Developmental & Comparative Immunology
April 2016, Volume 57, Pages 107-119
http://dx.doi.org/10.1016/j.dci.2015.12.014
http://archimer.ifremer.fr/doc/00302/41336/

First evidence for a *Vibrio* strain pathogenic to *Mytilus edulis* altering hemocyte immune capacities

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Abstract :

Bacterial isolates were obtained from mortality events affecting *Mytilus edulis* and reported by professionals in 2010–2013 or from mussel microflora. Experimental infections allowed the selection of two isolates affiliated to *Vibrio splendidus/Vibrio hemicentroti* type strains: a virulent 10/068 1T1 (76.6% and 90% mortalities in 24 h and 96 h) and an innocuous 12/056 M24T1 (0% and 23.3% in 24 h and 96 h). These two strains were GFP-tagged and validated for their growth characteristics and virulence as genuine models for exposure. Then, host cellular immune responses to the microbial invaders were assessed. In the presence of the virulent strain, hemocyte motility was instantaneously enhanced but markedly slowed down after 2 h exposure. By contrast, hemocyte velocity increased in the presence of the innocuous 12/056 M24T1. At the same time interval, 10/068 1T1 invaded hemocytes and was more rapidly internalized than the innocuous strain. Extracellular products (ECPs) prepared from 10/068 1T1 cultures significantly inhibited phagocytic activity while 12/056 M24T1 ECPs had no effect. Furthermore, the pathogenic strain and its ECPs inhibited oxidative burst unlike 12/056 M24T1 strain/ECPs which enhanced ROS production. Taken together, our results suggest that the mussel pathogen 10/068 1T1 may escape immune response by altering hemocytes functions.

Highlights

► Isolation of a *Vibrio* strain pathogenic to the blue mussel. ► Two differentially virulent *Vibrio* strains were GFP-labeled. ► The virulent strain altered hemocyte motility and quenched ROS production. ► ECPs from the virulent strain inhibited bead phagocytosis and ROS production. ► The virulent strain was rapidly internalized in hemocytes.

Keywords : innate immunity, molluscs, cell-mediated immune response, bacterial infections, green fluorescent protein

1. Introduction

Shellfish farms have been impacted by bacterial infectious diseases for many years, inducing repeated episodes of mortality and consequently important economic loss. The most common causative agent is represented by members of the genus *Vibrio* capable of infecting oysters, abalone, clams, and scallops at different life stages: larval, juvenile and adult (Beaz-Hidalgo et al., 2010a; Travers et al., 2015). Among bivalve species and until recently, mussels were not massively affected by bacterial pathogens (Gestal et al., 2008; Watermann et al., 2008). This relative resistance has been related to a robust innate immunity able to prevent efficiently bacteria infestation (Balbi et al., 2013; Ciacci et al., 2010; Tanguy et al., 2013). Nevertheless, since 2010, abnormal mortality events were also reported for farmed blue mussels (juveniles and adults) in France (Bechemin et al., 2014; Guichard et al., 2011). During this period, different *Vibrio* strains were isolated from moribund animals. Primary investigations on recent mortality events suggest that these episodes could be accounted for by multiple reasons, including a combination of particular environmental conditions together with biological factors (Bechemin et al., 2014).

Host-pathogen interactions implicate multiple processes initiated both by the pathogen, as a strategy to survive, and the host in an attempt to eliminate the invader (Gestal et al., 2008). Whilst *Vibrio* virulence has been frequently associated to secretion of extracellular products (Labreuche et al., 2010, 2006), lesions and infection kinetics remain poorly understood because of the diversity of bacteria and the number of hosts and age classes they can infect (Travers et al., 2015). The study of immune systems may help to understand responses of hosts facing invaders. As other bivalves, mussels are endowed of exclusive innate immune responses carried out by circulating hemocytes and soluble hemolymph factors (Canesi et al., 2002).

Mytilus edulis immunocytes form an heterogeneous cell population. They can be classified into 3 main groups of hemocytes, small semi-granular basophils, agranular hyalinocytes and more complex granulocytes (Le Foll et al., 2010). These cells are involved in various physiological functions but their key role consist in internal defense since they are able to recognize, bind, and phagocytize microbes. Phagocytosis is the primary mechanism for bacterial killing and elimination of foreign particles in these organisms. It can be divided into several stages, including chemotaxis, recognition, adhesion, endocytosis and destruction (Gosling, 2015). Mussel hemocytes are motile, they migrate to infected sites following the

detection of foreign materials (Donaghy et al., 2009), leading to pathogens recognition and adhesion. Internalization is the key stage of microbicidal activity. During this process, sophisticated cascades of reactions involving multiple molecular partners take place. As a consequence, phagocytes produce free radicals derived from oxygen and nitrogen, highly toxic to the ingested pathogens, and release lysosomal enzymes as well as antimicrobial peptides (Canesi et al., 2002; Mitta et al., 2000).

Nevertheless, the immune response is sometimes insufficient to defeat microbial aggressions. Bacteria can be pathogenic to host, having the ability to cause diseases and death. The degree of pathogenicity corresponds to virulence, a phenotype intimately dependent on host-pathogen interactions (Casadevall and Pirofski, 2009; Steinhaus and Martignoni, 1970). Experimental infections of *Mytilus sp.* and *in vitro* studies using hemocytes co-incubated with various *Vibrios* have generated data describing systemic, cellular and molecular responses of mussels to bacteria (Ciacci et al., 2010, 2009; Costa et al., 2009; Parisi et al., 2008; Tanguy et al., 2013). However, bacterial strains used in these studies, like *V. splendidus* LGP32 or *V. aestuarianus* 01/132, are pathogens of the pacific oyster *Crassostrea gigas* with, by contrast, no characterized virulence towards the blue mussel. Thus, it should be considered that, up to now, results reported from bacterial challenges of *Mytilus sp.* immunity were obtained in a context of low virulence.

In this work, we evaluated the virulence of bacterial isolates in the adult blue mussel by carrying out experimental infections and characterizing hemocyte responses. Experimental infections led to the selection of two isolates affiliated to *V. splendidus/V. hemicentroti* groups: a virulent and an innocuous strain. Corresponding GFP-tagged *Vibrio* strains were constructed and validated to be used in flow cytometry and fluorescence microscopy. Responses of blue mussel hemocytes exposed to virulent/non virulent *Vibrio* strains or to their extracellular products during different phases of phagocytosis were examined.

2. Material and methods

2.1. Mussel collection

Adult mussels, *M. edulis* with shell length ranging from 4 to 5 cm, were collected on the intertidal rocky shore of Yport (0°18'52"E:49°44'30"N, France) between December 2013 and December 2015, immediately transported to the laboratory and placed in a temperature-controlled (10°C) aerated Biotop Nano Cube 60 seawater tank (Sera, Heinsberg, Germany) , equipped with mechanical and activated biological filtering. The animals were fed with algae (*Isochrysis galbana*) and maintained in these conditions for at least one week before use.

2.2. Bacterial strains

2.2.1. Isolation from mussels (Laboratoire National de Référence, LNR, La Tremblade)

Strains used in this study are described in **Error! Reference source not found.**. Bacteria were isolated from mussel mortality events reported by professionals (French national surveillance network REPAMO) in 2010 and 2013, or from mussel microflora in absence of mortality in the context of Bivalife European project in 2011 and 2012. Briefly, crushed tissues were homogenized in 100 µl of Sterile Artificial Sea Water (SASW : 2.3 % (w/v) NaCl, 20 mM KCl, 5 mM $MgSO_4$, 2 mM $CaCl_2$) with a sterile pellet-pestle (Sigma) for 1 minute on average. Samples diluted 100x and 1000x in SASW were plated on Zobell agar (0.4% peptone, 0.1% yeast extract, 0.01% ferric citrate and 1.5 % agar in SASW, pH 7.6) and the predominant bacteria were isolated after 48h at 20°C. Pure cultures of bacterial strains were conserved frozen at -80°C in Zobell broth with glycerol 15%.

2.2.2. Genetic characterization: *gyrB* sequencing

Total DNA from a log-phase culture was extracted from cultured *Vibrio* by boiling in 100 µl of ultrapure water (Saulnier et al., 2009). The *gyrB* (gyrase B) gene was amplified using the universal bacterial primer pairs (gyrB274F GAAGTTATCATGACGGTACTTC and gyrB1171R CCTTTACGACGAGTCATTTC) and the methods previously described (Thompson et al., 2005). Amplicons with the expected size were purified using a Microcon PCR filter kit (Millipore). Purified PCR products were mixed (final volume 10 µl) with 0.4 µl ABI Prism Big Dye Terminator ready reaction mix (Applied Biosystems®) and 0.75 µM of primer. Cycle sequencing reactions were performed using a Gene Amp PCR System 2700 (Applied Biosystems®) and following the manufacturer's instructions. Separation of the DNA fragments was carried out in an ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystems).

