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Characterization of a phosphotriesterase-like lactonase from the hyperthermoacidophilic crenarchaeon *Vulcanisaeta moutnovskia*

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ABSTRACT

The phosphotriesterase-like lactonase (PLL) encoded by *Vmut_2255* in the hyperthermoacidophilic crenarchaeon *Vulcanisaeta moutnovskia* (VmutPLL), represents the only hyperthermophilic PLL homologue identified so far in addition to the previously characterized thermophilic PLLs from *Sulfolobus* spp. The *Vmut_2255* gene was cloned, heterologously expressed in *Escherichia coli*; the resultant protein purified and characterized as a 82 kDa homodimer (36 kDa subunits). The VmutPLL converted lactones and acyl-homoserine lactones (AHLs) with comparable activities. Towards organophosphates (OP) VmutPLL showed a promiscuous but significantly lower activity and only minor activity was observed with carboxylesters. The catalytic activity strictly depended on bivalent cations ($Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Zn^{2+}$). Furthermore, VmutPLL showed a pH optimum around 8.0, a temperature optimum of 80 °C, and thermostability with a half-life of 26 min at 90 °C. In this work, the stereoselectivity of a PLL enzyme was investigated for the first time using enantiopure lactones. The VmutPLL showed a slight preference but not an exclusive specificity for the (R)-enantiomers of capro- and valerolactone. The high thermal stability as well as the broad substrate spectrum towards lactones, AHLs and OPs underlines the high biotechnological potential of VmutPLL.

1. Introduction

Phosphotriesterase-like lactonases (PLLs) have first been identified in the crenarchaeal hyperthermoacidophiles *Sulfolobus solfataricus* and *Sulfolobus acidocaldarius* (Merone et al., 2005; Afriat et al., 2006; Porzio et al., 2007; Merone et al., 2008). This group of enzymes catalyze the hydrolytic cleavage of the intramolecular ester bond in lactones and acyl-homoserine lactones (AHLs) yielding the corresponding hydroxyacylic acids. In addition to lactones and AHLs, PLLs show a promiscuous but significantly lower phosphotriesterase activity towards organophosphate compounds (OP). In contrast, the canonical phosphotriesterases (PTE) (EC 3.1.8.1), first characterized and best investigated from *Pseudomonas diminuta*, are specific and show high activity for OPs and convert only marginally if at all other compounds like lactones (Donarski et al., 1989; Dumas et al., 1989; Benning et al., 2000). However, both PLLs and PTEs belong to the same protein family within the amidohydrolase superfamily as revealed by sequence and structural comparisons (Afriat et al., 2006). They share the same $(\beta/\alpha)_8$-barrel structural fold and catalytic activity strictly depends on bivalent metal-ions like Zn^{2+}, Mn^{2+}, Co^{2+} or Fe^{2+} (Seibert and Raushel, 2005). These metal ions are organized in a binuclear center located at the C-terminus of the β barrel and the coordinating residues (i.e. four histidines, one aspartate and one lysine) are highly conserved in PLLs and PTEs (Benning et al., 2000; Jackson et al., 2005; Elias et al., 2008). In the proposed catalytic cycle the metal ions activate a bridging water molecule through proton abstraction. The resulting hydroxide ion then performs a nucleophilic attack on the C_1 of the lactone ring and the phosphorus atom in OPs, respectively, resulting in hydrolysis (Aubert et al., 2004; Elias et al., 2008; Bigley and Raushel, 2013). However, the structural analysis also revealed some striking differences between PTEs and PLLs, which might account for the altered substrate specificities: In PLLs a unique hydrophobic

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channel accommodates the acyl chains of the (acyl-homoserine) lactones and a conserved Tyr97 is involved in the positioning of the lactone ring. These features have not been identified in PTEs. Furthermore, PLLs have a heterobinuclear metal center with Co^{2+} and Fe^{2+} whereas in PTEs homobinuclear metal ion centers, mostly with Zn^{2+}, are found which are proposed to cause differences in the geometry and coordination states of the transition intermediate during catalysis (Elias et al., 2008).

