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First evidence of a potential antibacterial activity involving a laccase-type enzyme of the phenoloxidase system in Pacific oyster *Crassostrea gigas* haemocytes

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Abstract:

Phenoloxidases (POs) are a group of copper proteins including tyrosinase, catecholase and laccase. In several insects and crustaceans, antibacterial substances are produced through the PO cascade, participating in the direct killing of invading microorganisms. However, although POs are widely recognised as an integral part of the invertebrate immune defence system, experimental evidence is lacking that these properties are conserved in molluscs, and more particularly in the Pacific oyster *Crassostrea gigas*. In the present study, *Vibrio splendidus* LGP32 and *Vibrio aestuarianus* 02/041 growths were affected, after being treated with *C. gigas* haemocyte lysate supernatant (HLS), and either a common substrate of POs, L-3,4-dihydroxyphenylalanine (L-DOPA), to detect catecholase-type PO activity, or a specific substrate of laccase, *p*-phenylenediamine (PPD), to detect laccase-type PO activity. Interestingly, a higher bacterial growth inhibition was observed in the presence of PPD than in the presence of L-DOPA. These effects were suppressed when the specific PO inhibitor, phenylthiourea (PTU), was added to the medium. Results of the present study suggest, for the first time in a mollusc species, that antibacterial activities of HLS from *C. gigas* potentially involve POs, and more particularly laccase catalysed reactions.

Highlights

► Bacterial growth inhibition was studied in two *Vibrio* oyster bacterial pathogens. ► Different *Crassostrea gigas* tissues, haemolymph fractions, and PO substrates, were tested. ► The inhibition occurred only in the presence of *C. gigas* HLS and PO substrates. ► Bacterial growth inhibition was suppressed with a PO inhibitor. ► Results suggest that HLS antibacterial activities involve laccase-type PO-catalysed reactions.

Keywords: Phenoloxidases; Antibacterial activity; *Vibrio*; Bivalve; Immunity

Abbreviations: POs, phenoloxidases; HLS, haemocyte lysate supernatant; L-DOPA, L-3,4-dihydroxyphenylalanine; PPD, p-phenylenediamine; PTU, 1-phenyl-2-thiourea; DETC, diethyldithiocarbamate; CTAB, cethyltrimethylammonium bromide; OD, optical density.

