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Vibrio harveyi uses both type III secretion system and quorum sensing for the colonization of the European abalone

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ABSTRACT

The vibriosis of the European abalone, *Haliotis tuberculata*, is characterized by the rapidity of the infection by the pathogen *Vibrio harveyi* ORM4, leading to death of animals only after two days. The lethality of the pathogen is linked to the production of the type III secretion system (T3SS) and to genes regulated by quorum sensing (QS). The aim of this study was to investigate the colonization of the European abalone by both virulent and avirulent *V. harveyi* strains, as well as the involvement of T3SS and QS during infection. Our results emphasize the importance of gills for the bacterial establishment as the bacterial concentration of the avirulent *V. harveyi* strain significantly decreased from 189.3 ± 98.6 CFU/mg to 0.8 ± 0.5 CFU/mg between 24 and 48 h post-infection (hpi). In opposition, the pathogen *V. harveyi* ORM4 was able to maintain itself on the gills, with a concentration of 461.9 ± CFU/mg at 48 hpi, which was allowed by the production of T3SS and a functional QS. Following the infection cycle of *V. harveyi* ORM4 inside *H. tuberculata*, we also demonstrated that QS is essential for the ability of *V. harveyi* ORM4 to colonize the abalone hemolymph and to maintain in it. In response to the presence of *V. harveyi*, an increase in reactive oxygen species production was recorded, while the phagocytosis activity remained unchanged. We also highlighted the involvement of both QS and T3SS to escape the immune system activity, and that an overproduction of T3SS induced hemocyte mortality. This study provides the evidence that both T3SS and QS are essential for the establishment of *V. harveyi* ORM4 inside the European abalone.

1. Introduction

Abalones are environmentally- and economically important marine mollusks, offering essential ecosystem services and a prized delicacy for humans [1]. In Europe, only the species *Haliotis tuberculata* is able to reach a size large enough to be harvested [2]. In the late 90s, wild and farm populations of the European abalone located along the French North Atlantic coast have suffered of mass mortalities, and dead animals presented white pustules on their foot [3]. The responsible agent for the disease was found to be a bacterial strain called ORM4 and belonging to the species *Vibrio harveyi*, a serious pathogen of marine vertebrates and invertebrates [3,4]. These mortalities have been reproduced in laboratory and occurred when seawater temperature exceeded 17 °C [5]. It has also been observed that sexually mature European abalone developed this vibriosis, whereas immature or post-spawning individuals remained resistant to *V. harveyi* [5,6]. Previous studies have identified the gills as the pathogen portal of entry into the European abalone. These organs are located close to the heart and the circulatory system, enabling *V. harveyi* ORM4 to spread rapidly within the animal [7–9]. Vibriosis of the European abalone is characterized by the rapidity of the infectious process of *V. harveyi* ORM4, since gills and hemolymph are colonized within the first 3 h of contact [7–9]. Subsequently, the pathogen is able to invade and multiply in the abalone circulatory system, and the first mortalities were recorded two days after contact [5,9].

Abbreviations: Type III secretion system, T3SS; quorum sensing, QS; reactive oxygen species, ROS; Trimethoprim, Trim; filtered sterile seawater, FSSW; anti-aggregant solution, AASH; 2′,7′-dichlorofluorescein diacetate, DCFH-DA; dichlorofluorescein, DCF.

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This efficiency of host colonization has been observed in other *Vibrio* species, such as *Vibrio* sp. PJ, which was detected in hemolymph and stomach already 3 h post-infection of the shrimp Kuruma *Penaeus japonicus* [10]. Furthermore, immunity genes in *Penaeus monodon* hemocytes were activated as soon as 6 h after an infection with *V. harveyi* [11].

The reasons underlying the rapid infection of the European abalone by *V. harveyi* have been studied, and the results demonstrated that infection is favored by an absence of response from the immune cells in hemolymph, caused by an activation delay of the p38 MAPK pathway [12]. After the pathogen establishment, the hemocytes showed a significant decrease in their phagocytosis activity and production of reactive oxygen species (ROS), as well as a reduction of their viability, suggesting a potential cell lysis induced by the pathogen [13].

During the past twenty years, the sensitivity of the European abalone to the pathogen has been well studied, while the factors involved in the establishment of *V. harveyi* ORM4 remain underexplored. Indeed, the majority of our knowledge about *Vibrio* virulence factors has been learned from the human pathogen *Vibrio cholerae*, but little is known about the colonization mechanisms of marine pathogens [14]. Recently, the development of genetic tools in *V. harveyi* ORM4 [15] allowed us to demonstrate that both functional type III secretion system (T3SS) and quorum sensing (QS) are required for *V. harveyi* ORM4 to induce European abalone mortalities [16]. T3SS is a syringe-like device commonly associated with the virulence of Gram-negative bacteria, which injects effector proteins from the bacterium cytoplasm directly into the cytoplasm of the target animal cell. The effectors then induce cell lysis by interacting with host proteins or signaling pathways [17–19]. The expression of T3SS genes is controlled by a major regulator encoded by the *exsA* gene, the deletion of which from *V. harveyi* ORM4 genome leads to a total loss of capacity to induce abalone mortality [16]. In addition, 18 of the 49 T3SS synthesis genes were shown to be over-expressed in *V. harveyi* ORM4 biofilm forming cells [20], suggesting a potential involvement of this lifestyle in the pathogenicity of the bacteria. Like other virulence systems, the expression of T3SS is under the QS system control [16,21–23]. Thanks to this cell-to-cell communication mediated by signal molecules, bacteria collectively synchronize behavioral changes across the population for their survival or adaptation inside hosts in response to variations in cell density [24,25]. This system was first discovered in *Vibrio fischeri* and since then, similar systems have been described in most bacteria [21,24]. The QS system of *Vibrio campbellii* ATCC BAA-1116 has been extensively studied, and is composed of three signal-sensing pathways converging to a single regulatory transduction pathway involving the LuxO protein [26]. Interestingly, all critical QS-genes have orthologs in the genome of *V. harveyi* ORM4 [20]. Recent work showed that the deletion of *luxO* in the latter strain led to an overexpression of the *exsA* gene, and to a loss of motility, biofilm production and pathogenicity against the European abalone [16].

On the basis of these advances and the accumulation of knowledge about the interactions between the abalone and *V. harveyi* ORM4, we sought to determine the role(s) T3SS and QS might have in the establishment of *V. harveyi* ORM4 in *H. tuberculata*, with a focus on gill and hemolymph colonization.

2. Materials and methods

2.1. Abalone and bacterial strains

Mature European abalones (6–7 cm) were brought from France Haliotis (Plouguerneau, France) and acclimated during 2 weeks in 100 L tanks with circulating seawater at 20 °C. Only mobile and adherent abalones showing no tissue damage or pedal muscle pustules were used for this study. *V. harveyi* ORM4, *V. harveyi* LMG 7890 (a strain avirulent towards abalone [12]) and their derivatives used in this study are listed in Table 1. Each of these bacteria contains one plasmid conferring trimethoprim resistance: either the empty pFD085 vector or this vector carrying a functional gene (*exsA* and *luxO* in pFD067 and pFD084, respectively) in order to complement the corresponding mutants (Table 1) [16]. The bacteria were cultivated in Luria-Bertani Broth (Lennox, Sigma, France) supplemented with NaCl (20 g/L, LBS final concentration) and Trimethoprim (Trim, 10 μg/mL) at 20 °C overnight under agitation.

