The evolutionary history of detoxifying enzymes may explain their ambiguous and overlapping specificity profiles.

**Background:** Serum paraoxonases (PONs) comprise a family of mammalian detoxifying lactonases with different substrate specificities.

**Results:** A newly identified bacterial PON and the reconstructed ancestor of mammalian PONs are quorum-quenching lactonases.

**Conclusion:** PONs originated from quorum-quenching lactonases that later acquired new detoxifying functions.

**Significance:** The evolutionary history of detoxifying enzymes may explain their ambiguous and overlapping specificity profiles.

Serum paraoxonases (PONs) are a family of detoxifying enzymes that were first identified in mammals. Three mammalian families are known, PON1, PON2, and PON3 that reside primarily in the liver. They catalyze essentially the same reaction, lactone hydrolysis, but differ in their substrate specificity. Although some members are highly specific, others have a broad specificity profile. The evolutionary origins and substrate specificities of PONs therefore remain poorly understood. Here, we report a newly identified family of bacterial PONs, and the reconstruction of the ancestor of the three families of mammalian PONs. Both the mammalian ancestor and the characterized bacterial PONX_OCCAL were found to efficiently hydrolyze N-acyl homoserine lactones that mediate quorum sensing in many bacteria, including pathogenic ones. The mammalian PONs may therefore relate to a newly identified family of bacterial, PON-like “quorum-quenching” lactonases. The appearance of PONs in metazoa is likely to relate to innate immunity rather than detoxification. Unlike the bacterial PON, the mammalian ancestor also hydrolyzes, with low efficiency, lactones other than homoserine lactones, thus preceding the detoxifying functions that diverged later in two of the three mammalian families. The bifunctionality of the mammalian ancestor and the trade-off between the quorum-quenching and detoxifying lactonase activities explain the broad and overlapping specificities of some mammalian PONs versus the singular specificity of others.

Serum paraoxonases (PONs) are a family of detoxifying enzymes that were thus far found in all mammals, in certain vertebrates and nematodes, but not in bacteria (1, 2). PONs are paralogous enzymes that catalyze the same reaction with different substrates. The three known mammalian PON families (PON1, PON2, and PON3) share ~65% sequence identity, whereas in other vertebrates, a single PON gene is found. The name of the family, paraoxonases, was derived from the ability of PON1 to hydrolyze the pesticide paraoxon (Fig. 1). However, in fact, PONs are lactonases (3, 4), and paraoxon turned out to be a substrate that is promiscuously hydrolyzed by PON1 and barely hydrolyzed by PON3 or PON2 (4). Nor are PONs “serum” enzymes. Although PON1 and PON3 are associated with serum HDLs (5), they are present predominantly in the liver, whereas PON2 is expressed in nearly all tissues (6). The physiological roles of PONs may include the detoxification of lactones (exogenous, e.g., from food, or endogenous lactones derived from oxidized lipids) and organophosphates, as well as antioxidative functions that relate to arteriosclerosis and cancer (7, 8). However, as is the case with other families of detoxifying enzymes (9–16), the physiological substrate(s) of PONs remain largely unknown. More recently, PONs have also been found to hydrolyze N-(3-oxo-dodecanoyl)-homoserine lactone (3OC12-HSL; Fig. 1) (17), a quorum-sensing molecule that regulates virulence and biofilm formation in many bacteria (18). This implied an additional, antimicrobial role for PONs and for PON2 in particular (19, 20).

Some PON family members (mainly PON2) appear to be highly specific, whereas others (PON1 and PON3) exhibit broad specificity. Biochemically, PON1 and PON3s seem like a “jack of all trades.” They hydrolyze a wide range of δ- and γ-lactones with lipophilic side chains (fatty acid lactones or lipo-lactones; Fig. 1) with $k_{cat}/K_m$ values of $10^4$–$10^6$ $\text{M}^{-1}\text{s}^{-1}$ (4, 21). PON1 and PON3 also hydrolyze the quorum-sensing lactone 3OC12-HSL, yet with much lower efficiency (17). PON1 and PON3 also exhibit paraoxonase and aryl-lactonase activities. In contrast, PON2 shows no detectable paraoxonase, aryl-lactonase, or lipo-lactonase activity but, as recently discovered, efficiently hydrolyzes the quorum-sensing molecule 3OC12-HSL (17, 20). The confusion is augmented by PONs being most commonly characterized by their paraoxonase and aryl-esterase activities (2), although these turned out to be purely coincidental (4). Such bewildering trends are apparent in other mammalian detoxifying enzymes and in multiparalogues families of plant enzymes (22). By analogy, 1-chloro-2,4-dinitrobenzene (a...
synthetic substrate of no physiological relevance) is taken by all GSTs. GST-A1–1 conjugates a broad range of substrates, but GSTA4–4 is selective for long chain alkenals, and GST-P1 is selective for ethacrynic acid (23).

In light of their phylogeny and their confusing substrate specificity patterns, it remains unclear at which evolutionary stage and from which origin, the ancestor of the vertebrate and mammalian PONs was recruited and for what purpose: detoxification or possibly a different function. Following Dobzhansky’s tenet (24), we surmised that the biochemical features of mammalian PONs might make sense in view of their evolutionary history. To this end, we attempted to identify new PON genes, and particularly bacterial PONs, and also reconstructed the ancestor of the mammalian PONs. Our findings indicate that the mammalian PONs diverged from an ancestor that was primarily a quorum-quenching HSLase and as such may relate to a newly identified family of bacterial PON-like quorum-quenching HSLases. Our findings also suggest that the bewildering pattern of substrate specificities in PONs may relate to the mechanism of their divergence and, specifically, to the bifuntionality of the reconstructed mammalian PON ancestor that is capable of hydrolyzing quorum-sensing lactones as well as lipophilic lactones.

