Appl Microbiol Biotechnol (2017) 101:609–619
DOI 10.1007/s00253-016-7782-3

A thermophilic-like ene-reductase originating from an acidophilic iron oxidizer

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Received: 17 May 2016 / Revised: 21 July 2016 / Accepted: 3 August 2016 / Published online: 19 August 2016

Abstract Ene-reductases originating from extremophiles are gaining importance in the field of biocatalysis due to higher-stability properties. The genome of the acidophilic iron-oxidizing bacterium "*Ferrovum*" sp. JA12 was found to harbor a thermophilic-like ene-reductase (*F*OYE-1). The *foye-1* gene was ligated into a pET16bp expression vector system, and the enzyme was produced in *Escherichia coli* BL21 (DE3; pLysS) cells in yields of 10 mg L^{-1}. *F*OYE-1 showed remarkable activity and rates on *N*-phenylmaleimide and *N*-phenyl-*2*-methylmaleimide (up to 89 U mg^{-1}, >97 % conversion, 95 % (*R*)-selective) with both nicotinamide cofactors, NADPH and NADH. The catalytic efficiency with NADPH was 27 times higher compared to NADH. At the temperature maximum (50 °C) and pH optimum (6.5), activity was almost doubled to 160 U mg^{-1}. These findings accomplish *F*OYE-1 for a valuable biocatalyst in the synthesis of succinimides. The appearance of a thermophilic-like ene-reductase in an acidic habitat is discussed with respect to its phylogenetic placement and to the genomic neighborhood of the encoding gene, awarding *F*OYE-1 a putative involvement in a quorum-sensing process.

Keywords Flavoprotein · Old yellow enzyme · Biocatalysis · Extremophil · *Ferrovum*

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7782-3) contains supplementary material, which is available to authorized users.

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Introduction

Ene-reductases (ERs) are flavin-dependent enzymes catalyzing the stereoselective bioreduction of activated C = C bonds at the expense of a nicotinamide cofactor (Fig. 1). Most investigated ERs are old yellow enzymes, which are categorized in the following two subclasses: classical and thermophilic-like. In 2010, Toogood and coworkers performed a sequence alignment between known and putative OYE homologs applying both mesophilic and thermophilic organisms, leading to the renaming of the thermophilic-like subclass (previous YqjM subclass). Thermophilic-like ERs usually originate from thermophile organisms (e.g., Adalbjörnsson et al. 2010; Schittmayer et al. 2011; Liu et al. 2012) and hold typical conserved residues as described recently (Litthauer et al. 2014).

Limiting values for the biocatalysis with ERs are usually enzyme stability, inhibition, substrate scope, and stereoselectivity as a mechanistic consequence (e.g., Stueckler et al. 2007; Stuermer et al. 2007; Hall et al. 2007, 2008a, b). Regarding enzyme stability and reaction rates together, temperature, pH, and cosolvent stability are substantial parameters. One approach to find highly stable enzymes for catalysis is the search in extremophile organisms, which may already possess the latter properties.

Nowadays, mainly thermophilic organisms are considered as potential sources of thermostable proteins. A significant number of genes encoding for thermophilic-like ERs have been cloned, expressed, and characterized from thermophiles, which were isolated, e.g., in hot springs and mining areas (Blehert et al. 1999; Kitzing et al. 2005; Opperman et al.

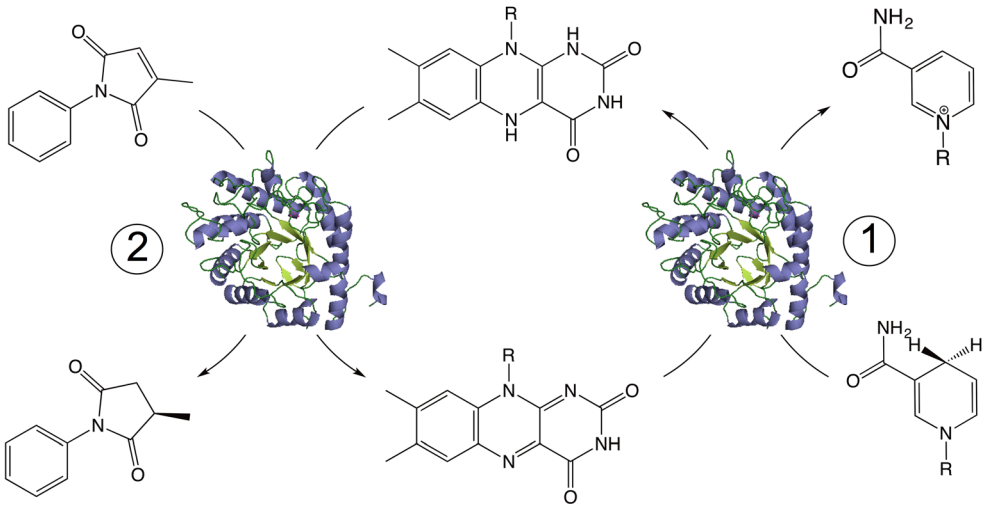


Fig. 1 Stereoselective bioreduction of *N*-phenylmaleimide by an ER. (1) The flavin becomes reduced by the nicotinamide as electron donor. (2) The reduced flavin cofactor transfers a hydride to the Cα of the substrate (reductive half-reaction). The second hydrogen is added to the Cβ in form of a proton from an essential Tyr residue conserved in the class of old yellow enzymes (oxidative half-reaction)

2008; Adalbjörnsson et al. 2010; Schittmayer et al. 2011; Liu et al. 2012; Litthauer et al. 2014; Tsuji et al. 2014; Xu et al. 2014). Interestingly, mesophilic rhodococci were also found to encode for thermophilic-like ERs, which appeared to have similar properties in catalysis but showed differences in terms of stability (Riedel et al. 2015). Other extremophiles such as acidophiles may also represent a valuable enzyme source for industrial applications. So far, no ER has been investigated out of an acidophilic organism.

