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Review Article

Evolution of Phosphotriesterases (PTEs): How Bacteria Can Acquire New Degradative Functions?

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The promiscuity of enzymes has often been considered a vestige activity based on the broad substrate spectrum of their progenitors. As such, divergent enzymes can be used as a fingerprint to track their evolutionary history. In the presence of structural mimics of active site or binding site ligands, and assisted by mutations in the associated binding site, this promiscuity contributes to acquisition of new catalytic functions. This phenomenon is often referred to as substrate-assisted gain-of-function and helps soil microbes to thrive on re-calcitrant xenobiotic molecules, hitherto unfamiliar to the microbial world. This review describes the evolution of organophosphorous hydrolases, which potentially and originally functioned as quorum-sensing 'quenching' lactonases and highlights their remarkable horizontal mobility within diverse bacterial species.

Keywords: Phosphotriesterase; Organophosphates; Evolution

Introduction

There is a growing worldwide concern over the steady accumulation of xenobiotic materials, which have been produced or used in industrial/agricultural processes over the past several decades. These compounds include various organic solvents, heavy metals, neurotoxic pesticides, halogenated aromatic compounds, explosives and carcinogenic industrial chemicals. Although the introduction of xenobiotics into the environment is lamentable, and the effects they have on the environment can be devastating, many different types of microbial systems rapidly acquired the ability to thrive on these compounds, using them as sources of carbon, phosphate, nitrogen, etc. There are a number of excellent reviews describing the complex metabolic pathways involved in the conversion of these otherwise recalcitrant and toxic chemicals into central metabolic intermediates. The well-structured genetic information and finely-tuned regulatory mechanisms of these degradative enzymes/pathways indicate that these traits evolved specifically to enable microbes to use these re-calcitrant compounds, generated through anthropogenic activities, as a nutrient source (Allpress and Gowland, 1998; Janssen *et al*., 2005; Khajamohiddin *et al*., 2006; Juhas *et al*., 2009; Carmona *et al*., 2009). Understanding the biology of these metabolic traits is relevant to our general understanding of remarkably rapid evolution and acquisition of these degradative genes.

Chemistry of Organophosphates

Organophosphates (OP) are esters or thiols derived from phosphoric, phosphonic or phosphoramidic acid (Sogorb and Vilanova, 2002). Gerhard Schradera, a German chemist working at the Bayer Company (IG Farben), developed the insecticide "Schraden" in 1941. Schraden was eventually not used as an insecticide owing to its extreme toxicity to mammals; rather it was principally synthesized for military purposes. However, this discovery led to the synthesis of the first OP insecticide tetraethyl pyrophosphate (TEPP) and that of an extensively used OP compound, parathion (O, O-dimethyl-O-*p*-nitrophenyl phosphorothioate). Since then, thousands of OP compounds have been synthesized for use as

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insecticides, nerve agents, solvents, plasticizers etc. (Minton and Murray, 1988).

As shown by the general formula (Fig. 1) of organophosphorus compounds, the mainly aryl or alkyl groups R_1 and R_2 can be directly attached to a phosphorus atom (phosphinates) or via an oxygen (phosphates) or sulphur atom (phosphothioates). In some cases, R_1 is directly bonded with phosphorus and R_2 with an oxygen or sulfur atom (phosphonates or thion phosphonates, respectively). However, in phosphoramidates these two groups are attached with mono- or di-substituted amino groups. The X group can be diverse aliphatic, aromatic, heterocyclic or halide moieties. The X group is also known as a leaving group because it is released from phosphorus on hydrolysis of the ester bond (Sogorb and Vilanova, 2002).

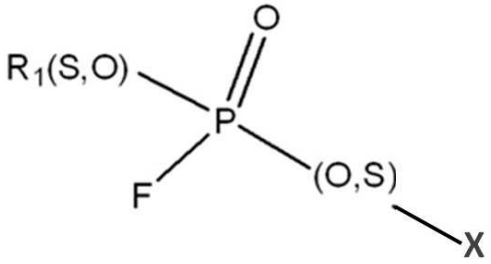

Fig. 1: General structural formula for organophosphates

The OP compounds irreversibly inhibit acetylcholinesterase (AchE) activity (Gaines, 1969; Eto, 1974; Green *et al*., 1977; Cremlyn *et al*., 1978; Ashani *et al*., 1991). Due to their neurotoxic nature, OP compounds have the potential to serve as chemical weapons, or nerve agents. Recent studies have revealed indications of their use during the Gulf War (Golomb *et al*., 2008). In November of 1992, the General Assembly approved by consensus a strict ban on the production, stockpiling and use of chemical warfare agents in the form of the Chemical Weapon Convention Treaty (CWCT).