**EXPERIMENTAL PROCEDURES**

**Identification of PON Sequences and Tree Reconstruction**—PON sequences were collected from the NCBI protein sequence database using BLAST. A more exhaustive search of expressed sequence tags and recently sequenced genomes revealed potential PONs in opossum, lizard (*Anolis carolinensis*), zebra finch, sea urchin, hydra, insects like *Drosophila melanogaster*, fungal, bacteria, a partial PON sequence in platypus, but none in plant genomes. Sequences in which essential active site residues (His-115, His-134, Cys-43, Cys-353, Glu-53, Asp-54, Asn-168, Asp-169, Asn-N224, Asp-268, and Asn-269) (25) were substituted were discarded. To minimize potential biases in the alignment, the redundancy was eliminated by using the Cd-hit program (26), such that the remaining sequences shared 30–90% identity. Sequence alignment was done by using PRANK (27) with the F+ option and Muscle (28). The evolutionary models most appropriate for analyzing our PON sequences were determined using Prottest (29). The alignments were used to generate trees with the Phyml program (30) by using the LG substitution matrix (31) combined with +I and +G parameters selected by Prottest. Bootstrap resample tests were performed 100 times to confirm reliability of the constructed trees, and the resulting bootstrap values for the corrected tree were calculated by the Raxml program (32). The Phyml generated trees were largely in accordance with the species tree. However, in the tree based on the MUSCLE alignment, chicken and frog PONs were grouped with the mammalian PONs. We therefore chose to proceed with the tree derived from the PRANK alignment. In this tree, the only two significant discrepancies were that PONX_TETNG from the spotted green pufferfish was not clustered with all other fish PONs but as an outgroup for amphibian and that PONX_NEMATODE was clustered with the bacterial PONs. These discrepancies were manually corrected to give the final tree for ancestral inference. The Shimodaira-Hasegawa test (see below) indicated the validity of the modified topology. The Drp35 (Protein Data Bank code 2DG1) and glucuronolactonase (Protein Data Bank code 3DR2) families that share the fold and catalytic calcium of PON were added as outgroups to the tree. Their sequences were collected by Blast search and structurally aligned with PON1 (Protein Data Bank code 1V04) using Mustang (33). Additional PON sequences were added to the structural profile by Clustalw2 (34), and the alignment was manually refined at gaps. These alignments were used to generate the trees by Phyml as described above. All the trees and the alignment files can be obtained via the corresponding author.

**Shimodaira-Hasegawa Test**—In all maximum likelihood trees, the PON-like sequences from fungal were placed as an outgroup to the bacterial PONs and not in proximity to the metazoan PONs as expected by the species closeness. Using the Shimodaira-Hasegawa test, the derived tree topology was compared with an artificially generated tree that coincides with the species tree (The tree file can be obtained via the corresponding author). The SH test is a likelihood-based statistical test for comparing alternative evolutionary trees (and thereby of hypotheses that relate to the alternative trees). The test indicates whether alternative trees are significantly less valid than the derived maximum likelihood tree. The test was performed with Raxml (32) using the SH test option.

**Ancestral Predictions**—Ancestral sequences were inferred using maximum likelihood methods: Fastml (35). The most probable ancestor of mammalian PONs, mpa-N9 (dubbed N9), was predicted from the phylogenetic tree that was reconstructed prior to the addition of the lizard and zebra finch PON. The inclusion of these sequences resulted in the prediction of N9* that differed from the initially predicted N9 in 11 positions. However, the enzymatic features of both these ancestors were essentially identical (data not shown).
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TABLE 1
The diversified positions in the PON1-N9 transition library spiked with ancestral mutations

All positions within or near the active site where the ancestral state (the amino acid found in the inferred mammalian ancestor, N9) differs from PON1 were identified and spiked as individual mutations at an average of 3.5 substitutions per library variants. Listed are the ancestral mutations included in the library and their frequency in the variants exhibiting the highest HSLase activity.

| Residue | PON1 (G3C9) | Mammalian PON ancestor-N9 | Frequency in 12 selected variants |
|---------|-------------|---------------------------|---------------------------------|
| 55      | Leu         | Ile                       | %                               |
| 56      | Glu         | Asp                       | 17                              |
| 74      | Ile         | Leu                       | 16                              |
| 75      | Met         | Lys                       | 16                              |
| 78      | Asp         | Ala                       | 8                               |
| 136     | Asp         | His                       | 8                               |
| 137     | Ser         | Met                       | 8                               |
| 189     | Pro         | Phe                       | 8                               |
| 190     | Phe         | Ile                       | 17                              |
| 193     | Ser         | Phe                       | 16                              |
| 194     | Thr         | Leu                       | 16                              |
| 197     | His         | Tyr                       | 58                              |
| 222     | Phe         | Ser                       | 58                              |
| 237     | Ile         | Val                       | 25                              |
| 239     | Glu         | Asp                       | 16                              |
| 240     | Leu         | Ile                       | 16                              |
| 244     | Lys         | Asn                       | 16                              |
| 271     | Ile         | Leu                       | 16                              |
| 291     | Ile         | Leu                       | 16                              |
| 293     | Tyr         | Phe                       | 16                              |
| 332     | Thr         | Ser                       |                                 |

Back-to-Ancestor PON1 Library—All substitutions separating PON1 from the mammalian ancestor N9 that are within and near the active site (±12 Å from the catalytic calcium) were identified (Table 1). These back-to-ancestral substitutions were combinatorially incorporated into the recombinant PON1 gene using the oligonucleotide spiking method (36). The oligonucleotides incorporated the ancestral amino acid at individual active site positions with the original sequence of PON1 flanking the mutated position. The oligonucleotide mix was added to ~100 ng of DNaseI-generated fragments of the PON1 gene (~70–150 bp length), and an assembly PCR was applied, thus resulting in the combinatorial incorporation of an average of 3.5 ancestral substitutions per PON1 gene. Following a nested PCR with external primers flanking the PON1 gene, the purified PCR product was digested and ligated into the expression vector.

Gene Cloning—The genes encoding PONX_FLABA, PON2_9SPHI, PONX_OCCAL, and N9* were synthesized by Genscript. The gene encoding N9 was synthesized by Entechelon GmbH. All PON genes, including the PON1-N9 transition library, were cloned into the pASK-IBA3plus (IBA) by replacing the ribosome binding and multiple cloning sites (spanning the Xbal and HindIII restriction sites) with the ribosome-binding site, the Trx tag, and the multiple cloning site of pET32b(+) (Invitrogen). Ligated DNA was transformed to *Escherichia coli DH5a* (Invitrogen) and plated on LB agar supplemented with 100 mg/ml ampicillin (LB-Amp). The plasmid was extracted and used for transformation into *E. coli* Origami B (DE3) cells (Novagen) for expression.