Herein, we describe the identification of the first ER from an acidophilic iron-oxidizing betaproteobacterium of the genus "*Ferrovum*" (strain JA12) by genome mining. Members of the genus *Ferrovum* have been detected in mining-associated habitats worldwide (e.g., Bond et al. 2000; Hallberg et al. 2006; Heinzel et al. 2009a; Kimura et al. 2011; González-Toril et al. 2011; Santofimia et al. 2013; Fabisch et al. 2013; Jones et al. 2015; Hua et al. 2015). However, isolation of *Ferrovum* cultures proofed to be demanding. In accordance to previous reports for other chemolithoautotrophic acidophiles (Harrison et al. 1980; Johnson and Kelso 1983; Johnson and McGinness 1991; Johnson 1995), *Ferrovum* cultures were contaminated with the heterotrophic acidophile *Acidiphilium* (Johnson et al. 2014; Tischler et al. 2013; Ullrich et al. 2015, 2016a, b). Moreover, due to yet unknown reasons, long-time maintenance of *Ferrovum*-containing cultures in the laboratory is difficult and some isolates and mixed-cultured were lost during the recent years (Rowe and Johnson 2008; Hedrich et al. 2011; Ullrich et al. 2016a). Thus, the type strain "*Ferrovum myxofaciens*" P3G represents the only isolate (Johnson et al. 2014). Nevertheless, high-quality genome sequences of several *Ferrovum* strains are available for analysis (Moya-Beltrán et al. 2014; Ullrich et al. 2016a, b), opening the possibility of genome mining in order to discover new biocatalysts.

High concentrations of Fe (II) in the habitat and immediate proximity of the metal and oxygen result in the formation of reactive oxygen species via Haber-Weiss (Haber and Weiss 1934) and Fenton reactions (Walling 1975). This can cause oxidative damage to the microbial cellular membranes, proteins, and DNA (Imlay 2003; Imlay 2013). However, genome analysis has revealed a wide repertoire of systems in *Ferrovum* spp. to cope with the extreme conditions of the acidic environment (pH <4) and oxidative stress (Ullrich et al. 2016a, b).

Ferrovum sp. JA12 derived from a pilot plant to biotechnologically remediate acid mine drainage from a lignite-mining site in Lusatia (Germany) (Heinzel et al. 2009a and b; Tischler et al. 2013). In the plant, *Ferrovum* is confronted with changes of temperatures (5 to 25 °C), acidic pH of the mine water (pH 3), and high concentrations of dissolved metal ions (ferrous iron 270 mg L^{-1}, manganese 6.3 mg L^{-1}, zinc 0.18 mg L^{-1}) (Heinzel et al. 2009b; Tischler et al. 2014).

Although their physiological role remains unknown, ERs are oxidoreductases that may play a role in the oxidative stress response (e.g., Fitzpatrick et al. 2003; Toogood et al. 2010). Thus, we expected their presence in acidophilic *Ferrovum* and therefore screened the genome. In this work, we describe the cloning, expression, characterization, and biocatalytic potential of a thermophilic-like ER (*F*OYE-1) obtained from an acidophilic organism. *F*OYE-1 is the first putative thermophilic-like ER from an acidophilic organism. It points towards novel stability properties against temperature and pH.

Material and methods

Cloning

Ferrovum sp. JA12 was cultivated as described previously (Tischler et al. 2013). Genomic DNA of *Ferrovum* sp. JA12 was extracted from harvested cells of the *Ferrovum*-containing mixed culture JA12 (Tischler et al. 2013; Ullrich et al.

2016a) using the MasterPure™ Gram Positive DNA Purification Kit (Epicenter Technologies Corp., WI, USA). The gene encoding the old yellow enzyme family protein *F*OYE-1 (WP_056929840) was amplified from genomic DNA using the specific primers (*F*OYE-fw 5′-CATA TGAGTTTACTCTTCTCC-3′ and *F*OYE-rev 5′-GCGG CCGCTTATCTTGCTCTTTGG-3′). PCR products were purified via gel electrophoresis using the innuPREP Gel Extraction Kit (Analytik Jena AG, Jena, Germany). PCR products were cloned into the pJET1.2/blunt vector using the CloneJET PCR Cloning Kit (Thermo Fisher, Darmstadt, Germany) to yield pJET_Foye_01. Double digestion with *Nde*I/*Not*I and ligation into pET16bP yielded the expression construct pET_Foye_01, which was transformed into *Escherichia coli* BL21 (DE3; pLysS). Recombinant proteins were obtained as *N*-terminal His_{10}-tagged proteins.

