane, ~50 μg).

those required for posttranslational modification of the γ subunit (*qhpDE*), the *qhpFGR* genes are also indispensable for the production of active QHNDH, which catalyzes the oxidative deamination of *n*-butylamine in the periplasm so that it can be assimilated as a sole carbon and energy source.

Western blot analysis using antibodies against the α/β and γ subunits of QHNDH indicated that the amounts of α, β, and γ subunit proteins produced in the periplasmic fractions were similar in the wild-type Pd1222 strain and in PdΔqhpG cells grown in the *n*-butylamine-containing medium supplemented with choline, although the position of the stained band of the γ subunit on the gel is slightly different (Figure 4A). In contrast, in the PdΔqhpR cells, the three subunits were almost undetectable. Interestingly, in the PdΔqhpF cells, both α and β subunit proteins were detected in the periplasm but the γ subunit protein was not. By comparison with the QHNDH activity data shown in Figure 3, these results indicate that the α, β, and γ subunits of QHNDH are produced in the PdΔqhpG cells, as in the wild-type Pd1222 cells, but form an inactive QHNDH complex, whereas they are either not produced at all or degraded very rapidly in the PdΔqhpR cells, and the γ subunit is not translocated into the periplasm of the PdΔqhpF cells, observations consistent with the hypothesis that the products of these genes have distinct functions, as discussed later.

Rescue of Gene-Disrupted Mutants. We next performed plasmid complementation of the disrupted genes to examine whether, in each case, this could rescue bacterial growth and production of active QHNDH. The gene-disrupted mutant strains PdΔqhpF, PdΔqhpR, and PdΔqhpG were transformed with pRK-P_{A800}-QhpF, pRK-P_{R600C}-QhpR, and pRK-P_{A800}-QhpG, respectively. To examine the effect of a weak constitutive level of *qhpR* expression, PdΔqhpR was also transformed with pRK-P_{weak}-QhpR. Both the level of growth in the *n*-butylamine-containing minimal medium (Figure 2B) and QHNDH activity after culture for 36 h (Figure 3) were significantly increased by plasmid complementation in all mutant strains. Using Western blot analysis, the α, β, and γ subunits of QHNDH could also be detected in the periplasmic fractions of all mutant strains (Figure 4A). These results led us to conclude that all of the *qhpFRG* genes are necessary for bacterial growth in the medium with *n*-butylamine as the sole carbon and energy source, most likely because they participate in the production of enzymatically active QHNDH. It should be noted that in the constructs used for plasmid complementation, the *qhpF* and *qhpG* genes were placed 3′ of the *n*-butylamine inducible promoter (P_{A800}) and the *qhpR* gene 3′ of

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its original promoter that is located within ~600 bp 5′ of *qhpR* and is in the complementary strand (P_{R600C}) (Table S2 of the Supporting Information), on the assumption that expression of the genes from the plasmids would be controlled in a manner similar to that in the genome of *P. denitrificans* [although the *qhpG* gene is regulated in the opposite direction vs that of the *qhpADCBEF* genes (see below)]. Remarkably, even the *qhpR* gene that was placed 3′ of a weak constitutive promoter (P_{weak}) could rescue growth in the PdΔqhpR mutant, although not as effectively as under the native promoter (P_{R600C}) (Figure 2B), indicating that the *qhpR* gene product serves as a master transcriptional activator for the expression of the other *qhp* genes.

Putative Role of QhpF as a Periplasmic Translocater of the γ Subunit. Our previous nucleotide and amino acid sequence analyses revealed that both the α subunit and the β subunit of QHNDH have N-terminal flanking signal sequences, which are assumed to direct translocation of the polypeptides into the periplasm by the general Sec or Tat translocon.[5,6] However, the γ subunit has no signal sequence, although it has a 28-residue N-terminal leader peptide that is necessary for the production of active QHNDH but must be removed in the subsequent maturation process by the subtilisin-like serine protease encoded by the *qhpE* gene.[10] Thus, the mechanism of periplasmic translocation of the γ subunit, which lacks a signal peptide, remains to be elucidated.

