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Characterization of the recombinant Rieske [2Fe–2S] proteins HcaC and YeaW from *E. coli*

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Abstract Three genes within the genome of *E. coli* K12 are predicted to encode proteins containing the typical Rieske iron–sulfur cluster-binding motifs. Two of these, *hcaC* and *yeaW*, were overexpressed in *E. coli* BL21 and Tuner (DE3) pLacI. The recombinant proteins were purified and analyzed by UV/Vis- and EPR-spectroscopy. HcaC and YeaW display the typical redox-dependent UV/Vis-spectra of iron–sulfur proteins. The EPR spectrum of reduced HcaC shows characteristic g-values of a Rieske cluster whereas the g-values for YeaW are close to the upper limit for this type of iron–sulfur cluster. Both iron–sulfur clusters could be reduced by dithionite, but not by ascorbate, confirming their classification as low-potential Rieske proteins as derived from the amino acid sequences. A phylogenetic analysis of the two proteins reveals that HcaC clearly segregates with the Rieske ferredoxins of class IIB oxygenases whereas the classification of YeaW remains doubtful.

Keywords *E. coli* · Low-potential Rieske iron–sulfur protein · Phenylpropionic acids · Phylogenetic analysis

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Introduction

Rieske proteins are a unique group of both soluble and membrane residing [2Fe–2S] cluster containing proteins found in prokaryotic and eukaryotic organisms. They are well-established as essential subunits of electron transfer systems such as the cytochrome bc_1 and b_6f complexes as well as oxygenases and hydroxylases. In contrast to plant-type ferredoxins their [2Fe–2S] cluster is coordinated by two cystein and two histidin residues instead of four cysteins (Cline et al. 1985; Gurbiel et al. 1989; Iwata et al. 1996). This coordination sphere is responsible for a higher, pH dependent midpoint potential of −150 to +350 mV and their distinctive EPR signal with g_y-values in a range of 1.89–1.91 (Rieske et al. 1964). Among the Rieske proteins two groups of soluble proteins with lower midpoint potentials of −150 mV to +5 mV can be distinguished. The first group of these low-potential Rieske proteins contains smaller proteins with a typical length of 90–110 amino acid residues, commonly referred to as Rieske ferredoxins. The second group consists of the catalytic subunits of the Rieske hydroxylases and Rieske oxygenases. These proteins are significantly larger than the Rieske ferredoxins and typically containing a mononuclear iron center, or a μ-oxodi-iron center as a second redox co-factor (Schmidt and Shaw 2001).

Until today no Rieske proteins have been physically detected in, or isolated from non-genetically modified *E. coli* cells. Failures to express Rieske

protein encoding genes from other organisms in *E. coli* even have been interpreted as an indication of the absence of the machinery required for the synthesis of this type of iron–sulfur cluster (Holton et al. 1996). This situation prompted us to search for Rieske protein encoding genes in the genome of *E. coli* K12 (GenBank accession no. U00096).

Initial evidence for the presence of Rieske proteins in *E. coli* was found in studies examining the 3-phenylpropionic acid metabolism (Diaz et al. 1998). Two genes encoding potential Rieske proteins, *hcaC* and *hcaA1* (in the literature also referred to as *hcaA*, or *hcaE*), are part of the *hca*-gene cluster of *E. coli*, responsible for the degradation of 3-phenylpropionic acid and related aromatic compounds. A third potential low-potential Rieske protein is encoded by the *yeaW* gene (EcoGene accession no. EG13509).

Here we present a sequence analysis of the *E. coli* Rieske proteins and a preliminary characterization of the recombinant HcaC and YeaW proteins.

Materials and methods

Searching for potential Rieske protein encoding genes

The predicted open reading frames within the genome of *E. coli* K12 (GenBank accession no. U00096) were searched with the simplified sequence motif **C**-x-**H**-$x_{(17–30)}$-**C**-x-x-**H** derived from the PROSITE "Rieske iron–sulfur protein signatures 1 and 2" **C**-[TK]-**H**-[LV]-G-**C**-[LIVSTP]-$x_{(13–26)}$-**C**-P-**C**-**H**-{H}-[GSA] and the "Bacterial ring hydroxylating dioxygenases alpha-subunit signature" **C**-x-**H**-R-[GAR]-$x_{(7,8)}$-[GEKVI]-[NERAQ]-$x_{(4,5)}$-**C**-x-[FY]-**H**. The resulting 14 initial hits were subsequently scanned against the PROSITE database (http://expasy.ch/prosite/).

