Gene cluster of *Arthrobacter ilicis* Rü61a involved in the degradation of quinaldine to anthranilate.

Characterization and functional expression of the quinaldine 4-oxidase genes *gqoxLMS*

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**Running title:** quinaldine catabolic gene cluster of *Arthrobacter ilicis*
SUMMARY

A genetic analysis of the anthranilate pathway of quinaldine degradation was performed. A 23 kb region of DNA from *Arthrobacter ilicis* Rü61a was cloned into the cosmid pVK100. Whereas *E. coli* clones containing the recombinant cosmid did not transform quinaldine, cosmids harboring the 23 kb region, or a 10.8 kb stretch of this region, conferred to *Pseudomonas putida* KT2440 the ability to cometabolically convert quinaldine to anthranilate. The 10.8 kb fragment thus contains the genes coding for quinaldine 4-oxidase (Qox), 1H-4-oxoquinidine 3-monoxygenase, 1H-3-hydroxy-4-oxoquinidine 2,4-dioxygenase, and *N*-acetylanthranilate amidase. The *qoxLMS* genes coding for the molybdopterin cytosine dinucleotide- (MCD-), FeSI-, FeSII-, and FAD-containing Qox were inserted into the expression vector pJB653, generating pKP1. Qox is the first MCD-containing enzyme to be synthesized in a catalytically fully competent form by a heterologous host, *P. putida* KT2440 pKP1; the catalytic properties and the UV/Vis- and EPR-spectra of Qox purified from *P. putida* KT2440 pKP1 were essentially like those of wild-type Qox. This provides a starting point for the construction of protein variants of Qox by site-directed mutagenesis.

Downstream of the *qoxLMS* genes, a putative gene whose deduced amino acid sequence showed 37 % similarity to the cofactor inserting chaperone XdhC was located. Additional ORFs identified on the 23 kb segment may encode further enzymes (a glutamyl tRNA synthetase, an esterase, two short-chain dehydrogenases/reductases, an ATPase belonging to the AAA family, a 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase/5-oxopent-3-ene-1,2,5-tricarboxylate decarboxylase-like protein, an enzyme of the mandelate racemase group, and hypothetical proteins involved in transcriptional regulation, and metabolite transport.)
INTRODUCTION

The genetic diversity and flexibility of prokaryotes has led to the evolution of an impressive variety of metabolic pathways to transform or degrade natural as well as numerous xenobiotic compounds. The genes coding for enzymes involved in degradative pathways are often organized as operons and supraoperonic clusters comprising 'pathway modules' (1-4).

*N*-heteroaromatic compounds are metabolized and even mineralized by various bacteria (for a review, see 5 and references therein). Quinaldine (2-methylquinoline) is utilized by *Arthrobacter ilicis* Rü61a as a source of carbon, nitrogen, and energy; its degradation proceeds via the “anthranilate pathway” (5, 6). The initial step, the hydroxylation of quinaldine in *para* position to the *N*-heteroatom, is catalyzed by the inducible enzyme quinaldine 4-oxidase (Qox)1. Qox is a molybdo-iron/sulfur-flavoprotein with an (LMS)2 subunit structure and has been classified to belong to the xanthine oxidase family of molybdenum enzymes (7-10; for reviews on molybdenum enzymes, see 11-13). Like many other bacterial molybdenum hydroxylases, e.g., quinoline 2-oxidoreductase (Qor) from *Pseudomonas putida* 86 (14, 15), isoquinoline 1-oxidoreductase (Ior) from *Brevundimonas diminuta* 7 (16), CO dehydrogenase from *Oligotropha carboxidovorans* (17), and aldehyde dehydrogenases from *Desulfovibrio gigas* and *D. desulfuricans* (18-21), Qox contains the molybdopterin cytosine dinucleotide form (MCD) of the molybdenum pyranopterin cofactor (7).

X-ray crystal structures of molybdenum hydroxylases have allowed to identify amino acid residues that might possibly be involved in substrate positioning and/or catalytic turnover (17-23). The catalytic relevance of these residues can be assessed by constructing protein variants carrying amino acid replacements, and their biochemical, spectroscopic and structural characterization. Replacement of a distinct amino acid residue in a protein can be performed by site-directed mutagenesis. However, a prerequisite for such a mutagenesis approach is the availability of a suitable system for the genetic manipulation and for the regulated, functional expression of the genes coding for the enzyme to be investigated. Whereas genes coding for molybdenum hydroxylases containing molybdopterin or the molybdopterin guanine dinucleotide form of the cofactor have been successfully expressed in *E. coli* (24-26), attempts to produce MCD-containing enzymes in *E. coli* clones failed (27, 28, unpublished results of our group). We have recently been working at the construction of expression clones for the
synthesis of Qor and Ior. Synthesis of catalytically fully competent Qor was only achieved when using a ΔqorMSL mutant of the donor strain *P. putida* 86 as recipient for the expression plasmid (29). Ior protein showing minor activity was synthesized from the respective expression plasmid when using the Qor-producing strain *P. putida* 86 as a host, suggesting that accessory gene(s) encoding product(s) essential for the synthesis or assembly of the enzyme is(are) part of the quinoline regulon in *P. putida* 86 (30).

Here we report on a gene cluster from *A. ilicis* Rü61a that comprises several genes coding for enzymes of the anthranilate pathway of quinoline degradation. The amino acid sequences deduced from the *qox* genes are compared to those of other molybdenum hydroxylases. Due to the broad substrate specificity of Qox, which in addition to different *N*-heteroaromatic compounds oxidizes aromatic aldehydes (8), sequence comparisons to the crystallized aldehyde oxidoreductases are of special interest. Moreover, we present the functional heterologous expression of the *qoxLMS* genes in *P. putida* KT2440 pKP1.
MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *A. ilicis* Rü61a was grown in mineral salts medium (8) at 30 °C with 0.5 ml/l quinaldine. *E. coli* HB101 (31), which served as host strain for recombinant cosmids, and both *E. coli* DH5α (32) and *E. coli* XL1-Blue MRF’ (Stratagene), used for cloning procedures with pUC18, were grown in Luria Bertani (LB) broth (32) at 37 °C. If appropriate, *E. coli* and *P. putida* KT2440 cultures contained the following antibiotics: ampicillin (60 μg/ml and 500 μg/ml for *E. coli* DH5α and *P. putida* KT2440 pKP1, respectively), tetracycline (15 μg/ml), and kanamycin (50 μg/ml). To investigate functional expression of the *qox* genes in the cosmid clone *E. coli* HB101 pVK55B/5, it was grown at 37 °C on mineral salts medium containing (per liter) 0.2 g MgSO₄ · 7 H₂O, 4 g (NH₄)₂SO₄, 5.25 g K₂HPO₄, 2.25 g KH₂PO₄, 2.7 mg FeCl₃ · 6 H₂O, and 8 g glucose as carbon source. The medium was supplemented with 15 μg/ml of each proline and leucine, and 2 ml/l vitamin solution (33); 0.1 ml/l quinaldine was added when the culture reached an optical density (600 nm) of about 0.8.

*P. putida* KT2440 pKP1 was grown in the mineral salt medium described by Tshisuaka et al. (15) with 8 mM benzoate as carbon and energy source and as XylS effector, and with 1 g/l (NH₄)₂SO₄. As an additional XylS effector, 2 mM 2-methylbenzoate was added at an optical density (600 nm) of 0.8-1.2. To generate biomass for protein purification, cells were grown in two 4 l glass fermenters to which benzoate was added repeatedly. At an optical density (600 nm) of about 3, cells were harvested by centrifugation at 14,000 × g for 15 min at 4 °C.

For the preparation of electrocompetent cells, *E. coli* DH5α, *E. coli* XL1-Blue MRF’ and *P. putida* KT2440 were cultured in TB medium (32).

For transfection of *E. coli* HB101, 20 ml LB broth with 0.2 ml 1 M MgSO₄ and 0.2 ml 20 % (w/v) maltose were inoculated with a single bacterial colony and grown to an optical density (600 nm) of 0.8. After harvesting the cells by centrifugation at 2000 × g and 4 °C for 10 min, cells were diluted to an optical density (600 nm) of 1 with icedcold, sterile 10 mM MgSO₄.

Analysis of the degradative potential of *P. putida* KT2440 pVK55B/5 and *P. putida* KT2440 pVK55/11. In order to determine Qox activity and to identify metabolites of quinaldine catabolism, recombinant cosmids were transferred to *P. putida* KT2440 (34) by electroporation. The clones *P.
**P. putida** KT2440 pVK55B/5 and **P. putida** KT2440 pVK55/11 were grown in mineral salt medium (8) with 30 mM succinate, 1 g/l \((\text{NH}_4)_2\text{SO}_4\) and 1 ml/l vitamin solution (33) at 30 °C. At an optical density (600 nm) of about 0.8, 0.1 ml/l quinaldine was added. Quinaldine conversion was monitored spectrophotometrically in the culture supernatant. Spectra were compared with those of authentic references diluted in the same medium. Qox activity was measured in the cell free extracts, obtained after cell disruption by sonification, and centrifugation at 48,000 \(\times\) g for 40 min at 4 °C.

