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Janek Bzdrenga, Julien Hiblot, Guillaume Gotthard, Charlotte Champion, Mikael Elias, et al.. SacPox from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* is a proficient lactonase. BMC Research Notes, 2014, 7 (1), 10.1186/1756-0500-7-333 . hal-01771510

HAL Id: hal-01771510

<https://amu.hal.science/hal-01771510>

Submitted on 19 Apr 2018

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RESEARCH ARTICLE

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SacPox from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* is a proficient lactonase

Janek Bzdrenga^{1†}, Julien Hiblot^{1†}, Guillaume Gotthard¹, Charlotte Champion¹, Mikael Elias^{2*} and Eric Chabriere^{1*}

Abstract

Background: *SacPox*, an enzyme from the extremophilic crenarchaeal *Sulfolobus acidocaldarius* (*Sac*), was isolated by virtue of its phosphotriesterase (or paraoxonase; Pox) activity, *i.e.* its ability to hydrolyze the neurotoxic organophosphorus insecticides. Later on, *SacPox* was shown to belong to the Phosphotriesterase-Like Lactonase family that comprises natural lactonases, possibly involved in *quorum* sensing, and endowed with promiscuous, phosphotriesterase activity.

Results: Here, we present a comprehensive and broad enzymatic characterization of the natural lactonase and promiscuous organophosphorus hydrolase activities of *SacPox*, as well as a structural analysis using a model.

Conclusion: Kinetic experiments show that *SacPox* is a proficient lactonase, including at room temperature. Moreover, we discuss the observed differences in substrate specificity between *SacPox* and its closest homologues *SsoPox* and *SisLac* together with the possible structural causes for these observations.

Keywords: Lactonase, PLL, *Quorum* sensing, Phosphotriesterase, Extremophile, Thermoacidophile

Background

Phosphotriesterase-Like Lactonases (PLLs) are natural lactonases (EC 3.1.1.25) (Figure 1C, D, E) with promiscuous phosphotriesterase activity (EC 3.1.8.1) (Figure 1A) [1,2]. They are structurally closely related to bacterial phosphotriesterases (PTEs) [3-6], such as *Brevundimonas diminuta* PTE (*BdPTE*; ~30% sequence identity) [7]. PTEs naturally hydrolyze neurotoxic organophosphorus (OPs) compounds (Figure 1A) such as paraoxon (the active metabolite of the insecticide parathion) with catalytic constants that approach the diffusion limit (*i.e.* $k_{cat}/K_M \sim 10^8 \text{ M}^{-1} \text{ s}^{-1}$) [7]. Because OPs have been massively used as pesticides since the 50's [8], PTEs are believed to have emerged in few decades from a PLL progenitor [2], providing a new source of phosphorus to bacteria, and consequently a selective advantage [8].

Both enzyme families exhibit the same (β/α)₈-barrel topology [9,10] and belong to the amidohydrolase superfamily [11,12]. Their structure consists of 8 β -strands forming a central barrel surrounded by 8 α -helices. The

active site is constituted by a bimetallic center (two metal cations) localized at the C-terminus of the barrel. Metal cations are coordinated by four histidines, an aspartic acid and a carboxylated lysine residue [9]. While the nature of the bimetallic center can vary depending on the enzyme nature and the purification procedure [3,5,13,14], the catalytic mechanism is presumed to be identical. The bimetallic center activates a water molecule into a hydroxide ion which performs a nucleophilic attack onto the electrophilic center [9,15].

The difference in substrate specificities of PLLs and PTEs seems mainly governed by variation in the connecting loops of the barrel [2,16]. Major differences between PTEs and PLLs reside in the active site loop size and conformation [1,2]. Indeed, loop 7 is shorter in PLLs than in PTEs whereas the loop 8 is larger, forming a hydrophobic channel that accommodates lactones aliphatic chain [9]. Loop 7/8 length and sequence also differ within the PLL family and led to the identification of two different subfamilies: PLLs-A and PLLs-B [2]. Both subfamilies exhibit different substrate specificities: PLLs-B are exclusively oxo-lactonases (Figure 1DE) whereas PLLs-A hydrolyze efficiently oxo-lactones and Acyl-Homoserine Lactones (AHLs, Figure 1C) [2]. AHLs are messenger molecules involved in a bacterial communication system