Bio-degradation of Organophosphates

Although nerve agents were initially synthesized prior to or during World War II, they were confined to the laboratories in which they were made. Their derivatives, however, were extensively used as insecticides and thus were released into the environment from early 1950's onwards. Currently, OP insecticides constitute about 70% of the insecticides used around the world (Prokop *et al*., 2006; Singh 2009). Persistent and indiscriminate use of OP insecticides has resulted in the accumulation of OP residues in both soil and aqueous environmental systems (Ragnarsdottir, 2000; Wang *et al*., 2007). In fact, OP poisoning is a growing global clinical problem with approximately 3 million poisonings and 300,000 human deaths occurring every year as a result of OP ingestion (Bird *et al*., 2008; Singh 2009). The level of pollution is high-lighted by the presence of OP compounds in ground water (46 to 2659 ng/L), rainwater and snow (Ragnarsdottir, 2000, Regnery and Püttmann, 2010). In addition, the Organisation for the Prohibition of Chemical Weapons, the implementing body of the CWCT, reports that just 44,131, or 61.99%, of the world's declared stockpile of 71,194 metric tons of chemical agent have been destroyed. The controlled destruction of these compounds continues to be a major challenge (Karpouzas and Singh, 2006).

Although incineration and chemical neutralization of OP compounds are the most advanced technologies, enzymatic methods of de-contamination are arguably among the safest. OP-degrading enzymes have been explored for decontamination of OP compounds and nerve agents (Karns, 1998; LeJeune *et al*., 1998; Raushel, 2002; Prokop *et al*., 2006, Theriot and Grunden, 2011, Landguard: www.orica-landguard.com). The OP hydrolyzing enzymes, classified here as organophosphate hydrolases (OPHs), have been identified in a variety of organisms, including mammals; however, it is the microbial enzymes that are the most attractive candidates for de-contamination of nerve agents and other OP-compounds. A number of recent reviews have appeared on bio-degradation of OP compounds. Most of these have either high-lighted degradation pathways available in various microbial systems or described enzymatic mechanisms for hydrolyzing the triester linkage found in structurally diverse groups of OP-compounds (Singh *et al*., 2005; Singh and Walker, 2006; Karpouzas and Singh, 2006; Yair *et al*., 2008; Singh, 2009; Theriot and Grunden, 2011). Here, we aim to provide a comprehensive review on the evolution of these degradative traits among soil bacteria.

Enzymology of Organophosphate Hydrolases

Organophosphate hydrolase (OPH) is the generic term used to describe all enzymes involved in hydrolytic cleavage of the triester bond found in structurally diverse group of OP compounds. Three structurally distinct OPHs are present among prokaryotes. Parathion hydrolase, isolated from *Flavobacterium* sp. ATCC27551 was the first phosphotristerase (PTE) reported from prokaryotes. Subsequently methyl parathion hydrolase (MPH) and organophosphate acid anhydrolase (OPAA) were isolated from taxonomically diverse micro-organisms (Zongli *et al*., 2001; DeFrank *et al*., 1993). The bacterial organophosphate hydrolases reported to date have shown structural similarity to one of these three structural groups. However, analysis of meta-genome revealed existence of a number of structurally diverse group of PTEs among culturable and non-culturable soil bacteria (Colin *et al*., 2015).

The structure of MPH is a homodimer comprising two crystallographically independent subunits. The monomer of MPH has an αβ/βα sandwich structure typical of the metallo-hydrolase/ oxidoreductase fold. Each sub-unit is composed of a â-lactamase-like domain, which includes the binuclear metal center. The N-terminal arm of each sub-unit appears to play a significant role in dimerization (Dong *et al*., 2005).