**DNA techniques.** Genomic DNA of **A. ilicis** Rü61a was isolated according to Hopwood *et al.* (35). Plasmid and cosmid DNA was isolated with the QIAGEN Plasmid Mini- and Midi Kit, respectively (Qiagen, Hilden, Germany). Gel extraction of DNA fragments for cloning was done with the Nucleo Spin® Extraction Kit of Macherey-Nagel (Düren, Germany), however, DNA fragments larger than 10 kb were size fractionated in 0.5 % low-melting agarose gels and extracted by agarase treatment. DNA restriction, dephosphorylation and ligation, and agarose gel electrophoresis were carried out using standard procedures (32). Electrocompetent cells were generated according to Dower *et al.* (36) and Iwasaki *et al.* (37).

**Construction of genomic libraries.** In order to generate an enriched gene library for **A. ilicis** Rü61a, genomic DNA, restricted with **SmaI**, was separated in agarose gels and vacuum-blotted to nylon membranes (parablot NY plus from Macherey-Nagel, Düren, Germany). Fragments in the size of 4 kb to 5 kb showing positive hybridization signals with the probe „b-DIG“ (see below) were extracted from an agarose gel and ligated to the **SmaI** digested, dephosphorylated vector pUC18 (38). **E. coli** XL1-Blue MRF’ transformants were screened by colony blotting, and identified by Southern hybridization of **SmaI** restricted plasmids, using the probe „b-DIG“.

For construction of a cosmid library, genomic DNA of **A. ilicis** Rü61a was partially restricted with **HindIII**. DNA fragments ranging in size from 15 kb to 25 kb were extracted from a 0.5 % low-melting agarose gel and ligated to the **HindIII** digested, dephosphorylated cosmid vector pVK100 (39). The cosmids were packaged *in vitro* into lambda phage particles using a commercial extract (DNA Packaging Kit from Roche, Mannheim, Germany). The preparation was used to infect **E. coli** HB101, which was selected for tetracycline resistance (\(\text{Tc}^r\)) and kanamycin sensitivity (\(\text{Km}^r\)). The clone library was screened by colony blotting and hybridization with a probe described below as „1.1 DpnI“.
DNA probes, and hybridization. The oligonucleotide probe „b-DIG“, which was 5´-end labeled with a Digoxigenin derivative, was a degenerated 29-mer: 5´-TTY ATG CAY CCN TTY CAR TTY ATH ACN CC-3´ (following the IUPAC ambiguity code), deduced from the amino-terminal amino acid sequence of the medium-sized subunit of Qox (FMHPFQFITP) (7). Prehybridization for two hours and hybridization overnight with „b-DIG“ was carried out at 54.5 °C. The membranes were stringently washed for 2 × 15 min in 2 × SSC, 0.1 % SDS at room temperature, 2 × 15 min in 0.5 × SSC, 0.1 % SDS at 54.5 °C, and 2 × 15 min in 0.2 × SSC, 0.1 % SDS at 54.5 °C. Screening an enriched gene library of A. ilicis Rü61a established in the vector pUC18 with “b-DIG” revealed a clone containing an insert of 4580 bp (pUC55/4.5). Isolation of an 1052 bp DpnI fragment from this insert led to the specific probe „1.1 DpnI“, which was used to screen the cosmid clone library. „1.1 DpnI“ hybridizes with the 5´-terminal half of qoxL (Fig. 1). After prehybridization for two hours and hybridization overnight with the probe „1.1 DpnI“ at 68 °C, the membranes were washed twice for 15 min in 2 × SSC, 0.1 % SDS at room temperature and twice for 15 min in 0.5 × SSC, 0.1 % SDS at 68 °C. Random primed labeling of the probe „1.1 DpnI“ using the DIG-High Prime DNA Labeling Kit (Roche, Mannheim, Germany), Southern and colony blotting, hybridization and colorimetric detection with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate were performed following the „DIG System User’s Guide for Filter Hybridization“ (40).

Subcloning procedures. Restriction of the recombinant cosmid pVK55B/5 with HindIII produced two fragments of 12,203 bp and 10,812 bp, respectively, beside the 23 kb vector. These two fragments were separately inserted into the HindIII cleavage site of pUC18, generating pUC55/12 and pUC55/11. The 10.8 kb fragment was also inserted into the cosmid vector pVK100 (forming pVK55/11) in order to propagate it in P. putida KT2440. When pUC55/11 was restricted with SmaI, three fragments were generated, the internal 4.58 kb fragment showing the positive signal with probe „b-DIG“., and two fragments of 2978 bp and 3254 bp, respectively (Fig. 1). The 4.58 kb fragment was cloned into the SmaI site of pUC18 (forming pUC55/4.5). The 2.97 kb fragment and the 3.25 kb fragment were removed from pUC55/11 by SmaI-HindIII and SmaI digestion, respectively. Both fragments were separately cloned into the multiple cloning site of pUC18, yielding pUC55/3 and pUC55/3.2. All pUC derivatives were transferred to E. coli DH5α by electroporation.
Expression cloning of qoxLMS genes. Using the cosmid pVK55B/5 as template, the qoxLMS genes were amplified with Pfu-polymerase. The forward primer was chosen to contain the assumed Shine-Dalgarno sequence (in italics) preceding the qox genes, and an EcoRI recognition site (underlined): 5’-ACGCCGAATTCGTGACGAAGTTAAGGAGACC-3’; the nucleotides set in boldface are complementary to nucleotides 17,492-17,511 of EMBL acc. no. AJ537472. The reverse primer was completely complementary to nucleotides 21,401-21,375 of EMBL acc. no. AJ537472: 5’-TTTGAATGCGCAGTGAGGAGATTTGC-3’. After EcoRI restriction the PCR product was ligated into the EcoRI and SmaI restricted plasmid pJB653 (41), generating pKP1. The recipient P. putida KT2440 was transformed by electroporation (36).

DNA sequencing and comparative sequence analysis. The genes and open reading frames shown in Fig. 1 were deduced from computer assisted analysis of sequences obtained from single strand sequencing of the inserts of pUC55/12, pUC55/3, pUC55/4.5 and pUC55/3.2; sequences of the qox genes were verified by sequencing both strands. Sequencing was carried out according to the method of Sanger et al. (42) on a sequencer no. 377 from Applied Biosystems. Analysis of the DNA sequences were performed with the HUSAR 4.0 program package (EMBL, GENIUSnet, DKFZ Heidelberg, Germany) using the BLAST family of programs (43) for database searches, GAP for calculating similarities and identities, and CLUSTAL (44) for calculating multiple alignments. Conserved protein domain sequences and fingerprint motifs were found at Pfam (Sanger Institute, Hinxton, Cambridge, UK) and PRINTS (45). Gene-coding sequences were identified by the program FRAMES.

Sequence data on the P. putida KT2440 genome were obtained from The Institute for Genomic Research through the website at http://www.tigr.org.

Assay for Qox activity, and polyacrylamide gel electrophoresis (PAGE). The activity of Qox was assayed spectrophotometrically by measuring the quinaldine-dependent reduction of the artificial electron acceptor iodonitrotetrazolium chloride (INT) as described by de Beyer and Lingens (7). Protein concentrations were estimated by the method of Bradford as modified by Zor and Selinger (46) using bovine serum albumin as standard protein. The Qox preparation used for the determination of $K_m$ app and $k_{cat}$ app for quinaldine and for INT showed a specific activity of 5 U/mg.
Non-denaturing PAGE was performed using the high pH discontinuous system according to Hames (47), preparing resolving gels containing a final acrylamide concentration of 7.5 % (w/v). SDS-PAGE (48) was used to check homogeneity of the Qox preparations; resolving gels contained 12.5 % (w/v) acrylamide, and 20 mM 1,4-dithio-D,L-threitol was added to the samples as reducing agent. Proteins were stained in Coomassie blue G-250 (0.1 % (w/v) in 50 % (w/v) trichloroacetic acid). For activity staining of Qox in non-denaturing PA gels, gels were immersed in the same buffer as used for the spectrophotometric assay, containing the substrate quinaldine and INT as electron acceptor.

