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First molecular evidence of cross-species induction of metalloprotease gene expression in *Vibrio* strains pathogenic for Pacific oyster *Crassostrea gigas* involving a quorum sensing system

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Abstract:

The aim of that study was to explore the hypothesis that a quorum sensing mechanism modulates metalloprotease gene expression of two *Vibrio* species pathogenic for oyster, at intraspecific (*Vibrio splendidus*) and interspecific (*V. splendidus* affecting *V. aestuarianus*) levels. Metalloprotease activities (Vsm for *V. splendidus* LGP32 and Vam for *V. aestuarianus* 02/041) and growth curves obtained by real-time PCR assay revealed cell density-dependent metalloprotease inductions triggered between 12 h and 16 h of culture. A quorum sensing assay was then developed using a conditioned medium prepared from supernatant of a mutant strain of *V. splendidus* LGP32 unable to produce metalloprotease. Specific real-time qPCR assays targeting metalloprotease genes *vsm* and *vam* were performed. A relative increase in expression was observed for *vsm* and, particularly, *vam* in the presence of the conditioned medium, probably controlled by a quorum sensing system. The study revealed intraspecific and interspecific modulation of expression in metalloprotease genes *vsm* and *vam* in *V. splendidus* and *V. aestuarianus*.

Our experiments showed for the first time that *V. splendidus* is able to produce an autoinducer-like substance that displays intra- and interspecific effects on the expression of two different metalloprotease genes: *vsm* and *vam*.

Highlights

► *V. splendidus* increases the expression level of *V. aestuarianus* metalloprotease vam. ► This mechanism is probably linked with quorum sensing. ► We show for the first time interspecific communication between these two *Vibrio*.

Keywords: Quorum sensing ; *Crassostrea gigas* ; *Vibrio* ; Metalloprotease ; Gene expression

1. Introduction

Vibrio splendidus and *V. aestuarianus* are two ubiquitous bacteria widely represented in marine ecosystems (Thompson, et al., 2005) and associated with the endogenous flora of various fish and shellfish (Arias, et al., 1999; Azandégbé, et al., 2010; Beaz Hidalgo, et al., 2008; Gomez-Gil, et al., 2010; Le Roux, Austin, 2006; Macián, et al., 2000; Montes, et al., 2006; Montes, et al., 2003; Pujalte, et al., 1999). These two *Vibrio* have been reported to be frequently involved in summer mortality events of the cultured Pacific oyster *Crassostrea gigas* in France in the last decade and most strains isolated from oyster during such summer mortality events were found to be virulent in experimental infections made by injection (Garnier, et al., 2007; Gay, et al., 2004a; Le Roux, et al., 2004; Saulnier, et al., 2009; Saulnier, et al., 2010).

Virulence processes of these two species are still not fully understood, but metalloproteases of the strains *V. splendidus* LGP32 (Vsm) and *V. aestuarianus* 01/032 (Vam) have been described as virulence factors (Binesse, et al., 2008; Labreuche, et al., 2010; Le Roux, et al., 2007). Furthermore, culture supernatants fluids from most of the virulent *V. splendidus* and *V. aestuarianus* strains isolated during abnormal summer mortality of *C. gigas* also have metalloprotease activities (Saulnier, et al., 2010). Indeed, the toxicity of extracellular products (ECPs) containing Vsm and of purified Vsm protein from *V. splendidus* LGP32 has been confirmed by experimental infection of oyster (Le Roux, et al., 2007) and tests on snail and mouse fibroblastic cell lines, while the LGP32-Δvsm-Δ1062 mutant ECPs had dramatically reduced toxicity (Binesse, et al., 2008). Concerning *V. aestuarianus* 01/032, a recombinant plasmid carrying the gene encoding the metalloprotease Vam was transferred to the non-virulent *V. splendidus*-related strain LMG20012T, which is naturally devoid of any protease activity (Labreuche, et al., 2010). Recombinant LMG20012T ECPs exhibited toxicity both *in vivo* on *C. gigas* oyster and *in vitro* on oyster hemocytes. Both *V. splendidus* LGP32 and *V. aestuarianus* 02/041 strains produce metalloproteases sharing 71 and 68 % of identity in their nucleotide and amino acid sequences respectively.

Furthermore, a complete genome analysis of the strain *V. splendidus* LGP32 (Le Roux, et al., 2009) revealed many genes potentially involved in quorum sensing mechanisms (Grimes, et al., 2009) and therefore potentially modulating virulence gene expression. Quorum sensing is a bacterial regulation system using extracellular signal molecules known as autoinducers to control expression of particular genes encoding bioluminescence, conjugation, virulence and biofilms (Bassler, 2002; Defoirdt, et al., 2005; Fuqua, Greenberg, 1998; Kaprelyants, Kell, 1996; Ren, et al., 2001; Schauder, Bassler, 2001; Wirth, et al., 1996). Synthesis of extracellular signal molecules appears critical for this process, and increases with bacterial concentration up to a significant threshold that induces the expression of target genes (Miller, Bassler, 2001; Waters, Bassler, 2005). Different quorum sensing systems have been described, notably depending on the signal molecules implicated: autoinducer AI-2, *cholerae* autoinducer-1 (CAI-1), acylated homoserine lactone (AHL), oligopeptides, quinolones, cyclic dipeptides and γ-butyrolactones (McDougald, et al., 2007). For example, *V. harveyi* bioluminescence, siderophore expression, a type III secretion system and a metalloprotease have all been proven to be modulated by quorum sensing mechanisms (Henke, Bassler, 2004b). Three different mechanisms involving three types of autoinducers and receptors have been found in this species: AI-2 (encoded by *luxS*, *luxQ*, *luxP*), CAI-1 (encoded by *cqsA*, *cqsS*) and AHL (encoded by *luxM*, *luxN*) (Defoirdt, et al., 2007). Based on the complete genome sequence of *V. splendidus* LGP32, it seems that this strain shares the three quorum sensing systems found in *V. harveyi*, which are based on three different autoinducers: AHL, CAI-1 and AI-2 1. In *V. aestuarianus* 02/041, a few genes

linked to quorum sensing have been found in its partial genome sequence (*luxS*, *luxO*, *luxU* and *luxR* – J.L. Nicolas, pers. com., Genoscope project, http://www.genoscope.cns.fr/spip/Whole-genome-sequencing-of-Vibrio.html, in progress).

