Resorcinol Hydroxylase of *Azoarcus anaerobius*: Molybdenum Dependence, Activity, and Heterologous Expression

Paula I. Darley^{1,2} · Jutta Hellstern^{1,3} · Bernhard Schink¹ · Bodo Philipp^{1,4}

Abstract

The obligately anaerobic, denitrifying bacterium *Azoarcus anaerobius* strain LuFRes1 grows with resorcinol (1,3-dihydroxybenzene) as sole carbon and energy source. Resorcinol is oxidized to hydroxyhydroquinone (1,2,4-trihydroxybenzene) by resorcinol hydroxylase (RH), an inducible membrane-bound enzyme. Sequence comparison places resorcinol hydroxylase into the group of anaerobic molybdopterin oxidoreductases and dimethyl sulfoxide reductase-like enzymes. In the large subunit, a molybdopterin-binding domain was predicted, and the small subunit most likely contains two [4Fe-4S] centers. Growth of molybdate-starved cells was inhibited by tungstate, and in vitro resorcinol hydroxylase activity was inhibited by arsenite and selenite that are known to inhibit molybdenum-containing enzymes. The two genes encoding resorcinol hydroxylase could be expressed in *Escherichia coli* but the products remained in inclusion bodies. All attempts to purify RH from *A. anaerobius* or to produce soluble, active RH in *E. coli* failed. Nevertheless, RH was produced as a C-terminally Strep-tagged protein from plasmid pSKM1 in *Thauera aromatica* AR1 transconjugants carrying a transposon insertion in the coding gene for the large (ΔrhL) or the small subunit (ΔrhS) of RH from cosmid R⁺. RH in the membrane fraction of wild-type transconjugant *T. aromatica* AR1/R⁺ showed a specific activity of 80 mU mg⁻¹, and the specific activity of RH in the membranes of the complemented mutants was in the same range (80–95 mU mg⁻¹). We conclude that RH of *A. anaerobius* is a membrane-bound molybdoenzyme consisting of two subunits which might require a further loosely bound subunit as membrane anchor.

Introduction

Bacteria degrade aromatic compounds under oxic and anoxic conditions. In aerobic degradation of aromatics, molecular oxygen acts as a co-substrate in substrate activation and ring fission [1]. In the absence of oxygen, different strategies are used for degradation of aromatics. First, the broad variety of aromatic compounds is transformed into few central intermediates, namely benzoyl-CoA, hydroxyhydroquinone, phloroglucinol, and resorcinol, which are subsequently

de-aromatized and further degraded [2–5]. Most anaerobic bacteria reduce the aromatic ring of the central intermediates before ring fission takes place by benzoyl-CoA reductases which are the most common enzymes involved in ring reduction. However, *Azoarcus anaerobius* and *Thauera aromatica* AR1, two nitrate-reducing bacteria, attack resorcinol or α -resorcyate (3,5-dihydroxybenzoate) anaerobically by an oxidative rather than a reductive reaction. *Azoarcus anaerobius* hydroxylates resorcinol to hydroxyhydroquinone (1,2,4-trihydroxybenzene, HHQ) [6], and *T. aromatica* AR1 transforms α -resorcyate as well by hydroxylation, followed by decarboxylation to HHQ [7]. HHQ is oxidized by a membrane-bound enzyme to hydroxybenzoquinone (2-hydroxy-1,4-benzoquinone, HBQ), a non-aromatic intermediate which is prone to ring fission, both in *A. anaerobius* and in *T. aromatica* AR1 [8].

The oxidative pathway involving HHQ is apparently widespread in anoxic habitats [9] and its regulation has been investigated in *A. anaerobius* LuFRes1 and *T. aromatica* AR1 [10, 11]. In vitro studies with *A. anaerobius* strain LuFRes1 demonstrated that resorcinol hydroxylase (RH)

✉ Bodo Philipp
bodo.philipp@uni-muenster.de

¹ University of Konstanz, Fachbereich Biologie,
78457 Constance, Germany

² Present Address: Cambivac Ltd, Cambridge CB22 3AT, UK

³ Present Address: Novartis Pharma AG, Lichtstrasse 35,
4056 Basel, Switzerland

⁴ Present Address: Institute for Molecular Microbiology
and Biotechnology, University of Münster, Corrensstr. 3,
48149 Münster, Germany

activity is membrane-associated and uses nitrate or potassium ferricyanide as electron acceptor [6]. A genomic fragment of *A. anaerobius* was isolated that is located on cosmid R⁺ and enables the *T. aromatica* strains AR1 and K172 to grow with resorcinol. By heterologous expression of mutagenized cosmids in both *T. aromatica* strains, we identified the genes encoding resorcinol hydroxylase, HHQ dehydrogenase, and putative enzymes for ring fission of HBQ [12]. Furthermore, genes involved in α -resorcyate degradation have been identified in *T. aromatica* AR1 [13]. Mutants defective in the genes *dbhL* and *dbhS* are not able to grow with α -resorcyate and are also deficient in α -resorcyate hydroxylase activity. Anaerobic hydroxylation reactions are often catalyzed by molybdenum-containing enzymes [14]. The genes encoding resorcinol hydroxylase as well as *dbhLS* from strain AR1 were found to be homologous to the genes for phloroglucinol-pyrogallol *trans*hydroxylase of *Pelobacter acidigallici*, a well-characterized molybdoenzyme [15]. The goal of the present study was to investigate in vivo and in vitro whether resorcinol hydroxylase is a molybdenum-containing enzyme.

Materials and Methods

Materials

All chemicals and biochemicals were purchased from Fluka, Merck, Serva, Sigma-Aldrich or Biozym and were of the highest quality available. Enzymes used for cloning and other materials used for molecular biology techniques were obtained from MBI Fermentas, Invitrogen, Stratagene, or Qiagen. PCR and sequencing primers were purchased from MWG. Custom sequencing was performed at GATC. Strep-tactin AP conjugate used for the western blots was purchased from IBA GmbH. Protein molecular mass marker was purchased from Bio-Rad and DNA size markers were from MBI Fermentas.