Table 1
Strains used in this study.

Strain name	Characteristics	Reference
V. harveyi ORM4	Wild-type strain isolated from moribund European abalone *Haliotis tuberculata*. Virulent towards *H. tuberculata*.	[3]
V. harveyi ORM4 (pFD085)	Derivative of *V. harveyi* ORM4 containing the empty plasmid pFD085. $Trim^R$	[16]
V. harveyi LMG 7890	Wild-type strain isolated from the kidney of a dead brown shark *Carcharinus plumbeus*. Non-lethal strain for *H. tuberculata*.	[12,46]
V. harveyi LMG 7890 (pFD085)	Derivative of *V. harveyi* LMG 7890 containing the empty vector pFD085. $Trim^R$	[16]
V. harveyi ORM4 Δ*exsA* (pFD085)	Derivative of *V. harveyi* ORM4 in which the *exsA* gene has been deleted and containing the empty vector pFD085. Avirulent towards *H. tuberculata*. $Trim^R$	[16]
V. harveyi ORM4 Δ*luxO* (pFD085)	Derivative of *V. harveyi* ORM4 in which the *luxO* gene has been deleted and containing pFD085. Avirulent towards *H. tuberculata*. $Trim^R$	[16]
V. harveyi ORM4 Δ*exsA* (pFD067)	Derivative of *V. harveyi* ORM4 Δ*exsA* containing pFD067 (*exsA* cloned into pJLS199) for the complementation of *exsA*. The complementation restores the virulence towards *H. tuberculata*. $Trim^R$	[16]
V. harveyi ORM4 Δ*luxO* (pFD084)	Derivative of *V. harveyi* ORM4 Δ*luxO* containing pFD084 (*luxO* cloned into pJLS199) for the complementation of *luxO*. The complementation restores the virulence towards *H. tuberculata*. $Trim^R$	[16]

2.2. Infection experiment

After 2 weeks of acclimation, 36 mature abalones per condition were placed equally in three 20 L tanks containing 10 L of seawater at 20 °C with a bubbling system. Abalones were infected for 24 h by balneation with 5×10^6 CFU/mL of each of the *V. harveyi* strains listed in Table 1 [16]. The control group was made of 30 individuals not exposed to bacteria. The seawater in the tanks was changed every day in every tanks. For each condition of infection, one tank was used for sampling after 24 h post-infection (hpi), another for sampling after 48 hpi, and the final tank was used to monitor mortality for 10 days, during which dead abalones were counted and removed daily.

2.3. Gills and hemolymph sampling

Gills and hemolymph were sampled from uninfected and infected abalones at 24 and 48 hpi. At least 5 animals were sampled per time point and infection condition. The hemolymph was collected from the cephalic arterial sinus with a 2.5 mL syringe (Terumo, Tokyo, Japan) and a needle (Terumo, Tokyo, Japan, 25G, 05 × 16 mm). Hemolymph samples were divided into two microtubes, one used for bacterial quantification and the other one to analyze hemocyte parameters. Then, gills were dissected in sterile conditions, transferred into a 2 mL grinding tube containing 5 to 6 ceramic beads (Ozyme France, Montigny-le-Bretonneux, France, 2.8 mm and/or 1.4 mm), and kept on ice until grinding. Between each dissection, the tools were rinsed with ethanol and sterile distilled water. The grinding tubes containing the beads were weighed before and after adding the gills, to obtain the mass of the samples taken. After gills sampling, 500 μL of filtered sterile seawater

(FSSW) were added to each sample. Gills were grounded using the FastPrep-24 5G (MP Biomedicals, Santa Ana, CA, USA) with two 30-s grinding cycles at 4.5 m/s and then placed on ice until bacterial quantification.

2.4. Bacterial quantification

The cell concentration of the different *V. harveyi* strains was obtained by plate counting from gills/hemolymph homogenates. 50 mg of shredded gills or 500 μL of hemolymph were diluted in FSSW (10^0 to 10^{-3}) and spread on LBS + Trim plates. All plates were incubated at 20 °C for 48 h and colonies were enumerated to obtain the bacterial concentration in colony forming units (CFU) per mg of gill or per mL of hemolymph.

2.5. Hemocyte parameters analysis by flow cytometry

Immediately after bacterial quantification, sampled hemolymph from at least 5 animals per time point and infection condition were filtered with a 80 μm nylon mesh to eliminate aggregates and debris. Each filtered hemolymph sample was then analyzed as an individual using the FACSVerse flow cytometer (Becton Dickinson, San Diego, CA, USA) and its blue laser (488 nm) as an excitation wavelength. In parallel, an anti-aggregant solution (AASH) was prepared according to Travers et al. 2008 [6], with 6.25 g/L of NaCl, EDTA 1.5 % in a 0.1 M phosphate buffer at pH 7.4, to prevent aggregation of abalone hemocytes.

2.5.1. Hemocytes viability and density

A total of 200 μL of each filtered hemolymph samples were added to 200 μL of AASH containing SYBR Green I (Molecular Probes, Eugene, OR, 10^{-2} dilution of the commercial stock solution) and Propidium Iodide (10 μg/mL) and then incubated 30 min at 20 °C. Analyses were performed using a flow cytometer (FCM) FacsVerse (Becton-Dickinson®, San Jose, USA), set on medium flowrate (around 80 μL min^{-1}) for 30 s. Flow rate was estimated each day by weighing H_2O tube before and after a 5 min run. The hemocyte cells were detected and counted according to their green fluorescence level due to SYBR Green DNA staining (FITC detector: 527/32 nm), and their forward/side scatter (FSC/SSC) values. Hemocyte concentration was calculated in cell per mL of hemolymph. Percentages of dead cells was obtained by counting cells stained by the Propidium Iodide indicating a loss of membrane integrity (Red fluorescence level, Per-CP-Cy5.5 detector: 700/54 nm), as compared to SYBR Green-stained cells.

2.5.2. Phagocytosis index

The phagocytic index was measured according to Travers et al., 2009 [12]. Briefly, a total of 250 μL of 80 μm-filtered hemolymph, collected from abalone infected for 24 and 48 h by balneation with the different strains, were mixed with 250 μL of FSSW and the solution was added to a 24-wells plates (Cellstar, Greiner Bio-one). After hemocytes adhesion during 15 min at 20 °C, 100 μL of 2 μm fluorescents beads (Polysciences, 1:200 in FSSW) were added in each well and incubated at 20 °C for 3 h. The supernatant was subsequently removed and 200 μL of a trypsin solution (2.5 mg/mL in ASSH) were added to detach adherent hemocytes before 10 min of plates agitation. Finally, 200 μL of ASSH solution were added to stop the reaction. Because of their fluorescence, beads are detected by the cytometer and non-ingested beads are distinguished from engulfed ones by their size. The phagocytosis index is defined as the percentage of active hemocytes; active hemocyte means an hemocyte with at least one engulfed bead [27].