Heterologous gene expression and purification

*F*OYE-1 was expressed in 3-L baffled flasks from recombinant BL21 (pLysS) cells harboring pET_Foye_01 using high-salt LB medium containing glucose and betaine, as described previously for another thermophilic-like ene-reductase OYE*Ro*2 (Riedel et al. 2015). Cell harvest, disruption, and protein purification via fast protein liquid chromatography, applying two tandem 1-mL HisTrapFF columns, was performed as described elsewhere (Riedel et al. 2015; Tischler et al. 2009). Protein fractions were pooled and concentrated using an ultrafiltration device with MWCO of 30.000 Da (Vivaproducts). Protein aliquots were stored at −20 °C in 50 mM phosphate buffer (KH_2PO_4/Na_2HPO_4; pH 7.1) with 40–50 % (*v*/*v*) glycerol. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) proved purity of recombinant *F*OYE-1.

Phylogenetic analysis

The genome of the beta-proteobacterium *Ferrovum* sp. JA12 (genome accession number NZ_LJWX00000000) was investigated for redox proteins through similarity searches applying BLASTp (Altschul et al. 1990, 1997) when an OYE family member (*foye-1*) was uncovered. Pairwise sequence analysis was performed with EMBOSS Needle (Rice et al. 2000). The maximum likelihood distance tree was computed by Mega 6.06-mac, applying ClustalW for multiple sequence alignment (500 bootstrap replications; Tamura et al. 2013). The protein sequence of *F*OYE-*1* is publicly available via the accession number WP_056929840.

Protein analysis

The standard assay for *F*OYE-1 activity was performed in a 1-mL cuvette at 30 °C in 50 mM phosphate buffer (KH_2PO_4/Na_2HPO_4; pH 7.1), applying 200 μM NADPH or NADH, respectively, and 1 mM maleimide. The reaction was started through addition of 30 nM protein in the final concentration. NAD(P)H consumption was followed spectrophotometrically under aerobic conditions at 340 nm ($\varepsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$). Background activity with molecular oxygen was subtracted from all activity values.

K_m and V_{max} values were determined from the double inverse representation (Lineweaver-Burk diagram), assuming an uncompetitive substrate inhibition. The inhibition constant K_{iA} was calculated from the inverse rate of turnover ($1/v$) against the inhibitor concentration (Dixon diagram).

For determination of specific activities on several compounds with activated C = C double bonds, 1 mM of the substrates, maleimide, *N*-methylmaleimide, cyclohexenone, ketoisophorone, fumaric acid, maleic acid, and vitamin C ((*R*)-3,4-dihydroxy-5-((*S*)-1,2-dihydroxyethyl)furan-2(5H)-one), were applied in the standard assay, which was then performed at 20 °C.

The temperature maximum was determined by preincubation of the standard assay at temperatures between 22.5 and 70 °C. Optimum pH was determined from the standards assay applying the Britton-Robinson buffer (Britton and Robinson 1931) in a range between 5.0 and 9.0. In both cases, the assay was started through the addition of 30 nM protein in the final concentration.

Thermal stability was determined as described previously (Riedel et al. 2015) and in a temperature range between 20 and 75 °C. The pseudo-first-order rate constants for enzyme inactivation (k_{inact}) were obtained from the logarithmic activity values plotted against the time axis. Half-lives ($t_{1/2}$) of thermal inactivation were calculated using the equation

$$t_{1/2} = ln(2) \Big/ k_{inact}.$$

Biotransformation and stereochemistry

Conversions of (prochiral) maleimides were performed about 4 h at 50 °C in 2-mL sealed glass vials containing the following components in 1-mL final volume: 25 mM potassium/sodium phosphate buffer (pH 7.1), 10 mM maleimide (substrate), 10 mM NAD(P)H, and 1.875 μM (81.56 μg mL^{-1}) enzyme. Vials were constantly shaken at 800 rpm in a thermomixer (Thermo Fisher Scientific). Reactions were stopped through the addition of 500 μL ethyl acetate, and shaking was performed on top of a thermomixer (1200 rpm, 20 min). For GC analysis, the ethyl acetate layer was dried with $MgSO_4$, centrifuged (12,000 rpm, 5 min), and transferred to GC vials. For HPLC analysis, the ethyl acetate was evaporated, the solid components were redissolved in an *n*-heptane/isopropanol mixture (95:5), and the solution obtained was used for injection.

GC analyses were carried out on a Shimadzu GC-2010 gas chromatograph equipped with an FID. A CP-Sil 5 CB column (50 m × 0.53 mm × 1.00 μm) was used to determine the conversion of *N*-phenyl-maleimide and *N*-phenyl-*2*-methylmaleimide to their corresponding reduced product. The injection temperature was 340 °C, nitrogen/air was used as a carrier gas, and linear velocity was 20 mL min^{-1}. The column oven temperature program is listed in the Supporting Information (Tables S1 and S2).

