



OPEN

SUBJECT AREAS:

HYDROLASES

X-RAY CRYSTALLOGRAPHY

Received

24 October 2014

Accepted

12 January 2015

Published

11 February 2015

Crystal structure of *VmoLac*, a tentative quorum quenching lactonase from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia*

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A new representative of the Phosphotriesterase-Like Lactonases (PLLs) family from the hyperthermophilic crenarchaeon *Vulcanisaeta moutnovskia* has been characterized and crystallized. *VmoLac* is a native, proficient lactonase with promiscuous, low phosphotriesterase activity. *VmoLac* therefore represents an interesting candidate for engineering studies, with the aim of developing an efficient bacterial quorum-quenching agent. Here, we provide an extensive biochemical and kinetic characterization of *VmoLac* and describe the X-ray structures of the enzyme bound to a fatty acid and to its cognate substrate 3-oxo-C10 AHL (Acyl-Homoserine Lactone). The structures highlight possible structural determinants that may be involved in its extreme thermal stability ($T_m = 128^\circ\text{C}$). Moreover, the structure reveals that the substrate binding mode of *VmoLac* significantly differs from those of its close homologues, possibly explaining the substrate specificity of the enzyme. Finally, we describe the specific interactions between the enzyme and its substrate, and discuss the possible lactone hydrolysis mechanism of *VmoLac*.

Phosphotriesterase-Like Lactonases (PLLs) compose a class of lactonases (EC 3.1.1.25) that has long been mistaken for the organophosphorus-degrading enzymes phosphotriesterases (PTEs; EC 3.1.8.1)¹. Indeed, several PLLs were initially isolated and characterized by virtue of their organophosphorous insecticide degrading abilities^{2–4}. In contrast with known PTEs that hydrolyze these compounds with high efficiency⁵, PLLs are less proficient^{3,4}. In fact, PLLs are native lactonases that are endowed with promiscuous phosphotriesterase activity^{1,6}, and it might be the progenitors of PTEs that may have diverged from PLLs upon the first use of organophosphorous insecticides in the 1950's¹.

The PLL family has been subdivided into two sub-classes based on their structures and catalytic preferences¹: the PLLs-A, such as *SsoPox*⁷, *SisLac*⁸ and *PPH*¹ are capable of hydrolyzing with high efficiency both Acyl-Homoserine Lactones (AHLs) and oxo-lactones, whereas the PLLs-B, e.g., *DrOPH* and *Gkl*^{9,10}, comprise exclusive oxo-lactonases. The biological function of PLLs remains unclear but some evidence (e.g., the enantiospecificity of PLL-As⁷) may indicate a role of some PLLs in quorum sensing. Indeed, the ability of these enzymes to hydrolyze AHLs enables them to interfere with bacterial communication¹¹, a property that may be used to develop new approaches to annihilate bacterial pathogens' virulence^{12–15}.

PLLs belong to the amidohydrolase superfamily¹⁶ and exhibit a $(\beta/\alpha)_8$ topology containing a bi-metallic active site that is coordinated by four histidines, an aspartic acid and a carboxylated lysine^{17,18}. The bi-metallic center acts as a Lewis acid and activates a bridging, putatively catalytic, water molecule into a hydroxide anion, which subsequently serves as a nucleophile for the organophosphorous compounds or lactone hydrolysis. The active site loops 7 and 8 mediate the substrate specificity^{17,19}: indeed, PTEs and PLLs mainly differ by the size and conformations of these loops²⁰. In particular, in the PLL *SsoPox*, the sole loop 8 position 263 modulates the promiscuous phosphotriesterase and lactonase activities, by altering the active site loop conformational landscape⁷.

VmoLac (YP_004245953) is a recently identified enzyme that was isolated from the hyperthermophilic crenarchaeon *Vulcanisaeta moutnovskia* strain 768-28³³. Interestingly, *VmoLac* shares ~50% sequence identity with PLL-A and ~30% with PLL-B representatives, and may therefore possess a unique active site configuration and



substrate specificity. Here we provide a biochemical and kinetic characterization of the phosphotriesterase, esterase and lactonase activities of *VmoLac*. Moreover, we provide the crystal structures of *VmoLac* in two crystal forms, both in complex with a fatty acid, and the crystal structure of *VmoLac* with a bound 3-oxo C10 acyl-homoserine lactone. Together, these data allow us to propose a lactone hydrolysis mechanism for *VmoLac*.

Methods

Sequence alignment. Phylogenetic analysis of PLLs were performed using *T-coffee* server (expresso)^{21,22}, with manual improvement. The 30 sequences used for the phylogenetic analysis are issued from a previous analysis and were subsequently updated (Table S1 & S2)²⁰. The *PhyML* software²³ was employed to generate phylogenetic tree using default parameters. The sequence alignment was drawn using *BioEdit* 7.1.3. The *ClustalW* server²⁴ was used to calculate protein sequence identities.

Protein purification. The protein was produced in *E. coli* BL21(DE₃)-pGro7/GroEL strain (TaKaRa). Purification procedure took advantage of the protein thermostability by performing an initial heat treatment of 30 minutes at 70°C. Proteins were then loaded on a StrepTrap HP chromatography column (GE Healthcare), followed by tag removal using TEV protease²⁵. Finally, a size exclusion chromatography column allowed to obtain pure protein (Superdex 75 16/60, GE Healthcare)^{4,26}. The protein molar extinction coefficient was calculated using the *PROT-PARAM* server²⁷ in order to quantify the protein concentration using a nanospectrophotometer (Nanodrop, ThermoFisher Scientific, France).

Kinetic measurements. The catalytic parameters were obtained using a microplate reader (Synergy HT, BioTek, USA) controlled by the Gen5.1 software in 96-well plates of 6.2 mm path length cell for a 200 μ L reaction⁸. Kinetics were performed at 25°C. The *Graph-Pad Prism* 5 software was used to obtain catalytic parameters by fitting the data to the Michaelis-Menten (MM) equation. The linear part of the MM plot was fitted to a linear regression using *Graph-Pad Prism* 5 software if V_{max} could not be attained.

Kinetics were performed in *activity buffer* (HEPES 50 mM pH 8, NaCl 150 mM and CoCl₂ 0.2 mM). The time course hydrolysis of pNP derivatives ($\epsilon_{405\text{ nm}} = 17\,000\text{ M}^{-1}\text{cm}^{-1}$) has been measured for OPs (Fig. 1A) and esters (Fig. 1B). For malathion (Fig. S2V), 2 mM DTNB was added to the buffer ($\epsilon_{412\text{ nm}} = 13\,700\text{ M}^{-1}\text{cm}^{-1}$). The time course hydrolysis of phenyl-acetate (Fig. S2VII) and dihydrocoumarin (Fig. S2X) were monitored at 270 nm ($\epsilon_{270\text{ nm}} = 1\,400\text{ M}^{-1}\text{cm}^{-1}$) and at 412 nm for the coumarin nerve agent derivative of cyclosarin (CMP-coumarin Fig. S2VI; $\epsilon_{412\text{ nm}} = 37\,000\text{ M}^{-1}\text{cm}^{-1}$). The lactonase activities were performed in *lactonase buffer* (Bicine 2.5 mM pH 8.3, NaCl 150 mM, CoCl₂ 0.2 mM, Cresol purple 0.25 mM and DMSO 0.5%) with different AHLs (Fig. 1C) [*i.e.* C4-AHL (*r*), 1 mM; C6-AHL (*r*), 2 mM; 3-oxo-C6-AHL (*l*), 2 mM; C8-AHL (*r*), 1 mM; 3-oxo-C8-AHL (*l*), 2 mM; and 3-oxo-C10-AHL (*l*), 2 mM] (Fig. S2XI–XVI) and oxo-lactones (Fig. 1D E) [*i.e.*, ϵ -caprolactone, 5 mM; γ -heptanolid (*r*), 5 mM; nonanoic- γ -lactone (*r*), 5 mM; nonanoic- δ -lactone (*r*), 5 mM; undecanoic- γ -lactone (*r*), 5 mM; dodecanoic- γ -lactone (*r*), 5 mM] (Fig. S2XVII–XXIII). The lactone ring hydrolysis was followed by acidification of the medium for which Cresol purple serves as pH indicator (pK_a 8.3 at 25°C, 577 nm).