1. Introduction

The Pacific oyster *Crassostrea gigas* (Thunberg, 1753) is an ecologically and economically important species that dominates overall other molluscs with respect to global world distribution and aquaculture production [1]. However, massive summer mortalities of *C. gigas* have become a widespread concern in the world in recent decades [2-4]. Studies have shown a positive correlation between *C. gigas* summer mortalities and the presence of pathogens (e.g. bacteria or viruses), and suggest that this could be related to a weakening of immune defence mechanisms in the host that would be potentially affected by environmental factors [4, 5]. Among immune defence mechanisms, phenoloxidases (POs; EC 1.14.18.1) are a group of copper proteins including tyrosinase (EC 1.14.18.1), catecholase (EC 1.10.3.1) and laccase (EC 1.10.3.2), which are the rate limiting enzymes in melanisation [6, 7], and play an important role in immune defence mechanisms in invertebrates [8]. In these organisms, POs exist as an inactive form, proPO. Pathogen associated molecular patterns (PAMPs), such as peptidoglycans or lipopolysaccharides from bacteria, or β-1,3-glucans from fungi, are recognized by pattern-recognition receptors (PRRs). This will trigger the activation of a cascade of serine proteases that activates PO-activating enzymes (PPAs), and therefore, the conversion of the pro-enzyme proPO into PO [8].
The three types of POs can oxidise o-diphenols, such as L-3,4-dihydroxyphenylalanine (L-DOPA; catecholase activity). However, among these three enzymes, only tyrosinases can hydroxylate monophenols, such as L-tyrosine (monophenoloxidase activity), and only laccases can oxidise m- and p-diphenols, or aromatic compounds containing amine groups, such as p-phenylenediamine (PPD; laccase activity) [9, 10]. Recently, POs, and more particularly catecholase and laccase, have been detected and identified in different tissues of *C. gigas*, i.e. the haemolymph, digestive gland, gills and mantle [11-14]. In addition, studies have shown that PO activity in *C. gigas* can be modulated by environmental factors, such as the presence of contaminants [15-17], and that the expression of a gene coding for a putative laccase in *C. gigas* can be modulated in the presence of hydrocarbons [18].
Different roles have been attributed to POs in bivalves [19-22], especially in haemolymphatic immune defence mechanisms [23, 24], and PO-generated reactive compounds are known to contribute to the destruction of microbial cells in several insects and crustaceans [25-27]. However, there is no direct experimental evidence indicating that POs in molluscs, and more particularly in *C. gigas,* conserve these properties and therefore, that they participate in immune defences against pathogen agents in this species. A better understanding of roles played by POs in *C. gigas* is needed to expand our knowledge on immune defence mechanisms in this organism, and therefore to a better understanding of the potential causes of summer mortality events.
The aims of this work were *(i)* to study the implication of PO activity(ies) on *C. gigas* immune defence system through antimicrobial assays and *(ii)* to identify which type(s) of PO activity(ies) is(are) implicated in this mechanism. For this purpose, different *C. gigas* tissues (i.e. digestive gland, gills and mantle) and haemolymph fractions (i.e. the acellular fraction [plasma] and the haemocyte lysate supernatant [HLS]), were analysed for their ability to inhibit *in vitro* the growth of oyster bacterial pathogens related to *Vibrio splendidus* and *Vibrio aestuarianus.* These bacterial pathogens were found to be associated to *C. gigas* summer mortality outbreaks in France [28]. Additionally, a common substrate of POs, L-DOPA, used to detect catecholase activity, a specific substrate of laccase, PPD, used to detect laccase activity, and a specific inhibitor of POs, 1-phenyl-2-thiourea (PTU), used to inhibit all types of POs, were tested for identification of PO activities, particularly in the haemocyte lysate supernatant (HLS).

2. Materials and methods

2.1.Oysters

Three-year-old Pacific oysters, *C. gigas* (mean ± SD; weight: 75.5 ± 8.7 g; length: 9 ± 3 cm) were purchased during October-November 2008 from shellfish farms in Aytré (Charente Maritime, France), on the French Atlantic coast, and were processed immediately after their arrival in the laboratory.

2.2.Preparation of oyster tissue extracts

After opening the oyster shells by cutting off the adductor muscle, haemolymph (0.5-1 ml) was withdrawn directly from the pericardial cavity, with a 1-ml syringe equipped with a needle (0.9 x 25 mm), and haemolymphs from 10 oysters were pooled [15]. Haemolymph samples were centrifuged (260 g, 10 min, 4°C) to separate the cellular fraction (i.e. haemocytes) from the acellular fraction (i.e. plasma) [12]. The digestive gland, gills and mantle from 10 oysters were dissected and pooled. The haemocytes, digestive gland, gills and mantle were homogenized at 4°C in Tris buffer (0.1 M Tris HCl, 0.45 M NaCl, 26 mM $MgCl_2$ and 10 mM $CaCl_2$) adjusted to pH 7 (1 ml of buffer for the HLS, 1 $ml.g^{-1}$ of fresh weight for the digestive gland and mantle and 0.5 $ml.g^{-1}$ of fresh weight for the gills). The digestive gland, gills and mantle were lysed using an Ultra-Turrax (T25 basic, IKA-WERKE) at 19 000 rpm for 30 sec and a Thomas-Potter homogenizer (IKA-Labortechnik, clearance 0.13-0.18 mm) at 200 rpm for 1 min, and centrifuged at 10 000 g for 10 min at 4°C. The resulting supernatants were collected. Haemocytes were lysed using Thomas-Potter homogenizer at 200 rpm for 1 min and centrifuged at 10 000 g for 10 min at 4°C, and the supernatant was collected. The resulting digestive gland, gills, mantle, plasma and HLS samples were filtered at 0.22 µm (Millipore membrane-Millipore Co., Bedford, MA, USA) to eliminate bacteria. Absence of bacterial development in filtered samples was tested by incubating the samples with Zobell medium and by measuring potential bacterial growth with a spectrophotometer at 620 nm wavelength during at least 7 h (data not shown). Aliquots (100 µl) were stored at -80°C. Each aliquot was used only once.