Cloning and over-expression of the *hcaC* and *yeaW* genes

Genomic DNA from *E. coli* DH5α cells was used as a template to amplify the coding regions of the *hcaC* and *yeaW* genes. The primers GATGAATCGAATTTATG CGTGT and AAAATGGACTCGTTATGCAGAT were used to amplify *hcaC* and the primers TATGAGC AATCTGAGCCCTGACT and CGCGATGTCATTA GTCCTTAAACA for *yeaW*.

The PCR products were verified by restriction digests and ligated into the pETBlue^{TM}-1 plasmid using the Perfectly Blunt® cloning kit (Novagen). An aliquot of the ligation-mixture was transformed into *E. coli* NovaBlue cells. Plasmids were isolated from individual clones and verified by sequencing. Subsequently, the plasmids were transformed into BL21 Gold (DE3) or Tuner^{TM}(DE3) pLacI cells (Novagen) and clones were screened for expression. Plasmids that contained the respective gene in reverse orientation were used as negative controls in the expression tests.

For large-scale over-expression of *hcaC* 1l M9 medium supplemented with 0.25% yeast extract (Schmidt et al. 1997), 100 μg/ml carbenicillin and 34 μg/ml chloramphenicol was inoculated with 1–4% of an overnight culture grown in the same medium and incubated over night at 37°C in a shaking incubator. The next morning the same volume of fresh medium was added and the culture was incubated for another 60 min at 37°C. Subsequently, two volumes of medium additionally containing 0.5 mM $FeSO_4$ and 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) were added to induce expression. The cells were incubated for another 3–4 h at 37°C and harvested by centrifugation. The cell pellet was washed once with 400 ml of 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, resuspended in 40–80 ml of the same buffer and disrupted by sonication. Insoluble material was removed by centrifugation for 30 min at 12,000*g* (4°C) and the soluble fraction was treated with Benzonase® (Merck) to degrade nucleic acids. Precipitated protein was removed by a second centrifugation for 30 min at 12,000*g* (4°C).

A similar protocol was used for over-expression of *yeaW*. However, the cells were grown at 25°C and in LB-medium supplemented with 100 μg/ml carbenicillin and 34 μg/ml chloramphenicol. The incubation time subsequent to the addition of IPTG was increased to 15–18 h at 25°C and the optical density (OD) at 600 nm was measured to monitor growth. The expression of the *yeaW* gene was induced at an $OD_{600\ nm}$ of 0.7 adding 0.5 mM $FeSO_4$, 1 mM L-Cystein and 1 mM IPTG.

Purification of the HcaC and YeaW proteins

The HcaC and YeaW proteins were partially purified by gel permeation chromatography on a Superdex^{TM}75

HiLoad™16/60 column (Pharmacia) (for HcaC) or a Superdex™200 HiLoad™ 16/60 column (for YeaW). The columns were equilibrated and eluted with 25 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 100 mM NaCl at a flow rate of 1 ml/min. Iron–sulfur protein containing fractions were identified by monitoring the UV absorbance at 336 nm and 281 nm.

Determination of the native molecular weight of YeaW

The Superdex™200 HiLoad™16/60 column was calibrated using the following proteins: Alcohol dehydrogenase (150.0 kD), apotransferrin (78.5 kD), ovalbumin (43.0 kD) and cytochrome *c* (12.4 kD).

Spectroscopic methods

EPR spectra were recorded with an X-band Bruker ER 200 D-SRC spectrometer equipped with an ESR 910 continuous-flow helium cryostat from Oxford Instruments.

UV/Vis spectra were recorded using a Varian Cary 50 Conc spectrometer equipped with a quartz cuvette (path length 1 cm).

The individual conditions are given in the figure legends.

Sequence analysis

The program "ClustalX" (version 1.83) (Thompson et al. 1997) was used to calculate the alignment of the *E. coli* Rieske proteins with a set of pro- and eukaryotic Rieske proteins (compare (Schmidt and Shaw 2001)). The following parameters were used: Pairwise alignments: Identity matrix, gap opening penalty: 10, gap extension penalty: 0.1, Multiple alignments: Blosum matrix, gap opening penalty: 15, gap extension penalty: 0.05, delay divergent sequences: 0%. Protein gap parameters: Residue-specific penalties: on, hydrophilic penalties: on, hydrophilic residues: GPSNDQERK, gap separation distance: 8 and end gap separation: off. Positions that contained a gap in any of the sequences were ignored for the calculation of the phylogenetic tree. The correction for multiple substitutions was applied. The program "Unrooted" (http://pbil.univ-lyon1.fr/software/unrooted.html) was used to draw the tree.

