Construction of a stable GFP-tagged *Vibrio harveyi* strain for bacterial dynamics analysis of abalone infection

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**Abstract**

*Vibrio harveyi* is a bacterial marine pathogen that can cause fatal disease in a large range of vertebrates and invertebrates, including the commercially important marine gastropod, *Haliotis tuberculata*. Since 1997, strains of this bacterium have regularly been causing high mortalities in farmed and wild abalone populations. The way in which the pathogen enters into abalone and the disease transmission mechanisms are thus far unknown. Therefore, a pathogenic strain, ORM4, was green fluorescent protein-tagged and validated both for its growth characteristics and for its virulence as a genuine model for abalone disease. The strain allows *V. harveyi* quantification by flow cytometry in seawater and in abalone haemolymph as well as the *in situ* detection of the parasite inside abalone tissues.

**Introduction**

In the last 20 years, many economically important marine organisms in Europe have been affected by an ever-growing number of pathologies. A large part of these emerging diseases that paralyse European aquaculture are caused by *Vibrio* bacteria (Paillard et al., 2004). Since 1997, one of these, *Vibrio harveyi*, has regularly been described as attacking French farms and field stocks of the European abalone, *Haliotis tuberculata*, causing up to 80% mortality within a few days (Nicolas et al., 2002). Although this marine gastropod of high commercial value is present in the eastern Atlantic from the Channel Islands in the north to the Senegal coasts in the south, the disease has so far only been found in French waters. *Vibrio harveyi* (synonym of *Vibrio carchariae*) (Gauger & Gomez-Chiarri, 2002) is a widespread marine pathogen of which several strains are already known to cause fatal disease both in fish and in crustaceans (Austin & Zhang, 2006). To avoid infesting other abalone stocks around the world, both the cellular and the molecular disease mechanisms of this particular *V. harveyi* strain, named ORM4, should be elucidated in detail.

Recently, this abalone vibriosis was shown, both in laboratory experiments and in field surveys, to be directly controlled by seawater temperature, and the absolute bacterial concentrations in the direct neighbourhood of the animals also seem to be of importance for successful infection (Travers et al., in press). This opportunistic pathogen successfully invades its host only during the abalone summer spawning period, when energy reserves are limited and immune systems are partially depressed (Travers et al., in press). However, how the pathogen enters into its host and the ways in which disease transmission occur are both still unknown.

Combining green fluorescent protein (GFP) – expression cassettes with the development of plasmids that are stably retained in *Vibrios* without any antibiotic selection (Dunn et al., 2006; Sawabe et al., 2006) has recently led to an understanding of disease dynamics in fish (O’Toole et al., 2004), of *Vibrio fischeri* symbiosis establishment in squid (Nyholm et al., 2000) and bacterial dynamics with filter-feeding oysters (Cabello et al., 2005). To begin unravelling the cellular and molecular disease mechanisms, we engineered a GFP-tagged *V. harveyi* ORM4, which can be easily...
followed by flow cytometry or epifluorescence microscopy. By comparing growth rates, plasmid retention and in vivo virulence, this GFP-tagged bacterium was found to represent a genuine model for the pathogenic parental strain. Subsequently, the fate of V. harveyi ORM4-GFP in contact with abalone and its capacity to proliferate within these animals or in the surrounding water were investigated. This allowed us to detect the pathogen in moribund abalone tissues and to monitor bacterial concentration variations in seawater and haemolymph, which led us to suggest three disease progression phases for this abalone.

Materials and methods

Bacterial strains
Escherichia coli CC118 λpir (conjugative helper strain) (Stabb & Ruby, 2002), E. coli strain DH5α carrying GFP-expression plasmid pSV102 (Dunn et al., 2006), V. harveyi strain ORM4 isolated from moribund abalone (Nicolas et al., 2002) and the GFP-expressing derivative, ORM4-GFP, were used. Bacterial concentrations were calculated by OD measurements at 490 nm according to the following formula

$$\text{CFU} = 6 \times 10^9 \times \text{OD} + 2 \times 10^8$$

This formula was determined experimentally by OD measurements and control bacterial plating and is only used between OD 0.1 and 1 to remain in the linear part of the correlation.

