Isolation of Vibrionaceae from wild blue mussel (*Mytilus edulis*) adults and their impact on blue mussel larviculture

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One sentence summary: Vibrionaceae were isolated from mass mortality events in wild-caught blue mussels, and were (highly) pathogenic in a newly developed, highly controlled immersion challenge test with blue mussel D-larvae.

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ABSTRACT

The blue mussel (*Mytilus edulis*) is known as a robust bivalve species, although its larviculture appears to be highly susceptible to diseases. In this study, we isolated 17 strains from induced mortality events in healthy wild-caught blue mussel adults and demonstrated that they caused between 17% and 98% mortality in blue mussel larvae in a newly developed, highly controlled immersion challenge test model. Eight of the isolates belong to the *Splendidus* clade of vibrios, while the other isolates belong to the genus *Photobacterium*. The genomes of the most virulent *Vibrio* isolate and the most virulent *Photobacterium* isolate were sequenced and contained several genes encoding factors that have previously been linked to virulence towards bivalves. In vitro tests confirmed that all 17 isolates were positive for these virulence factors. The sequenced genomes also contained a remarkably high number of multidrug resistance genes. We therefore assessed the sensitivity of all isolates to a broad range of antibiotics and found that there were indeed many strong positive correlations between the sensitivities of the isolates to different antibiotics. Our data provide an ecological insight into mass mortality in blue mussels as they indicate that wild mussels contain a reservoir of pathogenic bacteria.

Keywords: bivalve; vibriosis; *Photobacterium*; *Splendidus*; aquaculture; immersion challenge

INTRODUCTION

Mussels are the most important aquaculture species in Europe (based on live weight). The two main cultivated species are the blue mussel (*Mytilus edulis*) and the Mediterranean mussel (*Mytilus galloprovincialis*) (EC 2015). Today, blue mussel culture is 100% based on the capture of larvae or juveniles. Mussel spat is collected from the wild and transferred for grow-out to
different coastal rearing systems until they reach commercial size, after about 1.5–2 years of culture (Helm, Bourne and Lovatelli 2004). Irregular spat settlement has affected natural blue mussel populations in many European waters (FAO 2004). Fluctuations of natural conditions, predominantly temperature, can lead directly (impact on survival) and/or indirectly (impact on predator/pathogen populations) to fluctuations in natural spat availability, which is translated in unpredictable production levels. Furthermore, overexploitation and harmful spot collection methods have a detrimental impact on natural ecosystems, and in order to avoid this, more sustainable production methods are needed. In The Netherlands, a gradual change from bottom spot collection (28% reduction in 2014) towards suspended mussel spat collectors is being implemented, and alternative spot production methods are encouraged, driven by both ecological and economical incentives (Ministry of Economic Affairs 2015).

Hatchery production of seed provides a good alternative to spat collection from nature. The technology to produce blue mussel spat in hatcheries is available (FAO 2004) and has several advantages over the collection of natural spat (e.g. the possibility to establish breeding programs). However, commercial hatchery production is not yet widely applied due to the high costs associated with the production of microalgae as feed (Carrasco et al. 2014), the low market value of mussels in contrast to some other bivalves like oysters (FAO 2004) and because of mass mortalities that occur in dense larval cultures (Sainz et al. 2012). Mytilus edulis mussel larvae.

The cultures were stored at –80°C under constant agitation (100 min⁻¹). The growth of mussel-associated bacteria was stimulated by a unique addition of 100 mg/l tryptone (Biokar diagnostics, Pantin, France) and 50 mg/l yeast extract (Fisher Scientific, Waltham, USA) to the mussel rearing water at the start of the experiment. In a control treatment also receiving tryptone and yeast extract, 20 mg/l rifampicin (Sigma-Aldrich, Saint Louis, USA) was added to the rearing water every 2 days. Two additional controls consisted of a blank and addition of rifampicin only, respectively. During the experiment, the mussels were not fed, mortality was scored daily and dead animals were removed. Bacteria were isolated from tanks when mortality was occurring, more specifically from homogenised mussel tissue and from mussel haemolymph. For mussel tissue isolates, mussels were aseptically removed from their shell, rinsed with sterile seawater and subsequently homogenised. Sterile sea water was added to allow efficient homogenisation of the tissues. The recipient was consequently centrifuged and the supernatant was retained. For haemolymph samples, 500 μl aliquots were taken from the adductor muscle of live mussels after sedation with MgCl₂ (28 g l⁻¹, maintaining a salinity of 35 g l⁻¹) until valves did not close upon contact. Dilution series of all samples were prepared, and 50 μl aliquots were plated on Thiosulfate-Citrate-Bile salts-sucrose (TCBS) agar and incubated at 18°C for 24 h. Individual colonies with different morphology/size were picked and inoculated (1% v/v) into fresh LB broth containing 50 μg/ml rifampicin (Sigma-Aldrich, Saint Louis, USA) to the mussel rearing water at the start of the experiment. Debris and dead animals were removed and the cultures were thoroughly rinsed with filtered (0.2 μm) sea water. Aliquots (50 μl) were transferred to new TCBS plates, and this procedure was repeated twice. Duplicates as manifested by ERIC-PCR fingerprinting (Ruwandeepika et al. 2011) were excluded. The isolates that were retained for further testing are listed in Table 1.

