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# MINIREVIEW

# Paraoxonases as Potential Antibiofilm Agents: Their Relationship with Quorum-Sensing Signals in Gram-Negative Bacteria[▽]

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**The property of many bacteria to form biofilms constitutes a major health problem. Bacteria living in biofilms have a very high resistance to antibiotics. Biofilms may develop at a certain locations with the participation of secreted molecules, termed quorum-sensing signals, when a sufficient density of bacterial growth occurs. In Gram-negative bacteria, acyl homoserine lactones (AHL) have been identified as major quorum-sensing signals. The paraoxonases (PONs) constitute a family of enzymes comprising 3 members (PON1, PON2, and PON3) that have lactonase activity and are able to hydrolyze AHL. In this minireview, we summarize some existing basic knowledge on PON genetics, biochemistry, and function and describe recent research that reports evidence of the important roles that they may play in the organism's defense against biofilm formation. Finally, we propose some lines of future research that could be very productive.**

Many bacteria form organized communities of aggregated cells living in hydrated matrices of extracellular polymeric substances. These structures (termed "biofilms") are evolutionary adaptations by bacteria that enable them to survive in hostile environments and to colonize new ecological niches (7, 38). Bacteria living in biofilms have high resistance to antimicrobial agents, which constitutes a major health problem. Hence, investigation of the molecular mechanisms of biofilm formation and the effectiveness of new therapeutic agents in counteracting these mechanisms is becoming a productive line of research. Biofilms may develop with the participation of secreted molecules (termed "quorum-sensing signals") when a sufficiently high density of bacterial growth occurs at a certain location. In Gram-negative bacteria, acyl homoserine lactones (AHLs) have been identified as major quorum-sensing signals. The paraoxonases (PONs) constitute a group of lactonases ubiquitously expressed in human tissues. Recent evidence shows that they may play an important role in the fight against bacterial biofilm formation. In the present minireview, we summarize the current knowledge regarding PONs and their biological function while describing appropriate experiments to elucidate their possible role in antibacterial treatment.

## WHAT ARE THE PARAOXONASES?

The PON enzyme family comprises 3 members, PON1, PON2, and PON3, the genes coding for which are located adjacent to each other on chromosome 7q21–22 (72, 81). In humans, PON1 and PON3 genes are mainly expressed in the liver and kidney and their protein products are found in the circulation bound to high-density lipoprotein (HDL) (45, 55, 58, 79). Conversely, the PON2 gene is expressed a variety of tissues. Its protein product is an intracellular enzyme that is not, however, found in plasma (64). PON1 was first identified in 1953 by Aldridge, who, while examining the rates of hydrolysis of organophosphate insecticides in different tissues of rats and rabbits (5, 6), observed that rabbits exhibited a very high rate of paraoxon degradation in serum and that this compound was cleaved by an esterase. Aldridge segregated esterases into two categories according to whether they were inhibited by interaction with substrates (B-esterases) or whether they could catalytically hydrolyze substrates (A-esterases). Based on these original publications, the serum A-esterase was referred to as paraoxonase because of its ability to hydrolyze the toxic oxon metabolite of parathion (hence, "paraoxon"). When two closely similar enzymes were identified in the mid-1990s, the original paraoxonase was renamed PON1, while the two new enzymes were termed PON2 and PON3 (72).

Analysis of the ability to hydrolyze paraoxon was employed in the 1960s as the method to measure PON1 activity in several species and tissues. It soon became apparent that, in human serum, there were considerable differences in PON1 activity between individuals. Frequency distribution histograms suggested that there were three different phenotypes, corresponding to high-, intermediate-, and low-serum PON1 activities. Also, the frequencies at which the low-activity allele occurred differed considerably between populations of different geographical and ethnic origins, a higher frequency being observed in Caucasian than in African, Asian, or Australian aborigine populations (23).