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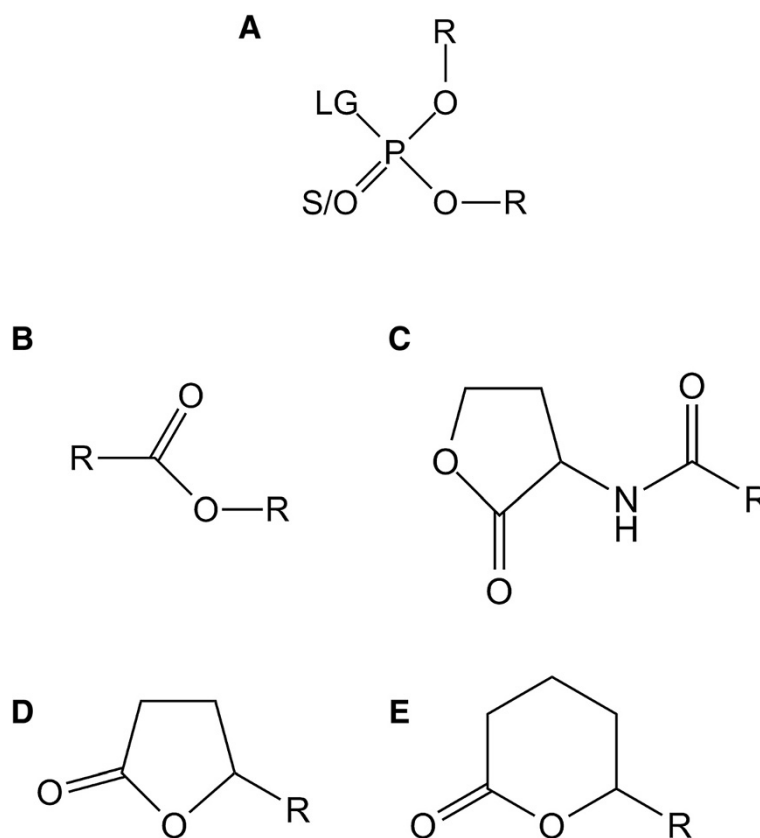


Figure 1 Chemical structure of *SacPox* substrates. Chemical structures of (A) phosphotriesters, (B) esters, (C) Acyl-Homoserine Lactones, (D) γ -lactones and (E) δ -lactones are presented. For phosphotriesters, R corresponds to different nature of substituents; LG corresponds to the leaving group. The 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 different nature of substituent. For AHLs and γ/δ -lactones, R corresponds to different size of acyl chain.

dubbed *quorum* sensing (QS) [17]. QS regulates the expression of numerous genes, and enables bacterial population to adopt a “group” behavior, including the expression of virulence factors of some pathogens [18,19]. The involvement of PLLs-A in *quorum* sensing has not yet been demonstrated, and these enzymes are often found with no other AHL components, including in archaeal species [20]. However, the fact that they hydrolyze specifically the natural enantiomer of AHL indicates that it may be their native substrate [16].

PLLs are promiscuous enzymes that catalyze two chemical reactions of potential biotechnological interest. Indeed, the inhibition or “quenching” of the QS is seen as a possibly promising strategy to develop innovative therapies [21-25]. Indeed, lactonases such as PLLs can inhibit QS (known as *quorum* quenching, *i.e.* QQ) [26,27] and thereby annihilate the virulence of micro-organisms possessing an AHL-based QS system [28]. Moreover, PLLs are endowed with relatively low phosphotriesterase activity, but might be optimized against OPs and subsequently used for degrading organophosphorus pesticides [3,5,6,9,29] and nerve

agents [30], for which no satisfactory remediation methods are currently available [31].

In addition, several PLLs members are thermostable [3,4,6,32-34]; *e.g.* PLLs from extremophilic crenarchaeon sources [3,4,16,34]. These counterparts exhibit industry-compatible properties (*e.g.* thermal and detergent resistance) [35-37]; making them good starting point for *in vitro* improvement protocols [37,38]. Several studies report the engineering of thermostable PLLs and improvement of catalytic efficiency against OPs, including for *SsoPox* [16,39], *DrOPH* (*Deinococcus radiodurans* organophosphorus hydrolase) [6,40] and *GkL* (*Geobacillus kaustophilus* lactonase) [41] but also for the lactonase activity of *SsoPox* [16], *MCP* (*Mycobacterium avium* subsp. *Paratuberculosis* K-10 lactonase) [42] and *GkL* [43].

Here we focus on *SacPox*, the PLL from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* (living conditions: 55–85°C, pH 2–3) [44]. *SacPox* was originally isolated and studied for its ability to hydrolyze OP compounds at high temperature [4]. The enzyme shares about 30% of sequence identity with *BdPTE* and

about 70% with its closest homologues, *i.e.* *SsoPox* from *Sulfolobus solfataricus* [3] and *SisLac* from *Sulfolobus islandicus* [33,45]. Being an enzyme from a hyperthermophile, *SacPox* is however less stable than *SsoPox* (half-life of 5 min at 90°C [4] and of 4 h at 95°C [3,46], respectively). The kinetic characterizations performed on *SacPox* revealed that it hydrolyzes OP, ester and lactone molecules at high temperature [4,13]. However, only few substrates have been tested, and no natural lactones were assayed as substrate. In this study, we performed a broad kinetic characterization of *SacPox* at room temperature (25°C) for several OPs, esters (Figure 1B) and lactone molecules including AHLs, γ -lactones and δ -lactones in the aim to evaluate the biotechnological potentialities of this enzyme.