The PTEs and PLLs have gained special interest due to their broad application potential: Firstly OP compounds like the phosphotriesters are highly toxic to higher animals by irreversibly inactivating the key enzyme of neurotransmission, the acetyl cholinesterase. Due to these toxic properties they were extensively utilized as pesticides/insecticides in agriculture and also as warfare agents (Raushel, 2002). Therefore, there is a high demand for OP detoxifying/degrading agents like PTE and PLL enzymes. Secondly AHLs, also named as autoinducer 1 (AI-1), are known as small signalling molecules, produced mainly by gram-negative bacteria, enabling cell to cell communication known as quorum sensing (QS). Although critically discussed in this respect, QS seems to play a role even in certain stages of biofilm formation (Kirisits and Parsek, 2006; De Kievit, 2009) and also in the expression of virulence factors and is thus of great importance in medicine, pharmacy and biotechnology where microorganisms cause deleterious effects (Hiblot et al., 2012b). The enzymatic degradation of AHLs catalyzed by PLLs provides high potential for the interruption of QS signalling pathways (i.e. quorum quenching, QQ) and hence for the control of microbial communities. Thirdly lactonases are also of interest for the selective synthesis of enantiopure hydroxyacids and lactones. Since the pioneering discovery of a novel lactonase for the resolution of racemic pantolactone to D-pantolactone, several bacterial and eukaryotic lactonases are employed in the synthesis of enantiopure compounds (Kataoka et al., 2000; Mochizuki, 2003; Sakamoto et al., 2005; Chen et al., 2009, 2010a, 2010b). D-Pantolactone is an important intermediate in the synthesis of D-pantothenate and its derivatives (Shimizu et al., 1992; Kataoka et al., 1995, 2007). The mentioned lactonases belong mostly to the Ca^{2+} phosphotriesterase superfamily and not to the amidohydrolase superfamily like PLLs (e.g. L-pantoic acid from L-pantoyl lactone). However, stereoselectivity and the potential of the PLLs in the production of enantiopure compounds has so far not been investigated. Lastly 'extremozymes', especially from hyperthermophiles, are often advantageous in process applications due to their high intrinsic thermal stability frequently accompanied by other resistance properties (towards salts and organic solvents) and they can also be produced in high amounts using simplified purification procedures compared to their mesophilic counterparts (Vieille and Zeikus, 2001; Merone et al., 2005; Hawwa et al., 2009b).

Vulcanisaeta mountnovskia is a hyperthermoacidophilic crenarchaeon that was recently isolated from a solfataric field close to Moutnovsky volcano in Kamchatka (Russia) (Gumerov et al., 2011). It is an obligate anaerobe and grows in a temperature range of 60–95 °C and a pH range of 3.5–6.5. It is metabolically versatile and shows a heterotrophic lifestyle utilizing proteinaceous substrates or sugars either in a fermentative manner or by means of sulfur/thiosulfate reduction (Gumerov et al., 2011). In the genome of *V. moutnovskia* (Gumerov et al., 2011) the only additional hyperthermophilic PLL homologue (VMUT_2255) has been identified. The crystallization and preliminary structural determination of the *V. moutnovskia* PLL has recently been reported but the crystallographic co-ordinates are not available (Hiblot et al., 2013a).

In this study, we report the cloning and expression of the *VMUT_2255* gene from *V. moutnovskia* as well as the purification and characterization of the encoded PLL (VmutPLL) with special emphasis on its substrate specificity and stereoselectivity with lactone substrates. Its thermostability has also been studied. The properties of the VmutPLL have been compared to the archaeal PLLs previously characterized from *Sulfolobus* spp. (Merone et al., 2005; Afriat et al., 2006; Elias et al., 2008; Merone et al., 2008; Hiblot et al., 2012b).

2. Materials and methods

2.1. Cloning and expression

Genomic DNA from *V. moutnovskia* was kindly provided by Prof. Dr. Elizaveta A. Bonch-Osmolovskaya (Winogradsky Institute of Microbiology, Russia). After PCR amplification using the primer set 5′-GACTGCATATGGTCAGGATATCGATTGCTGGAGGA-3′ and 5′-AATGCTCGAGCCTACCAGTAAATAACCTTCTTGG-3′ (*Nde*I/*Xho*I restriction sites underlined) with genomic DNA as the template (*Pfu* polymerase (Fermentas), the gene putatively encoding the VmutPLL (*Vmut_2255*) was cloned into the vector pET24a. For cloning procedures *E. coli* DH5α (Invitrogen) was used as the host strain. Successful cloning was confirmed by DNA sequencing (LGC Genomics).

For expression *E. coli* BL21 CodonPlus (DE3) RIL (Agilent technologies) transformed with the plasmid construct was grown in Luria-Bertani medium in the presence of 1 mM $MnCl_2$ containing chloramphenicol (50 μg/ml) and the appropriate antibiotic for the respective plasmids, i.e. kanamycin (50 μg/ml, pET24a). After growth at 37 °C to an OD_{600} of 0.4 expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) and cells were further grown overnight at 30 °C and harvested by centrifugation (16,000 × *g*, 30 min, 4 °C).

2.2. Purification of recombinant enzyme

Cells were resuspended (1 g wet weight/3 ml) in buffer A (50 mM NaH_2PO_4, pH 8.0 containing 300 mM NaCl and 1 mM $MnCl_2$) and disrupted by passing three times through a French pressure cell at 150 MPa. After centrifugation the supernatant was diluted 1:1 with buffer A and heat precipitation was performed at 80 °C for 20 min followed by centrifugation (16,000 × *g*, 30 min, 4 °C). The supernatant was applied to a Ni-TED column (5 ml, Macherey-Nagel) equilibrated with buffer A. His tagged proteins were eluted with a linear imidazole gradient of 100–250 mM in buffer A. Fractions containing His-tagged enzymes were collected and dialyzed overnight against 50 mM Tris–HCl pH 7.0 containing 1 mM $MnCl_2$. Samples were analyzed by SDS-PAGE and protein concentrations were determined using Bradford reagent (QuickStart™, BioRad) with bovine serum albumin as standard.

For further purification and the determination of the native molecular mass of VmutPLL, protein (2 mg) was applied to size exclusion chromatography on a Superdex TM 200 26/60 column equilibrated with 20 mM Tris–HCl, 1 mM $MnCl_2$, 300 mM NaCl, pH 7.2. Proteins were eluted with an isocratic flow of equilibration buffer (2 ml/min). Fractions containing lactonase activity were pooled and stored at 4 °C.

