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The Latent Promiscuity of Newly Identified Microbial Lactonases Is Linked to a Recently Diverged Phosphotriesterase†

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ABSTRACT: In essence, evolutionary processes occur gradually, while maintaining fitness throughout. Along this line, it has been proposed that the ability of a progenitor to promiscuously catalyze a low level of the evolving activity could facilitate the divergence of a new function by providing an immediate selective advantage. To directly establish a role for promiscuity in the divergence of natural enzymes, we attempted to trace the origins of a bacterial phosphotriesterase (PTE), an enzyme thought to have evolved for the purpose of degradation of a synthetic insecticide introduced in the 20th century. We surmised that PTE's promiscuous lactonase activity may be a vestige of its progenitor and tested homologues annotated as "putative PTEs" for lactonase and phosphotriesterase activity. We identified three genes that define a new group of microbial lactonases dubbed PTE-like lactonases (PLLs). These enzymes proficiently hydrolyze various lactones, and in particular quorum-sensing *N*-acyl homoserine lactones (AHLs), and exhibit much lower promiscuous phosphotriesterase activities. PLLs share key sequence and active site features with PTE and differ primarily by an insertion in one surface loop. Given their biochemical and biological function, PLLs are likely to have existed for many millions of years. PTE could have therefore evolved from a member of the PLL family while utilizing its latent promiscuous paraoxonase activity as an essential starting point.

The analyses of numerous sequence and structural alignments indicate putative evolutionary links between enzymes belonging to the same family or superfamily. However, the gaps (in the number of mutations) between family members are usually large, typically many more than 10 amino acid changes. The frequency of point mutations is low (on the order of one in 10^6, per gene, per generation), and the likelihood of a specific mutation occurring in a gene is accordingly lower (on the order of 10^{-9}) (*1*, *2*). The emergence of a completely novel enzymatic function via the simultaneous exchange of multiple amino acids is therefore not a likely event. The likelihood (*2*), or unlikelihood (*3*), of an event leading to the divergence of a novel enzyme via the simultaneous change of just two amino acids has been the subject of a recent debate, but the answer to this question lies not only in the mathematics of population genetics but also in the biochemistry of enzymes.

To minimize the mutational gaps between diverging enzymes, it has been proposed that the ability of a progenitor

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to promiscuously catalyze a low level of the "new" or evolving activity facilitates the divergence of a new function by providing an immediate selective advantage (*4*, *5*). Many examples illustrate that functional promiscuity is a common feature of proteins (*5*−*8*), and protein variants containing one or few mutations, thereby exhibiting a dramatically higher proficiency of an initially weak promiscuous function, are routinely selected in the laboratory (*9*, *10*). In many cases, improvements in existing promiscuous activities are not accompanied by parallel reductions in the levels of the original function, thus indicating the evolvability of promiscuous functions, and their unique importance as evolutionary starting points (*9*, *11*). Promiscuous activities are often shared by family members that have presumably diverged from one progenitor (for examples, see refs *12*−*15* and recent reviews in refs *6* and *9*). However, these findings are largely circumstantial, and to date, the direct role of catalytic promiscuity in the divergence of a naturally occurring enzyme has not been established.

The emergence of new enzyme variants degrading man-made chemicals that appeared on this planet only several decades ago provides a powerful demonstration of the evolvability of enzymes, and a fascinating model for the study of evolution of new functions whose divergence has not been masked by millions of years of drift (*16*−*19*). Newly evolved enzymes generally exhibit sequence homologies to more ancient or even housekeeping enzymes and had presumably diverged from them (*16*, *18*, *20*). Indeed, a newly recruited enzyme in the pentachlorophenol degradation pathway was found to possess a catalytic activity present in a remotely related housekeeping enzyme (*17*). However, tangible links of catalytic cross reactivity, indicating a gradual evolutionary transition from a long-existing enzyme to a new one, have not yet been identified. We surmise that promiscuous activities could provide important clues regarding the progenitor of a newly diverged family member (*9*). We implemented this hypothesis in the identification of a new group of lactonases and a putative progenitor of *Pseudomonas diminuta* PTE,[1] an enzyme that is thought to have evolved during the 20th century and belongs to the same superfamily.

PTE (phosphotriesterase) was first identified in *P. diminuta* taken from sewage and soil samples, and its gene (*opd*) is located on a large plasmid and comprises a part of a transposable element (*21*, *22*). The same gene, and closely related ones, were found in other bacteria such as *Flavobacterium sp.* (*23*) and *Agrobacterium radiobacter* (*24*). Paraoxon, a widely used pesticide introduced ca. 60 years ago, is by far PTE's best substrate, with rates approaching the diffusion limit ($k_{cat}/K_M \geq 4 \times 10^7$) (*25*). PTE belongs to the amidohydrolase superfamily (*26*). As such, it possesses a TIM (β/α)-barrel fold and an active site with two transition metal ions such as cobalt, manganese, or zinc (*27*). While the protein scaffold and key active site features of all amidohydrolases are conserved, the β/α loops that comprise the substrate binding site, and loops 1, 7, and 8 in particular, vary in sequence and length, as do the substrate and reaction of different family members (*26*). No naturally occurring substrate has been identified for PTE. Its proficiency with paraoxon and its genetic location on a mobile element and in soil bacteria indicate that PTE has probably evolved to hydrolyze organophosphates such as paraoxon (*28*, *29*), possibly in response to limiting amounts of inorganic phosphate, a common limiting factor for growth of bacteria in the soil (*30*). Indeed, PTE was recently identified in soil bacteria such as *Pseudomonas aeruginosa* that are capable of growing with organophosphates as a sole phosphorus source (*31*).