A strong synergistic effect of virulence has been described in experimental infections in which both of these strains were injected simultaneously rather than singly at the same dose (Saulnier, et al., 2010). In the present study, we explored the hypothesis of quorum sensing mechanisms involved in the modulation of expression of *vsm* and *vam* metalloprotease genes at both protein and transcriptomic levels, evaluating strains i/ intraspecifically (*V. splendidus*) and ii/ interspecifically (*V. splendidus* / *V. aestuarianus*). Expression of the two metalloprotease genes *vsm* and *vam* were quantified by real-time qPCR assays using two different growth media: Marine Broth on the one hand, and a conditioned medium prepared from a culture of a mutant strain of *V. splendidus* LGP32 unable to produce metalloprotease in culture supernatant fluids on the other hand.

2. Material and Methods

2.1. Growth and metalloprotease activity curves

2.1.1. Bacterial strains and culture conditions

All strains used in this study (listed in Table 1) were cultured in Marine Broth (MB, Difco). For 02/041 and LGP32-GFP growth curves and proteolytic activity curves of the culture supernatant fluid (CSF), pure cultures were prepared in parallel as follows. Seven ml MB were inoculated with colonies isolated on MB 15% agar medium plates from bacterial stock culture stored at -80°C and prepared as 15% (v/v) glycerol suspensions in MB. Bacteria were grown at 20°C for 24 h under constant shaking. The expected 10^9 bacteria per ml concentrations of bacterial suspensions at stationary phase were checked by the plate counting method. A 75-µl subsample of this bacterial stationary phase suspension was used to inoculate a 250 ml-flask of MB. This 250 ml MB culture was divided 30 times into 7 ml cultures that were maintained under constant shaking. Three tubes were sampled as biological triplicates at 3 h, 5 h, 7 h, 9 h, 10.5 h, 12 h, 16 h, 20 h, 45 h and 141 h post-inoculation for bacterial quantification using a qPCR assay and for the assessment of the proteolytic activity in the CSF.

2.1.2. Real-time qPCR assays to quantify bacteria in pure cultures

Standard curves for the quantification of each strain were established using 1:10 serial dilutions of *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 cells obtained from 20 h stationary phase pure cultures. Nine hundred µL of each dilution were centrifuged at 10 000 g for 5 min at 20°C. The resulting cell pellets were resuspended in 900 µl ultra pure water (Sigma). Total DNA was extracted from 100 µl by boiling for 10 min at 98°C and then placing at 4°C for a few minutes before PCR assay. Five µl of the solution were used in triplicate as PCR template. The quantification of bacterial cells by real-time PCR was compared each time with the enumeration of the bacterial cells used for DNA extractions, done using the plate counting method.

To obtain growth curves, this same DNA extraction procedure was applied to 200-µl samples of the pure cultures. Two real-time qPCR assays were then used for the specific quantification of *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 strains in pure culture samples, as already described in (De Decker, Saulnier, 2011; Saulnier, et al., 2009). Both qPCR were based on Taqman® chemistry and targeted the GFP and dnaJ genes, respectively. The sequences of oligonucleotides and Taqman® probes used here are given in (De Decker, Saulnier, 2011). Real-time PCR assay was conducted on an MX3000 Thermocycler (Stratagene) using a Brilliant QPCR Core Reagent Kit (Stratagene). The thermal cycle and Ct analysis were carried out in exactly the same way as described by (Saulnier, et al., 2009). Each reaction was run in triplicate in a final volume of 25 µl, containing various concentrations of DNA sample (5 µl), 5 mmol l^{-1} $MgCl_2$, 200 µmol l^{-1} of each dNTP, 300 nmol l^{-1} of each primer, 200 nmol l^{-1} of oligonucleotidic probe and 1.25 units of Hot Start Sure Taq polymerase (Qiagen).

2.1.3. Metalloprotease activity in culture supernatant fluids

Culture supernatant fluids (CSF) were obtained by centrifugation of each bacterial suspension at 10 000 g for 5 min. Proteolytic activity in 250 µl CSF was measured in the ten samples covering the two strains 02/041 and LGP32-GFP grown in pure cultures (see above) and determined using azocasein (Sigma Chemical Co., St. Louis, MO) as a substrate, according to the method described in (Saulnier, et al., 2010). To confirm metalloprotease activity in culture supernatants, CSF were assayed with azocasein under the same conditions, with an additional CSF preincubation step at 20°C for 30 min in the presence of 8 mmol l^{-1} 10-phenanthroline as a metalloprotease inhibitor.

2.2. Quorum sensing assay

2.2.1. Preparation of cell-free conditioned medium and cultures

To test the hypothesis that a quorum-sensing system regulates metalloprotease genes expression, *V. splendidus* LGP32 Δ*vsm*-Δ1062 was grown in 100 ml MB at 20°C for 20 h under constant shaking. Cells were centrifuged at 3500 g for 15 min and the resulting supernatant was filtered through a 0.22 µm-pore-size acetate filter. This cell-free and non-proteolytically active supernatant from stationary phase cultures of the mutant strain was used as culture media for both *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 strains.

To examine the effects of such a conditioned medium on metalloprotease gene expression, several cell pellets from centrifuged stationary phase cultures of *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041 were prepared for expected final concentrations of 10^5 and 10^8 CFU ml^{-1} and resuspended in 7 ml of either conditioned medium or MB medium.

For the quantification of bacteria and the analysis of *vsm* and *vam* gene expression levels by real-time PCR, Total DNA (see procedures above) and RNA (see procedures below) were extracted from 1.5 h post-inoculum cultures, respectively.