Bacterial Strains, Vectors, and Culture Conditions

Bacteria and vectors used in this work are summarized in Table 1. Unless stated otherwise, *A. anaerobius* strain

Table 1 Bacterial strains and vectors used in this study

Strain or vectors	Relevant characteristics	Source or reference
Strains		
<i>A. anaerobius</i>		
LuFRes1 (DSM12081)	Wild-type, Res ⁺	[38]
<i>T. aromatica</i>		
AR1 (DSM11528)	Wild-type, Res ⁻ , DHB ⁺ , HHQ pathway ⁺	[7]
<i>E. coli</i>		
DH5 α	F ⁻ , ϕ 80d λ lacZ Δ M15, Δ (lacZYA-argF)U169, <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>phoA</i> , <i>supE44</i> , λ^- , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	[16]
HB101	<i>supE44</i> , <i>hsdS20</i> (r _B ⁻ m _B ⁻), <i>recA13</i> , <i>ara-14</i> , <i>proA2</i> , <i>galK2</i> , <i>rpsL20</i> , <i>xyl-5</i> , <i>mtl-1</i> , <i>leuB6</i> , <i>thi-1</i> , <i>lacY1</i>	[16]
BL21 (DE3) Codon Plus-RIL	F ⁻ <i>ompT</i> <i>hsdS</i> (r _B ⁻ m _B ⁻) <i>dcm</i> ⁺ Tet ^r <i>gal</i> λ (DE3) <i>endA</i> Hte[<i>argU</i> <i>ileY</i> <i>leuWCm</i> ^r]	Stratagene
Rosetta (DE3)pLysS	F ⁻ <i>ompT</i> <i>hsdS</i> (r _B ⁻ m _B ⁻) <i>gal</i> <i>dcm</i> (DE3) pLysSRARE2 (Cm ^r)	Novagen
Plasmids		
pRK600	Cm ^r , ColE1, Tra ⁺	[39]
pASK-IBA3plus	Ap ^r , f1, <i>tetA</i> promoter, tag fused at C-terminus, used for construction of pPDI2	IBAGmbH
pASK-IBA5plus	Ap ^r , f1, <i>tetA</i> promoter, tag fused at N-terminus, used for construction of pPD2	IBAGmbH
pBBR-MCS5	Gm ^r , broad-host-range, used for construction of pSKM1	[40]
pPD2	Ap ^r , expression plasmid of N-terminus tagged protein	This study
pPID2	Ap ^r , expression plasmid of C-terminus tagged protein	This study
pSKM1	Gm ^r , complementing plasmid harboring <i>rhLS</i> with Strep-tag at C-terminus of RhS subunit	This study
Cosmids		
R ⁺	Tc ^r , pLAFR3 containing a 29.9-kp resorcinol gene cluster of <i>A. anaerobius</i>	[12]
Mu_21 (Δ <i>rhL</i>)	Tc ^r , Km ^r , R ⁺ <i>rhL</i> ::TN<KAN-2>, <i>rhL</i> transposon mutant	[12]
Mu_40 (Δ <i>rhS</i>)	Tc ^r , Km ^r , R ⁺ <i>rhS</i> ::TN<KAN-2>, <i>rhS</i> transposon mutant	[12]

Res⁺ growth on resorcinol, Res⁻ no growth on resorcinol, DHB⁺ growth on 3,5-dihydroxybenzoate, DHB⁻ no growth on 3,5-dihydroxybenzoate, HHQ pathway⁺ possess HHQ pathway, Km^r kanamycin resistant, Cm^r chloramphenicol resistant, Ap^r ampicillin resistant, Gm^r gentamicin resistant

LuFRes1 (DSM12081) and *T. aromatica* strain AR1 (DSM 11528) were cultured under denitrifying conditions in non-reduced mineral medium with resorcinol (2 mM) as the sole source of carbon and energy as described previously [12]. N₂O was added to cultures to a final concentration of 10% with a glass syringe through a hydrophobic sterile filter. Growth was measured as optical density at 578 nm (OD₅₇₈) either directly in test tubes with a Bausch and Lomb Spectronic 70 spectrophotometer or in 1 cm cuvettes with a Hitachi 100-40 spectrophotometer. Molybdenum-free medium was prepared by omitting the selenite-tungstate solution and the molybdate in the SL 10 solution. For these experiments, all glassware was pre-incubated overnight in 25% HCl, washed four times with pure Milli-Q water, incubated for more than 6 h in 4 N NaOH, and washed again 4 times with pure Milli-Q water.

Thauera aromatica AR1 transconjugants carrying the Mu_40 (ΔrhS) or Mu_21 (ΔrhL) cosmid [12] with or without the pSKM1 vector were grown under the same conditions in the presence of kanamycin (50 $\mu\text{g ml}^{-1}$) with or without gentamicin (10 $\mu\text{g ml}^{-1}$), respectively. For biochemical studies, all strains were cultured in 1 l infusion bottles and harvested at an OD₅₇₈ of 0.3–0.4. Cultures were supplemented with the carbon source and nitrate from 0.5 M sterile stock solutions. To study functional complementation of the *T. aromatica* AR1 carrying Mu_40 or Mu_21 cosmids, 100 ml serum bottles containing 50 ml anoxic mineral medium supplemented with resorcinol (2 mM), nitrate (8 mM), kanamycin (50 $\mu\text{g ml}^{-1}$), and gentamicin (10 $\mu\text{g ml}^{-1}$) were inoculated with 1% pre-culture. *Escherichia coli* HB101 (pRK600) was used as a helper strain and DH5 α harboring Mu_40 or Mu_21 cosmids or the pSKM1 vector, were used as donor strains in triparental matings as described previously [12]. All *E. coli* strains were grown aerobically at 37 °C in Luria–Bertani, LB [16] or Standard I medium from Merck. Antibiotics were used at the following concentrations: tetracycline, 20 $\mu\text{g ml}^{-1}$; chloramphenicol, 30 $\mu\text{g ml}^{-1}$; carbenicillin, 50 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; gentamicin, 10 $\mu\text{g ml}^{-1}$.

Construction of Expression and Complementation Plasmids

Routine manipulation of plasmid DNA, PCR, and construction of recombinant plasmids were performed as previously described [16]. The individual cloning steps were carried out in *E. coli* DH5 α .

Construction of Expression Plasmids

Genomic DNA of *A. anaerobius* strain LuFRes1 was isolated with Puregene DNA Purification Kit (Qiagen) and used as a template for amplification of resorcinol hydroxylase

genes by PCR. To minimize PCR errors a Hi-Fidelity DNA polymerase (Phusion, NEB) was employed. For cloning *rhLS* genes in pASK-IBA5plus expression plasmid the following primers were used: 5'-ATGGTACGTCCTCAGCGCCATGGTCATTGGCCGCGACGAG-3' (forward) and 5'-ATG GTACGTCCTCATATCACGCCTGCGCGCGCTGCTGG-3' (reverse) and for cloning in pASK-IBA3plus expression plasmid 5'-AGAATTTTGGTACCATGGTCATTGGCCGCGACGAGCTG-3' (forward) and 5'-AGGCCTCGAGAGACGCCTGCGCGCGCTGCTG-3' (reverse) oligonucleotides were used. The cleavage sites for the restriction enzymes in the above primers are underlined. The amplified fragments were either digested with *Esp3I* and inserted into the *Eco3II* site of pASK-IBA5plus or digested with both *KpnI* and *XhoI* and ligated into the *KpnI*-*XhoI* site of pASK-IBA3plus. The first approach generated the expression plasmid pPD2 while the latter yielded pPID2. Both plasmids were double-strand sequenced by a company (GATC, Constance, Germany).