Despite its most recent emergence, the evolutionary origins of PTE remain elusive. Its closest family member described to date, PHP (PTE homology protein), is 28% identical and 60% similar in sequence to PTE [similarity defined by protein−protein BLAST (blastp), http://www.ncbi.nlm.nih.gov/BLAST/]. Although considerably homologous in sequence (*29*) and in the structure of the bimetal catalytic site (*32*), PHP differs from PTE in three active site loops (1, 7, and 8) and exhibits no phosphotriesterase activity (*13*, *32*).

We have recently discovered that PTE exhibits considerable promiscuous lactonase activity, with k_{cat}/K_M values from 160 to 6.5×10^5 M^{-1} s^{-1} for different lactones and thiolactones (*13*). This led us to look at homologues of PTE that were tentatively annotated in the databases as "putative PTEs" and test both their phosphotriesterase and lactonase activity. Herein, we present the characterization of a group of PTE homologues that appear to be lactonases (dubbed PLLs), with promiscuous paraoxonase activity. Members of this group represent a feasible starting point from which PTE may have diverged, smoothly, with a gradual increase in paraoxoanse activity (and other phosphotriesterase activities) and the loss of the ancestral lactonase function.

MATERIALS AND METHODS

Cloning. The genes encoding AhlA and PPH were PCR-amplified from *Rhodococcus erythropolis* genomic DNA (kindly provided by Dr. Dessaux, Institut des science du Vegetal, Gif-sur-Yvette, France) and DNA library clone for *Mycobacterium tuberculosis* (kindly provided by the Pathogen Functional Genomics Resource Center, The Institute for Genomic Research, Rockville, MD), using primers appending EcoRI and PstI restriction sites. The amplified genes were cloned into pMAL-c2x (NEB) at its EcoRI and PstI sites, for expression as fusions with maltose binding protein (MBP), to give the pMAL-c2x-AhlA or -PPH plasmids, which were then used to transform *Escherichia coli* DH5α cells. As previously described with PTE, fusion to MBP increased the yield of the soluble, active enzyme, without altering the enzymatic parameters (*13*).

Expression and Purification of AhlA and PPH. Functional expression and activity in the presence of different divalent metals were tested by transforming the plasmids into BL21(DE3) *E. coli* cells carrying a plasmid encoding the GroEL/ES chaperones (pGro7/GroEL; TaKaRa). Expression in the presence of GroEL/ES increased the levels of soluble, active AhlA and PPH, by ≥5- and 10−15-fold, repectively.

[1] Abbreviations: AHL, *N*-acyl-homoserine lactone; AhlA, *N*-acyl-homoserine lactone acylase from *Rhodococcus erythropolis*; OpdA, phosphotriesterase from *Agrobacterium radiobacter*; PHP, phosphotriesterase homology protein from *Escherichia coli*; PLL, phosphotriesterase-like lactonase; PPH, putative parathion hydrolase from *Mycobacterium tuberculosis*; SsoPox, phosphotriesterase from *Sulfolobus solfataricus*; PTE, phosphotriesterase from *Pseudomonas diminuta*; TBBL, 5-thiobutyl-γ-butyrolactone.

Colonies were picked, grown overnight in 3 mL of LB medium containing 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, 0.2% arabinose, and 0.5 mM $MnCl_2$, $ZnCl_2$, or $CoCl_2$, then diluted 1:60 in the same medium, and grown for 40 h at 30 °C with shaking. Cells were lysed with BugBuster (Novagen), in the presence of assembly buffer, consisting of 50 mM Tris-HCl (pH 8.5) and 0.1 mM $ZnCl_2$, $MnCl_2$, or $CoCl_2$. Enzymatic activity was measured in the crude lysates with 4-thiobutyl-γ-butyrolactone (TBBL) (*33*) and paraoxon. For large-scale production, LB medium (5 mL) containing 100 μg/mL ampicillin, 34 μg/mL chloramphenicol, 0.2% arabinose, and 0.5 mM $ZnCl_2$ or $MnCl_2$ was inoculated with a single colony of *E. coli* BL21 (DE3)/pGro7/GroEL cells freshly transformed with pMAL-c2x-AhlA or -PPH plasmid and grown overnight. The resulting culture was added to 500 mL of the same medium and grown overnight at 30 °C. The subsequent steps were all performed at 4 °C. Cells were harvested by centrifugation and resuspended in lysis buffer [50 mM Tris-HCl (pH 8.0), 10 mM $NaHCO_3$, 1 mM PMSF, the histidine-tagged protease inhibitor Cocktail (Sigma) diluted 1:500, and 100 μM $ZnCl_2$ (AhlA) or $MnCl_2$ (PPH)]. After centrifugation, the supernatants were passed through an amylose column (NEB) equilibrated with column buffer [50 mM Tris (pH 8.0), 0.25 M NaCl, and 100 μM $ZnCl_2$ or $MnCl_2$]. The fusion proteins were eluted with column buffer supplemented with 10 mM maltose. The enzymatic activity of the collected fractions was analyzed with TBBL, and the fractions containing the highest activity were pooled together and dialyzed against assembly buffer. The purity of the fusion enzymes was established by 12% SDS−PAGE, and they were stored at 4 °C. SsoPox was cloned, expressed, and purified as previously described (*34*). We have made several attempts to assess the metal content of the purified enzymes. However, because of low solubility, the purified AhlA and PPH preparations could not be concentrated to a level that affords accurate and reproducible atomic absorption data. Attempts to reassemble the apoenzymes (following removal of the metal with a chelator; see Figure 2) also failed, presumably due to the irreversible misfolding of the apo forms.