PTE has been classified as a metallo-dependent hydrolase and possesses an (αβ)8 TIM barrel structure (Benning *et al*., 2001). It has been shown to be the membrane-associated lipoprotein found as part of the multi-protein complex (Gorla *et al*., 2009, Parthasarathy *et al*., 2016). Though PTEs shares no sequence or structural homology with MPH, they contain a similar bi-nuclear zinc center. The two metals are separated by 3.4 Å and coordinated to the protein via the side chains of four histidine residues (His55, His57, His201, and His230) and Asp301. The two metals are bridged *via* a carbamylate group from Lys169 and a nucleophile hydroxide ion. Detailed investigations have been done to unravel the catalytic mechanism of PTE (Aubert *et al*., 2004; Wong and Gao, 2007). The phosphoryl-oxygen bond of the organophosphate is polarized through its association with the metal ion at the active site, and the nucleophilic attack occurs from the bridging hydroxide ion. As the chemical step proceeds, a proton is abstracted from a coordinating histidine and is released to the solvent as the leaving group is released from the active site. A water molecule subsequently enters into the binding pocket and the metal coordination sphere originally occupied by the nucleophilic hydroxide ion, resulting in displacement of the phosphodiester product and restoring the resting state of PTE. The hydrophobic residues and the metal centers are similar in both MPH and PTE (Vanhooke *et al*., 1996; Dong *et al*., 2005). The presence of similar hydrophobic binding pocket, metal coordination spheres and catalytic residues suggests that MPH and PTE may have common catalytic mechanisms (Fig. 2).

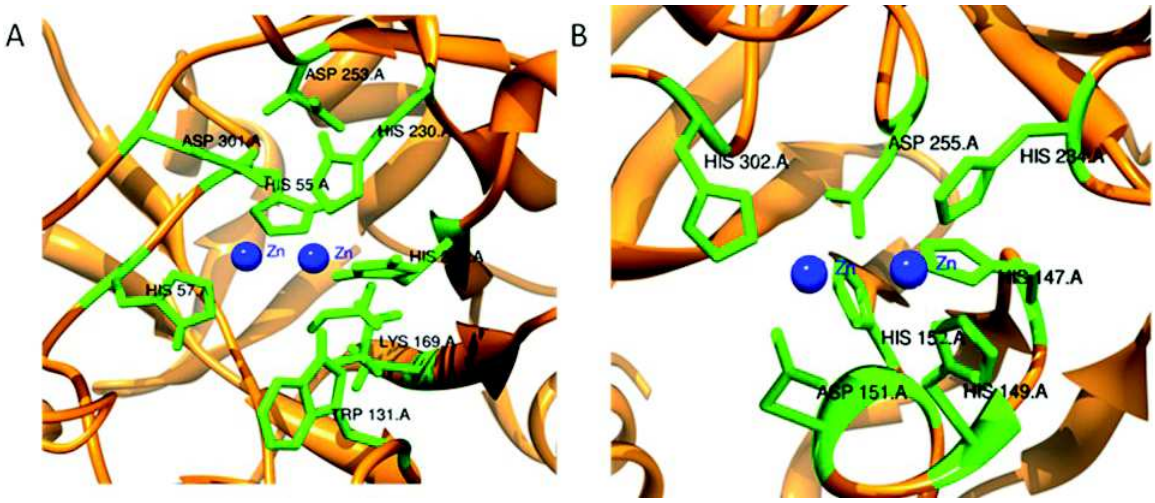

Fig. 2: Enzymatic hydrolysis of organophosphates. The active site residues found in *B. diminuta* phosphotriesterase (A) and methyl parathion hydrolase of *Pseudomonas* sp. WBC3 (B) are highlighted in green color. The zinc ion found in bi-metallic center of the active are shown in blue color

Evolution of Catalytic Function

It is generally accepted that ancient organisms possessed small genomes with few gene products in comparison with contemporary organisms. Jensen (1976) suggested this was possible because primitive enzymes had broad substrate specificity, allowing them to react with a wide range of related substrates producing a family of related products. Gene duplication then provided the genetic reservoir necessary for the narrowing of substrate specificity, leading to the collection of specialized enzymes seen in most organisms today. The increasing number of deduced amino acid sequences made available by genomic sequencing projects has revealed many large gene families. As can be seen within the family of phosphotriesterase-related proteins, predictions of homology relationships based on similarity in function are not always straight-forward. In the case of the phosphotriesterase-related proteins, the *PON* family tree roughly corresponds to the phylogenetic relationships of the organisms, suggesting that this is an orthologous family of paralogues in which the duplication event occurred before phylogenetic divergence (Fig. 3). The absence of any significant homology between the OPH, MPH and OPAA groups suggest that the shared function of OP hydrolysis is the result of convergent evolution (Fig. 4).