**Purification of Qox from *P. putida* KT2440 pKP1.** X gram of cells (wet weight), suspended in 1.5 × X ml 100 mM Tris-HCl buffer, pH 7, containing 10 μM phenylmethane sulfonyl fluoride and 1.25 U/ml benzon nuclease, were immersed in an ice bath and disrupted by sonification. Crude extract, obtained by centrifugation at 48,000 × g for 40 min at 4 °C, was loaded onto a 20 ml DEAE-Sepharose CL-6B (Amersham Biosciences, Freiburg, Germany) column, equilibrated in 50 mM Tris-HCl buffer, pH 7. Proteins were eluted with a linear gradient from 0 M to 1 M NaCl in the equilibration buffer. Fractions showing Qox activity were pooled, and ammonium sulfate was added to a final concentration of 0.75 M. After centrifugation for 30 min at 20,000 × g and 4 °C, the supernatant was loaded onto a 5 ml column containing Phenyl-Sepharose CL-4B (Amersham Biosciences, Freiburg, Germany), which had been equilibrated in 100 mM Tris-HCl buffer, pH 7, containing 0.75 M (NH₄)₂SO₄. After washing the column with 85 mM Tris-HCl buffer, pH 7, containing 0.55 M (NH₄)₂SO₄, and elution with a linear gradient from the washing buffer to 50 mM Tris-HCl, pH 7, the active fractions were pooled and applied to an anion exchange column UNO™-Q 1 (Biorad, München, Germany) that had been equilibrated in 50 mM Tris-HCl buffer, pH 7. The proteins were eluted with a linear gradient from 0.15 M to 1 M NaCl in the equilibration buffer after washing the column with 0.15 M NaCl in the same buffer. For gel filtration, the enriched Qox was loaded onto the HiLoad 26/60 Superdex 200 prep grade column (Amersham Biosciences, Freiburg, Germany). The column was equilibrated and run at 1 ml/min with 50 mM Tris-HCl, pH 7, containing 0.25 M NaCl. Fractions showing Qox activity were pooled, concentrated by ultrafiltration (membrane cut-off 10 kDa), and stored at -80 °C.
Determination of the nucleotide moiety of the molybdenum cofactor. For identification of the nucleotide moiety of the molybdenum cofactor, the enzymes were incubated at 95 °C for 10 min in the presence of sulfuric acid (3 %, by vol.); hydrolysis leads to the release of nucleotides from MCD and FAD. After centrifugation for 10 min at 20,000 × g, the supernatant was analyzed by isocratic HPLC on a Lichsorb C-RP-18 column (5 μm particle size, 4 × 250 mm) at a flow rate of 1 ml/min with 0.2 % acetic acid, 0.5 % methanol (by vol.) in water as eluent. The compounds were identified by their retention times, as well as their UV-spectra (obtained with a photodiode array detector, Waters 996), and by co-chromatography with authentic reference compounds (CMP, cytidine, AMP, GMP).

EPR spectroscopy and sample preparation. The samples were filled into EPR quartz tubes (Wilmad) and immediately frozen in liquid nitrogen within one minute. Each sample of Qox protein from \textit{P. putida} KT2440 pKP1 was first reacted with a 5-10 fold excess of substrate (quinaldine) and measured at 77 K in a nitrogen finger dewar or between 10 K and 65 K in a continuous He-flow ESR900 cryostat (Oxford). In a second step a 60-fold excess of substrate was added to the sample in the quartz tube and measured again. Finally, the sample was exposed to an excess of dithionite (20-fold) for complete reduction. The samples were handled under anaerobic conditions. EPR spectra at X-band frequencies (9.5 GHz) were recorded on a Bruker ESP300 spectrometer. The magnetic field and the microwave frequency were determined with a NMR gaussmeter and a microwave counter, respectively. The modulation amplitude for spectra recording generally was 0.5 mT. For measurements at 65 K and 77 K several spectra at different microwave powers (0.2–10 mW) were recorded to avoid saturation broadening. The spectra below 25 K were recorded at about 10 mW microwave power. To improve the signal/noise ratio X-band spectra were accumulated up to 50 times. All spectra were base-line corrected.

Nucleotide sequence accession number. The nucleotide sequence of the 23,015 bp insert of pVK55B/5, which includes the genes coding for QoxL, QoxM and QoxS, is deposited in the EMBL Nucleotide Sequence Database under the accession number AJ537472.\(^2\)
RESULTS

Degradative capacities of *P. putida* KT2440 cosmid clones, and synthesis of active quinaldine 4-oxidase by *P. putida* KT2440 cosmid clones and by *P. putida* KT2440 pKP1.

*P. putida* KT2440 containing the cosmid pVK100 is able to grow on catechol, but it does not utilize quinaldine or the subsequent intermediates of the anthranilate pathway, namely, 1H-4-oxoquinaldine, 1H-3-hydroxy-4-oxoquinaldine, N-acetylanthranilate, and anthranilate. After transformation of *P. putida* KT2440 with the recombinant cosmids that hybridized with “1.1 DpnI”, five of the resulting *P. putida* KT2440 pVK100 (Tcr, Kms) clones were able to convert quinaldine to anthranilate cometabolically, suggesting that the genes coding for the enzymes catalyzing the first four steps of the anthranilate pathway are located on the inserts of the cosmids. One of these clones, designated *P. putida* KT2440 pVK55B/5, was chosen for further investigations. Its cosmid pVK55B/5 harbors the 23 kb insert shown in Fig. 1. *P. putida* KT2440 pVK55/11, which contains the 10.8 kb region depicted in the lower part of Fig. 1, also shows cometabolic conversion of quinaldine to anthranilate. The specific activity of Qox in cell free extracts of both *P. putida* KT2440 pVK55B/5 and *P. putida* KT2440 pVK55/11 was 0.04 U/mg. In contrast, an *E. coli* HB101 pVK55B/5 clone did not transform quinaldine, and crude extracts did not show Qox activity. This observation is in accordance with other reports on futile attempts to express genes coding for MCD-containing hydroxylases in *E. coli* (27, 28).

The expression plasmid pKP1 has been constructed from the broad host range expression vector pJB653 (41) and a fragment comprising the *qoxLMS* genes preceded by their putative Shine Dalgarno sequence. Whereas the *Pseudomonas* cosmid clones mediate expression of the *qox* genes and further catabolic genes from their own promoters, expression of *qoxLMS* on pKP1 is regulated by the plasmid-encoded XylS protein that activates the operator region of the *Pm* promoter of the plasmid. Quinaldine 4-oxidase indeed was produced by *P. putida* KT2440 pKP1 when growing the strain in the presence of XylS effectors. The specific activity of Qox in crude extracts was 0.06 U/mg protein. With respect to its electrophoretic mobility in non-denaturing PAGE, the Qox protein produced by the expression clone did not differ from Qox produced by the wild-type strain *A. ilicis* Rü61 (Fig. 2A).
Purification of Qox from *P. putida* KT2440 pKP1. Qox was purified in a four step procedure to near electrophoretic homogeneity (Fig. 2B). The results of the enzyme purification are listed in Table 2. The enzyme was purified 85-fold from the crude extract with a yield of 7%. Qox from *P. putida* KT2440 pKP1 consists of three subunits with molecular mass of about 20 kDa, 35 kDa, and 80 kDa, as observed for the wild-type enzyme (8).

Properties of the Qox protein from *P. putida* KT2440 pKP1. The UV/Vis spectrum of Qox purified from *P. putida* KT2440 pKP1 (Fig. 3) is characteristic for molybdo-iron/sulfur flavoproteins. The ratios $E_{280nm}/E_{450nm}$ and $E_{450nm}/E_{550nm}$ were 5.6 and 3.3, respectively, indicating stoichiometric amounts of FAD:Fe:S of 1:4:4 in the nearly pure enzyme (49).

HPLC analysis of the non-protein part of the enzyme after acidic hydrolysis indicated the presence of CMP and AMP with retention times of 4.6 min and 7.9 min, respectively. GMP or free cytidine were not detected in the sample. While the CMP is released from the molybdopterin cytosine dinucleotide cofactor, AMP derives from the FAD cofactor.

The apparent $K_m$ values of Qox purified from the expression clone were very similar to those of the wild-type Qox; its $k_{cat}$ values were even higher (Table 3).

Analysis of redox-active centers in Qox from *P. putida* KT2440 pKP1 by EPR spectroscopy. EPR spectroscopy is capable to selectively monitor the paramagnetic states of the various redox centers present in the Qox enzymes. In this way typical fingerprint spectra of redox centers (e.g., the rapid or very rapid species of the Mo(V)-cofactor) can be compared between the enzyme preparations to yield information on the presence and integrity of these centers. Here the comparison focuses on Qox of the wild-type specimen *A. ilicis* Rü61a and the enzyme produced by *P. putida* KT2440 pKP1. The spectra obtained at 65 K after addition of substrate are shown in Fig. 4. For Qox isolated from *P. putida* KT2440 pKP1 (Fig. 4B and C), addition of substrate in small excess leads mainly to the formation of the FAD signal and small traces of the very rapid (vr) signal (Fig. 4B). At higher substrate concentration, very rapid signals comparable to those of wild-type Qox are observed (Fig. 4C). The EPR parameters ($g_1=2.024$, $g_2=1.945$, $g_3=1.935$) are identical to those of Qox from *A. ilicis* Rü61a (Fig. 4A) indicating that very similar Mo(V)-conformations are prevailing in both enzymes. Only minor traces of a rapid (r) species are detectable. In comparison, the $g_2$- and $g_3$-components of the very rapid signals found in Qor from *P. putida* 86 and Ior from *B. diminuta* are clearly different (9).
FAD signals in all Qox samples display a line width of 1.6 mT typical for the anionic (red) FAD radical. The neutral (blue) FAD radical species found in Qor or in xanthine oxidase shows a larger line width of about 1.9 mT. The FAD signal intensity was shown to depend critically on the oxygen status of the Qox preparation (9).