A 3D homology model of the HcaC protein was calculated using the Swiss Model-Server (http://swissmodel.expasy.org).

Results

Sequence analysis of the *E. coli* Rieske proteins

Three genes encoding proteins containing the typical [2Fe–2S] cluster binding motif of a low-potential Rieske protein could be detected (Schmidt and Shaw 2001) (Fig. 1) within the genome of *E. coli* K12. Among these, *hcaA1* and *hcaC* had been previously described (Diaz et al. 1998), whereas the third one, *yeaW* was previously unrecognized to encode a Rieske protein.

HcaC is the smallest of the three proteins consisting of 106 amino acid residues with a predicted molecular mass of 11.2 kD. YeaW and HcaA1 are considerably larger: YeaW consists of 374 residues and the predicted molecular mass is 42.6 kD for the monomer. HcaA1 contains 453 residues with a predicted molecular weight of 51 kD.

The Rieske [2Fe–2S] cluster binding motifs within HcaA1 and YeaW are located close to the N-terminus of the proteins whereas the C-terminal parts are expected to contain the residues for the coordination of a mononuclear iron centre (Karlsson et al. 2003).

In comparison to the previously characterized naphthalene dioxygenase (NDO) (Karlsson et al. 2003) the two residues corresponding to His208 and His213 are well conserved in HcaA1 and YeaW. The third iron-centre binding ligand corresponding to Asp362 could be clearly identified only in the HcaA1 sequence (Fig. 2).

Overexpression and characterization of HcaC and YeaW

YeaW was over-expressed in Tuner (DE3) pLacI cells whereas for the HcaC protein best results were achieved using BL21 Gold (DE3) cells.

Both over-expressed proteins could be purified by gel permeation chromatography (Fig. 3). HcaC eluted with a volume corresponding to the predicted molecular mass of the monomer. The native molecular mass of YeaW was determined to 120–125 kD