As described above, the *qhpF* gene encodes a protein annotated as an efflux ABC transporter. In addition to the QhpF homologues that are conserved with the full set of *qhp* genes summarized in Table 1, two bacterial multidrug ABC transporters, *E. coli* MsbA (PDB entry 3B5W)[24] and *Staphylococcus aureus* Sav1866 (PDB entry 2HYD),[25] were found to show marked sequence similarities with QhpF, both sharing 24% identities, when a BLAST search was conducted with the database limited to PDB. Furthermore, the modeled structures of human multidrug resistance-associated protein 1 (MRP1/ABCC1)[26] and cystic fibrosis transmembrane conductance regulator (CFTR; alternative name, cAMP-dependent chloride channel)[27] were also found to show moderate structure-based sequence similarities to *qhpF* (Figure S4 of the Supporting Information). From the point of view of structural similarity of the substrates being transported, the *Bacillus subtilis* ABC transporter AlbCD (=YwhQP[28]), which is believed to export an extracellular bacteriocin subtilosin A, a 35-residue head-to-tail cyclized peptide with three intrapeptidyl thioether cross-links,[29] is the closest to QhpF [which probably translocates the cross-linked γ subunit of QHNDH (see below)], and these two proteins share ~20% sequence identity in the ABD portion of the two-component protein AlbCD.

To examine whether the QhpF protein actually functions as an ABC transporter, the two highly conserved residues (Asp500 and Glu501) in the presumed ABD of QhpF (Figure S4 of the Supporting Information) were mutated, either individually or simultaneously, into Asn and Gln residues, respectively, and tested using plasmid complementation of the mutant strain PdΔqhpF. As shown in Figure 2C, the rate of growth of PdΔqhpF in the *n*-butylamine medium was either not increased at all or only slightly increased by transformation with the mutant plasmids. QHNDH activities in cells grown in the *n*-butylamine medium supplemented with choline were also undetectable or very low (Figure 3), demonstrating that the two acidic residues Asp500 and Glu501, which are highly conserved in the presumed ADB region of QhpF, are very important for the function of this protein, as reflected in either rescue of bacterial growth or QHNDH enzyme activity. As observed for PdΔqhpF cells, Western blot analysis revealed the absence of the γ subunit from the periplasm of PdΔqhpF cells transformed with either pRK-P_{A800}-$QhpF^{D500N}$, pRK-P_{A800}-$QhpF^{E501Q}$, or pRK-P_{A800}-$QhpF^{D500N/E501Q}$, in marked contrast to the α and β subunits, which were detected at normal levels in the periplasm (Figure 4B). In the multidrug ABC transporter MsbA of *E. coli*,[24,30] ATP binding and hydrolysis have been reported to be coupled with conformational changes in the transmembrane domain of the transporter, which facilitate translocation of the substrate from the cytoplasm to the periplasm. When mutations were introduced into the conserved residues in the ABD region of QhpF, the γ subunit protein could not be transported into the periplasm. On the basis of these findings, it is very likely that QhpF serves as an efflux ABC transporter for translocation of the γ subunit of QHNDH into the periplasm. Although it remains to be determined whether QhpF is able to distinguish the cross-linked γ subunit from that without cross-links, the γ subunit that is about to be transported by QhpF has presumably already undergone multiple cross-linkings by the cytoplasmic radical SAM protein QhpD (ORF2)[9] and subsequent cleavage of the N-terminal leader peptide by the subtilisin-like protease QhpE (ORF5).[10] It may be necessary that the *qhpF* gene be transcribed and translated in the same hexacistronic operon as other *qhp* genes (as shown above) to ensure swift and specific translocation into the periplasm of the γ subunit, which is produced in large amounts, together with the α and β subunits, upon induction with an amine.