Interestingly, the MPH and its close homologue OPHC-3 of *Pseudomonas pseudoalcaligenes* are shown to have sequence similarity (61%) with β-lactamases. In view of such high similarity the β-lactamases is assumed as a common progenitor of MPH and OPHC (Fig. 4). In contrast, OPAA is a prolidase that cleaves the C-N bond of dipeptides with prolyl residues at the carboxy-terminus (Cheng *et al*., 1996). It has been suggested that the activity of OPAA on organophosphorous compounds is due to a fortuitous similarity between these compounds and X-pro dipeptides (Cheng *et al*., 1998). If this is the case, it would support the hypothesis that there is no evolutionary link between OPAA, MPH and PTE and suggests they emerged from different progenitors.

Since functions can be fulfilled in a number of ways, a variety of different proteins can acquire similar functions. Between the creation of OPs as insecticides by Farbenfabriken Bayer AG in 1937 and the first gene cloned in 1985, a time frame of 60-70 years can be placed on the evolution of OP-hydrolyzing activity. While this may challenge traditional views on the pace of evolution, experimental studies with an evolved β-galactosidase operon in *E. coli* indicate it is certainly possible to acquire a novel function within a relatively short period of time given the proper environmental

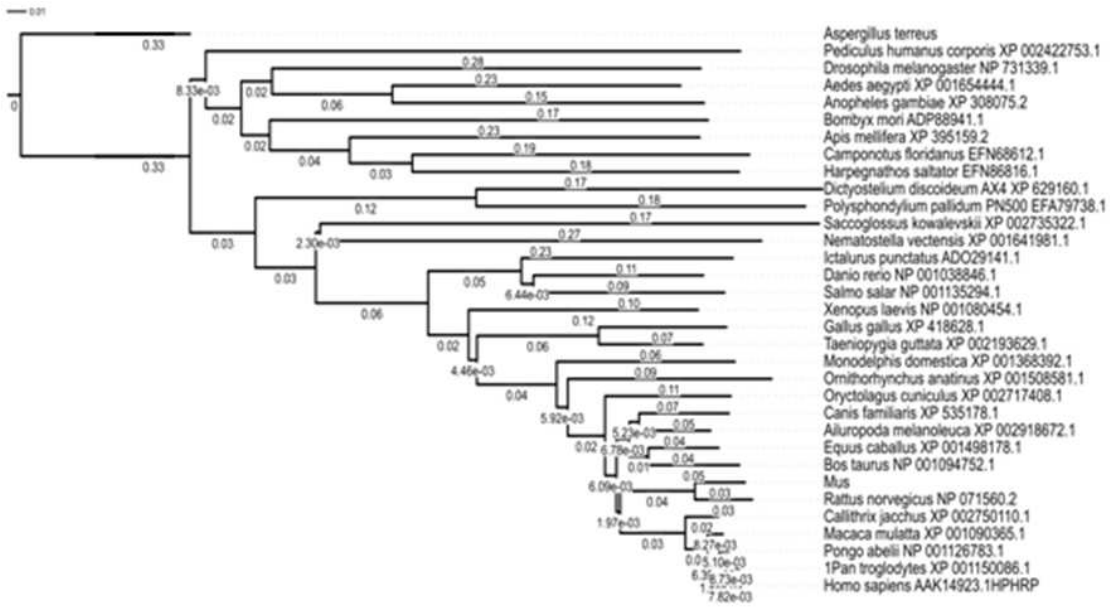

Fig. 3: Phylogenetic tree of eukaryotic Phosphotriesterases. The *B. diminuta* organophosphate degrading (*opd*) gene homologues were collected from the NCBI database and used to construct phylogenetic tree. using online tool 'interactive tree of life' (www.itol.embl.de)