For enantioselectivity, HPLC analyses were carried out on a Shimadzu 20-series HPLC. The enantiomeric excess of *N*-phenyl-2-methylmaleimide was determined on a Chiralcel OD column (250 × 4.6 mm) running in *n*-heptane/isopropanol (95:5) at 40 °C at a flow rate of 1 mL min^{-1}. HPLC column retention times are listed in the Supporting Information (Table S3).

Results

Genome localization and phylogenetic analysis

Genome mining of *Ferrovum* sp. JA12 combined with a conserved domain search (Marchler-Bauer et al. 2015) revealed an 1062-bp-sized open reading frame (*foye-1*), which was predicted to encode an NADH:flavin oxidoreductase of the old yellow enzyme family. The encoding gene of this putative ER is located within a cluster of several open reading frames (ORFs), which appear to function in membrane transport systems (Fig. 2a). Closely downstream of *foye-1*, three genes encoding for type II secretion system (TypeIISS) proteins are positioned. TypeIISSs are abundant in Gram-negative bacteria to translocate a wide range of proteins from the periplasm across the outer membrane (Korotkov et al. 2012). The TypeIISS protein F is part of the inner membrane platform directing the interactions between the periplasmic filamentous pseudopilus, the outer membrane complex, and the cytoplasmic secretion ATPase. Interestingly, two Flp pilus assembly protein-encoding genes were identified almost directly downstream of *foye-1* (Fig. 2a).

Gene-encoding proteins of the general secretion pathway (Sec-SRP; Pugsley et al. 1997) were uncovered upstream of *foye-1*. The subunits SecD and SecF belong to a translocase that releases mature peptides into the periplasm across the inner membrane (Bieker et al. 1990). The subunit YajC is bound in a complex to SecD and SecF and has a key function in regulating the secretion through the integral membrane (Duong and Wickner 1997).

To summarize, *foye-1* is located in a central position between membrane transport systems. Additionally, a transcriptional regulator of the LuxR family was found directly downstream of *foye-1*. This regulator plays a key role in quorum sensing—a cell-to-cell communication in order to express a variety of target genes encoding virulence factors, antibiotics synthesis, motility, plasmid transfer, and biofilm formation (Ng and Bassler 2009).

A pairwise sequence alignment at protein level with previously cloned and characterized thermophilic-like ERs showed highest similarity with two mesophilic reductases, *Rm*OYE from *Ralstonia (Cupriavidus) metallidurans* (55 % identity, 71 % similarity) and *Dr*OYE from *Deinococcus radiodurans* (50 and 65 %; Litthauer et al. 2014) as well as with the thermostable (stability >70 °C) *Ts*OYE originating from *Thermus scotoductus* (47 and 65 %; Opperman et al. 2008). These findings are underlined by the phylogenetic analysis (Fig. 2b), which places *F*OYE-1 within the class of thermophilic-like ER, where it clusters directly next to mesophilic enzyme *Rm*OYE. Apparently, a closer relationship also exists to the mesophilic OYE*Ro*2 from *Rhodococcus opacus* 1CP (45 % identity, 59 % similarity; Riedel et al. 2015). Interestingly, *D. radiodurans* and *R. metallidurans* are betaproteobacteria thriving in (poly)extreme habitats including acidic and heavy metal-rich habitats (Brim et al. 2000; Mergeay et al. 2003), similar to those habitats, which also harbor acidophiles of the genus *Ferrovum.*

*F*OYE-1 reveals typical conserved residues, which occur in the thermophilic-like subclass' active side shown previously in multiple sequence alignments (Litthauer et al. 2014; Riedel et al. 2015). The histidine pair His177 and His180 (*F*OYE-1 numbering) is responsible for the hydrogen bonding of the substrates' carbonyl oxygen, whereas Tyr182 donates a proton for the oxidative half-reaction. The conserved Cys25 is known to modulate the flavin reduction potential (Spiegelhauer et al. 2010). The residue Tyr27 is involved in the binding of several ligands as well as the conserved Ile66, Ala102, and Tyr199. At position 352, the typical arginine finger (Arg352) was observed.

Cloning, expression, and purification

The restriction sites *Nde*I and *Not*I were successfully introduced at the 5′ and 3′ end in the *foye-1* gene from amplification using the aforementioned primers and ligation into the blunted pJET1.2 vector. The 1062-bp-sized gene was ligated into the His_{10}-tagged pET16bP vector, allowing for high soluble expression yields after efficient transformation into *E. coli* BL21 (DE3; pLysS) host. Expression in high-salt LBNB media (0.5 M NaCl) amounted 9.8 mg soluble *F*OYE-1 protein per liter cell culture.