Construction of Complementation Plasmid

A fragment carrying both resorcinol hydroxylase genes and the Strep-tag was obtained from plasmid pPID2 digested with both *KpnI* and *HindIII* and ligated into the *KpnI*-*HindIII* site of the broad host-range vector pBBR-MCS5; the constructed plasmid was designated pSKM1.

Resorcinol Hydroxylase Gene Expression in *E. coli*

The *E. coli* Rosetta (DE3)pLysS strain was used to transform the expression plasmids pPD2 and pPID2. The growth conditions (media, temperature, oxygen, molybdate) and the concentration of the inducer were varied in order to establish optimal conditions for the production of soluble proteins. Cells were grown aerobically at 30–37 °C and induced at OD₅₇₈ = 0.3–0.5 with anhydrotetracycline (200 $\mu\text{g l}^{-1}$) for further 3 h.

Testing for Complementation

Plasmid pSKM1 was mated into *T. aromatica* AR1 transconjugants carrying Mu_21 (ΔrhL) or Mu_40 (ΔrhS) cosmid via triparental-mating as described before [12] and selected by growth under denitrifying conditions in non-reduced minimal medium with resorcinol and 3,5-dihydroxybenzoate (2 mM each) as the sole carbon and energy source plus nitrate (16 mM), kanamycin (50 $\mu\text{g ml}^{-1}$) and gentamicin (10 $\mu\text{g ml}^{-1}$). The transconjugants were analyzed for colony shape and motility, and 16S rDNA gene sequencing confirmed that the transconjugants were indeed *T. aromatica* AR1. After three transfers with both carbon sources, the resulting transconjugant strains were tested to determine if the subclone complemented the mutations by growing the

cultures in the presence of kanamycin and gentamicin with resorcinol. The ability of the transconjugants to grow with resorcinol was confirmed by substrate depletion analysis, and immunoblotting analysis was employed to confirm RH expression in the complemented mutants. Growth and substrate depletion were analyzed as described previously [12].

Preparation of Cell-Free Extracts and Detection of Resorcinol Hydroxylase Activity

Cell-free extracts for biochemical tests were prepared anoxically as described before [12]. Resorcinol hydroxylase activity was determined anoxically at 30 °C in a total volume of 1 ml containing 50 mM potassium phosphate buffer, pH 7.0, 1 mM $K_3Fe(CN)_6$, and 0.5 to 1.5 mg total protein. The reaction was started by addition of 1 mM resorcinol, and the reduction of $K_3Fe(CN)_6$ was monitored in a Hitachi 100-40 spectrophotometer at 420 nm ($\epsilon = 0.9 \text{ mM}^{-1} \text{ cm}^{-1}$). One unit of activity is defined to catalyze the reduction of 1 μmol of $K_3Fe(CN)_6 \text{ min}^{-1}$ at 30 °C.

Purification of Resorcinol Hydroxylase

All purification steps were performed in an anoxic chamber (Coy Laboratories) in a N_2/H_2 (95%/5%) atmosphere at 15–20 °C. Resorcinol hydroxylase (RH) was purified from anaerobically grown cells of *T. aromatica* AR1/Mu_40 (pSKM1) transconjugant. The cell pellet (1 g) was washed with buffer A (100 mM potassium phosphate buffer, pH 7.6) and resuspended in 3 ml buffer B (100 mM potassium phosphate buffer, pH 7.6, with 150 mM NaCl). Cell-free extract was prepared as described previously [12]. Membranes were resuspended and solubilized in 3 ml buffer B supplemented with 3% (w/v) Triton X-100 and incubated for 2 h on ice. Cell debris was removed by ultracentrifugation ($100,000 \times g$, 1 h) and the clear supernatant was diluted to a final volume of 9 ml with buffer B and applied to a Strep-Tactin Superflow column (1 ml) equilibrated with buffer C [buffer B supplemented with 1% (w/v) Triton X-100]. Non-bound protein was washed off with buffer B prior to elution of RH with *d*-desthiobiotin (2.5 mM) in buffer B. The enzyme (fraction 3 and 4) was concentrated in 500 μl Vivaspin centrifugation devices with a 30 kDa cut-off.

Protein Determination

Protein concentrations were determined according to Bradford [17] with bovine serum albumin as standard.

High-Performance-Liquid-Chromatography (HPLC)

HPLC analyses were used as described previously for quantifying resorcinol [6] and nitrate/nitrite [12].

Electrophoresis and Western Blotting

SDS-PAGE was performed in a vertical electrophoresis device (Mini-Protean II, Germany) after Laemmli [18]. Gels consisted of a 5% stacking gel and a 10% or 12% resolving gel and were run at 120 mV voltage. Protein samples were pre-treated with SDS-loading buffer (50 mM, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100 mM DTT) for 3 min at 100 °C, and 6 μg protein was loaded per lane. Gels were stained with Coomassie Brilliant Blue R250 (0.1%) for 4 h and de-stained (10% glacial acetic acid, 40% methanol) for another 3–4 h. Digital pictures were taken immediately after de-staining. For western blotting, protein samples were subjected to SDS-PAGE and transferred (400 mA, 90 min) onto a nitrocellulose membrane (0.2 μm). Resorcinol hydroxylase immunoblot analysis was performed using Strep-tactin alkaline phosphatase conjugate, and the signals were detected with NTB and BCIP according to the supplier's recommendation.

Sequence Analysis

Nucleotide and amino acid sequences were analyzed using tools provided by the National Center for Biotechnology Information (<https://www.ncbi.nlm.nih.gov/blast/>), and the ExpASY molecular biology server (<https://www.expasy.ch/>). Transmembrane helices in proteins were predicted using TMHMM server 2.0 (<https://www.cbs.dtu.dk/services/TMHMM/>).

Sequence Accession Numbers

The amino acid sequences for RhS and RhL were deposited in the NCBI (GenBank) under the accession numbers ABK58619 and ABK58620, respectively.