QhpG Is Involved in Quinone Cofactor Formation. The *qhpG* gene-disrupted mutant PdΔqhpG showed no QHNDH activity (Figure 3). However, all three of the QHNDH subunit proteins were produced in the periplasmic fraction (Figure 4A) when the growth of the PdΔqhpG mutant was stimulated by the addition of choline to the culture medium. The reason for the presence of QHNDH subunits lacking enzyme activity in the mutant cells may be the absence of the quinone cofactor from the γ subunit (Figure 4C), as revealed by the redox cycling staining method developed for quinoproteins.[31] Plasmid complementation of the *qhpG* gene restored bacterial growth (Figure 2B), QHNDH activity (Figure 3), and quinone staining (Figure 4C), showing unequivocally that the QhpG protein is involved in the formation of the quinone cofactor. The slight difference in the position of the stained band of the γ subunit shown by Western blotting (Figure 4A) is also suggestive of the absence of CTQ from the γ subunit in the PdΔqhpG mutant cells. It is also noteworthy that the γ subunit produced by PdΔqhpG cells gives only a single band on a Western blot, whereas the periplasmic fractions of wild-type Pd1222 and plasmid-rescued mutant strains show multiple (apparently three) bands for the γ subunit on a stained blot, although quinone detection reveals only one band. Fujieda et al. reported that a silent (inactive) form of QHNDH was produced alongside active enzyme in *P. denitrificans* grown in a medium containing *n*-butylamine.[32] The silent form of QHNDH was shown to contain an oxime (C6═NOH) of CTQ, which may be formed by reaction with hydroxylamine in the cells and can be slowly reactivated by incubation with amine substrates. It is therefore very likely that the multiple γ subunit bands shown by Western blot analysis can be explained, at least in part, by the presence of the γ subunit containing the CTQ-oxime, which is