2.5.3. Reactive oxygen species (ROS) production

The ability of hemocytes to produce ROS in the different infection conditions was measured according to Lambert et al., 2003 [28]. Briefly, 300 μL 2′,7′-dichlorofluorescein diacetate (DCFH-DA, 0.1 mM final concentration) diluted in FSSW was added to 300 μL of 80 μm-filtered hemolymph, and each sample was analyzed exactly 10 min after. Oxidation of DCFH-DA by ROS produces dichlorofluorescein (DCF), a green fluorescent molecule which is quantified by flow cytometry using the FITC detector (527/32 nm). Results are given as intra-hemocyte green fluorescence levels expressed in arbitrary units (A.U.).

2.6. Statistical analysis

Statistical analysis (comparison of bacterial quantification and hemocyte response) was performed using the Kruskal-Wallis test followed by a pairwise Wilcoxon test to compare the difference between groups in the RStudio software (R-4.2.0 version). A p-value of <0.05 was considered significant.

3. Results

3.1. Validation of abalone sensitivity to V. harveyi

In order to study the colonization of abalones, one had to ensure that infected animals presented the same sensitivity to bacteria as demonstrated previously [16]. European abalone mortalities following a 24 h exposure to the different strains of *V. harveyi* were followed for 10 days (Fig. 1). The first mortalities generated by *V. harveyi* ORM4 occurred 4 days after exposure and only 30 % of European abalones survived after 10 days. On the other hand, *V. harveyi* LMG 7890 and deletion mutants of *V. harveyi* ORM4 caused either no mortality (Fig. 1A) or only 8 % of mortality after 10 days (Fig. 1B).

The complementation of *exsA* and *luxO* mutations restored the virulence of *V. harveyi* ORM4 (Fig. 1A and B). These results are in agreement with the previous ones [12,16] and demonstrated that the animals used in this study were mature and sensitive to *V. harveyi* ORM4, which requires both functional QS and T3SS systems for its virulence.

3.2. Gills colonization

In order to investigate the barrier role of abalone gills during *V. harveyi* infection and their ability to prevent bacterial establishment, 210 animals were infected using a balneation method to mimic the natural process of *Vibrio* infection. At 24 and 48 hpi, 10 animals from each infection condition were randomly sampled to collect the gills. Bacteria were enumerated by plating gills homogenates on LBS medium supplemented with trimethoprim, to which all bacteria used in this experiment are resistant. No or a very small number of colonies were obtained from non-infected animals (Fig. 2), showing that trimethoprim resistance is an appropriate selection method to discriminate the used *V. harveyi* strains from other bacteria present in these samples.

V.harveyi LMG 7890 was observed in gills at 24 hpi, but after 48 h the bacterial concentration was significantly reduced from 189.3 ± 98.6 CFU/mg to 0.8 ± 0.5 CFU/mg (Fig. 2A and D, p-value <0.01). As a *result*, *V. harveyi* LMG 7890 was considered absent from these organs at 48 hpi (Fig. 2A). Therefore, these results demonstrated that the inability of *V. harveyi* LMG 7890 to cause mortality to European abalone is at least partly due to its rapid curing by the host, as observed here with the gills (Fig. 2A, compare time points 24 and 48 h). In contrast, the *V. harveyi* ORM4 load in gills was 1232.0 ± 287.9 and 491.9 ± 126.7 at 24 and 48 hpi, respectively (Fig. 2A and D).

The *exsA* deletion resulted in a decreased capability to colonize gills, since *V. harveyi* ORM4 Δ*exsA* concentration was 3-fold lower than that of the wild-type strain in this organ for both time of sampling (Fig. 2B). The complementation of the *exsA* deletion did not completely restore *V. harveyi* ORM4 ability to colonize gills at 48 h, so we cannot draw conclusions for this time (Fig. 2B). On the other hand, the *luxO* deletion led to a 3-fold reduction in the number of bacteria detected in the abalone gills at 24 hpi, as compared to the wild-type (Fig. 2C). However, the *V. harveyi* ORM4 Δ*luxO* concentration significantly decreased when comparing the time points at 24 and 48 h, from 399.8 ± 72.2 CFU/mg to

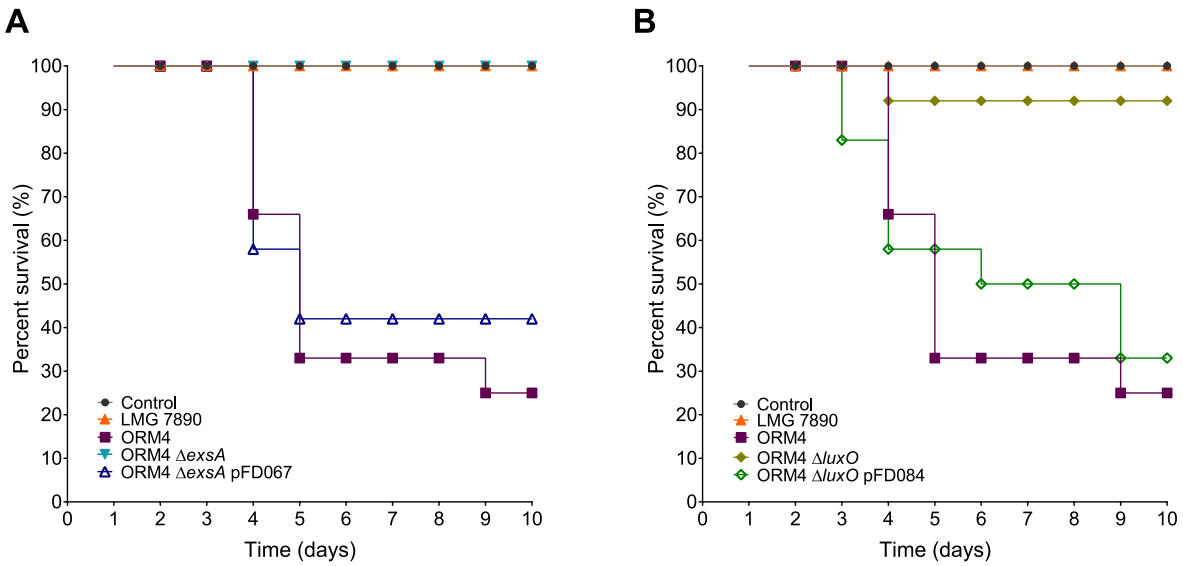


Fig. 1. Survival rate of European abalones after 10 days of infection with *V. harveyi*. Strains LMG 7890, ORM4 (A and B), ORM4 Δ*exsA*, ORM4 Δ*exsA* pFD067 (A), ORM4 Δ*luxO*, or ORM4 Δ*luxO* pFD084 (B) were used to infect 12 abalones by balneation, and surviving animals were counted daily. Note that black (control tanks without added bacteria), orange (tanks infected with *V. harveyi* LMG 7890) and blue (tanks infected with *V. harveyi* ORM4 Δ*exsA*) curves overlap. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

94.9 ± 81.2 CFU/mg of gills (Fig. 2D, p-value <0.01). In this mutant, the complementation by the *luxO* gene restored the gill colonization by *V. harveyi* ORM4 (Fig. 2C).