Enzyme Kinetics. The esterase, phosphotriesterase, and lactonase hydrolyses of AhlA and PPH were analyzed by monitoring absorbance changes in 200 μL reaction volumes using 96-well plates and a microtiter plate reader (Synergy HT; optical length of ~0.5 cm) at 25 °C. For SsoPox, lactonase and paraoxonase activities were monitored in a 1 cm path length cell, using a reaction volume of 600 μL, in a Cary 5E spectrophotometer (Varian) at 70 °C. For each substrate, reactions were performed at the same concentration of organic solvent, regardless of substrate concentration. The substrates that were used are listed below, with the monitoring wavelength, extinction coefficient for the 0.5 cm pathway, and final organic solvent content: paraoxon (405 nm, ϵ = 9200 OD/M, 0.25% methanol), *p*-nitrophenol acetate and decanoate (405 nm, ϵ = 9200 OD/M, 1% DMSO), 7-acetoxycoumarine ester (408 nm, ϵ = 21 500 OD/M, 1% DMSO), 2-naphthyl acetate (320 nm, ϵ = 600 OD/M, 1% methanol), dihydrocoumarine (DHC) (270 nm, ϵ = 700 OD/M, 1% DMSO), and TBBL (or its analogues TEBL and THBL) together with 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as an indicator (*33*) (412 nm, ϵ = 7000 OD/M, 1% acetonitrile and 0.5% DMSO). The hydrolysis of other lactone substrates was monitored by following the appearance of the carboxylic acid products using a pH indicator as described previously (*35*). The reaction mixtures contained 0.01−1 mM lactone substrates in 0.2 M NaCl and 2.5 mM bicine buffer (pH 8.3), supplemented with 0.2−0.3 mM cresol purple as a pH indicator (577 nm, ϵ = 1550−2500 OD/M, 1% DMSO). Initial rates (v_0) were corrected for the background rate of spontaneous hydrolysis in the absence of enzyme. Kinetic parameters were obtained by fitting initial rates directly to the Michaelis−Menten equation [$v_0 = k_{cat}[E]_0[S]_0/([S]_0 + K_M)$] with Kaleidagraph. Error ranges relate to the standard deviation of the data obtained from at least two independent measurements.

Structure Modeling. The model of PPH was obtained by querying ModBase, a database of structural models (*36*). The models in this database are generated using MODPIPE, an automated software, pipeline, that calculates models on the basis of known structural templates and sequence−structure alignments (*36*). Molecular graphics and the structural alignment (using Matchmaker with 100% secondary structure weighting) were created with the UCSF Chimera package, from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, CA (supported by NIH Grant P41 RR-01081) (*37*).

RESULTS

Gene Identification. A BLAST search for homologues of *P. diminuta* PTE yielded the completely identical *opd* gene from *Flavobacterium sp. (23) and other genes with very high levels of homology (86−96% identical) that were identified as phosphotriesterases, such as opdA from A. radiobacter (24). The remaining genes were in the range of being 26−35% identical with PTE and could be divided into two groups. The first group contained genes with shorter loops 1, 7, and 8, relative to PTE, among them PHP from E. coli and other organisms. The second group contained genes that aligned well with one another (32−66% identical and 48−81% similarity), and have only one loop (#7) shorter than PTE. The second group included five genes from different species of Mycobacterium, two genes from Sulfolobus species, and single genes from various other bacteria. A search for conserved regions in PLLs using http//: blocks.fhcrc.org identified six motifs, five of which are shared with PTE (see Figure 2 of the Supporting Information) and one that is unique to PLLs (this unique motif is marked with a black frame in Figure 1).*

Three members of the second group, annotated as "putative parathion hydrolases" or "PTE homology proteins" (as most of the other genes identified in the BLAST search), were examined: AhlA from *R. erythropolis* and PPH from *M. tuberculosis*, which are 28 and 34% identical with *P. diminuta* PTE, respectively, and SsoPox, which has been recently identified and biochemically characterized (*34*). This thermostable enzyme from the archeon *Sulfolobus solfataricus* is 31% identical in sequence to PTE and was found to hydrolyze various phosphotriesters at rates $>10^4$-fold lower than that of PTE (*34*).

The sequence alignment of these genes with both PTE and PHP, the structures of which have been determined (*27*, *32*), indicated that the residues that coordinate the two catalytic metals are entirely conserved (Figure 1): His55, -57, -201,