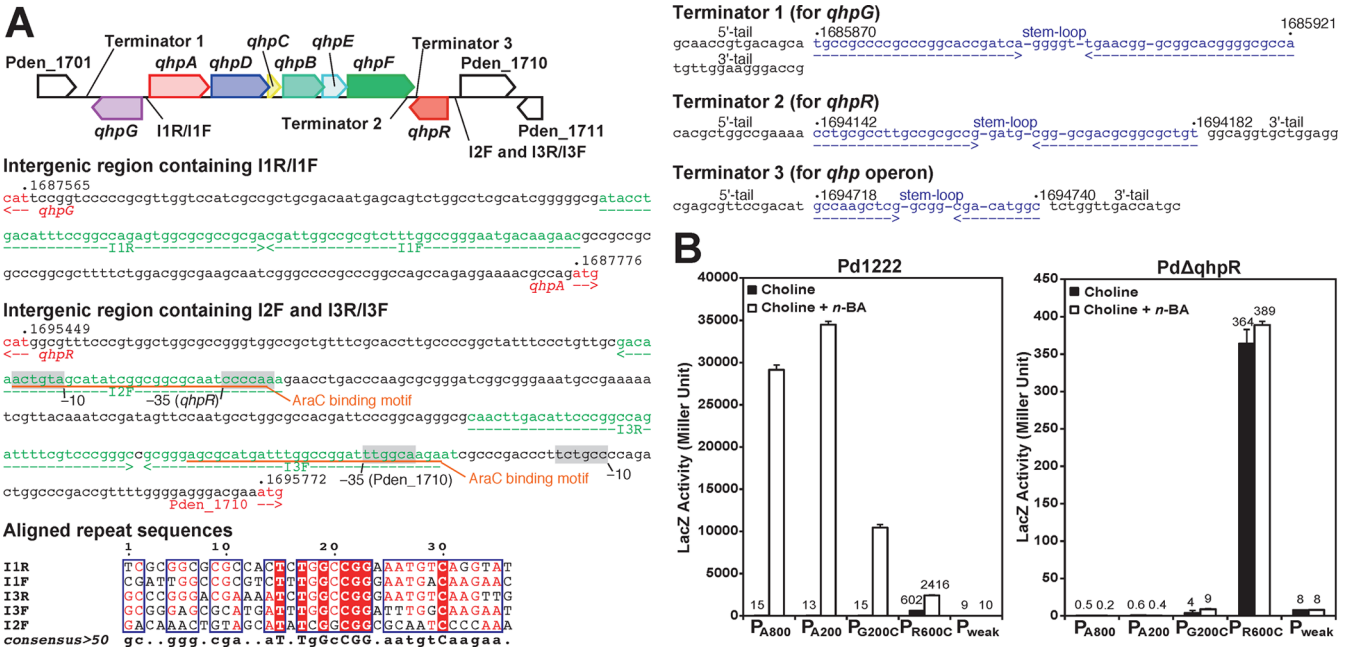

Figure 5. Identification of terminator, promoter, and TFBS sequences, and measurement of promoter activities. (A) Nucleotide sequences of possible terminators, promoters, and TFBSs (AraC-binding motifs) identified by PePPER are shown below the *qhp* operon structure. The seven-digit numbers denote the nucleotide number, in Pd1222 chromosome 1, of the nucleotide marked with a dot above it. Aligned IR (I1R/I1F and I3R/I3F) and single-repeat (I2F) sequences are also shown at the bottom. (B) Promoter activities assayed by the ONPG method. The wild-type Pd1222 and mutant PdΔqhpR strains transformed with plasmids carrying the *lacZ* gene fused 3′-downstream of the promoters indicated were cultured at 30 °C for 24 h in minimal mineral medium supplemented with 20 mM choline (black bars) or 20 mM choline with 0.5% (w/v) *n*-butylamine (white bars). After the cells had been collected by brief centrifugation, they were permeabilized by chloroform/SDS treatment[18] and LacZ activities in the cell extracts were measured with ONPG as the substrate. Each bar represents the mean ± the standard error from two independent experiments.

presumably formed after the biogenesis of active QHNDH has been completed in the periplasm.

As already described, BLAST analysis indicated that QhpG is a flavin-dependent monooxygenase. More specifically, BLAST searching against a data set limited to the PDB identified the myxobacterial chondrochloren halogenase CndH (PDB entry 3E1T)[33] as being homologous to QhpG (23% identical). Using CndH as a reference structure, the *Streptomyces venezuelae* chloramphenicol halogenase CmlS (PDB entry 3I3L),[34] *p*-hydroxybenzoate hydroxylase from *Ps. fluorescens* (PDB entry 1CC4),[35] and kynurenine 3-monooxygenase from *Saccharomyces cerevisiae* (PDB entry 4J33)[36] were found to be structurally homologous to QhpG; analysis through the Dali server gave high *Z* scores of 43.8, 34.7, and 30.1, respectively, for the three proteins. All these proteins belong to a large family of flavoprotein monooxygenases.[37] We also found that the flavoprotein LodB from the marine bacterium *Marinomonas mediterranea* shows moderate sequence similarity to QhpG (19% identical). It has recently been reported that LodB is involved in the activation of the newly identified CTQ-containing enzyme LodA (L-lysine ε-oxidase);[38] in the absence of LodB, LodA does not contain the quinone cofactor and remains in an inactive form.[39] In the structure-based sequence alignment of these proteins (Figure S5 of the Supporting Information), Lys76 of CndH, an essential residue for halogenation that is highly conserved in halogenases,[33] is not conserved in any of the hydroxylases, or in QhpG or LodB, suggesting that QhpG (and also LodB) can be categorized as an FAD-dependent hydroxylase (monooxygenase) but not a halogenase.

Although the mechanism of the biosynthesis of the CTQ cofactor of QHNDH is largely unknown, QhpG may catalyze the initial hydroxylation of the indole ring of the CTQ precursor Trp43 in the γ subunit to form a hydroxytryptophan intermediate, acting as a peptidyl tryptophan monooxygenase. It appears likely that QhpG functions in the cytoplasm, as its N-terminal region lacks a signal sequence, which would be needed for periplasmic translocation. In a preliminary experiment, we found that QhpG expressed in *E. coli* contained FAD and interacted with the γ subunit only if it had thioether cross-links (T. Nakai and T. Okajima, unpublished data). It has been reported that, in the biogenesis of the tryptophan tryptophylquinone (TTQ) cofactor of methylamine dehydrogenase (MADH), a partially assembled cofactor (pre-TTQ) with a monohydroxylated βTrp57 at C7 of the indole ring is initially formed.[40] Although the enzyme catalyzing the hydroxylation of the peptidyl tryptophan in pre-MADH is unknown at present, the hydroxytryptophan intermediate is subsequently converted into the mature cofactor TTQ by the diheme protein MauG, which is encoded in a region of the genome near the genes for MADH subunits.[41] By analogy with this process of TTQ biogenesis in MADH, the final oxidation to CTQ of the hydroxytryptophan intermediate (pre-CTQ) produced by QhpG in QHNDH may be catalyzed by the two *c*-type hemes that are contained within the α subunit, presumably after the periplasmic formation of a complex between the cross-linked γ subunit and the α subunit with its two hemes. As described below, the *qhpG* gene, which is encoded in the complementary strand, is transcribed independently of other *qhp* genes, but its expression is, like theirs, controlled by QhpR.