2.3.Chemicals

PO substrates (dopamine, L-3,4-dihydroxyphenylalanine [L-DOPA]) and inhibitors (1-phenyl-2-thiourea [PTU], diethyldithiocarbamate [DETC]), laccase substrate (p-phenylenediamine [PPD]) and inhibitor (cethyltrimethylammonium bromide [CTAB]), mushroom tyrosinase and *Trametes versicolor* laccase were purchased from Sigma (France).

2.4.Bacterial strains and effect of L-DOPA and PPD-derived compounds on bacterial growth

Virulent *V. splendidus* LGP32 strain [4, 29] and *V. aestuarianus* 02/041 strain [4] isolated in experimental cohabitation trials (Ifremer, La Tremblade, France) and from the Ifremer experimental hatchery at Argenton (Brittany, France), respectively, were used in antibacterial assays. Bacteria were grown at 25°C for 20 h in Marine Broth (Difco) under constant shaking until they reached the stationary phase of growth. The bacterial culture concentrations were evaluated spectrophotometrically at an OD of 620 nm. Cells were centrifuged at 3200 g for 10 min, the supernatant discarded and the resulting pellet resuspended in sterile artificial seawater to obtain an OD of 1 that corresponded to a concentration of $1\text{-}2.10^9$ colony forming units per ml for both bacterial strains.

Concentrations given in the following protocol correspond to final concentrations in the medium reaction, before adding Marine Broth. Resuspended bacteria cells (5 µl at OD of 1) were separately added to 100 µl of prepared oyster tissue extracts and 100 µl of L-DOPA (1.25 mM) or PPD solution (1.50 mM). The same protocol was used with PTU (1 mM)-treated HLS (100 µl). Two types of controls were performed either replacing substrates with 100 µl of Tris buffer as sterility controls of samples or replacing samples with 100 µl of Tris buffer to monitor the potential inhibitory effect of substrates on bacterial growth. After a 90 min incubation at 25°C, the samples were secondly diluted in Marine Broth medium (Difco) by a 20-fold factor: they were incubated with 4 ml of Marine Broth and grown at 25°C with constant shaking. Then, A_{620nm} readings were carried out at 1-2 h intervals for 3-7 h. The different samples were maintained at 25°C in a rotor (10 rpm) during all the experiment.

2.5.Phenoloxidase assays

Catecholase-type PO activity was measured spectrophotometrically by recording the formation of o-quinones, by using the method described previously [13]. For all conditions, assays were performed with three 10-oyster pools. Each pool was tested in triplicate wells. PO assays were conducted in 96-well microplates (Nunc, France). L-DOPA or PPD were used as substrates, at final concentrations of 10 mM and 50 mM, respectively. L-DOPA (10 mM) and PPD (50 mM) were prepared just before being used in Tris buffer and methanol, respectively. At 25°C, 10 µl of sample was incubated with 80 µl of L-DOPA and 50 µl of Tris buffer. Immediately after L-DOPA addition, PO-like activity was monitored during 4 h at 490 nm, by using a VersaMax™ microplate reader (Molecular Devices). Because of solubility constraints, the protocol was slightly modified in the case of PPD: the sample was incubated with 7 µl of PPD (50 mM diluted in methanol) and 123 µl of buffer (no effect of methanol was observed on the enzymatic reactions). PO-like activity was monitored during 2 h at 420 nm. Results were systematically corrected for non-enzymatic autoxidation of the substrate.
PO inhibition assay was performed by preincubating 10 µl of PO inhibitor (prepared at various concentrations in Tris buffer) with 10 µl of sample for 20 minutes, at 25°C. Then, PO assay was carried out with L-DOPA or PPD. Assays were performed with three 10-oyster pools. Each pool was tested in triplicate wells. Enzymatic oxidation (in the presence of PO inhibitor) was systematically corrected for non-enzymatic autoxidation of the substrate (in the presence of PO inhibitor).
Specific activities (SA) were expressed in international units (IU) per mg of total protein. One IU is defined as the amount of enzyme that catalyzes the appearance of 1 µmole of product per min [30], using molar extinction coefficients of L-DOPA and PPD reaction products of 3600 M^{-1} cm^{-1} [31] and 43 160 M^{-1} cm^{-1} [32, 33] under these conditions, respectively. The protein concentration of oyster tissue extracts was determined by the slightly modified Lowry method, as described previously [34]. Serum albumin (Sigma-Aldrich, France) was used as standard.