2.2.2. Analysis of *vsm* and *vam* gene expression in pure cultures by real-time qPCR

Total bacterial RNA was isolated from 500 µl of MB or conditioned pure culture (see above) added with 1 ml of the stabilizing and protecting RNA Bacteria Reagent (Qiagen). Bacteria RNA extractions were performed using a commercial kit (RNeasy Mini Kit, Qiagen) including a DNase treatment.

Culture samples were centrifuged at 10 000 g for 5 min. Bacterial disruption and lysate homogenization were performed in three steps: i/ using an appropriate buffer containing proteinase K (10 mg μl^{-1}) and lysozyme in DEPC-treated water, ii/ using an appropriate buffer containing β-mercaptoethanol (10 µl ml^{-1}) and iii/ adding 250 µl absolute ethanol. Seven hundred µl of these lysates were purified according to manufacturers' instructions (RNeasy Mini Kit, Qiagen).

The expression levels of metalloprotease genes *vsm* and *vam* were determined by real-time qPCR on an MX3000 Thermocycler (Stratagene). Approximately 100 ng of RNA transcripts were reverse-transcribed in a final volume of 20 µl, using an optimized blend of random primers (Qiagen) and a mix of two reverse transcriptases (Quantiscript Reverse Transcriptase, Qiagen), according to the manufacturer's instructions (Quantitect Reverse Transcription Kit, Qiagen). cDNAs were amplified by real-time PCR using specific primers designed with Primer 3 software, under general conditions of 60°C optimal annealing temperature for pairs of oligonucleotides, and an amplicon size of 159 bp (Table 2). The cDNA regions for primer design were deduced from a GenBank targeted sequence alignment. The primers were checked for specificity using a BLAST search to determine homology with known sequences. Two primer pairs per gene were tested for their efficacy and specificity in real-time PCR, leading to the selection of a primer pair for the subsequent gene expression analysis done in this study.

The real-time PCR assay was performed in triplicate in a total volume of 25 µl, using Brilliant SYBR Green qPCR master mix (Stratagene), with 200 nmol l^{-1} of each primer and exactly the same thermal cycle conditions as described in (De Decker, Saulnier, 2011). PCR efficiencies ($E=10^{(-1/slope)}$) and linear regressions were calculated by drawing standard curves from a serial dilution analysis of cDNAs obtained from overnight cultures of either *V. splendidus* LGP32-GFP or *V. aestuarianus* 02/041 strains, testing each dilution in triplicate. Normalized relative gene expression levels were calculated with the formula: $F=(E+1)^{40-Ct}/N$ where i/ E = qPCR efficacy, ii/ threshold cycle (Ct) value corresponds to the PCR cycle number at which an increase in reporter fluorescence above the baseline signal was first detected, iii/ sensitivity of qPCR metalloprotease gene detection is obtained at Ct=40 and arbitrarily considered to correspond to one copy of the targeted gene present in the qPCR well, iv/ N is the number of 10^9 CFU-equivalent genomes determined by absolute real time PCR quantification of 02/041 and LGP32 strains.

3. Results

3.1. Growth curves and metalloprotease activity

Standard curves of the virulent LGP32-GFP and 02/041 strains are shown in Figure 1. The *V. splendidus* LGP32-GFP linear regression curve had a regression coefficient (r^2) of 0.997 and a PCR reaction efficacy of 103.9%. The *V. aestuarianus* 02/041 linear regression curve had an r^2 of 0.998 and a PCR reaction efficacy of 103.6%.

The growth curves quantified by real-time qPCR, show similar general patterns. Strains reached comparable concentrations after 20 h culture (*V. aestuarianus*: 2.3 10^8 CFU-equivalent genomes number ml^{-1}; *V. splendidus*: 3.9 10^8 CFU-equivalent genomes number ml^{-1}) (Figure 2). Similar curves were obtained by the plate counting method (data not shown). The linearity of the exponential phase of growth enabled us to calculate generation times, which were of 41 min for *V. splendidus* LGP32-GFP and 60 min for *V. aestuarianus* 02/041 in these experimental conditions.

Large differences in metalloprotease activity were found between the strains from 16 h culture time. *Vibrio aestuarianus* 02/041 produced 5.5 times more metalloprotease than *V. splendidus* LGP32-GFP, i.e. 433 units per number ($\times 10^9$) of cells in the 16 h *V. aestuarianus* 02/041 CSF and 79 units per number ($\times 10^9$) of cells in the 16 h *V. splendidus* LGP32-GFP CSF. Production by *V. splendidus* LGP32-GFP was almost stable after 16 h: 66 ± 4 units per number ($\times 10^9$) of cells, whereas *V. aestuarianus* 02/041 production increased logarithmically from 16 h to 141 h to reach 1016 ± 18 units per number ($\times 10^9$) of cells.

For both bacteria, metalloprotease activity was only detectable after 12 h or more of culture and was particularly observed in the 16 h cultures. These cell density-dependent metalloprotease inductions were triggered at concentrations between 2.9 10^8 and 3.8 10^8 CFU-equivalent genomes number ml^{-1} for *V. splendidus* LGP32-GFP and between 7.2 10^7 and 1.9 10^8 CFU-equivalent genomes number ml^{-1} for *V. aestuarianus* 02/041.

3.2. Quorum sensing assay

Two kinds of media were inoculated at 10^5 and 10^8 CFU ml^{-1} with either *V. splendidus* LGP32-GFP or *V. aestuarianus* 02/041: Marine Broth and the conditioned medium prepared from the stationary phase of mutant cultures (see Materials and Methods). Bacteria in 1.5 h cultures were quantified by real-time qPCR targeting recombinant *gfp* and *dnaJ* genes.

PCR primers were designed to specifically quantify the expression of metalloprotease genes *vsm* and *vam*. Real-time qPCR efficacies and r^2 correlations were calculated by drawing standard curves, giving 99.3% and 0.952, respectively for *vsm*, and 103.7% and 0.998 for *vam*. Size of amplified PCR products was verified by visualizing the expected 159 bp amplicon on agarose gel electrophoresis (data not shown).