When the Qox samples reacted with substrate are measured at lower temperatures (25 K) the broad features of FeS-clusters are appearing. Interestingly, mainly the signals of FeSII characterised by a larger g-anisotropy with respect to FeSI-signals can be observed as shown in Fig. 5A for the enzyme from *P. putida* KT2440 pKP1. Complete reduction with dithionite generally leads to a loss of FAD radical signals as well as of Mo(V)-species, but the FeS-centers are fully converted to their paramagnetic reduced states. The spectrum of fully reduced Qox from *P. putida* KT2440 pKP1 obtained at 25 K is compared to that of Qox from *A. ilicis* Rü61a in Fig. 5B and C, respectively. It is evident that the axial spectrum of the FeSI-center typical for Qox from *A. ilicis* Rü61a is also present in the enzyme produced by *P. putida* KT2440 pKP1. The overall spectral pattern is very similar for the enzymes. There are, however, some subtle differences visible. The FeSI1-component is identical whereas the axial signal FeSIax of Qox from *P. putida* KT2440 pKP1 displays a slightly altered line shape caused by a small but unresolved increase in rhombicity of g2 and g3. The most prominent difference concerns the g3-component of FeSII which is shifted to higher g-factors for Qox from *P. putida* KT2440 pKP1 (Fig. 5B), indicated by the arrow. In addition its line shape and that of the g1-component are clearly asymmetric (marked by asterisks) as compared to the corresponding features of Qox from *A. ilicis* Rü61a (Fig. 5, trace C). It is noted that for the partially reduced enzyme (Fig. 5A) the related lines appear more symmetric with the g3-component also slightly shifted to higher g-factors. For other related enzymes (Qor, Ior), small shifts of the g-factors of FeS-clusters have been reported depending on the mode of reduction (9). An indication of a magnetic interaction between the FeS-centers as found for Ior is not observed.

**Sequence analysis of the qox genes.** The genes and potential open reading frames identified on the 23 kb insert of pVK55/5 are presented in Fig. 1 and Table 4. Genes coding for the three subunits of Qox were identified by comparing their amino-terminal amino acid sequences deduced from the nucleotide sequence with those determined by Edman degradation (7). The calculated *M*<sub>r</sub> for the three peptides are 84,115 for QoxL, 30,608 for QoxM and 18,539 for QoxS. The calculated mol % G+C content of
qoxL, qoxM, and qoxS is 60.1, 62.2, and 58.8, respectively. For the whole 23 kb fragment, a G+C content of 61.8 mol % was calculated. This value is in good agreement with the mol % G+C content of 61.5 reported for *A. ilicis* (50). Strain *P. putida* KT2440, used as a host for expression cloning of the qox genes, shows a similar G+C content of 61.6 mol % (51).

The transcriptional order of the genes coding for the subunits of Qox is 5´-qoxL-M-S-3´. Among the heterotrimeric molybdoenzymes no conservation in gene arrangement is obvious. While in many cases the genes coding for the three subunits of these enzymes are transcribed in the order 5´-medium-small-large-3´ (28, 52-54), other enzymes are known whose genes are arranged in the order 5´-large-small-medium-3´ (55) or even with a gap between the gene for the large subunit and the genes for the medium and the small subunit (54). These divergencies may lead to the assumption that there is no ancestral common transcriptional unit for these enzymes.

The qoxL gene is 2388 bp in length, coding for a protein of 795 amino acids (aa). A potential ribosome-binding site (AAGGAGA) is located fourteen nucleotides upstream of the start codon ATG. 146 nucleotides upstream of the qoxL start codon a putative -35 region was detected (TTGACG) which, however, is not followed by a recognizable -10 region in the usual distance of 16-19 nucleotides (56). QoxL exhibits the well conserved motifs assumed to be involved in binding the pyranopterin cofactor (MoCoI-MoCoV) (19, 57) (Fig. 6A). The glutamate residue E736 of QoxL corresponds to E869 and E869 of the *D. gigas* and *D. desulfuricans* aldehyde oxidoreductases (MOP and MOD) (19, 21), respectively, to E730 of the B-subunit of xanthine dehydrogenase from *Rhodobacter capsulatus* (XDHBRc) (23) and to E1261 of bovine xanthine dehydrogenase/oxidase (XOb) (22). This glutamate residue is conserved in all enzymes of the xanthine oxidase family; it is assumed to activate the water ligand by proton abstraction and to form a transient bond to the metal during catalysis (19, 21). Residues forming hydrogen bonds to the pyranopterin (MOP: R533 and Q807; MOD: R535 and Q807; XDHBRc: R342 and Q663) and to the water ligand of the molybdenum (MOP: G697; MOD: G699; XOb: A1079; XDHBRc: A529; QorL: A546) are also conserved in QoxL (R362, Q671, G526) (Fig. 6A).

*QoxM* starts at position 19,901 (EMBL acc. no. AJ537472); its start codon ATG overlaps with the TGA stop codon of qoxL. *QoxM* ends after 873 nucleotides with the stop codon TAG, coding for a
protein of 290 aa. Thirteen nucleotides upstream of the start codon a putative ribosome-binding site was identified (AAGGAGA).

The motifs $^{30}$AGGQT$^{34}$ and $^{109}$TIGG$^{112}$, which correspond to the typical loop-forming FAD-binding sites of the vanillyl-alcohol oxidase family (58), were identified in QoxM, indicating that it harbors the FAD cofactor (Fig. 6B). The motif TIGG is described to create a pocket for the adenosine and to contact the pyrophosphate moiety of the FAD molecule in XDH from *R. capsulatus* (23). In XDHARc, T206 of the amino-terminal motif (first motif in Fig. 6B) as well as the double glycine of this motif also interact with the pyrophosphate (23). In QoxM, Q33 corresponds to this residue; the medium-sized subunits of Qor from *P. putida* 86 (28) and of nicotine dehydrogenase from *A. nicotinovorans* (59) likewise contain a glutamine residue in this position. Tyrosine 193 of the medium-sized subunit of the CO dehydrogenase from *O. carboxidovorans* (CoxM), shielding the central part of the FAD isoalloxazine ring from the solvent (17), is not conserved in QoxM (P192 by sequence comparison), but in some other molybdenum hydroxylases.

QoxS starts two nucleotides downstream of the stop codon of qoxM with the start codon ATG at position 20,776 and ends with the stop codon TGA at position 21,300. Fourteen nucleotides upstream a putative ribosome-binding site was detected (AAGGGAG). QoxS, which consists of 174 aa, shows the two cysteine rich motifs strictly conserved in the prokaryotic molybdenum hydroxylases, which probably bind the two different [2Fe2S] clusters (60). The N-terminal motif following the sequence $^{40}$CX$_4$CGXCX$_{15}$C$^{60}$ is homologous to the plant-type ferredoxin signature pattern; the second motif $^{117}$CGXCX$_{31}$CXC$^{154}$ has a binding motif that is typical for molybdenum hydroxylases. While the latter motif is presumed to bind the FeSI center, which is “proximal” to the molybdenum cofactor, the first motif may bind the “distal” FeSII center (57).

**Sequence analysis of the qoxLMS flanking regions.** Within the 23 kb region, fifteen putative ORFs were identified upstream of the qoxLMS gene cluster, and one ORF downstream of qoxS (Fig. 1; Table 4). The hypothetical protein encoded by the nearly complete ORF 1, which starts 574 nucleotides downstream of qoxS but lacks some C-terminal residues, is related to chaperone like proteins presumed to be involved in recruitment of the molybdenum cofactor. The deduced protein shows 37 % similarity to XdhC from *Rhodobacter capsulatus* which has been proven to be required for insertion of MPT into xanthine dehydrogenase (61). 41 % similarity was found to PucA, which is part of the
purine catabolic gene cluster of *Bacillus subtilis*; PucA and XdhC show 22 % aa identity in their C-terminal half (62).

1958 nucleotides upstream of the *qoxL* start codon the gene coding for 1H-3-hydroxy-4-oxoquinaldine-2,4-dioxygenase (Hod) was identified, which is transcribed in the opposite direction to the *qox* gene cluster. Hod catalyzes the third step in the anthranilate pathway of quinaldine degradation, namely the cleavage of 1H-3-hydroxy-4-oxoquinaldine to *N*-acetylanthranilate and carbon monoxide; it belongs to a unique group of oxygenases without requirement for cofactors or metal ions (63-66).

ORF 2, which starts 1257 nucleotides upstream of the *hod* start codon, is identical to ORF 491 reported by Betz et al. (67). The 42,216 Da hypothetical protein (388 aa) deduced from this ORF shows high similarities to monoxygenases belonging to the single-component flavoproteins, e.g., the 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase from *Pseudomonas* sp. MA1 (68) and a salicylate hydroxylase of *Sphingomonas* sp. (acc. no. BAA19150). Its N-terminal region includes a typical ADP binding site that may bind the ADP portion of an FAD cofactor (69, 70). Upstream of the ORF 2 start codon a putative -35 region (TTGACG), identical to that found in front of *qoxL*, was identified, which is followed by a putative -10 region (TATATAA) in a distance of 16 bp. No possible -35/-10 promoter regions upstream of the *hod* gene are obvious so we may speculate that ORF 2 and the *hod* gene form an operon.