Sequences were aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html.) Phylogenetic tree was built using Mega6 (http://www.megasoftware.net/mega6/mega.html.). The tree was drawn using the Neighbor-Joining method with the Kimura two-parameter model (Saitou and Nei, 1987). Reliability of topologies was assessed by the bootstrap method (Felsenstein, 1985) with 1000 replicates. The Genbank accession numbers gene sequences obtained in this study or already published in GenBank are presented in **Error! Reference source not found.**.

2.2.3. Characterization of virulence by *in vivo* injections

Bacteria were grown overnight in Marine broth or Luria Bertani NaCl 20 g.L^{-1} at 22°C with constant agitation (80 rpm). Cells were washed twice with filtered sterile seawater (FSSW) and centrifuged at 1200 g for 10 min. The supernatant was discarded and the resulting pellet resuspended in FSSW to obtain an OD_{600nm} of 1. Mussels were anesthetized for 2–3 h at 20°C in a magnesium chloride solution ($MgCl_2$, Sigma Aldrich) at a final concentration of 50 g.L^{-1} (1/4: v/v seawater/freshwater) and under aeration. Subsequently, a volume of 100 µl of bacterial suspension was injected into the posterior adductor muscle. A group of mussel was injected with FSSW to serve as negative controls. After injection, the animals were transferred to tanks (3 replicate tanks, 10 mussels per tank) filled with 2L of UV-treated and filtered seawater supplemented with 50 ml of phytoplankton and maintained under static conditions at 20°C with aeration. Mortality was monitored each day over a four day period. Animals were considered to be dead when the valves did not close following stimulation. Newly dead mussels were removed from the tanks.

2.2.4. GFP-tagging by triparental mating

The pVSV102 plasmid (Dunn et al., 2006) carrying GFP/ kanamycin-resistance expression cassette was transferred from *E. coli* to *Vibrio* strains (10/068 1T1, 12/056 M24T1) by triparental mating (Stabb and Ruby, 2002) using the conjugative helper strain CC118 λpir as described by Dunn et al. (2006). Donor, helper and receptor cells were grown overnight to the stationary phase in Luria Bertani (LB) (*E. coli* strains) and LBS [LB complemented with salt, NaCl 20 g.L^{-1} (f.c.), for *Vibrio* strains] with addition of 40 µg.mL^{-1} kanamycin for DH5α-pVSV102. 100 µL of each culture was combined in a microfuge tube, washed in LBS without antibiotics and centrifuged at 1200 g for 10 min. The resulting pellet was suspended into 10 µL of LBS and dropped on a fresh LBS agar plate and then incubated for 16 hours at 28°C. The bacterial spot was suspended in 800 µL of LBS, serially diluted, plated on LBS plates containing 100 µg.L^{-1} kanamycin and incubated at 18°C. Donor bacteria were counter-selected by growing at 18°C, whereas the helper strain and the acceptor strain, which did not receive conjugative, plasmids were killed by the antibiotic selection. Therefore, clones were isolated by inoculation into new LBS antibiotic plates and green fluorescent were verified by epifluorescence microscopy.

2.2.5. Validation of GFP-tagged strains

GFP expression conservation: To ensure plasmid conservation, fluorescent bacteria strains were grown overnight in LBS 100 µg.L^{-1} kanamycin at 22°C. This culture was diluted in LBS without antibiotics and grown at 22°C overnight. Every day, a new culture was started with an aliquot of the previous day's culture, which was analyzed by flow cytometry, after dilution.

Growth curves: Parental bacterial strains and GFP-tagged strains were cultivated in LBS at 22°C with constant shaking at 80 rpm. At regular intervals, the bacterial concentrations in the cultures were evaluated spectrophotometrically at an optical density (OD) of 600 nm.

In vivo injection: To compare virulence between parental and derivative fluorescent strains, GFP-tagged strains were injected intramuscularly into mussels according to the protocol described above for parental strains.

2.3. Contact with hemocytes

2.3.1. Haemolymph collection

Haemolymph was withdrawn from the posterior adductor muscle sinus, by gentle aspiration with a 1 mL syringe equipped with a 22G needle. Quality of samples was systematically checked by microscopic observation before using in bioassays.

2.3.2. Preparation of bacteria and their extracellular products

For *in vitro* experiments, two *Vibrio* strains were used: 10/068 1T1 and 12/056 M24T1 (parental or GFP-tagged). Bacteria were cultivated overnight in Marine broth or Luria Bertani NaCl 20 g.L^{-1} at 22°C, centrifuged at 3000 g for 10 min. Supernatants were filtered (0.22µm) and conserved at -20°C until use and bacteria were diluted in sterile physiological water (NaCl 9 g.L^{-1}) at 10^8 cfu.ml^{-1} for immediate utilization.

2.3.3. Motility

Hemocyte motility was assessed via live-cell nuclei tracking. The protocol was adapted from Rioult et al. (2013). Briefly cells exposed to bacteria (10^8 cfu.ml^{-1}) or in marine physiological saline solution (MPSS) pH 7.8, 0.2 µm filtered (Rioult et al., 2014) for the control were incubated with 5 µM of the nuclei-specific fluorescent probe Hoechst 33342 for 15 minutes at 15°C. A culture dish was placed on the stage of a TE-2000 inverted microscope (Nikon, Champigny-sur-Marne, France) equipped for epifluorescence excitation (HBO arc lamp with 377/50 nm bandpath filter) and time-lapse imaging. A Peltier temperature controller (PDMI-2 and TC-202A; Harvard Apparatus, Holliston, MA) keeps preparation at

15°C for extended live cell imaging. Wild-field epifluorescence time-lapse imaging was performed with a x10 objective (numerical aperture 0,3). A VCM-D1 shutter (Uniblitz, Vincent Associates, NY) was added in the illumination pathway to cut off the excitation light between two image recordings. Camera and shutter were controlled by Metamorph (Molecular Device, Sunnyvale, CA) as acquisition software. A CCD Coolsnap EZ camera (Photometrics, Tucson, AZ) captured 12-bit digital of 1392 × 1040 pixels greyscale images every 30 s for 2 hours (409 nm long path emission filter). Camera and software were calibrated to express distance in microns. Time-series image stacks were imported into Metamorph Analysis software. The track Objects application (available with Multi-Dimensional Motion Analysis option) was started. Typically, for each biological replicate, 20 nuclei were randomly chosen to be tracked. Extracted data were transferred to a spreadsheet and, for each cell, the mean distance travelled during 30 seconds was calculated and multiplied by 2 to express velocity in $\mu m.min^{-1}$.

2.3.4. Phagocytosis assay

The phagocytic ability of hemocytes was determined by flow cytometry using fluorescent beads or GFP tagged bacteria. Internalization of beads or bacteria was verified by microscopic observation.

The protocol was adapted from Costa et al. (2009) with some modifications. Briefly, fluospheres (Fluosphere Carboxylate-Modified Microspheres, 2.00 µm, yellow-Green, Life technologies) were added to haemolymph to generate minimally a 10:1 bead to hemocyte ratio and cells were incubated for 2, 4 and 6 hours at 15°C in the dark. The effect of bacterial extracellular products (ECPs) was tested by mixing 100 µl of ECPs to cell suspension one hour before adding beads. After incubation, supernatants were gently aspirated and attached cells were removed by adding cold Alsever's solution (300 mM NaCl, 100 mM Glucose, 30 mM sodium Citrate, 26 mM citric acid, 10 mM EDTA, pH 5.4) and analyzed by Beckman Coulter flow cytometer. Phagocytosis was defined as cell internalization of 3 beads or more.

The capacity to phagocyte bacteria was also evaluated. Cells were exposed to GFP-tagged *Vibrio* strains at 10:1 ratio (bacteria:cell) for 2, 4 and 6 hours at 15°C in the dark. Before analysis on flow cytometry, propidium iodide was added to quantify the percentage of viable cells.

2.3.5. ROS production

Crude haemolymph was placed into individual wells of 24-well tissue-culture plates (Greiner) and cells allowed to adhere for 15 minutes at 15 °C. The haemolymph was removed and replaced with 400 µl MPSS (0.2 µm filtered) alone for the control or containing 0.2 µM phorbol 12-mystriate 13-acetate (PMA, Sigma) or 100 µl of ECPs or heat killed bacteria (15 min at 100°C). For *Vibrio* challenges, 400 µl of bacterial suspension diluted in physiological water at 10^8 cfu.ml^{-1} were added to hemocytes. After two hours of incubation at 15°C, CM-H_2DCFDA at 0.2 µM f.c. was added in each well and plates were incubated 30 minutes at 15°C in the dark. Fluorescence was analyzed on flow cytometer (Beckman Coulter).