The normalized relative gene expression levels were calculated using the formula: $F=(E+1)^{40-Ct}/N$. No expression of *vsm* and *vam* was measured in MB at the lower bacterial concentration (Figure 3), while significant relative gene expression F levels were measured in MB at the

higher concentration for both *V. splendidus* LGP32-GFP and *V. aestuarianus* 02/041: 8 and 1.1 10^4, respectively.

In the comparison of MB and conditioned media, no expression of *vsm* was measured at the lower bacterial concentration in either of the media, while an increase in F value was observed (from 8 to 35) at the higher concentration, corresponding to an increase in the quantity of *vsm* transcripts by a factor of 4.4 in the conditioned medium.

For *V. aestuarianus* 02/041, no metalloprotease gene expression was detected at lower bacterial concentrations in MB medium whereas a significant increase by a factor of at least 2.4 10^5 was observed at a similar concentration in the conditioned medium. A higher F value was also found at the higher concentration (F value of 1.1 10^4 in MB and 2.4 10^6 in the conditioned medium) which corresponds to an increase of the transcript levels by a factor of 218. So the relative expression level of *vam* at the fold higher bacterial concentration is 6.7 10^4 higher than *vsm* in the conditioned medium.

4. Discussion

In this study we explored the modulation of *vsm* and *vam* gene expression using a conditioned medium hypothetically containing autoinducers that modulate virulence factors *via* a quorum sensing system. Several different arguments led us to hypothesize that such a quorum sensing system could be at work, modulating metalloproteases in *V. splendidus* and *V. aestuarianus*:

i/ Vsm and Vam metalloproteases have been described as important virulence factors in ECPs of *V. splendidus* LGP32 and *V. aestuarianus* 01/032, respectively (Binesse, et al., 2008; Labreuche, et al., 2010; Le Roux, et al., 2007). Furthermore, metalloprotease activities have been characterized in most of the virulent *V. splendidus* and *V. aestuarianus* strains isolated during summer mortality events of oyster *Crassostrea gigas* in France in a large scale epidemiological survey conducted between 2003 and 2007 (Saulnier, et al., 2010). Metalloprotease gene expression modulated by quorum sensing has been described in a number of different *Vibrio* species: *V. cholerae*, *V. harveyi*, *V. vulnificus* and *V. anguillarum* (Denkin, Nelson, 2004; Henke, Bassler, 2004b; Mok, et al., 2003; Shao, Hor, 2001; Zhu, et al., 2002).

ii/ Natural co-infection by *V. splendidus* and *V. aestuarianus* has been described, as have synergistic effects in experimental infections, observed both intra-specifically between different *V. splendidus* strains (Gay, et al., 2004b) and inter-specifically between *V. splendidus* and *V. aestuarianus* (Saulnier, et al., 2010). Such synergistic effects could be linked to bacterial communication *via* a quorum sensing system that modulates virulence factor expression.

iii/ A quorum sensing system was identified *in silico* using the complete genome sequence of *V. splendidus* LGP32 (Le Roux, et al., 2009). Concerning *V. aestuarianus* 02/041, the partially annotated genome sequencing has already allowed the identification of an autoinducer synthase AI-2-like, signal transducer and transcriptional regulator (unpublished results). CAI-1 is produced by a number of *Vibrio* species and this system is presumed to be used for intragenus communication whereas AI-2 seems to be linked to interspecies quorum sensing communication (Higgins, et al., 2007). Thus, because of their genome content, both *V. splendidus* and *V. aestuarianus* could putatively have the capacity to communicate interspecifically *via* a quorum sensing mechanism.

iv/ Some experimental observations based on the analysis of both growth curves and metalloprotease production curves (Figure 2) support the existence of a quorum sensing system in both *V. splendidus* and *V. aestuarianus* strains used in this study.

V. splendidus and *V. aestuarianus* growth curves showed very slight differences in our culture conditions; in particular, there was a shorter generation time in *V. splendidus* LGP32-GFP than in *V. aestuarianus* 02/041: about 40 min and 60 min, respectively. After 20 h of culture, both strains reached the stationary phase at similar concentrations between 10^8 and 10^9 CFU ml^{-1}. The bacterial quantification by qPCR was compared with plate counts for each point in time studied and showed convergent results until 45 h of culture. This last result suggests that no bias of quantification by qPCR occurred, since this latter technique is able to detect dead as well as live bacteria if they still contain an intact genome. Nevertheless, higher accuracy and narrower standard deviations were obtained from the biological triplicates using qPCR assay compared with the plate counting method, which was more laboratory intensive. In the first 45 h of growth, the qPCR assay used in this study was deemed useful because of its rapidity, specificity, accuracy and reliability.

Our method for measuring metalloprotease production is based on the assessment of azocaseinase activity, which is assumed to be due to a metalloprotease-like enzyme due to the effects of zinc-dependent metalloprotease inhibitors such as phenanthrolein. First, it was interesting to notice that metalloprotease-like activity measured in *V. aestuarianus* 02/041 CSF at 45 h incubation time and related to the corresponding number of cells, reached levels more than 6 times higher (772 ± 6 units per number ($\times 10^9$) of cells) than in *V. splendidus* LGP32 CSF (124 ± 2 units per number ($\times 10^9$) of cells). Second, the first detection of metalloprotease activities occurred in CSF from both strains from 12 to 16 h in MB cultures, corresponding to 10^8 CFU ml^{-1} concentrations. Earlier in the culture period, metalloprotease activities were undetectable in the CSF, but *V. splendidus* and *V. aestuarianus* metalloprotease production was clearly triggered when cultures reached a threshold concentration of 7.2 10^7 CFU ml^{-1} for both cultures. These results suggest that the production of these virulence factors involves a cell-density dependent phenomenon and is likely induced by a quorum sensing system.

Our study therefore confirms the presence of a bacterial quorum sensing system, acting through the release of soluble communicator molecules that allow intercellular communication contributing to the regulation of cell division and gene expression. We tested the hypothesis of a quorum-sensing system regulating metalloprotease gene expression (virulence) *in vitro*, using the 0.22 µm filtered cell-free and non-proteolytically active supernatant from a 20 h culture of the *V. splendidus* LGP32 Δ*vsm*-Δ1062 strain as a conditioned medium likely to contain autoinducer-like substances.