Abalone specimens

One hundred and fifty juveniles of H. tuberculata were transferred from the ‘France Haliotis’ hatchery (Plouguerneau, France) to the LEMAR laboratory in June 2006 (24.5 ± 1.5 mm) and 90 in January 2008 (42.5 ± 1.0 mm) and laboratory acclimated in 50-L polyethylene tanks with an open seawater circuit at 14–15 °C under continuous aeration. During the acclimation period, animals were fed ad libitum on a mixed marine algae diet of Gracilaria sp., Laminaria digitata and Palmaria palmata. Two weeks before experimentation, abalone were transferred into 5- or 30-L experimental tanks with 19 °C stagnant seawater. Seawater was aerated, totally renewed each day and its temperature was monitored continuously. This daily total water renewal is necessary for the survival of abalone in small laboratory tanks. Experiments were performed in triplicate on groups of 20 abalone.

DNA transfer and selection of GFP-tagged Vibrio

The pSV102 plasmid (Dunn et al., 2006) harbouring GFP and kanamycin-resistance expression cassettes was transferred from E. coli to V. harveyi ORM4 by triparental mating (Stabb & Ruby, 2002) using the conjugative helper strain CC118 λpir as described by Dunn et al. (2006). In short, 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 V. harveyi] with addition of 40 µg mL⁻¹ kanamycin for DH5α-pSV102. One hundred microlitres of each culture was combined in a microfuge tube, washed in LBS without antibiotics and resuspended into 10 µL of LBS. This small volume was dropped on a fresh LBS agar plate and incubated overnight at 28 °C. The next day, the bacterial spot was resuspended in 800 µL of LBS, serially diluted, plated on LBS plates containing 100 µg mL⁻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 kanamycin selection. Unfortunately, the V. harveyi strain ORM4 is not bioluminescent like many other V. harveyi strains, and thus this feature could not be used for positive selection. Therefore, green fluorescent colonies were detected under UV illumination after 2 days at 18 °C and each fluorescent colony was tested by epifluorescence microscopy to verify whether we were dealing with bona fide GFP-expressing and, most importantly, moving (flagella-baring) bacteria. Surviving E. coli donor cells were unable to swim (microscopy examination), in contrast to the Vibrionaceae.

Estimation of conservation of GFP expression

One fluorescent colony was grown overnight at 28 °C with 100 µg mL⁻¹ kanamycin to ensure 100% plasmid conservation. This stationary culture was diluted in LBS without antibiotics and grown at 28 °C overnight. Every day, a new culture was started with an aliquot of the previous day’s culture, which was plated, after dilution in filtered and sterile seawater (FSSW), on LBS agar to verify the number of fluorescent over nonfluorescent colonies. Fluorescent CFUs were counted 48 h later by optical observation under UV light. The percentage of bacteria that expressed GFP was calculated after each of the 16 passages.

Growth curves of GFP-tagged Vibrios

To verify eventual growth differences between the parental ORM4 and derivative ORM4-GFP, proliferation of both strains at 28 °C was compared by regular measurements of the culture’s OD(λ=490 nm) (Mithras LB940, Berthold Technologies) over time in three independent experiments.

Bacterial immersion and injection challenges

Vibrio harveyi ORM4 or V. harveyi ORM4-GFP bacteria, grown overnight in LBS at 28 °C, were washed twice with FSSW. For comparison of parental and GFP-expressing strains, the seawater in 5-L containers at 19 °C, containing...
Bacterial quantification in seawater and abalone fluids by flow cytometry

Bacterial standard curves (estimated by OD and checked by plating) ranging from $10^4$ to $10^7$ ORM4-GFP mL$^{-1}$ were prepared in FSSW as well as in 0.5 μm of filtered seawater in which control abalone had lived. These were analysed on a FACS calibur flow cytometer (Becton Dickinson, France) equipped with a 488-nm laser. Total fluorescent bacterial counts were assessed by counting at a low rate at least 10,000 events or accumulating events during 3 min. Fluorescent bacteria were identified by their fluorescent peak (FL-1) and their relative size [forward scatter (FSC)] and complexity [side scatter (SSC)]. No differences could be detected between the FSSW and the ‘natural seawater’ standard curves. All samples were filtered through an 80-μm nylon mesh to remove debris and aggregates before flow cytometry analysis.