2.6.Statistical analysis

All values are reported as mean ± standard deviation (SD). Statistical analysis was carried out with STATISTICA 7.0. Values were tested for normality (Shapiro test) and homogeneity of variances (Bartlett test). For normal values, a nested ANOVA test was used to analyse the results, with condition as fixed factor, and pool as random factor. Pool was nested within each combination of condition [35]. When no significant differences were observed between pools and the null hypothesis (H_0: no difference between conditions) was rejected, significant differences were tested using Tukey's HSD test. For non normal values, a Kruskal-Wallis test was carried out, followed by Dunn's multiple comparisons test [35]. Statistical significance was designed as being at the level of $p<0.05$.

3. Results

3.1.Effect of PO-generated reactive intermediates on the growth of V. splendidus LGP32 strain in the presence of L-DOPA and oyster tissue extracts

In a previous study, catecholase-type PO activity was detected in the haemolymph of *C. gigas* with L-DOPA and dopamine as substrates [13]. However, in the present study, dopamine displayed a toxic effect, while L-DOPA displayed no toxic effect on LGP32, when used alone at 1.25 mM concentration (data not shown). Therefore, further studies were carried out with L-DOPA (1.25 mM).
When the digestive gland, gills, mantle and acellular fraction of the haemolymph were incubated with LGP32 strains, no spectrophotometric changes were noticed in the absence nor in the presence of L-DOPA at 620 nm, the wavelength for bacterial growth determination (data not shown). Interestingly, while optical density measures were not reduced in the presence of the HLS alone (Fig. 1a), they were significantly reduced in the presence of HLS and L-DOPA (Fig. 1a), indicating that a bacterial growth inhibition occurred. This effect was statistically significant after 5 and 6 h of incubation in presence of Marine Broth. Since inhibitory effects were noticed only with HLS (and PO substrate), further studies were conducted with this oyster extract.

3.2.Effect of PO-generated reactive intermediates on the growth of V. aestuarianus in the presence of L-DOPA and HLS

In order to confirm results obtained with LGP32, the effect of L-DOPA and HLS on an oyster bacterial pathogen phylogenetically distinct from *V. splendidus*, i.e. *V. aestuarianus* 02/041, was tested. In the presence of L-DOPA, a significant 02/041 growth inhibition was observed after 6 h of incubation (Fig. 1b).

3.3.Effect of PO-generated reactive intermediates on the growth of V. splendidus and V. aestuarianus in the presence of PPD and HLS

We decided next to test a PO substrate different from L-DOPA. In a previous study, laccase-type PO activity was detected in the haemolymph of *C. gigas* in the presence of the non phenolic substrate, PPD, but neither in the presence of the methoxy phenolic substrate, 4-hydroxy-3,5-dimethoxybenzaldehyde azine (syringaldazine), nor in the presence of the non phenolic substrate, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) [13]. However, since PPD exerted an effect on bacterial growth in the absence of HLS in the present study (data not shown), results were expressed as the percentage of inhibition due to HLS (Table 1). Interestingly, when PPD was used as substrate, a later, but similar, inhibition of LGP32 and 02/041 growths than in the presence of L-DOPA (Fig. 1), was observed: 18 ± 1 and 29 ± 1 % of inhibition for LGP32 at 6 and 7 h and 30 ± 6 % of inhibition for 02/041 at 7 h (Table 1).