2.3. Synthesis of Lactones

In order to address substrate stereoselectivity of the lactonase enzyme, a valuable practical method for the synthesis of enantiopure lactones was established which can be further extended. The γ-caprolactones and γ-valerolactones have been prepared by catalytic hydrogenation of the corresponding γ-ketoalkanoic acid methylesters. The 4-ketohexanoic acid methylester required for the preparation of γ-caprolactones has been prepared in a two-step reaction from maleic acid dimethylester via a Michael addition to propionylsuccinic acid dimethylester and subsequent hydrolysis/decarboxylation. The enantiomerically pure (R)- and (S)-γ-caprolactones as well as the (R)- and (S)-γ-valerolactones

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were formed by a convenient one-step synthesis (Starodubtseva et al., 2008) using the $RuCl_3$–BINAP–HCl catalytic asymmetric hydrogenation/lactonization of 4-ketohexanoic acid methylester and levulinic acid methylester, respectively.

2.4. Enzyme-assay

2.4.1. Esterase activity

The esterase activity was determined spectrophotometrically (JASCO, JenaAnalytics) in a continuous reaction assay by monitoring the p-nitrophenolate anion release from p-nitrophenyl substrates (pNP-acetate, pNP-butyrate (Sigma-Aldrich)) as well as from organophosphate esters (paraoxon, methyl-paraoxon, parathion, methyl-parathion (Sigma-Aldrich)) at 405 nm and 348 nm, respectively. Absorption at 348 nm represents the pH independent isobestic point of the p-nitrophenolate ion. The molar extinction coefficient under the different temperature and pH conditions was determined from standard calibration curves (Fig. S1). Measurements (i.e. determination of kinetic constant as well as substrate specificity) with organophosphate substrates (Sigma-Aldrich) were performed in a total volume of 500 μl at 70 °C in 50 mM Tris–HCl pH 8.0 containing 1 mM $CoCl_2$ and 0–4 mM substrate (or as indicated). For para-nitrophenyl esters (pNP-acetate, pNP-butyrate), due to the thermal and pH instability under alkaline conditions, the determination of the kinetic parameters were performed at 50 °C in 50 mM PIPES pH 6.5 with 1 mM $MnCl_2$ and substrate concentrations 0–4 mM. All assays were done in duplicate or triplicate. Organophosphate substrates and pNP-ester compounds were added from 50 mM and 10 mM stock solutions in 100% acetonitrile, respectively.

The pH optimum of the enzyme was determined with methyl-paraoxon as substrate at 50 °C using each 50 mM Na-acetate (pH 4.5–6.5), PIPES (pH 6.5–7.5), and Tris–HCl (pH 7.5–10) at 50 °C without divalent metal ions to avoid precipitation.

The temperature dependence was assayed between 40 °C and 90 °C with methyl-paraoxon in 50 mM Tris–HCl pH 8.0. The thermal stability was tested by incubating the enzymes (0.2 mg/ml) in 50 mM Tris–HCl pH 8.0 at the respective temperatures. At the time points indicated samples were taken and analyzed for residual activity in 50 mM Tris–HCl pH 8.0 at 70 °C with methyl-paraoxon as substrate. For determination of the cation specificity (cation concentration 1 mM) the pH was lowered to 6.5 by using 50 mM PIPES to avoid metal ion dependent precipitation.

2.4.2. Lactonase activity

Lactonase mediated hydrolytic cleavage of lactones yielding the open chain deprotonated form of the acid and a proton was measured according to Khersonsky and Tawfik (2005) at 577 nm by monitoring the decrease in pH using m-cresol-purple as a pH indicator (pKs 8.3 at 25 °C). The decrease in absorbance was followed in 200 μl reaction volumes using 96-well plates (0.6 cm path length cell) and a microplate reader (Infinity 200 M, TECAN, Switzerland) at 40 °C. The low temperature was used to prevent significant temperature induced autohydrolysis of AHLs. The reaction mixture contained 2.5 mM BICINE pH 8.3, 200 mM NaCl, 1 mM $CoCl_2$, 0.5% (v/v) DMSO and 0.2 mM cresol-purple as well as the different lactone substrates (up to 30 mM or as indicated) or AHL-substrates (1 mM), respectively. AHLs were added from 50 mM stock solutions in DMSO. It was ensured that DMSO had no effect on enzyme activities. Due to the low buffer capacity of lactonase-buffer the enzyme was diluted 1:50 in the same buffer (~0.02 mg/ml) to avoid pH changes and added at a final concentration of 0.2 mg/ml to the assay mixture. The absorption coefficient of cresol purple was determined to be 2.9 mM^{-1} cm^{-1} using a standard calibration curve with acetic acid (0.1 mM) which fits to previously reported values (Fig. S1) (Khersonsky and Tawfik, 2005; Hiblot et al., 2012b).

2.5. Molecular modelling

The structure of the VmutPLL was constructed with the *Sulfolobus* PLL structure (PDB code 2VC7) as a template using SWISS-MODEL online server (Arnold et al., 2006). The quality of the model produced was evaluated by *Z*-score (68%) and by its Ramachandran plot.