Expression and Purification—For expression of N9, N9*, the newly identified bacterial PONs, and selected variants from the PON1-N9 transition library, a single colony of freshly transformed *E. coli* Origami B (DE3) cells (Novagen) was grown in LB medium containing 100 mg/liter ampicillin (LB-Amp, 2 ml). Cultures were grown at 37 °C overnight and were used to inoculate 50 ml of fresh LB-Amp medium containing 1 mM CaCl$_2$. Cells were grown at 30 °C to A$_{600}$ nm of ~1, and overexpression was induced by adding 200 µg/liter anhydrotetracycline. Cultures were shaken at 20 °C for ~16 h, and the cells were harvested and subjected to two cycles of freezing at ~20 °C and thawing at 37 °C. The thawed cells were lysed in lysis buffer (50 mM Tris, pH 7.5, 1 mM CaCl$_2$, 50 mM NaCl, 1:500 PIC (Protease inhibitor mixture, Sigma), 5 units/ml benzonase nuclease (Novagen), 1 mg/ml lysozyme, 0.1% Triton X-100) at 4 °C for 6 h. The clarified lysates were incubated with nickel-nitrilotriacetic acidagarose resin overnight. The resin was rinsed with IMAC buffer (50 mM Tris, pH 7.5, 1 mM CaCl$_2$, 0.1% Triton X-100) containing 10 mM, 20 mM, and finally 40 mM imidazole. The purified enzyme was eluted with IMAC buffer containing 250 mM imidazole. Enzymatically active fractions were pooled and dialyzed against activity buffer (50 mM Tris, pH 8.0, 1 mM CaCl$_2$, 0.1% Triton X-100).

Applying the above procedure, all reconstructed ancestors gave high yields of soluble and active protein. The bacterial PONs proved more problematic. PONX_OCCAL was successfully purified and characterized, although at relatively low yields (~1 mg of protein to 1 liter of culture). In the case of PONX_FLABA, PON2_9SPHI, and PONX_9RHIZ ≤10% of the activity detected in the lysate could be detected after purification, by either nickel-nitrilotriacetic acid or anion exchange. Lowering the lysis and purification temperature to 4 °C, the addition of glycerol to the lysate or of detergents such as tergitol, octyl glucoside, or dodecyl maltoside and other measures we attempted (e.g., co-expression with GroEL/ES), did not yield purified enzymes with significant activity. Recombinant forms of mammalian PON1 and PON3 were expressed in *E. coli* (37, 38). Human PON2 was expressed in baculovirus and purified as described (3).

Enzymatic Assays—Substrate stock solutions (×100) were prepared in acetone/ethanol. Hydrolysis of TBBL, p-nitrophenyl acetate, phenylacetate, dihydrocumarine, paraoxon, and methylparaoxon was monitored in activity buffer as described (3, 39). The hydrolysis of various lactones and HSLs was monitored with a pH indicator in BSCP buffer (2.5 mM bicine, 50 mM NaCl, 1 mM CaCl$_2$, 50 mM m-cresol purple, 0.1% Triton X-100) at 580 nm. The extinction coefficient was determined by titration with acetic acid for each kinetic run (~2,000 A$_{580}$ nm/M$_{substrate}$). The background hydrolysis with no enzyme was measured for each substrate concentration and subtracted from the enzyme-catalyzed rates. All kinetic measurements were performed at room temperature, and error ranges were derived from at least two independent measurements. Kinetic parameters were obtained by measuring initial rates at substrate concentrations ranging from 0.3 × $K_m$ up to 3 × $K_m$ and fitting the data directly to the Michaelis-Menten equation using Kaleidagraph. In cases where solubility limited substrate concentrations, data were fitted to the pseudo-first order equation ($v_0 = \frac{[S]_0[E]_0}{k_{cat}[K_m]}$), and $k_{cat}/K_m$ was deduced. All of the data presented are the averages of at least two independent experiments. The errors represent
standard deviations for the applied fit, or the maximal deviation seen between two independent measurements, whichever value was larger.

**PAO-JP2 Luminescence Assay**—Screening of variants from the PON1-N9 library for 3OC12HSLase activity was performed in crude bacterial lysates using a *Pseudomonas aeruginosa* PAO1-JP2 strain that harbors a reporter luciferase gene under the *LasI* promoter (40). In this assay luminescence levels correspond to the concentration of 3OC12HSL, but the hydrolyzed lactone yields no response. PAO-JP2 cells were grown overnight at 37 °C and used to inoculate (1:100) LB medium plus trimethoprim (300 μg/ml). From each library variant, 5 μl of clarified lysate (see above) was preincubated for 20 min in 50 μl of activity buffer containing 0.2 μM of 3OC12HSL. The reaction mixtures were added to 450 μl of freshly inoculated PAO-JP2 cultures, and the cultures were shaken for 3 h at room temperature. Luminescence levels were measured in 460/40 nm to determine the concentration of intact (nonhydrolyzed) 3OC12HSL. The latter were determined from a standard calibration curve generated from PAO1-JP2 cells incubated with 3OC12HSL at 0–20 nm concentration.

**Structural Modeling**—Structural models of the bacterial PONs were generated using the 3D-JIGSAW homology modeling server (41).

**RESULTS**

**Bacterial PONs, a New Family of Quorum-quenching Lactonases**—We sought to identify new PON sequences that may clarify this family’s history. To date, PONs were identified in mammals and sporadically in vertebrates and invertebrates including nematodes (1). However, our search of the NCBI protein sequence database revealed five new bacterial PON-like sequences: PONX_FLABA from *Flavobacteriales* bacterium HTCC2170, PON2_9SPHI from *Microscilla marina* ATCC 23134, PONX_OCCAL from *Oceanicaulis alexandrii* HTCC2633, PONX_Pha from *Phaeobacter gallaeciensis*, and PONX_9RHIZ from *Parvibaculum lavamentivorans*. These sequences were defined as PONs based on ≥30% sequence identity with human PON1 and absolute conservation of the active site residues. PON1 is a six-bladed β-propeller with two calcium atoms: a structural and a catalytic one, in the central tunnel of the propeller. Catalysis is mediated by the catalytic calcium, its ligating residues, and the His-115–His-134 dyad (25). These elements are utterly conserved in the newly identified bacterial PONs (Fig. 2a).