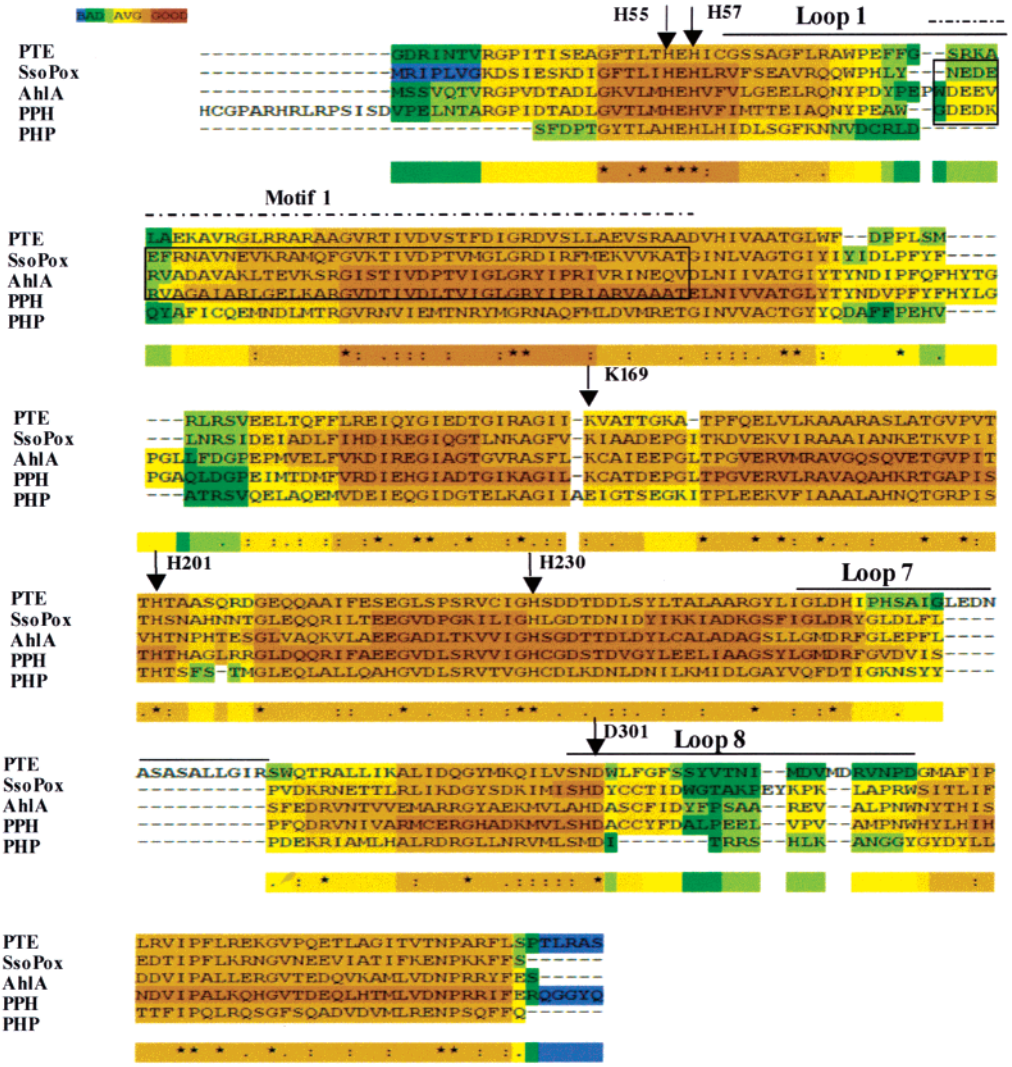


FIGURE 1: Sequence alignment (T-Coffee) of *P. diminuta* PTE, *E. coli* PHP, and the newly identified PLLs from *S. solfataricus* (SsoPox), *M. tuberculosis* (PPH), and *R. erythropolis* (AhlA). The loops of the first, seventh, and eighth β/α modules, the length of which distinguishes PLLs from PTE and PHP, are marked. Note that while PHP differs from PTE in the length of three active site loops (the loops connecting the first, seventh, and eighth β/α modules), AhlA, PPH, and SsoPox differ primarily in the length of the seventh loop. Marked also are conserved active site residues with numbering according to PTE. Five of the residues that coordinate the two catalytic metals are conserved throughout (His55, -57, -201, and -230 and Asp301). The sixth ligating residue, the carbamylated Lys169, is conserved in PTE and all PLLs but is replaced with a Glu in PHP (*32*) (a model showing the three-dimensional alignment of these residues appears in Figure 4A). A conserved motif identified in AhlA, PPH, and SsoPox, and in the remaining PLLs, but not in PTE or PHP, is also marked (identified using http://blocks.fhcrc.org/). Other PLLs identified via these characteristics, and a phylogenetic analysis of all genes related to PTE, are provided in Figure 1 of the Supporting Information.

and -230 and Asp301 are conserved in AhlA, PPH, and SsoPox. The sixth ligating residue, the carbamylated Lys169, is also conserved in all three genes but is replaced with a Glu in PHP (*32*). Additionally, PHP differs from PTE in the length of three active site loops (the loops connecting the first, seventh, and eighth β/α modules). However, AhlA, PPH, and SsoPox exhibit only minor differences in the lengths of loops 1 and 8 (one or two residues) and differ primarily in the length of loop 7 (Figure 1).

Cloning, Expression, and Initial Characterization. The *AhlA* and *PPH* genes were amplified from the respective genomic sources and cloned for expression in *E. coli*. To reveal their metal dependency, the enzymes were overexpressed with different divalent cations (Mn^{2+}, Zn^{2+}, and Co^{2+}) that prevail in the amidohydrolase superfamily (*26*). Lactonase activity was measured with the chromogenic substrate TBBL and phosphotriesterase activity with paraoxon. AhlA's lactonase activity was 2−10000-fold higher than the paraoxonase activity, depending on which metal was added, and was highest with zinc. The maximum paraoxonase activity was observed with manganese. PPH's lactonase activity was 2−1000-fold higher than that of paraoxonase with all three metals, and both activities were the highest with manganese. SsoPox was previously examined for its paraoxonase activity only and found to be most active with cobalt (*34*). The detailed analysis of these enzymes was therefore performed after addition of Zn^{2+} for AhlA, Mn^{2+} for PPH, and Co^{2+} for SsoPox as previously described (*34*). AhlA and PPH were produced in large quantities in *E. coli* under overexpression of the GroEL/ES chaperone (*38*) and affinity purified using the maltose binding protein tag. Expression as a fusion protein with maltose binding protein [as previously done with PTE (*13*)], and in the presence of chaperones, yielded 1 mg of pure enzyme per liter of *E. coli*

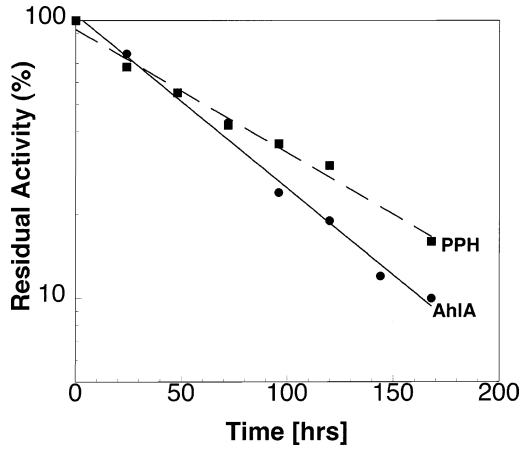


FIGURE 2: Inactivation of *M. tuberculosis* PPH and *R. erythropolis* AhlA in the presence of the metal chelating agent EDTA. The residual lactonase activity observed with these enzymes following their dialysis against 50 mM Tris and 50 mM EDTA (pH 8.0), at 4 °C, was measured up to the time point where ≥90% loss of activity was observed. Data were fit to a first-order reaction to give a k_{inac} of 0.014 h^{-1} for AhlA and 0.01 h^{-1} for PPH.

culture, while in the absence of these two, the yield was nearly nil. The apparent molecular mass of the unique band observed on SDS−PAGE (data not shown) was ∼78 kDa for both enzymes, in agreement with the predicted molecular mass for the MBP-fused proteins.