3. Results

Using PLL sequences from *S. solfataricus* and *S. acidocaldarius* as query, BLAST searches identified VmutPLL, as the sole hyperthermophilic PLL homologue (despite very few haloarchaeal sequences) in the archaeal domain in addition to sequences from *Sulfolobus* species. The protein sequence showed 52% identities to the PLLs characterized from *S. solfataricus*, *S. acidocaldarius* and *S. islandicus* as well as 31–41% identities to the well-studied bacterial PTE/PLLs from *P. diminuta* (29%), *Deinococcus radiodurans* (32%), *Geobacillus stearothermophilus* (32%), *Mycobacterium tuberculosis* (41%) and *Rhodococcus erythropolis* (38%) (Afriat et al., 2006; Hawwa et al., 2009a, 2009b). In agreement with the high aminoacid sequence identity the model produced for the VmutPLL superimposed to the *Sulfolobus* PLL and reveals a highly conserved active site with small differences of Tyr230 and Thr273 (Phe229 and Leu274 respectively in *Sulfolobus* PLL). One noticeable structural difference in the VmutPLL enzyme was the shortening of the loop (Pro266–Val 270, VmutPLL numbering) by two aminoacid residues located at the entrance of the active site.

3.1. Cloning and overexpression of VmutPLL in E. coli

To biochemically characterize the encoded protein, the gene *vmut_2255* (VmutPLL) was amplified by PCR and cloned into the expression vector pET24a and expression was carried out overnight in *E. coli* BL21(DE3) pRIL. The recombinant C-terminally His-tagged enzyme was purified to homogeneity in the presence of 1 mM Mn^{2+} using heat precipitation and Ni-TED affinity chromatography (Fig. S2). 1.8 mg protein of VmutPLL was obtained per g (wet weight) of *E. coli* cells.

Under denaturing conditions VmutPLL showed an apparent molecular mass of 36 kDa as revealed by SDS-PAGE (Fig. S2), which corresponds well to the theoretically calculated mass of 36.52 kDa (VmutPLL). The apparent molecular mass under native conditions was 81.5 kDa as determined by gel filtration, suggesting a homodimeric structure.

3.2. Enzyme characterization

3.2.1. pH optimum, temperature dependence and thermostability

The determination of pH optimum, temperature optimum and temperature stability of VmutPLL was performed using methyl-paraoxon (1 mM) as substrate (see Section 2). The enzyme exhibits a pH optimum at pH 8.0 and the residual specific activity at pH 7.0 and pH 9.0 was 30% and 80%, respectively (Fig. S3). In accordance with the growth temperature of *V. moutnovskia* (60–90 °C), the temperature optimum of VmutPLL was determined to be at 80 °C (Fig. 1A). The enzyme was thermostable showing only 30% loss in specific activity upon incubation for 3 h at 70 °C (Fig. 1B). The half lives at 80 °C and 90 °C were 2 h and 26 min, respectively.

3.2.2. Cation-dependency

VmutPLL required bivalent metal ions (1 mM) for activity. Addition of EDTA of 0.05–10 mM to the reaction assay resulted in a

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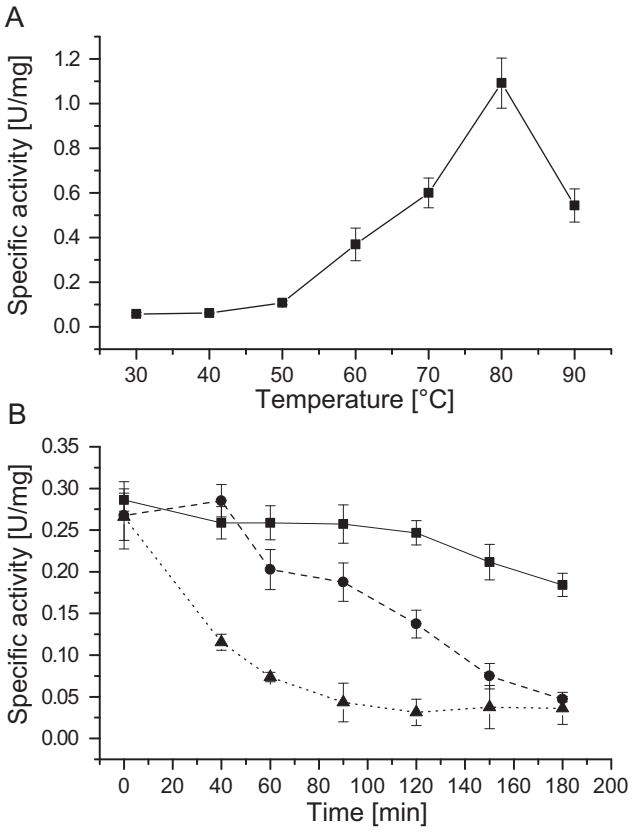

Fig. 1. Effect of temperature on the specific activity and thermostability of VmutPLL. (A) The temperature optimum was determined following PTE activity using methyl-paraoxon (0.5 mM) as substrate (Tris–HCl 50 mM, pH 8.0 at the respective temperature). (B) To determine thermal stability VmutPLL was diluted with buffer (Tris–HCl 50 mM, 1 mM $MnCl_2$ pH 8.0) to a final concentration of 0.2 mg/ml and incubated in sealed reaction tubes at 70 (•), 80 (■) and 90 °C (▲). Samples were withdrawn at the indicated time points and assayed at 70 °C under PTE standard conditions.