3.4.Effect of PO inhibitors on PO-generated reactive intermediates

In the absence of HLS, CTAB (0.5, 1 and 2 mM) and DETC (1, 3, 5 and 10 mM), but not PTU (1, 5 and 10 mM), exerted an inhibition on LGP32 bacterial growth (data not shown).

Therefore, PO inhibitor effects were conducted only with the specific PO inhibitor, PTU. In the presence of PTU, the inhibition of LGP32 growth, produced in the presence of HLS and L-DOPA or PPD as substrates, was completely abolished, independently of the incubation time considered (Fig. 2a and b).

3.5.PO activity in HLS and plasma

Enzymatic tests were performed to quantify PO activity in two haemolymph fractions from *C. gigas*, i.e. the plasma and the HLS. Catecholase-type and laccase-type PO activities were detected in all samples with L-DOPA and PPD as substrates, and were inhibited in the presence of PTU (Table 2).

4. Discussion

The aim of the current work was *(i)* to study the implication of PO activity (ies) from *C. gigas* on immune oyster defence capacities, through antimicrobial assays, and *(ii)* to identify which type(s) of PO activity(ies) may be implicated on antibacterial activities. No oyster tissue (i.e. digestive gland, gills and mantle) or haemolymph fraction (i.e. plasma and HLS) exerted an antibacterial effect on *V. splendidus* LGP32 growth, in the absence of added PO substrates. Interestingly, in the presence of L-DOPA, only HLS inhibited bacterial growth. The higher PO activity detected in the HLS could contribute to the antibacterial properties observed in this haemolymph fraction, in comparison to the plasma (Table 2). Obviously, we were unable to estimate the substrate concentrations and enzyme activities required for an effective antimicrobial response against pathogens from the environment, but, among the different tissues (i.e. digestive gland, gills and mantle) and haemolymph fractions (i.e. plasma and HLS), haemocytes are known to play a critical role in bivalve defence responses [36, 37]. We may hypothesize that, in *C. gigas*, the PO activity in haemocytes is likely to restrict bacterial dissemination *in vivo*, and be complemented by other cellular immune reactions such as phagocytosis or encapsulation.

In the presence of L-DOPA, antibacterial effects on *V. splendidus* LGP32 and *V. aesturianus* 02/041 growth were observed (Fig. 1). Even if quinones formed with HLS alone or with HLS + L-DOPA could generate an optical signal at 620 nm, the significant reduction in absorbance obtained at 620 nm with HLS + L-DOPA at times 5 and 6 h in Fig. 1a, and at 6 h in Fig. 1b, can only be interpretated as an inhibition of the bacterial growth. Additionally, results with both strains, belonging to phylogenetically distinct units, suggest that PO-generated reactive intermediates could exert an effect on different oyster pathogens. Previous studies reported that PO cascades may have not only antibacterial effects but also virucidal effects [38]. Therefore, the effect of HLS and PO substrates in the presence of other bacteria or viruses, pathogens for *C. gigas*, should be further investigated.

It is important to notice however that, in the present study, the inhibition of bacterial growth by HLS was reported only when L-DOPA or PPD were added to the reaction medium. These results differ from previous studies conducted in different invertebrate species, where HLS elicited an inhibitory effect on bacteria growth, and in the absence of PO substrates [36, 37, 39, 40]. Nonetheless, results from the present study are in agreement with studies carried out in insects and crustaceans, such as the tobacco hornworm *Manduca sexta*, the mealworm beetle *Tenebrio molitor* and the freshwater crayfish *Pacifastacus leniusculus*, where antibacterial substances produced by POs have been reported in *in vitro* experiments [25-27]. Additionally, this is the first time to our knowledge that antibacterial activities of HLS from a mollusc species have shown to potentially involve POs, comforting the hypothesis of Zhao et al. [25], who states that PO-catalysed formation of quinones and semiquinones is a universal defence mechanism to kill microbial pathogens.