Results and Discussion

Analysis of the Amino Acid Sequences of the Two Subunits of Resorcinol Hydroxylase

Based on genetic evidence, the *rhLS* genes of the *A. anaerobius* genome have been suggested to encode RH [12]. According to the nucleotide sequence, the polypeptides encoded by the *rhLS* genes representing the large and the small subunits of the hydroxylase consist of 915 and 298 amino acids and have predicted molecular masses of 102.7 and 33.2 kDa, respectively. The *rhL* and *rhS* genes are separated by a 49 nucleotide non-coding DNA sequence. Data Bank analysis and alignment studies for RhS and RhL revealed identities of 49% and 52% and similarities of 68% and 66%, respectively, to the functionally analyzed small

and large subunits of *P. acidigallici* pyrogallol–phloroglucinol *trans*hydroxylase (aTH). Sequence analysis of RhL showed that RH, as well as pyrogallol–phloroglucinol *trans*hydroxylase, belongs to the DMSO reductase (DMSOR) family (Fig. 1a). Enzymes within the DMSOR family are characterized by the presence of one molybdenum atom and two molecules of molybdopterin guanosine dinucleotide (MGD) and share a molybdenum-containing large subunit, such as DMSO reductase [19, 20], formate dehydrogenase [21, 22], and dissimilatory nitrate reductase [23], but may also have one or two additional small subunits as found in pyrogallol–phloroglucinol *trans*hydroxylase [15] or formate dehydrogenase-N [22]. Crystal structures have been solved for DMSO reductase [20] and pyrogallol–phloroglucinol *trans*hydroxylase [15]. Of the 30 amino acids shown to contact the molybdenum cofactor (MoCo) in DMSO reductase and in the alpha subunit of pyrogallol–phloroglucinol *trans*hydroxylase, 12 and 24 residues, respectively, are identical in the large subunit of resorcinol hydroxylase, indicating that the MoCo should be bound exclusively in RhL (data not shown). In pyrogallol–phloroglucinol *trans*hydroxylase, Ser175 is the molybdenum-coordinating residue, while Tyr560 has been proposed not to be involved in the catalytic mechanism but to be an active site “protector” since its side chain can adopt two different conformations. When a substrate or an inhibitor enters the channel to the active site, a recognition process takes place allowing tyrosine 560 to change its conformation in order to open or lock the access to the molybdenum [15]. Interestingly, Ser196 of RhL aligns to Ser175 of aTH, and Tyr572 aligns to Tyr560 (Fig. 1a).

An amino acid sequence alignment of the deduced *A. anaerobius* *rhS* gene product and the small subunit of pyrogallol–phloroglucinol *trans*hydroxylase of *P. acidigallici* is shown in Fig. 1b. Eight out of eleven cysteines present in *A. anaerobius* RhS aligned to the eight cysteines of the *P. acidigallici* protein previously shown to be involved in iron-sulfur cluster coordination [15]; the same cysteines also aligned with eight cysteines of the beta subunit of dimethylsulfide dehydrogenase of *Rhodovulum sulfidophilum*. The beta subunit of dimethylsulfide dehydrogenase, an iron-sulfur cluster enzyme, is predicted to be involved in electron transfer during phototrophic growth with dimethylsulfide. These *in silico* findings strongly suggest the presence of a molybdenum cofactor in RhL and at least two 4Fe–4S clusters in RhS.

Although resorcinol hydroxylase activity is localized in the membrane fraction, the sequences do not show any indications for a transmembrane domain.

Effect of Molybdate on *A. anaerobius* Growth and Resorcinol Hydroxylase Activity

Molybdenum cofactor-containing enzymes catalyze the transfer of an oxygen atom derived from or incorporated

into water, to or from a substrate in a two-electron redox reaction [24, 25]. For supporting the sequence-based prediction of resorcinol hydroxylase being a molybdenum-containing enzyme, physiological experiments with molybdenum-starved cells were performed. As *A. anaerobius* is a denitrifying bacterium, it will also have at least one further molybdenum-containing enzyme, i.e., nitrate reductase [26]. We therefore looked for the effect of molybdate on *A. anaerobius* resorcinol metabolism *in vivo*, and *in vitro* by assaying membrane fractions prepared from cells grown with resorcinol in the absence of molybdate for resorcinol hydroxylase activity.

Azoarcus anaerobius was cultivated in molybdate-free medium with resorcinol as carbon and energy source with either nitrate or N₂O as electron acceptor for more than seven subsequent transfers (5% transfer each). Even after 10 transfers in medium without molybdate, the cells still grew and metabolized resorcinol (Fig. 2a), however, at much lower rate than in the presence of molybdenum (Fig. 2b). In addition, these cells accumulated nitrite indicating that its further reduction was somehow impeded. Although every effort was made to avoid exogenous introduction of molybdate, trace amounts present in the medium components, glass, and syringe needles used to inoculate and take samples from the cultures could presumably not be completely eliminated. Addition of the molybdate antagonist tungstate (0.02 mM) inhibited growth with resorcinol completely (Fig. 3a, b). In cultures grown with nitrate, no nitrite was formed. The few bacterial cells visible were heavily deformed and immotile, and formed aggregates. Addition of molybdate (0.02 mM) caused an increase in OD₅₇₈ (Fig. 3a, b) and nitrite formation, and the cells returned to normal shape.

In similar experiments with acetate as growth substrate, growth with nitrate as electron acceptor was slightly retarded after addition of tungstate (Fig. 3c). Addition of molybdate had no influence on the final OD₅₇₈. With nitrous oxide as electron acceptor, the final OD₅₇₈ was not affected by addition of tungstate or molybdate (Fig. 3d). Interestingly, cells grown in minimal medium with resorcinol but lacking molybdate also formed large cell aggregates after 2 weeks of incubation without shaking. Cells grown with resorcinol in the presence of molybdate did not form such aggregates, even after 3 months of incubation without shaking.

Tungsten is known to be a molybdenum antagonist [26] due to its similar physical and chemical properties [27]. Growth inhibition of *A. anaerobius* by tungstate was observed only in cultures with resorcinol, independent of the used electron acceptor. Growth with acetate plus nitrate was slightly delayed, probably due to impairment of the nitrate reductase [26].