Metal chelation led to inactivation of both AhlA and PPH. The residual enzymatic activity was measured after the enzymes had been dialyzed in the presence of EDTA and the lactonase activity had been assayed with 0.1 mM TBBL at varying time points (Figure 2). Both enzymes were completely inactivated only after 10 days, as observed previously for SsoPox (*34*). The pseudo-first-order rates (k_{inac}) were derived by measuring the residual activity as a function of time and were found to be 0.018 h^{-1} for AhlA and 0.016 h^{-1} for PPH. Thus, the metal ions appear to be very tightly bound and are crucial for the enzymatic activity of all three enzymes. At this stage, however, the metal content of the purified enzymes could not be verified (see Materials and Methods). It appears, though, that the metal ion added to the growth medium dominates the purified enzyme preparations. Indeed, overexpression in growth media with no metal ion supplementation gave only 1% lactonase activity in the case of PPH and 20% in the case of AhlA.

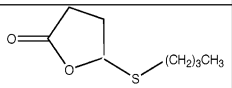
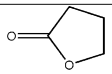
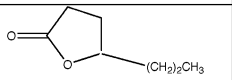
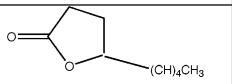
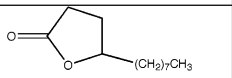
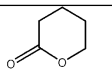
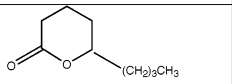
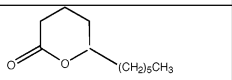
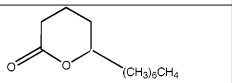
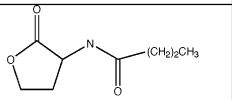
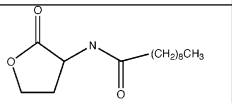
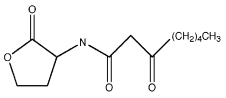
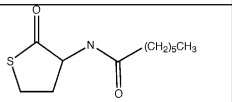
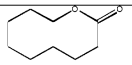
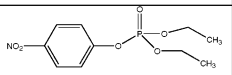
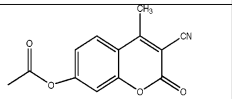
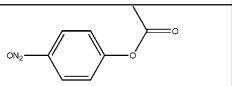
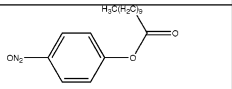
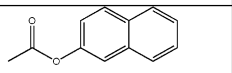
Substrate Specificity of PLLs. A variety of compounds were examined as substrates for two of the three enzymes, as described in Table 1. The lactonase activity was routinely tested with the chromogenic lactone TBBL, using DTNB as an indicator (*33*). The hydrolysis of other aliphatic lactones was monitored by the release of their carboxylic acid products using purple cresol, a pH indicator (Figure 3). The hydrolysis of paraoxon and various aryl esters was followed spectrophotometrically. The kinetic parameters determined for these substrates indicated that AhlA hydrolyzes lactones with K_M values ranging from ≤10 to 150 μM and k_{cat}/K_M values ranging from 1.05×10^3 to $>1.7 \times 10^6$ M^{-1} s^{-1}. PPH hydrolyzes lactones with K_M values ranging from ≤20 to 230 μM and k_{cat}/K_M values ranging from 1.4×10^4 to 5×10^5 s^{-1} M^{-1}. The highest k_{cat}/K_M values are generally observed with six-membered ring lactones (which are inherently more reactive) and lactones with longer and more hydrophobic side chains. High k_{cat}/K_M values were observed with *N*-acyl-homoserine lactones, in particular for AhlA, with a preference for 3-oxo side chains. An *N*-acyl-thiolactone analogue, derived from homocysteine rather than serine, exhibited no detectable activity.

Both AhlA and PPH exhibit weak phosphotriesterase activity. For hydrolysis of paraoxon by AhlA, only the k_{cat}/K_M ratio could be estimated (0.5 s^{-1} M^{-1}). PPH exhibited higher activity, and both parameters could be obtained independently ($k_{cat} = 0.01$ s^{-1}, $K_M = 1.4$ mM, and $k_{cat}/K_M = 8.6$ M^{-1} s^{-1}). Several aryl ester substrates and an aromatic lactone (dihydrocoumarin) were also examined, but no rate enhancement could be detected (Table 1).