3.3. Hemolymph colonization

To follow the infectious cycle of *V. harveyi* ORM4 in the European abalone, we then examined the ability of the different strains to colonize and maintain in the hemolymph. *V. harveyi* LMG 7890, the avirulent strain for the European abalone was presented at a bacterial concentration of up to 1.82×10^4 CFU/mL at 24 h, before dropping to 3.73×10^2 CFU/mL at 48 hpi (Fig. 3A and D). In contrast, the pathogen *V. harveyi* ORM4 was detected at concentrations ranging between 10^4 to 10^5 CFU/mL of hemolymph at both sampling times (Fig. 3A and D). The *exsA* deletion did not alter the ability of *V. harveyi* ORM4 to colonize the hemolymph (Fig. 3B). In opposition to these results, a defective QS in *V. harveyi* ORM4 induced a decrease of *V. harveyi* ORM4 concentration in hemolymph after 48 h of infection, which was 3-fold lower than that of the wild-type strain (Fig. 3D). The complementation of the *luxO* mutant restored the bacterial concentrations to levels similar to those measured in hemolymph from abalones infected with the wild-type strain (Fig. 3C).

3.4. Hemocyte mortalities in response to V. harveyi infections

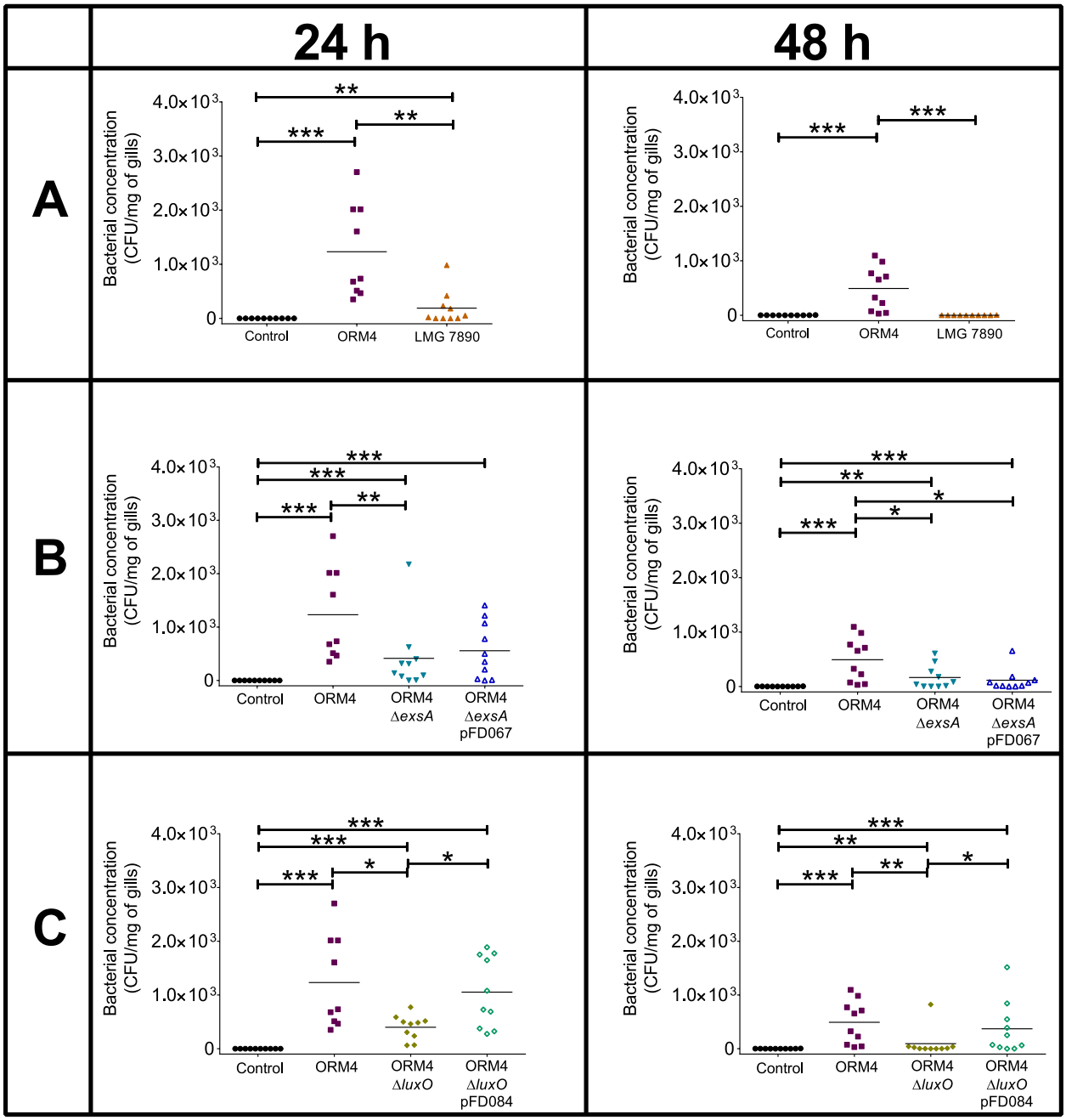
As *V. harveyi* ORM4, its T3SS and QS mutants, and *V. harveyi* LMG 7890 are all able to penetrate into the circulatory system of the abalone (the hemolymph) (Fig. 3), we next sought to quantify the hemocyte mortalities induced by those bacteria. Flow cytometry was used to quantify dead hemocytes (labeled with propidium iodide) and total hemocytes (live + dead) were labeled with SYBR Green. The calculated hemocyte density in hemolymph was between 1×10^6 and 3.5×10^6 cells/mL and it remained stable between the non-infected control and abalones infected with *V. harveyi* LMG 7890, *V. harveyi* ORM4 Δ*exsA* and *V. harveyi* ORM4 Δ*luxO* (Fig. 4A). Infections with these bacteria also did not induce hemocyte mortality (Fig. 4B).

On the other hand, *V. harveyi* ORM4 appeared to prevent the hemocytes from multiplying. Indeed, in the presence of the pathogen, the hemocyte mortality tended to increase (Fig. 4B) and hemocyte density to halve compared with the control (Fig. 4A) at 48 hpi. In addition, it should be noted that in the presence of *V. harveyi* ORM4 Δ*exsA* pFD067, the hemocyte density tended to increase at 24 hpi (Fig. 4A). This density significantly decreased at 48 h (Fig. 4A, p-value <0.05), which may be associated with the increase in hemocyte mortality recorded at the same time in presence of this bacterium (Fig. 4B, p-value <0.001). In parallel, based on the hemocyte (Fig. 4A) and *V. harveyi* concentrations (Fig. 3) in the hemolymph, these results highlight the average hemocytes: bacteria ratio was 100:1 for the various infection settings.

3.5. Hemocyte parameters in response to V. harveyi infection

Since *V. harveyi* strains only partially affect the viability of hemocytes (Fig. 4B), we finally sought to characterize various hemocyte parameters following infection. We evaluated by flow cytometry the response of hemocytes by measuring their ability to phagocyte fluorescents beads, and their ROS production by quantifying the production of DCF by host cells (Fig. 5). No fluctuations in the hemocyte phagocytosis index was recorded in infected animals as compared to the control experiment (Fig. 5A). In line with this observation, the complementation of both *exsA* and *luxO* genes did not affect this parameter (Fig. 5A).