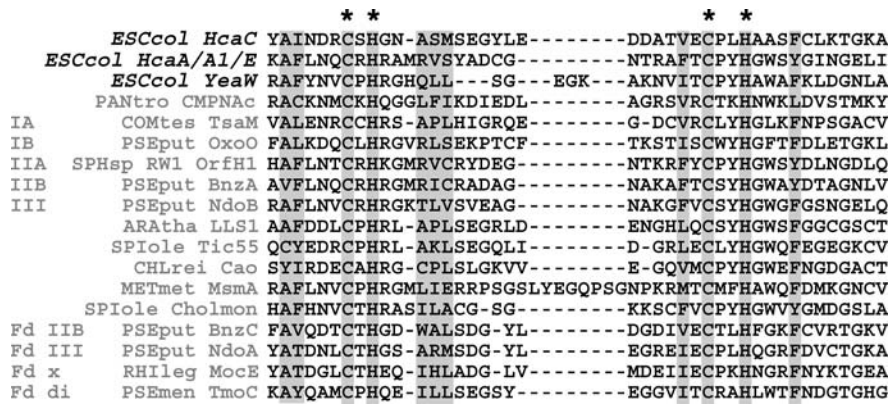

Fig. 1 Alignment of the Rieske [2Fe–2S] cluster-binding motifs and adjacent sequences of selected Rieske proteins. The positions of the conserved ligands of the [2Fe–2S] cluster are marked with asterisks. Shading indicates other conserved positions or residues considered to be conservative replacements in the majority of the sequences. Abbreviations: ESCol, *Escherichia coli*; PANtro CMPNAc, cytidine monophosphate-*N*-acetylneuraminate hydroxylase from chimpanzee (*Pan troglodytes*); COMtes TsaM, toluenesulfonate methyl-monooxygenase from *Comamonas testosteroni*; PSEput OxoO, 2-oxo-1,2-dihydroquinoline 8-monooxygenase from *Pseudomonas putida*; SPHsp RW1 OrfH1, α-subunit of the putative ring-hydroxylating dioxygenase from *Sphingomonas* sp.; PSEput BnzA, α-subunit of the benzene 1,2-dioxygenase from *Pseudomonas putida*; PSEput NdoB, α-subunit of the naphthalene dioxygenase from *Pseudomonas putida*; METmet MsmA, α-subunit of the methanesulfonic acid monooxygenase from *Methylosulfonomonas methylovora*; PSEput BnzC, ferredoxin component of the benzene-1,2-dioxygenase from *Pseudomonas putida*; PSEput NdoA, ferredoxin component of the naphthalene dioxygenase from *Pseudomonas putida*; RHIleg MocE, ferredoxin component of the rhizopine degradation pathway of *Rhizobium leguminosarum*; PSEmen TmoC, ferredoxin component of the toluene-4-monooxygenase from *Pseudomonas mendocina*; ARAtha LLS1, lethal leaf spot 1 protein from *Arabidopsis thalia*; SPIole Tic55, 52 kD protein involved in protein translocation across the inner membrane of chloroplasts from spinach (*Spinacia oleracea*); CHLrei Cao, chlorophyllid a oxygenase from *Chlamydomonas reinhardtii*; SPIole Cholmon, choline monooxygenase from spinach (*Spinacia oleracea*)

suggesting a trimeric structure (theoretical molecular mass 127.8 kD) (data not shown).

The purified proteins were characterized by UV/Vis- and EPR-spectroscopy (Figs. 4 and 5).

HcaC and YeaW display similar UV/Vis spectra possessing typical characteristics of Rieske proteins with absorbance maxima at 320 and 460 nm and a diminished absorbance in the reduced state (Fig. 4a). The iron–sulfur clusters of both HcaC and YeaW could be reduced by dithionite but not by ascorbate, indicating a midpoint potential significantly lower than +60 mV. This clearly identifies HcaC and YeaW as low-potential Rieske proteins. EPR spectroscopy of HcaC revealed the characteristic rhombic spectrum and g-values of a Rieske iron–sulfur cluster (Fig. 4b, Table 1).

Concerning YeaW, EPR spectroscopy also confirmed the presence of a Rieske [2Fe–2S] cluster (observable in the reduced state in the g ∼ 2.0-region) and a mononuclear iron centre (observable in the oxidized state in the g ∼ 4.3-region) (Fig. 5). Thus, the recombinant YeaW exists in a homo-oligomeric structure in which at least part of the monomers contain both predicted cofactors. However, close inspection of the EPR spectra reveals unusual features. The signal of the mononuclear iron centre (g ∼ 4.3) shows a very narrow line width (compare (Wolfe et al. 2001)) and the g_y-line of the [2Fe–2S] cluster is close to the upper limit for a Rieske cluster (Fig. 5, Table 1). These features may reflect the unique nature of the enzyme, especially since similar g_y-values have been reported for other oxygenases (Table 1), but then could also indicate a subtle distortion of the protein structure. Such a distortion could be due to partial misfolding under the unphysiological conditions of over-expression or may indicate the absence of a subunit essential for the stability of the native conformation. A possible candidate for a missing subunit would be the hypothetical product of the *yeaX* gene located immediately downstream of *yeaW*.