Identification of AraC-like Promoters and Role of QhpR as a Transcriptional Activator. We searched for promoters, terminators, ribosomal binding sites, and TFBSs in the noncoding and coding regions around the *qhp* genes using a

dx.doi.org/10.1021/bi401625m | *Biochemistry* 2014, 53, 895−907

web-based regulon mining system, PePPER. Three regions were identified as candidates for possible rho-independent transcriptional terminators:[23] a 10 bp IR at 137 bp 3′ of *qhpF* [described above (labeled Terminator 3 in Figure 5A)], a 15 bp IR (ΔG = −6.6 kcal/mol) at 271 bp 3′ of *qhpR* in the complementary strand (Terminator 2), and a 22 bp IR (ΔG = −12.9 kcal/mol) at 378 bp 3′ of *qhpG* in the complementary strand (Terminator 1); all were followed by a stretch of 15 AT-rich nucleotides (Figure 5A). These findings are consistent with the prediction that the *qhpADCBEF*, *qhpG*, and *qhpR* genes are transcribed independently. Also shown in Figure 5A are two IRs (I1F/I1R and I3F/I3R) and a single consensus sequence (I2F) identified by PePPER, which may be able to function as an AraC-like transcriptional promoter. In addition, two presumed TFBSs were found at I2F and I3F.

To evaluate the promoter activities of these regions, ONPG assays were conducted using the following *lacZ*-fused DNA fragments: P_{A800}, P_{A200}, P_{G200C}, and P_{R600C}, covering 800 bp 5′ of *qhpA*, 200 bp 5′ of *qhpA*, 200 bp 5′ of *qhpG* (complementary strand), and 600 bp 5′ of *qhpR* (complementary strand), respectively. As a negative control, P_{weak} was also fused with *lacZ*. Wild-type Pd1222 and PdΔqhpR mutant cells were transformed with these plasmids, cultured at 30 °C for 24 h in minimal mineral medium containing 20 mM choline or choline with 0.5% (w/v) *n*-butylamine, and subjected to the ONPG assay. As expected, no induction by *n*-butylamine was observed in the PdΔqhpR cells carrying these plasmids (Figure 5B), clearly showing that QhpR is the transcriptional activator that responds to induction by the amine. P_{R600C} showed a slightly higher promoter activity in the wild-type Pd1222 cells than in the PdΔqhpR cells even in the absence of the inducer (i.e., there was constitutive activity), suggesting that QhpR does not act as a transcriptional repressor. Also, P_{R600C} in the wild-type cells showed higher activity in the presence of *n*-butylamine than in its absence, suggesting that QhpR may be self-inducible. P_{A200} in the wild-type cells showed even higher *n*-butylamine-inducible activity than P_{A800}, indicating that the promoter for the *qhpADCBEF* operon is located within 200 bp 5′ of the *qhpA* gene. Moreover, P_{G200C} in the wild-type cells exhibited approximately 30% of the *n*-butylamine-inducible promoter activity of P_{A200}, showing that the same region probably serves as a transcriptional promoter for both the *qhpADCBEF* operon and, in the reverse direction, the *qhpG* gene. Combining these findings with the results of the PePPER analysis, we conclude that QhpR is a transcriptional activator responding to amine induction and that, after changing its conformation upon binding of the inducer, it can activate transcription of the *qhpADCBEF* operon and the *qhpG* gene, by binding to the IR (I1F/I1R) located within P_{A200} (P_{G200C}), as well as that of *qhpR* itself and aldehyde dehydrogenase (Pden_1710) genes by binding to another IR (I3F/I3R) and/or to the possible TFBSs found at I2F and I3F. The consensus sequence (TxTGGCC-GGxxxTGxCAxG, where x is any nucleotide) of these IRs (Figure 5A) may be described as a "QhpR box". Transcriptional activation of the aldehyde dehydrogenase gene by QhpR is advantageous for the bacterium when it is utilizing *n*-butylamine as a carbon source.