Over the subsequent years, several assays were developed to examine PON1 phenotype polymorphisms (32). Plotting the values of hydrolysis of one substrate (phenyl acetate, diazoxon, or sarin) against those of a second substrate (paraoxon) provided a clear resolution of the differences between individuals with low-level (termed AA), intermediate-level (termed AB),

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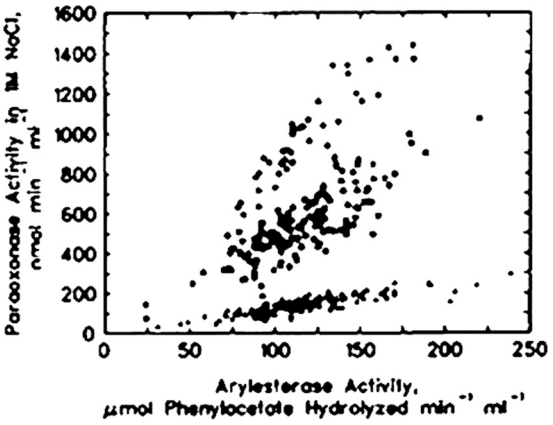


FIG. 1. Relationship between PON1 paraoxonase and arylesterase activities in different individuals. Plots of paraoxonase activity with 1 M NaCl versus arylesterase activity show three groups of individuals corresponding to the three paraoxonase phenotypes (AA, AB, and BB). (Reprinted from reference 28 with permission of the publisher.)

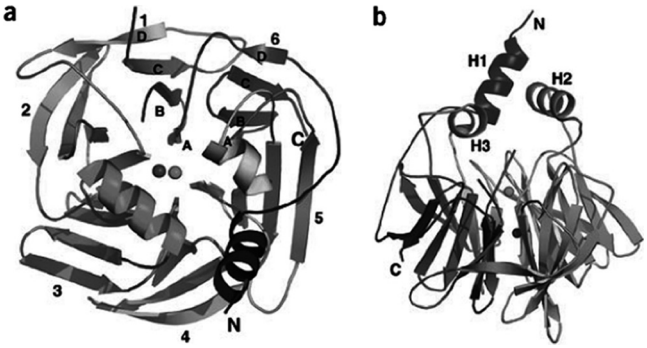


FIG. 2. Chemical structure of PON1. (a) View of the six-bladed β-propeller from above. The top of the propeller is, by convention, the face carrying the loops connecting the outer β-strand of each blade (strand D) with the inner strand of the next blade. The N and C termini and the two calcium atoms in the central tunnel of the propeller are shown. (b) A side view of the propeller with the three helices at the top (H1 to H3). (Reprinted from reference 39 with permission of the publisher.)

and high-level (termed BB) PON1 activities (19). Eckerson et al. (27, 28) noted that the B isozyme was more active in the presence of NaCl. Using this property, the authors plotted the arylesterase activity values against the paraoxonase activity values measured in the presence of 1 M NaCl. This system was shown to be the most efficient in differentiating between the different PON1 polymorphisms (Fig. 1).

The genetic bases of the PON1 phenotype polymorphisms were first defined by Adkins et al. (1), who sequenced the coding region for PON1 from human cDNA libraries and identified two polymorphic sites: Arg/Gln at position 192 ($PON1_{192}$ polymorphism, with two alleles termed Q and R) and Leu/Met at position 55 ($PON1_{55}$ polymorphism, with two alleles termed L and M). The $PON1_{192}$ polymorphism correlated clearly with the AB phenotype system (see above), with QQ individuals segregating with the AA phenotype and RR individuals segregating with the BB phenotype.

In 1997, Garin et al. (34) evaluated the influence of $PON1_{192}$ and $PON1_{55}$ polymorphisms on the enzyme's activity as well as its concentration. They observed considerable differences in relation to the $PON1_{55}$ genotype; individuals carrying a leucine at position 44 (L isoform) had higher serum PON1 concentrations than those with a methionine (M isoform) at this position. In contrast, the $PON1_{192}$ polymorphism affected the enzymatic activity but had only a slight impact on the PON1 concentration in serum. These findings suggested a possible divergence between the enzyme's concentration and its activity. More recently, several polymorphisms in the promoter region of the *PON1* gene have been described. However, only $PON1_{-108}$, $PON1_{-909}$, and $PON1_{-1741}$ appear to be significantly associated with changes in the enzyme's concentration or activity in serum.