Methods

Sequence alignment

The sequence alignment was performed based on the previously published PLL sequence alignment [2], using the *T-coffee* server (expresso) [47,48] and manually improved with the *seaview* software [49]. It contains 29 different sequences (Additional file 1: Table S1). The sequence alignment was represented using the *BioEdit* 7.1.3 software [50]. Protein sequence identities were computed using *ClustalW* server [51]. The phylogenetic tree was performed using *PhyML* [49] and default parameters.

Protein production and purification

The protein production and subsequent purification steps were performed analogously to previously described [16,33,34,45,52-54]. In brief, the protein was heterologously produced in *Escherichia coli* strain BL21 (DE₃)-pGro7/GroEL (TaKaRa) at 37°C in ZYP medium [55]. When OD_{600nm} reaches 0.8, protein production was induced with addition of arabinose (0.2%, w/v) and CoCl₂ (2 mM) and temperature transition to 25°C for 20 hours. Cells were harvested by centrifugation, and pelleted cells were suspended in *lysis buffer* (50 mM HEPES pH 8, 150 mM NaCl, 0.2 mM CoCl₂, lysozyme 25 mg/ml, PMSF 0.1 mM, DNase I 10 mg/ml), stored at -80°C during 2 hours; then sonicated 3 times during 30 seconds (Branson Sonifier 450, 80% intensity and microtype limit of 8) and centrifuged. Taking advantage of the high stability of *SacPox*, the supernatant was heated at 70°C during 30 minutes and centrifuged before proceeding a STREP-TRAP affinity chromatography step (GE Healthcare, Uppsala, Sweden). The sample was then cleaved by the Tobacco Etch Virus protease (TEV, ratio 1:20, w/w [56]) during 20 hours at 30°C prior to be loaded a second time on STREP-TRAP affinity chromatography. The flow through containing the cleaved protein was then concentrated and loaded on a size exclusion column (S75-16-60; GE Healthcare, Uppsala, Sweden). The protein

purity and identity were checked by SDS-PAGE and mass spectrometry analysis (MS platform Timone, Marseille, France). The protein concentration was determined using a nanospectrophotometer (Nanodrop, Thermofisher Scientific, France) using its molar extinction coefficient (*SacPox* $\epsilon_{280\text{ nm}} = 35\,307.7\text{ M}^{-1}\text{ cm}^{-1}$) calculated by the *PROT-PARAM* server [57].

Kinetic characterization

General procedures

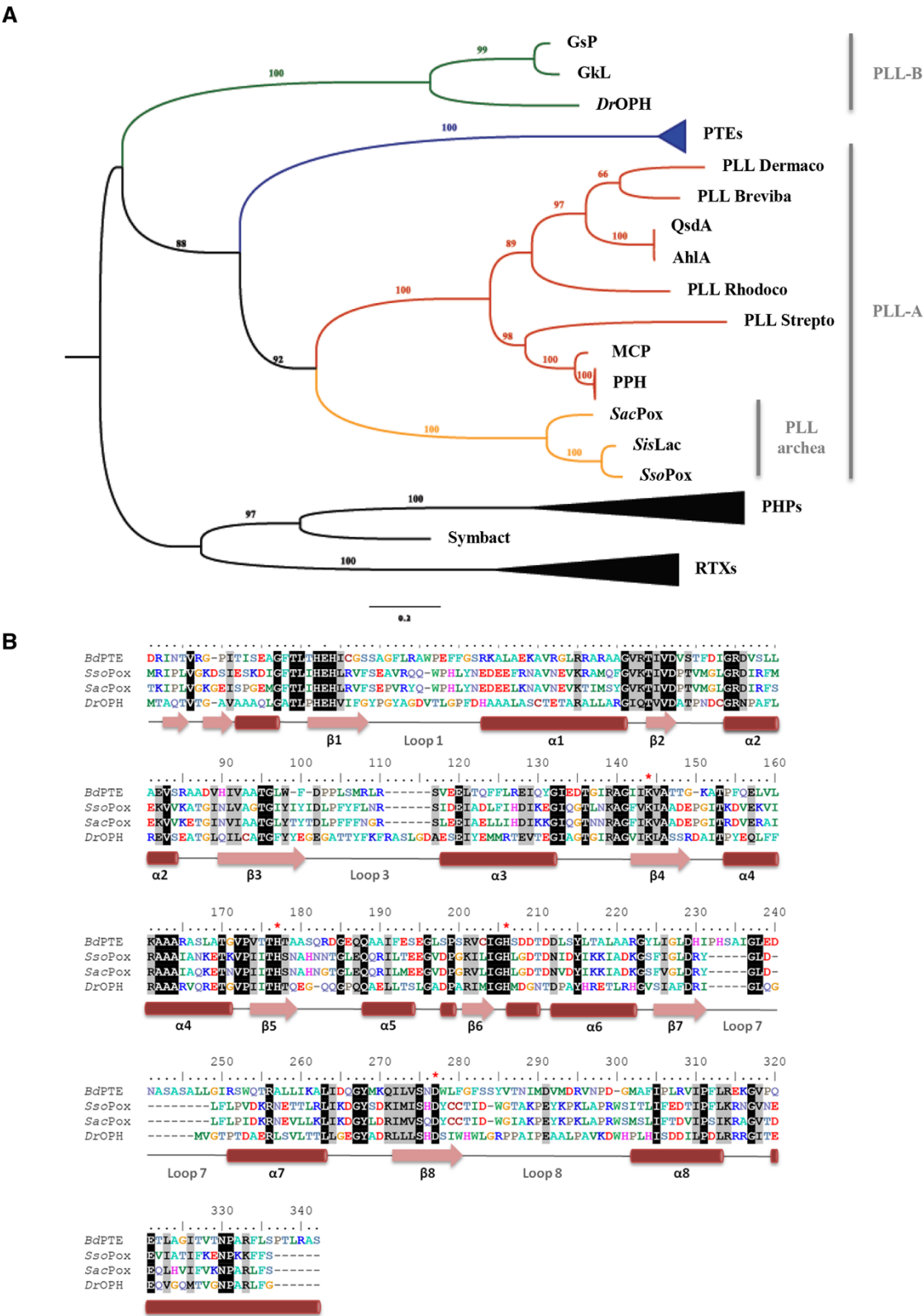
Catalytic parameters were evaluated at 25°C and recorded with a microplate reader (Synergy HT, BioTek, USA) and the Gen5.1 software as previously explained [16,33,52,54]. The reaction was performed in a 200 μL volume using a 96-well plate with a 6.2 mm path length as previously described [33]. The collected data were subsequently fitted to the Michaelis-Menten (MM) equation [58] using *Graph-Pad Prism 5.00* (GraphPad Software, San Diego California USA, www.graphpad.com). In cases where V_{max} could not be reached, the catalytic efficiency was obtained by fitting the linear part of MM plot to a linear regression using *Graph-Pad Prism 5.00* software.