Melting temperature (T_m) determination. Circular Dichroism (CD) spectra were recorded with a Jasco J-810 spectropolarimeter equipped with a Pelletier type temperature control system (Jasco PTC-4235) both monitored by the *Spectra Manager* software. Samples were placed in 1-mm-thick quartz cell and the melting temperature of the protein was determined by following its denaturation at 222 nm in 10 mM sodium phosphate buffer at pH 7.5. Temperature was increased from 20 to 90°C (at 1°C min⁻¹) and increasing concentrations of guanidinium chloride (4–6 M) were applied. A theoretical T_m without guanidinium chloride was then determined by extrapolating at the y-intercept by a linear fit using the *Graph-Pad Prism* 5 software.

Crystallisation. *VmoLac* was concentrated to 20 mg/mL for crystallization trials. Crystals were obtained in different conditions from Structure Screen 1 + 2 (Molecular Dimensions) after 2 weeks at 293 K in drops (2 : 1 and 1 : 1 protein : reservoir ratio) using the sitting drop vapor diffusion method in a 96-well plate. In addition of what was previously published (*i.e.* 400 mM ammonium dihydrogen phosphate)²⁸, crystals were obtained under conditions G9 (100 mM sodium citrate pH 5.6, 10 mM ferric chloride and 10% v/v jeffamine M-600), C4 (100 mM sodium HEPES pH 7.5, 800 mM sodium and potassium dihydrogen phosphate) and E7 (100 mM Tris pH 8.5, 1.5 M ammonium sulfate and 12% v/v glycerol). The crystals from the G9 and E7 conditions had the same P6₄ space group and the ones issued from the C4 condition had an alternative P6₂₂ group space. The crystals from E7 were soaked into the well solution supplemented by 2 mM of 3-oxo-C10 AHL for 1 min prior to flash cooling.

Data collection, structure resolution and refinement. The crystals were transferred into a cryo-protectant solution (reservoir solution plus 20% (v/v) glycerol) before being flash cooled in liquid nitrogen. An x-ray diffraction dataset was collected at 100 K using synchrotron radiation at the ID29 beam line (ESRF, Grenoble) and a PILATUS-6M detector. X-ray diffraction data were integrated and scaled with the

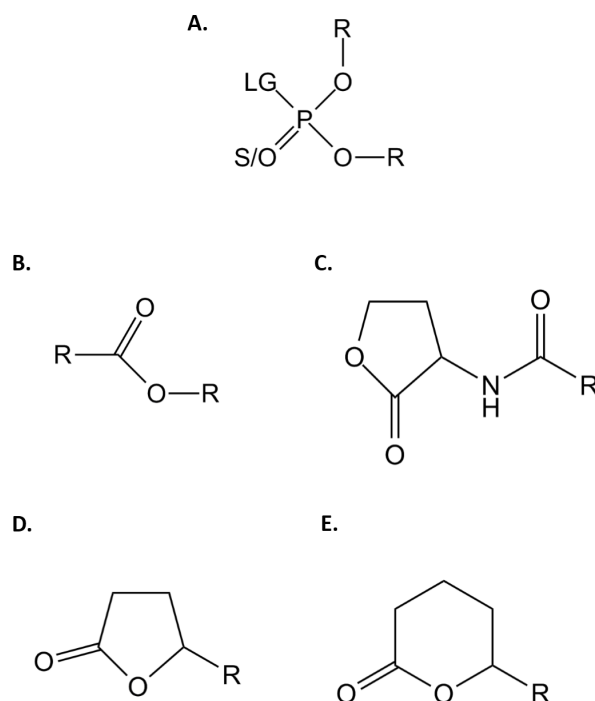


Figure 1 | Chemical structure of the tested substrates. Chemical structures of (A.) phosphotriesters, (B.) esters, (C.) Acyl-Homoserine Lactones, (D.) γ -lactones and (E.) δ -lactones. For phosphotriesters, R corresponds to different nature of the substituents; LG corresponds to the leaving group, which can be F, S-R, O-R or CN. The terminal substituent could be S atom if the molecule is a thionophosphotriester or an O atom if the molecule is an oxonophosphotriester. For esters, R corresponds to the different nature of the substituent. For AHLs and γ/δ -lactones, R corresponds to the different size of the acyl chain.

XDS package²⁹ (Table 1). The phases were obtained by molecular replacement using *PHASER* and the *SsoPox* structure as a starting model (PDB ID 2vc5)²⁸. The model was subsequently built with *Coot*³⁰ and refined using *REFMAC5*³¹. A total of 2 monomers (a dimer) was found per asymmetric unit in the P6₄ space group crystals while only one monomer per asymmetric unit was observed in the P6₂₂ space group crystals. One of these dimers was highly agitated in the crystal, resulting in a poor electron density. The models and structure factors were deposited under the Protein Data Bank (PDB) codes 4RDZ, 4RE0 and 4RDY.

Anomalous X-ray scattering data. Two datasets were collected at 1.7 and 1.8 Å resolution at energies lower (7.700 keV) and higher (7.725 keV) than the Co-K absorption edge (7.7093 keV) (Table S3).

Structure analysis. Crystal structures of *SsoPox* (PDB ID 2VC5 and 2VC7), *SisLac* (PDB ID 4G2D), *DrOPH* (PDB ID 2ZC1) and *GkL* (PDB ID 3OJG) were employed for structural comparisons. Illustrations, analysis and comparisons were performed with *PyMOL*, vacuum electrostatic potentials and surface representation were determined using a solvent probe of 1.4 Å radius. The dimer interface surface together with the number of hydrogen bonds and salt bridges were computed using *PISA*³². The root mean square deviations (RMSD) were calculated on α -carbon using the *align* command under the *PyMOL* interface.

Results

***VmoLac* is a highly thermostable PLL-A.** The *VmoLac* protein sequence was aligned with 30 sequences of PLLs, PTEs, resiniferatoxin-binding protein (RTXs) and phosphotriesterase homology proteins (PHPs) representatives (Fig. 2B, Table S1). The phylogenetic tree that was built from this sequence alignment, indicates that *VmoLac* belongs to the clade of the PLLs-A (Fig. 2A). However, *VmoLac* constitutes the most distant known representative of this clade, and shares only approximately 52% sequence identity with *SsoPox*, 41% with PPH, < 30% with identified PLLs-B and 29% with *BdPTE* (Table S2). The sequence alignment highlights the strict conservation of essential active site residues between *VmoLac* and the different clades;

Table 1 | Data collection and refinement statistics of *VmoLac* structures

Data collection			
	<i>VmoLac</i> -P6 ₄	<i>VmoLac</i> -P6 ₂₂	<i>VmoLac</i> -3-oxo-C10-AHL
PDB ID	4RDZ	4REO	4RDY
Beamline	ID29	ID29	ID29
Wavelength (Å)	0.9537	0.9537	0.9537
Detector	PILATUS 6M	PILATUS 6M	PILATUS 6M
Oscillation (°)	0.1	0.1	0.1
Number of frames	1800	1800	1200
Resolution (Å)	1.8	2.35	2.0
Space group	P6 ₄	P6 ₂₂	P6 ₄
Unit-cell parameters (Å)	a = 174.96; b = 174.96; c = 62.07; α = 90; β = 90; γ = 120	a = 134.67; b = 134.67; c = 126.40; α = 90; β = 90; γ = 120	a = 174.74; b = 174.74; c = 61.55; α = 90; β = 90; γ = 120
No. of observed reflections (last bin)	1 001 921(150 041)	544 316(34 394)	490 250(67 169)
No. of unique reflections (last bin)	100 625(14 960)	28 744(1 722)	72 593(9 826)
Completeness (%) (last bin)	100.0(100.0)	99.9(100.0)	99.8(99.9)
R _{meas} (%) (last bin)	7.4(63.2)	11.2(89.0)	12.2(92.1)
I/σ(I) (last bin)	25.30(4.62)	28.19(4.07)	17.94(2.89)
Redundancy (last bin)	99.56(100.29)	18.94(19.97)	6.75(6.83)
CC(1/2)	99.9(91.0)	99.9(90.2)	99.8(71.2)
Refinement statistics			
R _{free} /R _{work}	0.16007/0.13001	0.17063/0.14090	0.17444/0.13866
No. of total model atoms	6244	2880	5844
Ramachandran favored (%)	98.94	96.86	98.43
Ramachandran outliers (%)	0.00	0.31	0.00
Generously allowed rotamers (%)	0.70	1.45	1.28
Rmsd from ideal			
Bond lengths (Å)	0.024	0.022	0.022
Bond angles (°)	2.150	2.173	2.039