## PON1 IS A LACTONASE

What was the original activity of members of the PON family? PON1 hydrolyzes a broad range of substrates, including esters, lactones (32, 44, 86), organophosphates such as the nerve agents soman and sarin (19), lipid peroxides, and estrogen esters (32, 85). PON1 has been studied extensively in relation to the prevention of arteriosclerosis. In addition, PON1 metabolizes certain drugs and has been proposed for therapeutic use in drug inactivation (8, 56). Identifying the native function of PON1 has, for a considerable time, been hampered by confusion with respect to the structure and mechanism of action of this enzyme. Purified PON1 preparations are unstable and often contaminated, but the method of directed evolution has been productive in determining PON1 structure and function. Essentially, directed evolution seeks to replicate the evolutionary process in the laboratory by artificially inducing mutations in the gene of interest, followed by selection and amplification of the variants that show an enhancement of the desired characteristics. In a report from a study using this technique, Harel et al. (39) described PON1 as a six-bladed beta propeller with a unique active site lid that is also involved in HDL binding (Fig. 2). The active site and the deduced catalytic mechanism suggested that PON1 is reminiscent of the secreted phospholipase A2. Despite the huge increase in the complexity of living organisms in the course of evolution, relatively little of novelty has been produced at the molecular level since primordial times (52). PONs are probably ancestral enzymes. They appeared very early in evolution and are present in many organisms, from invertebrates to mammals (32). Jensen (46) proposed that, in contrast to more evolutionarily modern enzymes, primitive enzymes possessed very broad specificities and that it is this catalytic versatility that enabled a relatively few enzymes to perform the multitude of functions necessary to maintain the ancestral organism (12, 15, 51, 66). Hence, research on PON1 function was focused on trying to distinguish the native or "ancestral" function of this enzyme from all the other secondary or "adapted" functions. Again, directed evolution studies, together with structure-function studies, established the primordial function of PON1 as that of a lipolactonase (2–4, 53) that subsequently evolved new substrate specificities. These studies also established that the preferred substrates of PON1 are 5- and 6-membered ring lactones, typically with aliphatic side chains (52).

New data on the PON1 mechanism of lactone hydrolysis

have been reported by Tavori et al. (84) using modeling and docking simulation techniques. These methods use theoretical models of the ligands being evaluated (in this case, lactones), which are allowed to interact with models of the protein's three-dimensional structure, thus enabling the nature of the target ligand, as well as the fitness of the evaluated ligand within the protein, to be determined. The authors suggested that the PON1 active site may be reached by a range of lactones that have similar orientations in the active center. The carboxylate moiety is directed toward the hydrophylic inner part of the active center, while the ligand's aromatic ring is facing the hydrophobic part. The results from Tavori et al. also revealed an inverse correlation between docking energy and the lactone rate of hydrolysis and a direct correlation with the length of the lactone side chain.

## PON2 AND PON3

Currently, not much is known about the PON2 and PON3 proteins. Their genes were identified in 1996 when Primo-Parmo et al. (72) characterized a large number of cDNA sequences in the Genome Data Base that had significant homology to, but were not identical with, human PON1. The percentages of identity among human PON1, PON2, and PON3 genes are similar (about 70%), and the genes are believed to derive from a common precursor (13). Ng et al. (64) demonstrated that PON2 is not present in the circulation, although its gene expression was detected in several human tissues. The authors concluded that this result represents ubiquitous intracellular distribution of the enzyme. They also reported that cells transfected with the human PON2 gene had a higher antioxidant capacity than those cells that were not transfected. PON3 is present in HDL and prevents lipoproteins from oxidation *in vitro*. Both PONs are able to hydrolyze lactones but not paraoxon or other xenobiotics (24, 74, 86). These data highlight the idea that the term "paraoxonase" is a misnomer, and many investigators feel that the family name ought to be changed.