OP hydrolase and esterase kinetics

Standard assays for organophosphates (Figure 1A) and esters (Figure 1B) were performed in *activity buffer* (50 mM HEPES pH 8, 150 mM NaCl, 0.2 mM CoCl₂) by measuring the *p*-nitrophenolate release over time at 405 nm ($\epsilon_{405\text{ nm}} = 17\,000\text{ M}^{-1}\text{ cm}^{-1}$). For ethyl-paraoxon (Additional file 1: Figure S1I), the *activity buffer* has also been supplemented with SDS (w/v) at 0.01% or 0.1% for detergent essays. Malathion (Additional file 1: Figure S1V) hydrolysis was followed at 412 nm in *activity buffer* added of 2 mM DTNB to follow the release of free thiols ($\epsilon_{412\text{ nm}} = 13\,700\text{ M}^{-1}\text{ cm}^{-1}$). The time course hydrolysis of dihydrocoumarin (Additional file 1: Figure S1X), CMP-coumarin (Additional file 1: Figure S1VI) and phenylacetate (Additional file 1: Figure S1VII) were respectively monitored at 270 nm ($\epsilon_{270\text{ nm}} = 1\,400\text{ M}^{-1}\text{ cm}^{-1}$), 412 nm ($\epsilon_{412\text{ nm}} = 37\,000\text{ M}^{-1}\text{ cm}^{-1}$) and 270 nm ($\epsilon_{270\text{ nm}} = 1\,400\text{ M}^{-1}\text{ cm}^{-1}$).

Lactonase kinetics

Kinetics monitoring the lactone hydrolysis were performed according to a previously described protocol [33]. The lactone hydrolysis was monitored in the *lactonase buffer* (2.5 mM Bicine pH 8.3, 150 mM NaCl, 0.2 mM CoCl₂, 0.25 mM Cresol purple and 0.5% DMSO) with different AHLs (Figure 1C) [*i.e.* C4-AHL (*r*), C6-AHL (*r*), C8-AHL (*r*), 3-oxo-C8-AHL (*l*), 3-oxo-C10-AHL (*l*)] (Additional file 1: Figure S1XI-XVI) and oxo-lactones (Figure 1D,E) [*i.e.* ϵ -caprolactone, γ -heptanolide (*r*), Nonanoic- γ -lactone (*r*), Nonanoic- δ -lactone (*r*), Undecanoic- γ -lactone (*r*), Undecanoic- δ -



(See figure on previous page.)