2.4. Statistical analyses

Statistical analysis was performed by using SigmaPlot 12 (Systat Software Inc., Chicago, IL). Replicates were averaged and the values were tested for normality (Shapiro-Wilk) and paired comparisons were performed by Student's t-tests or by Mann-Whitney rank sum tests in case of unequal variance. Statistical significance was accepted for $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$

3. Results

3.1. Bacteria pathogenicity and genetic characterization

Bacterial strains were isolated from *M. edulis* during mortalities events reported by professionals or from mussel microflora in absence of mortalities (**Error! Reference source not found.**). Fifteen strains of *Vibrio* isolates were tested at OD_{600}=1 for their pathogenicity toward mussels by experimental infection (Figure 1). First mortalities appears 24 hours post-injection and were comprised between 0 % and 76,6 % indicating different degrees of virulence between bacteria. The most virulent strain was 10/068 1T1 and caused respectively 76,6 ± 8% and 90 ± 5% (n=3) mortalities after 24 and 96 hours. For 10/060 2T1 and 12/040 11T2 mortalities increased progressively from 5-10% after 24h to 40-47,5% after 96 hours. Strains 10/058 2T1, 10/058 3T1, 11/100 M22T3, 11/148 M6T2, 12/037 M22T1, 12/037 M24T1, 12/037 M7T1, 12/037 M23T1, 12/056 M1T1, 12/056 M24T1, 13/026 2T3, 13/026 5T3 can be considered as innocuous (mortalities between 0 and 6,6 % after 24h). It is important to notice that some of these strains can induce limited mortalities after 96 hours.

To confirm the virulence of 10/068 1T1 strain, different OD_{600} doses were injected to mussels (Figure 2). After 24 hours, a dose-dependent mortalities was observed (36,6 ± 12%, n=3 mortalities at OD_{600}=0.1).

Phylogenetic analyses of bacteria isolates based on the housekeeping gene *gyrB* revealed that the majority of isolated strains belongs to *V. Splendidus* group and can be affiliated to the *V. splendidus / V. hemicentroti* species, or *V. lentus / V. atlanticus* species (Figure 3). No clear correlation between phylogeny and virulence can be noticed. Conversely, few virulent strains (3) were identified, all isolated from mussel mortality events (2010 and 2012), and phylogenetically close to non virulent strains isolated from normal flora.

3.2. Validation of GFP-tagged strains

Two *Vibrio* strains were GFP-tagged, the virulent 10/068 1T1 and the non virulent 12/056 M24T1. To validate their use as genuine models for *M. edulis*, the fluorescence stability and potential effects of the GFP-plasmid on bacteria growth capacities and virulence were analyzed. Plasmid stability tests, determining the proportion of plasmid-bearing cells remaining overtime, were conducted by culturing the GFP-labeled strains in the absence of antibiotic selection. Both strains 10/068 1T1 and 12/056 M24T1 showed a high stability of GFP encoding plasmid after 14 passages in a non-selective culture (Figure 4a). Bacterial physiology and phenotype after GFP-tagging were also studied. No obvious difference was noticed in growth characteristics (Figure 4b) and colony size or aspect when plated on LBS (not shown). Furthermore, bacteria virulence did not change after transformation, both parental and GFP-tagged 10/068 1T1 strains leading to 90% mussel mortality in 4 days while parental and labeled 12/056 M24T1 strains caused in this experiment respectively 20% and 23% mortalities (Figure 4c).

3.3. Hemocyte motility

Hemocyte migration was followed *in vitro* by nuclei tracking during 2h with a recording rate of 1 image/30 sec. Off-line tracking was carried out on a set of 20 nuclei selected randomly in the microscopic field. In control conditions, velocity was stable with values of about 1 µm/min. In the presence of *V. splendidus*-related 10/068 1T1 in the imaging chamber, cell velocities were higher, 3.3 µm/min at the beginning of the recording, and increased after 30 min to reach 4,8 µm/min. Then, migration speed decreased and get closer to control velocity after 2 hours of recording. In contrast, the motility of hemocytes exposed to the innocuous strain 12/056 M24T1 increased progressively and exceeded 5µm/min at the end of the recording (Figure 5a). Standard error of the mean (SEM) values followed the same distribution than mean velocity (Figure 5b). These results indicate that hemocytes co-incubated with *V. splendidus*-related 10/068 1T1 had transiently elevated velocities at the

beginning of the experiments and rapidly converged towards a reduced motility in a bell-shaped time course. Conversely, 12/056 M24T1 continuously activated hemocyte speed during motility recordings and provoked a dispersion of single-cell velocities.

3.4. Phagocytosis assays

The ability of hemocytes to engulf latex beads was investigated at different time intervals (Figure 6); the percentage of cells containing 3 or more beads increased with incubation time and ranged from 40% at 2h to 56% at 6h. The preincubation with 10/068 1T1 ECPs altered significantly the phagocytic capacity at 2h with a decrease from 40% to 22% and also at 4h and 6h ($p<0.05$). 12/056 M24 T1 ECPs did not affect phagocytosis by comparison to control. The phagocytic capacity of bacteria was evaluated by challenging hemocytes with virulent and non-virulent strains at different exposure time (Figure 7). The percentage of cells containing one bacterium was significantly higher for hemocytes exposed to 12/056 M24T1 than 10/068 1T1, and rates decreased at 6h. However, cells engulfed 2 bacteria and more were significantly less important for 12/056 M24T1 than 10/068 1T1 and an increase was observed after 6 h exposure. Furthermore, during phagocytosis hemocytes were viable at 95% for both strains (data not shown).

3.5. ROS production

The capacity of *M. edulis* hemocytes to produce reactive oxygen species was investigated by *in vitro* exposure to a chemical activator used as a positive control (phorbol 12-mystriate 13-acetate, PMA) and bacterial strains (living or heat killed) or their extracellular products (ECPs) (Figure 8). Immunocytes respond actively and significantly to all treatments. PMA activated a respiratory burst at very low concentration (0.2µM), demonstrating that mussel hemocytes are able to produce toxic radicals. Bacterial strains or corresponding ECPs also induced ROS production. However, significant differences were obtained according to experimental conditions. Hemocyte exposure to non virulent *Vibrio* (12/056 M24T1) or to 12/056 M24T1 ECPs activated ROS production at levels similar or higher than PMA, respectively. Conversely, when exposed to the strain 10/068 1T1 or its ECPs, hemocytes generated oxygen radicals at amounts significantly inhibited by comparison to PMA-stimulated levels without any reduction of cell viability (data not shown). Heat-killed 10/068 1T1 did not significantly modify the response compared to PMA.

4. Discussion

Unlike other bivalve species, *Mytilus edulis* is not known to be particularly affected by any major bacterial disease (Beaz-Hidalgo et al., 2010a; Travers et al., 2015; Watermann et al., 2008). It has been suggested that mussel resistance to bacterial infection was due to the presence of potent immune defense mechanisms (Balbi et al., 2013; Ciacci et al., 2010, 2009; Tanguy et al., 2013). However, while many studies explored mussel immune responses towards Gram + and Gram – bacteria including *Vibrios* (Costa et al., 2009; Parisi et al., 2008), strain virulence for *Mytilus sp. per se* was never tested. This lack of data is all the more regrettable that, since 2010, abnormal mortality events have touched farmed blue mussels (juveniles and adults) in France, where different bacterial strains were isolated (Guichard et al., 2011).

In this context, we performed *1)* for a first time, an evaluation of virulence and a genetic characterization of bacterial strains isolated from *Mytilus edulis*, *2)* a construction and validation of two stable GFP-tagged *V. splendidus*-related strains for their use in flow cytometry, and *3)* a description of functional activity of mussel hemocytes challenged by virulent and non virulent bacteria or by their extracellular products.

4.1. Pathogenicity of *V. splendidus*-related strains towards the blue mussel

To evaluate virulence of isolates, experimental infection assays were carried out by injecting bacteria into mussels, intramuscularly. Among the tested strains, 10/068 1T1 showed a high and dose dependent degree of virulence (76.6% and 90% mortalities after 24h and 96h) whereas 12/056 M24T1 was found innocuous. Phylogenetic analysis using *gyrB*, one of the more polymorphic housekeeping genes used for *V. Splendidus* clade (Le Roux et al., 2004), revealed the affiliation of both virulent and non virulent bacteria 10/068 1T1 and 12/056 M24T1 to the *V. splendidus* and *V. hemicentroti* type strains. Even if more realistic protocols as immersion or cohabitation challenges are needed to confirm the pathogenic potential of this strain, our injection protocol yet allows a marked differentiation of phylogenetically close strains with contrasted pathotypes (76.6% of mortality induced in 24 hours vs 0%).

Different strains related to the *Vibrio Splendidus* clade were implicated in mortalities of various bivalves, including the pacific oyster (Gay et al., 2004; Lacoste et al., 2001; Saulnier et al., 2010), the atlantic scallop (Lambert et al., 1999; Nicolas et al., 1996), the carpet shell clam (Beaz-Hidalgo et al., 2010b) and the greenshell mussel (Kesarcodi-Watson et al., 2009). An epidemiological study of *V. splendidus* strains associated with *Crassostrea gigas* mortalities demonstrated genetic diversity within this group and suggested its polyphyletic

nature (Le Roux et al., 2004). In fact, the *V. Splendidus* group includes 16 species, many of them containing virulent and non virulent strains (for instance *V. celticus, V. crassostreae, V. cyclitrophicus, V. tasmaniensis* and *V. splendidus*) (Travers et al., 2015). Concerning *M. edulis*, we conclude that, as in other mollusk species affected by *V. splendidus*-related strains, a virulent pathotype cannot be discriminated through housekeeping gene sequencing since virulent strains appear phylogenetically close to innocuous ones. To define this pathotype, further studies based on a large collection of strains including ecological populations (Hunt et al., 2008) are needed.