however, some discrepancies are visible in the regions of loops 7 and 8 of *VmoLac* that might account for differences in the substrate specificities. In particular, from the sequence alignment, loop 8 is shortened in *VmoLac* compared to his closest homologues archaeal PLLs. Moreover, a biochemical analysis of *VmoLac* using circular dichroism measurements, performed at various temperatures and guanidinium chloride concentrations, allowed us to determine the melting temperature of *VmoLac*: $T_m = 128 \pm 7^\circ\text{C}$ (Fig. S1). This extremely high value is consistent with *VmoLac* originating from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia* which grows between 60 and 98°C ³³, as previously concluded in works evaluating enzyme thermophilicity³⁴.

Kinetic characterization of *VmoLac*. Phosphotriesterase activity. The ability of *VmoLac* to hydrolyze various insecticides (e.g., ethyl/methyl-paraoxon, ethyl/methyl-parathion and malathion; Fig. 1 & S2) was evaluated (Table 2). The catalytic efficiency of *VmoLac* against ethyl- and methyl-paraoxon is very low ($k_{\text{cat}}/K_M = 2 \text{ M}^{-1}\text{s}^{-1}$), a much lower efficiency than that of other PLLs-A such as *SsoPox* and *SisLac* ($\sim 10^2 \text{ M}^{-1}\text{s}^{-1}$ for ethyl-paraoxon and $\sim 10^3 \text{ M}^{-1}\text{s}^{-1}$ for methyl-paraoxon)^{4,8}. The enzyme showed no detectable activity against thiono-phosphotriester substrates (i.e., ethyl/methyl-parathion and malathion). This behavior may relate to the previously described thiono-effect, in which some PLLs exhibit profound preference for oxono-phosphotriesters, whereas PTEs do not³⁵. *VmoLac* also hydrolyzes CMP-coumarin, a cyclohexyl sarin fluorescent analogue, albeit with very low efficiency ($k_{\text{cat}}/K_M = 63.9 \text{ M}^{-1}\text{s}^{-1}$). Overall, *VmoLac* is a poor phosphotriesterase. The catalytic efficiency of *VmoLac* for phosphotriesters is ~ 200 -fold higher at 70°C ³⁴, similar to what was observed for other extremophilic archaeal PLLs^{4,8}.

Esterase activity. The *VmoLac* kinetic parameters were recorded for several esters (e.g., substrates phenyl-acetate, *p*NP-acetate and

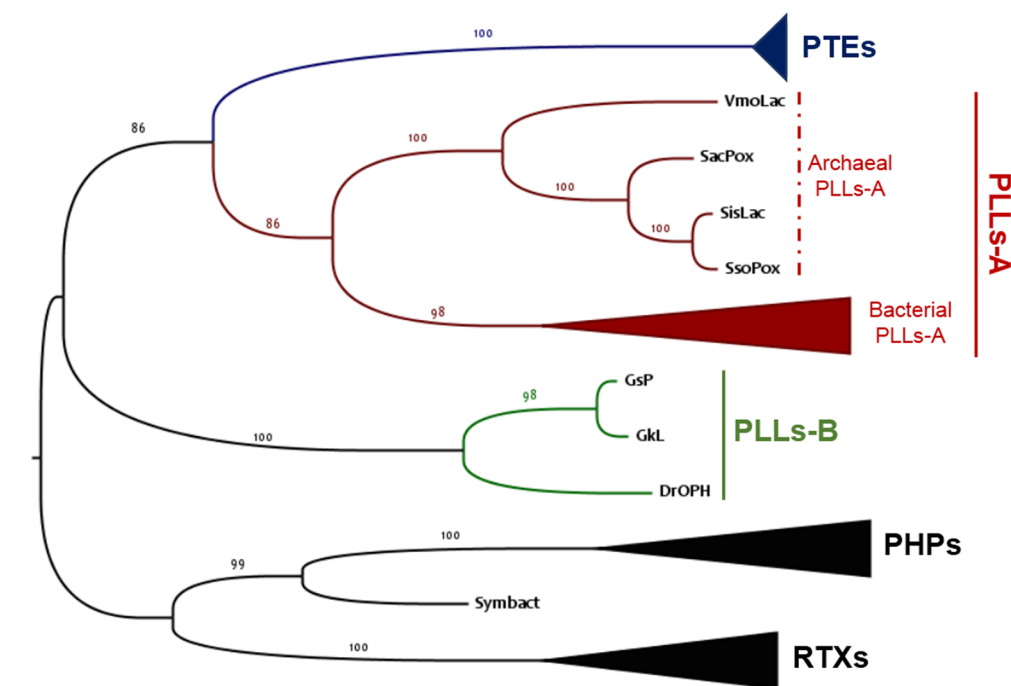
*p*NP-decanoate; Table 2, Fig. 1 & S2). This enzyme exhibits low catalytic efficiency against *p*NP-acetate ($k_{\text{cat}}/K_M = 5.48 \pm 0.84 \text{ M}^{-1}\text{s}^{-1}$) but no activity against *p*NP-decanoate. Its catalytic efficiency is ~ 40 -fold higher at (50°C)³⁴. Notably, *VmoLac* shows some activity against phenyl-acetate ($k_{\text{cat}}/K_M = 58.15 \pm 0.95 \text{ M}^{-1}\text{s}^{-1}$), whereas other PLL-As do not^{4,8}.

Lactonase activity. The catalytic parameters of *VmoLac* were evaluated for a broad range of lactones, including AHLs, oxo-lactones and dihydrocoumarin (Table 2 & Fig. 1 & S2). No activity could be detected against dihydrocoumarin and AHLs with short aliphatic substituents (4 to 6 carbon atoms). However, AHLs with longer substituents are better substrates for *VmoLac*, including C8 and C10-AHLs ($k_{\text{cat}}/K_M = 2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$). Oxo-lactones are the best substrates for *VmoLac*. Indeed, γ -caprolactone and γ -heptalactone are degraded with approximately the same catalytic efficiencies ($k_{\text{cat}}/K_M \sim 3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$), and oxo-lactones with long aliphatic chains such as nonanoic- γ -lactone and undecanoic- δ -lactone, are hydrolyzed with high catalytic efficiencies (k_{cat}/K_M up to $\sim 10^6 \text{ M}^{-1}\text{s}^{-1}$). Intriguingly, γ and δ -dodecanoic lactone substrates have an allosteric profile of hydrolysis ($K_h \sim 2 \text{ 100 } \mu\text{M}$ and $840 \mu\text{M}$, respectively). Overall, *VmoLac* exhibits moderate catalytic efficiency for AHLs and shows higher rates for oxo-lactones, with a clear preference for long aliphatic chains.

Crystal structures of *VmoLac*. The structure of *VmoLac* is a homodimer with the overall dimensions of the monomers being approximately $42 \times 47 \times 56 \text{ \AA}$, consistent with previous observations in solution³⁴. As expected, *VmoLac* is roughly globular and exhibits a (β/α)₈ barrel topology that is similar to that of others PLLs such as *SsoPox*¹⁷, *SisLac*⁸, *DrOPH*³, *GsP*¹⁰ and *GkL*³⁶ (Fig. S3). Indeed, the overall structure is very similar to the structure of *SsoPox* (root-mean-square deviation (r.m.s.d.) for α -carbon atoms (over 314 atoms) of 0.87 \AA ; Fig. 3A). The most significant differences



A.



B.