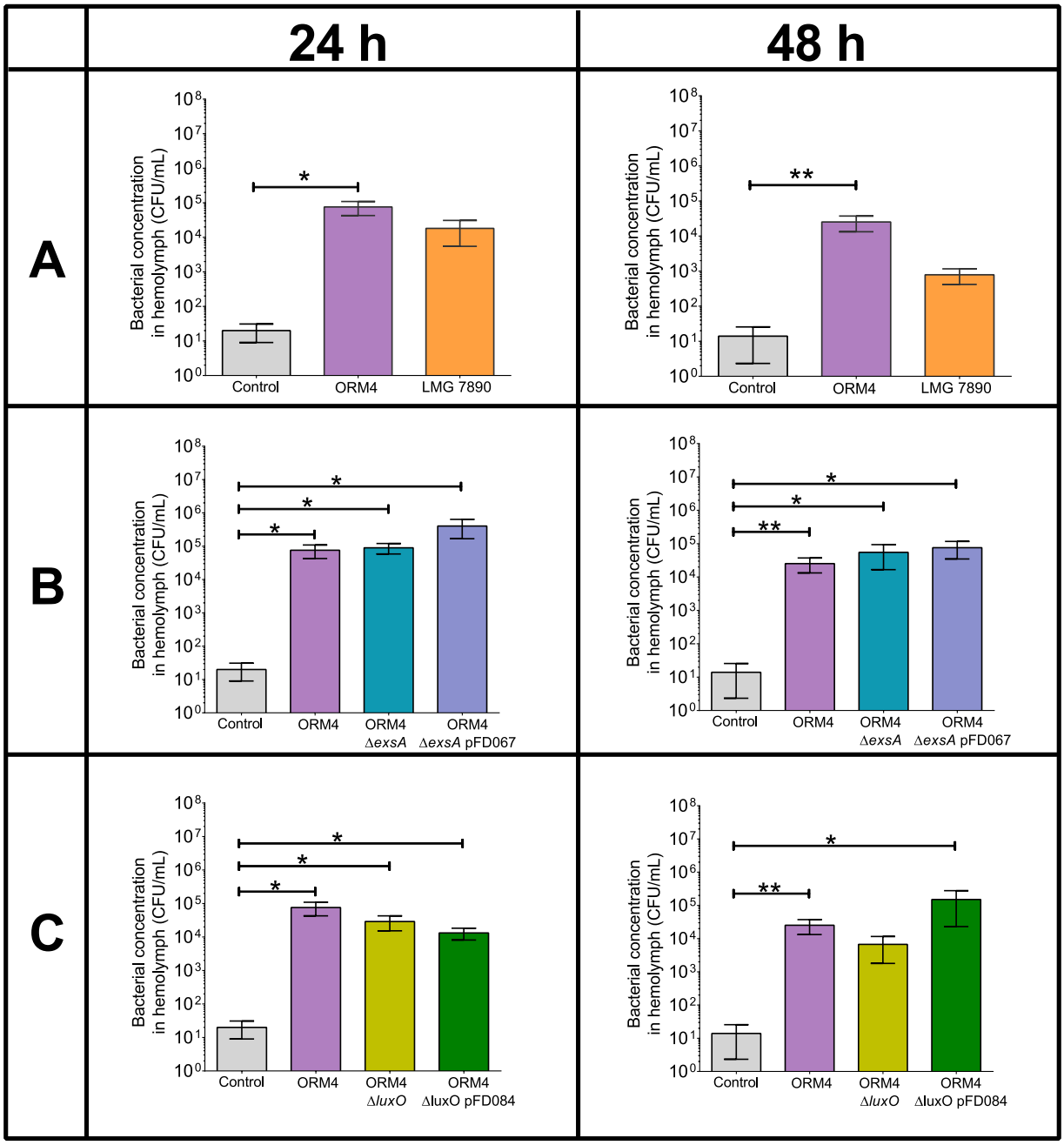
In contrast, ROS production by hemocytes was influenced by the presence of *V. harveyi* (Fig. 3). Indeed, as a result of infection with the non-pathogenic strain LMG 7890, the ROS production was 2-fold higher than in control animals after the two sampling times (Fig. 5B). When infected by the virulent strain *V. harveyi* ORM4, ROS production was greatly increased by hemocytes compared with the control condition, with an 11-fold increase at 24 hpi, and a 3-fold increase after 48 h of infection (Fig. 5B). Interestingly, hemocytes from abalone infected with *V. harveyi* ORM4 Δ*exsA* showed a significant (p-value <0.05) 1.4-fold higher ROS production as compared to *V. harveyi* ORM4 at 24 h (Fig. 5B). This increased ROS production was maintained at 48 h, although this increase was not significant (Fig. 5B). On the other hand, the overexpression of *exsA* led to a significant increase in ROS production (Fig. 5B). Finally, the absence of a functional QS by the deletion of *luxO* did not alter the response of immune cells, since these hemocyte parameters were similar to the ones observed during an infection with *V. harveyi* ORM4 (Fig. 5B).



D

Time	Bacterial concentration (CFU/mg of gills)						
	Non infected	*V. harveyi* LMG 7890	*V. harveyi* ORM4	*V. harveyi* ORM4 Δ*exsA*	*V. harveyi* ORM4 Δ*exsA* pFD067	*V. harveyi* ORM4 Δ*luxO*	*V. harveyi* ORM4 Δ*luxO* pFD084
24 h	0.4 ± 0.2	189.3 ± 98.6	1,232.0 ± 287.9	415.8 ± 205.3	557.6 ± 167.6	399.8 ± 72.2	1,054.0 ± 207.9
48 h	1.1 ± 0.5	0.8 ± 0.5	491.9 ± 126.7	166.2 ± 68.5	115.5 ± 62.8	94.9 ± 81.2	371.8 ± 154.7

Fig. 2. *V. harveyi* bacterial counts in gills of infected European abalones after 24 and 48 h. LMG 7890, ORM4 (A), ORM4 Δ*exsA*, ORM4 Δ*exsA* pFD067 (B), ORM4 Δ*luxO* and ORM4 Δ*luxO* pFD084 (C) were enumerated by plating gill homogenates on LBS containing with trimethoprim. Calculated mean (±SEM) of the bacterial concentrations measured in at least 7 animals (D). Statistical differences were obtained using a Kruskal-Wallis test followed by a pairwise Wilcoxon test. Significant differences between infection conditions are represented by stars (* *p*-value <0.05; ** *p*-value <0.01; *** *p*-value <0.001).



D

	Bacterial concentration in hemolymph (CFU/mL)						
Time	Non infected	*V. harveyi* LMG 7890	*V. harveyi* ORM4	*V. harveyi* ORM4 Δ*exsA*	*V. harveyi* ORM4 Δ*exsA* pFD067	*V. harveyi* ORM4 Δ*luxO*	*V. harveyi* ORM4 Δ*luxO* pFD084
24 h	20.0 ± 10.9	18,188.0 ± 12 700.2	110,713.1 ± 46 284.4	89,696.0 ± 31 237.3	403,438.0 ± 235 682.6	29,000.0 ± 13 747.7	13,186.0 ± 5 052.3
48 h	14.0 ± 11.7	790.0 ± 372.5	25,354.6 ± 12 048.3	55,280.0 ± 38 583.1	76,000.0 ± 41 241.9	6,704.0 ± 4 911.9	150,510.0 ± 127 462.1

Fig. 3. Bacterial concentration of *V. harveyi* observed in hemolymph of infected European abalones after 24 and 48 h. LMG 7890, ORM4 (A), ORM4 Δ*exsA* (B), Δ*luxO* (C) and their respective complemented were enumerated by plating samples homogenates on LBS supplemented in trimethoprim. Depicted here are the bacterial mean (±SEM) in hemolymph of 5 individuals (15 for the wild-type, because more abalone were sacrificed for diverse controls) for each time and infection conditions, the values of which are listed in table (D). Statistical differences were obtained using a Kruskal-Wallis test followed by a pairwise Wilcoxon test. Significant differences between infection conditions are represented by one or more stars (* *p*-value <0.05; ** *p*-value <0.01; *** *p*-value <0.001).