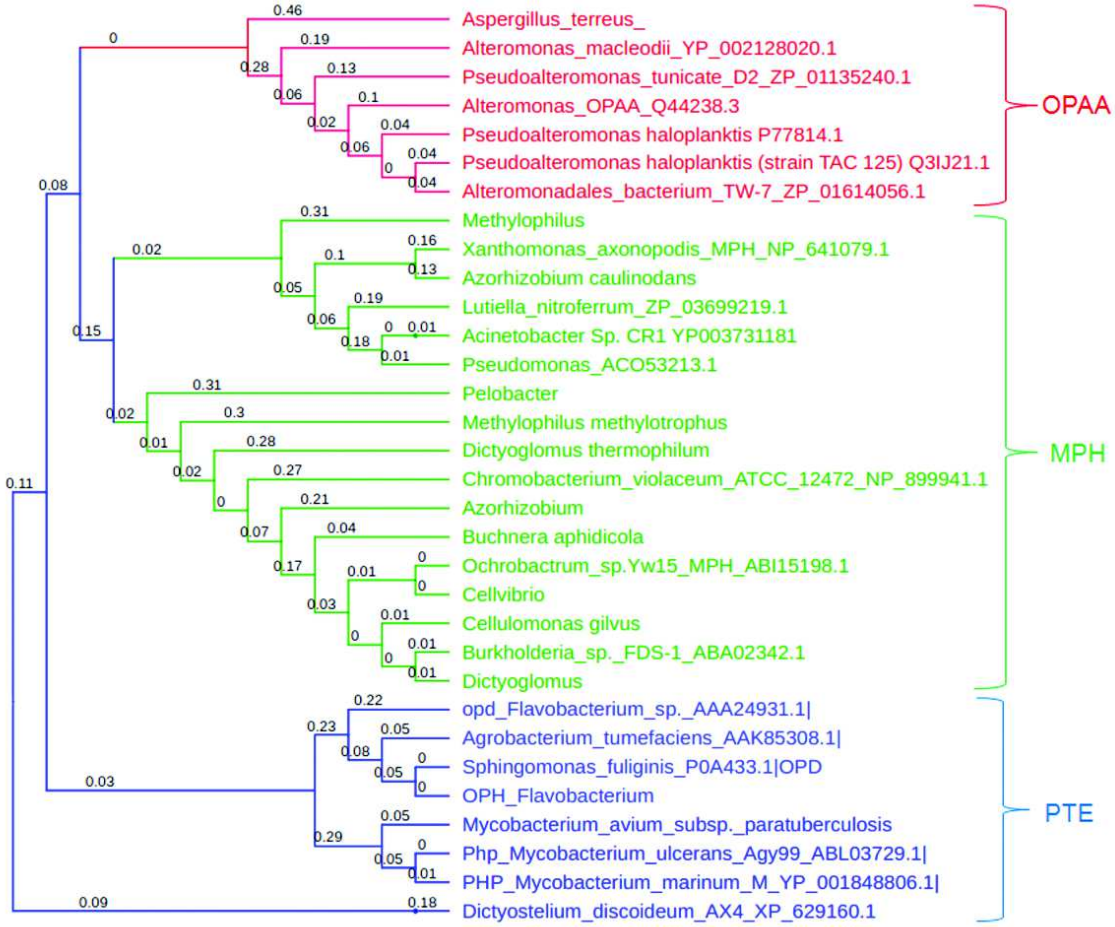

Fig. 4: Phylogenetic tree of bacterial organophosphate hydrolases. The homologues of *opd* (PTE), *mpd* (*MPH*) and *opaA* (OPAA) coding sequences available in NCBI database were used to construct phylogenetic tree using online tool 'interactive tree of life' (www.itol.embl.de)

conditions (Campbell *et al*., 1973; Hall, 1999). OPH provides an additional challenge in that not only did it evolve the OP-hydrolyzing function in such a relatively short time, but it also reached what has been described as an evolutionary endpoint, catalysis at the limit of diffusion control (Albery and Knowles, 1976; Caldwell *et al*., 1991). This framework for the evolution of catalysis predicts that *Bd* PTE should have absolute specificity for paraoxon. This is not the case, as *Bd* PTE has appreciable reaction rates with a variety of OP compounds. In evaluating the evolutionary optimization of catalysis, it may be important to consider whether the substrate is a physiological substrate. The distinction between physiological and non-physiological substrates is also important to consider when evaluating the evolutionary dynamics of catalysis (Demetrius L, 1998). For most enzymes, the significant component for evolution of catalysis is predicted to be competition between physiological substrates and related compounds for the active site of the enzyme. However, with xeno-biotics, significant evolutionary pressure may derive from competition between the various metabolic targets of the compound. For example, OP compounds bind and inhibit acetyl-cholinesterase in humans, and the evolutionary dynamic of PON1 may be determined by competition with acetyl-cholinesterase. An obvious analogy is missing in bacterial cells, as they do not possess cholinesterases. However, Buchbinder *et al*. (1998) have suggested that the *E. coli* PTE (*ec*-PTE), which has no OP-hydrolyzing capacity, may belong to the family of proteins from which the functional phosphotriesterases, such as *bd*-PTE, evolved. Similar to *bd* PTE, the *ec*-PTE structure consists of a long, elliptical α/β barrel with a binuclear zinc center in the cleft at the carboxy end of the barrel near the