**FIGURE 2. Sequence and structural comparison of the newly identified bacterial PONs with mammalian PON1.** a, sequence alignment of human PON1 with the newly identified bacterial PONs. The calcium-ligating residues and other essential active site residues are marked in red. b, a homology model of the newly identified bacterial PON, PONX_OCCAL, aligned with the crystal structure of mammalian PON1 (Protein Data Bank codes 1V04 and 3SRG). The two calcium ions that are essential for maintaining the structure and catalytic activity of PON are marked as yellow spheres, and the catalytic histidine dyad (His-115 and His-134) are turquoise sticks. Also annotated are the N-terminal helix (H1) and the adjacent active site helix (H2) that mediate PONs association with lipids (25, 81). c, Michaelis–Menten plots comparing PONX_OCCAL hydrolysis rates of the quorum-sensing lactones C12HSL and 3OC12HSL with those of the reconstructed mammalian ancestor N9 and human PON2. d, the computed propensity of the N termini of the bacterial PONX_OCCAL as being a transmembrane region was calculated by the prediction algorithm TMHMM (72) and is plotted as pink bars. The sequence stretch that overlaps the N-terminal helix (H1) of mammalian PON1 shows the highest probability.
Synthetic genes encoding the newly identified bacterial PON-like sequences were expressed in *E. coli* under a range of conditions. However, only PONX_OCCAL could be purified while maintaining enzymatic activity (see “Experimental Procedures”). PONX_OCCAL was found to be a proficient quorum-quenching lactonase with marked specificity toward 3-oxo-HSLs (Fig. 2c and Table 2). The *k*<sub>cat</sub>/K<sub>m</sub> for 3OC12-HSL was ∼2.4 × 10<sup>5</sup> M<sup>−1</sup>s<sup>−1</sup> and ∼80-fold higher than with the corresponding HSL with an unmodified acyl chain C12-HSL (Fig. 2c and Table 2). The activity of PONX_OCCAL also decreased with substrates with shorter acyl chains, down to 120-fold lower specific activity with 3OC6-HSL (Table 3). However, the bacterial PONX_OCCAL distinctly lacks the lipophilic lactonase activity seen in PON1 and 3 (Table 2). It also lacks the promiscuous paraoxonase activity present in mammalian PONs and exhibits very weak aryl-esterase and aryl-lactonase activity relative to mammalian PON1 (Table 4).

**TABLE 2**

| Lactone substrate | PON variant | N9 | PON1 | PON2 | PON3 | PONX_OCCAL |
|-------------------|-------------|----|------|------|------|-------------|
| **3OC12HSL**      |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 31.6±3.3 | 13.0±0.006 | 43.5±2.14 |
| K<sub>m</sub> (mM) | 0.17±0.04 | 0.05±0.014 | 0.18±0.01 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (1.9±0.3)X10<sup>5</sup> | (2.7±0.08)X10<sup>5</sup> | (2.4±0.07)X10<sup>5</sup> |
| **C12HSL**        |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 6.4±0.8 | ND | ND | 2.2±0.14 |
| K<sub>m</sub> (mM) | 0.41±0.014 | ND | ND | 0.05±0.01 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (1.6±0.15)X10<sup>4</sup> | <<(0.13±0.08)X10<sup>3</sup> | (4.6±1)X10<sup>4</sup> |
| **3OC10HSL**      |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 15.9±0.5 | ND | ND | ND |
| K<sub>m</sub> (mM) | 0.09±0.01 | ND | ND | ND |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (1.8±0.3)X10<sup>5</sup> | (5.9±0.06)X10<sup>3</sup> | (2.8±0.8)X10<sup>4</sup> | (2.1±0.01)X10<sup>4</sup> |
| **δ-nonalactone** |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 40.3±0.04 | ND | 28±0.05 |
| K<sub>m</sub> (mM) | 1.65±0.2 | ND | 3±0.007 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (2.2±1.1)X10<sup>3</sup> | (8.8±0.8)X10<sup>4</sup> | (1±0.1)X10<sup>4</sup> |
| **δ-undecalactone** |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 76±2.5 | ND | 14.9±1.8 |
| K<sub>m</sub> (mM) | 1.28±0.17 | ND | 1.15±0.2 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (1.8±0.5)X10<sup>3</sup> | (3.2±0.4)X10<sup>4</sup> | (2.5±0.5)X10<sup>4</sup> |
| **γ-nonalactone** |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 76±2.5 | ND | 2.8±0.8 |
| K<sub>m</sub> (mM) | 2.1±0.007 | ND | 2.8±0.8 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (3.6±0.1)X10<sup>4</sup> | (1.1±0.07)X10<sup>5</sup> | (5.3±0.8)X10<sup>3</sup> |
| **γ-undecalactone** |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 74.2±0.4 | ND | 17.4±0.6 |
| K<sub>m</sub> (mM) | 1.02±0.08 | ND | 0.57±0.04 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (7.3±0.6)X10<sup>4</sup> | (1.4±0.033)X10<sup>5</sup> | (3±0.1)X10<sup>4</sup> |
| **TBBL** |             |    |      |      |      |             |
| *k*<sub>cat</sub> (s<sup>−1</sup>) | 10.7±0.17 | ND | 9.6±0.4 |
| K<sub>m</sub> (mM) | 0.07±0.002 | ND | 0.3±0.04 |
| *k*<sub>cat</sub>/K<sub>m</sub> (s<sup>−1</sup>M<sup>−1</sup>) | (1.5±0.02)X10<sup>5</sup> | (4.6±0.01)X10<sup>5</sup> | (3±0.3)X10<sup>4</sup> |
All currently known HSLases are bacterial, and these enzymes belong to completely different superfamilies than PONs (42, 43). The enzymatic profile of PONX_OCCAL reveals that this newly identified bacterial PON is a quorum-quenching lactonase with highly similar enzymatic features to AiiA, the firstly discovered HSLase from the metallo-β-lactamase superfamily (43, 44), and to phosphotriesterase-like lactonases (PLLs), the second known family of bacterial HSLases from the amidohydrolase superfamily (42).