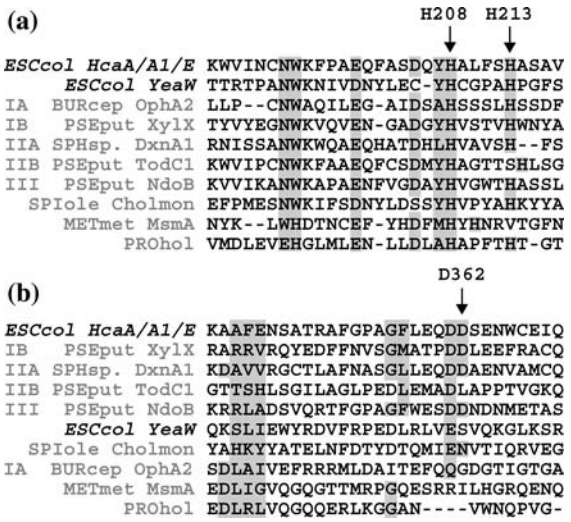

Fig. 2 Partial alignments of putative mononuclear iron binding sites of selected Rieske oxygenases: (**a**) Region corresponding to the residues 188–217 and (**b**) region corresponding to the residues 341–370 of the naphthalene dioxygenase from *Pseudomonas putida*. Shading indicates conserved residues and similarities. Arrows mark the residues liganding the mononuclear iron in the naphthalene dioxygenase (NdoB) structure. Abbreviations: BURcep OphA2, phthalate dioxygenase from *Burkholderia cepacia*; PSEput XylX, toluene-1,2-dioxygenase from *Pseudomonas putida*; PSEput TodC1, toluene 2,3-dioxygenase from *Pseudomonas putida*; PROhol, chlorophyllide a oxygenase from *Prochlorothrix hollandica*; SPHsp. dxnA1, dioxin dioxygenase (α-subunit) from *Sphingomonas* sp. All other abbreviations as denoted under Fig. 1

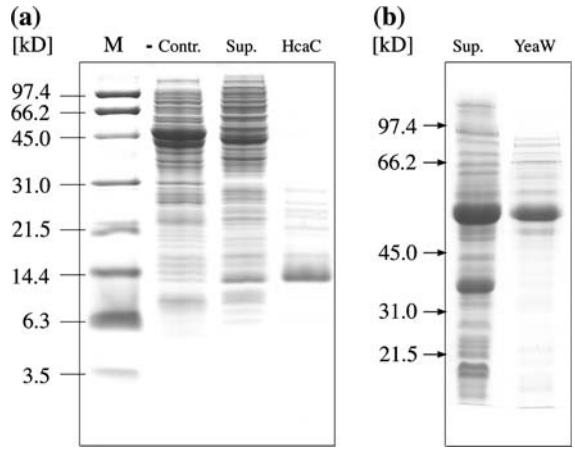

Fig. 3 SDS-polyacrylamide gel electrophoresis of the partially purified HcaC (**a**) and YeaW (**b**) proteins. Abbreviations: M, Molecular weight marker; Contr., Soluble protein fraction from an expression strain containing the pETBlue-1 plasmid with the *hcaC* gene in reverse orientation as negative control; Sup., Soluble protein fraction from the expression strain; HcaC, Protein purified by gel permeation chromatography. The identity of the purified protein was verified by automated Edman degradation. YeaW, Partially purified YeaW protein

Phylogenetic analysis

The phylogram in Fig. 6 illustrates the association of *E. coli* HcaA1 and HcaC Rieske proteins with previously described Rieske oxygenases and ferredoxins of the class-II-oxygenases (Schmidt and Shaw 2001; Mason and Cammack 1992). The branching point of HcaA1 does not allow an unequivocal assignment to the class IIA or IIB (Mason and Cammack 1992) oxygenases. In contrast, HcaC segregates unquestionably with the ferredoxin components of the class IIB oxygenases, though within this group it seems to be the most distantly related member. The experimental identification of HcaC as a Rieske protein strongly supports the classification of HcaA1 as a class IIB oxygenase, since only IIB, but not IIA oxygenases are associated with Rieske ferredoxins as electron donors (Schmidt and Shaw 2001). YeaW does not segregate into one of the well defined oxygenase classes. Instead it forms a lose cluster with two rather distinct enzymes: The choline monooxygenase from spinach chloroplasts, and the methanesulfonic acid monooxygenase from methylotrophic *Methylosulfonomonas methylovora* (Fig. 6).

Predicted structure of HcaC

Using the coordinates of the Rieske-ferredoxin from *Burkholderia cepacia* (PDB code: 1FQT) as template we were able to calculate a homology model for HcaC. The tertiary structure of the HcaC model could be largely matched to the template structure, except for three loops where the HcaC and FQT sequences differ considerably (Fig. 7). Two of these regions are situated in proximity to the iron–sulfur cluster binding site. Thus, subtle differences in the structures of the iron–sulfur clusters of both proteins may occur.