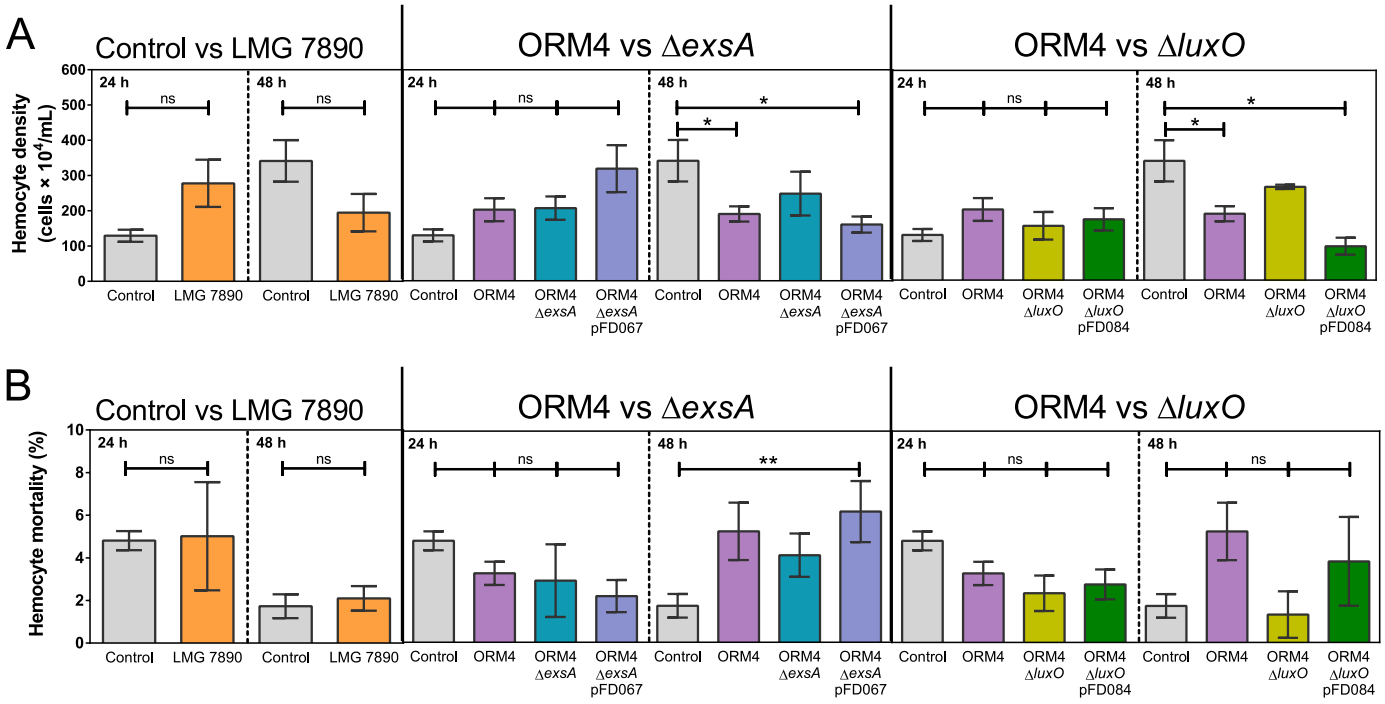


Fig. 4. Influence of *V. harveyi* on hemocytes during *in vivo* infections of the European abalones. The represented mean (±SEM) of hemocyte density (A) and mortality (B) was measured by flow cytometry in hemolymph sampled from 5 individuals (15 for the wild-type, because more abalone were sacrificed for diverse controls) for each time and infection condition. A Kruskal-Wallis test followed by a pairwise Wilcoxon test was done to compare the different conditions. Significant differences between infection conditions are represented by one or more stars (* p-value <0.05; ** p-value <0.01).