The results from the growth and metalloprotease activity experiments (Figure 2) allowed us to accurately determine the relevant culture concentrations to study. The first concentration, of 10^8 CFU ml^{-1}, corresponds to the culture concentration with a positive metalloprotease activity for both *Vibrio*. The second concentration, of 10^5 CFU ml^{-1}, was lower than the previously recorded 7.2 10^7 CFU ml^{-1} threshold for metalloprotease activity detection in CSF. Moreover, these results (Figure 2) allowed us to determine the minimal optimal age of the cultures to study: cultures aged 1.5 h offered two advantages: i/ avoidance of the significant production of endogenous autoinducers by bacteria in each inoculum used, which could mask the effect of exogenous autoinducers potentially present in the conditioned medium; and ii/ maintainance of bacterial concentrations close to those of the inocula, since generation times of 41 min and 60 min had already been determined for the studied *V. splendidus* and *V. aestuarianus* strains, respectively.

The normalized relative quantification of *vsm* and *vam* expression level in different 1.5 h cultures allowed us to corroborate the quorum sensing hypothesis. Firstly, concerning *V. splendidus* LGP32 cultures, no *vsm* mRNA was detected in either conditioned or MB media at the low bacterial concentration (10^5 CFU ml^{-1}). A 4.4-fold increase was revealed in the conditioned culture compared with the MB media at the high bacterial concentration (10^8 CFU ml^{-1}). These results indicate that the *V. splendidus* mutant produced autoinducer-like activity available to *V. splendidus* LGP32 and capable of regulating *vsm* expression.

Secondly, concerning *V. aestuarianus* 02/041 cultures, the first detection of *vam* metalloprotease mRNA occurred 1.5 h after the addition of the cell-free conditioned medium, corresponding to 2.10^4 CFU ml^{-1}, whereas no *vam* mRNA was detected at the same concentration in MB cultures. The use of conditioned medium provoked spectacular fold increases of *vam* transcripts of at least 2.4 10^5 and 218 at low and high (10^8 CFU ml^{-1}) bacterial concentrations, respectively, when comparing the effect of the two media. *V. aestuarianus* is thus capable of responding to molecules produced by this *V. splendidus* strain present in culture supernatant fluids. Finally, a 6.7 10^4-fold increase of F value was found when comparing the relative metalloprotease gene expression level of *V. aestuarianus* to *V. splendidus* at high bacterial concentration in the conditioned medium. These results showed that the *V. splendidus* mutant produced an autoinducer-like activity that could act interspecifically through the *V. aestuarianus* 02/041 signaling system, thereby regulating and inducing *vam* expression even at the lowest concentration under the threshold value for metalloprotease activity detection. These results could partly explain why the *V. aestuarianus* 02/041 strain exhibited higher virulence when injected simultaneously with the *V. splendidus* LGP32 strain into *C. gigas* oysters, than when *V. aestuarianus* was injected alone at the same concentration (Saulnier, et al., 2010). Our experiments confirmed for the first time that *V. splendidus* LGP32 is able to produce an autoinducer-like substance that displays both intra- and interspecific effects on the expression of the two metalloprotease genes, *vsm* and *vam*.

A number of different approaches could be taken in future research to identify which autoinducers are produced by *V. splendidus* and which are able to modulate *V. splendidus* and *V. aestuarianus* metalloprotease gene expression. First, biosensor strains that report autoinducer activities could be employed. Such strains do not produce their own autoinducers but contain a reporter gene that can be induced when supplied with an exogenous signal molecule. Different biosensor strains are available targeting a variety of autoinducers, for example *V. harveyi* JAF375 (HAI-1^-, AI-2^-, CAI-1^+), *V. harveyi* JMH597 (HAI-1^-, AI-2^+, CAI-1^-) and *V. harveyi* JMH612 (HAI-1^+, AI-2^-, CAI-1^-) (Bassler, et al., 1997; Defoirdt, et al., 2008; Henke, Bassler, 2004a; Yang, et al., 2011). Furthermore, normal-phase high-performance liquid chromatography (HPLC) could be carried out to identify the autoinducers present in ECPs. Finally, mutagenesis could be performed targeting genes involved in the quorum sensing system, as allelic exchange strategies have been seen to be efficient on *V. splendidus* strain LGP32 using suicide vectors (Le Roux, et al., 2007).

To conclude, these results suggested that a quorum sensing system causes the modulation of *vsm* and *vam* metalloprotease genes expression in our culture conditions. This quorum sensing phenomenon had intra- and interspecific effects on virulence factor expression when using *V. splendidus* and *V. aestuarianus* strain models. This common cell-to-cell communication between these two bacteria could contribute to the synergistic effect previously described between these two *C. gigas* oyster pathogens (Saulnier, et al., 2010). More bacterial strains commonly found around or in oyster's environments, will have to be tested in further cross-stimulation assays in order to assess the specificity of the quorum sensing system involved in the metalloprotease virulence factor expression. Using a quorum sensing inhibitor could reveal

much information about the virulence modulation and residual pathogenicity of marine bacteria pathogenic for oysters. Our experimental approaches performed in pure cultures could be extended to *in vivo* studies. Knowing the abundance and complexity of the oyster endogenous bacterial flora and the physiological permeability of internal hemolymph tissue to surrounding bacterioplankton (De Decker, Saulnier, 2011), bacterial interaction studies involving the quorum sensing system could contribute to our knowledge on bacterial competition or collaboration mechanisms leading to bacterial pathogenesis (Duan, et al., 2003). These findings offer many new directions for study of the modulation of bacterial virulence mechanisms involved in *C. gigas* mortality events.

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References

Arias, C.R., Macian, M.C., Aznar, R., Garay, E., Pujalte, M.J., 1999. Low incidence of *Vibrio vulnificus* among *Vibrio* isolates from sea water and shellfish of the western Mediterranean coast. J Appl Microbiol. 86, 125-134.