presumptive active site. Although the aspartate and all four histidine residues that coordinate Zn^{2+} in OPH are conserved, significant differences between the two structures are found in the region corresponding to the active site. The active site of *bd*-PTE has been described with the non-hydrolyzed ligand, diethyl-4 methylbenzylphosphonate (Vanhooke *et al*., 1996). The ligand occupies a site near the binuclear metal center in a fairly hydrophobic pocket defined by Trp 131, Tyr 309, His 257, Leu 271, Phe 306 and Met 317. Of these residues, Met 317 is the only relatively conservative substitution (Leu 250), while Phe 306, Tyr 309 and Leu 271 have no corresponding residues in *ec*-PTE (Buchbinder *et al*., 1998). Existence of such structural homologues may provide a structural framework necessary for having competitive interactions with substrates (organo-phosphates). If a mutation at an appropriate position facilitates acquiring a new catalytic function, the presence of a substrate, the OP compounds, serves as a positive selection pressure to stabilize its presence.

Lactonases to Phosphotriesterases

Promiscuous activities play a key role in the evolution of enzymes. They actually serve as a starting point for acquiring a new function through gene duplication (Harper *et al*., 1988; Benning *et al*., 1994; Lai *et al*., 1995; Kolalowski *et al*., 1997; Rastogi *et al*., 1997;). In fact, these promiscuous activities are considered to be the vestiges of the function of their corresponding ancestral protein (Lai *et al*., 1995; Kolalowski *et al*., 1997). The phospho-triesterases have been shown to have phosphodiesterase, carboxyl esterase, and lactonase activities (McDaniel *et al*., 1988; DeFrank *et al*., 1993; Cheng *et al*., 1997). In general, family members that have diverged from a common ancestor often share promiscuous activities (Poelarends *et al*., 2005; Roodveldt *et al*., 2005b; Yew *et al*., 2005; Elias *et al*., 2008). Afriat *et al*. have elegantly shown the existence of reciprocal promiscuities between lactonases and tri-esterases (Afriat *et al*., 2006). Based on the structural differences, especially in the loops 1, 7 and 8 that comprise substrate-binding sites, they have classified OPH homologues into three groups. In the first group, designated as phospho-triesterases (PTE), with more than 86% identity to *bd* PTE, the existence of promiscuous lactonase activity has been demonstrated. The *ec* PTE is in the second group of OPH homologues, as it contains shorter substrate-binding loops. The third group of enzymes has only one loop (loop 7) shorter than PTE (Fig. 5). The second and third groups have less than 35% homology to the PTE family. These proteins,