Purification of *N*-terminal His_{10}-tagged protein was performed via immobilized nickel ion chromatography, and the protein was found to be stable during storage at −20 °C in 50 mM phosphate buffer (pH 7.2) containing 40 % glycerol. A subsequent SDS-PAGE analysis indicated purification to homogeneity and the expected subunit molecular mass of 43.5 kDa calculated from the deduced amino acid sequence (395 amino acid residues with a theoretical molecular masse of 43,504 Da; Fig. 3). *F*OYE-1 showed a deep yellow color as

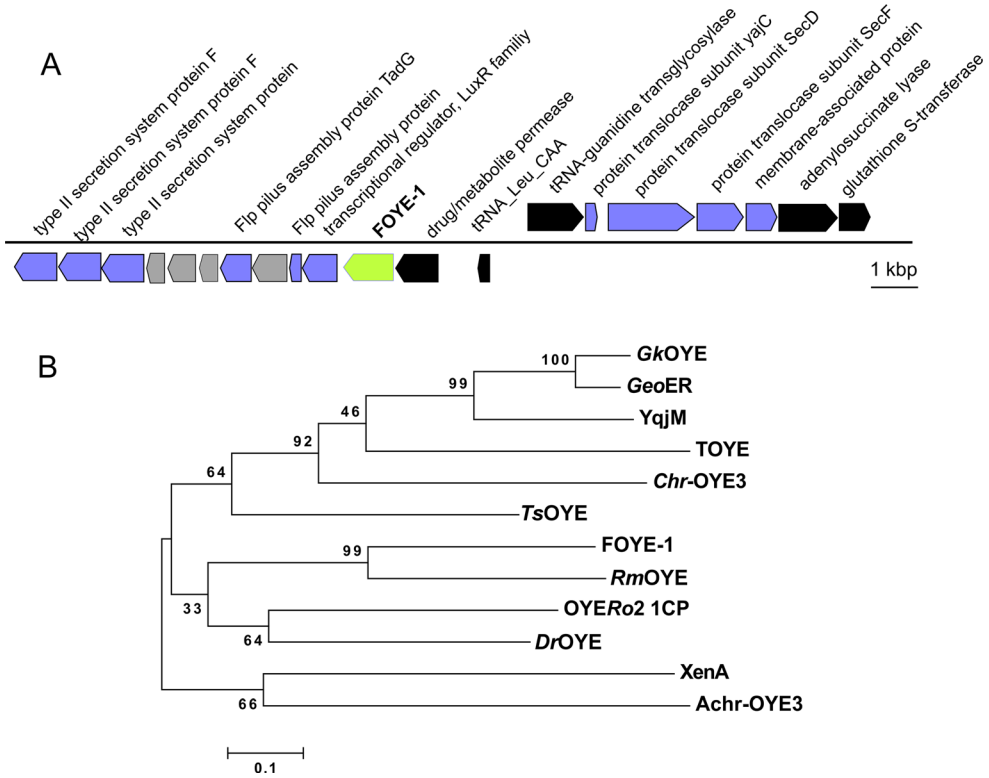


Fig. 2 Genomic neighborhood and phylogenetic analysis of *F*OYE-1. **a** Genomic neighborhood of *foye-1* (WP_056929840) in the genome of "*Ferrovum*" sp. JA12. The *lime green shading* indicates the gene encoding *F*OYE-1. The *slate blue shading* indicates the various type II secretion system protein-encoding genes. The *gray shading* represents the hypothetical proteins. The *black shading* indicates the proteins presumably without a relationship to *F*OYE-1. **b** Phylogenetic tree of *F*OYE-1 and previously characterized thermophilic-like ERs. Maximum likelihood distance tree (Mega 6.06-mac computed) was performed with 500 bootstrap replications; percentages of bootstrap values are indicated. NCBI accession numbers are given in parentheses, *Dr*OYE *Deinococcus radiodurans* (AAF11740), XenA *Pseudomonas putida* (AAF02538), *Rm*OYE *Cupriavidus metallidurans* (ABF11721), OYE*Ro*2 *Rhodococcus opacus* 1CP (ALL54975), *Ts*OYE *Thermus scotoductus* (CAP16804), TOYE *Thermoanaerobacter pseudethanolicus* (ABY93685), YqjM *Bacillus subtilis* (BAA12619), *Gk*OYE *Geobacillus kaustophilus* (BAD76617), *Achr*-OYE3 *Achromobacter* sp. JA81 (AFK73187), *Chr*-OYE3 *Chryseobacterium* sp. CA49 (AHV90721), and *Geo*ER *Geobacillus* sp. no. 30 (BAO37313)

it was discovered previously for the mesophilic ene-reductase OYE*Ro*2 (Riedel et al. 2015). A spectral analysis of OYE*Ro*2 showed a tightly bound flavin in a 1:1 ratio related to the protein (Riedel et al. 2015). Similar results were obtained for *F*OYE-1 (data not shown).

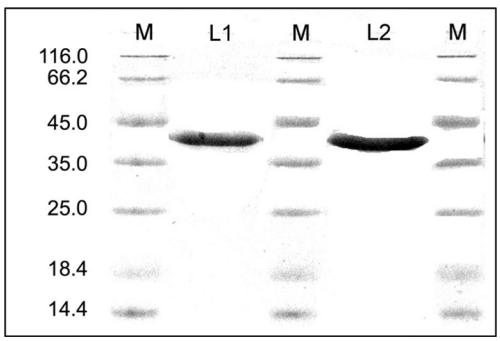
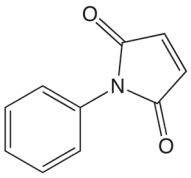
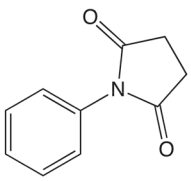
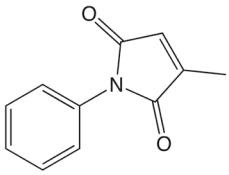
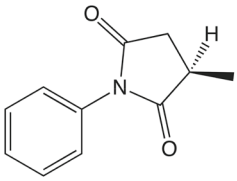