## *N*-ACYL HOMOSERINE LACTONES, QUORUM SENSING, AND BACTERIAL BIOFILMS

Implantation of artificial devices, such as prostheses, in the human body and the extensive use of catheters constitute some of the greatest advances in medicine over the past decades. However, an important side effect of these clinical-surgical maneuvers is the development of infections that are difficult to treat, because bacterial infections often develop biofilms of complex biochemical composition at the site of the intervention and become resistant to antibiotic treatments. The biofilm matrix commonly consists of a mixture of polysaccharides, proteins, and nucleic acids, but the precise structure and composition of biofilms vary considerably with the resident species (22). Polysaccharides can be composed of one or more types of monosaccharides, usually *O*- or *N*-acylated. Among the most common extracellular polysaccharides are poly-ß-1,6-*N*-acetylglucosamine (an adhesin for maintenance of biofilm structural stability), cellulose, and alginate. Extracellular DNA is also important in the maintenance of some biofilms, such as those of *Pseudomonas aeruginosa.* In these bacteria, young biofilms can be dissolved by DNase treatment, whereas mature biofilms cannot. This suggests that the structural stability of the more recent biofilms is strongly dependent on DNA and that this is not so in older biofilms. Within the biofilms, bacteria can develop specialized and coordinated phenotypes and include differences in the expression of surface molecules and virulence factors, antibiotic resistance, and nutrient utilization (9, 14, 21, 57, 78, 91).

Biofilm formation can be distinguished as having different stages: (i) bacterial attachment to a surface; (ii) cell growth and extracellular matrix deposition; and (iii) detachment of some cells from the colony into the surrounding medium. The initial interaction between the cells and the supporting surface is based on physicochemical forces and enhanced by host- and tissue-specific adhesions that are located on the bacterial cell surface or in cellular appendices such as pili and fimbriae (48). The second step of biofilm development involves the multiplication of bacteria on the surface and the concomitant synthesis of an extracellular polymeric matrix from one bacterial type or from several different species. In addition to providing a structural matrix for the colony, the biofilm contributes to antimicrobial resistance, either by acting as a physical barrier to the entry of the drugs or by making it difficult for the antibacterial agents to diffuse into the biofilm (59). Mature biofilms may contain millions of cells gathered into pillar- and mushroom-shaped structures (37). These structures are surrounded by fluid-filled channels, which facilitate the exchange of nutrients and waste products. Thus, mature biofilm colonies are highly complex, organized structures with numerous microenvironments exhibiting differing pH, oxygen concentration, nutrient availability, and cell density characteristics (33). The final stage of biofilm development is the detachment of cells from the colony followed by movement to, and colonization of, new environments. This stage is also highly complex from the molecular point of view and involves several environmental signals, signal transduction pathways, and effectors (50).

An important concept underlying bacterial biofilm formation is that of quorum sensing. This phenomenon occurs when the density of bacteria in a specific environment reaches a certain level. Defined as the communication and coordination of the bacterial behavior via the accumulation of signaling molecules, the mechanism relies upon a process such that, when the concentration of these signaling molecules reaches a critical threshold, activation or repression of certain target genes occurs (73). In Gram-negative bacteria, AHLs have been identified as the major signaling molecules in this communication system (68). Despite the great number of species and strains, only a few varieties of AHLs have been reported to be involved in quorum sensing (Table 1). The first signal molecule of this kind was identified in a *Vibrio fischeri* isolate as *N*-(3′-oxohexanoyl)-L-homoserine lactone (3-oxo-$C_6$-AHL) and is synthesized by the LuxI protein (also referred to as autoinducer synthase) and secreted into medium. Another protein encoded in the same operon is LuxR, which, upon interaction with AHL, modulates the transcription of certain genes and induces phenotypic changes (26, 29). One of the best-documented bacterial species in relation to quorum sensing and biofilm formation is probably *P. aeruginosa*. This microorganism colonizes the lungs of patients with cystic fibrosis and forms a biofilm on the epithelial cells of the airways (88).