Azandégbé, A., Garnier, M., Andrieux-Loyer, F., Kérouel, R., Philippon, X., Nicolas, J., 2010. Occurrence and seasonality of *Vibrio aestuarianus* in sediment and *Crassostrea gigas* haemolymph at two oyster farms in France. Dis Aquat Org. 91, 213-221.

Bassler, B.L., 2002. Small talk. Cell-to-cell communication in bacteria. Cell. 109, 421-424.

Bassler, B.L., Greenberg, E.P., Stevens, A.M., 1997. Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. J Bacteriol. 179, 4043-4045.

Beaz Hidalgo, R., Cleenwerck, I., Balboa, S., De Wachter, M., Thompson, F.L., Swings, J., De Vos, P., Romalde, J.L., 2008. Diversity of Vibrios associated with reared clams in Galicia (NW Spain). Syst Appl Microbiol. 31, 215.

Binesse, J., Delsert, C., Saulnier, D., Champomier-Verges, M.C., Zagorec, M., Munier-Lehmann, H., Mazel, D., Le Roux, F., 2008. The metalloprotease Vsm is the main toxic factor for *Vibrio splendidus* extracellular products. Appl Environ Microbiol. 74, 7108-7117.

De Decker, S., Saulnier, D., 2011. Vibriosis induced by experimental cohabitation in *Crassostrea gigas*: Evidence of early infection and down-expression of immune-related genes. Fish Shellfish Immun. 30, 691-699.

De Decker, S., Normand, J., Saulnier, D., Pernet, F., Castagnet, S., Boudry, P., 2011. Responses of diploid and triploid Pacific oysters *Crassostrea gigas* to *Vibrio* infection in relation to their reproductive status. J Invertebr Pathol. 106, 179-191.

Defoirdt, T., Verstraete, W., Bossier, P., 2008. Luminescence, virulence and quorum sensing signal production by pathogenic *Vibrio campbellii* and *Vibrio harveyi* isolates. J Appl Microbiol. 104, 1480.

Defoirdt, T., Bossier, P., Sorgeloos, P., Verstraete, W., 2005. The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio*

harveyi on their virulence towards gnotobiotically cultured *Artemia franciscana*. Environ Microbiol. 7, 1239-1247.
Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2007. Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. Trends Biotechnol. 25, 472-479.
Denkin, S.M., Nelson, D.R., 2004. Regulation of *Vibrio anguillarum* empA metalloprotease expression and its role in virulence. Appl Environ Microbiol. 70, 4193-4204.
Duan, K., Dammel, C., Stein, J., Rabin, H., Surette, M.G., 2003. Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. Mol Microbiol. 50, 1477-1491.
Fuqua, C., Greenberg, E.P., 1998. Self perception in bacteria: quorum sensing with acylated homoserine lactones. Curr Opin Microbiol. 1, 183-189.
Garnier, M., Labreuche, Y., Garcia, C., Robert, M., Nicolas, J.L., 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microb Ecol. 53, 187-196.
Gay, M., Berthe, F.C., Le Roux, F., 2004a. Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. Dis Aquat Org. 59, 49-56.
Gay, M., Renault, T., Pons, A.M., Le Roux, F., 2004b. Two *Vibrio splendidus* related strains collaborate to kill *Crassostrea gigas*: taxonomy and host alterations. Dis Aquat Org. 62, 65-74.
Gomez-Gil, B., Roque, A., Lacuesta, B., Rotllant, G., 2010. Diversity of vibrios in the haemolymph of the spider crab *Maja brachydactyla*. J Appl Microbiol. 109, 918.
Grimes, D., Johnson, C., Dillon, K., Flowers, A., Noriea, N., Berutti, T., 2009. What genomic sequence information has revealed about *Vibrio* ecology in the ocean—A review. Microb Ecol. 58, 447.
Henke, J.M., Bassler, B.L., 2004a. Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. J Bacteriol. 186, 6902-6914.
Henke, J.M., Bassler, B.L., 2004b. Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J Bacteriol. 186, 3794-3805.
Higgins, D.A., Pomianek, M.E., Kraml, C.M., Taylor, R.K., Semmelhack, M.F., Bassler, B.L., 2007. The major *Vibrio cholerae* autoinducer and its role in virulence factor production. Nature. 450, 883.
Kaprelyants, A.S., Kell, D.B., 1996. Do bacteria need to communicate with each other for growth? Trends Microbiol. 4, 237-242.
Labreuche, Y., Le Roux, F., Henry, J., Zatylny, C., Huvet, A., Lambert, C., Soudant, P., Mazel, D., Nicolas, J.-L., 2010. *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. Fish & Shellfish Immunology. 29, 753.
Le Roux, F., Austin, B., 2006. *Vibrio splendidus*. In: Thompson, F.L., Austin, B., Swings, J. (Eds.), The Biology of Vibrios. ASM Press, Washington, DC, pp. 285-296.
Le Roux, F., Binesse, J., Saulnier, D., Mazel, D., 2007. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Appl Environ Microbiol. 73, 777-784.
Le Roux, F., Gay, M., Lambert, C., Nicolas, J.L., Gouy, M., Berthe, F., 2004. Phylogenetic study and identification of *Vibrio splendidus*-related strains based on *gyrB* gene sequences. Dis Aquat Org. 58, 143-150.
Le Roux, F., Mohamed, Z., Chakroun, N., Binesse, J., Saulnier, D., Bouchier, C., Zidane, N., Ma, L., Rusniok, C., Lajus, A., Buchrieser, C., Médigue, C., Polz, M.F., Mazel, D., 2009. Genome sequence of *Vibrio splendidus*: an abundant planctonic marine species with a large genotypic diversity. Environ Microbiol. 11, 1959-1970.

Macián, M., Garay, E., González-Candelas, F., Pujalte, M., Aznar, R., 2000. Ribotyping of *Vibrio* populations associated with cultured oysters (*Ostrea edulis*). Syst Appl Microbiol. 23, 409-417.

McDougald, D., Rice, S., Kjelleberg, S., 2007. Bacterial quorum sensing and interference by naturally occurring biomimics. Anal Bioanal Chem. 387, 445.