Fig. 3 SDS-PAGE analysis of *F*OYE-1 samples. *M* marker proteins, *lane 1* purified *F*OYE-1 (5 μg protein), and *lane 2* purified *F*OYE-1 (7.5 μg protein)

Biocatalytic potential

Asymmetric reduction of conjugated C = C double bonds leads to the creation of up to two stereogenic centers in the respective products and is therefore a key reaction in asymmetric synthesis. In the case of *F*OYE-1, the transformation of different maleimides creating (stereoselective) succinimides was found to be highly efficient. *F*OYE-1 showed high conversion on *N*-phenylmaleimide (>99 %, using NADPH) and also on the *N*-phenyl-2-methylmaleimide (>98 %, using NADPH; Table 1). Changing the cofactor to NADH gave 10 % lower conversion for *N*-phenylmaleimide (>89 %) and a comparable conversion value for the *N*-phenyl-2-methylmaleimide (>97 %). The product *N*-phenyl-2-methylsuccinimide was obtained in a stereoselective manner, affording 94–96 % enantiomeric excess of the (*R*)-enantiomer. Both substrates were also completely converted (>99 %) by the previously studied and phylogenetic closely related thermophilic-like ene-reductases *Dr*OYE,

Table 1 (Asymmetric) bioreduction of activated maleimides using *F*OYE-1

Substrate	Product	Cofactor[a]	Conversion [%][b]	*ee* [%][c]
		NADPH	> 99	-
		NADH	> 89	-
		NADPH	> 98	> 94 (*R*)
		NADH	> 97	> 96 (*R*)

[a] Stoichiometric amounts of cofactors

[b] Determined after 4 h by GC analysis using dodecane as an internal standard

[c] Determined by HPLC analysis

*Rm*OYE, and OYE*Ro*2 (Litthauer et al. 2014; Riedel et al. 2015). Stereochemistry was only studied for OYE*Ro*2, which was also shown to be (*R*)-selective on maleimides (data not published).

The specific activities on several substrates were tested under standard assay conditions at 20 °C (Table 2) and showed that the enzyme is highly active on maleimide and *N*-methylmaleimide (65–71 U mg^{-1}). No activity was measured for ketoisophorone and very low activity on cyclohexenone (0.51 U mg^{-1}). However, the dicarboxylic acids fumaric acids and maleic acid gave considerably higher activities (15.5 and 8.3 U mg^{-1}). Interestingly, *F*OYE-1 was also active on the lactone vitamin C ((*R*)-3,4-dihydroxy-5-((*S*)-1,2-dihydroxyethyl)furan-2(5H)-one) with an activity of 1.00 U mg^{-1}.

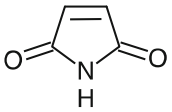
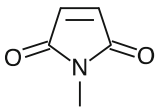
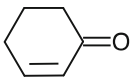
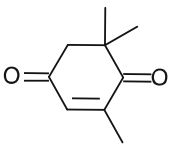
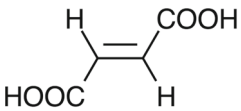
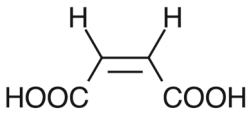
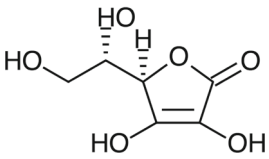
Steady state kinetic analysis with saturated concentrations of maleimide revealed a plateau at 160 to 200 μM, applying both cofactors, NADPH and NADH. At NAD(P)H concentrations higher than 200 μM, the activity decreased, indicating an uncompetitive substrate inhibition (Supporting Information Figs. S1–S4). The K_m values for NADPH and NADH are equal in a range between 72 and 77 μM (Table 3). However, V_{max} amounted to 135 U mg^{-1} for NADPH, which is 23 times higher as using NADH as an electron donor. Consequently, the turnover number k_{cat} (98 molecules per second) is 23 times higher when NADPH is used as the cofactor and the catalytic efficiency K_{cat}/K_m amounted to 1.36 μM^{-1} s^{-1}, which is 27 times higher compared to NADH (Table 3). The catalytic efficiencies are comparable to those of OYE*Ro*2, where K_{cat}/K_m for NADH was even one magnitude lower (NADPH 2.3 μM^{-1} s^{-1}, NADH 0.003 μM^{-1} s^{-1}; Riedel et al. 2015).

Uncompetitively inhibited reactions indicate an inhibition constant K_{iA} from the inverse abscissa intersection point of the plot of the inverse rate of turnovers ($1/v$) against the inhibitor concentration [NAD(P)H] (Supporting Information Figs. S3 and S4). It was shown that the K_{iA} of both electron donors revealed similar dimensions (265–327 μM).