A recent study on normal microflora, *i.e.* microflora of healthy animals, associated with *M. galloprovincialis* reveals a high diversity of strains belonging to 5 major *V. splendidus* groups (Kwan and Bolch, 2015). In accordance with these results, the majority of analyzed strains are close to *V. splendidus, V. tasmaniensis, V. lentus or V. atlanticus*, even if we didn't find *V. toranzoniae*-affiliated genotypes. Efforts to distinguish *V. Splendidus*-related innocuous bacteria that compose natural microflora of mussel from virulent pathotypes are now necessary.

4.2. Construction and validation of GFP-tagged bacteria

To facilitate the study of interactions between *V. splendidus*-related strains and mussel hemocytes, bacteria were tagged with the Green Fluorescent Protein. Fluorescent 10/068 1T1 and 12/056 M24T1 strains showed a high plasmid stability. They constitute useful tools for flow cytometry and epifluorescence microscopy. However, in some cases, the addition of the marker gene generated changes in bacterial physiology or behavior (Aboubaker et al., 2013; Allison and Sattenstall, 2007; Knodler et al., 2005). To investigate undesired virulence-interfering effects of GFP expression, parental and genetic-engineered bacteria were compared. For both strains, our results indicate that GFP-labeling disturbed neither growth characteristics nor degrees of virulence of bacteria, thus validating the use of 10/068 1T1 and 12/056 M24T1 GFP expressing strains as genuine models for challenging *Mytilus edulis*.

4.3. *Mytilus* hemocyte responses to *Vibrio* strains

Hemocytes form the first defense line of the immune system in bivalves. Similarly to cells of the vertebrate monocyte/macrophage lineage, activated hemocytes achieve pathogen elimination through chemotaxis, phagocytosis and cytotoxic processes, essentially (Liu, 2008).

In an early phase of response to microbial threat, hemocytes migrate toward infected sites. Herein, we explored the influence of *V. splendidus*-related strains on the motility of *Mytilus edulis* hemocytes, by using the nuclei tracking method (Rioult et al., 2013). In the absence of stimulation, hemocyte velocity was stable (1 µm/min). In the presence of the innocuous strain 12/056 M24T1, we observe a progressive speed up of cell motility over 2 hours recordings that probably corresponds to a chemoactivation. Chemotactic and chemokinetic responses increase the probability of physical contact between hemocytes and invaders (Schneeweiss and Renwrantz, 1993), accelerating their detection and recognition. In our experiments however, hemocyte exposure to virulent bacteria 10/068 1T1 triggered more complex responses. The first phase lasting 30 min consists in an immediate acceleration in cell migration (4,7µm/min). Thereafter, hemocyte instantaneous velocity progressively decreased to reach control values after 2 h. This deceleration may be a consequence of bacteria virulence capable to alter cell migration. Only few studies evoked the mobility of bivalve hemocytes, their high clumping potential and chemotactic activity towards pathogens or their extracellular products (Canesi et al., 2002; Pruzzo et al., 2005; Rioult et al., 2013). In blue mussels, Boyden chamber assays revealed that ability of blood cells to generate both chemotactic and chemokinetic reactions depends on the nature of bacteria secreted molecules (Schneeweiss and Renwrantz, 1993). Short-term (40 min) chemoactivation have been reported from oyster hemocytes that migrate to accumulate around bacteria (Alvarez et al., 1995).

After migration, vertebrate as invertebrate phagocytes, on encounter with foreign objects surfaces, respond by sending out finger-like pseudopods to engulf detected particles (Bayne, 1990). This process requires a major reorganization of cytoskeletal elements in the region of the cell where phagocytosis has been triggered (Russell, 2001) and the resulting filaments must be removed from the base of the forming phagosome to enable its closure (Sarantis and Grinstein, 2012). In our study, we first explored the effect of 10/068 1T1 ECPs on *M. edulis* hemocyte capacity to engulf latex beads.

After 2-hours incubations, results indicate a reduction of phagocytic activity to 22%, instead of 40% in control conditions, demonstrating the phagocytosis-inhibition ability of secreted products. A similar significant trend was obtained at 4-6h after contact while 12/056 M24T1 ECPs had no effect on phagocytosis. The decrease of phagocytosis may be a consequence of a loss of pseudopodia and reduction of adhesion capacity. This is reminiscent of cell detachments and unspreadings frequently described for bivalve hemocytes challenged with pathogenic *Vibrio* strains or with their extracellular products. For example, adhesion and phagocytic activity of pacific oyster hemocytes were altered in contact with *V. aestuarianus*

01/32 ECPs or *V. tubiashii* 07/118 T2 ECPs (Labreuche et al., 2006; Mersni-Achour et al., 2014) in a manner that was dependent on ECPs doses (Labreuche et al., 2006). The same hemocyte responses were reported for *Mytilus edulis* and *Mya arenaria* hemocytes challenged with the oyster pathogen *V. splendidus* LGP32 (Araya et al., 2009; Tanguy et al., 2013). Nevertheless, precise molecular mechanisms involved in bacteria alteration of hemocyte phagocytosis are still unclear. Involvement of metalloprotease activities have been suggested since such enzymes are present in ECPs of virulent *Vibrios* and since the metalloenzyme Vsm, secreted by *V. splendidus* LGP32 is toxic to oysters (Binesse et al., 2008). So, it will be interesting to explore the effect of 10/068 1T1 ECPs on adult mussel.

Direct hemocyte phagocytosis of virulent and non virulent GFP-tagged bacteria was also quantitatively investigated. The number of hemocytes strictly containing one engulfed *Vibrio* decreased with time. This number was lower for cells exposed to the virulent strain 10/068 1T1 than for hemocytes incubated with the innocuous strain 12/056 M24T1. In appearance, this result may suggest a more efficient destruction of *Vibrio* 10/068 1T1. Interestingly however, analysis of hemocytes containing 2 or more *Vibrios* results in opposite findings. The percentage hemocytes with 2 or more engulfed bacteria increased time-dependently but was higher for the virulent strain. This latest observation is rather consistent with a faster internalization of the pathogenic 10/068 1T1 strain in hemocytes, compared to 12/056 M24T1.

In some extent, internalization by host cells may be advantageous for invaders to effectively establish infection and to colonize tissues (Sarantis and Grinstein, 2012). Such mechanisms were observed in some *Vibrio* species, usually considered as extracellular pathogens but having obviously intracellular stages (Duperthuy et al., 2011). This is especially the case of, the coral pathogen *V. shiloii* which invades epithelial cells (Banin et al., 2000) and of *V. splendidus* LGP32, able to attach and invade oyster hemocytes through OmpU porin (Duperthuy et al., 2011). In a good agreement with these studies, it can be proposed that the virulent strain 10/068 1T1 invades mussel hemocytes actively, although its interaction with the cell inner environment remain to be determined.

Among the events taking place during the immune response, the oxidative burst plays an important role in microbe destruction. ROS are lethal weapons used by phagocytes to kill microbial invaders, directly, by causing oxidative damage to biocompounds, or indirectly, by stimulating pathogen elimination (Paiva and Bozza, 2014). In *Mytilus edulis*, we confirmed the capacity of hemocyte to produce oxygen radicals after stimulation by PMA at low doses (García-García et al., 2008). When exposed to non virulent bacteria, mussel blood cells also

produced ROS at levels equivalent to the PMA treated group, 12/056 M24T1 ECPs also enhanced oxidative bursts. Increases of ROS production by hemocytes exposed to bacteria or to their extracellular products have been previously reported in the blue mussel (Tanguy et al., 2013) and other bivalves species (Buggé et al., 2007; Lambert et al., 2003).

In the presence of virulent bacteria or of their ECPs, but not after hemocyte exposure to heat killed virulent bacteria, ROS production was significantly inhibited by comparison to PMA-stimulated levels. These data reveal the capacity of the pathogenic *V. splendidus*-related strain 10/068 1T1 to alter cell activation by itself but also through secreted products. As a consequence, it is possible that the pathogenic bacterial strain 10/068 1T1 actually quenches ROS production in *M. edulis* hemocytes as a mean to survive within host cells. In this respect, a wide variety of microbes have developed strategies to promote their survival within hostile cellular environment, in particular by inhibition of ROS-mediated host responses (Spooner and Yilmaz, 2011). For example Densmore et al. (1998) have demonstrated an inhibition of oxidative burst in trout phagocytes, previously stimulated by PMA in the presence of *R. salmoninarum* ECPs. In invertebrates, *L. anguillarum* does not induce any oxidative burst in *Crassostrea virginica* hemocytes (Bramble and Anderson, 1997) and quenches ROS production in lobster hemocytes (Moss and Allam, 2006). Likewise, the pathogenic strain *V. harveyi* does not elicit ROS production when added to shrimp hemocytes contrary to a probiotic strain of *V. alginolyticus* which induces cell activation (Muñoz et al., 2000). In addition, *V. splendidus* LGP32 exhibits intracellular survival capabilities and escape from host cellular defenses by avoiding acidic vacuole formation and by limiting ROS production (Duperthuy et al., 2011).