Results of the present study suggest also that natural PO substrate components have been inadvertently removed during HLS preparation, or alternatively, that they are in insufficient amount in bacterial culture medium to produce cytotoxic compounds. If we admit that, (1) as suggested by Cerenius et al. [27], some minutes are enough to obtain a bacteriotoxic effect from products generated by the phenoloxidase cascade (i.e. that the post-incubation dilution in the culture medium does not play a role in the bacteriotoxic effect), that, (2) as suggested by the present study, the prophenoloxidase cascade is essentially present in haemocytes, but that (3) the "natural" PO substrate is present in the acellular fraction of the haemolymph or in another tissue, we may hypothesize that the addition of an artificial substrate would be necessary to observe a bacteriotoxic effect with the HLS. Thus, it could be interesting to carry out the same type of experiments from the present study with plasma and HLS filtrated at 0.22 µm (to get rid of commensal bacterial flora), or with haemolymph treated with antiobiotics and with pathogenic bacteria resistant to these antiobiotics (i.e. in order to keep haemocytes intact).

Since PO substrates may also be used by other enzymes such as peroxidases, the next step in our study consisted on verifying that the inhibition of bacteria growth observed with HLS, and L-DOPA or PPD as substrates, was due to a PO-like activity. For this, we followed LGP32 growth in the presence of HLS, PO substrates, and the specific inhibitor of PO, PTU. Antibacterial effects were inhibited in the presence of PTU. These results are in agreement with previous studies carried out in different invertebrate species where the antibacterial activity in the presence of PO substrates was inhibited by PTU [25, 27, 41]. Whether tyrosinase, catecholase or laccase are implicated in L-DOPA reactions remains unresolved. Recently, a gene encoding a laccase has been identified and characterized at a molecular level in *C. gigas* [GenBank accession EU678320; 42]. Moreover, previous studies suggest the absence of tyrosinase activity and the presence of laccase activity in haemocytes, suggesting that laccase could be implicated in the L-DOPA reactions observed in the present study [13]. Nevertheless, the inhibitory effect observed in the presence of the PO inhibitor, PTU, was transitional. This observation could be due to the method used to quantify bacterial concentrations. Indeed, the spectrophotometric method is a simple and rapid technique, i.e. not time consuming. However, this technique is not very sensitive, i.e. the quantification can only be carried out only at the end of the exponential growth phase but, during this phase, the inhibition of bacterial growth could be masked by an important proliferation of bacteria that would be resistant to the initial antibacterial treatment. Other non spectrophotometrical-based techniques such as RT-qPCR could be used in this type of studies to increase the sensitivity to detect living organisms at low concentrations. Furthermore, because of their high sensitivities, the RT-qPCR technique would allow to use low concentrated inocula, which will in turn increase the sensitivity of the antimicrobial test [43].

Antibacterial effects with PPD (1.50 mM) as substrate were higher than with L-DOPA (1.25 mM) as substrate. This could be due to the fact that in PPD oxidation reactions, reactive semiquinones are produced while in L-DOPA oxidation reactions, quinones, which are more stable, are produced. Reactive semiquinones may react with O_2 to yield a superoxide radical ($O_2^{-\bullet}$) that may contribute to the production of other semiquinones or other oxygen radicals, that can be deleterious for *Vibrio* species [44]. In both cases, these results suggest that PO-catalysed reactions are an integral component of *C. gigas* defence system involving the production of intermediates, e.g. quinones that kill invading microorganisms.