Seven nucleotides downstream of the *hod* stop codon starts ORF 4 that codes for a hypothetical protein with a *M*<sub>r</sub> of 32,002. The 293 aa protein exhibits 49 % similarity to a putative protein from *Pseudomonas* sp. CA10 (acc. no. BAB32459.1), which is assumed to belong to a family of esterases/lipases/thioesterases. The ORF 4 amino acid segment spanning positions 59 to 158 is related to a domain of type-B carboxylesterases (Pfam signature PF00135), although the described consensus pattern for this family (Prosite acc. no. PDOC00112) is not completely conserved. Since on the 10.8 kb fragment of pVK55/11, which confers to *P. putida* KT2440 the ability to convert quinaldine to anthranilate, ORF 4 is the sole hypothetical gene supposed to code for a hydrolase, it apparently acts as an *N*-acetylanthranilate amide hydrolase in quinaldine degradation. No potential -35/-10 promoter region was identified upstream of ORF 4, so the putative operon comprising ORF 2 and *hod* might also involve ORF 4.
Sixty-seven nucleotides downstream of ORF 4 starts ORF 5 coding for a putative protein of 423 aa with a $M_r$ of 43,695. For the ORF 5 gene product, a transmembrane protein belonging to the family of general substrate transporters is predicted (InterPro entry IPR005828). These proteins share a common structural feature of 12 transmembrane $\alpha$-helices with a cytoplasmatic loop after the sixth transmembrane $\alpha$-helix. Prediction of transmembrane regions of the ORF 5 protein by the program SOSUI (71) indeed revealed twelve $\alpha$-helices corresponding to those of the transport proteins; the conserved feature RXGR(R/K), which is proposed to form a $\beta$-bend that links two $\alpha$-helices (72), was found at positions 80-84 (RWGLK). This bend is located between $\alpha$-helix two and $\alpha$-helix three as suggested by the secondary structure prediction (using SOSUI (71)). However, contrary to representative proteins of this family, the RXGR(R/K) motif is not duplicated in the deduced ORF 5 protein. A hydropathy plot (73) of the ORF 5 protein also suggested that it is a transmembrane protein.

One subfamily of the general substrate transporter family comprises the benzoate transporters including, e.g., the 4-hydroxybenzoate transporters PcaK from $P.$ putida PRS2000 and Acinetobacter calcoaceticus ADP1 (74, 75), the cis,cis-muconate transporter MucK from $A.$ calcoaceticus ADP1 (76), and the benzoate transporter BenK from the same strain (77, 78). All these transporters are located in or close to operons coding for the enzymes and proteins of the respective aromatic degradation pathway. The proximity of ORF 5 to other genes involved in quinaldine degradation may suggest that this putative transmembrane protein could be involved in transport of quinaldine or of one of the metabolites across the cell membrane.

ORF 6 encodes a protein with a $M_r$ of 32,349 composed of 295 aa, which shows the 5-element fingerprint for glutamyl-tRNA synthetases (Prints acc. no. PR00987). The segments $^{16}$HVGN$^{19}$ and $^{233}$RLAKR$^{237}$ of the deduced protein correspond to the conserved motifs HIGH and KMSKS (or KLSKR), respectively, that in the class I aminoacyl-tRNA synthetases are involved in ATP binding (79, 80).

Twenty-five nucleotides downstream of the ORF 6 stop codon starts ORF 7 coding for a hypothetical protein of 372 aa, which has 87 % similarity and 82 % identity to the ethyl chrysanthemate-hydrolyzing esterase from Arthrobacter globiformis SC-6-98-28 (81). Like this esterase the putative ORF 7 protein shows significant similarities to $\beta$-lactamases, other esterases and
the D,D-carboxypeptidase from *Streptomyces* R61 (82). The serine and the lysine residues in the amino-terminal motif SXXK, suggested to be involved in substrate binding and proton transfer during catalysis in β-lactamases and β-lactam sensitive enzymes (82, 83), are conserved in the ORF 7 protein in position 59 and 62, respectively.

When comparing the putative protein encoded by ORF 8 of 203 aa (deduced *M*<sub>r</sub> of 21,472) to proteins in databases, a high degree of similarity to transcriptional regulators of the TetR family was detected. Analysis of the ORF 8 protein predicted a helix-turn-helix (HTH) motif at the amino terminal part comprising the residues 32 to 53. Moreover, the signature pattern (Prosite acc. no. PS01081) described to surround this motif in proteins of the TetR family is nearly completely conserved.

The hypothetical proteins encoded by ORF 9 and ORF 14 with a length of 279 aa and 253 aa, respectively, are assumed to be members of the large and diverse superfamily of short chain dehydrogenases/reductases (SDR), which is defined by a common folding pattern rather than by function (84). They can be assigned to the “classical family” of SDRs according to the classification of Kallberg *et al.* (84). The members of this family catalyze NAD(P)(H)-dependent oxidation/reduction reactions on a wide spectrum of substrates, e.g., alcohols, steroids, or aromatic compounds. The binding sites for the cosubstrates NAD(H) or NADP(H) in the N-terminal part of the SDR proteins are represented by the conserved pattern GXXXGXG; this motif is indeed found in the sequences deduced from ORF 9 and ORF 14. The motif YXXXK, comprising the conserved tyrosine and lysine residues involved in catalysis, is also present in both amino acid sequences. The conserved residues R39 in the ORF 9 protein and D42 in the ORF 14 protein suggest that the cosubstrate of the former protein is NADP(H), whereas the latter may utilize NAD(H) (84).

ORF 10 which is located downstream of ORF 9 probably codes for a protein of 178 aa with a *M*<sub>r</sub> of 20,331 that shows up to 40 % similarity to the amino-terminal part of (p)ppGpp 3´-pyrophosphohydrolases belonging to the RelA/SpoT family. This N-terminal part of the RelA/SpoT family enzymes includes a so-called HD-domain (Pfam acc. no. PF01966) (85) that contains highly conserved histidine and aspartate residues; these are presumed to coordinate divalent cations. It has been proposed that all the enzymes harboring a HD-domain catalyze divalent-cation-dependent
phosphohydrolase reactions (85). The HD superfamily comprises not only multi-domain enzymes like the RelA/SpoT proteins, but also ‘stand-alone’ proteins that essentially consist of a single HD-domain (85). Since about half of the ORF 10 protein (residues 30-120 out of 178 aa) resemble the HD-domain, it may belong to the latter group.

ORF 11 and ORF 12 are transcribed in the reverse direction to ORF 10 and code for putative proteins of $M_r$ of 43,306 and 37,043, respectively. The N-terminal part (aa 18-306) of the ORF 11 protein (414 aa) shares conserved sequence stretches with putative membrane proteins of unknown function grouped by sequence homology in an orthologous group (COG4292; www.ncbi.nlm.nih.gov/COG/new/release/cow.cgi?cog=COG4292). Prediction of putative transmembrane regions using the program SOSUI (70) indicated ten membrane spanning helices for the ORF 11 protein. BLAST searches for the ORF 12 protein showed high similarities to ATPases associated with diverse cellular activities (AAA ATPases) from a variety of organisms. Members of the AAA ATPases family usually share a ring-shaped oligomeric structure and represent a type of molecular chaperone. They are involved either in protein folding and assembly, protein transport, or in disassembly or degradation of proteins (86, 87). The hypothetical ORF 12 protein consists of 336 aa and harbors one AAA domain where the two strictly conserved Walker motifs A and B are located. Walker motif A or ’P-loop’ is found in position 105-113, Walker motif B in position 160-167 of the ORF 12 protein. These two sequences are responsible for ATP binding and hydrolysis (86, 88). Additionally the consensus pattern for AAA ATPases (Prosite acc. no. PS00674) was detected in the central part of the AAA domain in position 204-224.

The 376 aa hypothetical protein encoded by ORF 13 with a $M_r$ of 40,153 is located upstream of ORF 12 in the opposite orientation. Beside the 43 % similarity to the C-terminal part of an ATP/GTP-binding protein (ORF 666) of A. nicotinovorans (acc. no. CAD47885), the amino acid sequence shows slight similarities to different putative proteins from Streptomyces coelicolor A3(2) (89) but for these proteins no hypothetical function can be deduced.

ORF 15 codes for a hypothetical protein with a $M_r$ of 31,820. The 302 aa protein may be identified as a member of the family of fumaracetoacetate hydrolases; this family also includes the bifunctional enzyme 2-hydroxyhepta-2,4-diene-1,7-dioate (HHDD) isomerase/5-oxopent-3-ene-1,2,5-tricarboxylate (OPET) decarboxylase, which catalyzes two steps in the catabolic pathway of
homoprotocatechuate (90). The ORF 15 protein indeed exhibits a high degree of similarity to known HHDD isomerases/OPET decarboxylases (see Table 4).