5. Conclusion

In this study, we selected two *V. splendidus*-related strains and constructed GFP-tagged models. The 10/068 1T1 strain was isolated from mortality events reported by professionals and proven as virulent to blue mussel. The 12/056 M24T1 strain was isolated from normal flora and found as innocuous. Functional immune responses of hemocytes challenged by different strains were explored through cell motility, phagocytosis and oxidative burst. Virulent bacteria generated stronger immunocyte responses. Overall, 10/068 1T1 enhanced instantaneously cell migration but adversely affected cell motility after 2 hours exposures. These bacteria were also able to quench ROS production and to alter phagocytosis capacity through ECPs secretion. It has been shown that virulent bacteria were rapidly internalized by hemocytes, suggesting more the involvement of a pathogen-controlled invasion strategy than

an immune cell-driven phagocytosis process. Taken together, our data support the hypothesis that some bivalve pathogens escape cellular immune response by dysregulation of some hemocyte bactericidal activities. Further investigations are necessary to establish whether hemocytes also constitute carriers for tissues infection.

Acknowledgements

This work received fundings from the State/Region Plan Contract (CPER) allocated through the Research Federation FED 4116 SCALE (Sciences Appliquées à L'Environnement),), from the FP7-European project BIVALIFE 'Controlling infectious diseases in oysters and mussels in Europe' (Grant Agreement 266157) and DGAl support through National Reference Laboratory activities for mollusk diseases (Ifremer-La Tremblade). Yosra Ben Cheikh was a recipient for a Ph.D. grant from the Conseil Regional de Haute-Normandie. The authors are indebted to the marine fish farm Aquacaux (Octeville, France) for valuable technical assistance. Dr. Eric Stabb (Dept. Microbiology, University of Georgia, Athens, USA) is kindly acknowledged for providing us with his excellent *Vibrio* vectors and helper strain. We also thanks LGPMM technical unit, Manon Garrigues and Philippe Haffner for their contribution.

Supplementary data

Video 1: Epifluorescence time-lapse microscopy of hemocytes plated in a culture dish and co-incubated at 15°C with the 10/068 1T1 *Vibrio* strain. Nuclei were stained with a vital DNA dye Hoechst 33342 (5µM). Recording time, 2 hours. Imaging interval time, 30 sec.

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Figure legends

Figure 1. Cumulative mortalities recorded after experimental bacterial infections of adult mussels. Bacterial strains were grown for 24 h in Marine broth before resuspension into filtered sterile seawater (FSSW) and adjustment to OD_{600} = 1. One hundred microliters of bacterial suspension were injected intramuscularly to anesthetized mussels (10 animals/replicate). Mussels injected with FSSW were used as control. Data are means ± SEM of cumulative mortalities triplicate tanks

Figure 2. Effect of inoculum size on mussel mortality. Dose-response effect of *V. splendidus*-related strain 10/068 1T1 on adult mussels estimated 24 hours post-injection. Mussels injected with FSSW were used as control. Data are means ± SEM of triplicate tanks.

Figure 3. Phylogenetic analysis of bacteria strains based on *gyrB* gene sequencing. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The analysis was carried out by the neighbor-joining method on 34 nucleotide sequences and 529 positions in the final dataset. Evolutionary analyses were conducted in MEGA6. Virulence of bacteria, assessed by the percentage of mortality induced 24 hours post-injection is indicated by the horizontal bars.

Figure 4. Plasmid expression stability in *Vibrio* strains and effect of GFP expression on growth capacities and virulence. (a) Fluorescence conservation in GFP-tagged bacteria over time. Strains were cultivated in non-selective LBS medium and fluorescence was estimated by flow cytometry. (b) Growth curve of parental and GFP-tagged bacteria at 22°C in LBS medium (n=3± SEM). (c) Comparison of virulence capacity of parental and GFP-tagged strains on adult mussels (n=3± SEM).

Figure 5. Effect of *V. splendidus*-related strains on hemocyte motility. *M. edulis* hemocytes were challenged by *V. splendidus*-related strains 10/068 1T1 or 12/056 M24T1 at 10^8 cfu.ml^{-1} or incubated in MPSS (control) during 2h at 15°C. (a) Mean velocity of 60 nuclei (3 independents experiences) obtained from centroid XY coordinate changes in the microscopic

field between two successive images, separated by 30 seconds. (b) Standard errors of the mean taken from (a).

Figure 6. Effect of bacterial ECPs on bead phagocytosis ability. (a) Flow cytometry analysis of hemocyte incubated *in vitro* with latex beads during 2, 4 and 6 hours. The percentage of cells containing 3 beads or more was measured (mean ±SEM, n=6, $p<0.05$, Student's t-test). (b) Phase contrast (Ph) and fluorescence (Fitc) microscopy observation of hemocytes after 2 hours phagocytosis (bar 10 µm).

Figure 7. Hemocyte phagocytic ability towards GFP-tagged bacteria. (a) Comparison between the internalization of virulent and non-virulent bacteria after *in vitro* exposure during 2, 4 and 6 hours. Left Y-axis and solid bars refer to the percentage of hemocytes containing 2 or more bacteria. Right Y-axis and dashed bars refer to the percentage of hemocytes containing at least one *Vibrio*. Significant values respectively to non-virulent strain are represented with *** ($p<0.001$, Student's t-test). Data are means ±SEM, n=6. (b) Phase contrast (Ph) and fluorescence (Fitc) microscopy observations of hemocyte after internalization of *Vibrio* strains 10/068 1T1 or 12/056 M24T1, bar 10 µm.

Figure 8. ROS production in *M. edulis* hemocytes**.** Hemocytes were pre-exposed 2 hours to heat killed or living virulent bacteria at 10^8 cfu.ml^{-1}, non-virulent bacterial strain at 10^8 cfu.ml^{-1}, or their extracellular products or PMA 0,2 µM. Cell fluorescence was quantified by flow cytometry after incubation with CM-H_2DCFDA at 0.2 µM f.c. Data are means of fluorescence ±SEM, A.U., n=6. * indicates significant differences from control (Mann-Whitney rank sum test, $p< 0.05$) and § marks significant differences from PMA (Student's t-test § $p<0.05$, §§§ $p<0.001$.