TABLE 1. Acyl homoserine lactones (AHL) utilized by various Gram-negative bacteria for quorum sensing

| Bacterium | Signaling molecule |
|---|---|
| *Aeromonas hydrophila* | *N*-Butanoyl-AHL |
| *Aeromonas salmonicida* | *N*-Butanoyl-AHL |
| *Agrobacterium tumefaciens* | *N*-(3-Oxo-$C_8$)-AHL |
| *Burkholderia cepacia* | *N*-$C_8$-AHL |
| *Erwinia carotovora* | *N*-(3-Oxohexanoyl)-AHL |
| *Pseudomonas aeruginosa* | *N*-(3-Oxo-$C_{12}$)-AHL |
| *Pseudomonas chlororaphis* | *N*-$C_6$-AHL |
| *Rhodobacter spheroides* | 7,8-*cis*-*N*-(Tetradecanoyl)-AHL |
| *Vibrio fischeri* | 3-Oxo-$C_6$-AHL |

Chronic infection by *P. aeruginosa* results in progressive lung damage and, eventually, death by respiratory failure in many patients with cystic fibrosis. Due to the architecture of the biofilms formed, antibiotic treatment is very ineffective for these patients (9, 63).

*P. aeruginosa* biofilm formation operates through two quorum-sensing AHLs, *N*-butyryl-L-homoserine lactone ($C_4$-AHL) and *N*-(3′-oxododecanoyl)-L-homoserine lactone (3-oxo-$C_{12}$-AHL) (70, 71). These molecules are synthesized by LuxI-like enzymes and detected by receptors similar to LuxR. The autoinducer 3-oxo-$C_{12}$-AHL is synthesized by LasI and detected by LasR, while $C_4$-AHL is synthesized by RhlI and detected by Rh1R (31, 69, 89). Currently, the consensus is that Gram-negative bacteria utilize various AHLs to regulate the mechanisms that underlie the characteristics of biofilms and other adaptative mechanisms. For example, serine protease and metalloprotease production in *Aeromonas hydrophila* is regulated by *N*-butanoyl-AHL, carbapenem synthesis by *Erwinia carotovora* is regulated by 3-oxo-$C_6$-AHL, plasmid conjugation in *Agrobacterium tumefaciens* is regulated by 3-oxo-$C_8$-AHL, and *Rhodobacter sphaeroides* regulates bacterial aggregation with 7,8-*cis*-*N*-(tetradecanoyl)-AHL (76). Conversely, quorum sensing in Gram-positive bacteria is not regulated by a LuxI-LuxR-like system and does not involve an AHL signal autoinducer. An excellent review of AHL biochemistry and the relationships with biofilm formation has been published recently (22).

## BIOFILM DISPERSAL AND ANTIBIOFILM AGENTS: THE ROLE OF PON

Investigation of the molecular mechanisms of biofilm formation and the manner in which destruction can be induced has increased exponentially over the past few years. From the clinical point of view, one of the most significant reasons for this interest is the threat to antibiotic treatment posed by the emergence of progressive bacterial resistance. In *P. aeruginosa*, evidence exists that bacterial infection presenting as biofilms versus individual plankton forms is regulated by bis-(3′-5′)-cyclic GMP (c-di-GMP), which is synthesized by diguanylate cyclase or degraded by phosphodiesterase (40, 62). Several factors (such as carbon starvation or nitric oxide exposure) may contribute to dispersal of biofilms by interrupting the activity of these enzymes (35, 36). In addition, *P. aeruginosa* produces *cis*-2-decanoic acid, which induces dispersion of established biofilms (18). This bacterium also produces alginate lyase, which degrades alginate and promotes the detachment of cells from cultured biofilms (42). Another well-documented biofilm matrix-degrading compound is dispersin B. It is an enzyme that degrades biofilm polysaccharides and is produced by *Aggregatibacter actinomycetemcomitans* (47, 49). Proteases have also been implicated in biofilm cell detachment. In *Staphylococcus aureus*, deletion of the genes encoding the extracellular proteases aureolysin and Sp1 resulted in an increase in biofilm formation (11). In these bacteria, the addition of exogenous deoxyribonucleases induced detachment of the bacteria from the microplate wells on which they had been plated (60, 75). Some organic compounds and molecular mediators that have been investigated in relation to their potential to degrade biofilms include homoserine lactones, quinolones, furanosylborate, glycopeptidolipids, and phenazines (10, 14, 20, 42, 47, 65, 88).