ORF 16 codes for a putative protein composed of 434 aa with a $M_r$ of 47,172 that shows significant similarity to members of the enolase superfamily. These enzymes catalyze diverse overall reactions which however are initiated by a common step, i.e., abstraction of the $\alpha$-proton of a carboxylic acid to form an enolic intermediate (91, 92). The members of the enolase superfamily were assigned to three subgroups by Babbitt et al. (91): (i) mandelate racemases (ML), (ii) muconate lactonizing enzymes (MLE), and (iii) enolases. All members of the superfamily show a two domain structure, one common N-terminal domain and one catalytic TIM-barrel domain that contains residues acting as general acid/base catalysts, and residues that coordinate one (MR, MLE) or two (enolase) metal ion(s). Both domains were predicted for the ORF 16 protein; residues 4-137 and 174-424 resemble the common N-terminal domain (Pfam acc. no. PF02746) and the TIM-barrel domain (Pfam acc. no. PF01188), respectively. Residues which in the catalytic domain of MR and MLE from *P. putida* coordinate a metal ion are also conserved in the ORF 16 protein (MR, acc. no P11444: D195, E221, E247; MLE, acc. no. AAA66202: D198, E224, D249; ORF 16 protein: D248, E274, E301) (91, 92). Moreover, amino acid residues described to be involved in catalysis of MR and MLE were also identified in the ORF 16 protein. In the MR reaction, K166 acts as the $(S)$-specific general base that abstracts the $\alpha$-proton from $(S)$-mandelate. One carboxylate oxygen of the resulting enolic intermediate is thought to be stabilized by functioning as a ligand to the Mg$^{2+}$ and by binding to K164, whereas the second carboxylate oxygen is stabilized through a strong hydrogen bond to E317. In MLE, these functions probably are taken over by the residues K169, K167, and E327 (91). Sequence alignment with the ORF 16 protein revealed K220, K218, and E382 as homologous residues. As enolases are devoid of the KXXK motif of MR and MLR (91), it is unlikely that the ORF16 protein belongs to the enolase subgroup. In the reverse MR reaction, H297 is the $(R)$-specific catalytic base that abstracts the $\alpha$-proton from $(R)$-mandelate, assisted by D270 (91, 93). These two residues are not conserved in MLE, but they actually were found in the ORF 16 protein in positions H351 and D324. Based on the pattern of conserved residues, we suggest that the gene product of ORF 16 belongs to the mandelate racemase subgroup of enzymes. This subgroup apart from MR includes (D)-galactonate
dehydratase, (D)-glucarate dehydratase, (L)-rhamnoate dehydratase, and some reading frames with unassigned functions (91, 93).

DISCUSSION

The qoxLMS genes from Arthrobacter ilicis Rü61a were expressed in a Pseudomonas host, yielding catalytically competent Qox protein. Active quinaldine 4-oxidase was produced by cosmid clones containing the whole 23 kb insert, or a 10.8 kb fragment (Fig. 1), and by P. putida KT2440 pKP1, where expression of the qox genes is regulated by the Pm promotor which in turn is induced by benzoate-activated XylS protein. The specific activity for Qox in the crude extract of P. putida KT2440 pKP1 was as high as that found in crude extracts of the wild-type strain A. ilicis Rü61a. The specific activity of Qox purified from P. putida KT2440 pKP1 even exceeded that determined by Stephan et al. (8) for preparations of wild-type Qox. The biochemical and spectroscopic properties of the Qox protein purified from P. putida KT2440 pKP1 were similar to those of the wild-type enzyme. EPR spectroscopy revealed identical spectral patterns of the FAD-radical signal and the catalytically competent very rapid species in both enzymes (Fig. 4), indicating that the environment of the centers and particularly the mode of substrate/product binding at the site of the Mo(V)-cofactor are very similar. The sole appearance of the FeSII signals at lower temperatures in the enzyme of P. putida KT2440 pKP1 are in accordance with the findings of redox and rapid freeze experiments of Qox from A. ilicis Rü61a for which the redox potential of FeSII was 180 mV higher than that of FeSI. Consequently, the FeSII signal was observed first under single turnover conditions in kinetic EPR experiments (10). Hence, it is concluded that the difference of the redox potentials of both FeS centers in Qox from P. putida KT2440 pKP1 should be similar. The axial type FeSI signal is not found for other proteins of the xanthine oxidase family. Its presence in Qox from the expression clone points to a conserved structural environment of this cluster which also has been shown to be proximal to the molybdenum cofactor (10, 57, 94). The spectral signature of the FeSII center of Qox of P. putida KT2440 pKP1 is similar to that of A. ilicis Rü61a but shows some slight differences in g-factor and line shape. These changes seem to be related to the simultaneous presence of substrate and dithionite reduced clusters. Since this cluster presumably is located close to the surface of the domain it is more
susceptible to solvent effects and generally shows a relaxation behaviour different from FeSI cluster (10, 11, 57, 95). On the whole, our results suggest that the Qox protein produced by the expression clone is identical to the wild-type enzyme, implying that the host strain *P. putida* KT2440 is able to provide all the accessory functions that are required for the assembly of this complex enzyme. Qox is in fact the first MCD-containing enzyme to be synthesized in a catalytically fully competent form by a heterologous host. There is no overexpression of the qoxLMS genes in *P. putida* KT2440 pKP1, however overexpression was not our primary goal, but we intended to construct a system that allows the genetic manipulation of the *qox* genes by mutagenesis approaches, and the production of protein variants of Qox.

Assembly of the Fe/S protein (QoxS) and the flavoprotein (QoxM) is thought to involve ubiquitously conserved pathways. However, biosynthesis of the MCD form of the molybdenum pyranopterin cofactor and its insertion into QoxL requires not only proteins involved in molybdenum uptake and MPT biosynthesis, but also a tailoring enzyme forming MCD from MPT, and maybe even a specific chaperone for MCD insertion (for reviews on molybdate uptake and biosynthesis of the molybdenum cofactor, see: 96, 97). Sequence comparisons of known genes of the moa, moe and mod operons of *E. coli* with the *P. putida* KT2440 genome revealed corresponding sequences with significant similarities, suggesting that strain KT2440 is able to synthesize the MPT cofactor. The successful expression of fully active Qox and the release of CMP upon acidic hydrolysis of the enzyme indicated that *P. putida* KT2440 is also able to provide the MCD cofactor, and moreover, to insert it into the maturing Qox protein.

It is remarkable to note that the gene product of ORF 1 (Fig. 1), which due to its similarity to the XdhC protein (61) is thought to be involved in cofactor insertion during Qox assembly in the wild-type strain, is not required for formation of functional Qox by the *P. putida* expression clone.

On the basis of X-ray crystal structure analyses of aldehyde oxidoreductases from *D. gigas* (MOP) and *D. desulfuricans* (MOD), CO dehydrogenase from *O. carboxidovorans* (Cox), xanthine oxidase/dehydrogenase from bovine milk (XOb) and xanthine dehydrogenase from *R. capsulatus* (XDHBRc), amino acid residues responsible for coordination of the molybdenum pyranopterin cofactor as well as residues probably involved in the catalytic mechanism were described (17-23). Additionally, mutagenesis of xanthine dehydrogenase from *Emericella* (formerly, *Aspergillus*)
nidulans (HxA) defined residues contributing to substrate specificity and substrate positioning at the active site (98). Thus, comparison of Qox with these proteins may provide a first view of the molecular features of the active site of Qox. Non-polar residues described to give access to the active site in MOP and MOD (MOP: F425, F494, L497, L626; MOD: F427, F496, L499, L628) have counterparts in the QoxL sequence (G253, F323, I327, L459). Although the preferred substrates of the aldehyde oxidases (i.e., aliphatic aldehydes) differ from those of Qox (N-heteroaromatic compounds and aromatic aldehydes) with regard to their molecular structure, a hydrophobic entrance to the active site appears to be necessary for both types of enzymes.

Glutamate 833 of HxA is conserved among xanthine dehydrogenases (XOb: E802, XDHBRC: E232) and is proposed to influence the substrate specificity of the enzymes. MOP and MOD have a phenylalanine in this position (F425 and F427, respectively). Alignments of MOP, MOD, QoxL, and QorL revealed G253 for QoxL and A259 for QorL as corresponding residues (Fig. 6A), thus small residues seem to be necessary at this position for the accommodation of the bicyclic azaarenes in the active site.

Of particular interest is a conserved arginine found in the vicinity of the MOP and MOD substrate binding sites (MOP: R501, MOD: R503); these residues correspond to R911 in the E. nidulans xanthine dehydrogenase HxA. This amino acid has been proposed to be involved in positioning the substrate relative to the molybdenum center, since mutations of R911 of HxA yielding G911 or Q911 changed the hydroxylation position of 2-hydroxypurine from C-8 to C-6 (97). In QoxL and QorL this arginine is not conserved, it is exchanged to hydrophobic residues in both enzymes (QoxL: I330, QorL: V339, Fig. 6A).