SsoPox was previously shown to hydrolyze various phosphotriesters, among them paraoxon, with a k_{cat}/K_M of 4000 M^{-1} s^{-1} (*34*). This enzyme was also shown to hydrolyze various aryl esters, such as *p*-nitrophenyl butanoate and 2-naphthyl acetate with a k_{cat}/K_M of 400 M^{-1} s^{-1}. We found that SsoPox proficiently hydrolyzes lactones. We tested a series of lactones, the hydrolysis of which can be detected with chromogenic reagents (*33*). TEBL (5-thioethyl-γ-butyrolactone) exhibited a k_{cat} of 9.00 ± 0.07 s^{-1}, a K_M of 15 ± 1 μM, and a k_{cat}/K_M of $0.700 \pm 0.003 \times 10^6$ $M^{-1}s^{-1}$. TBBL (5-thiobutyl-γ-butyrolactone) exhibited a k_{cat} of 29 ± 7 s^{-1}, a K_M of 80 ± 3 μM, and a k_{cat}/K_M of $(3.0 \pm 0.9) \times 10^5$ M^{-1} s^{-1}. THBL (5-thiohexyl-γ-butyrolactone) exhibited a k_{cat} of 6.00 ± 0.06 s^{-1}, a K_M of 70 ± 2 μM, and a k_{cat}/K_M of $(0.800 \pm 0.006) \times 10^5$ M^{-1} s^{-1}. Since SsoPox is hyperthermophilic, and its optimum activity occurs above 95 °C, it proved impossible to test other lactones using the pH indicator assay, due to shifts in the pK_a values of both the buffer and the indicator at this temperature. At any rate, its activity with 5-thioalkyl-γ-butyrolactones assayed at 70 °C resembles that of AhlA and PPH (Figure 3), and although its phosphotriesterase activity is the highest of those of the three enzymes that were tested, it is still 100-fold lower than its lactonase activity. We also applied a bioassay to test the rate by which SsoPox hydrolyzes AHLs (Figure 3 of the Supporting Information) (*39*−*41*). A comparison with AhlA indicated that, at 70 °C, SsoPox hydrolyzes *N*-(β-ketocaproyl)-DL-homoserine lactone and *N*-butyro-DL-homoserine lactone at a rate which is ≥10-fold higher than that of AhlA at ambient temperature. This suggests that SsoPox exhibits k_{cat}/K_M values for quorum sensing lactones that are well over 10^6 M^{-1} s^{-1}.

PTE's Lactonase Activity. We had previously reported that PTE hydrolyzes the aromatic lactone dihydrocoumarin (DHC) with a k_{cat}/K_M of 6.5×10^5 M^{-1} s^{-1} and the chromogenic oxolactone 5-thioethyl-γ-butyrolactone (TEBL) with a k_{cat}/K_M of 1.1×10^3 M^{-1} s^{-1} (*13*). For a better comparison with PLLs, we have now measured PTE's lactonase activity with TBBL [$k_{cat} = 0.90 \pm 0.04$ s^{-1}, $K_M = 260 \pm 30$ μM, and $k_{cat}/K_M = (3.00 \pm 0.07) \times 10^3$ M^{-1} s^{-1}] and with several other oxolactones, such as γ-butyrolactone, δ-nonanoic lactone, δ-valerolactone, γ-nonanoic lactone, *N*-butyryl homoserine lactone, *N*-(3-oxooctanoyl) homoserine lactone, and *N*-decanoyl homoserine lactone. However, no activity beyond background was detected with any of these lactones using PTE concentrations of up to 2.5 μM.

Table 1: Kinetic Parameters for *R. erythropolis* AhlA and *M. tuberculosis* PPH[a]

Name	Structure	*R. erythropolis* (AhlA)			*M. tuberculosis* (PPH)		
		k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $M^{-1}s^{-1}$	k_{cat}, s^{-1}	K_M, μM	k_{cat}/K_M, $M^{-1}s^{-1}$
TBBL		17.0±0.8	<10	>2x10^6	14.5±0.8	230±20	6.0±0. 6x10^4
γ-butyro lactone		1.00±0.02	100±5	1.00±0.08x10^4	1.3 ±0.01	120±8	1.0±0.1x10^4
γ –heptanolide		6.5±0.4	150±20	4.2±0.3x10^4	1.00±0.01	60±8	1.60±0.2x10^4
γ-nonanoic lactone		1.40±0.02	15±2	1.1±0.1x10^5	7.0±0.4	100±10	8.0±0. 5x10^4
γ-dodecanoic lactone		13.4±0.2	20±1	0.70±0.02x10^6	4.0±0.2	40±5	1.00±0.02x10^5
δ-valero lactone		2.00±0.12	100±10	2.6±0.1x10^4	1.9±0.10	70±6	2.65±0.04x10^4
δ-nonanoic lactone		14.5±0.2	60±2	2.5±0.2x10^5	3.0±0.15	90±5	3.3±0.4x10^4
δ-undecanoic lactone		11.0±0.3	20±2	0.550±0.005x10^6	13.0±0.1	30±2	4.30±0.07x10^5
δ-dodecanoic lactone		10.00±0.07	15±1	0.75±0.06x10^6	10.20±0.06	20±2	4.6±0.5x10^5
N-butyryl, DL, homoserine lactone		11.0±0. 8	75±8	1.50±0.06x10^5	1.60±0.07	60±8	3.0±0.3x10^4
N-decanoyl, DL, homoserine lactone		0.66±0.03	<10	>0.66x10^5	2.60±0.02	150±10	1.7±0.1x10^4
N-(3 oxooctanoyl), L, homoserine lactone		24.5±0.4	30±3	0.72±0.05x10^6	3.05±0.06	60±1	0.550±0.004x10^5
N-hexanoyl, DL, homocysteine lactone		ND	ND	ND	ND	ND	ND
dihydro coumarine		ND	ND	ND	ND	ND	ND
paraoxon				0.50±0.04	0.0120± 0.0003	1400±30	8.65±0.07
7-acetotoxy coumarin		ND	ND	ND	ND	ND	ND
P-nitrophenyl acetate		ND	ND	ND	ND	ND	ND
P-nitrophenyl dodecanoide		ND	ND	ND	ND	ND	ND
2-naphthyl acetate		ND	ND	ND	ND	ND	ND

[a] ND means no activity detected.