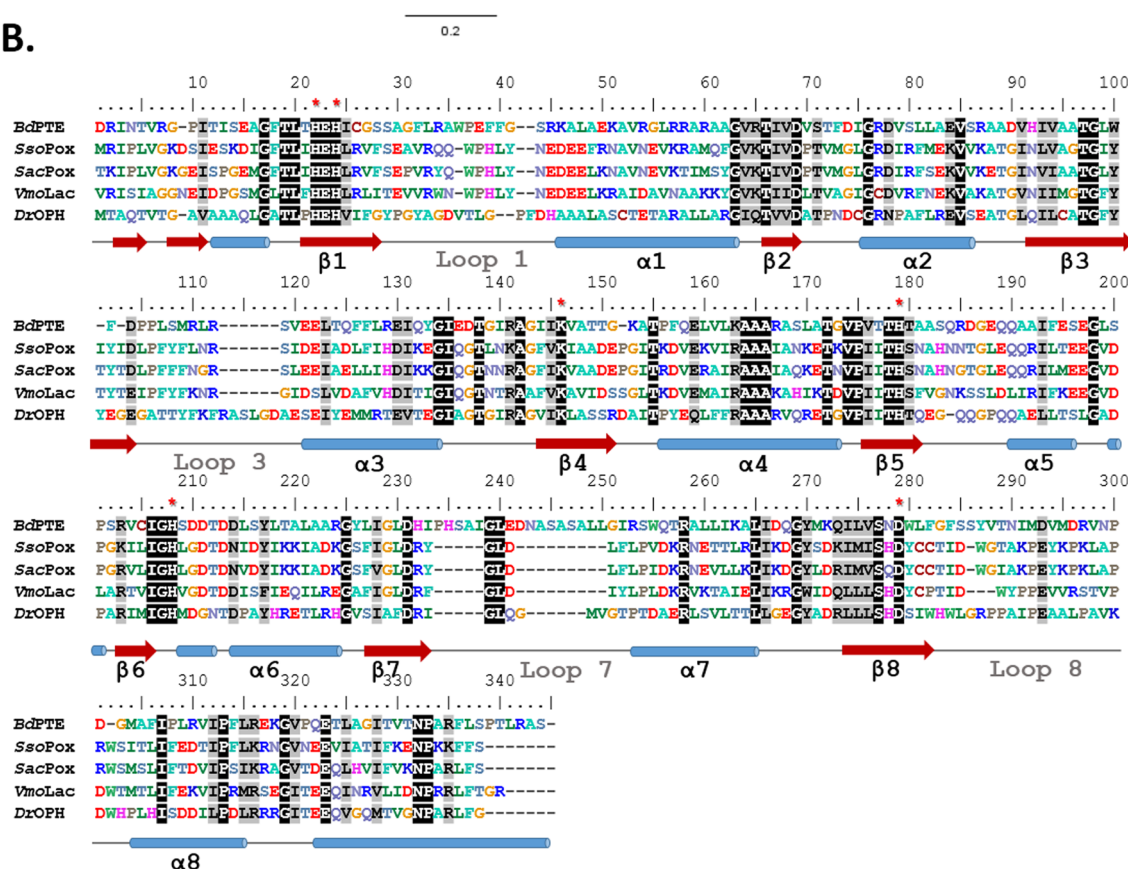


Figure 2 | Phylogenetic analysis of PLLs. (A). Phylogenetic tree of PLL representatives and close enzymes families. Members of PLL-B are in green, while those of PLL-A are in red. For clarity, the clades with members of PTEs (blue), bacterial PLLs-A, PHP and RTX are collapsed. The sequences that were used to generate this tree are listed in supplementary Table S1. (B). Sequence alignment of *BdPTE* from *B. diminuta*, *SsoPox* from *S. solfataricus*, *SacPox* from *S. acidocaldarius*, *VmoLac* from *V. moutnovskia* and *DrOPH* from *D. radiodurans*. The conserved amino acid residues are highlighted in black and similar residues in gray. The conserved active site residues that were involved in metal coordination are highlighted by red stars. The secondary structures are represented according to the *SsoPox* structure (with red arrows depicting β -sheets and blue cylinders depicting α -helices).



Table 2 | Enzymatic characterisation of VmoLac enzyme

	Substrates	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($M^{-1}s^{-1}$)
Phosphoesters	ethyl-paraoxon (I)	$(1.08 \pm 0.06) \times 10^{-3}$	581 ± 61	1.86 ± 0.22
	methyl-paraoxon (II)	ND	ND	2.32 ± 0.15
		1.25 [#]	2 790 [#]	442.58 [#]
	ethyl-parathion (III)	ND	ND	ND
	methyl-parathion (IV)	ND	ND	ND
Esters	malathion (V)	ND	ND	ND
	CMP-coumarin (VI)	0.13 ± 0.01	$2\,050 \pm 257$	63.90 ± 10
	Phenyl-acetate (VII)	ND	ND	58.15 ± 0.95
	pNP-Acetate (VIII)	$(2.45 \pm 0.19) \times 10^{-2}$	$4\,471 \pm 593$	5.48 ± 0.84
		1.66 [#]	8 190 [#]	201.74 [#]
Lactones	pNP-Decanoate (IX)	ND	ND	ND
	C8 AHL (XIV)	0.59 ± 0.04	262 ± 54	$(2.23 \pm 0.48) \times 10^3$
	3-oxo C8 AHL (XV)	0.35 ± 0.01	186 ± 37	$(1.88 \pm 0.38) \times 10^3$
	3-oxo C10 AHL (XVI)	0.47 ± 0.03	231 ± 55	$(2.05 \pm 0.50) \times 10^3$
	γ caprolactone (<i>r</i>)	112.30 ± 8	3709 ± 497	$(3.03 \pm 0.46) \times 10^4$
	γ caprolactone (<i>R</i>)	3.04 [#]	550 [#]	5.56×10^3 [#]
	γ caprolactone (<i>S</i>)	1.89 [#]	750 [#]	2.53×10^3 [#]
	γ heptalactone (XVIII)	27.25 ± 3.1	872 ± 220	$(3.12 \pm 0.86) \times 10^4$
	γ nonalactone (XIX)	44.49 ± 0.89	47 ± 11	$(9.48 \pm 2.18) \times 10^5$
	γ undecalactone (XX)	8.86 ± 0.30	120 ± 22	$(7.36 \pm 1.37) \times 10^4$
	γ dodecalactone* (XXI)	0.16 ± 0.03	2.1 ± 0.6	$(7.77 \pm 2.40) \times 10^4$
	δ nonalactone (XXII)	88.91 ± 1.56	1220 ± 59	$(7.29 \pm 0.37) \times 10^4$
	δ undecalactone (XXIII)	60.78 ± 2.36	105 ± 24	$(5.80 \pm 1.32) \times 10^5$
	δ dodecalactone* (XXIV)	28.08 ± 0.44	839 ± 13	$(3.35 \pm 0.74) \times 10^5$
		dihydrocoumarin (X)	ND	ND

Roman numerals correspond to the related chemical structure of the substrate that is presented in Fig. S1. Kinetics were measured as triplicates, and standard deviation values are given for each parameter.

*substrate γ dodecalactone and δ dodecalactone showed allosteric curves with $K_i = 2109 \pm 554 \mu M$ and $K_i = 839 \pm 13 \mu M$, respectively. The data were obtained with cobalt as cofactor. ND corresponds to not determined values because of a no or too low catalytic rate. VLH corresponds to Very Low Hydrolysis observed without the possibility of recording a value.

[#]Data from Kallnik *et al.* (2014): The lactonase activity was determined at 40 °C pH 8.3 in presence of $CoCl_2$, phosphotriesterase activity at 70 °C pH 8 in the presence of $CoCl_2$ and esterase activity at 50 °C pH 6.5 in the presence of $MnCl_2$. *r* corresponds to a racemic mixture of *R* and *S* enantiomers.

between the VmoLac structure and the SsoPox and SisLac structures reside in the active site loop 8, which is shorter for VmoLac according to the sequence alignment (Fig. 2B). Indeed, loop 8 in the VmoLac structure is well structured into an α -helix, whereas this secondary structure is broken by proline residues in SsoPox and SisLac (Fig. 3B). Remarkably, loop 8 carries the same number of proline residues (3) in all three of the counterparts, but their distribution is different: while being distributed over loop 8 in SsoPox and SisLac, the 3 proline residues are located at the start and end of loop 8 in VmoLac, enabling a structure in α -helix (Fig. 3B).