With the settings determined on these standard curves, three independent samples of seawater surrounding the abalone (duplicate tanks) during the bacterial challenges were analysed after 1, 6, 12 and 24 h and, subsequently, twice a day during the experiment.

Fluorescent ORM4-GFP present in the haemolymph of moribund abalone was quantified both by flow cytometry and by bacterial plating.

Microscopic observation of bacteria in tissues and fluids of moribund abalone

Haemolymph was withdrawn from the cephalic sinus of moribund abalone (fallen animals that retain muscular reactivity) and different organs were dissected ‘aseptically’: gills, gonads, digestive gland and muscle.

To verify the presence of ORM4-GFP in the tissues of moribund animals, small pieces of the organs were also directly crushed between two microscopy slides and examined with an inverted fluorescent Leica DM-IRB microscope equipped with a Retiga 2000R Fast 1394 CC camera (QImaging) and IMAGE-PRO PLUS 6.2 software (Media Cybernetics). Controls on tissue squashes of abalone without bacteria or abalone infected with non-GFP ORM4 were also performed and were found to be negative.

Results

Validation of GFP-tagged V. harveyi ORM4 as equivalent to wild-type ORM4

To facilitate the use of the GFP-transformed ORM4 as a genuine model for wild-type ORM4, the growth characteristics of the two strains were compared. As can be seen in Fig. 1a, no obvious differences from the wild-type strain were observed in vitro when grown in liquid LBS medium. Also, no noticeable differences could be observed in colony size or colony aspect when plated on LBS or thiosulfate–citrate–bile salts–sucrose agar plates (not shown). To ensure that the GFP-encoding plasmid was stably retained under nonselective growth, ORM4-GFP was cultured for 16 days, with daily reinoculations of fresh medium. This feature was important to ensure that most of the bacteria retained fluorescence during infection experiments (5–7 days). After 7 days of nonselective culture, around 80% of bacteria had retained their high fluorescence, and 12 days of continuous culture was needed before counting < 20% GFP-expressing bacteria (Fig. 1b). To compare the virulence status of GFP-tagged V. harveyi with that of the wild-type strain, in vivo bacterial immersion challenges were performed. No differences could be found in the mortality rate after inoculation of 5-L tanks with either of the V. harveyi strains. Both strains lead to 85% abalone mortality in 5 days (Fig. 1c). Similar results were also obtained by direct intramuscular injections, which led to 100% death within 4 days with as little as $10^5$ cells of either strain per animal (not shown).

Quantification of GFP-tagged V. harveyi by flow cytometry

GFP-tagged V. harveyi were identified by flow cytometry through their intrinsic green fluorescence emission (FL1). A density plot of GFP green fluorescence against side scatter light (SSC representing complexity) was used to set the gate through which particles were identified as being V. harveyi on the FSC (representing size)–SSC plot (Fig. 2a). The linear correlation between calculated ORM4 and the concentration standard of ORM4-GFP in 0.5 μm filtered seawater in which abalone had lived, determined by flow cytometry, is similar to that in FSSW and represented by the equation ($y = 0.96x$, $R^2 = 0.997$). This bacterial concentration standard allowed us to determine the sensitivity of the flow cytometer and to ensure linearity. The sensitivity threshold was set to $10^3$ bacteria mL$^{-1}$ (Fig. 2b), and thus counts corresponding to $< 10^3$ bacteria mL$^{-1}$ were not considered further. All the dilutions were verified by bacterial plating and a complete concordance between the numbers obtained by OD, flow cytometry and the
plating method was found down till the $10^5$ flow-cytometry limit. Below this level, the fluorescence-assisted cell sorting values overestimated numbers due to nonbacterial particles in the abalone seawater. Note that in FSSW, the sensitivity declined to 100 bacteria mL$^{-1}$.