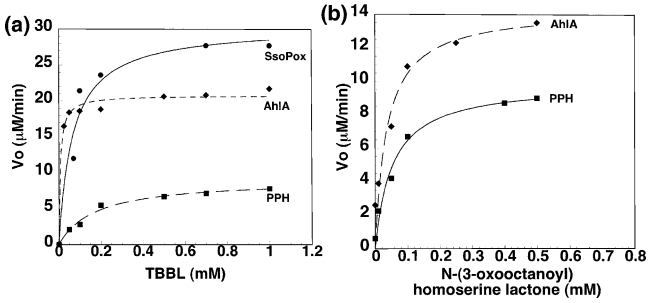


FIGURE 3: Michaelis−Menten analyses of the lactonase activities of AhlA, PPH, and SsoPox. (a) Lactonase activity assayed at pH 8.0 with TBBL and AhlA ($[E]_0 = 0.02$ μM, at 25 °C), PPH ($[E]_0 = 0.011$ μM, at 25 °C), or SsoPox ($[E]_0 = 0.014$ μM, at 70 °C). (b) Lactonase activity assayed at pH 8.0 with *N*-(3-oxooctanoyl)-homoserine lactone and AhlA ($[E]_0 = 0.01$ μM, at 25 °C) and PPH ($[E]_0 = 0.027$ μM, at 25 °C).

DISCUSSION

All three PLLs tested here proficiently hydrolyze lactones, with a preference for relatively hydrophobic lactones, including *N*-acyl-homoserine lactones. The lactonase specificity factors (k_{cat}/K_M) range from 6×10^4 to 1.7×10^6 M^{-1} s^{-1}, with distinctly low K_M values (10−230 μM). The phosphotriesterase activity exhibited by these enzymes is not only much lower (10^2−10^6-fold) but also varies to a much larger degree ($k_{cat}/K_M = 0.5$−4000 M^{-1} s^{-1}). The arylesterase activity could be detected only in SsoPox (*34*). This pattern is indicative of the lactonase being the common denominator and, hence, the native, or primary, activity of PLLs. Indeed, promiscuous functions often appear in one family member, but not others, while the native function is obviously shared by all family members (*9*).

The biological roles of PLLs appear to be linked to quorum sensing. The locus of AhlA in the *R. erythropolis* genome indicated a role in the degradation of *N*-acyl-homoserine lactones (AHLs), microbial quorum-sensing molecules (*42*). *R. erythropolis* degrades AHLs and specifically AHLs with 3-oxo substituents, using these as sole carbon and energy sources. It has been proposed, however, that AHL degradation does not include lactone hydrolysis (*42*). Nevertheless, our biochemical analysis indicated that AhlA is a lactonase, with high specific activity toward AHLs, and 3-oxo-AHLs in particular [the k_{cat}/K_M value with 3-oxooctanoate AHL is 11-fold higher than that of its closest homologue lacking the 3-oxo group (*N*-decanoyl homoserine lactone); Table 1]. SsoPox is also capable of degrading both linear and 3-oxo AHLs with rates that are approximately 10-fold higher than that of AhlA. Other PLLs are likely to be involved in the degradation and inactivation of quorum-sensing AHLs, either for their utilization as carbon, nitrogen, or energy sources or for quenching the signals of quorum sensing. Quorum-sensing signals of the AHL type have also been reported in the kingdom of archaea (*43*) to which *S. solfataricus* belongs. While other roles cannot be excluded (lactones are abundant natural products), PLL's most common physiological role might be in processing quorum-sensing lactones. Given their distribution in archea and bacteria, and their biochemistry and physiology, PLLs have probably evolved many millions of years ago, and well before PTE.

Several other "quorum-quenching" lactonases are already known, including AiiA from *Bacillus thuringiensis*. This enzyme belongs to a completely different superfamily, that of metallo-β-lactamases (*44*, *45*). Mammalian serum paraoxonases (PONs) belong to yet another family of calcium-dependent, six-bladed β-propellers (*46*). PONs can also hydrolyze, albeit with low efficiency, quorum-sensing AHLs (*47*). Intriguingly, all these lactonases exhibit promiscuous phosphotriesterase activity. Some PONs do (*35*), and so do PLLs. In addition, a methyl parathion hydrolase has been identified in the metallo-β-lactamase superfamily (*48*) to which AiiA belongs. AiiA has been now tested, and it appears to exhibit weak promiscuous phosphotriesterase activity (H.-S. Kim, personal communication). It could well be that this parathion hydrolase (*48*) has evolved from a related lactonase of the same superfamily. Because the three families are so different, in scaffold and catalytic mechanism, the promiscuous phosphotriesterase activity could stem from a certain overlap between the transition states of lactone and phosphotriester hydrolysis. Thus, like many other catalytic cross reactivities (*5*, *7*), the lactonase−phosphotriesterase cross-reactivity may also be inherent to a particular active site chemistry. Because all active sites are hydrophobic clefts