Salt bridge network analysis. VmoLac is a highly charged protein: 43 (Asp + Glu) and 38 (Lys + Arg), for a total of 315 residues, most of them located at the protein surface. About 2/3 of these residues (54) are involved in salt bridges: VmoLac contains 40 salt bridges per monomer compared to 36 salt bridges per monomer for SsoPox (using a cut-off distance of 6.5 Å). Electrostatic potential calculation suggests that one face of the protein, the active site side, is essentially negatively charged, whereas the opposite face contains positively charged clusters (Fig. S4). The protein may therefore possess a dipolar moment. One region of the enzyme is little electrostatically charged: the active site, hydrophobic channel. The salt bridges are uniformly localized on the protein surface, and most of them are involved in complex networks of interactions (Fig. S5), classical features for hyperthermophilic enzymes¹⁸.

Dimer interface analysis. The protein dimer interface involves 47 residues. This interface is mostly hydrophobic but also involves 17 hydrogen bonds and 15 salt bridges (Fig. 3C). The contacting area of 1 708 Å² is very similar to the values that have been reported for other PLLs, e.g., 1 770 Å² for SisLac, 1 750 Å² for SsoPox, 1 728 Å² for GkL, 1 632 Å² for GsP and 1 473 Å² for DrOPH^{3,8,10,17,36}.

Interestingly, VmoLac was crystallized in two different space groups. Both structures are homodimeric. The structure that was solved in P6₄ reveals a homodimer in the asymmetric unit, as previously observed for other PLLs, such as SsoPox (Fig. 3D). The other

structure, solved in P622, contains only one protein molecule in the asymmetric unit. The dimer can be reconstructed by symmetry, and the observation of the other neighboring molecules reveals another, weaker interaction within the crystal packing (Fig. 3E & 3F). The contacting surface in this interaction is much smaller (616 Å²) and involves 4 hydrogen bonds and 4 salt bridges. The interface also involves numerous contacts *via* water molecules (19). Intriguingly, this contact mode between the two protein molecules connects the two active sites hydrophobic channels (Fig. 3E; Fig. S6).

Comparison of the VmoLac structures. As expected, both of the structures are nearly identical (r.m.s.d for α -carbon atoms (over 315 atoms) of 0.34 Å), but some slight differences can be observed in the active site region (loops 7 and 8; Fig. S7). In particular, loop 8 residues Y265, V269, V270 and T273 occupy slightly altered conformations, whereas loop 7 undergoes a larger conformational displacement. The largest rearrangement involves Y230, whose conformation differs by 2.2 Å between the structures. This is consistent with a higher mobility of active site loops 7 and 8, as compared to the rest of the structure.

VmoLac is a bi-cobalt metalloenzyme. The two metal cations are coordinated by four histidines (23, 25, 171 & 200), an aspartic acid (257) and a carboxylated lysine residue (238). Both of the metal cations are bridged by a putatively catalytic water molecule. The chemical nature of the bound metal cations was investigated by X-ray anomalous scattering at the Co-K edge (1.6049 Å) and below (1.6101 Å) (Table S3). The presence of two peaks for each metal in the Bijvoet difference Fourier maps at the Co-K edge (Fig. S8) and their drop to nearly no signal under the K edge unambiguously indicate that VmoLac possesses a bi-cobalt active site.

Active site of VmoLac. A clear extra density was observed in the active sites of both of the VmoLac structures (Fig. S9B), although no potential substrates were present in the crystallization conditions. Because of a relatively good resolution of the structures (1.8 and

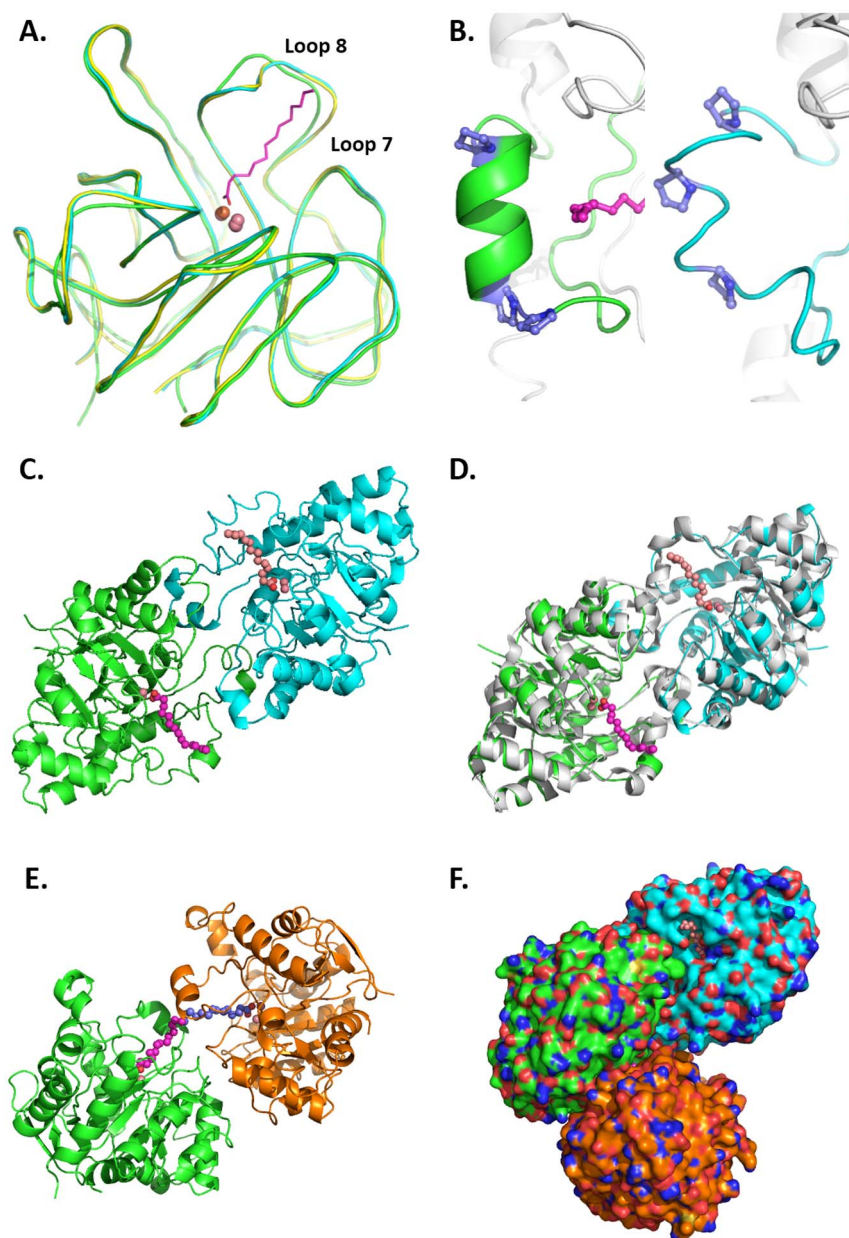


Figure 3 | *VmoLac* structure comparison with that of *SsoPox* and *SisLac*. (A). Structural superposition of *VmoLac*, *SsoPox* (blue, 2VC5) and *SisLac* (yellow, 4G2D) enzymes. Iron and cobalt are represented as orange and pink spheres, respectively. Myristic acid co-crystallized with *VmoLac* is represented as a purple stick. (B) Loop 8 structure in *VmoLac* (left panel) and *SsoPox* (right panel) context. Prolines are represented as blue sticks. The structure is in gray while loop 8 is in color, green for *VmoLac* and blue for *SsoPox*. Iron and cobalt are represented as orange and pink spheres, respectively. Myristic acid co-crystallized with *VmoLac* is represented as a purple stick. (C). Classical dimer of *VmoLac*. Each monomer is colored in green and blue with respective co-crystallized myristic acid represented in light purple and pink spheres. Cobalt cations are represented as pink spheres. (D). Superposition of *VmoLac* dimer as in C. with *SsoPox* dimer (2VC5) represented in light gray. (E). Alternative crystal packing interaction of *VmoLac*. Each monomer is colored in green and orange with respective co-crystallized myristic acid represented in light purple and dark purple spheres. The cobalt cations are represented as pink spheres. (F). Representation of the dimer, and the crystal contact of *VmoLac* with colorations as previously described.