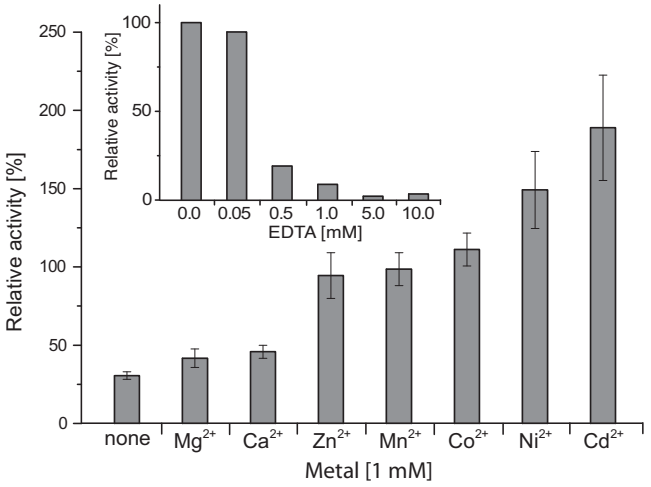

Fig. 2. Metal ion dependence of VmutPLL. PTE activity was followed with methyl-paraoxon as substrate in the absence or presence of 1.0 mM of metal ions, i.e. Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+}. Assays were performed in PIPES buffer (50 mM) at pH 6.5 and 70 °C because of the precipitation of Mn^{2+} at higher pH-values. 100% relative activity accounts for 0.086 U/mg protein determined with $MnCl_2$. The insert diagram shows the effect of different EDTA concentrations on enzyme activity. Measurements were performed as described above (50 mM PIPES pH 6.5, 70 °C) in the presence of 1 mM $MnCl_2$. 100% relative activity refers to the control without EDTA (0.086 U/mg protein).

nearly complete loss of activity. Highest activities were obtained with Cd^{2+} followed by Ni^{2+}, Co^{2+}, Mn^{2+} and Zn^{2+} whereas Mg^{2+} and Ca^{2+} had only minor stimulating effects (Fig. 2).

3.2.3. Substrate specificity and kinetic properties

The substrate specificity of VmutPLL was analyzed using a variety of lactones among others also the newly synthesized enantiopure lactones (R- and S-γ-caprolactone, R- and S-γ-valerolactone, whiskey lactone and γ-butyrolactone) (Table 1). Furthermore, also various phosphotriesters (paraoxon, methyl-paraoxon, parathion, methyl-parathion, diazinon and fensulfothion (Table 3), carboxylesters (pNP-acetate, pNP-butyrate, pNP-C8, pNP-C10, pNP-C12 (Table 1)) and acyl-homoserine lactones (N-butyryl-DL-AHL, N-C6-AHL, N-C8-AHL, 3-oxo-C8-AHL, 3-oxo-C10-AHL, N-C12-AHL, 3-oxo-C12-AHL, γ-β-keto-caproylactone (Table 2)) were tested as substrates. The VmutPLL converted preferentially lactones and AHLs and with significantly lower activity also phosphotriesters (i.e. paraoxon, methyl-paraoxon). Only minor activities could be observed with carboxylesters pNP-acetate. These results clearly identify VmutPLL as a PLL enzyme.

3.2.4. Lactonase activity

Under apparent V_{max} conditions VmutPLL exhibits highest activity with (R)-γ-valerolactone (4.59 ± 0.06 U/mg) followed by whiskey lactone (3.25 ± 0.28 U/mg) (R)-γ-caprolactone (1.58 ± 0.25 U/mg) and γ-butyrolactone (2.37 ± 0.2 U/mg). The R-enantiomers of γ-valerolactone and γ-caprolactone were preferentially converted compared to the respective (S)-enantiomers (2.21 ± 0.05 U/mg, 0.76 ± 0.014 U/mg). However, the catalytic efficiencies determined from the Lineweaver–Burk plots gave slightly different results indicating highest catalytic efficiencies for (R)-γ-caprolactone followed by whiskey lactone, (S)-γ-caprolactone, (R)- and (S)-γ-valerolactone as well as γ-butyrolactone (Table 1).

It is known that PTEs are highly promiscuous and to elucidate the substrate spectrum several AHLs were tested as substrates for VmutPLL (Table 2). The VmutPLL activity increased with increasing length of the aliphatic side chains in the AHLs from not detectable for C4 substrates to 1–3 U/mg for C12 substrates, indicating a preference for medium-length aliphatic side chains (C10) (Table 2). In addition, the presence of an oxo group in the C3 position of the acyl side chain of the tested medium-chain AHLs (C10 and C12) resulted in an increased enzyme activity.