The regulation of catabolic operons coding for enzymes of aromatic degradation pathways has been the subject of intensive research (for a review, see 99). The regulatory proteins involved were found to belong to a variety of families, among them the family of tetracycline repressors (TetR), to which the protein encoded by ORF 8 seems to be related. Genes and operons regulated by TetR like repressors encode proteins with very diverse functions (100-103). An example is agmatine utilization by P. aeruginosa, which is catalyzed by the products of aguA and aguB, transcribed in one operon and negatively regulated by AguR (104). The calculated $M_r$ of the ORF 8 protein of 21,472 resembles the average $M_r$ of 21,000 to 25,000 reported for the TetR proteins. In the case of the archetypal TetR
repressor, its DNA binding site is located between the \textit{tetR} gene and the vicinal gene \textit{tetA} that is transcribed in the opposite direction. The \textit{tetA} gene, which codes for an energy-dependent tetracycline/Mg\textsuperscript{2+} antiporter, is the target of the TetR mediated regulation (105). Such an arrangement has been found for other \textit{tetR}-like genes and their targets. However, we have not been able to identify possible DNA binding sites for the regulator between ORF 8 and ORF 9, or in front of \textit{qoxL} or ORF 2. Nevertheless, involvement of the putative ORF 8 protein in regulation of transcription of these genes can not be excluded, since the DNA sequences recognized by the HTH-motifs appear to be very diverse among different TetR like regulators.

\textbf{Conclusions.} Identification, sequencing, cloning, and functional, heterologous expression of the \textit{qoxLMS} gene cluster has been achieved in this work. The available expression system will allow the genetic manipulation of the \textit{qox} genes by site-directed mutagenesis. Of special interest is the investigation of the functional role of the glutamate residue E736, which corresponds to the glutamate residues strictly conserved in enzymes of the xanthine dehydrogenase family and which has been predicted to be involved in the catalytic mechanism. Residues thought to be involved in substrate positioning are also important targets for mutagenesis studies; their alteration might give us an idea about the molecular basis of substrate specificity and regioselectivity of hydroxylation.

The identification of a number of putative genes that might be functionally related to quinaldine oxidation may open up further investigations on the anthranilate pathway and its regulation.

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FOOTNOTES

1 Abbreviations: Qox: quinaldine 4-oxidase, Qor: quinoline 2-oxidoreductase, Ior: isoquinoline 1-oxidoreductase, MCD: molybdopterin cytosine dinucleotide, Tc: tetracycline, Km: kanamycin, IUPAC: International Union of Pure and Applied Chemistry, EMBL: European Molecular Biology Laboratory, PCR: polymerase chain reaction, INT: iodinotetrazolium chloride, PAGE: polyacrylamide gel electrophoresis, HPLC: high pressure liquid chromatography, EPR: electron paramagnetic resonance, NMR: nuclear magnetic resonance, UV/Vis: ultra violet/visible, aa: amino acid(s), MOB: aldehyde oxidoreductase from Desulfovibrio gigas, MOD: aldehyde oxidoreductase from Desulfovibrio desulfuricans, XDHRe: xanthine dehydrogenase from Rhodobacter capsulatus, XOb: Xanthine oxidase from cow’s milk, ORF: open reading frame, Hod: 1H-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase, TetR: tetracycline repressor, HTH: helix-turn-helix, SDR: short chain dehydrogenase, COG: cluster of orthologous group, HHDD: 2-hydroxyhepta-2,4-diene-1,7-dioate, OPET: 5-oxopent-3-ene-1,2,5-tricarboxylate, MR: mandelate racemase, MLE: muconate lactonizing enzyme, MPT: molybdopterin cofactor, HxA: xanthine dehydrogenase from Emericella nidulans, Ap: ampicillin

2 EMBL Accession Number AJ537472

LEGENDS TO FIGURES

Fig. 1:
23 kb DNA fragment of A. ilicis Rü61a cloned into the cosmid pVK100, yielding pVK55B/5. Genes coding for the subunits of Qox and for Hod are named accordingly. Genes coding for proteins proposed to be involved in quinaldine degradation are in light grey. For a detailed description of the ORFs, see text. The arrows indicate the DNA fragments subcloned for sequencing. Striped boxes give the positions of the probes „1.1 DpnI“ and „b-DIG“.
Fig. 2:

(A)
Non-denaturing polyacrylamide gel of crude extracts stained for Qox activity. Lane 1: *P. putida* KT2440 pKP1; Lane 2: *A. ilicis* Rü61a; Lane 3: *P. putida* KT2440 pJB653.

(B)
SDS-PAGE of purified quinaldine 4-oxidase (lane 1) from *P. putida* KT2440 pKP1 dissociated into its three subunits (82, 35, and 22 kDa), indicating electrophoretic homogeneity of the preparation. Lane 2: standard proteins: β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumine (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease Bsp98I (25 kDa), β-lactoglobuline (18.4 kDa).

Fig. 3:
UV/Visible spectrum of Qox purified from *P. putida* KT2440 pKP1 (1.2 mg/ml in 50 mM Tris-HCl, pH 7, containing 0.25 M NaCl).

Fig. 4:
EPR spectra of Qox from *A. ilicis* Rü61a (A) and *P. putida* KT2440 pKP1 (B, C) measured at 65 K. The sample in B was exposed to a ca. 10-fold excess of substrate; in A,C to a 60-fold excess. (vr = very rapid species, r = rapid species)

Fig. 5:
EPR spectra of Qox from *P. putida* KT2440 pKP1 reduced with a 10-fold excess of substrate (A) and fully reduced by subsequent addition of dithionite (B) in comparison to the spectrum of Qox from *A. ilicis* Rü61a (C). Spectrum A was recorded at 15 K, B and C at 25 K. The assignment of EPR lines to the two 2Fe2S-centers I and II is indicated.

Fig. 6:
Comparison of sequence stretches of different molybdenum hydroxylases.

(A) Conserved motifs presumed to be involved in binding the pyranopterin cofactor or in positioning of the substrate. Sequence stretches of QoxL are compared to corresponding sequences of the large subunit of quinoline 2-oxidoreductase from *P. putida* 86 (QorL) (acc. no. CAA66828), the large subunit of CO dehydrogenase from *O. carboxidovorans* (CoxL) (acc. no. P19919), aldehyde
oxidoreductases from \textit{D. gigas} (MOP) (acc. no. A57429), and \textit{D. desulfuricans} (MOD) (acc. no. CAB64929), xanthine oxidase from cow’s milk (XOb) (acc. no. P80457), and xanthine dehydrogenase from \textit{R. capsulatus} (XDHBRc) (acc. no. CAA04469). Residues that are completely conserved are in shaded boxes. Residues supposed to be involved in coordination of the molybdenum pyranopterin cofactor and in catalysis which are discussed in the text are marked with an asterisk (*) below the sequence.

Based on our sequencing results, residues 465 and 466 in QorL were corrected (EV, instead of DC reported previously (28) / acc. no. CAA66828).

(B) Sequence stretches of the medium sized subunit QoxM comprising the loop-forming FAD-binding sites as described for the vanillyl-alcohol oxidases, compared to the corresponding segments of QorM, CoxM, XDHARc, and XOb (for an explanation of the abbreviations, see (A)); NdhM: medium sized subunit of the nicotine dehydrogenase from \textit{A. nicotinovorans} (acc. no. CAD47954).
Table 1. Bacterial strains and plasmids

| Bacterial strains and plasmids | Description | Source or reference |
|-------------------------------|-------------|---------------------|
| *Arthrobacter ilicis* Rü61a  | Wild-type strain utilizing quinaldine as sole source of carbon, nitrogen and energy | 106          |
| *E. coli* HB101              | F- leuB6 Δ(gpt-proA)62 glnV44 recA13 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL20 (Str') Δ(mcrC-mrr) | 31          |
| *E. coli* DH5α               | endA1 (rκ mκ') glnV44 thi-1 gyrA (Nal') relA1 Δ(lacIZYA-argF)U169 deoR (ϕ80lacΔ(lacZ)M15) hsdR17 recA1 | 32          |
| *E. coli* XL1-Blue MRF′      | F′::Tn10 proA′ B′ lacIΔ(lacZ)M15 recA1 endA1 gyrA96(Nal') thi hsdR17 (rκ mκ') glnV44 relA1 lac | Stratagene |
| *Pseudomonas putida* KT2440  | r′ derivative of *P. putida* mt-2 | 34, 51 |
| pVK100                       | IncP Tc′ Km′ | 39          |
| pUC18                        | colE1 lacZ Ap′ | 38          |
| pJB653                       | Broad-host-range cloning vector: RK2 replicon, Pm promoter, xylS for transcriptional regulation, Ap′ | 41          |
| pVK55B/5                     | 23 kb insert of *A. ilicis* Rü61a DNA in *Hind*III site of pVK100 | This work |
| pVK55/11                     | 10.8 kb *Hind*III fragment of pVK55B/5 in *Hind*III site of pVK100 | This work |
| pUC55/11                     | 10.8 kb *Hind*III fragment of pVK55B/5 in *Hind*III site of pUC18 | This work |
| pUC55/3.2                    | 3.2 kb *SmaI* fragment of pUC55/11 in *SmaI* site of pUC18 | This work |
| pUC55/4.5                    | 4.58 kb *SmaI* fragment of *A. ilicis* Rü61a genomic DNA in *SmaI* site of pUC18 | This work |
| pUC55/3                      | 2.9 kb *SmaI/Hind*III fragment of pUC55/11 in *SmaI* and *Hind*III sites of pUC18 | This work |
| pKP1                         | *qoxLMS* (PCR amplificate of the structural genes of quinaldine 4-oxidase) inserted in *EcoRI* and *SmaI* sites of pJB653 | This work |
Table 2: Purification of quinaldine 4-oxidase from *P. putida* KT2440 pKP1