BLAST analysis against the UniProtKB/Swiss-Prot database identified two QhpR homologues: OruR (ornithine utilization regulator of *Ps. aeruginosa*)[42] and VqsM (global regulator of quorum-sensing signaling and virulence in *Ps. aeruginosa*),[43] which are 24% and 21% identical with QhpR, respectively. Both OruR and VqsM belong to the AraC/XylS family of transcriptional activators,[44] and QhpR possesses the characteristic features of this family of proteins. Multiple-sequence alignment of these AraC/XylS family proteins, including the *Vibrio cholerae* virulence-related regulator ToxT,[45] which is also an AraC/XylS member, is shown in Figure S6 of the Supporting Information. The mode of transcriptional activation of the *qhpADCBEF*/*qhpG* and *qhpR*/Pden_1710 gene pairs by QhpR, as described above, is very similar to that reported for ToxT, which binds to the centrally located IR between two genes that are transcribed in the opposite direction.[45]

Bacterial Distribution of *qhp* Genes. To study the distribution of the *qhp* genes, a PSI-BLAST search was first performed with QhpC (γ subunit) of *P. denitrificans* Pd1222 (GI: 119384442) as a query sequence; of the 236 bacterial hits (137 organisms) obtained, only those homologues that contain all the residues (Cys7, Glu16, Cys27, Asp33, Cys37, Cys41, Trp43, and Asp49) that are posttranslationally modified in the γ subunit were selected manually (leaving a total of 213 hits), because posttranslational modification of these residues is essential for the formation of the active QHNDH complex. Finally, using the *qhpGADCBEFR* genes of *P. denitrificans* Pd1222 as query sequences, homologous genes in each bacterial species were searched for by BLAST (Table 1). In summary, more than 52 species belonging to the Gram-negative *Proteobacteria* and four species belonging to the Gram-positive *Bacilli* were identified, ∼70% of which contain homologues of the full set of *qhpGADCBEFR* genes. In particular, the four genes *qhpADCB* (and their reverse, *BCDA*, in the complementary strand) are highly conserved as a group in this gene order and without insertions; the only exceptions are one Gram-negative species, *D. toluolica*, and the four species of Gram-positive bacteria belonging to the *Bacilli*. It is also noteworthy that several Gram-negative bacteria possess two sets of the *qhp* genes, encoded either in the same strand or in complementary strands. These findings strongly suggest that the *qhp* genes have evolved by duplication of the core set of *qhpADCB* genes. The *qhpD* gene, encoding the radical SAM enzyme QhpD, which participates in intrapeptidyl thioether cross-link formation in the γ subunit,[9] is as essential as the structural genes (*qhpABC*) that encode QHNDH components. In contrast, the locations of other accessory genes (*qhpEFG*) are somewhat variable among bacteria even within the same class, although they also have essential roles in QHNDH biogenesis, as demonstrated in this study and previous[10] studies.

In the BLAST search, five species of *Proteobacteria* (*Magnetospirillum* sp. SO-1, *Burkholderia* sp. TJI49, *T. phenylacetica* B4P, *D. toluolica* Tol2, and *G. electrodiphilus*) and four species of *Bacilli* (*A. aneurinilyticus* ATCC 12856, *B. azotoformans* LMG 9581, *Brevibacillus* sp. phR, and *Geobacillus thermoglucosidans* TNO-09.020) were found to lack a *qhpG* homologue in the vicinity of the identified qhp gene clusters (Table 1). Similarly, there were several species lacking *qhpEF* homologues: *T. linaloolentis* DSM 12138 had neither *qhpE* nor *qhpF*, *Burkholderia* sp. TJI49 did not have a *qhpE* homologue, and *T. aminoaromatica* S2 showed no *qhpF* homologue. These results were assumed to be a result of incomplete BLAST searching using only one query sequence (*qhpEFG* of *P. denitrificans* Pd1222). Therefore, to identify *qhp* homologues in the genomes of these bacterial species, we further conducted BLAST searches using the target genes of the nearest bacterial species as respective query sequences (e.g., the *qhpG* homologue of *Bu. cepacia* GGR was used as a query sequence