Optimal reaction conditions and enzyme stability

The temperature dependency of *F*OYE-1 enzyme activity was investigated in the range between 20 and 70 °C in steps of 10° (Fig. 4a) using maleimide as a substrate. The highest activity of 160 U mg^{-1} was achieved at 50 °C. At 20 and 70 °C, the lowest activities were observed (39–51 U mg^{-1}). This finding implies that the enzyme activity could be tripled by raising the temperature from standard assay conditions to 50 °C (Fig. 4a, black graph). However, the temperature maximum of most proteins is rarely the optimum temperature for biocatalysis since the maximum is not constant over time. This is indicated in Fig. 4a (gray graph), showing the residual enzyme activity after 30-min incubation time at distinct temperatures. The maximum shifted from 50 to 30 °C. The thermal denaturation process of the protein was observed for an overall time of 2 h at temperatures between 30 and 70 °C

Table 2 Specific activities of *F*OYE-1 on activated C = C bonds

Substrate	Specific activities [U mg^{-1}]
	65.4 ± 1.4
	70.6 ± 2.1
	0.5 ± 0.1
	n.d.
	15.5 ± 0.8
	8.3 ± 0.8
	1.0 ± 0.1

Specific activity was determined in a 1-mL cuvette at 20 °C in 50 mM phosphate buffer (KH_2PO_4/Na_2HPO_4; pH 7.1) applying 200 μM NADPH and 1 mM of the respective substrates

n.d. not detectable

Table 3 Kinetic parameters of uncompetitive substrate inhibition of *F*OYE-1 towards nicotinamide cofactors

Substrate	K_m (μM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	K_{cat}/K_m (μM^{-1} s^{-1})	k_{iA} (μM)
NADH	76.92	5.74	4.17	0.05	327
NADPH	72.27	135.13	98.19	1.36	265

The standard assay was performed in a 1-mL cuvette at 30 °C in 50 mM phosphate buffer (pH 7.1) applying 30 nM (1.31 μg mL^{-1}) *F*OYE-1, 1 mM maleimide, and 0–300 μM NADPH or NADH, respectively. All values have a maximal experimental error of 10 %

(Table 4). The logarithmic fits can be found in the Supporting Information (Fig. S5). The half-lives of thermal inactivation ($t_{1/2}$) amounted to 5 h at 50 °C and 2.5 h at 60 °C. At 65 °C, the protein started to denaturate more rapidly and $t_{1/2}$ decreased to 26 min. *F*OYE-1 was still active at 75 °C for 2 min.

To determine pH dependency, the universal Britton-Robinson buffer (BRB) was used in a pH range between 5 and 10 (Fig. 4b). Since BRB can be applied for the entire pH range, any secondary influence of different ion types and ion strengths can be excluded. The pH maximum was determined to be at 6.5. With lower pH values (5.0), the residual activity dropped to 20 %.

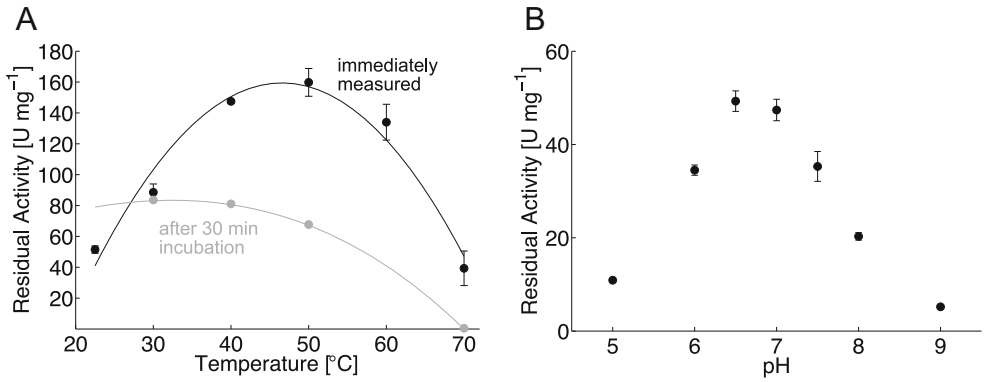


Fig. 4 Temperature maximum and pH optimum of purified *F*OYE-1. **a** Activity–temperature plot, a mixture of 200 μM NADPH, 1 mM maleimide, and 50 mM KH_2PO_4/Na_2HPO_4 (pH 7.1) was incubated at various temperatures (20 to 70 °C). The reaction was started through the addition of 30 nM enzyme stored at room temperature (*black graph*). The enzyme was simultaneously incubated at given temperatures for 30 min before the reaction was started (*gray graph*). **b** Activity–pH plot measured at 30 °C, 200 μM NADPH, 1 mM maleimide, and 40 mM Britton-Robinson buffer (pH 4.5 to 9.0) and 30 nM enzyme

Discussion

Herein, we described the discovery of *F*OYE-1—the first thermophilic-like ER originating from an acidophilic organism. *F*OYE-1 displays a close phylogenetic relationship to the mesophilic *Dr*OYE, *Rm*OYE, and OYE*Ro*2, which were found to be not thermostable. Regarding this finding, it was to our surprise that *F*OYE-1 is highly stable at temperatures up to 60° (80 % residual activity after 140 min), demonstrating that the present ER is indeed thermophilic and thus its phylogenetic classification to thermophilic-like ERs is reinforced. The temperature adaptability might be caused by the temperature changes; the organism source *Ferrovum* underlies in the pilot plant during discontinuous operation periods or long delay times in the winter (Heinzel et al. 2009b).