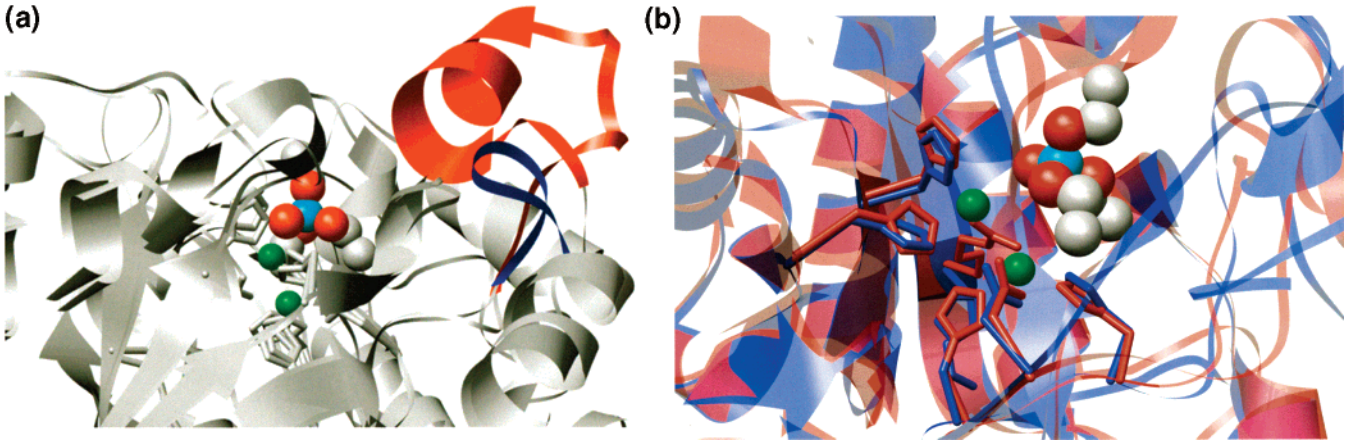


FIGURE 4: Structural overlay of *P. diminuta* PTE [PDB entry 1EYW (red)] and a computational model of a representative PLL [PPH from *M. tuberculosis* (blue)]. Shown are the backbones of both enzymes, the active site zinc atoms (green), and an organophosphate substrate analogue (spheres) in PTE's active site. (a) Replacement of short loop 7 in PLLs (e.g., PPH, blue) with the elongated loop of PTE (red) provides an active site "cap" which narrows the active site mouth and may thereby increase PTE's activity and selectivity toward paraoxon. (b) Overlay of the binuclear catalytic center. Five of the zinc-ligating residues (His55, -57, -201, and -230 and Asp301) align perfectly, while the sixth residue (carbamylated Lys169; hidden in this view) differs only in its side chain rotamer.

and contain a set of similar chemically active side chains (*49*), such cross reactivities are inevitable (*50*), and presumably because phosphotriesters are not natural compounds, the paraoxonase promiscuity had no physiological relevance. It was therefore not selected against and remained latent in these enzymes.

The identification of AhlA, PPH, and SsoPox as lactonases with promiscuous paraoxonase activity provides a tangible evolutionary link to *P. diminuta* PTE and explains how this enzyme could have evolved into a highly proficient paraoxonase in a matter of just a few decades or perhaps years. Although PTE is distant in sequence from the currently identified PLLs, differences of up to 70% within one family are the norm (as indeed seen between the currently identified PLLs). Thus, having a PLL member that is highly homologous to PTE is a reasonable assumption. The distinct functional and sequence homologies described here indicate that the native lactonase activity of PPLs comprises the promiscuous activity of PTE, and vice versa. The promiscuous paraoxonase activity of PPLs varies over several orders of magnitude, even for the three members analyzed here. Despite being a proficient lactonase, SsoPox exhibits the highest paraoxonase activity and therefore seems the closest to PTE (also phylogenetically; Figure 1 of the Supporting Information). It is also the closest to a PTE-like phenotype in having considerable arylesterase activity. Thus, gradual, possibly haphazard, changes in PLLs that increase their promiscuous paroxonase activity, without compromising their lactonase function, may have facilitated the divergent evolution of PTE (*9*, *11*). We further hypothesize that an insertion in loop 7 completed the transition from a PLL into PTE. Structural modeling suggests that the core of the active sites of PLLs and PTE is essentially identical, and the replacement of short loop 7 of PLLs with elongated loop 7 containing a short α-helix seen in PTE may result in "capping" of the active site and narrowing of its "mouth" (Figure 4). In TIM barrels, and in the amidohydrolase superfamily in particular, loops 7 and 8 are most often involved in contacting substrates and in determining substrate specificity (*26*, *51*). Indeed, deletions from and insertions into loops, or loop swapping, are believed to be a primary mechanism of creating enzyme diversity (*52*, *53*). Adaptation toward new substrates via an insertion or deletion of an active site loop has also been demonstrated with haloalkane dehydrogenase (*54*), although the evolutionary routes of this enzyme (which presumably adopted to degrade 1,2-dichloroethane, the production of which began in 1922) are largely unknown (*19*).

To conclude, the functional and structural homologies noted above indicate that PTE evolved from an as yet unknown PLL, using its promiscuous paraoxonase activity as an essential starting point. As demonstrated by the properties of SsoPox, the paraoxonase activity of PLLs can be considerably high, even without compromising the lactonase activity, and perhaps while acquiring other activities such as aryl esterase. In that respect, SsoPox resembles a "generalist" intermediate (*11*, *55*) from which PTE may have emerged. The recent specialization as a phosphotriesterase, as seen in *P. diminuta* PTE, possibly through an insertion into loop 7, did not completely eradicate the ancestor's lactonase activity. Indeed, this presumed vestige of PTE's past has enabled us to trace the footsteps of its divergence and assign a function to the newly identified PLLs. We thus portray a highly reasonable scenario for the virtually overnight divergence (on evolutionary time scales) of PTE. The same scenario may have occurred independently in lactonases belonging to another superfamily (*44*, *45*, *48*). Most importantly, this scenario obeys an oft-forgotten essence of evolutionary processes: They usually occur smoothly while maintaining fitness throughout. Such "tinkering" (*56*) scenarios (see also refs *57*−*59*) stand in contrast to unreasonable models which assume "leaps in thin air", such as the evolution of completely novel activities via multiple and simultaneous amino acid changes (*3*) (for another critical assessment of this model, see ref *2*).

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SUPPORTING INFORMATION AVAILABLE

Sequence alignment of PTE homologues dubbed PLLs (PTE-like lactonases) and a phylogenetic tree of PTE and homologous sequences (Figure S1), sequence motifs found in PTE and PLLs (Figure S2), and a bioassay for *N*-acyl-homoserine lactone (AHLs) hydrolysis by SsoPox and AhlA using an indicator strain (Figure S3) with experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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