Figure 2 Phylogenetic analysis of the PLL family. **A.** Phylogenetic tree of PLLs, PTEs, and close homologues. Members of PLL-B are colored in green while within the PLL-As, mesophilic and archaeal PLLs are respectively colored in red and orange. The clades of PHPs, PTEs and RTXs were collapsed for clarity. All the sequences used for this tree are listed in Additional file 1: Table S1. **B.** Sequence alignment of *BdPTE* from *B. diminuta*, *SsoPox* from *S. solfataricus*, *SacPox* from *S. acidocaldarius* and *DrOPH* from *D. radiodurans*. Conserved amino acid residues are highlighted in black and similar residues in grey. Conserved active site residues involved in metals coordination are highlighted by red stars. Secondary structures are represented according to *SsoPox* structure (with pink arrows depicting β -sheets and red cylinders depicting α -helices).

lactone (*r*), Dodecanoic- γ -lactone (*r*) and Dodecanoic- δ -lactone (*r*) (Additional file 1: Figure S1XVII-XXIV). Cresol purple (pK_a 8.3 at 25°C) is a pH indicator (577 nm) used to monitor the acidification of the medium following lactone ring hydrolysis ($\epsilon_{577nm} = 5\ 500\ M^{-1}\ cm^{-1}$).

Structural modeling and structural analysis

The *SacPox* structure was modelled using the *ESyPred3D* server using *SacPox* protein sequence as query and *SsoPox* structure (2VC5) as template [59]. Structures were analyzed and figure made using PyMol [60].

Results

First classified within the bacterial PTEs, *SacPox* shares in fact only 33.8% sequence identity with *BdPTE* (Additional file 1: Table S2). *SacPox* indeed belongs to the PLLs-A (Figure 2A) [2]: it shares 76.1% of sequence identity with its closest homologues *SsoPox* and *SisLac*, and only 30.6% identity with the PLL-B *DrOPH*. Together with *SisLac* and *SsoPox*, *SacPox* comprises the creanarcheal clade of the PLLs-A (Figure 2A). The sequence alignment highlights the strict conservation of essential active site residues between the different clades (Figure 2B).

Enzymatic characterization

Phosphotriesterase activity

SacPox ability to hydrolyze insecticides ethyl/methyl-paraoxon, ethyl/methyl-parathion and malathion has been evaluated (Table 1). The best *SacPox* phosphotriester substrate, methyl-paraoxon is processed with moderate catalytic efficiency ($k_{cat}/K_M = 1.10(\pm 0.17) \times 10^3\ M^{-1}.s^{-1}$), low

rate ($k_{cat} = 0.307\ s^{-1}$) and low K_M (278.3 μM). Very similar catalytic efficiencies were recorded for *SsoPox* and *SisLac*: k_{cat}/K_M of $1.27 \times 10^3\ M^{-1}.s^{-1}$ and $4.26 \times 10^3\ M^{-1}.s^{-1}$, respectively [33,52]. Ethyl-paraoxon comprise a slower substrate, ($k_{cat}/K_M = 2.81 \times 10^2\ M^{-1}.s^{-1}$), highlighting the enzyme preference for OP substrates with small substituents. No hydrolysis could be measured for ethyl-parathion and malathion, whereas a low catalytic efficiency was recorded for methyl-parathion ($k_{cat}/K_M = 4.31\ M^{-1}.s^{-1}$). This specificity profile illustrates the clear preference of *SacPox* for oxono-phosphotriesters rather than thiono-phosphotriesters; as previously observed for *SsoPox* [52] and *SisLac* [33]. Moreover, whereas anionic detergents like SDS can significantly stimulate *SsoPox* phosphotriesterase activity [52], the same treatment on *SacPox* yields only a 2-fold increase in catalytic efficiency with ethyl-paraoxon as substrate. Finally, we show that *SacPox* hydrolyzes CMP-coumarin ($k_{cat}/K_M = 4.38 \times 10^2\ M^{-1}.s^{-1}$), albeit with 20-fold lower catalytic efficiency than *SsoPox* [52].

Esterase activity

The ability of *SacPox* to hydrolyze phenyl-acetate, *pNP*-acetate and *pNP*-decanoate (Additional file 1: Figure S1VII-IX) has been evaluated (Table 2). While no activity could be detected against *pNP*-decanoate, *SacPox* exhibits low catalytic efficiencies against both phenyl-acetate and *pNP*-acetate ($k_{cat}/K_M \approx 50\ M^{-1}.s^{-1}$). This weak activity against classical esters differs from previous studies on the close homologues *SsoPox* and *SisLac*, for which activity has only been recorded on *pNP*-acetate [33].

Lactonase activity

The catalytic parameters of *SacPox* for various lactone substrates have been measured, including against oxo-lactones (lipophilic aroma), AHLs and dihydrocoumarin (Table 3). Our results indicate a preference of *SacPox* for oxo-lactone substrates; *i.e.* γ -heptanolide and nonanoic- γ -lactone ($k_{cat}/K_M \approx 2.5 \times 10^4\ M^{-1}.s^{-1}$), while AHLs are about 10 times worse substrates (*i.e.*; C8 AHLs, $k_{cat}/K_M \approx 5 \times 10^3\ M^{-1}.s^{-1}$). Furthermore, it seems that *SacPox* prefers AHLs vs 3-oxo-AHLs since the K_M for C8 aliphatic chains is 5-fold lower than that for 3-oxo-C8 AHLs. Overall, long aliphatic chain substrates AHLs are better substrates for the enzyme. Indeed, short aliphatic chain AHLs are not hydrolyzed by *SacPox*. Interestingly, this preference is not retained for oxo-lactones, for which molecules with short