2.35 Å), this unexpected density could be attributed to a fatty acid with good confidence. However, the precise nature of this fatty acid is uncertain: the number of carbon atoms is clear from the electronic density maps (14), but possible unsaturation is difficult to interpret from density maps. Therefore, this density was attributed to the simplest fatty acid that fit the observed density and that was present in the expression host *E. coli*: myristic acid. The carboxylate of the fatty acid molecule is tightly bound to the bi-metallic active site (2.2 \AA_α – 2.3 \AA_β), while the long aliphatic fatty chain that sits in the hydrophobic channel is mainly formed by loop 8 residues (Fig. 4A). Notably, the bound fatty acid adopts a

slightly different binding mode in both of the monomers, and movements of residues on the active site loop 7 can be observed (Ile229 and Tyr230) (Fig. S10).

Complex with 3-oxo C10 AHL. Crystals of *VmoLac* were soaked with 3-oxo-C10 AHL, and the lactone sits in the hydrophobic channel of the active site (Fig. 4B & S9A & S10). The omit density map cannot be confounded with that of the fatty acid, since the bound lactone is shorter, and the lactone ring is visible in the electronic density map. Moreover, we note that both structures, solved in the same space group ($P6_4$), exhibit significant

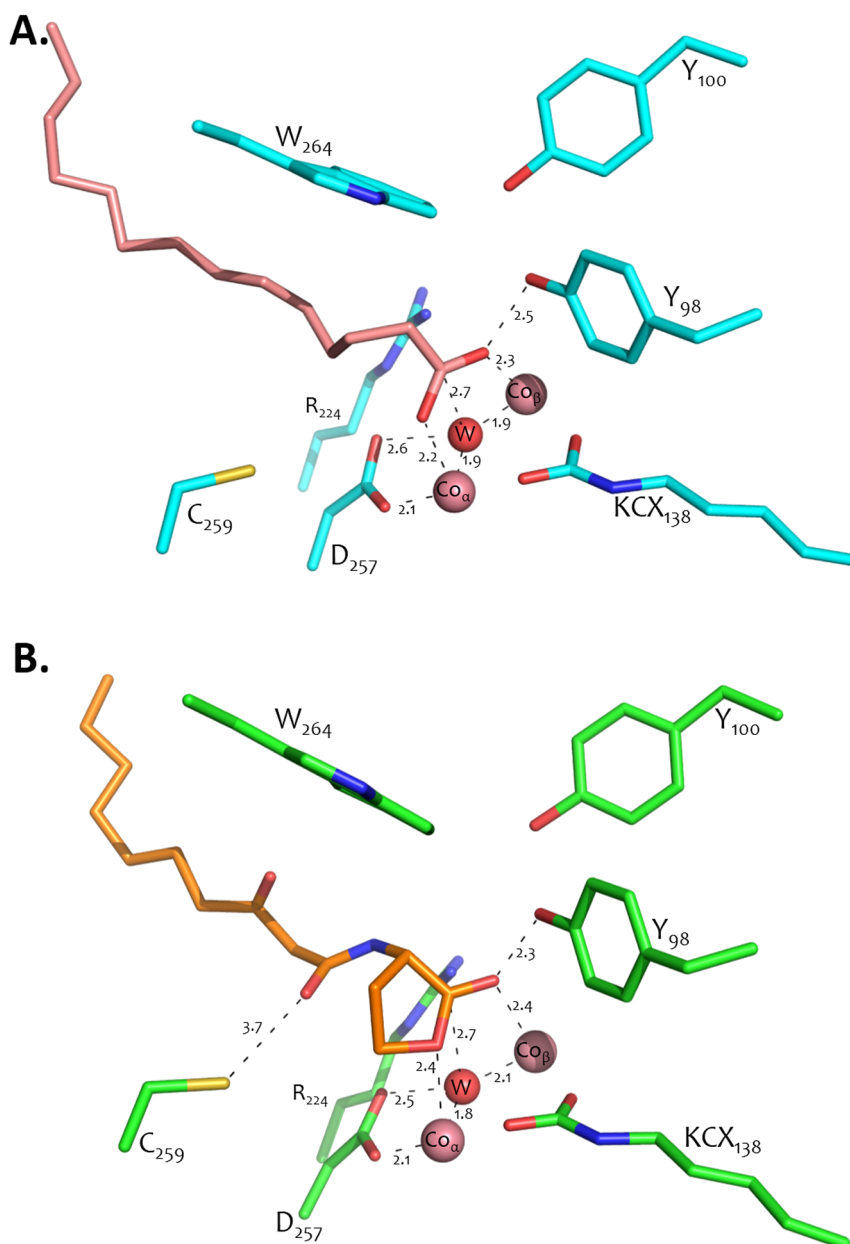


Figure 4 | *VmoLac* active site ligand binding. (A). Active site residue representation of *VmoLac* dimer structure. The residues are represented as blue sticks. The myristic acid in the active site is represented by light pink sticks. The cobalt cations and the water molecule are represented by light pink and red spheres, respectively. (B). Active site residue representation of the complex *VmoLac* structure with 3-oxo-C10 AHL. The residues are represented as green sticks. The AHL is represented as orange sticks; the cobalt cations and the water molecule are represented by light pink and red spheres, respectively.

conformational differences, in particular in loop 7, where Y230 undergoes a significant reorientation (1.7 Å) (Fig. S11). Noteworthy, the carboxylate group of the bound fatty acid nearly superimposes with the lactone group of the bound AHL.

The overall binding of the 3-oxo-C10 AHL is very similar to the previously observed binding of a lactone mimic, the inhibitor C10 homocysteine lactone in the *SsoPox* structure¹⁷ (Fig. 5A & S9A). The lactone ring sits on the bi-metallic active site, while the carbonyl oxygen is bound to the β -Co (2.4 Å), and the ester oxygen contacts the α -Co (2.4 Å). The carbonyl oxygen of the lactone ring also interacts with the Y98 O-H (2.3 Å) and the R224 guanidinium group (3.5 Å) (Fig. 4B). The 1-oxo group weakly interacts with C259 (3.7 Å), and the long aliphatic chain sits in the hydrophobic channel that is formed by loop 8. A deeper comparison highlights the fact that the lactone ring is closer to the bi-metallic center in the *VmoLac* structure compared to the thiolactone ring

in *SsoPox*. This discrepancy could be due to the larger van der Waals radius of sulfur compared to that of the oxygen atom, and/or by the lower polarity of sulfur compared to that of the oxygen atom. It could be also due to the significant differences found between both active sites: in particular, the substitution L28_{*VmoLac*}/V27_{*SsoPox*} may contribute to the observed different conformation of the bound lactone and of W264 (W263 in *SsoPox*). The latter residue is known to be critical for substrate binding and catalytic efficiency in *SsoPox*³⁷: while W263 positions and stacks the lactone ring onto the bi-metallic active site in *SsoPox*, it interacts very differently with the lactone ring in the *VmoLac* structure (Fig. 5A). Whereas several substrate binding residues are strictly conserved between both enzymes, and adopt similar conformations, such as R224_{*VmoLac*}/R223_{*SsoPox*}, W277_{*VmoLac*}/W278_{*SsoPox*}, Y100_{*VmoLac*}/Y99_{*SsoPox*}, Y98_{*VmoLac*}/Y97_{*SsoPox*}, L73_{*VmoLac*}/L72_{*SsoPox*}, L227_{*VmoLac*}/L226_{*SsoPox*}, I262_{*VmoLac*}/I261_{*SsoPox*}, other residues differ