Specific activity on cyclohexenone is very low (0.5 U mg^{-1}), while specific activities on maleimide and *N*-methylmaleimide are 130 and 142 times higher under similar conditions. Activity on fumaric and maleic acid is 29 and 16 times higher as with cyclohexenone. These results again hint towards a close relationship between *F*OYE-1 and the mesophilic enzyme OYE*Ro*2, showing similar activities on ketoisophorone, cyclohexenone, and maleimides (Riedel et al. 2015).

Conversion rates and stereochemistry of *F*OYE-1 for the production of valuable succinimides are comparable for NADH and NADPH (89–99 %), respectively, and therefore cofactor independent. In both cases, the (*R*)-enantiomeric succinimide is produced with 94–96 % enantiomeric excess. Succinimides have a broad application spectrum as pharmaceuticals like anticonvulsant drugs, core structural units in biologically active compounds as hirsutellones with antibacterial activity, haterumaimides with antitumor activity, and tandospirones with anxiolytic and antidepressant effects (Riedel et al. 2015; Chauhan et al. 2013; Vízcaíno-Milla et al. 2015).

Table 4 Kinetic parameters for thermal inactivation of *F*OYE-1

T (°C)	r^2	$t_{1/2}$ (min)
50	0.73	301.4
60	0.78	161.2
65	0.91	25.7
67	0.95	17.5
70	0.95	5.0
75	0.77	2.3

The catalytic efficiency applying NADPH is considerably higher than with NADH as implied by the high V_{max}. Further mechanistic and structural studies are needed to uncover the reason of slower NADH oxidation, since here, only the nicotinamide oxidation was followed. Since the catalytic efficiency is not affecting the conversion rates (Table 1), aforementioned reactions should be performed with inexpensive NADH or other cost-efficient, synthetic (mimic) cofactors operating well with the presented ene-reductase as it was shown for ERs previously (Paul et al. 2013; Riedel et al. 2015; Paul and Hollmann 2016).

Optimal activity of the ER was uncovered at circum neutral pH (6.5). This is hardly surprising, since many acidophilic bacteria have been reported to employ strategies to protect their cytoplasm against the very low external pH and the uncontrolled inflow of protons (Baker-Austin and Dopson 2007; Slonczewski et al. 2009). *Ferrovum* sp. JA12 has also been predicted to maintain a circum neutral cytoplasmic pH (Ullrich et al. 2016a).

Inspection of the genomic neighborhood of *F*OYE-1 showed the putative ER gene within a cluster of several ORFs, which appeared to function in membrane transport systems. This led to the assumption that *F*OYE-1 becomes translocated through the outer membrane and operates as an extracellular protein. However, this finding was disproved, since the protein does not show the respective recognition

sequence and becomes rapidly inactivated in acidic solutions (see "Optimal reaction conditions and enzyme stability" section).

More fascinating, *foye-1* is located directly downstream of *luxR*. The encoding protein plays a major role in cell-to-cell communication (Fuqua and Greenberg 2002). The natural autoinducer molecules for LuxR are acylated derivatives of homoserine lactone (Fuqua and Greenberg 2002), which are synthesized by an acyl-homoserine lactone synthase (LuxI). *Ferrovum* sp. JA12 genome lacks the *luxI* gene. This finding supports the notion that *F*OYE-1 might be responsible for the supply of homoserine lactones, which can be produced by *trans*-hydrogenation of the respective furanones. Coincidently, *F*OYE-1 was found to be highly efficient in the biocatalysis of (prochiral) maleimides with activities up to 160 U mg^{-1}. These compounds are structurally closely related to furanones by changing only the cyclic nitrogen against an oxygen atom. Additionally, the enzyme was active with vitamin C ((*R*)-3,4-dihydroxy-5-((*S*)-1,2-dihydroxyethyl)furan-2(5H)-one), which is a typical furanone.

To summarize, *F*OYE-1 is the first discovered and characterized ER in the thermophilic-like subclass originating from an acidophilic organism. Despite that its phylogenetic relationship is closest to three mesophilic ERs, which are not stable at higher temperatures, *F*OYE-1 is stable up to 60 °C. Next to rather low activities on cyclohexenone and ketoisophorone, it is highly active on maleimides. Regarding genomic neighborhood and substrate specificity, *F*OYE-1 might be involved in quorum-sensing processes by replacing LuxI, an acyl-homoserine lactone synthase that supplies the autoinducer for LuxR, the signal receptor.

Compliance with ethical standards

Funding This project was financial supported by the Saxon Ministry of Science and Fine Arts and the European Union (EU) in the framework of the European Social Fund (ESF; project numbers 100,101,363 and 100,236,458).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Author contributions

AS and SRU carried out the genome mining, cloning, and phylogenetic analysis. AS and DT carried out the recombinant protein production, purification, enzyme characterization (temperature and pH optimum, thermal stability), and kinetics (data acquisition and analysis). Substrate specificity, product analysis, and stereochemistry were established and analyzed by AS and CEP. AS, SUR, and DT drafted the manuscript. MM, MS, and CEP critically revised the manuscript. All authors read and approved the final manuscript.