Table 1 Phosphotriesterase kinetic parameters

	$k_{cat}\ (s^{-1})$	$K_M\ (\mu M)$	$k_{cat}/K_M\ (M^{-1}.s^{-1})$
Paraoxon	0.12 ± 0.01	434 ± 54	$2.81\ (\pm 0.38) \times 10^2$
Paraoxon 0.01% SDS	0.28 ± 0.01	537 ± 48	$5.22\ (\pm 0.51) \times 10^2$
Paraoxon 0.1% SDS	0.25 ± 0.01	405 ± 21	$6.10\ (\pm 0.34) \times 10^2$
Methyl Paraoxon	0.31 ± 0.02	278 ± 40	$1.10\ (\pm 0.17) \times 10^3$
Parathion	ND	ND	ND
Methyl Parathion	ND	ND	4.31 ± 0.20
Malathion	ND	ND	ND
CMP-Coumarin	0.28 ± 0.02	642 ± 89	$4.38\ (\pm 0.68) \times 10^2$

ND correspond to Not Detected hydrolysis. Results have been obtained with cobalt as cofactor.

Table 2 Esterase kinetic parameters

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\text{M}^{-1}\cdot\text{s}^{-1}$)
Phenyl-acetate	0.35 ± 0.05	$8\ 181 \pm 1750$	42.3 ± 11.1
pNP-acetate	0.13 ± 0.01	$2\ 107 \pm 313$	60.1 ± 9.9
pNP-decanoate	ND	ND	ND

ND correspond to Not Detected hydrolysis. Results have been obtained with cobalt as cofactor.

or without aliphatic chain are efficiently hydrolyzed ($k_{\text{cat}}/K_M \approx 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$). As previously observed for *SsoPox* and *SisLac* [16,33], this feature may reveal a potential alternative binding mode of these compounds in *SacPox* active site. Finally, contrary to *SsoPox* and *SisLac* [16,33], *SacPox* does not hydrolyze dihydrocoumarin.

Structural analysis

Numerous attempts to crystallize *SacPox* were made, with no success (Elias, Hiblot, Gotthard & Chabriere, unpublished). A previous structural model was generated by homology modeling based on *BdPTE* structure [4] (~33.8% sequence identity with *SacPox*), but yielded little insights given the moderate sequence identity with the template and the very significant differences in the active site loops between these two representatives of distinct enzyme families [1,9,16]. Here we generated a homology-based model using the structure of *SsoPox* as template (76.1% of sequence identity; Additional file 1: Table S2).

As expected, the *SacPox* model structure almost perfectly superimposes to the *SsoPox* crystal structure (Figure 3A). Residues forming the active site are all conserved and residues involved in loops 7 and 8 occupy nearly identical conformation in *SacPox* and *SsoPox* but

Table 3 Lactonase kinetic parameters

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M ($\text{M}^{-1}\cdot\text{s}^{-1}$)
C4 AHL	ND	ND	ND
C6 AHL	ND	ND	ND
C8 AHL	0.94 ± 0.02	178 ± 26	$5.28 (\pm 0.77) \times 10^3$
3-oxo C6 AHL	ND	ND	ND
3-oxo C8 AHL	0.89 ± 0.07	836 ± 178	$1.07 (\pm 0.25) \times 10^3$
3-oxo C10 AHL	1.03 ± 0.04	213 ± 33	$4.88 (\pm 0.77) \times 10^3$
γ heptanolide	10.25 ± 0.50	388 ± 62	$2.64 (\pm 0.44) \times 10^4$
Nonanoic- γ -lactone	2.64 ± 0.07	109 ± 19	$2.44 (\pm 0.44) \times 10^4$
Undecanoic- γ -lactone	0.34 ± 0.01	578 ± 78	$5.89 (\pm 0.84) \times 10^2$
dodecanoic- γ -lactone	0.53 ± 0.03	242 ± 60	$2.21 (\pm 0.57) \times 10^3$
Nonanoic- δ -lactone	4.55 ± 0.21	348 ± 53	$1.31 (\pm 0.21) \times 10^4$
Undecanoic- δ -lactone	1.05 ± 0.05	168 ± 37	$6.22 (\pm 1.40) \times 10^3$
Dodecanoic- δ -lactone	3.34 ± 0.07	185 ± 27	$1.81 (\pm 0.27) \times 10^4$
ϵ caprolactone	15.04 ± 0.47	$1\ 031 \pm 83$	$1.46 (\pm 0.13) \times 10^4$
Dihydrocoumarine	ND	ND	ND

ND correspond to Not Detected hydrolysis. Results have been obtained with cobalt as cofactor.