Starting material was 22 g of wet biomass

| Fraction                  | Activity (Units) | Protein (mg) | Sp. Activity (U mg⁻¹) | Purification (-fold) | Yield (%) |
|---------------------------|------------------|--------------|-----------------------|----------------------|-----------|
| Crude extract             | 82               | 1390         | 0.059                 | 1                    | 100       |
| DEAE-Sepharose CL-6B      | 60               | 75           | 0.79                  | 13.5                 | 72        |
| Phenyl Sepharose CL-4B    | 23               | 17           | 1.37                  | 23.2                 | 28        |
| UNO™ Q 1                  | 15.5             | 3.5          | 4.54                  | 77                   | 19        |
| HiLoad 26/60 Superdex 200 | 5.6              | 1.12         | 5                     | 85                   | 7         |
Table 3: Kinetic parameters of the Qox proteins

| Source of Qox | $K_m$ app (quinaldine) (M) | $k_{cat}$ app (quinaldine) (s$^{-1}$) | $K_m$ app (INT) (M) | $k_{cat}$ app (INT) (s$^{-1}$) |
|---------------|---------------------------|--------------------------------------|-------------------|---------------------------|
| *A. ilicis* Rü61a (grown on quinaldine) | $3.4 \times 10^{-5}$ | 16.7 | $1.9 \times 10^{-5}$ | 15.6 |
| *P. putida* KT2440 pKP1 (grown on benzoate) | $3.5 \times 10^{-5}$ | 29.4 | $1.7 \times 10^{-5}$ | 24.9 |
| gene/ORF no. | (hypothetical) gene product                                      | % similarity/% identity to published aa sequences (accession number) |
|-------------|------------------------------------------------------------------|-------------------------------------------------------------------|
| qoxL        | large subunit of quinaldine 4-oxidase                            | 47/36 put. nicotine dehydrogenase (BAA81228), 45/36 put. aldehyde oxidase (CAC48532) |
| qoxM        | medium subunit of quinaldine 4-oxidase                          | 46/34 put. nicotine dehydrogenase (Q9Y9R9), 42/32 put. CO dehydrogenase (Q97W13) |
| qoxS        | small subunit of quinaldine 4-oxidase                           | 59/51 put. CO dehydrogenase (Q98L90), 57/50 put. CO dehydrogenase (BAC50930) |
| ORF 1       | putative chaperone for Moco insertion                            | 68/56 ORF377 (AAK64260), 41/32 put. XdhA (O32147), 43/32 put. Xdh accessory factor (AAN68092) |
| hod         | 1H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase                     | 46/38 1H-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (CAA75080), 43/25 put. dehalogenase (BAC52230) |
| ORF 2       | putative flavin monooxygenase                                   | 58/36 MhpC oxygenase (AAB60878), 43/36 2-methyl-3-hydroxypyridine-5-carboxylic acid oxygenase (NP_107231) |
| ORF 4       | putative esterase                                               | 49/40 put. esterase (Q9AQ20), 38/30 put. carboxylesterase (AAK46863) |
| ORF 5       | putative transporter                                            | 39/27 permease (CAB50057), 36/28 put. transport transmembrane protein (CAD18765) |
| ORF 6       | putative glutamyl t-RNA synthase                                | 64/56 glutamyl t-RNA synthetase (NP_599489), 61/55 put. glutamyl t-RNA synthetase (BAC17013) |
| ORF 7       | putative β-lactamase related protein or esterase                | 87/82 esterase (AAA99492), 43/38 put. esterase (CAB59658) |
| ORF  | Description                                      | Match Details                                                                 |
|------|--------------------------------------------------|-------------------------------------------------------------------------------|
| 8    | putative TetR-type repressor                    | 42/35 transcriptional regulator (AAK24175), 41/35 put. transcriptional regulator (CAC33063) |
| 9    | putative member of SDR superfamily              | 47/38 put. dehydrogenase (BAB74590), 48/41 put. ketoacyl reductase (CAA98318) |
| 10   | HD-domain protein (phosphohydrolase)            | 43/30 (N-terminal) put. guanosine-3,5-bis(diphosphate) 3-pyrophosphohydrolase (AAO89860), 40/31 (N-terminal) guanosine-3,5-bis(diphosphate)-3-pyrophosphohydrolase (AAG08723) |
| 11   | putative transmembrane protein                  | 51/43 put. integral membrane protein (CAB90976), 36/28 probable transmembrane protein (CAD13758) |
| 12   | putative AAA ATPase                             | (C-terminal) 43/31 cell division control protein 48 (CAB49317), 41/27 AAA family ATPase (AAK42941) |
| 13   | unknown                                         | (C-terminal) 43/32 put. ATP/GTP-binding protein (CAD47885)                      |
| 14   | putative member of SDR superfamily              | 73/69 probable oxidoreductase (CAB55718), 70/65 put. 3-oxoacyl-(acyl-carrier protein) reductase (CAC01650) |
| 15   | putative HHDD isomerase/OPET decarboxylase      | 66/59 put. HHDD isomerase (CAB55708), 58/48 put. HHDD isomerase/OPET decarboxylase (AAM39022) |
| 16   | putative protein of mandelate racemase subgroup | 75/66 rTS-beta protein (AAM43290), 67/56 mandelate racemase (AAL52888)         |

1 BLAST P matches with hypothetical proteins without any predicted function have been excluded in these sequence comparisons.
Fig. 2

(A)  

(B)  

1  2  3  

1  2
Fig. 3
### Moco I

| Protein | Sequence                  |
|---------|---------------------------|
| QoxL    | 243 LNVGSPFGSKGD          |
| Mop    | 415 NPGGSTFGYKFS          |
| Mod    | 417 NTTGTFGTRKFS          |
| QorL   | 249 PDVGFQFGQKAH           |
| CoxL   | 265 PDIGGGFGNKG           |
| XDHRec | 222 RRGGGFGGKES           |
| Xob    | 752 KRIGGGFGGKET          |

### Moco II

| Protein | Sequence                  |
|---------|---------------------------|
| QoxL    | 321 SPFGG...QI.MKIGLH.IGM |
| Mop    | 492 SFEGD....LTLRAGQFGA   |
| Mod    | 494 SFEGD....LTLRAGYIGA   |
| QorL   | 327 NNLPTQVLVESHRGNAVILG  |
| CoxL   | 343 DACADPS...KWPAGMNICT  |
| XDHRec | 301 ADLSL...PVDRAMLHADG   |
| Xob    | 871 RDLSH...SIMERALFMHDN  |

### Moco III

| Protein | Sequence                  |
|---------|---------------------------|
| QoxL    | 357 SG.GPVRF.GF          |
| Mop    | 528 WGC.SAFA.GY          |
| Mod    | 530 WAG.AAFR.GY          |
| QorL   | 366 PI.GAYR.GV           |
| CoxL   | 381 SGYAVYRCFSF         |
| XDHRec | 337 SN.TAFR.GF           |
| Xob    | 907 SN.TAFR.GF           |

### Moco IV

| Protein | Sequence                  |
|---------|---------------------------|
| QoxL    | 482 DVPFGQGHHT           |
| Mop    | 650 WEDHQQGADI           |
| Mod    | 652 WEDHQQGADA           |
| QorL   | 503 LASSQGGHE            |
| CoxL   | 525 TSQGGGHE            |
| XDHRec | 485 GTEMGQGLHA           |
| Xob    | 1035 GTEMGQGLH           |

### Moco V

| Protein | Sequence                  |
|---------|---------------------------|
| QoxL    | 670 GQHGFGFAHG          |
| Mop    | 806 GQYGGLQOG           |
| Mod    | 806 GQYGGLQOG           |
| QorL   | 677 GRIQGILQA           |
| CoxL   | 677 GQYHGGLTEA          |
| XDHRec | 662 GQIEGAVYQQG         |
| Xob    | 1153 GQVEGAFVQGG        |

### (B)

| Protein | Sequence |
|---------|----------|
| QoxM    | 30 AGGCT  |
| QorM    | 31 AGGCS  |
| NdhM    | 31 AGGCS  |
| CoxM    | 32 AGGHS  |
| XDHRec  | 203 AGGTD |
| Xob     | 259 VGYTE  |

**Fig 6**

(A)

Moco I

Moco II

Moco III

Moco IV

Moco V
Gene cluster of Arthrobacter ilicis Rü61a involved in the degradation of quinaldine to anthranilate. Characterization and functional expression of the quinaldine 4-oxidase genes qoxLMS
Katja Parschat, Bernhard Hauer, Reinhard Kappl, Roswitha Kraft, Jürgen Hüttermann and Susanne Fetzner

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