Specific activities of RH in membrane fractions prepared from *A. anaerobius* cells grown in the absence of molybdate were significantly lower (7 mU mg⁻¹) than those from

Fig. 1 Sequence alignments of RH subunits. Alignment was performed using the ClustalW program [41]; amino acids identical in all three sequences are shaded in red, and amino acids identical in at least two sequences are shaded in yellow. **a** Sequence alignment of RhL and members of the DMSO reductase family from *P. acidigallici* large subunit of pyrogallol-phloroglucinol transhydroxylase (aTH) and *R. sphaeroides* DMSO/TMAO-reductase (Dms). Ser175 from alpha subunit of pyrogallol-phloroglucinol transhydroxylase is indicated with asterisk and Tyr 560 with a triangle. **b** Sequence alignment of RhS and other iron-sulfur proteins: *A. anaerobius* small subunit of resorcinol hydroxylase (RhS), *P. acidigallici* beta subunit of pyrogallol-phloroglucinol transhydroxylase (bTH) and *R. sulfidophilum* dimethylsulfide dehydrogenase beta subunit (bDh). The cysteines shown to bind the first, second and third [4Fe-4S] in bTH are shaded light gray, green and cyan, respectively. Aa *A. anaerobius*, *Pa Pelobacter acidigallici*, *Rs Rhodovulum sulfidophilum* (Color figure online)

Fig. 1 (continued)

B

RhS_Aa	--MKKNLIIVAAEENNNMLTT-----RDEIVGNDFFG-----	34
bTH_Fa	--MEQYVMVIVAAEQDCHNCFMG-----MDEELNEWFG-----	34
bDh_Rs	MVERQISMVLLNNIGQTTSACKLQWTNRNGREYMYNNVETIPGPGYRNYESGG	60
RhS_Aa	-----AAQFVHGHNVIRIHRK-----VDEQD	57
bTH_Fa	-----TASMQR-GHRNRIERR-----EAGTY	56
bDh_Rs	GFDEEGALKIGITPSAEDYGI PWEYNTEALMTGIDPLRPNVKPTWGANWNEDEGRGEY	120
RhS_Aa	GMVDAARPTMNNNDCAFVVFAGDGSVTRPFGIIVIIIPQKARKKDIUGAEPFNV	116
bTH_Fa	FRNDINRPTFSCGENAPGV-KGNNAVYQNEGGIULIDPEKARKKELLDITQFGVM	114
bDh_Rs	ENSYYFLLRIENNDANFGLLACARNALIKQEDGIVLVQERCRTYRYCITAEPRKV	180
RhS_Aa	FWTELQLPQENIFDAHLID-QGNK--QPRQQSCTPGVYVAVQVEDSEMQLIKHLE	173
bTH_Fa	YWKLEENVAQSTIMCAHLIDDESXAPFMRCAHNGGSFVYFLKTTPEAMAKKEEGLE	174
bDh_Rs	YFTEQISKAEKCTCYFRIEKGFTACAKQVG--RIPIFYGLDDEAGFVHLERYKVA	238
RhS_Aa	V-LRDELINRERVYRNLYRFDKCFIGGSVVAEIDGVLDGISARVVLKRRQELASRES	232
bTH_Fa	V-IRFELGKSRVYRNLYRFEKNVYTAGILVQGD---QFEAKVVLKSGGKEVASAET	229
bDh_Rs	IFLHFGWGKSTSTVFPFLAPFR-----IDDGTEETRVFLAYLK	280
RhS_Aa	DTEDFRFDGDSASGVQVEVAHPKLGSAFAAASLTDQYLGILRLQGGTSTSSPSA	292
bTH_Fa	NFTDFRFDALD--NGEYTVIEDADGKSYSDTVVIDDSVDLGFIKL-----	274
bDh_Rs	ELFGKAVVFAETLTERAKKQSGAESLMDTLIGYRHPMFILS-----	325
RhS_Aa	QQRQA	298
bTH_Fa	-----	
bDh_Rs	-----	

cells that had not been starved for molybdate (95 mU mg^{-1}). While cyanide, a prominent inhibitor of molybdenum-containing enzymes [28], did not inhibit RH (results not shown), arsenite and selenite (1 mM each) decreased the RH activity by 97 and 61%, respectively. Both inhibitors can destabilize the molybdenum bonding inside the protein by forming a complex with the thiol groups of the molybdenum cofactor [30]. Taken together, these results clearly support the prediction of the sequence alignments that *A. anaerobius* resorcinol hydroxylase is a molybdenum-containing enzyme.

Expression of Resorcinol Hydroxylase in *E. coli*

Previously, it was reported that resorcinol hydroxylase activity could be measured in the *A. anaerobius* membrane fractions solubilized with 1% (w/v) CHAPS [6]. Since all attempts to purify this enzyme from solubilized *A. anaerobius* membranes failed, heterologous expression was chosen for purification and characterization of this enzyme. The genes *rhLS* coding for the two subunits of RH were amplified as single fragments and ligated in pSK-IBA5p-lus and in pASK-IBA3plus plasmids in order to generate N- and C-terminus streptavidin (Strep)-tagged proteins, respectively. Western blot analysis showed that cell extracts prepared from *E. coli* harboring pPID2 presented much lower expression levels of C-terminally tagged RH (data not shown). In contrast, a more efficient expression of RH genes was detected in *E. coli* if the enzyme was produced as N-terminus-tagged protein from plasmid pPD2. Nevertheless, both systems produced RH exclusively in inclusion

bodies and neither variation in induction procedure, media, nor temperature yielded a soluble protein. The formation of inclusion bodies could be explained in this case by the inability of *E. coli* to properly coordinate the synthesis of the enzyme with the synthesis and integration of MoCo cofactor and 4Fe-4S clusters.

Complementation of *T. aromatica* AR1 ΔrhL and *T. aromatica* AR1 ΔrhS with the Intact Resorcinol Hydroxylase Genes from the pSKM1 Plasmid

Thauera aromatica AR1/Mu_21 (designated ΔrhL hereafter) and *T. aromatica* AR1/Mu_40 (designated ΔrhS hereafter) transconjugants [12] have transposons inserted in the genes coding for the large subunit and small subunit, respectively. Neither one was able to grow with resorcinol (2 mM), but both grew with benzoate (2 mM) in the presence of kanamycin. To examine whether plasmid-born alleles could complement *T. aromatica* AR1 RH mutants, *T. aromatica* AR1 ΔrhL and *T. aromatica* AR1 ΔrhS transconjugants were transformed with pSKM1 carrying the entire *rhLS* locus plus the Strep-tag attached at the C-terminus of *rhS*. *Thauera aromatica* AR1 ΔrhL (pSKM1), and *T. aromatica* AR1 ΔrhS (pSKM1) transconjugants could grow with resorcinol as carbon and energy source under denitrifying conditions when cultivated in the presence of kanamycin and gentamicin. This indicated that both the mutated cosmid and the complementation plasmid could be maintained and that each transposon insertion mutation within the cosmid was complemented