Discussion

By now it has been proved beyond any doubt that *E. coli* is indeed able to synthesize Rieske iron–sulfur clusters (Kletzin et al. 2005; Schneider et al. 2002; Henninger et al. 1999; Jaganaman et al. 2007) (and

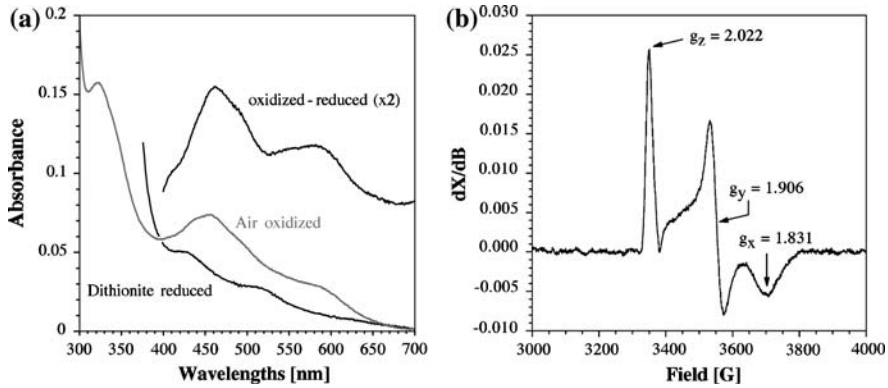

Fig. 4 UV/Vis (**a**) and EPR (**b**) spectrum of the partially purified HcaC protein. Both spectra were recorded in 50 mM Tris/HCl pH 7.5. The protein concentrations were 1.5 mg/ml (**a**) and 3 mg/ml (**b**). The protein was reduced by addition of dithionite to a final concentration of 5 mM. The strong increase in the absorbance of the reduced protein at wavelengths below 400 nm is an artifact caused by the reductant. The EPR spectrum was recorded at a temperature of 15.0 K, power: 1.0 mW, modulation amplitude: 12 G, modulation frequency: 100 kHz, microwave frequency 9.4807 GHz

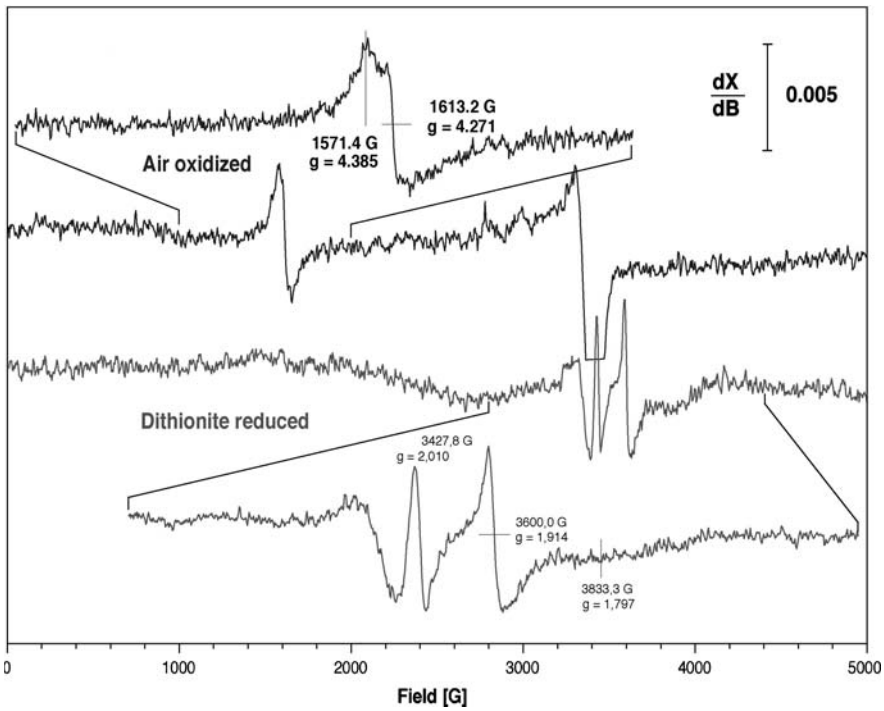

Fig. 5 EPR spectra of the partially purified YeaW protein. The spectra were recorded in 50 mM Tris/HCl pH 7.5 at a protein concentration of 4 mg/ml, temperature 10.0 K, power: 2.0 mW, modulation amplitude: 10 G, modulation frequency: 100 kHz, microwave frequency: 9.6438 GHz

many others). Hence, the question remains open why the endogenous Rieske proteins of *E. coli* have not been discovered already a long time ago. A likely explanation would be that the expression of *E. coli* Rieske proteins encoding genes is tightly regulated and these genes are expressed only at a very low level, or under specific conditions. This has been reported for the *hca*-genes which are induced by phenylpropionate and repressed by glucose (Diaz et al. 1998).