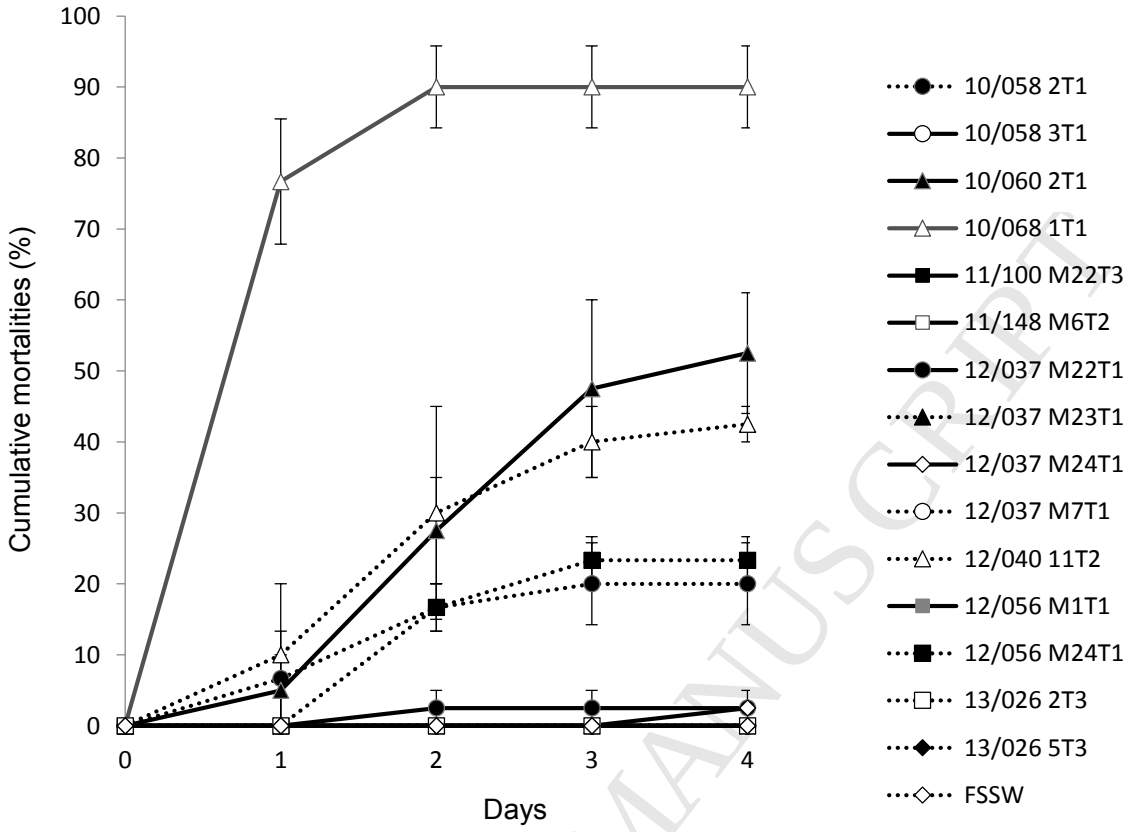
Table 1. Strains used in this study

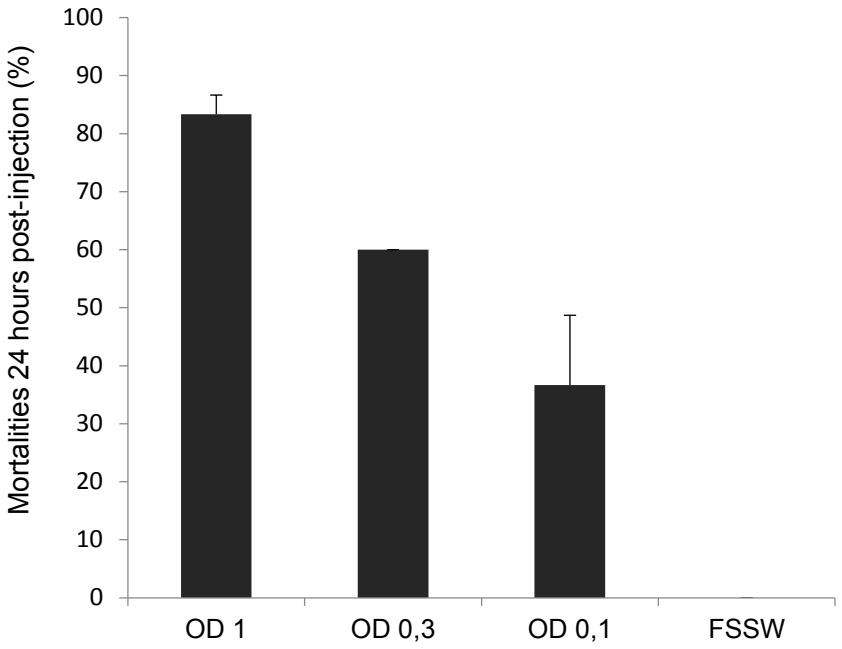
Strain	Isolation from	Context	Date
10/058 2T1	Mussel, baie de Somme (80) France	Mussel mortality	2010, May 18
10/058 3T1	Mussel, baie de Somme (80) France	Mussel mortality	2010, May 18
10/060 2T1	Mussel, baie d'Authie (62) France	Mussel mortality	2010, May 19
10/068 1T1	Mussel, baie de Camaret (29) France	Mussel mortality	2010, May 31
11/100 M22T3	Mussel, d'Agnas (17) France	Absence of mortality	2011, June 29
11/148 M6T2	Mussel, d'Agnas (17) France	Absence of mortality	2011, October 11
12/037 M22T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, May 22
12/037 M23T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, May 22
12/037 M24T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, May 22
12/037 M7T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, May 22
12/040 11T2	Mussel, Oye Plage (62) France	Mussel mortality	2012, May 24
12/056 M1T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, June 05
12/056 M24T1	Mussel, d'Agnas (17) France	Absence of mortality	2012, June 05
13/026 2T3	Mussel, Oye Plage (62) France	Mussel mortality	2013, April 08
13/026 5T3	Mussel, Oye Plage (62) France	Mussel mortality	2013, April 08

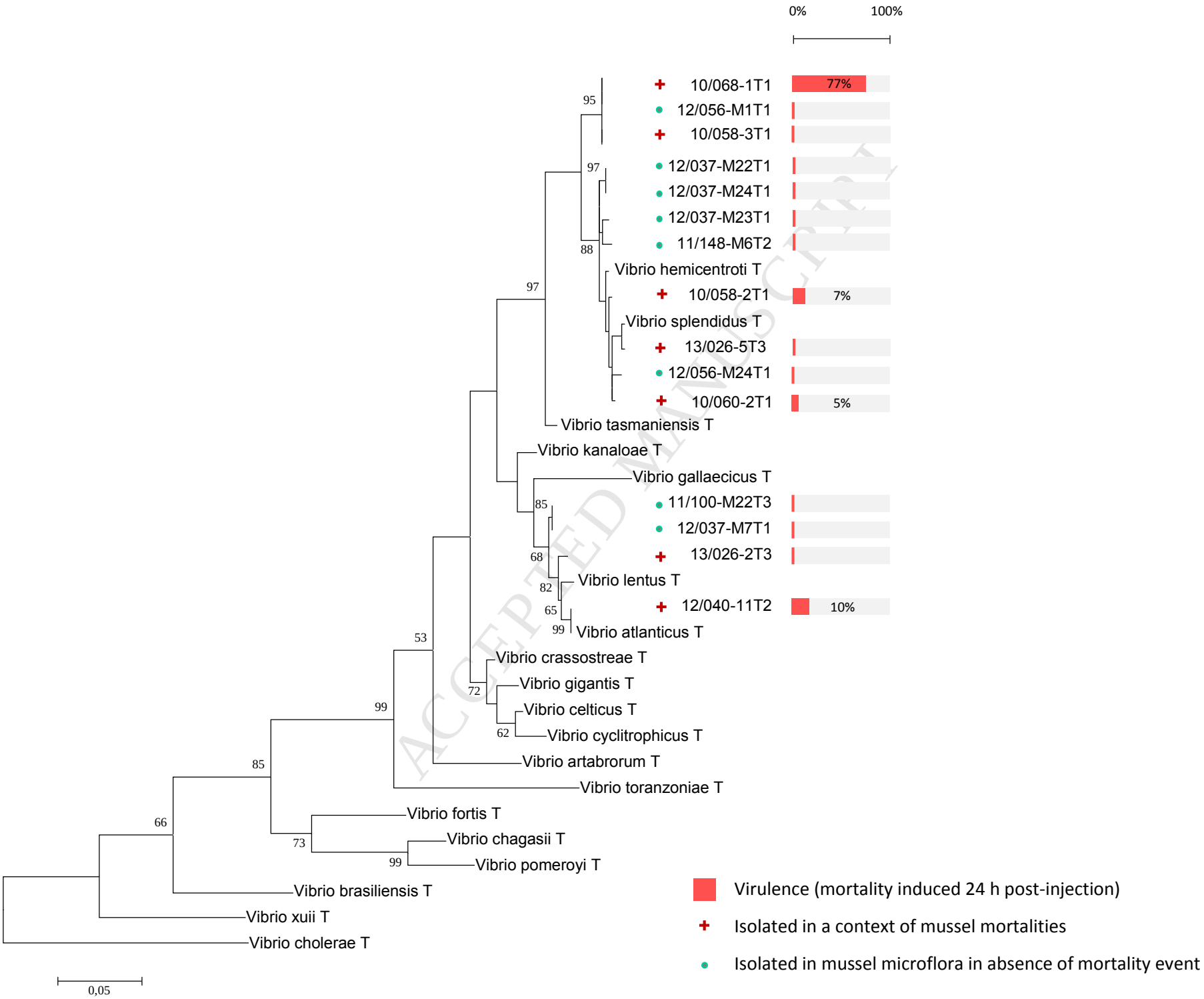
Table 1.Gyrase B gi reference

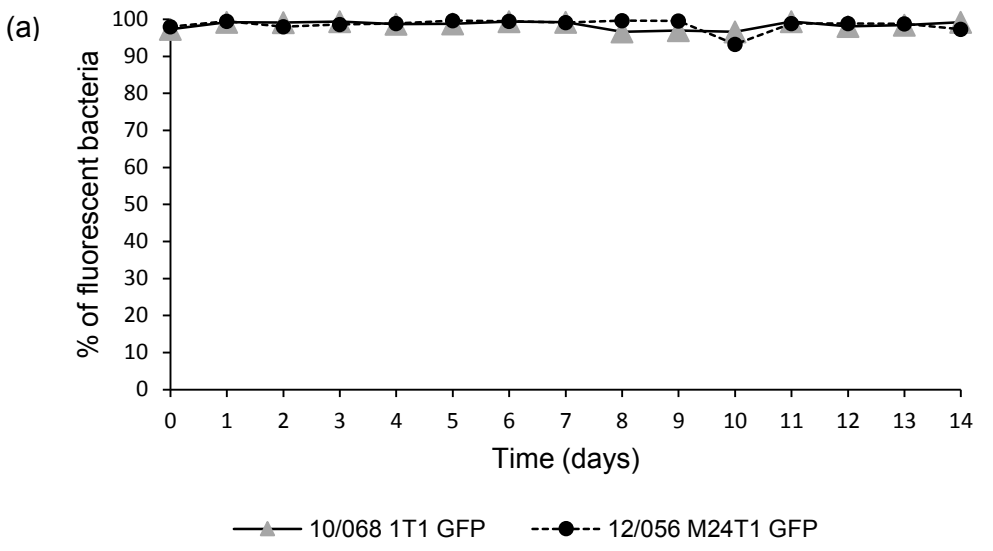
gi number	reference strains
gi|164456642	*Vibrio brasiliensis* strain LMG 20546T
gi|164456646	*Vibrio chagasii* strain LMG 21353T
gi|164456648	*Vibrio cholerae* strain IID 6019
gi|164456652	*Vibrio crassostreae* strain LMG 22240T
gi|164456656	*Vibrio cyclitrophicus* strain LMG 21359T
gi|164456666	*Vibrio fortis* strain LMG 21557T
gi|164456672	*Vibrio gigantis* strain LMG 22741T
gi|164456684	*Vibrio kanaloae* strain LMG 20539T
gi|164456686	*Vibrio lentus* strain LMG 21034T
gi|164456718	*Vibrio pomeroyi* strain LMG 20537T
gi|164456730	*Vibrio splendidus* strain LMG 19031T
gi|164456734	*Vibrio tasmaniensis* strain LMG 20012T
gi|164456742	*Vibrio xuii* strain LMG 21346T
gi|564274123	*Vibrio artabrorum* strain CAIM 1845 T
gi|564274125	*Vibrio atlanticus* strain CAIM 1847 T
gi|564274131	*Vibrio celticus* strain CAIM 1849 T
gi|754496442	*Vibrio gallaecicus* strain CECT 7244T
gi|564274143	*Vibrio toranzoniae* strain CAIM 1869 T
KU145472	10/058 2T1
KU145473	10/058 3T1
KU145474	10/060 2T1
KU145475	10/068 1T1
KU145476	11/100 M22T3
KU145477	11/148 M6T2
KU145478	12/037 M22T1
KU145479	12/037 M23T1
KU145480	12/037 M24T1