also in *SisLac* structures (Figure 3B). Noteworthy, loop 8 is partially structured into an α -helix, as seen in X-ray structures of *SsoPox* and *SisLac*. A substitution (I266 in *SacPox*; T265 in *SsoPox* and *SisLac*) in loop 8 may slightly alter the shape of the aliphatic channel. But overall, the active site of *SacPox* and *SsoPox* are nearly identical (Figure 2B). Furthermore, four other substitutions between *SacPox* and its close homologues can be seen in loop 8: *SacPox* exhibits a K at position 268, instead of an R residue (R267 in *SisLac*), Y271 instead of L (L270 in *SisLac*), K278 instead of R (R277 in both *SisLac* and *SsoPox*), and M281 instead of I (I280 in *SsoPox*) (Additional file 1: Figure S2). While the structural model suggests that these substitutions are not affecting directly the binding cleft of *SacPox*, they might modulate loop 8 conformation and its dynamics. Indeed, it was shown in the close homologue *SsoPox* that a single substitution in loop 8 (W263 in *SsoPox*, equivalent to W264 in *SacPox*) increases the conformational flexibility of loop 8, thereby conferring higher promiscuity to the enzyme [16]. The effect is in fact so dramatic that the substitution in *SsoPox* of W263 by any of the 19 other natural amino acids yields a variant with improved phosphotriesterase activity [16]. Additionally, loop 8 being involved in the accommodation of the aliphatic substituent of lactones substrates [9], mutations in this loop can also affect the lactonase activity [16].

Discussion

Here we show that *SacPox* is a proficient lactonase ($\sim 10^4 \text{ M}^{-1}\cdot\text{s}^{-1}$) and can hydrolyze both oxo-lactones and AHLs. Nevertheless, *SacPox* have a slightly different substrate specificity than its close homologues [16,33]. Indeed, *SacPox* exhibits slightly lower catalytic efficiencies, prefers AHLs over 3-oxo-AHLs and does not show any activity against dihydrocoumarin. Interestingly, as noted for *SisLac* and *SsoPox* [16,33], *SacPox* clearly prefers long chain AHLs, but can efficiently hydrolyze short chain or oxo-lactones without aliphatic substituents. This feature could reflect a putatively different binding mode of AHLs and oxo-lactones into PLLs active sites. We note that the biological role of lactonases such as PLLs is yet unclear, especially in extremophilic archaea where no AHL-based quorum sensing systems have been identified so far.

SacPox also exhibits promiscuous esterase and phosphotriesterase activities, a common feature of PLLs. Similarly to *SsoPox* and *SisLac* [33,52], *SacPox* prefers OPs with small substituents. Moreover, *SacPox* also shows a clear preference for oxono-phosphotriesters, rather than thiono-phosphotriesters, a feature previously dubbed thiono-effect [52]. Interestingly, *SsoPox*, *SisLac* and *SacPox* exhibit similar catalytic efficiencies against OPs ($10^{2-3} \text{ M}^{-1}\cdot\text{s}^{-1}$) at 25°C, efficiencies that are close to those measured at much higher temperatures [4].