Likewise, the expression of *yeaW* may be induced only under very specific circumstances. Since the function and the classification of YeaW remain uncertain, the search for a suitable inductor may turn out to be quite tedious. The two most closely related enzymes, the choline monooxygenase from spinach, and the

Table 1 g-values of selected Rieske proteins

	Organism	Protein	g_z[a]	g_y[a]	g_x[a]	Reference
Rieske proteins involved in respiratory and photosynthetic electron transfer reactions	*Saccharomyces cerevisiae* Mitochondria	Rieske subunit of the cytochrome bc_1 complex	2.028	1.899	1.750	Kessl et al. (2003)
	Spinacia oleracea Chloroplasts	Rieske subunit of the cytochrome b_6f complex	2.03	1.90	1.75	Riedel et al. (1991)
	Paracoccus denitrificans	Rieske subunit of the cytochrome bc_1 complex	2.02/2.03 Oxidized/reduced quinone pool	1.89	1.80/1.76	Yang and Trumpower (1986)
	Sulfolobus acidocaldarius	SoxL	2.035	1.895	1.768	Schmidt et al. (1995)
	Pyrobaculum aerophilum	ParR	2.030	1.888	1.795	Henninger et al. (1999)
Rieske ferredoxins	*Escherichia coli*	HcaC	2.022	1.906	1.831	This study
	Pseudomonas putida	Ferredoxin component of the benzene dioxygenase	2.026	1.890	1.834	Geary et al. (1984)
	Xanthobacter strain Py2	Ferredoxin component of the alkene monooxygenase	2.016	1.918	1.776	Small and Ensign (1997)
	Alcaligenes faecalis	Ferredoxin subunit of the arsenite oxidase	2.03	1.89	1.76	Anderson et al. (1992)
	Sulfolobus sp. strain 7	Sulredoxin	2.01	1.91	1.79	Iwasaki et al. (1995)
	Acidianus ambivalens	AaRFd	2.03	1.90	1.75	Kletzin et al. (2005)
Rieske oxygenases and hydroxylases	*Escherichia coli*	YeaW	2.010	1.914	1.797	This study
	Pseudomonas putida	2-oxo-1,2-dihydro-quinoline 8-mono-oxygenase	2.01	1.91	1.76	Roschea et al. (1995)
	Pseudomonas putida	Benzene dioxygenase	2.018	1.917	1.754	Geary et al. (1984)
	Pseudomonas sp. strain LB400	Biphenyl 2,3-dioxygenase	2.01	1.92	1.74	Haddock and Gibson (1995)
	Spinacia oleracea Chloroplasts	Choline monooxygenase	2.008	1.915	1.736	Rathinasabapathi et al. (1997)
	Sus scrofa	CMP-N-acetyl-neuraminic acid hydroxylase	2.01	1.91	1.78	Schlenzka et al. (1996)

[a] Listed with the same precision as in the original publications

methanesulfonic acid monooxygenase from *Methylosulfonomonas methylovora* catalyze considerably different reactions and represent distinct quaternary structures (Rathinasabapathi et al. 1997; de Marco et al. 1999). The choline monooxygenase is a homo-dimer or -trimer in accordance with other oxygenases of class IA and IB (Butler and Mason 1997), whereas the methanesulfonic acid monooxygenase shows the typical $(\alpha\beta)_n$ subunit composition (de Marco et al. 1999) of class II and III oxygenases (Schmidt and Shaw 2001). Hence, catalytic activities as well as the exact quaternary structure of YeaW remain open for further investigations.

In summary, this study provides first experimental evidence for the existence of Rieske iron–sulfur