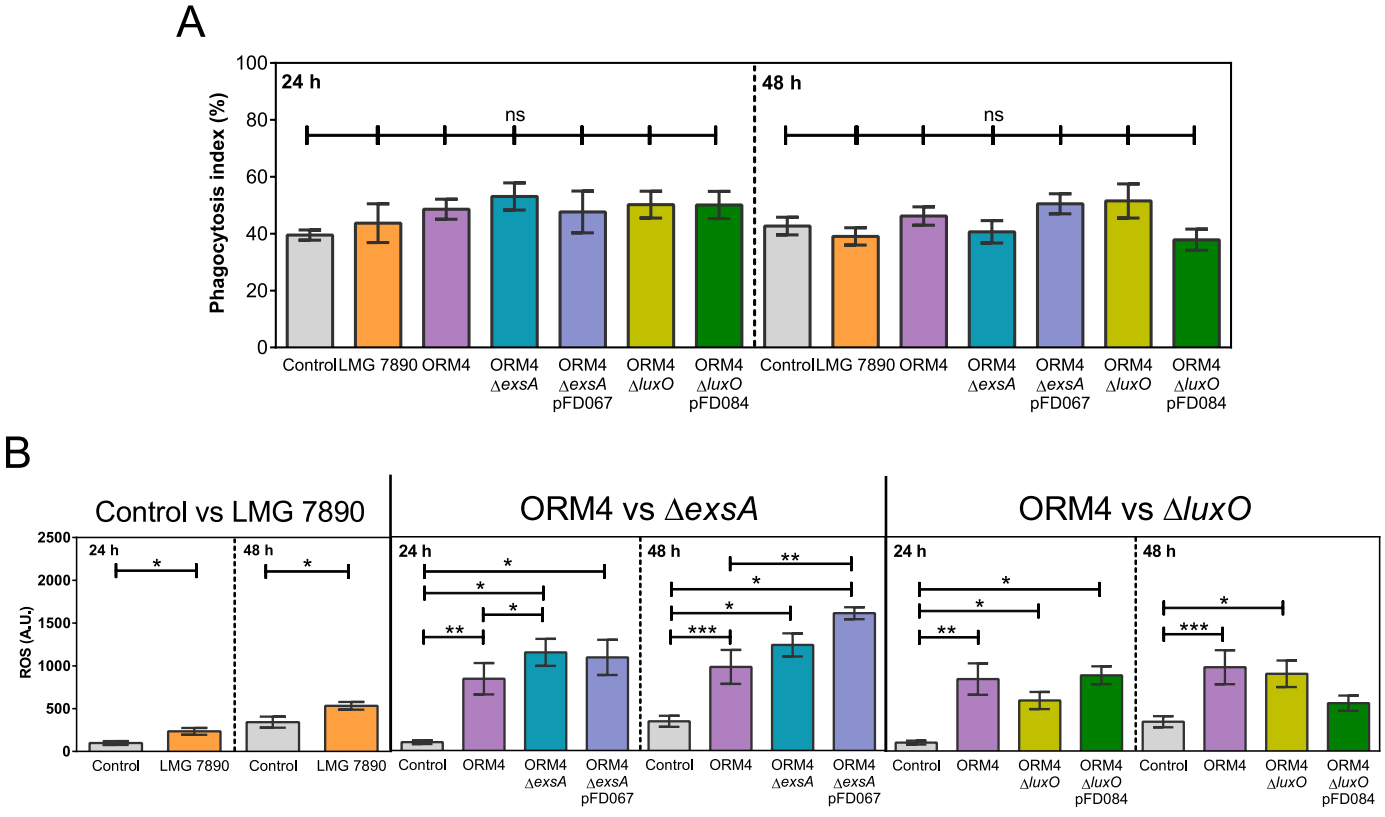


Fig. 5. Variations in hemocyte parameters measured by flow cytometry in response to an infection with *V. harveyi* after 24 and 48 h. Phagocytosis index (A) and ROS production (B) by hemocytes were measured by flow cytometry from hemolymph collected from 5 individuals (15 for the wild-type, because more abalone were sacrificed for diverse controls) for each time and infection condition. Statistical differences were obtained using a Kruskal-Wallis test followed by a pairwise Wilcoxon test. Significant differences between infection conditions are represented by one or more stars (* p-value <0.05; ** p-value <0.01, *** p-value <0.001).

4. Discussion

The purposes of this study were to characterize the colonization of the European abalone by a pathogenic and a non-pathogenic strain of *V. harveyi*, as well as to infer the involvement of both the QS and T3SS in the establishment of *V. harveyi* ORM4.

Firstly, to be an efficient pathogen, *V. harveyi* ORM4 must be able to colonize abalone and therefore bypass the host immune system to maintain itself. Previous studies showed that using a *V. harveyi* ORM4 concentration of 5×10^6 CFU/mL to infect European abalone by immersion was sufficient to detect the pathogen in the animal within the first few hours following the infection. Here we demonstrated for the first time that the *V. harveyi* LMG 7890 strain avirulent towards abalone was able to initiate the colonization of both the gills and hemolymph, but not to establish a long-term presence in abalone (Figs. 2A and 3A), explaining at least in part its inability to cause abalone mortality (Fig. 1, [16]). Interestingly, the genome of *V. harveyi* LMG 7890 harbors genes for a T3SS. Whether this secretion system is functional or not in LMG 7890 has not yet been investigated, nor were explored potential ORM4-specific T3SS effectors, which might explain the opposite virulence phenotypes between LMG 7890 and ORM4. Travers and collaborators [12] demonstrated that *V. harveyi* LMG 7890 is able of killing abalone only when injected at a minimum concentration of 10^5 CFU/animal [12]. During our experiments, although a higher concentration of *V. harveyi* LMG 7890 was recorded in the hemolymph at 24 h (1.8×10^4 CFU/mL), this decreased drastically at 48 h (7.9×10^2 CFU/mL). Taken together, this highlights the role and the importance of gills as a barrier to bacterial establishment during infection.

In contrast, *V. harveyi* ORM4 was able to maintain itself at high concentration in both the gills (1.2×10^4 CFU/mg and 4.9×10^2 CFU/mg at respectively 24 and 48 hpi) and hemolymph (1.1×10^5 CFU/mL and 2.5×10^4 CFU/mL at respectively 24 and 48 hpi) for a longer period, ultimately leading to animal death (75 % of mortalities after 10 days). In mollusks, the gills, which are the initial barrier that putative pathogenic bacteria would confront [7,9], were shown to display strong antibacterial activities [29]. This has been demonstrated in the gills of the mussel *Mytilus galloprovincialis* in which the expression of genes encoding pathogen recognition proteins and immune effectors are activated in presence of *Vibrio splendidus* [30], and in the oyster *Crassostrea gigas* which produces several antibacterial proteases inside the mucus covering the gills [31]. In the European abalone, hemocytes and epithelial cells play a key role in the immunity in gills and are able to phagocyte both pathogenic and non-pathogenic *V. harveyi* during *in vitro* experiments [7]. However, the fact that only *V. harveyi* ORM4, but not LMG 7890, is able to trigger host mortality suggests a specific response of *V. harveyi* ORM4 to counteract the host immune system. An hypothesis that can be put forward to explain the persistence of *V. harveyi* ORM4 is the bacterium ability to overproduce biofilm in the presence of abalone hemolymph, in contrast to *V. harveyi* LMG 7890 [16]. This lifestyle is associated with host colonization, as is the case during an infection with *V. cholerae* [32]. This hypothesis is supported by the utilization of the QS mutant during our experiments, which is unable to produce biofilm in microplates and did not show a biofilm overproduction in presence of host sample [16]. In line with this observation, we demonstrated here that, in the absence of a functional QS, *V. harveyi* ORM4 is almost unable to colonize gills (bacterial concentration divided by a factor 5 after 48 h, p-value <0.05) and followed the same pattern as the non-pathogenic strain with a strong decrease in bacterial concentrations between the two sampling times.

Our results therefore suggest that biofilm production is intimately linked with virulence, as observed with *V. cholerae* during human infection [32]. Indeed, some studies showed an upregulation of virulence genes in *Vibrio* when grown in biofilm [33,34]. Recently, we demonstrated that genes encoding proteins associated with the structure of T3SS are up-regulated in *V. harveyi* ORM4 biofilm-forming cells [20]. Here, we showed that this secretion system is involved in the ability of *V. harveyi* ORM4 to colonize abalone gills, since the *exsA* deletion caused an altered colonization (bacterial concentration divided by a factor 3 after 48 h, p-value <0.05). However, the link between biofilm formation and T3SS remains to be determined, as an *exsA* mutant of *V. harveyi* ORM4 is still able to produce a biofilm in microplate [16].