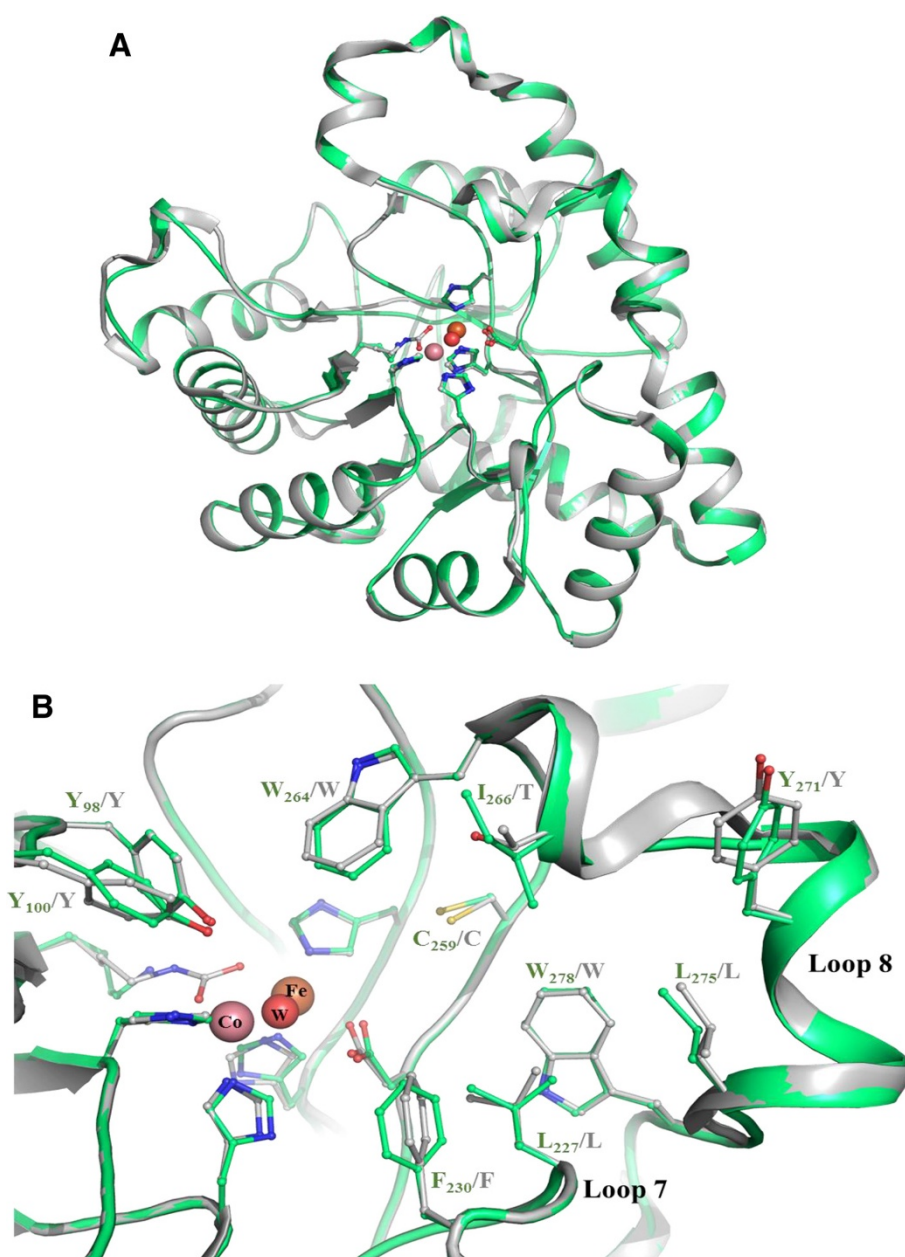


Figure 3 Structural model of SacPox. **A.** Structural superposition of SsoPox structure (2VC5; grey) and the SacPox model (green). Cobalt, iron and the catalytic water molecule are respectively represented by pink, orange and red spheres. Bimetallic center coordinating residues are represented as sticks. **B.** Active site view of superimposed SsoPox structure (grey) and the SacPox model (green). Several active site residues are represented as sticks. Numbering is made according to SacPox sequence.

The structural model shows that SacPox structure is very close to that of SsoPox (Figure 2A). Most critically, the active sites of both enzymes are essentially identical (Figure 2B), with the exception of position 266 (I in SacPox, T in SsoPox and SisLac). This substitution might partly account for the observed differences in substrates specificity between these enzymes, and would thereby represent an interesting target for future mutagenesis studies. But four other substitutions in loop 8 between

these close homologues might be involved as well, and comprise also interesting options for mutagenesis studies (K268R, Y271L, K278R and M281I). A recent study on SsoPox highlighted how profound the effect on catalysis of a single substitution on loop 8 (W263) can be [16]. Therefore, substitution T266I, and/or the four others on loop 8, might contribute to the observed differences between SacPox and SsoPox in substrate specificity, in combination with other factors that cannot be assessed

by a structural model such as subtle changes in active site loops conformation and dynamics [16,33]. Indeed, the observed differences in the detergent stimulation between both enzymes (*SacPox* is only weakly stimulated by SDS, as compared to *SsoPox*) could well be a manifestation of different dynamics of their respective active site loops.

Conclusions

To conclude, we here demonstrate that albeit being initially isolated, characterized, and named after its ability to degrade the insecticide paraoxon (pox; [4]), *SacPox* is putatively a native lactonase, capable of hydrolyzing these compounds with significant catalytic efficiencies at 25°C (up to $10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$). The extensive kinetic characterization reveals some substrate specificity differences between *SacPox* and its close homologues *SisLac* and *SsoPox*, and the proposed structural model of *SacPox* suggests putative candidates (e.g. I266) that could account for these observations. Such positions might constitute interesting targets for future engineering studies, with the aim of improving or altering the catalytic properties of *SacPox*.

Additional file

Additional file 1: Figure S1. Chemical structure of phosphoesters (I-VI), esters (VII-IX) and lactones (X-XXIV). **Figure S2.** Superposition of *SsoPox*, *SisLac* and *SacPox* structural models. **Table S1.** Accession numbers of the sequences used in the phylogeny study. **Table S2.** Sequence identity matrix.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

JH, GG and ME planned the experiments. JB, CC performed the experiments. JH, JB, ME and EC analysed the results. JB, JH and ME wrote the paper. All the authors offered a critical review of the paper.

Acknowledgements

We are grateful to 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. J.H. and C.C. are founded by DGA, France. G.G. is founded by APHM, France.

Received: 4 February 2014 Accepted: 27 May 2014

Published: 3 June 2014

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doi:10.1186/1756-0500-7-333

Cite this article as: Bzdrenga et al.: *SacPox* from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* is a proficient lactonase. *BMC Research Notes* 2014 **7**:333.

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