3.2.5. Phosphotriesterase activity

VmutPLL showed activity with the phosphotriesters methyl-paraoxon (1.54 ± 0.29 U/mg), paraoxon (0.43 ± 0.04 U/mg) as well as with methyl-parathion (0.49 ± 0.20 U/mg) and parathion (0.41 ± 0.16 U/mg) at 70 °C and pH 8.0 in the presence of 1 mM $CoCl_2$ as well as 0–0.5 mM phosphotriester substrate. Only minor or no activity could be detected with diaxinon and fentosulfothion as substrate (0.12 ± 0.03 U/mg, and 0.07 ± 0.08 U/mg, respectively) (Table 3). A detailed characterization was performed with methyl-paraoxon, however, in the presence of up to 0.5 mM no substrate saturation could be observed. Therefore, only the apparent V_{max} and K_m values were determined (2.05 ± 0.67 U/mg and 2.79 ± 0.70 mM, extrapolated from Lineweaver–Burk plots).

3.2.6. Esterase activity

VmutPLL also showed low but detectable activity with pNP-acetate. No activity was observed for pNP-substrates with longer acyl chains. A detailed characterization was performed for pNP-acetate (0.25–4 mM substrate, pH of 6.5 and 50 °C). However, also for this substrate no substrate saturation was achieved in the applied substrate concentration range and the apparent V_{max} and

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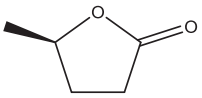
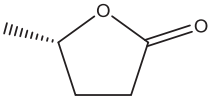
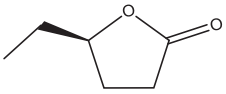
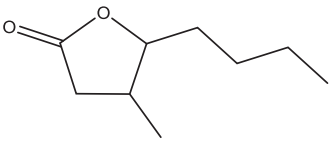
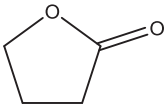
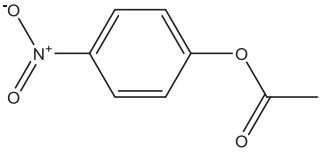
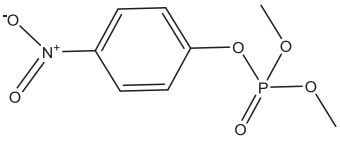
Table 1
Kinetic parameters of VmutPLL with different substrates. Lactonase activity was determined at 40 °C pH 8.3 in the presence of $CoCl_2$. PTE activity was assayed with methyl-paraoxon at pH 8.0, 70 °C in the presence of 1 mM $CoCl_2$. Esterase activity was assayed with pNP-acetate at 50 °C, pH 6.5 in the presence of 1 mM $MnCl_2$.

Substrate	Structure	K_{cat} [s^{-1}]	K_m [mM]	K_{cat}/K_m [s^{-1} M^{-1}]
γ-(R)-valerolactone		6.23 ± 0.42	4.56 ± 0.51	1327.1 ± 61.3
γ -(S)-valerolactone		2.68 ± 0.00	1.95 ± 0.18	1379.95 ± 110.67
γ-(R)-caprolactone		3.04 ± 0.01	0.55 ± 0.02	5563.72 ± 140.96
γ-(S)-caprolactone		1.89 ± 0.11	0.75 ± 0.04	2531.22 ± 298.99
Whiskey lactone		4.20 ± 0.06	0.93 ± 0.07	4538.63 ± 402.78
γ-butyrolactone		2.79 ± 0.07	11.57 ± 0.58	241.7 ± 11.18
pNP-acetate		1.66 ± 0.34	8.19 ± 1.10	201.74 ± 20.48
Methyl-paraoxon		1.25 ± 0.40	2.79 ± 0.7	442.58 ± 50.14

K_m values were determined (2.73 ± 0.55 U/mg and 8.19 ± 1.10 mM, respectively, extrapolated Lineweaver–Burk plots).

4. Discussion

The substrate spectrum of VmutPLL preferentially utilizing lactones and AHLs with a promiscuous but significantly lower activity towards organophosphates as substrates clearly defines the enzyme as PLL (Afriat et al., 2006). Minor activity was also observed with carboxylesters as substrates as previously reported for *Sulfolobus* PLLs (Merone et al., 2005; Porzio et al., 2007). PLLs constitute a distinct family within the amidohydrolase superfamily comprising functionally diverse metallo-dependent enzymes with a $(\beta/\alpha)_8$ barrel fold (Seibert and Raushel, 2005; Afriat et al., 2006; Elias et al., 2008). The VmutPLL shows extensive sequence identity (>50%) with characterized PLLs from *S. solfataricus* and *S. islandicus* from which the crystal structures are available (Elias et al., 2008; Hiblot et al., 2012b), as well as with the *S. acidocaldarius* enzyme (Porzio et al., 2007). The *Sulfolobus* PLLs exhibit a very similar substrate spectrum and also the bacterial PLLs characterized so far, e.g. from *D. radiodurans* and *G. stearothermophilus*, share comparable substrate preferences (Afriat et al., 2006; Hawwa et al., 2009a, 2009b). In addition to sequence features and substrate specificity, also the cation dependence is in accordance with the classification as PLL within the amidohydrolase family. The strict cation dependence ($Cd^{2+} > Ni^{2+} > Co^{2+} > Mn^{2+} > Zn^{2+}$) of VmutPLL confirmed by addition of EDTA has also been observed for the *Sulfolobus* PLLs which also show highest activity with Cd^{2+} ions (Afriat et al., 2006; Porzio et al., 2007). However, in SsoPox as well as in *Agrobacterium radiobacter* PTE a heterobinuclear metal center with Fe^{2+} and Co^{2+} has been identified (Jackson et al., 2006; Elias et al., 2008).