Bacterial dynamics during abalone challenge

To start determining the *V. harveyi*-GFP infection cycle in its host *H. tuberculata*, abalone and bacteria were placed together in a 30-L closed seawater system for 24 h. During this time, a 68% reduction was observed in the bacterial concentration both by plating and flow cytometry (from $1.6 \times 10^5 \pm 0.1 \times 10^6$ bacteria mL$^{-1}$ to $5.1 \times 10^4 \pm 0.2 \times 10^4$ bacteria mL$^{-1}$). As seawater was changed on a daily basis, bacterial concentrations ‘fell down’ after the first 24 h of immersion to reach concentrations below the flow cytometer-detection threshold (Fig. 3a).

Concentration peaks in seawater started to be observed on day 4 ($1.1 \times 10^5 \pm 0.8 \times 10^5$ bacteria mL$^{-1}$), day 5 ($4.2 \times 10^4 \pm 2.5 \times 10^4$ bacteria mL$^{-1}$), day 7 ($1.6 \times 10^4 \pm 1.4 \times 10^4$ bacteria mL$^{-1}$) and day 8 ($4.0 \times 10^3 \pm 1.1 \times 10^3$ bacteria mL$^{-1}$), although the daily seawater renewal eliminated the free-swimming *V. harveyi*-GFP each time (Fig. 3a). In parallel to the *V. harveyi*-GFP concentration in the surrounding water, abalone mortalities were recorded. The bacterial challenge led to 44% mortality in 8 days, with the first mortalities appearing on day 4 when bacterial concentration peaks became noticeable in seawater (Fig. 3b). Please note that on day 6, no bacterial concentration peak or mortalities were noticed (see Discussion).

In the haemolymph of moribund abalone, *V. harveyi*-GFP concentration reached $5 \times 10^6$ bacteria mL$^{-1}$ ($\pm 0.6 \times 10^6$, minimum $2.9 \times 10^5$, maximum $1.4 \times 10^6$). Haemolymph was also plated to ensure that most bacteria were fluorescent and that the fluorescence events counted by flow cytometry corresponded to the total plating counts. Huge concentrations of free-swimming fluorescent bacteria were observed by inverted microscopy in the haemolymph of moribund abalone (Fig. 4c). In the gills of moribund abalone, *V. harveyi*-GFP appeared fixed onto the cilia of the epithelial cells (Fig. 4a) and the bacteria followed their movement synchronously. In muscle, bacterial clusters were detected that may correspond to haemolymphatic sinuses (Fig. 4b). A few bacteria, apparently not associated with a particular region, were also observed in the digestive gland.

Discussion

In this study, we reported the construction and validation of a stable GFP-tagged *V. harveyi* strain as well as its detection by flow cytometry. By combining a GFP-tagged *V. harveyi* and flow cytometry during abalone infection, we obtained the first evidence to propose different disease progression phases for the *V. harveyi* strain ORM4.

Fluorescent molecules combined with flow cytometry are useful tools to explore with single-cell resolution the complex interactions between bacteria and their environment. They can be used to monitor or localize microorganisms in complex systems, such as host–pathogen interactions (Valdivia & Falkow, 1998). Moreover, unlike other fluorescent detection
methods, neither staining nor fluorescent probe addition is required, and samples are therefore analysed directly without additional manipulation.