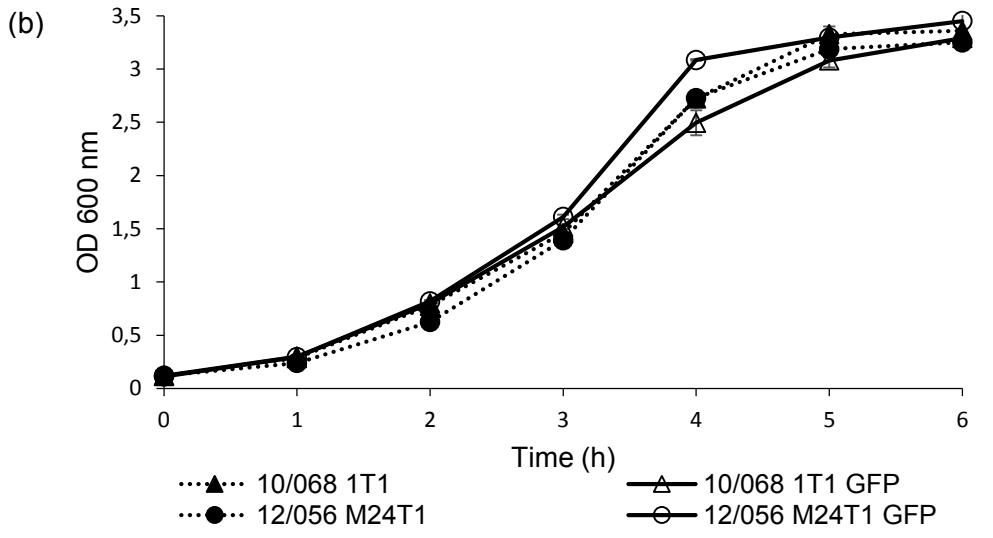
KU145481	12/037 M7T1
KU145482	12/040 11T2
KU145483	12/056 M1T1
KU145486	12/056 M24T1
KU145484	13/026 2T3
KU145485	13/026 5T3

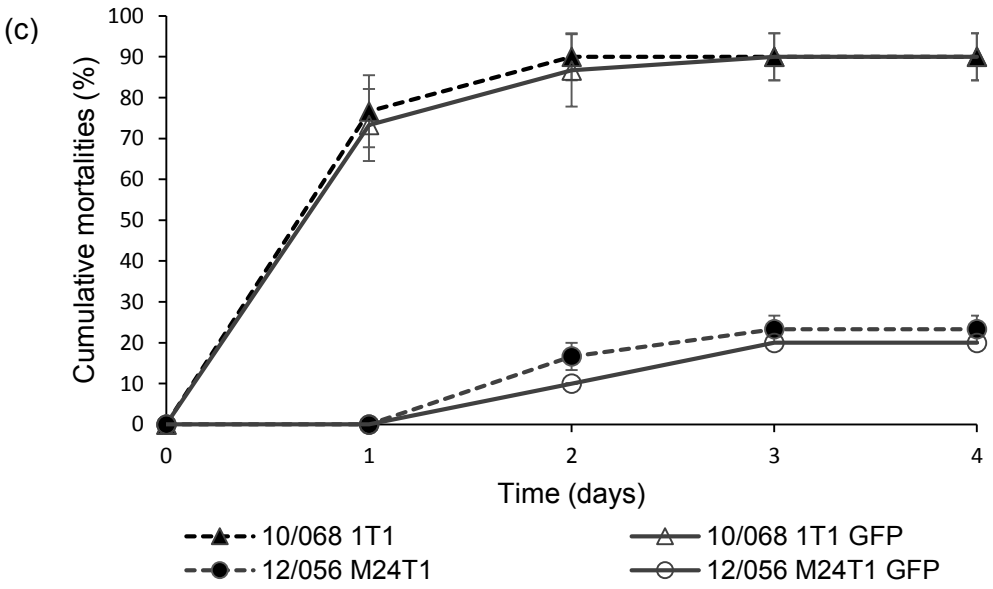




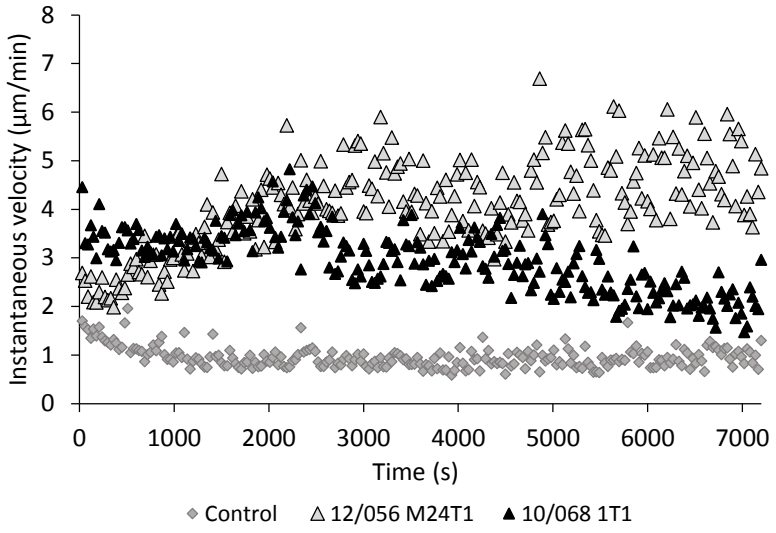



(a)
% of fluorescent bacteria
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Time (days)
10/068 1T1 GFP
12/056 M24T1 GFP

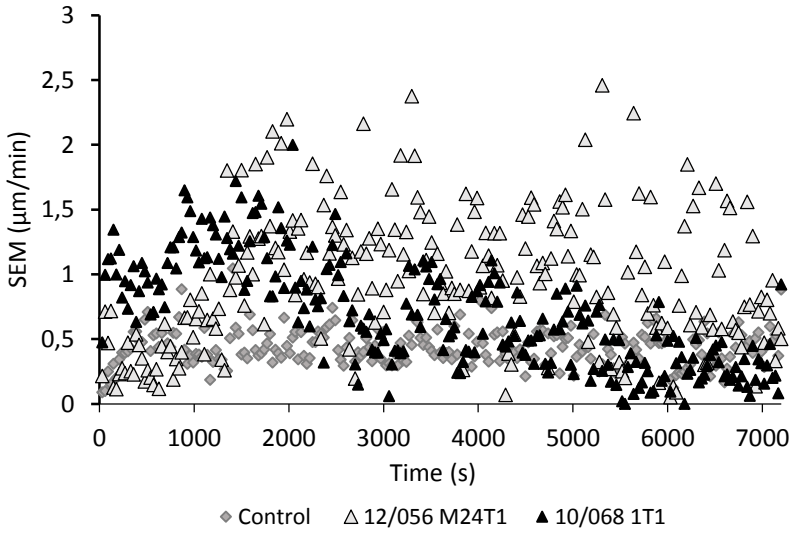

(b)
OD 600 nm
3,5
3
2,5
2
1,5
1
0,5
0
0 1 2 3 4 5 6
Time (h)
10/068 1T1
12/056 M24T1
10/068 1T1 GFP
12/056 M24T1 GFP


(c)
Cumulative mortalities (%)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4
Time (days)
10/068 1T1
12/056 M24T1
10/068 1T1 GFP
12/056 M24T1 GFP