Roles of laccases have been described in plants, fungi, bacteria and insects [45-47]. However, this is the first time to the best of our knowledge that a laccase-type PO activity is shown to be implicated in immune defence mechanisms in a mollusc species. This can be related to the recent identification of a gene coding a multicopper oxidase/laccase in the Pacific oyster, *C. gigas* [42]. Nevertheless, many questions remain unresolved. For example, whether the intracellular activities represent actual intracellular laccases, extracellular laccases in the process of being exported, or a combination of the two, remains to be determined. Additionally, no activators of PO activity (e.g. trypsin, lipopolysaccharides, laminarin, zymosan) were used in the present study [12]. Therefore, further investigations are

required on the role and mechanisms of action of laccases in invertebrates, and more particularly in molluscs of high economic value, such as *C. gigas*.

Conclusion

In summary, results in the presence of a specific chemical inhibitor of PO or of proPO activation, PTU, and of the enzyme substrates L-DOPA or PPD, suggest that a constitutive innate antibacterial immune response attributable to the activity of PO is capable of limiting *V. splendidus* LGP32 and *V. aestuarianus* 02/041 infections in *C. gigas,* although this effect appears in our *in vitro* trial as transitional. Antibacterial effects could be due to products of the haemocyte enzyme PO cascade, including quinone, superoxide anion and hydrogen peroxide. Given the potentially important roles that these enzymes, in particular laccases, might play in bivalve immune defence mechanisms, more detailed investigation of their activities is clearly warranted.

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Figure 1 Effect of HLS on *V. splendidus* LGP32 (a) and *V. aestuarianus* 02/041 (b) growth, with or without L-DOPA (1.25 mM). Absorbance readings were taken from the cultures at different intervals and plotted against time. Mean ± SD A_{620nm}, n=9 (3 replicates of 3 pools of 10 oysters), N= 3 experiment replicates. *= statistical difference for $p<0.05$ between 'LGP32' and 'LGP32+HLS+L-DOPA', or between '02/041' and '02/041+HLS+L-DOPA' conditions.

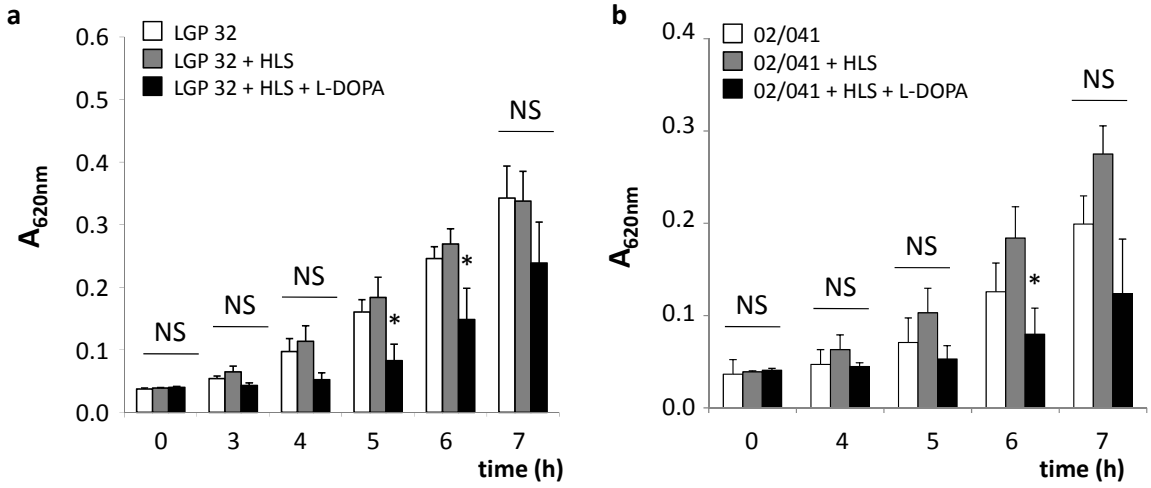

Figure 2 Effect of HLS and the specific PO inhibitor, PTU (1 mM), on *V. splendidus* LGP32 growth, with 1.25 mM L-DOPA (a) or with 1.50 mM PPD (b) as substrates. Absorbance readings were taken from the cultures at different intervals and plotted against time. Mean ± SD A_{620nm}, n=9 (3 replicates of 3 pools of 10 oysters), N= 3 experiment replicates. *= statistical difference for $p<0.05$ between 'LGP32+HLS+substrate' and 'LGP32+HLS+substrate+PTU'.