Evidence showing that the PON enzyme family may play an important role in activity against biofilm formation in *P. aeruginosa* has been reported recently. These bacteria utilize AHLs in the form of quorum-sensing signals to promote biofilm formation and to regulate several virulence factors (80, 87). Epithelial cells and resident macrophages are important defense mechanisms of the lungs against external toxic agents and bacteria. PONs are strongly expressed in lung epithelial cells (Fig. 3), in great part on the external cell membrane, and, as stated above, they are essentially lactonases (61, 77). Thus, it seems logical to infer that PONs are perhaps able to hydrolyze AHL and interrupt quorum-sensing signals from the bacteria located in proximity of the respiratory epithelium. This property has previously been demonstrated *in vitro* by researchers (17, 83) who observed that airway epithelial cells could inactivate 3-oxo-$C_{12}$-AHL but not $C_4$-AHL and that this capacity was present in cell membranes but not when PONs were secreted into the airway fluid. Further studies from the same group using cultured lung epithelial cells exposed to 3-oxo-$C_{12}$-AHL demonstrated that a lactonase was responsible for these outcomes (67). These investigators also demonstrated that serum, which is an environment rich in PON1, degraded 3-oxo-$C_{12}$-AHL and decreased *P. aeruginosa* biofilm growth and that this capacity was lost when serum from PON1 knockout (PON1-KO) mice was employed. Further, the addition of back-purified PON1 to serum from PON1-KO mice restored the ability to degrade 3-oxo-$C_{12}$-AHL and also reduced biofilm formation (25, 67). These data showed that, in mouse serum, PON1 is both required and sufficient to degrade this 3-oxo-$C_{12}$-AHL and to inhibit the growth of bacterial biofilms. The same authors showed that PON2 and PON3 are also able to degrade 3-oxo-$C_{12}$-AHL, with PON2 being the most efficient with respect to this function. Other authors have confirmed that PONs can degrade AHL and that PON2 has the greatest lactonase activity of the three enzymes (25, 90). Stoltz et al. (82) used a quorum-sensing reporter strain termed PAO1-qsc-102-lacZ that produces β-galactosidase in response to quorum-sensing signals. They showed that β-galactosidase levels were 2 to 2.5 times higher in bacteria harvested from PON2-KO mice lung epithelia compared to control results. Also, 3-oxo-$C_{12}$-AHL has been shown to downregulate PON2 expression in cultured airway epithelial cells (43). These results demonstrate that both PON1 and PON2 are important for the control of *P. aeruginosa* quorum sensing. Recently, a new experimental model employed in *Drosophila melanogaster* studies has been