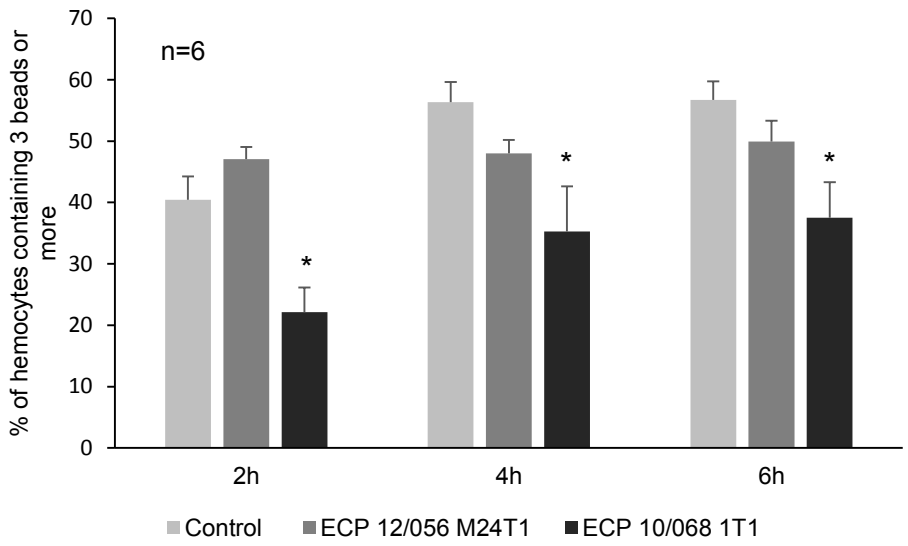
(a)


Instantaneous velocity (µm/min)
0
1
2
3
4
5
6
7
8
0
1000
2000
3000
4000
5000
6000
7000
Time (s)
Control
12/056 M24T1
10/068 1T1

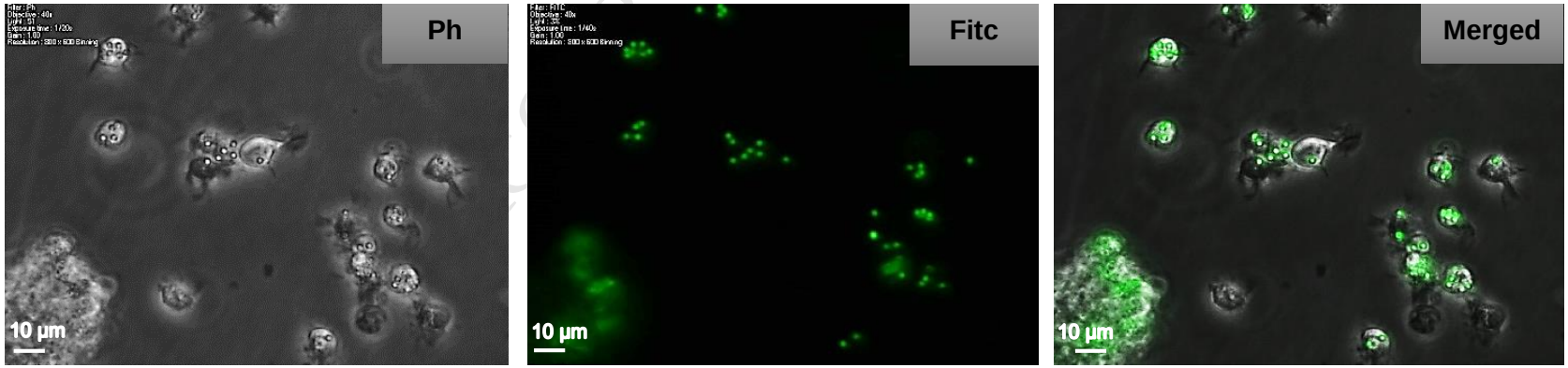
(b)


SEM (µm/min)
0
0,5
1
1,5
2
2,5
3
0
1000
2000
3000
4000
5000
6000
7000
Time (s)
Control
12/056 M24T1
10/068 1T1

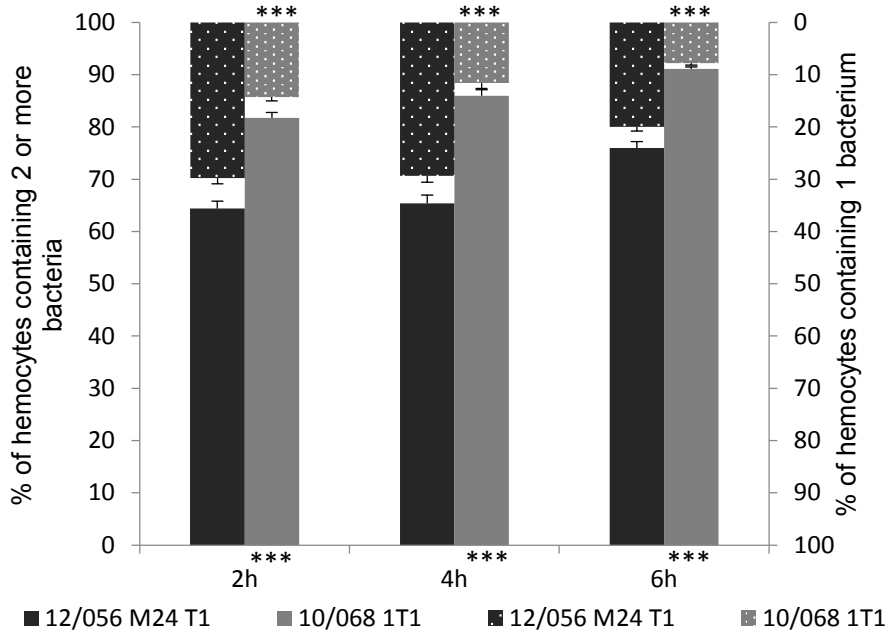
(a)


n=6
% of hemocytes containing 3 beads or more
70
60
50
40
30
20
10
0
*
*
*
2h
4h
6h
Control
ECP 12/056 M24T1
ECP 10/068 1T1

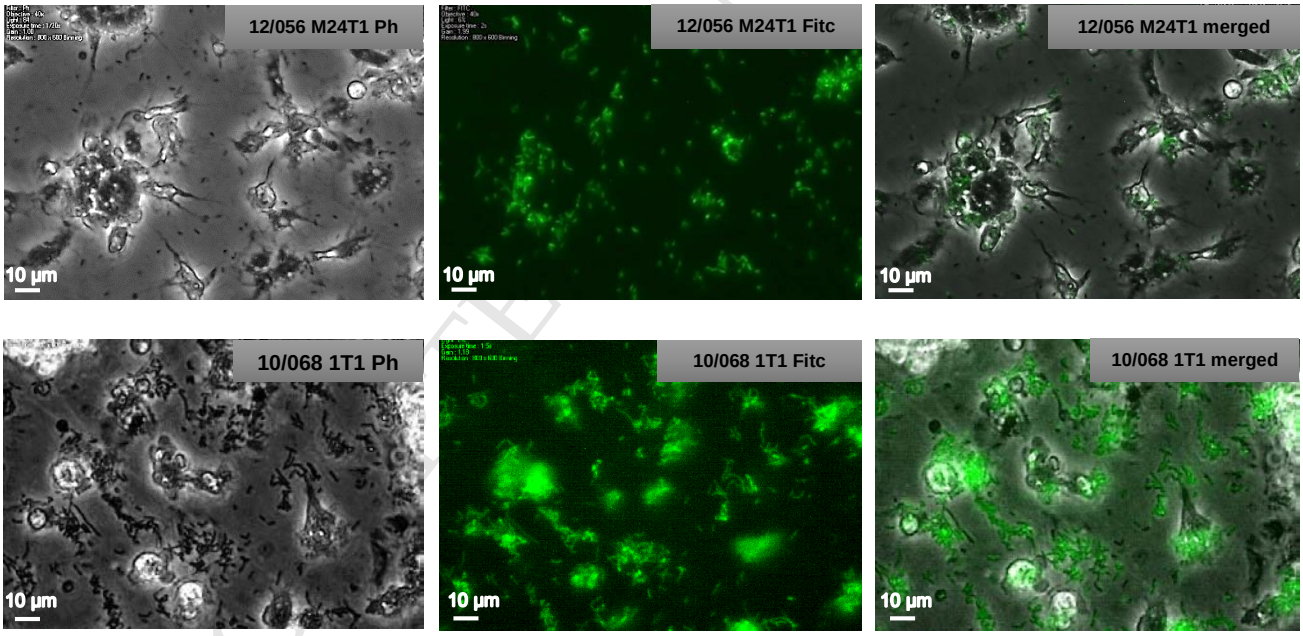
(b)


Ph
10 µm
Fitc
10 µm
Merged
10 µm

(a)


% of hemocytes containing 2 or more bacteria
% of hemocytes containing 1 bacterium
100
90
80
70
60
50
40
30
20
10
0
***
2h
4h
6h
12/056 M24 T1
10/068 1T1
12/056 M24 T1
10/068 1T1

(b)


12/056 M24T1 Ph
12/056 M24T1 Fitc
12/056 M24T1 merged
10/068 1T1 Ph
10/068 1T1 Fitc
10/068 1T1 merged
10 µm