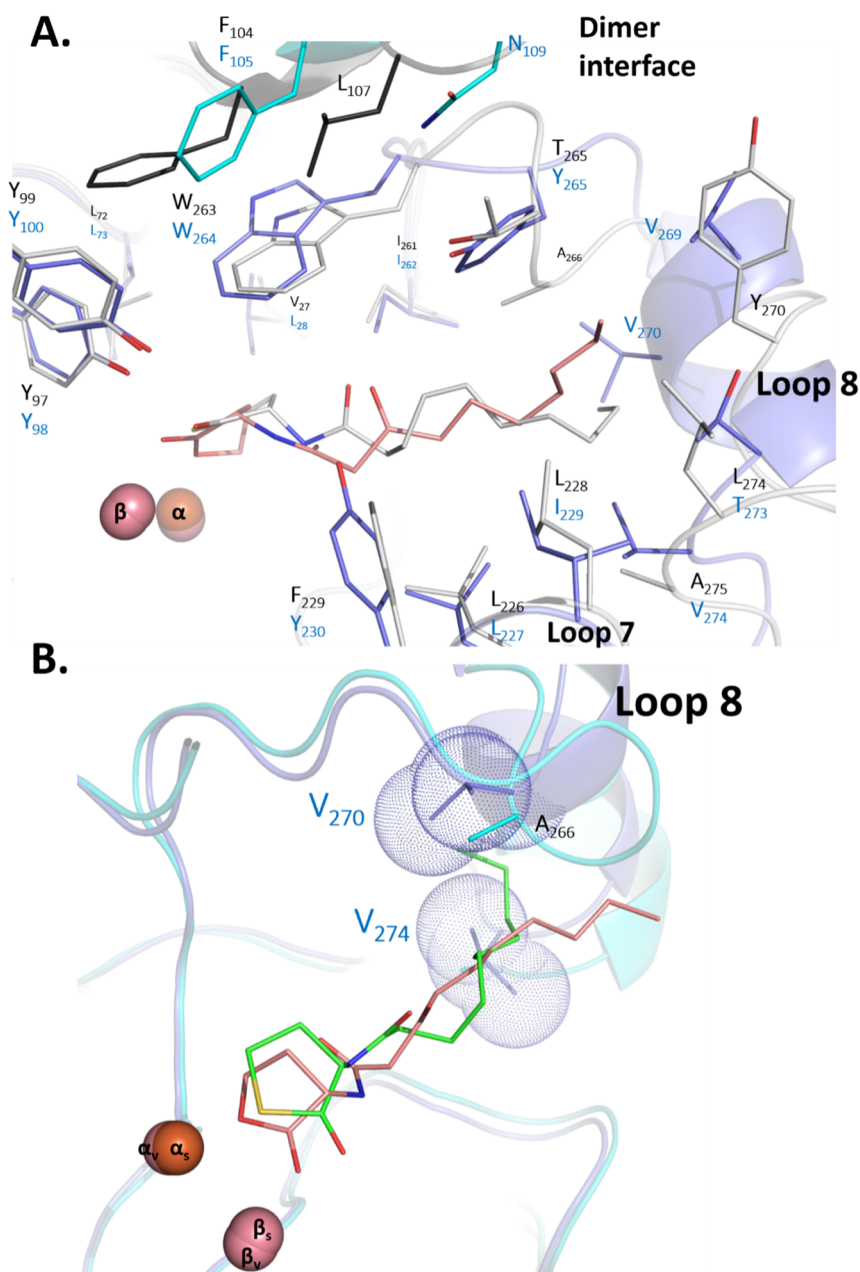


Figure 5 | Comparison between the *VmoLac* and *SsoPox* structures. (A). The *VmoLac* structure (in blue sticks and cartoon, residues labeled in blue) bound to 3-oxo-C10 AHL (pink sticks) was superposed onto the *SsoPox* structure (white sticks and cartoon, residues labeled in black; PDB ID: 2vc7) bound to C10 homocysteine thiolactone (white sticks). The second monomer of the homodimer is in cyan and black, for *VmoLac* and *SsoPox*, respectively. Active site metal cations are shown as spheres. (B). Zoom-in the binding of 3-oxo-C10 AHL (pink sticks; *VmoLac* structure) and C10 homocysteine thiolactone (green sticks; *SsoPox* structure) into the active sites of *VmoLac* (violet sticks) and *SsoPox* (cyan sticks). Active site metal cations of *VmoLac* (v) and *SsoPox* (s) are shown as spheres. The van der Waals surfaces of V270 and V274 side chains are shown as blue dots.

and contribute to the remodelling of the binding site, such as Y230_{*VmoLac*}/F229_{*SsoPox*} and I229_{*VmoLac*}/L228_{*SsoPox*}.

Moreover, the difference in the loop 8 conformation between both enzyme yield to a very different binding crevice (Fig. 5A), where very different residues are involved in the substrate binding, such as Y265_{*VmoLac*}/T265_{*SsoPox*}, V274_{*VmoLac*}/A275_{*SsoPox*}, T273_{*VmoLac*}/L274_{*SsoPox*}, and also V269_{*VmoLac*}, V270_{*VmoLac*}, A266_{*SsoPox*} and Y270_{*SsoPox*}. This different crevice results in the aliphatic chain binding outside loop 8 in *VmoLac*, whereas it binds inside loop 8 in *SsoPox*¹⁷(Fig. 5B). In particular, residues V274 and V270 sterically prevents the existence of a hydrophobic channel under loop 8 in *VmoLac*, as opposed to the channel existing in the *SsoPox* structure.

Discussion

***VmoLac* is a proficient lactonase.** The *VmoLac* enzyme is a recently discovered PLL-A member from the extremophilic archaeon *V. moutnovskia* that lives between 60 and 98°C^{33,38}. Consequently, *VmoLac* is highly stable and exhibits a maximum of activity at 80°C³⁴. We determined that *VmoLac* exhibits a T_m value of $128 \pm 7^\circ\text{C}$, a more than 20°C higher value than its hyperthermostable counterpart *SsoPox*. The crystal structures indicate that *VmoLac* shares many structural determinants with its hyperthermostable PLL counterparts¹⁸, and other thermostable enzymes³⁹, such as a very large number of surface salt bridges and a large, mainly hydrophobic homodimer interface. The structure of *VmoLac* also shows that the active site loop 8 is rigid and structured into an