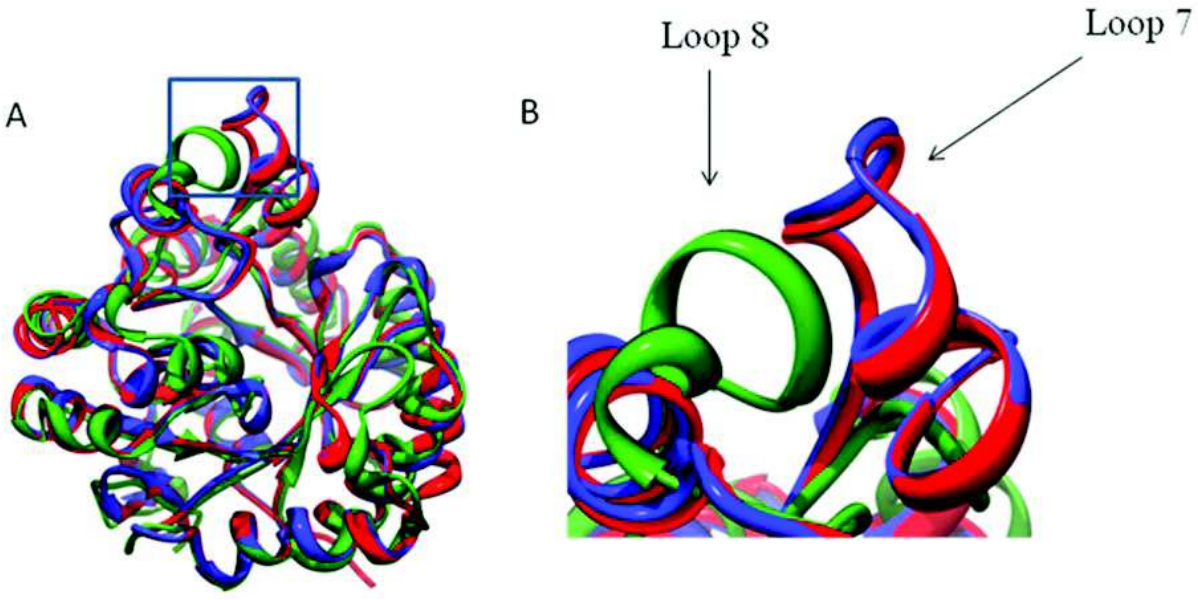

Fig. 5: Structural basis for natural lactonase and promiscuous phosphotriesterase activity. Superimposition of PTEs of *B. diminuta* (red) and *A. radiobacter* (blue) with the phosphotriesterase like lactonases PLLs, (green) of *S. solfataricus* (SsoPox), Absence of 15 residue long loop 7 in SsoPox, is shown with arrow mark. The crystal structures obtained from Protein data bank (www.pdb.org) were superimposed using UCSF chimera and the images were traced using persistence of Vision Raytracer Version 3.6 (www.povray.org)

annotated in the database as putative parathion hydrolases, include AhlA from *R. erythropolis*, PPH from *M. tuberculosis* and SsoPox of *Sulfolobus solfataricus* and have been characterized as phophotriesterase-like lactonases (PLLs) (Afriat *et al*., 2006), essentially based on their fairly limited homology to *bd*-PTE. All of them proficiently hydrolyze lactone with distinctively low Km (10-230 mM) values and a very weak phospho-tristerase activity (10^2- to 10^6-fold). Arylesterase activity was shown only by SsoPox. In principle, promiscuous activity is that activity shown by a family member not seen with other members of the family. If activity is shown by all the members, it is considered to be indicative of native function (Khersonsky *et al*., 2006). If this is taken into consideration, the PLLs are lactonases with promiscuous phospho-triesterase activity and the PTEs are phospho-triesterases with promiscuous lactonase activity. The substrate-binding loops contribute the main structural difference between PTEs and PLLs (Afriat *et al*., 2006).

Indeed insertions, deletions and loop-swapping are believed to be a primary mechanism for creating enzyme diversity (Tawfik, 2006; Park *et al*., 2006; Soskine and Tawfik, 2010). A number of studies have used PLLs as templates for directed evolution and succeeded in either enhancing the substrate range, catalytic efficiency (Jeng *et al*., 2009) or converting PLLs to catalyze altogether new reactions (Mandrich and Manco, 2009). Among the proposed PLLs the archaeal triesterase alone is shown to have arylesterase activity (Afriat *et al*., 2006). As *ec*-PTE has also shown arylesterase activity, the PLL, SsoPox, due to the existence of similar activity and structural similarities, is proposed to be a "generalist" molecule that served as a template for evolution of phospho-triesterases found in mesophilic organisms (Afriat *et al*., 2006; Merone *et al*., 2008). In fact, the recent discovery of a phosphotriesterase-like carboxyesterase, (*Mlo*PLC) from *Mesorhizobium loti* and its transformation into a diesterase through *in vitro* evolution supports the hypothesis proposed by Afriat *et al*., 2006 (Mandrich and Manco, 2009).