Fig. 2 Degradation of resorcinol by *A. anaerobius* grown in the absence (a) and in the presence (b) of 0.02 mM molybdate. Resorcinol (open circles) is degraded with concomitant consumption of nitrate (open triangles), release of nitrite (filled triangles), and increase in OD at 578 nm (filled circles), measured in 1 cm cuvettes with a Hitachi 100-40 spectrophotometer. The results shown are mean values obtained from three independent experiments

by the plasmid-born wild-type allele. Growth and substrate depletion by *T. aromatica* AR1 ΔrhS (pSKM1) were compared to *T. aromatica* AR1 ΔrhS and wild-type transconjugant *T. aromatica* AR1/R⁺, as negative and positive controls, respectively (Fig. 4a, b). Growth was coupled to resorcinol degradation and to simultaneous reduction of nitrate to nitrite; similar results were obtained with *T. aromatica* AR1 ΔrhL (pSKM1) (not shown). Figure 4c shows the RH expression level in the complemented *T. aromatica* AR1 ΔrhS (pSKM1) as detected in Western blot analysis of cell-free extracts prepared from cells grown with resorcinol. Furthermore, membrane and soluble fractions from both *T. aromatica* AR1 ΔrhL (pSKM1) and ΔrhS (pSKM1) were assayed for RH activity (Table 2). In the membrane fractions of both complemented mutants, the activities measured were comparable to those reported for the membranes of *T. aromatica* AR1/R⁺ (80 mU mg⁻¹) [12]. The specific activities in the soluble fractions were very low, which is in agreement with low RH levels detected by immunoblot analysis in these fractions (not shown).

Enrichment of Resorcinol Hydroxylase

As RH could obviously functionally be expressed in *T. aromatica* AR1 ΔrhL and *T. aromatica* AR1 ΔrhS transformed with plasmid pSKM1, protein purification was attempted with this system (Table 2). In particular, *T. aromatica* AR1 ΔrhS (pSKM1) cells were used since they showed higher expression levels than those of *T. aromatica* AR1 ΔrhL (pSKM1). Despite the fact that some resorcinol hydroxylase activity could be measured in the soluble fractions of complemented *T. aromatica* transconjugants, no protein was purified by affinity chromatography. The fact that the protein did not bind to the affinity column suggests that the tag may not be accessible and could be buried inside the protein. In contrast, RH could be solubilized from membranes under strictly anoxic conditions with detergent. Most of the protein was released from the membranes during Triton X-100 treatment; however, from a starting activity of 70 mU mg⁻¹ measured in the membrane fraction, only 11 mU mg⁻¹ activity was recovered in the solubilisate, indicating about 80% loss of activity during this step. Nevertheless, solubilized RH bound to the column and was eluted mainly in fractions 3 and 4 (Fig. 5a, lanes E3 and E4). These two fractions were concentrated using commercially available Vivaspin centrifugation devices with 30 kDa cut-off. As shown in Fig. 5b, the final preparation mainly contained two polypeptides with apparent molecular masses of 100 and 35 kDa, which are in agreement with the molecular masses predicted from the amino acid sequences of the large and small subunits of RH, respectively. Some further faint bands were observed as well, and the amounts of these polypeptides differed from one preparation to another. It is likely that RH had undergone some proteolytic degradation during purification. Similar cases of proteolysis were reported for other molybdenum-containing enzymes such as aldehyde oxidases [31–33]. The concentrated protein was colorless and inactive, suggesting that the cofactors were lost during purification and/or detergent treatment. One of the intriguing features of the *A. anaerobius* resorcinol hydroxylase is that even though activity was always detected in the membrane fraction, no membrane-binding elements were identified in either subunit. These findings lead us to speculate that the enzyme might contain a third subunit that functions as a membrane anchor. To verify this hypothesis, the complementation plasmid pSKM1 was engineered to produce a C-terminal strep-tagged small subunit which will allow a pull-down assay of a further subunit. Nonetheless, SDS-PAGE analysis of the purified resorcinol hydroxylase from the complemented resorcinol hydroxylase-deficient mutant showed only two major bands, indicating that RH consists of only two subunits or, if RH contains a third subunit, then it does not form a tight complex with RhLS.

Fig. 3 Growth of *A. anaerobius* without tungstate (filled diamonds), with 0.02 mM tungstate (filled triangles), and with 0.02 mM tungstate supplemented with 0.02 mM molybdate after 1 day of incubation (filled squares). Growth was followed as OD₅₇₈ directly in culture tubes in a Bausch and Lomb Spectronic 70 spectrophotometer.

Conclusions

Through the past years, more than 50 known molybdenum-containing enzymes have been identified that catalyze a variety of oxygen atom transfers, hydroxylations, and other oxidation–reduction reactions, and are involved in anabolic, catabolic and energy-conserving metabolic pathways [14]. Molybdenum hydroxylases constitute a large and widely dispersed group of enzymes that catalyze hydroxylation of either aromatic or aliphatic substrates using water as the oxygen source. Thus, they are especially important in anaerobic degradation of stable compounds, such as hydrocarbons [34] or sterols [35]; in aerobic microorganisms the degradation of these compounds requires molecular oxygen. With respect to the required redox potential for such hydroxylations they seem to be restricted to nitrate reducers while fermenting and sulfate-reducing bacteria employ different strategies [36]. In the present study, we show that *A. anaerobius* resorcinol hydroxylase is most likely composed of two subunits predicted to have an active Mo-molybdopterin guanine

with 2 mM resorcinol (a and b) or 5 mM acetate (c and d) as carbon and energy source and 10 mM nitrate (a and c) or 10% nitrous oxide in the headspace (b and d) as electron acceptor. The results show a representative experiment out of at least two independent biological replicates

dinucleotide site in the large subunit and two [4Fe–4S] centers in the small subunit. Apparently, this result excludes the presence of a third loosely membrane-bound subunit and raises the question of how the membrane association of RH is achieved. Although heterologous expression of a prokaryotic MoCo enzyme in *E. coli* is possible, e.g., with xanthine dehydrogenase of *Rhodobacter capsulatus* [37], all our attempts failed to purify resorcinol hydroxylase from *A. anaerobius* or from *E. coli*, expressing it both as N- or C-terminally tagged protein. Nevertheless, resorcinol hydroxylase was purified in the present study from a complemented RH-deficient mutant by affinity chromatography, as the complementation plasmid was engineered with a Strep-tag attached to the C-terminus of the small subunit. This enzyme was obtained inactive and in low amounts, most likely due to partial proteolytic degradation which was observed during purification and/or due to loss of cofactors during protein solubilization and did not allow further characterization. Reconstitution of the native protein is difficult because MoCo cannot be reconstituted in vitro. The two putative iron-sulfur clusters