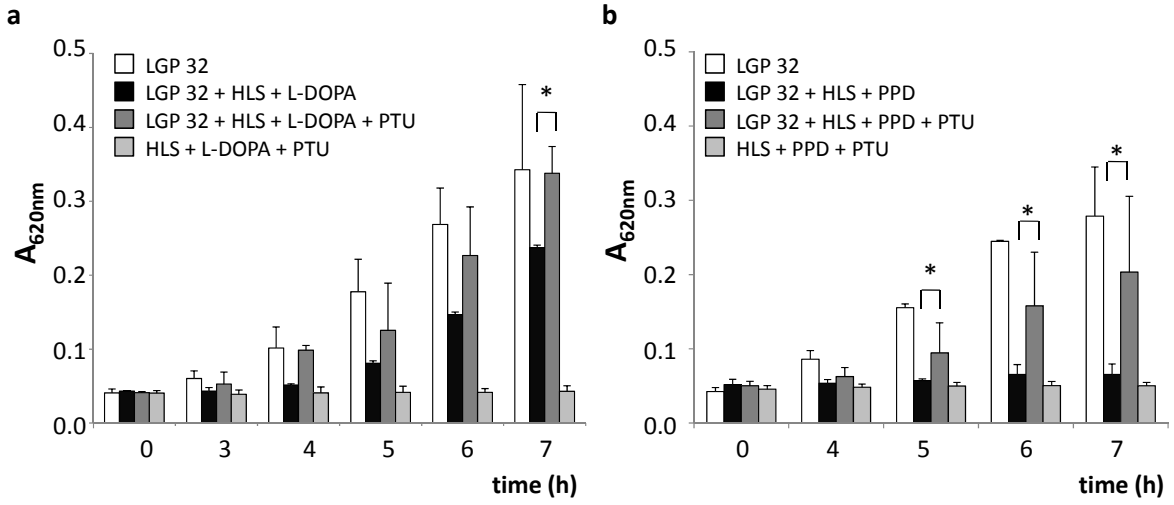

Table 1 Effect of PO-generated reactive intermediates on *V. splendidus* LGP32 and *V. aestuarianus* 02/041 growth, with PPD (1.50 mM) as substrate. Inhibition due to HLS (in %) corresponded to: [1 – (absorbance 'bacteria + sample + substrate' / absorbance 'bacteria + substrate')]*100. Mean ± SD, n=9, *= statistical difference ($p<0.05$) between the conditions 'bacteria + sample + substrate' and 'bacteria + substrate'. Negative values correspond to bacterial growth stimulation.

Time (h) / Treatments	3	4	5	6	7
LGP32 + HLS + PPD (1.50 mM)	-15 ± 9	-14 ± 3	-2 ± 14	18 ± 1 *	29 ± 1 *
02/041 + HLS + PPD (1.50 mM)	-22 ± 2	-17 ± 6	-14 ± 6	4 ± 6	30 ± 6 *

Table 2 Catecholase- and laccase-type phenoloxidase specific activities (in µmol.min^{-1}.mg prot^{-1}) in haemocyte lysate supernatant (HLS) and plasma, by using L-DOPA (10 mM) and PPD (50 mM) as substrate and PTU (5 mM) as inhibitor. Values represent the average of three replicates ± SD (n=9), *= statistical difference ($p<0.05$) between the conditions 'sample + substrate' and 'sample + substrate + PTU'.

Sample	L-DOPA (10 mM)	PPD (50 mM)
HLS	285.24 ± 41.53	164.68 ± 50.44
HLS + PTU (5 mM)	96.17 ± 5.54 *	0.00 ± 32.45 *
Plasma	48.96 ± 10.57	5.05 ± 1.25
Plasma + PTU (5 mM)	11.08 ± 9.64 *	0.00 ± 4.05 *