Table 2
Comparison of lactonase activities of PLLs from *V. moutnovskia* (VmutPLL), *S. solfataricus* (SsoPOX) and *S. islandicus* (SisLac). The specific activity (U/mg protein) of VmutPLL was determined using the lactonase assay (40 °C, pH 8.3, 1 mM $CoCl_2$) with different AHLs as substrate. Specific activities for SsoPox and SisLac are derived from previously published kinetic data determined at 25 °C in the presence of $CoCl_2$ (Hiblot et al., 2012b, 2013b).

Substrate	Specific activity [U/mg]		
	VmutPLL	SsoPox	SisLac
N-C4-DL-AHL	ND	ND	ND
N-C6-DL-AHL	ND	0.60 ± 0.02	ND
N-C8-DL-AHL	0.85 ± 0.00	1.67 ± 0.07	1.15 ± 0.15
3-oxo-C8-DL-AHL	ND	0.07 ± 0.01	6.9 ± 0.15
3-oxo-C10-DL-AHL	1.37 ± 0.25	7.53 ± 0.17	17.94 ± 0.61
N-C12-DL-AHL	1.11 ± 0.05	2.83 ± 0.05	3.20 ± 1.33
3-oxo-C12-DL-AHL	3.90 ± 0.28	1.68 ± 0.22	0.66 ± 0.07
N-β-ketocaproyl-AHL	0.16 ± 0.05	ND	ND

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Table 3
Comparison of PTE activities of PLLs from *V. moutnovskia* (VmutPLL), *S. acidocaldarius* (SacPox), *S. solfataricus* (SsoPOX) and *S. islandicus* (SisLac) with different OP compounds. The specific activity (U/mg) of VmutPLL was determined using the esterase assay (70 °C, pH 8.3, 1 mM $CoCl_2$) in the presence of different OPs (0.5 mM). Specific activities for SacPox, SsoPox and SisLac are derived from previously published kinetic values determined at 25 °C in the presence of $CoCl_2$.

Substrate	Specific activity [U/mg]			
	VmutPLL	SacPox[a]	SsoPox[b]	SisLac[c]
Paraoxon	0.43 ± 0.04	0.24 ± 0.06	0.42 ± 0.02	2.39 ± 0.15
Methyl-paraoxon	1.54 ± 0.29	1.38 ± 0.01	1.125 ± 0.24	12.46 ± 2.12
Parathion	0.41 ± 0.16	0.04 ± 0.01	0.019 ± 0.001	ND
Methyl-parathion	0.49 ± 0.20	0.54 ± 0.06	1.125 ± 0.24	0.016 ± 0.0003
Diazinon	0.12 ± 0.03	0.1 ± 0.01	0.011 ± 0.0004	ND
Fensulfothion	0.187 ± 0.02	ND	ND	ND

[a] Data were taken from reference "Merone et al. (2005)".
[b] Data were taken from reference "Porzio et al. (2007)".
[c] Data were taken from reference "Hiblot et al. (2012b)".

Bioinformatic analyses revealed that – besides few mesophilic haloarchaea – PLLs in archaea seem to be restricted to crenarchaeal aerobic. thermoacidophilic *Sulfolobus* species (order Sulfolobales). The distantly related hyperthermoacidophilic *V. moutnovskia* belongs to the order Thermoproteales and appears to be the only archaeon also harbouring a PLL homologue. In contrast to *Sulfolobus*, *V. moutnovskia* is an obligate anaerobe but shares the (hyper)thermophilic, heterotrophic as well as acidophilic lifestyle (temperature range 60–95 °C; pH range 3.5–6.5) (Gumerov et al., 2011). VmutPLL represents the closest relative to the *Sulfolobus* enzymes and both show sequence identities of around 40% to bacterial PLLs e.g. from *Mycobacterium* and *Rhodococcus* (Afriat et al., 2006).

From the distribution and from the position in previously reported phylogenetic tree it is tempting to speculate that the archaea gained these genes from high GC gram positive bacteria via lateral gene transfer (LTG) either to a common ancestor of *Sulfolobales* and *Thermoproteales* or to one of the two orders with a subsequent additional LGT to the other (Afriat et al., 2006; Porzio et al., 2007). Furthermore, also based on phylogenetic studies, it has been proposed that the specific phosphotriesterases e.g. from *P. diminuta*, which preferentially converts organophosphate compounds, have rapidly evolved from PLLs in the last 70 years due to the elevated application of non-natural organophosphates in agriculture and as war fare agents (Afriat et al., 2006; Draganov, 2010).