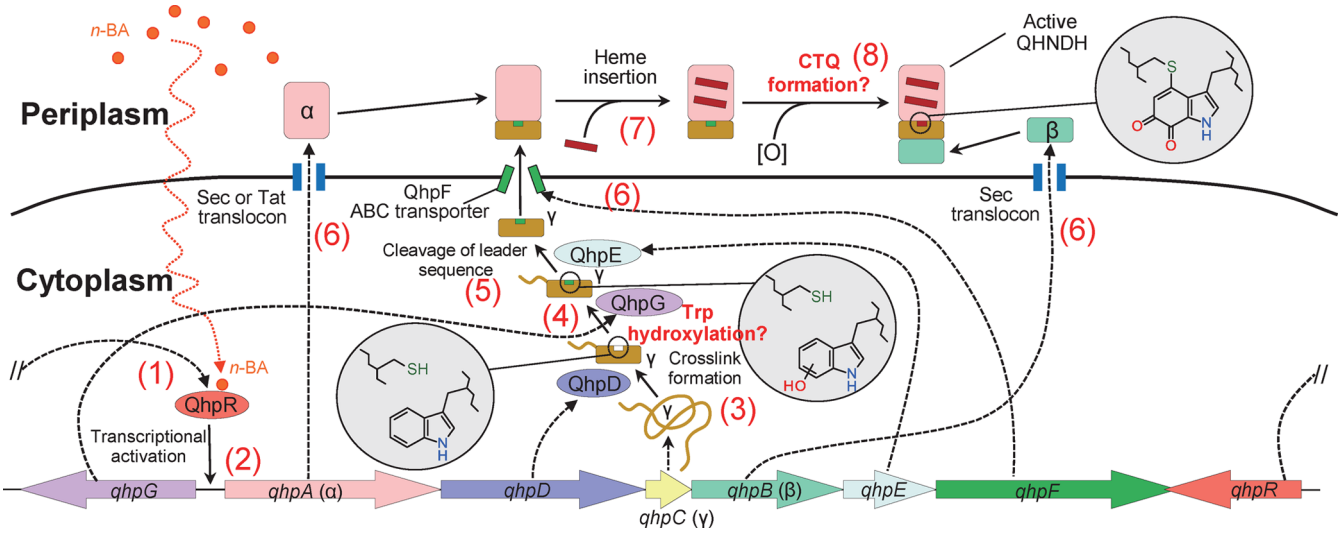

Figure 6. Postulated pathway of QHNDH biogenesis, showing the proteins encoded by *qhp* genes. Numbers in parentheses denote the predicted sequence of the events, as described in the text. *n*-BA stands for *n*-butylamine.

to search for *qhpG* in the genome of *Burkholderia* sp. TJI49). However, we again found no *qhp* homologues with high degrees of sequence similarity, not only in the vicinity of the identified *qhp* clusters but also in the complete genomes, strongly suggesting that the functions of the QhpEFG proteins may be conducted by other proteins in bacteria lacking these genes.

The locations of the *qhpR* genes relative to the *qhpADCB* genes were also quite variable, implying that different regulatory mechanisms controlling gene expression operate in different bacteria. We note that, in the Gram-positive *Bacilli*, QhpR homologues located in the vicinity of QhpC (γ subunit) in the reverse orientation (Table 1) are response regulators in bacterial two-component systems; the cognate sensor histidine kinases are encoded 3′ of these response regulators. It is tempting to speculate that the histidine kinase senses the extracellular presence of an amine and then phosphorylates the partner QhpR protein, which in turn activates the expression of *qhp* genes in a manner similar to that of ToxT. The active QHNDH enzyme thus produced (the mechanism for its biogenesis is as yet unknown) may be secreted from the cells of *Bacilli*, which lack a periplasm, to degrade the extracellular amine.