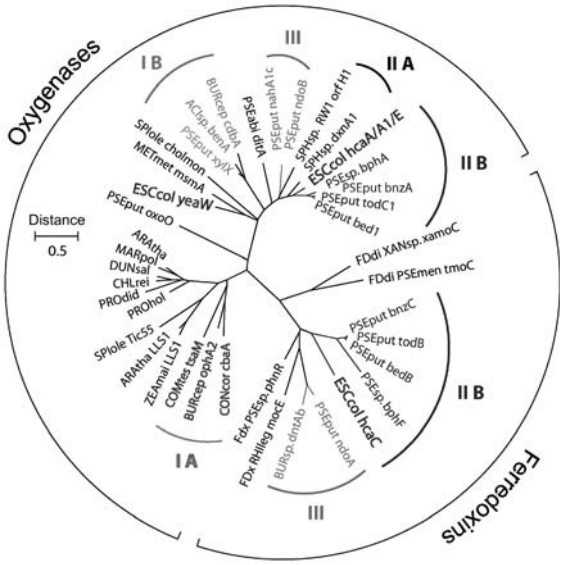

Fig. 6 Unrooted phylogenetic tree of the Rieske oxygenases and ferredoxins. I, II, III/A, B: Classification of the proteins according to Mason and Cammack (1992). Abbreviations: ACIsp. benA, benzoate-1,2-dioxygenase from *Acinetobacter* sp. (strain ADP1); BURcep ophA2, phthalate dioxygenase from *Burkholderia cepacia*; BURcep CbdA, 2-chlorobenzoate 1,2-dioxygenase from *Burkholderia cepacia*; CONcor cbaA, 3-chlorobenzoate-3,4/4,5-dioxygenase from *Conidiobolus coronatus*; PSEput nahA1c, naphthalene dioxygenase (α-subunit) from *Pseudomonas putida* (strain G7); PSEabi ditA, 7-oxo-dehydroabietic acid dioxygenase (large subunit) from the diterpenoid-degrading bacterium *Pseudomonas abietaniphila* BKME-9; PSEsp. bphA, biphenyl dioxygenase (α-subunit) from *Pseudomonas* strain LB400; PSEput bed1, benzene dioxygenase (α-subunit) from *Pseudomonas putida* (strain ML2); SPHsp. dxnA1, dioxin dioxygenase (α-subunit) from *Sphingomonas* sp.; PROhol, PROdid, CHLrei, DUNsal, MARpol, ARAtha: Chlorophyllid a oxygenases from *Prochlorothrix hollandica, P. didemni, Chlamydomonas reinhardtii*; *Dunaliella salina*, *Marchantia polymorpha* and *Arabidopsis thaliana*; ZEAmai LLS1, lethal leaf spot 1 protein from maize (*Zea mays*); ARAtha LLS1, lethal leaf spot 1 protein from *A. thaliana*; BURsp. dntAb, ferredoxin component of the 2,4-dinitrotoluene dioxygenase from *Burkholderia* sp.; PSEsp. phnR, ferredoxin of unknown function in *Pseudomonas* DJ77; PSEsp. bphF, ferredoxin component of the biphenyl dioxygenase from *Pseudomonas* strain LB400; PSEput bedB, ferredoxin component of the benzene-1,2-dioxygenase from *Pseudomonas putida* (strain ML2); PSEput todB, ferredoxin component of the toluene Dioxygenase from *Pseudomonas putida*; XANsp. xamoC, ferredoxin component of the alkene monooxygenase from *Xanthobacter* strain Py2. Other abbreviations as denoted in the legends of Figs. 1 and 2

proteins in *E. coli*. Two of them, HcaC and HcaA1 are components of a class IIB oxygenase involved in the phenylpropanoid metabolism (Diaz et al. 1998),

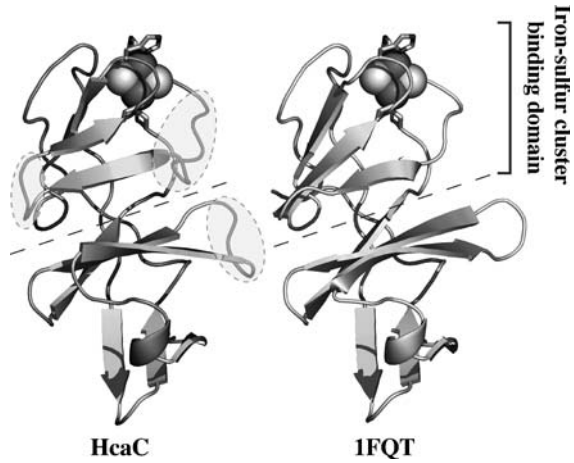

Fig. 7 Homology model of HcaC (left) and structure of the Rieske-ferredoxin from *Burkholderia cepacia* (PDB code: 1FQT) (right) used as template. The shaded areas indicate differences between the template and the predicted structure of HcaC. Two of the deviant loop regions are located within the iron–sulfur cluster binding domain

whereas the function of the third one, YeaW remains to be established.

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