Miller, M.B., Bassler, B.L., 2001. Quorum Sensing in Bacteria. Annu Rev Microbiol. 55, 165-199.

Mok, K., Wingreen, N., Bassler, B., 2003. *Vibrio harveyi* quorum sensing: a coincidence detector for two autoinducers controls gene expression. EMBO J. 22, 870-881.

Montes, M., Farto, R., Perez, M.J., Armada, S.P., Nieto, T.P., 2006. Genotypic diversity of *Vibrio* isolates associated with turbot (*Scophthalmus maximus*) culture. Res Microbiol. 157, 487.

Montes, M., Farto, R., Pérez, M.J., Nieto, T.P., Larsen, J.L., Christensen, H., 2003. Characterization of *Vibrio* strains isolated from turbot (*Scophthalmus maximus*) culture by phenotypic analysis, ribotyping and 16S rRNA gene sequence comparison. J Appl Microbiol. 95, 693.

Pujalte, M.J., Ortigosa, M., Macián, M.C., Garay, E., 1999. Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. Int Microbiol. 2, 259-266.

Ren, D., Sims, J., J., Wood, T., K., 2001. Inhibition of biofilm formation and swarming of *Escherichia coli* by (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone. Environ Microbiol. 3, 731-736.

Saulnier, D., De Decker, S., Haffner, P., 2009. Real-time PCR assay for rapid detection and quantification of *Vibrio aestuarianus* in oyster and seawater: A useful tool for epidemiologic studies. J Microbiol Meth. 77, 191-197.

Saulnier, D., De Decker, S., Haffner, P., Cobret, L., Robert, M., Garcia, C., 2010. A large-scale epidemiological study to identify bacteria pathogenic to Pacific oyster *Crassostrea gigas* and correlation between virulence and metalloprotease-like activity. Microb Ecol. 59, 787-798.

Schauder, S., Bassler, B.L., 2001. The languages of bacteria. Gene Dev. 15, 1468-1480.

Shao, C.-P., Hor, L.-I., 2001. Regulation of metalloprotease gene expression in *Vibrio vulnificus* by a *Vibrio harveyi* LuxR homologue. J Bacteriol. 183, 1369-1375.

Thompson, J.R., Pacocha, S., Pharino, C., Klepac-Ceraj, V., Hunt, D.E., Benoit, J., Sarma-Rupavtarm, R., Distel, D.L., Polz, M.F., 2005. Genotypic diversity within a natural coastal bacterioplankton population. Science. 307, 1311-1313.

Waters, C.M., Bassler, B.L., 2005. QUORUM SENSING: Cell-to-Cell Communication in Bacteria. Annu Rev Cell Dev Bi. 21, 319-346.

Wirth, R., Muscholl, A., Wanner, G., 1996. The role of pheromones in bacterial interactions. Trends Microbiol. 4, 96-103.

Yang, Q., Han, Y., Zhang, X.H., 2011. Detection of quorum sensing signal molecules in the family *Vibrionaceae*. J Appl Microbiol. 110, 1438.

Zhu, J., Miller, M.B., Vance, R.E., Dziejman, M., Bassler, B.L., Mekalanos, J.J., 2002. Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae*. P Natl Acad Sci USA. 99, 3129-3134.

Tables

Table 1 : Bacterial strains used in this study

Strain	Description	Metalloprotease activity	Reference or source
02/041	*V. aestuarianus*	+	(Garnier, et al., 2007)
LGP32-GFP	*V. splendidus* LGP32 *gfp* (Cm^r)	+	(De Decker, et al., 2011)
LGP32-Δ*vsm*-Δ1062	*V. splendidus* LGP32 Δ*vsm*-Δ1062	-	(Binesse, et al., 2008)

Table 2 : Primer pairs used to specifically target *vsm* and *vam* cDNA, and their amplification efficiency in real-time qPCR expression analysis.

Targeted genes	Primers name	Oligonucleotide sequences (5' – 3')	qPCR efficiency (%)	Amplicon length (bp)
vsm	S-METVS2F	ACCTAAGCGGGAACCAATCT	99.3	159
	S-METVS2R	GGTAAGGGCGACAGTCGTAA		
vam	S-METVA2F	CAAACTTGGGAAGGCTTGAA	103.7	159
	S-METVA2R	GACCGTTTTCACCGCACTAT		

Figure 1 : Standard curves for *V. splendidus* LGP32-GFP (black squares) and *V. aestuarianus* 02/041 (gray diamonds) real-time PCR in 1:10 serial dilutions of their DNA, corresponding to 1.7 10^3 to 1.7 10^8 and 2.6 10^2 to 2.6 10^7 CFU-equivalent genomes number ml^{-1} of the initial samples, respectively. Standard curves were generated by plotting the CFU-equivalent genomes number ml^{-1} on the mean threshold cycle (Ct) value obtained from triplicate assays.