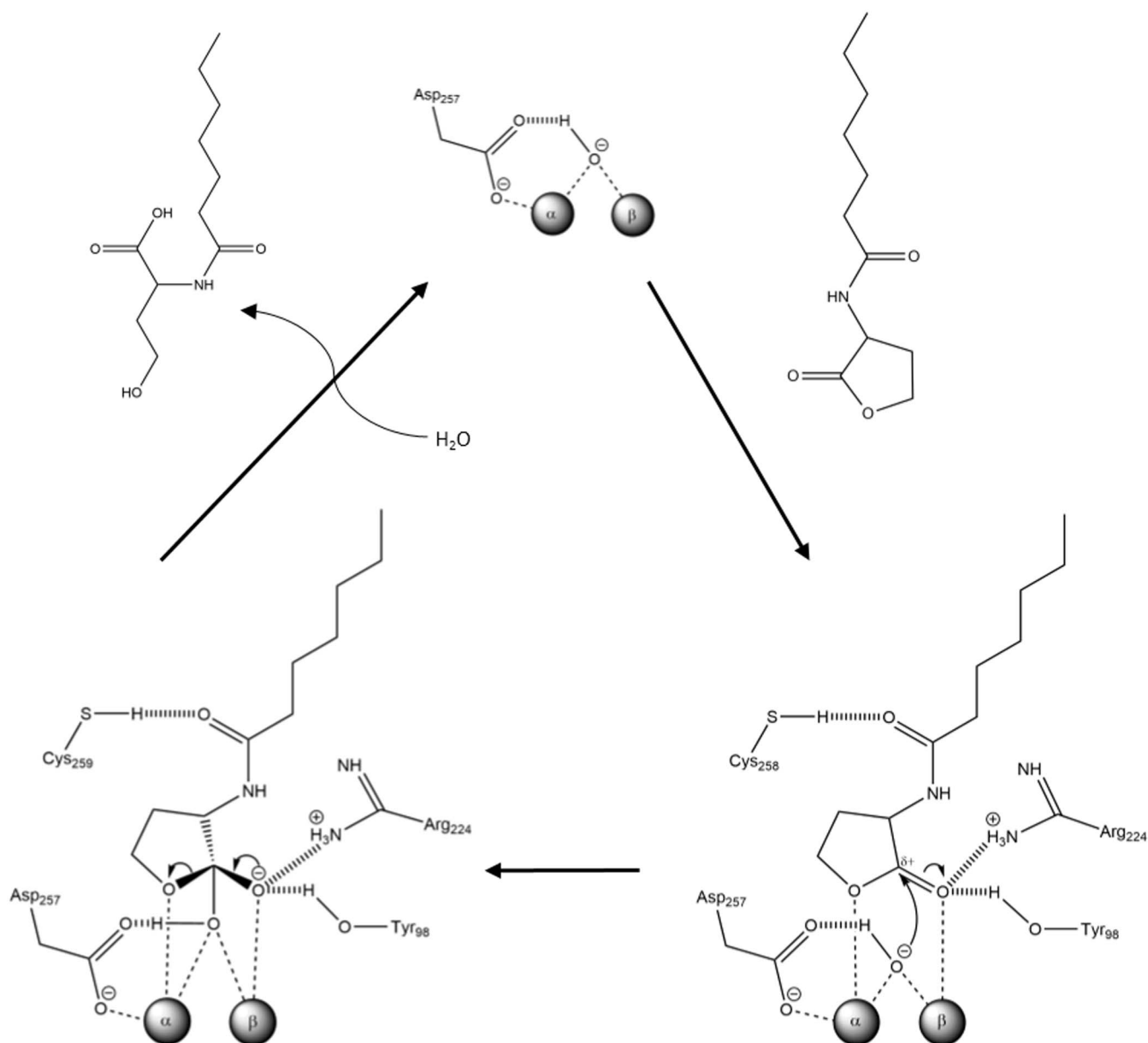


Figure 6 | Putative catalytic mechanism of *VmoLac* lactonase.

α -helix. This feature differs considerably with the closest PLLs ($\sim 50\%$ sequence identity), where loop 8 is unstructured and floppy^{8,37}, and might partly explain the superior thermal stability of *VmoLac*.

Kinetic characterization indicates that *VmoLac* is a proficient lactonase with promiscuous, poor esterase and phosphotriesterase activities. *VmoLac* clearly prefers long chain substrate, a consistent fact with the finding of a bound fatty acid (modeled as myristic acid) in the native structure. The biological role of *VmoLac* is uncertain: quorum quenching lactonases are found in Bacteria, Archaea and Eukarya. In many cases, these enzymes are found with no other AHL components^{1,6}, as it seems to be for *VmoLac*. Therefore, the role of these enzymes in such organisms might be to interfere with quorum sensing of other organisms or to utilize AHLs as carbon and nitrogen source.

Similar to other crenarchaeal PLLs^{4,8,40}, *VmoLac* exhibits a clear preference for oxonophosphotriesters compared to thiono-phosphotriesters. Notably, this preference dubbed thiono-effect⁴ is present in *VmoLac* despite the different nature of its bi-metallic active site: *VmoLac* has a bi-cobalt active site, whereas *SsoPox* possesses an iron/cobalt bi-metallic center¹⁷. This observation suggests that the

thiono-effect might be independent of the chemical nature of the bi-metallic center. We note that the biologically relevant metal content of *VmoLac* might be different, since cobalt cations were used during the enzyme purification.

VmoLac also possesses unique substrate specificity within PLLs: (i) a very low hydrolysis rates towards OPs, (ii) the ability to hydrolyze phenyl-acetate, (iii) the inability to hydrolyze dihydrocoumarin and short chain lactones and (iv) an allosteric behavior with some long chain oxo-lactones. The allosteric behaviour of *VmoLac* with some substrates might originate from one or a combination of the following features: the spatial proximity between active sites in the homodimer, a hypothetical alternate contact mode between monomers as seen in the crystal, or from the structural specificities of the *VmoLac* active site discussed below.

The structure of *VmoLac* indicates that the enzyme binding pocket is different from its counterparts and may account for the observed kinetics differences. A significant difference relates to L28_{*VmoLac*}/V27_{*SsoPox*} and W264_{*VmoLac*}/W263_{*SsoPox*}, the latter being a key residue in *SsoPox* that governs the active site specificity and flexibility³⁷. This residue interacts differently with the bound lactone: whereas in



SsoPox, the indole ring of W263 makes extensive van der Waals contacts with the bound lactone ring, W264 in VmoLac interacts only weakly with the bound lactone. Given the critical role of this residue in both enzymes, being a key active site and dimer interface residue, this difference may significantly contribute to the observed differences in activity between VmoLac and its homologues.

Another major difference concerns the active site loop 8, which is involved in the substrate binding. Whereas in SsoPox and SisLac^{8,17}, loop 8 is unstructured, possibly due to 3 well-distributed proline residues along this loop, loop 8 of VmoLac forms an α -helix. This difference has major consequences on the VmoLac binding crevice: the residues V270 and V274 sterically prevents the existence of a channel under loop 8. As a result, the aliphatic acyl chain of the bound substrate interacts with the outer surface of loop 8, whereas the chain is fitting in the hydrophobic channel formed under loop 8 in SsoPox¹⁷. This major difference in substrate binding yields different interactions between acyl chains and the enzymes: in particular, residues Y265_{VmoLac}, V269_{VmoLac}, T273_{VmoLac}, I229_{VmoLac} are involved.

Lactone hydrolysis mechanism. The structure of VmoLac bound to its substrate 3-oxo-C10 AHL enabled us to identify the specific interactions of the enzyme with the bound lactone. The obtained complex is similar to that of SsoPox bound to C10-HTL (Homoserine Thio-Lactone)¹⁷. We surmise here that the hydrolysis mechanism of lactones by VmoLac is therefore close to those that have been previously proposed for SsoPox¹⁷. Indeed, the bonding of the lactone ring onto the bi-metallic center may make the lactone sp² carbon more electrophilic and free the activated bridging water molecule. The latter may subsequently attack the lactone sp² carbon, via a tetrahedral, negatively charged, intermediate that is stabilized by the β -metal. The electron pair on the oxyanion folds back, allowing the breakage of the ester bond and the formation of a carboxylic acid, and an alcoholate (Fig. 6). This alcoholate may require acidic assistance. In the homologue GkL^{36,41}, and in the other lactonase AiiA^{42,43}, an aspartate residue (corresponding to Asp257 in VmoLac) has previously been proposed to protonate the leaving alcoholate, which remains to be clarified in VmoLac.

The potential of VmoLac as quorum-quenching agent or as organophosphorous compound biodecontaminant. VmoLac is an extremely thermostable enzyme, and is likely the most thermostable PLL that has been characterized thus far. Thermostability is a key feature in the use of enzymes in industrially compatible process for incorporation in chemical synthesis or other usages because thermostability is linked to easier storage, manipulation, solvent resistance and thermal resistance³⁹. Due to its dual catalytic activities, i.e., lactonase and phosphotriesterase, VmoLac could represent an interesting target for *in vitro* evolution with the aims of developing a quorum quenching agent and an efficient organophosphorous biodecontaminant.

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Acknowledgments

We are grateful to Prof. Dan S. Tawfik and Dr. Moshe Goldsmith for the kind gift of CMP-coumarin. This work was granted by DGA, France (REI. 2009 34 0045). J.B. is a PhD student granted by DGA. The BMBB/BTI startup fund is greatly acknowledged.

Author contributions

J.H., E.C. and M.E. designed experiments. J.H., J.B. and C.C. performed the experiments. J.H., E.C. and M.E. analysed the results. J.H., E.C. and M.E. wrote the paper. All of the authors offered a critical review of the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Hiblot, J., Bzdrenga, J., Champion, C., Chabriere, E. & Elias, M. Crystal structure of VmoLac, a tentative quorum quenching lactonase from the extremophilic crenarchaeon *Vulcanisaeta moutnovskia*. *Sci. Rep.* **5**, 8372; DOI:10.1038/srep08372 (2015).



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