GFP is one of the most widely exploited proteins in biochemistry, cellular biology and microbiology. The originally 238-amino acid protein isolated from the jellyfish *Aequoria victoria* emits green light when excited by deep blue light, without requiring either substrates or cofactors. GFP is often regarded as a ‘neutral’ protein that does not influence the processes in the cell. It is used in a plethora of articles without doubting this ‘dogma’ and often without verifying whether it possibly changes the cell’s or the tissue’s metabolism. In the case of some *E. coli* and *Pseudomonas putida* strains, it was shown that GFP expression confers to the bacteria an increased susceptibility to certain antibiotics (Allison & Sattenstall, 2007), and, in the case of *Salmonella enterica*, Knodler et al. (2005) showed that the production of the fluorescent GFP could affect the ability of the pathogenic bacteria to establish an intracellular niche in epithelial cells and macrophages.

Therefore, to avoid adverse effects by GFP expression as much as possible, we carefully compared our GFP-tagged *V. harveyi* with the nontagged strain. The results obtained (similar doubling time, no phenotypic changes, good conservation of the plasmid in nonselective media and similar toxicity to abalone) led us to suppose that production of the GFP does not have any detectable impact on the physiology of our GFP-tagged strain. Experiments with strains with the pVSV104 plasmid but mutated for GFP expression could help to completely rule out even the smallest effects of GFP or the vector backbone on the phenotype.

Infection in abalone with the ORM4 strain probably occurs in three phases: (1) a phase of adhesion and/or penetration, corresponding to the decrease in the free-living bacteria we observed in the first 24 h; (2) an incubation phase (days 2–3), where bacteria multiply (probably) inside the host without causing any visible effects; and, finally, (3) a
phase (days 4–8) of acute mortalities, where abalone death is accompanied by the appearance of huge peaks of free-living bacteria. Note that these bacteria have to originate from moribund or dead abalone as the ORM4 strain is unable to proliferate in seawater alone (data not shown). The absence of both moribund abalone and bacterial concentration peaks underlined the cause–effect relationship between dying abalone and the presence of *V. harveyi* in surrounding seawater.

During the infection process, different organs are colonized. In moribund abalone, high concentrations of bacteria are detected in the haemolymph (up to 10⁹ bacteria mL⁻¹) and the haemolymphatic sinuses in muscle in moribund abalone. Gill epithelial cells show clear accumulations of OMR4-GFP that seem to be adhered onto their cilia. Cabello *et al.* (2005) showed, with a GFP-tagged *Vibrio parahaemolyticus* that free bacteria in the seawater were actively filtered and retained by the oyster *Tiostrea chilensis*, leading to a decrease of about 85% of free-living bacteria in 24 h in the tanks containing living oysters. Therefore, gills are an ideal compartment for adhesion and entrance, as they are in direct contact with the seawater. Moreover, gills are covered with mucus, which could either chemotactically attract or serve as a nutrient for the *Vibrios* (Rosenberg & Falkovitz, 2004; Sharon & Rosenberg, 2008).

The haemolymph compartment is frequently targeted by *Vibrios* (Pruzzo *et al.*, 2005) and, for instance, in the salmon and shrimp diseases caused by *V. harveyi* (Austin & Zhang, 2006), the infection always ends in a fatal septicaemia. Also, in our moribund abalone, such a terminal septicaemia was observed and the haemolymph contained extremely high concentrations of *V. harveyi*: between 5 × 10⁸ bacteria mL⁻¹ (± 0.6 × 10⁸) and 1.4 × 10⁹ bacteria mL⁻¹ as determined both by flow cytometry and plating.

Our new tool allowed a clear detection of some target organs in moribund abalone (gills and haemolymph), but our preliminary data do not allow us to arrive at firm conclusions about the minor localizations, the entrance routes and bacterial tissue progression.

In conclusion, we constructed and validated a GFP-tagged *V. harveyi* strain as genuine model. This bacterium allowed us to quantify by flow cytometry, fluorescent *V. harveyi* concentrations in the surrounding seawater, in abalone haemolymph, and to visualize it in the tissue during a terminal infection. This GFP-tagged bacterium represents an ideal tool for an in-depth study with precise time-course experiments and a follow-up of the infection by histology and flow cytometry on tissue homogenates to determine the infiltration routes that pathogenic *V. harveyi* take to kill its host.

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**Authors’ contribution**

C.P. and M.K. should be considered as last coauthors.
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