The structural homology between the mammalian PONs and the bacterial one is not limited to the scaffold and the active site. The newly identified bacterial PONs have a predominantly hydrophobic sequence of ~20 amino acids at their N termini that may correspond to a membrane anchoring helix (Fig. 2d). This sequence overlaps with the HDL-anchoring helix of mammalian PONs and therefore represents an interesting case of evolutionary tinkering. The N-terminal helix of PON1 (dubbed H1) mediates binding of the enzyme to HDL together with another surface helix dubbed H2 (25). H2 is also predicted to adopt a similar structure in PONX_OCCAL (Fig. 2d) and in the other bacterial PONs (data not shown). It seems therefore that the structural features that mediate the ability of PONs to associate with lipids were already present in their bacterial ancestors.

The PON Family Phylogenetic Tree—We generated a maximum likelihood tree that included the newly identified bacterial PONs and other PON sequences collected from the NCBI nonredundant database and by an exhaustive search of available expressed sequence tags and recently sequenced genomes. Only sequences that showed ≥30% amino acid identity to mammalian PONs and contained the essential active site residues were included. Despite being of much relevance, large insertions in the opossum PON (that seem to derive from sequencing errors and misassignment of exon-intron junctions) and the lack of a fully sequenced PON in the platypus genome prevented their inclusion in the tree.

The tree indicated that PONs appear in bacteria and then in nematodes, multicellular eukaryotes that belong to the animal phyla. Notably, all intermediate species between bacteria and nematodes were absent in the tree (Fig. 3a). The lack of sequence data and the fact that the bacterial PONs share only ~30% sequence identity with other PONs resulted in the low bootstrap value for the bacterial branch (~0.2; Fig. 3a). However, the presence of all essential active site residues in all bacterial PONs (Fig. 2a), their predicted structural similarity with mammalian PONs (Fig. 2b), and the efficient quorum-quenching lactonase activity of PONX_OCCAL (Fig. 2c and Tables 2 and 3) indicated that these bacterial sequences belong to the PON family. The topology of the tree, including the location of the bacterial clade were further validated as discussed below.

The species gap could, in principle, be bridged by the fungal PON-like genes we have identified. However, in some of these sequences, essential active site residues such as His-115 were replaced. Foremost, the fungal sequences were located in the tree as an outgroup to all PON sequences including the bacterial PONs, and alongside Drp35 and Gluconolactonases (enzyme families sharing six-bladed β-propeller of PONs; detailed trees and the alignment files can be obtained via the corresponding author). The outgroup location suggested that the fungal PON-like sequences comprise an independent clade and that these fungal sequences do not bridge the gap between the bacterial and mammalian PONs. This conclusion was validated by the Shimodaira-Hasegawa test (45), which compared the maximum likelihood tree to an artificially generated tree that coincided with the species tree (i.e., the null hypothesis). The test results indicated that the artificial tree is significantly less likely than the original tree. Overall, PONs phylogeny begins to be consistent with the “tree of life” only for vertebrates, starting with actinopterygii (ray-finned fish) and ending with mammals (Fig. 3a).

Reconstruction of the Mammalian PON Ancestor—To bridge the gap between bacterial and vertebrate PONs, we attempted to reconstruct two ancestral nodes: N6, the ancestor of vertebrate PONs, and N9, which preceded the divergence of the three mammalian PON families (Fig. 3a). As originally proposed by Zuckerandl and Pauling (46), with knowledge of the

| Variant   | C12  | 3OC12 | C10  | 3OC10 | C8   | 3OC8 | C6   | 3OC6 |
|-----------|------|-------|------|-------|------|------|------|------|
| N9        | 1.37 ± 0.25 | 16.06 ± 0.08 | 5.81 ± 0.22 | 10.84 ± 0.57 | 9.44 ± 0.03 | 2.38 ± 0.28 | 0.56 ± 0.02 | 0.67 ± 0.29 |
| PON1      | ND   | 2.81 ± 0.15 | 0.66 ± 0.08 | 1.84 ± 0.08 | ND   | ND   | ND   | ND   |
| PON2      | 0.09 ± 0.02 | 7.00 ± 0.90 | ND   | 5.65 ± 0.06 | ND   | ND   | 2.15 ± 0.23 | ND   |
| PON3      | ND   | 0.41 ± 0.07 | ND   | 0.27 ± 0.04 | ND   | ND   | ND   | ND   |
| PONX_OCCAL| 1.70 ± 0.02 | 24.16 ± 1.44 | 0.88 ± 0.13 | 5.01 ± 0.41 | 0.28 ± 0.12 | 0.70 ± 0.06 | 0.09 ± 0.02 | 0.20 ± 0.02 |

TABLE 3
Kinetic parameters of HSL substrates
Specific activities (unit = 1 μmol of substrate hydrolyzed/minute/mg enzyme, at [S]0 = 0.25 (mM)) for various quorum sensing lactones (HSLs). ND, no activity beyond background rate was detected at the highest enzyme concentration applied (0.5 μM enzyme).

| Table 4 |
|---------|

| Variant   | p-Nitrophenyl acetate | Dihydrocoumarin | Phenylacetate | Methylparaoxon | Paraaxon |
|-----------|----------------------|-----------------|--------------|----------------|---------|
| N9        | 1.59 ± 0.14          | 10.87 ± 1.22    | 0.91 ± 0.27  | 0.52 ± 0.01    | 0.06 ± 0.008 |
| PON1      | 2.10 ± 0.60          | 385.49 ± 57.79  | 206.94 ± 39.41 | 0.18 ± 0.09    | 1.28 ± 0.04  |
| PON2      | 0.81 ± 0.07          | 3.39 ± 1.30     | ND            | ND             | ND       |
| PON3      | 0.76 ± 0.07          | 64.87 ± 5.45    | ND            | ND             | ND       |
| PONX_OCCAL| 3.62 ± 0.22          | 3.86 ± 1.98     | ND            | ND             | ND       |
amino acid substitution pattern (or the substitution matrix) and given an alignment and a tree, the ancestral state for each position—namely the amino acid from which all extant sequences within a given tree topology are most likely to have diverged—can be predicted. The most probable ancestor (mpa) comprises the most probable amino acids for all positions along the entire protein chain (for reviews see Refs. 47 and 48).