Fig. 4 Growth (a) and resorcinol degradation (b) by *T. aromatica* AR1/R⁺ (filled triangles), *T. aromatica* AR1 ΔrhS (filled circles) and *T. aromatica* AR1 ΔrhS (pSKM1) (filled squares). Western blot analysis (c) of Strep-tagged C-terminus of *rhS* in crude extracts of *T. aromatica* AR1 ΔrhS (pSKM1) at the time intervals indicated. The marker protein S had a mass of 37 kDa. Results shown are mean values obtained from three independent experiments

Table 2 Expression and localization of resorcinol hydroxylase activities in *T. aromatica* AR1 ΔrhL and *T. aromatica* AR1 ΔrhS transconjugants harboring pSKM1 plasmid

<i>T. aromatica</i> AR1 transconjugants	Cell fraction	Specific activity (mU mg ⁻¹ protein)
ΔrhL (pSKM1)	Membranes	81 ± 9 ^a
	Soluble	5 ± 2 ^a
ΔrhS (pSKM1)	Membranes	95 ± 12 ^b
	Soluble	7 ± 1 ^b

^aMean ± standard deviation, two determinations

^bMean ± standard deviation, four determinations

predicted to be embedded in the small subunit of RH could be involved in electron flow or could play a role only in maintaining proper protein folding.

Fig. 5 Purification of RH. a Western blot analysis of purified RhS from *T. aromatica* AR1 ΔrhS (pSKM1) expressing a C-terminal Strep-tagged *rhSL*. Lane M, molecular mass marker; lane MP2, membrane fraction containing Strep-tagged *rhSL* that remained in the membrane fraction after solubilization with Triton X-100; lane SP2, solubilized membranes with Triton X-100; lane FT, flow through; lane W5, last washing before elution of RhSL with desthiobiotin; E1–4 elution fractions. Molecular mass of standard protein (M) and the position of RhS are indicated. b SDS-PAGE analysis of RH purified from *T. aromatica* AR1 ΔrhS (pSKM1). Molecular masses of standard proteins (M) and the positions of RhS and RhL are indicated

Author Contributions All authors developed the concept of this study. PD and JH carried out the experiments and wrote a first draft. BS and BP wrote the final version of the manuscript on which all authors agreed.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interests.

References

- Harwood CS, Parales RE (1996) The beta-ketoadipate pathway and the biology of self-identity. *Annu Rev Microbiol* 50:553–590. <https://doi.org/10.1146/annurev.micro.50.1.553>
- Fuchs G, Boll M, Heider J (2011) Microbial degradation of aromatic compounds—from one strategy to four. *Nat Rev Microbiol* 9:803–816. <https://doi.org/10.1038/nrmicro2652>