Pathogens can cause animal death by septicemia through proliferation in hemolymph, requiring outwitting the natural defenses of the host. When confronted to *V. harveyi* LMG 7890, the European abalone is able to wipe out the bacterial strain, avoiding deadly damage (from 1.8×10^4 CFU/mL to 7.9×10^2 CFU/mL at 48 hpi). During our experiments, we showed that an abalone infection with the non-pathogenic *V. harveyi* only marginally induced an increase in ROS production at 24 h, and that even if the bacterium was present, no other hemocyte parameter was modified (Figs. 4 and 5). Interestingly, the bacterial concentration of *V. harveyi* LMG 7890 in hemolymph is 32-fold lower at 48 hpi than at 24 hpi (Fig. 3D) and this can be correlated with the observed increase in ROS production (Fig. 5B). A previous work showed that *V. harveyi* LMG 7890 is eliminated in 3 h by rapid immune response activation through phagocytosis and ROS production [12]. In the course of this study, a ratio of 1 hemocyte per 25 bacteria was used during *in vitro* bacterial challenges. On the other hand, during our study, the hemolymph of infected abalones had less than one bacterium for each hemocyte, according to our measurements of bacterial and hemocyte concentrations. In line with these observations, we showed here that the absence of phagocytosis is associated with a small load of *V. harveyi* LMG 7890 in the hemolymph, but the animal contains the infection by preventing its proliferation through ROS production. In contrast, an increase in ROS production was observed during exposure to *V. harveyi* ORM4 (Fig. 5B), with the other hemocyte parameters measured remaining unchanged (Figs. 4 and 5A). The ratio of hemocytes to bacteria determined for the avirulent strain was the same in the presence of the pathogenic strain (hemocyte: bacteria ratio = 100:1). Thus, the low bacterial load could result in the absence of an observed immune response. In addition, during an infection, one of the first responses of the immune system is the phagocytosis of the invaders by the host cells [35]. Therefore, virulent strains frequently alter the phagocytosis activity of hemocytes in order to escape the host immunity [36]. In contrast, ROS production by hemocytes increased by 2- to 3-fold when they were exposed to *V. harveyi* ORM4, as compared to ROS production by LMG 7890-exposed hemocytes (Fig. 5B). Additionally, the concentration of *V. harveyi* ORM4 in hemolymph only decreased by a factor of 4 between the two sampling times and remained high after 48 h of infection (Fig. 3D). During an infection, *Vibrio* are known to develop different strategies to escape immune cell actions, as observed with the synthesis of a capsule by *Vibrio parahaemolyticus* or *Vibrio vulnificus* to prevent phagocytosis or bacterial degradation [37,38], or by the production of enzymes to prevent the oxidative burst induced by ROS [39,40].

In parallel, to become an efficient pathogen, *Vibrio* causes damages in host by the secretion of hemolysins and other proteins, a phenomenon widely described in *V. harveyi* [41] or *V. parahaemolyticus* [42], which present a cytotoxicity and enterotoxicity against host cells. In addition, *Vibrio* can produce extracellular proteases which degrade proteins in the host tissue, as those used by *V. harveyi* against the shrimp *Artemia franciscana* [41] or by *V. alginolyticus* [43]. In Gram-negative bacteria, proteins can be exported via secretion systems, such as the T3SS which enables the injection of effector proteins directly into target cells [17–19]. In *V. harveyi* ORM4, the *exsA* deletion leads to a loss of pathogenicity against the European abalone [16]. In view of this system function, we hypothesized its implication in abalone cell mortality [7,13], which would allow *V. harveyi* to remain in the hemolymph [9,12]. Surprisingly, *V. harveyi* ORM4 cells with an impaired T3SS and unable to cause abalone mortality were observed at the same concentration in hemolymph as the wild-type strain (Fig. 3B and D). However, it is important to note that complementation of the *exsA* gene with a multicopy plasmid causes a slight increase in hemocyte mortality (Fig. 4B). It is therefore conceivable that this system could be associated

with the hemocyte mortality previously observed [13], but later in the infection, enabling ORM4 to induce sepsis and host death [9,12,16]. As observed with *V. harveyi* ORM4, the presence of *V. harveyi* ORM4 Δ*exsA* induced a production of ROS by hemocytes (Fig. 5B). Nevertheless, this hemocyte response is insufficient to affect the concentration of the *exsA* mutant in the hemolymph, at least at 24 and 48 hpi (Fig. 3B and D). Again, it is conceivable that the load of *exsA* mutant cells can decrease after 72 h, a timepoint not tested in this study. At the beginning of an infection with *V. parahaemolyticus*, proteins secreted by the T3SS help to inactivate the p38 MAPK pathway [44], thus preventing the proper functioning of a wide range of biological processes that are under its regulation, such as inflammation, apoptosis, cell differentiation, growth, and death [45]. An activation delay of this pathway, associated with the decrease in immune response, was also observed in hemocytes of abalones infected *in vitro* by *V. harveyi* ORM4 [12]. On the other hand, the T3SS is mainly involved in cell death by injection of cytotoxic proteins [17–19]. Here we showed that this apparatus in *V. harveyi* ORM4 did not affect hemocyte viability during the first few days of infection. However, the virulent strain requires the *exsA* gene to induce mortality in Europe abalone (Fig. 1) [16]. Here we showed that even in the absence of the principal activator of T3SS gene expression, *V. harveyi* ORM4 is able to pass through abalone immunity to settle in the hemolymph. Taken together, these results suggested that T3SS can be required by *V. harveyi* ORM4 later in the infection when the bacterium is well established.

Finally, we demonstrated that a defective QS had an important impact on the ability of *V. harveyi* ORM4 to maintain itself in hemolymph since its concentration declined by a factor of 4 after 48 h of infection when compared with the wild-type. In parallel to the bacterial decrease, an increase in ROS production by hemocytes was observed in the presence of this mutant (Fig. 5B). In *V. harveyi*, the QS regulates hundreds of genes [22] and a disrupted system leads to numerous altered phenotypes in bacteria, as observed for *V. harveyi* ORM4 in which swimming motility, biofilm formation, host detection, T3SS gene expression and lethality against *H. tuberculata* [16] were altered. It is assumed that the autoinducers accumulate to let the bacteria know they are "inside" the host rather than "outside" in the seawater, as observed with *V. fischeri* which produces bioluminescence only under conditions in which there is a positive advantage for the light in the squid *E. scolopes* [24,25]. As described before, in *V. harveyi* LuxO is activated in the presence of the host, inducing an overproduction of biofilm [16]. The decrease in *V. harveyi* ORM4 Δ*luxO* concentration in hemolymph as well as the increase in hemocyte-ROS production between the two sampling times (Figs. 3D and 5B) let us hypothesize that the QS enables *V. harveyi* ORM4 to sense host molecules, thus regulating genes associated with its establishment, including those enabling it to override host defenses such as ROS production.

Finally, it is important to note that in the absence of a functional QS or T3SS, *V. harveyi* ORM4 is able to colonize hemolymph at high bacterial concentrations (Fig. 3B, C and D), but does not induce mortality in European abalone (Fig. 1) [16]. This implies that, similar to *V. harveyi* LMG 7890, the animal may contain the infection up to a certain threshold of bacterial concentration.

5. Conclusion

In conclusion, we show for the first time that an abalone-avirulent strain of *V. harveyi* is able to initiate the colonization but not to establish a long-term presence inside *H. tuberculata*. In opposition, pathogenic *V. harveyi* can efficiently colonize the gills and hemolymph and escape the host immunity. Here we show that a functional QS is essential for the establishment of *V. harveyi* ORM4 in the European abalone, and contributes to the bacterium resistance to the host immune activity. Then, the *exsA* gene, which encodes the principal activator of T3SS, is essential for gill colonization at 24 h and causes hemocyte mortality when it is overexpressed. Further investigations will be required to fully characterize the mode of action of these two systems, which makes them essential to the virulence of *V. harveyi* ORM4 against the European abalone.

CRediT authorship contribution statement

Amandine Morot: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Christophe Lambert:** Data curation, Formal analysis, Methodology, Writing – review & editing. **Adeline Bidault:** Formal analysis, Methodology, Writing – review & editing. **Alain Dufour:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **Sophie Rodrigues:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **François Delavat:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition. **Christine Paillard:** Conceptualization, Supervision, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

None.

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Data availability

Data will be made available on request.

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