Although ancestral reconstruction is an established methodology, it has caveats and limitations. For example, most reconstructions address a single sequence, the mpa (49–51), or several mpa-related sequences (52). However, because at most positions alternative amino acids are predicted, the prediction actually comprises an ensemble, or a cloud of sequences. The fidelity of prediction depends on this ensemble showing a homogeneous phenotype. In this case, we sampled the ancestral ensembles for both N6 (the vertebrate PON ancestor) and N9 (the mammalian ancestor) using a newly developed library approach. The N6 library exhibited large variability, in particular with respect to the HSLase activity, and thus the phenotype of N6 could not be reliably inferred. In contrast, randomly selected variants from the N9 ancestral ensemble and the most probable ancestor (mpa-N9), the properties of which are described here, exhibited essentially identical enzymatic activities.

Enzymatic Characterization of the Mammalian Ancestor—

The predicted mammalian ancestor (mpa-N9, or N9 thereafter) exhibited high quorum-quenching lactonase activity with 3OC12-HSL; the $k_{cat}/K_m$ value of $2 \times 10^5 \text{M}^{-1}\text{s}^{-1}$ was essentially the same as PONX_OCCAL and human PON2 (Table 2). This value is 20-fold higher than that exhibited by PON1 and 100-fold higher than for PON3. However, unlike PON2 and PONX_OCCAL, N9 specificity with respect to the acyl chain of HSLs (length, and 3-oxo modification) was much broader. For example, N9 activity with 3OC10-HSL is essentially the same as with 3OC12-HSL. Overall, the reconstructed mammalian ancestor exhibits higher activity than PON2 with all HSLs, including unmod-

mpa-N9 differs from the consensus sequence of all mammalian PONs in 22 positions, and its sequence identity with the closest extant mammalian sequence is <82% (Fig. 3b). The N9 predicted sequence was further validated by comparing the enzymatic properties of N9 with those of N9*, the mammalian ancestor predicted from a tree that included the lizard and zebra finch PON sequences (Fig. 4a). These two sequences (~55% similarity to mammalian PONs) were discovered when this work was at an advanced stage. They are included in the tree presented here (Fig. 3a) but were lacking in our initial tree from which N9 was inferred. N9 differed in 11 positions from N9* (Fig. 4b and c) yet exhibited essentially identical enzymatic activities (data not shown).

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FIGURE 3. PONs phylogenetic tree, and a sequence alignment of the mammalian PONs and their inferred ancestor, N9. a, PONs tree. Red branches mark the mammalian PONs, and blue ones the newly identified bacterial PONs (trees containing other enzyme families that share PONs six-bladed β-propeller fold as outgroup can be obtained via the corresponding author). b, the core sequence alignment that relates to the PON tree. Residue numbering is according to human PON1. The sequence identity of N9 with the extant mammalian PONs is in the range of 70% (PON3_MOUSE) to 82% (PON2_HUMAN). The alignment also shows the consensus sequence for mammalian PONs, that differs in 22 positions (marked in orange) including two positions that are within the active site (positions 193 L/F and 240 V/I).

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Indeed, N9 exhibits γ-lactonase activity, which is only several-fold lower than that of PON1 and largely resembles that of PON3 (k_{cat}/K_m = 5 × 10^5 M^{-1} s^{-1}). In contrast, the δ-lactonase activity of N9 (k_{cat}/K_m ≈ 2 × 10^6 M^{-1} s^{-1}) is ≤40-fold lower than that of PON1 and PON3 (Table 2). Indeed, lypophilic δ-lactones appear to be the best substrates of PON1 (3). It seems therefore that the evolution of higher rates of hydrolysis of δ-lactones in PON1 and PON3 came at the expense of their HSLase activity. The trade-off between these two lactone classes may stem from the fact that δ-lactones differ from HSLs in their ring structures (six-membered versus five-membered rings), as well as in their side chain locations and compositions.

The Trade-off Underlining PONs Divergence—To examine the trade-off between the HSLase and lipo-lactonase activities, we attempted to identify the minimal number of sequence changes that may allow the contemporary PON1 to revert back to the ancestral state of an efficient HSLase. An alignment of the mammalian ancestor N9 and human PON1 revealed 21 sequence exchanges in residues within and near the active site (Table 1).

The identified PON1-to-ancestor or back-to-ancestor substitutions were "spiked" as individual mutations into the PON1 gene. This resulted in a library where each gene variant contained a different subset of ancestral substitutions, at an average of ~3.5 ancestral substitutions per gene (for details regarding this strategy, see Ref. 53). The resulting library was screened for HSLase activity using the PAO-JP2 luminescence assay and 3OC12HSL as substrate. Sequencing of the 12 variants showing the highest HSLase activity revealed that ~60% of these variants contained the F222S substitution within the active site wall of PON1, and 25% contained I237V, a second shell residue, whereas other second and third shell substitutions appeared sporadically (Table 1).

Several library variants, including P2F1, that corresponded to PON1 with only two back-to-ancestor substitutions, F222S and I237V, were purified and characterized. Their k_{cat}/K_m values with 3OC12HSL as substrate were increased by up to 20-fold and became similar to those of N9 (k_{cat}/K_m ~ 2 × 10^6 M^{-1} s^{-1}) (Table 5).

Along with the increase in HSLase activity, the lipo-lactonase activities of all the three characterized variants were concomitantly reduced, by up to 30-fold, particularly with δ-nonalactone (Tables 2 and 5). This result supports the hypothesis that the trade-off between the HSLase and lipo-lactonase activities underlined the duplication and ultimately the specialization of PON1 and PON3 as lipo-lactonases.

Notably, P2F1 activities toward C12HSL and 3OC10HSL were still ≤11–40-fold lower than those of N9 (Table 2). Thus, additional active site substitutions are needed to recapitulate the ancestor’s generalist character.

The structural basis underlining the trade-offs between the HSLase and lipo-lactonase activities remains unknown. Unfortunately, structures of PON2 and PON3 are unavailable, because our attempts and those of others to crystallize the human enzymes, as well as recombinant versions, failed. Although mpa-N9 is relatively soluble and stable, it has thus far failed to give crystals in all attempted screens.

The Promiscuous Paraoxonase Activity—In the case of the paraoxonase and aryl-esterase activities, no consistent trends could be detected (Table 4). It seems that the activities with...
these promiscuously hydrolyzed substrates are the highest in PON1, probably because these substrates were a priori identified with this enzyme. The coincidental nature of the paraoxonase and aryl-esterase activities is also reflected in the large variations seen with slightly different substrates. For example, as previously reported, the high paraoxonase activity associated with PON1 is weak in PON3 and undetectable in PON2 (17).