3. Carmona M, Zamarró MT, Blázquez B, Durante-Rodríguez G, Juárez JF, Valderrama JA, Barragán MJ, García JL, Díaz E (2009) Anaerobic catabolism of aromatic compounds: a genetic and genomic view. *Microbiol Mol Biol Rev* 73(1):71–133. <https://doi.org/10.1128/MMBR.00021-08>
4. Boll M, Fuchs G, Heider J (2002) Anaerobic oxidation of aromatic compounds and hydrocarbons. *Curr Opin Chem Biol* 6:604–611. [https://doi.org/10.1016/S1367-5931\(02\)00375-7](https://doi.org/10.1016/S1367-5931(02)00375-7)
5. Schink B, Philipp B, Müller J (2000) Anaerobic degradation of phenolic compounds. *Naturwissenschaften* 87:12–23. <https://doi.org/10.1007/s001140050002>
6. Philipp B, Schink B (1998) Evidence of two oxidative reaction steps initiating anaerobic degradation of resorcinol (1,3-dihydroxybenzene) by the denitrifying bacterium *Azoarcus anaerobius*. *J Bacteriol* 180:3644–3649
7. Gallus C, Schink B (1998) Anaerobic degradation of α -resorcyate by *Thauera aromatica* strain AR-1 proceeds via oxidation and decarboxylation to hydroxyhydroquinone. *Arch Microbiol* 169:333–338. <https://doi.org/10.1007/s0020300050579>
8. Philipp B, Schink B (2000) Two distinct pathways for anaerobic degradation of aromatic compounds in the denitrifying bacterium *Thauera aromatica* strain AR-1. *Arch Microbiol* 173:91–96. <https://doi.org/10.1007/s002039900112>
9. Pacheco-Sánchez D, Rama-Garda R, Marín P, Martirani-Von Abercron SM, Marqués S (2019) Occurrence and diversity of the oxidative hydroxyhydroquinone pathway for the anaerobic degradation of aromatic compounds in nitrate-reducing bacteria. *Environ Microbiol Rep* 11:525–537. <https://doi.org/10.1111/1758-2229.12752>
10. Pacheco-Sánchez D, Molina-Fuentes Á, Marín P, Medina-Bellver JI, González-López Ó, Marqués S (2017) The *Azoarcus anaerobius* 1,3-dihydroxybenzene (resorcinol) anaerobic degradation pathway is controlled by the coordinated activity of two enhancer-binding proteins. *Appl Environ Microbiol*. <https://doi.org/10.1128/AEM.03042-16>
11. Pacheco-Sánchez D, Molina-Fuentes Á, Marín P, Díaz-Romero A, Marqués S (2019) DbdR, a new member of the LysR family of transcriptional regulators, coordinately controls four promoters in the *Thauera aromatica* AR-1 3,5-dihydroxybenzoate anaerobic degradation pathway. *Appl Environ Microbiol*. <https://doi.org/10.1128/AEM.02295-18>
12. Darley PL, Hellstern JA, Medina-Bellver JI, Marques S, Schink B, Philipp B (2007) Heterologous expression and identification of the genes involved in anaerobic degradation of 1,3-dihydroxybenzene (resorcinol) in *Azoarcus anaerobius*. *J Bacteriol* 189:3824–3833. <https://doi.org/10.1128/JB.01729-06>
13. Molina-Fuentes Á, Pacheco D, Marín P, Philipp B, Schink B, Marqués S (2015) Identification of the gene cluster for the anaerobic degradation of 3,5-dihydroxybenzoate (α -resorcyate) in *Thauera aromatica* Strain AR-1. *Appl Environ Microbiol* 81:7201–7214
14. Hille R, Retey J, Bartlewski-Hof U, Reichenbecher W, Schink B (1999) Mechanistic aspects of molybdenum-containing enzymes. *FEMS Microbiol Rev* 22:489–501. <https://doi.org/10.1111/j.1574-6976.1998.tb00383.x>
15. Messerschmidt A, Niessen H, Abt D, Einsle O, Schink B, Kroneck PM (2004) Crystal structure of pyrogallol-phloroglucinol *trans*-hydroxylase, an Mo enzyme capable of intermolecular hydroxyl transfer between phenols. *Proc Natl Acad Sci USA* 101:11571–11576. <https://doi.org/10.1073/pnas.0404378101>
16. Sambrook J, Russell DW (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
17. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 72:248–254
18. Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 227:680–685. <https://doi.org/10.1038/227680a0>
19. Schindelin H, Kisker C, Hilton J, Rajagopalan KV, Rees DC (1996) Crystal structure of DMSO reductase: redox-linked changes in molybdopterin coordination. *Science* 272:1615–1621. <https://doi.org/10.1126/science.272.5268.1615>
20. Schneider F, Lowe J, Huber R, Schindelin H, Kisker C, Knaiblein J (1996) Crystal structure of dimethyl sulfoxide reductase from *Rhodobacter capsulatus* at 1.88 Å resolution. *J Mol Biol* 263:53–69. <https://doi.org/10.1006/jmbi.1996.0555>
21. Boyington JC, Gladyshev VN, Khangulov SV, Stadtman TC, Sun PD (1997) Crystal structure of formate dehydrogenase H: catalysis involving Mo, molybdopterin, selenocysteine, and an Fe₄S₄ cluster. *Science* 275:1305–1308. <https://doi.org/10.1126/science.275.5304.1305>
22. Jormakka M, Tornroth S, Byrne B, Iwata S (2002) Molecular basis of proton motive force generation: structure of formate dehydrogenase-N. *Science* 295:1863. <https://doi.org/10.1126/science.1068186>
23. Dias JM, Than ME, Humm A, Huber R, Bourenkov GP, Bartunik HD, Bursakov S, Calvete J, Caldeira J, Carneiro C, Moura JJ, Moura I, Romão MJ (1999) Crystal structure of the first dissimilatory nitrate reductase at 1.9 Å solved by MAD methods. *Structure* 7:65–79. [https://doi.org/10.1016/S0969-2126\(99\)80010-0](https://doi.org/10.1016/S0969-2126(99)80010-0)
24. Menendez C, Otto A, Igloi G, Nick P, Brandsch R, Schubach B, Bottcher B, Brandsch R (1997) Molybdate-uptake genes and molybdopterin-biosynthesis genes on a bacterial plasmid—characterization of MoeA as a filament-forming protein with adenosinetriphosphatase activity. *Eur J Biochem* 250:524–531. <https://doi.org/10.1111/j.1432-1033.1997.0524a.x>
25. Kisker C, Schindelin H, Rees DC (1997) Molybdenum-cofactor-containing enzymes: structure and mechanism. *Annu Rev Biochem* 66:233–267. <https://doi.org/10.1146/annurev.biochem.66.1.233>
26. Zumft WG (1997) Cell biology and molecular basis of denitrification. *Microbiol Mol Biol Rev* 61:533–616
27. Blaschke M, Kretzer A, Schäfer C, Nagel M, Andreesen JR (1991) Molybdenum-dependent degradation of quinoline by *Pseudomonas putida* Chin IK and other aerobic bacteria. *Arch Microbiol* 155:164–169
28. Johnson JL (1988) Molybdenum. *Methods Enzymol* 158:371–382
29. Coughlan MP, Johnson JL, Rajagopalan KV (1980) Mechanism of inactivation of molybdenum enzymes by cyanide. *J Biol Chem* 255:2694–2699
30. Voet D, Voet JG (1994) Biochemie, 1st edn. VCH Verlagsgesellschaft mbH, Weinheim
31. Koshiba T, Saito E, Ono N, Yamamoto N, Sato M (1996) Purification and properties of flavin- and molybdenum-containing aldehyde oxidase from coleoptiles of maize. *Plant Physiol* 110:781–789. <https://doi.org/10.1104/pp.110.3.781>
32. Stell JG, Warne AJ, Lee-Woolley C (1989) Purification of rabbit liver aldehyde oxidase by affinity chromatography on benzamidine sepharose 6B. *J Chromatogr* 475:363–372. [https://doi.org/10.1016/S0021-9673\(01\)89690-4](https://doi.org/10.1016/S0021-9673(01)89690-4)
33. Yoshihara S, Tatsumi K (1985) Guinea pig liver aldehyde oxidase as a sulfoxide reductase: its purification and characterization. *Arch Biochem Biophys* 242:213–224. [https://doi.org/10.1016/0003-9861\(85\)90495-3](https://doi.org/10.1016/0003-9861(85)90495-3)
34. Heider J, Szaleniec M, Sünwoldt K, Boll M (2016) Ethylbenzene dehydrogenase and related molybdenum enzymes involved in oxygen-independent alkyl chain hydroxylation. *J Mol Microbiol Biotechnol* 26:45–62. <https://doi.org/10.1159/000441357>
35. Dermer J, Fuchs G (2012) Molybdoenzyme that catalyzes the anaerobic hydroxylation of a tertiary carbon atom in the side

- chain of cholesterol. *J Biol Chem* 287:36905–36916. <https://doi.org/10.1074/jbc.M112.407304>
36. Philipp B, Schink B (2012) Different strategies in anaerobic biodegradation of aromatic compounds: nitrate reducers versus strict anaerobes. *Environ Microbiol Rep* 4:469–478. <https://doi.org/10.1111/j.1758-2229.2011.00304.x>
 37. Hall J, Reschke S, Cao H, Leimkühler S, Hille R (2014) The reductive half-reaction of xanthine dehydrogenase from *Rhodobacter capsulatus*: the role of Glu232 in catalysis. *J Biol Chem* 289:32121–32130. <https://doi.org/10.1074/jbc.M114.603456>
 38. Springer N, Ludwig W, Philipp B, Schink B (1998) *Azoarcus anaerobius* sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium. *Int J Syst Bacteriol* 48:953–956. <https://doi.org/10.1099/00207713-48-3-953>
 39. Herrero M, de Lorenzo V, Timmis KN (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J Bacteriol* 172:6557–6567. <https://doi.org/10.1128/jb.172.11.6557-6567.1990>
 40. Kovach ME, Elzer PH, Hill DS, Robertson GT, Farris MA, Roop RM, Peterson KM (1995) Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175–176. [https://doi.org/10.1016/0378-1119\(95\)00584-1](https://doi.org/10.1016/0378-1119(95)00584-1)
 41. Li KB (2003) ClustalW-MPI: ClustalW analysis using distributed and parallel computing. *Bioinformatics* 19:1585–1586. <https://doi.org/10.1093/bioinformatics/btg192>