Proposed Pathway of QHNDH Biogenesis. On the basis of this study and previous studies,[9,10] a possible pathway for QHNDH biogenesis is proposed, as illustrated in Figure 6. The sequence is as follows. (1) Upon induction with *n*-butylamine, transcription of the *qhpADCBEF* operon and *qhpG* is activated by QhpR. (2) QhpA, -D, -C, -B, -E, -F, and -G proteins (and probably QhpR as well) are immediately expressed. (3) Triple intrapeptidyl thioether cross-links are formed within the nascent γ subunit (QhpC) by the radical SAM enzyme QhpD. (4) The FAD-dependent monooxygenase QhpG may catalyze the hydroxylation of Trp43 (CTQ precursor) in the cross-linked γ subunit to form pre-CTQ. The assumption that step 3 precedes step 4 is based on the observations that the *qhpD*-knockout mutant of *P. denitrificans* Pd1222 produced a γ subunit containing neither thioether cross-links nor chemically modified Trp43 (CTQ precursor),[9] and also that recombinant QhpG interacted only with the γ subunit containing thioether cross-links (T. Nakai and T. Okajima, unpublished data). (5) The 28-residue leader sequence of the γ subunit is cleaved off by the subtilisin-like protease QhpE. However, it is currently unclear whether step 4 or 5 occurs first. (6) The α (QhpA), β (QhpB), and γ (QhpC) subunits are translocated into the periplasm, probably through the Sec or Tat translocon (for α and β) and the ABC transporter QhpF (for γ). (7) Two hemes are inserted into the α subunit in the periplasm. (8) Finally, most likely following formation of a heterodimer (αγ) or heterotrimer (αβγ), formation of the CTQ cofactor may be completed with the assistance of the *c*-type diheme contained in the α subunit. However, there is no direct evidence for steps 4 and 6−8 at present. The formation of multiple thioether cross-links in the γ subunit at an early stage of QHNDH biogenesis may be very important in allowing the γ subunit polypeptide, otherwise a featureless coil, to adopt a folded structure. This is probably necessary for interaction with the putative peptidyl tryptophan hydroxylase, QhpG, and also for formation of a complex with the α subunit, with the precursor Trp and Cys residues properly positioned for the subsequent synthesis of CTQ.

In conclusion, we have identified three additional genes essential for the biogenesis of QHNDH. The sophisticated and complex pathway of QHNDH biogenesis presented here, which is found not only in numerous Gram-negative species but also in some Gram-positive bacteria, thus requires a total of eight genes: three encoding the polypeptide subunits of the enzyme and five participating in the posttranslational modification and periplasmic translocation of the γ subunit, and in transcriptional activation of the genes in the pathway.

■ ASSOCIATED CONTENT

Supporting Information

Oligonucleotide sequences, promoter sequences, plasmid constructs, protein motifs, and multiple-sequence alignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

T.N., K.T., and T.O. participated in the design of the research. T.N., T.D., and T.O. conducted experiments. T.N., T.D., K.T., and T.O. performed data analysis. All authors wrote, or contributed to the writing of, the manuscript.

Funding

This work was supported by funding from the Japan Society for the Promotion of Science Grants in Aid for Scientific Research, Challenging Exploratory Research 24658288 (to T.O.) and Category C 23570135 (to T.N.), and by the Operational Program Education for Competitiveness-European Social Fund (Project CZ.1.07/2.3.00/20.0165) (to I.F. and K.T.).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

QHNDH, quinohemoprotein amine dehydrogenase; CTQ, cysteine tryptophylquinone; ORF, open reading frame; SAM, *S*-adenosylmethionine; K_m, kanamycin; LB, Luria broth; MCS, multiple cloning site; ABD, ATP-binding domain; ABC, ATP-binding cassette; ONPG, *o*-nitrophenyl β-D-galactopyranoside; TFBS, transcription factor binding site; IR, inverted repeat; TTQ, tryptophan tryptophylquinone; MADH, methylamine dehydrogenase.

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