We found, however, that all mammalian PONs, including the ancestor, show similar and relatively high activity with methyl paraoxon. Beyond the scope of this article, we note that the data presented here indicate that determination of the methyl and ethyl paraoxon hydrolysis rates may serve as a rapid way to identify PON2 levels (for which no easily monitored substrates are available) and for distinguishing PON1 from PON2.

**DISCUSSION**

**PONX_OCCAL, a Newly Identified Bacterial Quorum-quenching Lactonase and a Missing Link to Contemporary PONs**—PONX_OCCAL represents a third, newly identified family of bacterial quorum-quenching lactonases that joins the two previously known families of AiiAs (43, 44) and PLLs (54). PONX_OCCAL hydrolyzes a variety of HSLs with 3OC12-HSL being the optimal substrate (Table 2). Its activity with 3OC12HSL is strikingly similar to that of the mammalian ancestor N9 (Fig. 5), suggesting that bacterial and mammalian PONs share a common HSLase origin. This notion is also reinforced by the fact that many of the more ancient vertebrate PON genes are predicted as PON2s (1) and the fact that PON2 is primarily a quorum quenching HSLase (Table 2) (19, 20).

However, crucial gaps exist between the vertebrate and bacterial PON clades (Fig. 3). These gaps are not bridged by the PON-like fungal genes because they comprise an independent clade. A likely explanation for this phylogenetic gap is horizontal gene transfer, presumably of a bacterial HSLase to invertebrates. Cases of horizontal gene transfers from bacteria to multicellular eukaryotes (55) including nematodes (56) have been described. Such gene transfers may be promoted by endosymbiosis between bacteria and their eukaryotic hosts (55). The recruitment of a quorum quenching HSLase from an endosymbiotic bacterial species, or a pathogen, may have provided metazoa a new means of innate immunity. However, given the limited sampling of PON genes and given that PON sequences were thus far identified only in a small group of bacteria, vertical
TABLE 5
Enzymatic characterization of variants from the N9-PON1 transition library showing the highest HSLase activity with 3OC12-HSL
Turnover number ($k_{\text{cat}}$), Michaelis constant ($K_m$), and catalytic efficiency ($k_{\text{cat}}/K_m$) for lactone substrates.

| Substrate | PON1 (PON1-L55I,M75K, S137M, F222S, E239D) | P2A12 (PON1-F222S, I237V, Y293F) | P2F1 (PON1-F222S, I237V) |
|-----------|-------------------------------------------|---------------------------------|----------------------------|
| 3OC12HSL  | $k_{\text{cat}}$ (s$^{-1}$) 35.15±2.4     | 75.9±3.4                       | 46.2±3.2                   |
|           | $K_m$ (mM) 0.25±0.04                  | 0.37±0.06                      | 0.4±0.06                   |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (9.1±0.4)X10$^3$ | (1.35±0.14)X10$^5$ | (2.07±0.02)X10$^5$ |
| C12HSL    | $k_{\text{cat}}$ (s$^{-1}$) 1.3±0.17    | ND                             | 0.27±0.08                   |
|           | $K_m$ (mM) ND                        | 0.27±0.08                      | (4.92±0.92)x10$^3$         |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (4.92±0.92)x10$^3$ | (3.42±0.2)x10$^2$         | (4.06±0.56)x10$^2$ |
| 3OC10HSL  | $k_{\text{cat}}$ (s$^{-1}$) 145±5.5      | (6.86±0.02)X10$^4$            | (1.37±0.08)X10$^4$         |
|           | $K_m$ (mM) 1.65±0.2                   | (6.7±0.18)X10$^3$            | (1.6±0.02)X10$^4$         |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (8.8±0.8)X10$^4$ | (6.9±0.22)x10$^3$         | (2.91±0.15)x10$^3$ |
| δ-nonalactone | $k_{\text{cat}}$ (s$^{-1}$) 40.3±0.04  | 46.6±4.6                      | 9.95±0.6                   |
|           | $K_m$ (mM) 1.28±0.17                  | 3.4±0.84                      | 1.6±0.16                   |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (3.2±0.4)X10$^4$ | (1.4±0.21)x10$^4$         | (6.2±2.6)x10$^3$         | (7.52±0.3)x10$^3$ |
| δ-undecalactone | $k_{\text{cat}}$ (s$^{-1}$) 64.4±1.2   | Not measured                  | Not measured               | Not measured               |
|           | $K_m$ (mM) 0.6±0.007                  | Not measured                  | Not measured               | Not measured               |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (1.1±0.07)X10$^5$ | (1.4±0.033)X10$^5$         | (4.96±0.36)X10$^4$ | (4.22±0.64)X10$^4$ | (8.52±0.36)X10$^4$ |
| γ-nonalactone | $k_{\text{cat}}$ (s$^{-1}$) 37.8±0.14  | 35.35±0.6                     | 36.1±0.1                   |
|           | $K_m$ (mM) 0.26±0.007                 | 0.71±0.06                     | 0.86±0.13                  |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (1.4±0.033)X10$^5$ | (4.96±0.36)X10$^4$         | (4.22±0.64)X10$^4$ | (8.52±0.36)X10$^4$ |
| γ-undecalactone | $k_{\text{cat}}$ (s$^{-1}$) 291±12     | Not measured                  | Not measured               | Not measured               |
|           | $K_m$ (mM) 0.64±0.03                   | Not measured                  | Not measured               | Not measured               |
|           | $k_{\text{cat}}/K_m$ (m$^{-1}$s$^{-1}$) (4.6±0.01)X10$^5$ | Not measured                  | Not measured               | Not measured               |
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gene transfer combined with loss of the PON genes or “reverse horizontal gene transfer” from metazoa to bacteria remain options. What is clear, however, is that PONs represent the penetration of homoserine lactones hydrolysis, a function that has thus far been considered a purely bacterial enzymatic function, into metazoa and mammals.

The Acquisition of New Detoxification Functions Underlines the Appearance of the Mammalian Paralogues—Following vertical divergence within vertebrates, the PON gene diverged to give three mammalian families, PON1, 2, and 3. Similarly to other detoxifying enzymes, these paralogues are missing in all other vertebrates, including platypus and opossum that branched before mammals. Thus, all nonmammalian genomes seem to possess only one PON gene (exceptions are zebrafish and branchiostoma, which possess several PON sequences, probably because of extensive genome duplications (57, 58)). Divergence via gene duplication has been observed along the mammalian phylogenies of most detoxifying enzymes (e.g., mammalian CYP450s (59) and sulfotransferases (supplemental Fig. S7 in Ref. 53)). Environmental changes and/or exposure to pathogens are the likely drivers of this extensive duplication and diversification (59). In the case of PON, the acquisition of detoxification functions that were unrelated to the original innate immunity function seems to have underlined the birth of the new paralogues.