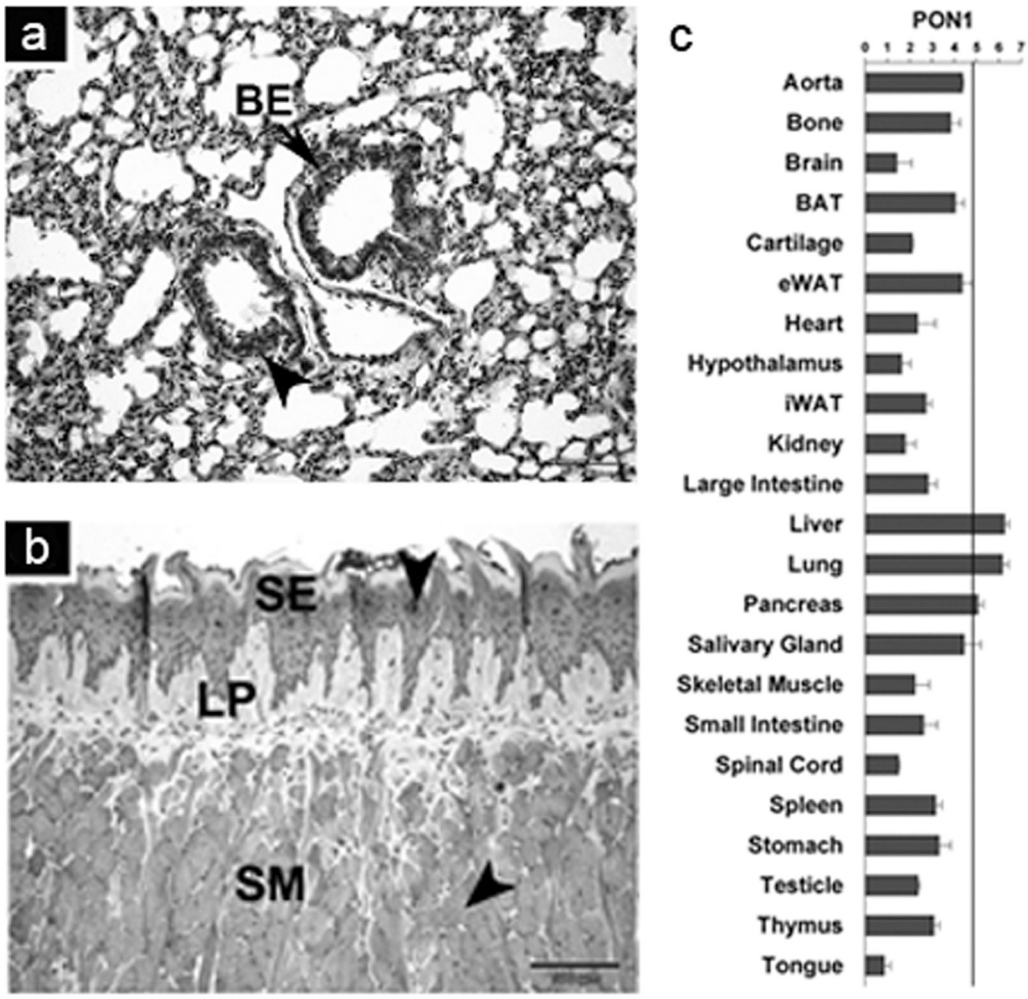


FIG. 3. (a) PON1 protein expression in mouse bronchiole epithelium. (b) PON1 protein expression in the mouse tongue. Bar, 100 μm. (c) PON1 gene expression in several tissues of the mouse. This enzyme is strongly expressed in the lung. BE, bronchiole epithelium; LP, lamina propria; SE, squamous epithelium; SM, skeletal muscle. The scale at the top of panel c represents arbitrary units. The arroheads indicate positive staining. (Adapted from references 61 and 77 with permission of the publishers.)

used to investigate the role of the PON family in protecting against biofilm formation. Arthropods do not have PON, and human PON1 transgenic flies have been shown to exhibit increased survival following infection with *P. aeruginosa* and *Serratia marcescens*, another AHL-sensing bacterium (30).

## DIRECTIONS FOR FUTURE RESEARCH

Bacterial antibiotic resistance is one of the most serious health problems worldwide. The biofilm "lifestyle" of many bacteria is very important in many chronic and device-associated bacterial infections, since biofilms tolerate, or are resistant to, essentially almost all antibiotics. This implies that increasing the knowledge of quorum-sensing biochemistry and how the mechanisms can be modulated could have significant therapeutic implications in the near future. In the present minireview, we have sought to highlight that members of the PON family of enzymes may play a key role in an organism's defense mechanisms against biofilms. Most evidence reported to date has been obtained from studies in *P. aeruginosa.* A productive line of research would be to explore whether PON enzymes are also effective in preventing biofilm formation in other AHL-dependent Gram-negative bacteria. In addition, since PONs are polymorphic enzymes, the possibility exists that genotypes influence the individual's ability to combat these bacteria. In patients with cystic fibrosis, the possibility exists that a defect in the cystic fibrosis transmembrane conductance regulator (CFTR) gene impairs natural PON1-driven protection (54) and that PON1 of the host may also be involved in the positive selection of LasR mutant bacteria (which have decreased quorum-sensing signal production), since these mutants are known to be selected under conditions of oxidative stress (41).

In general, there has been a paucity of clinical studies. For example, the relationships between circulating PON levels and the severity of various chronic infectious diseases have yet to be established. Another interesting approach, taking into account that PON enzymes are proposed to be antiatherogenic, would be to investigate whether alterations in their circulating levels can provide a link between periodontal disease and the

increased risk of arteriosclerosis, as reported from studies of these patients (16). Finally, the various PON recombinants that are currently available are not very stable when administered *in vivo*, and this hampers the development of intervention studies. Investigation of the possibility that PON enzymes may become an effective therapeutic tool against infectious diseases, and may contribute to increasing the efficacy of antibiotic treatment, is an important line of research with a promising future.

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