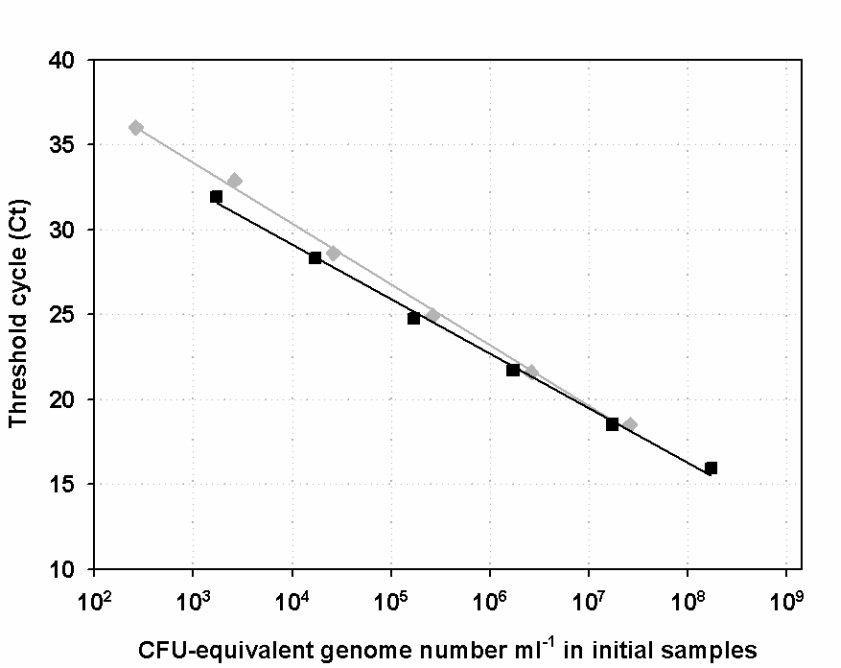

Figure 2 A : Growth curves of the two virulent *Vibrio* strains LGP32-GFP (black line) and 02/041 (gray line), quantified by real-time qPCR (CFU-equivalent genomes number ml^{-1}). Plotted values are means (±SD) obtained from three different cultures (n = 3 for each point).

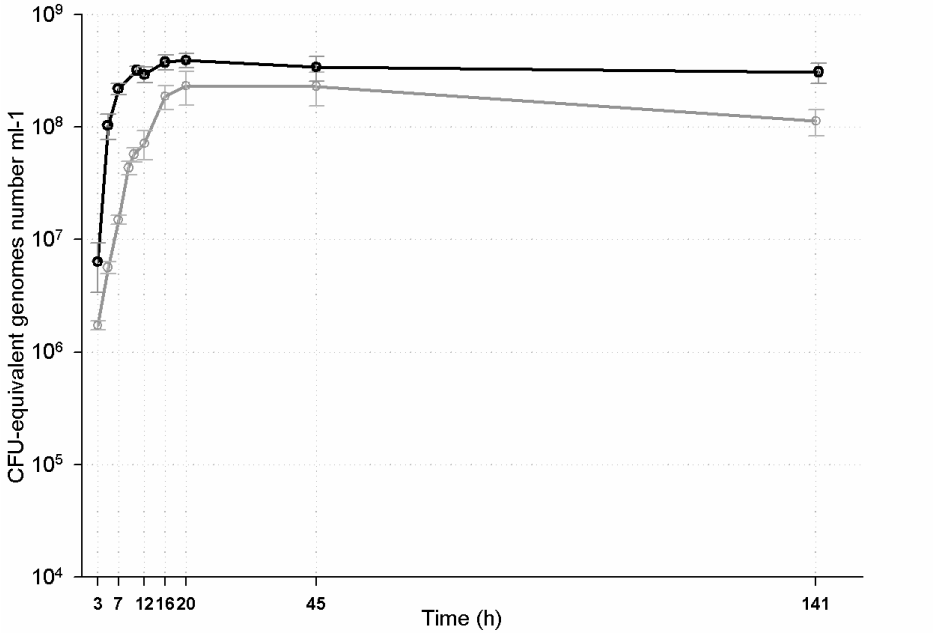

Figure 2 B : Metalloprotease-like activity in culture supernatant fluids from LGP32-GFP (black line) and 02/041 (gray line) *Vibrio* strains. Results are expressed as the metalloprotease activity per number of 10^9 CFU-equivalent genomes quantified by qPCR (units per number of cells). Metalloprotease activity was calculated as the difference between azocaseinase activity obtained in the absence or presence of phenanthrolein. One unit of protease activity was defined as the amount of enzyme that caused an increase of 0.01 absorbance units after 2 h of incubation at 37°C. Plotted values are means (±SD) obtained from three different culture samples (n = 3 for each point).

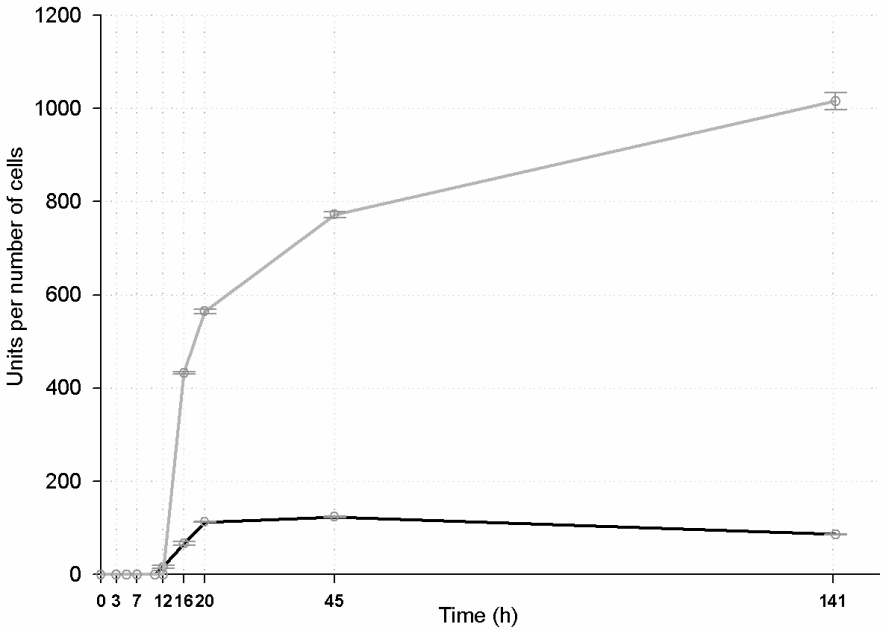

Figure 3 : *V. splendidus vsm* (black) and *V. aestuarianus vam* (grey) normalized relative gene expression levels (mean F ± SD) calculated by the formula: $F=(E+1)^{40-Ct}/N$ quantified by RT-qPCR in Marine Broth medium cultures (A) and in conditioned medium cultures (B). L: low bacterial concentration ranged between 2.0 10^4 and 1.6 10^5 CFU-equivalent genomes number ml^{-1}; H: high bacterial concentration ranged between 2.8 10^7 and 1.2 10^9 CFU-equivalent genomes number ml^{-1} quantified by real-time qPCR.