The Bifunctional Ancestor of Mammalian PONs—N9, the reconstructed ancestor of the three PON families, was found to be a bifunctional enzyme that exhibits both considerable HSLase and lipo-lactonase activities (Table 2 and Fig. 5). Beyond being bifunctional, N9 is a “generalist” with a much broader HSLase activity than the bacterial PON and its descendant PON2 (Tables 2 and 3 and Fig. 5). This generalist character may be an intrinsic prediction bias assigned to ancestors that “average” the different specificities seen in the descendent families (60, 61). However, N9 was found to reliably represent the kinetic features of the ancestral sequence ensemble from which it was derived. Additionally, identical phenotypes were exhibited by both N9 and N9*, which differ in 11 positions. Foremost, the generalist feature is not a mere by-product of high 3OC12-HSLase activity because restoring the 3OC12-HSLase activity of the ancestor in PON1 did not recover the broad specificity of the ancestor (PON1-F222S,I237V; Table 5).

The Ancestor’s Bifunctionality and PONs Overlapping Specificities Coincide with “Divergence before Duplication”—Of the three PON families, PON2 retained the original HSLase activity and presumably the innate immunity function for which PON might have been originally recruited. In contrast, PON1 and PON3 diverged and became lyophilic lactonases, presumably for detoxification of exogenous and endogenous lactones. Such asymmetrical divergence is a hallmark of Ohno’s model (62): the original copy (by this model, the contemporary PON2) maintained the ancestral function, whereas the new copies acquired new functions (PON1/3) (Fig. 5). However, inconsistently with Ohno’s model, both PON1 and PON3 show considerable HSLase activity. Further, N9 exhibits similar HSLase activities to the bacterial PONX_OCCAL as well as lipo-lactonase activities that are undetectable, neither in PONX_OCCAL nor in PON2.

Whether innovation precedes or follows duplication remains the subject of an intense debate (63, 64). Although controversial (65, 66), the analysis of certain families indicated that the new function could have emerged prior to duplication (65–68). Ancestors of several enzyme families were found to exhibit weak, latent activities that later became the primary activities of the contemporary enzymes (51, 69). However, these include cases where cross-reactivity is a coincidence exploited later by evolution (70) and not the explicit outcome of selection as presumed for PONs. At a minimum, the partially diverged mammalian ancestor of PONs versus the entirely specific bacterial PON shows that the divergence of new detoxifying functions in mammalian PONs could have significantly progressed prior to duplication (64, 68). Thus, a trajectory in which a specialized HSLase progenitor (PONX_OCCAL-like) first diverged into a bifunctional enzyme, and only then duplicated, is a tangible possibility (64, 67, 68).

Divergence via Weak Trade-offs and Bifunctional Ancestors May Be Generally Applicable to Detoxifying Enzymes—The reconstructed ancestor possesses relatively high level of lipo-lactonase activity ($k_{cat}/K_m = 10^3 \text{ M}^{-1} \text{s}^{-1}$), whereas the HSLase activity remained as high as that of the bacterial PON (Fig. 5). This weak trade-off may circumvent the requirement for duplication as a prerequisite for the divergence of new functions, as outlined by Ohno’s model (64, 68). Nonetheless, it could be that divergence mechanisms that provide “something for nothing” may only be applicable to certain types of proteins. Enzymes in secondary metabolism (51, 66, 69, 71), primarily in plants (22), and detoxifying enzymes, are only transiently active upon exposure to sporadic challenges and as such may remain multifunctional intermediates (73, 74). Bifunctionality may also explain the facile reversal to the ancestral HSLase phenotype by merely two ancestral mutations (Table 5) (69). On the other hand, in core metabolic enzymes that directly affect growth such as the yeast alcohol dehydrogenase, the mildest trade-off in enzymatic activity may affect organismal fitness. Thus, divergence is more likely to progress through an ancestor that possesses a single existing function—ethanol synthesis in this case—whereas the newly diverged parologue specialized in the new function of catalyzing ethanol (50).

Strong Trade-offs Underline PON1 Specialization as Lipo-lactonase—“Something for nothing” is also limited to the early stages of divergence. Further improvement in the lipo-lactonase activity was accompanied by large losses in the HSLase activity. In PON1 and PON3, the $\delta$-lactonase activity became 15–40-fold higher, and the quorum-quenching lactonase activity became 20–100-fold lower than that of the N9 ancestor (Fig. 5). This trade-off was confirmed by the substantial loss of lipo-lactonase activity accompanying ancestral mutations that restored the ancestral HSLase activity (Table 5). This shift from weak trade-off at the early stages of divergence to strong trade-off at the advanced stages has also been observed in the laboratory (75). Thus, optimization of the lipo-lactonase activity is likely to have been promoted by duplication of a bifunctional ancestor that evolved under weak trade-offs. Duplication enabled selection to act on PON1 and PON3 toward improvement of the lipo-lactonase activity while removing the burden of maintaining the quorum-quenching lactonase activity.
Apart from strong activity trade-offs, duplication may also be driven by the acquisition of additional roles (2) including functions that do not relate to the lactonase activity (8, 76). Duplication may also be driven by changes in regulation (77) and localization (78). Indeed, in conjunction with its presumed innate immunity function, PON2 that is most likely to have descended from the ancestral copy is found in nearly every tissue. In contrast, the duplicated and diverged detoxifying PON1/3 are found primarily in liver and serum (5).

Finally, in light of their evolutionary history, the substrate specificities of the mammalian PONs make sense. Being newly diverged from a bifunctional ancestor, PON1 and PON3s seem like “jacks of all trades and masters of none.” This is because, despite ~100 million years of evolution (79), they still maintain the relics of their ancestral HSLase activity alongside their newly diverged detoxifying lactonase activities. In contrast, PON2 has respecialized toward the ancestral HSLase activity and also acquired new functions (8, 80) independently of its HSLase function.

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