VmutPLL showed activity with γ-lactones in a similar range as observed for AHLs (Table 1). The enzyme converted the C_6 caprolactone (γ-lactone ring with an ethyl (C2) side chain substituent) with highest catalytic efficiency. Shorter side chains (i.e. methyl group in γ-valerolactone and no side chain in butylolactone) resulted in decreased catalytic efficiency. The lower efficiency with whiskey lactone as a substrate might either be due to the longer C4 side chain or to the second methyl side chain substituent at the γ-lactone ring. Also, *S. islandicus* PLL (SisLac) showed comparable activities with AHLs and γ-lactones. SisLac also converted γ-lactones with longer acyl side chains than in C_6 caprolactone as well as γ-lactones (Hiblot et al., 2012b). From the structural model (constructed as described in Section 2) it would appear that the loop (Pro266–Val 270) at the entrance to the active center must move to allow the substrates to enter into the active site. Although the shortened loop in VmutPLL compared to SsoPox seems to have no major influence on the substrate specificity which is similar in both enzymes, the relevance of structural difference can only be analyzed once a structure is available for the VmutPLL enzyme with the appropriate substrate bound into the active site.

The VmutPLL showed also activity with different AHL substrates with a preference for medium acyl side chain AHLs. Highest activity was observed with 3-oxo-C12-DL-AHL (Table 3). Also, *Sulfolobus* PLLs exhibit substantial activities for different AHLs, apparently with a preference for slightly shorter chain length (3-oxo-C10-DL-AHL) (Ng et al., 2011; Hiblot et al., 2012b, 2013b).

Lactones and AHLs are abundant natural compounds. The product of both, the lactone and AHL cleavage reaction, is the corresponding hydroxyacylic acid. Interestingly, in the direct gene neighbourhood of *Vmut_2255* genes putatively encoding fatty acid activating CoA ligases (*Vmut_2254*, *Vmut_2252*) and a 4-hydroxybutyryl-CoA dehydratase (*Vmut_2253*) were identified. These genes might link the lactonase reaction to fatty acid and lipid metabolism via e.g. β-oxidation. In *S. solfataricus* and *S. islandicus*, in addition to CoA ligases, esterase/lipase encoding genes are clustered with the PLL gene suggesting a function in lipid metabolism as well. In *S. acidocaldarius* the presence of genes putatively encoding glutamine synthetase (*Saci_2141*) and γ-aminobutyrate metabolism dehydratase/isomerase (*Saci_2143*) might additionally indicate a role of PLL in amino acid/nitrogen metabolism. Also, AHLs might serve as carbon, energy and nitrogen sources (Afriat et al., 2006). However, the overall activities with AHLs and lactones are quite low and the physiological role of PLL remains to be shown. The function of PLL in QQ and of AHLs in cell to cell communication especially in thermophilic archaea seems to be unlikely due to their expected thermolability (Surette and Bassler, 1998). Related genes, i.e. *luxR*/*luxI* homologues, have so far only been identified in mesophilic *Methanosaeta harundinacea* 6Ac (Zhang et al., 2012). In addition, it has not yet been investigated if e.g. *Sulfolobus* or *Vulcanisaeta* respond to AHL treatment.

In this study enantiopure (R)- and (S)-forms of γ-lactones were synthesized (described in Section 2), and tested for the first time as substrates for PLLs revealing that VmutPLL did not show pronounced stereoselectivity for the substrate lactones although the VmutPLL exhibited a slight preference for the R-enantiomers of capro- and valerolactone.

The VmutPLL exhibited a temperature optimum of 80 °C and thermostability with $t_{1/2}$ of 26 min at 90 °C which is in accordance with the hyperthermophilic lifestyle of *V. moutnovskia*. For SisLac and SsoPox a temperature optimum higher than 90 °C was described whereas the SacPox showed an optimum at 75 °C (Merone et al., 2005; Porzio et al., 2007; Hiblot et al., 2012a, 2012b). The thermal stability of VmutPLL was higher than described for SisLac and SacPox (8.5 min and 5 min at 90 °C, respectively) (Porzio et al., 2007; Hiblot et al., 2012b). For SsoPox a $t_{1/2}$ of 4 h at 95 °C has been reported (Merone et al., 2005). Thus, VmutPLL is the first hyperthermophilic PLL from another archaeal lineage than *Sulfolobus* and from bioinformatics it can be concluded that none of the other hyperthermophilic bacteria and archaea, for which the genome sequence is available to date, harbour PLL homologues. Due to their intrinsic thermal stability, often accompanied by stability against organic solvents and by the ease of purification, *Sulfolobus* spp. PLLs previously gained special interest, and also the VmutPLL with its similar thermophilic properties seems an interesting

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candidate for biotechnological application including the decontamination of organophosphates. The activities of VmutPLL for the organophosphates paraoxon (0.1 U/mg) and methyl-paraoxon (1-2 U/mg) are in a similar range as described for SacPox and SsoPox and all three enzymes convert other phosphotriesters like parathion, methyl-parathion, diazon or fensulfothion in a significantly lower rate (Merone et al., 2005; Porzio et al., 2007). Notably, the PLLs from *Flavobacterium* and *P. diminuta* have been successfully subjected to directed evolution to broaden the OP substrate spectrum (Raushel, 2002). Moreover, first attempts using whole cells as well as enzymes for OP decontamination, respectively, have already been performed (Singh and Walker, 2006). Therefore, also the archaeal (hyper)thermophilic PLLs including VmutPLL are promising candidates for directed evolution in order to improve their potential for the decontamination of organophosphates, for quorum quenching as well as for stereospecific lactone bioconversion.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jbiotec.2014.04.026.

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