Metallo-β-lactamase to Methyl Parathion Hydrolase

A scenario with the evolution of methyl parathion hydrolases (MPH) appears to be in no way different from the evolution of the PTEs. They appear to have evolved from ß-lactamases with which they share considerable structural homology. The N-acyl-L-homoserine lactone (AHL) lactonases are members of the metallo-β-lactamase super-family and contain two zinc ions in their catalytic center (Aravind, 1999;

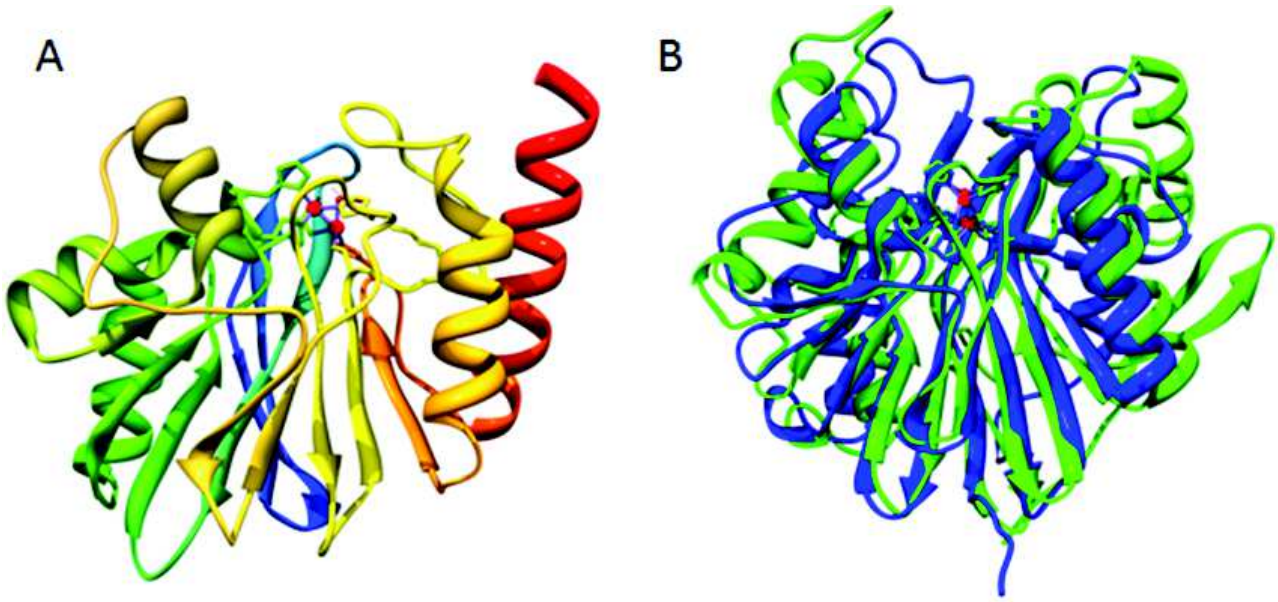

Fig. 6: Structural comparison between metallo ß-lactamase from *Serratia marcescens* (A) and superimposed crystal structures of AHL Lactonase (blue) from *Bacillus thuringiensis* and methyl parathion hydrolase (green) from *Pseudomonas* sp. WBC-3 (B). The crystal structures obtained from Protein data bank (www.pdb.org) were superimposed using UCSF chimera and the images were traced using persistence of Vision Raytracer Version 3.6 (www.povray.org)

Daiyasu *et al*., 2001; Crowder *et al*., 2006). The recently solved crystal structure of *Bacillus thuringiensis* AHL lactonase (Liu *et al*., 2008) has a striking similarity with the crystal structure of MPH (Dong *et al*., 2005) (Fig. 6). The AHL lactonase is also shown to have promiscuous triesterase activity (Afriat *et al*., 2006). If these findings are taken together with the aforementioned experimental evidence gathered to show the structural relationship between lactonases and phosphotriesterases, the proposal that metallo β lactamases were the progenitors of MPH is worthy of consideration.

Ample evidence available on the possible evolution of phosphotriesterases from quorum 'quenching' hydrolases and the existence of these traits on transposable elements suggests that their recent 'evolution' is a consequence of OP-induced gain-of-function. An increased presence of these OP compounds in agricultural soils due to repeated and excessive use would have created the necessary positive selection pressure to distribute the newly acquired functionality among microbial populations. The existence and transmission of such traits on self transmissible plasmids like pCMS1 would greatly facilitate lateral gene transfer.

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