Selection of natural rifampicin-resistant mutants of the isolates

Natural rifampicin-resistant mutants of the isolates were selected as described before (Pande et al. 2013). Briefly, the isolates were grown overnight in Luria-Bertani broth containing 35 g l⁻¹ NaCl (LB₅₅), whereupon 10% (v/v) was transferred to fresh LB₅₅ broth containing 50 mg ml⁻¹ rifampicin. The suspensions were incubated for 5 days at 18°C under constant agitation (100 min⁻¹). Grown cultures were inoculated (1% v/v) into fresh LB₅₅ broth with 50 mg ml⁻¹ rifampicin and incubated overnight at 18°C. The cultures were stored at ~80°C in 40% glycerol until further use. The rifampicin-resistant mutants were used in all further experiments.

Bacterial growth conditions

Isolates were inoculated on TCBS agar supplemented with rifampicin (50 mg l⁻¹) and grown overnight at 18°C. A single colony was picked and inoculated into 5 ml of fresh LB₅₅ medium and grown overnight at 18°C with constant agitation. Cell density

MATERIALS AND METHODS

Isolation of Vibrionaceae from induced mass mortality events in Mytilus edulis

Mass mortality was induced in blue mussel (Mytilus edulis) adults as described previously (Eggermont et al. 2014). Briefly, blue mussel adults harvested from the Oosterschelde river (The Netherlands) were transported in thermostable containers to the laboratory. Debris and dead animals were removed and the mussels were thoroughly rinsed with filtered (0.2 μm) sea water before being acclimatised for 48 h. Groups of 20 mussels were randomly stocked in duplicate in covered aerated rectangular tanks filled with 4l of filtered (0.2 μm) natural sea water. The growth of mussel-associated bacteria was stimulated by a unique addition of 100 mg/l tryptone (Biokar diagnostics, Pantin, France) and 50 mg/l yeast extract (Fisher Scientific, Waltham, USA) to the mussel rearing water at the start of the experiment. In a control treatment also receiving tryptone and yeast extract, 20 mg/l rifampicin (Sigma-Aldrich, Saint Louis, USA) was added to the rearing water every 2 days. Two additional controls consisted of a blank and addition of rifampicin only, respectively. During the experiment, the mussels were not fed, mortality was scored daily and dead animals were removed. Bacteria were isolated from tanks when mortality was occurring, more specifically from homogenised mussel tissue and from mussel haemolymph. For mussel tissue isolates, mussels were aseptically removed from their shell, rinsed with sterile seawater and subsequently homogenised. Sterile sea water was added to allow efficient homogenisation of the tissues. The recipient was consequently centrifuged and the supernatant was retained. For haemolymph samples, 500 μl aliquots were taken from the adductor muscle of live mussels after sedation with MgCl₂ (28 g l⁻¹, maintaining a salinity of 35 g l⁻¹) until valves did not close upon contact. Dilution series of all samples were prepared, and 50 μl aliquots were plated on Thiosulfate-Citrate-Bile salts-sucrose (TCBS) agar and incubated at 18°C for 24 h. Individual colonies with different morphology/size were picked and suspended in 500 μl sterile sea water. Aliquots (50 μl) were transferred to new TCBS plates, and this procedure was repeated twice. Duplicates as manifested by ERIC-PCR fingerprinting (Ruwandeepika et al. 2011) were excluded. The isolates that were retained for further testing are listed in Table 1.
Table 1. Selected bacterial strains isolated from diseased blue mussel (M. edulis) adults.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Origin</th>
<th>Identification</th>
<th>Most closely related strain based on 16S rDNA Amplicon</th>
<th>GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME2</td>
<td>Homogenised tissue</td>
<td>Photorbacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.9 1167 KT792703</td>
</tr>
<tr>
<td>ME3A</td>
<td>Haemolymph</td>
<td>Vibrio sp.</td>
<td>Vibrio hemicentroti</td>
<td>GQ386822 96.6 1107 KT792704</td>
</tr>
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<td>ME3B</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 96.8 1107 KT792705</td>
</tr>
<tr>
<td>ME4A</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 96.9 1000 KT792706</td>
</tr>
<tr>
<td>ME4B</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1010 KT792707</td>
</tr>
<tr>
<td>ME5</td>
<td>Haemolymph</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
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</tr>
<tr>
<td>ME6</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.2 985 KT792709</td>
</tr>
<tr>
<td>ME7</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.1 933 KT792710</td>
</tr>
<tr>
<td>ME8</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1031 KT792711</td>
</tr>
<tr>
<td>ME9</td>
<td>Haemolymph</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1085 KT792712</td>
</tr>
<tr>
<td>ME10</td>
<td>Haemolymph</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1089 KT792713</td>
</tr>
<tr>
<td>ME13A</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1130 KT792714</td>
</tr>
<tr>
<td>ME13B</td>
<td>Homogenised tissue</td>
<td>Photobacterium sp.</td>
<td>Photobacterium sanguinicancri</td>
<td>GQ386822 97.0 1135 KT792715</td>
</tr>
</tbody>
</table>

was determined at 600 nm using a spectrophotometer (Thermo electronic genesis 20, Thermo Fisher Scientific, Belgium).

Identification of the isolates

Identification of bacterial isolates was done as described earlier (Gomez-Gil et al. 2012). Briefly, DNA of the strains was extracted with a commercial kit (Wizard Genomic DNA purification kit; Promega Benelux, The Netherlands) according to the manufacturer’s instructions. The DNA concentration was adjusted to 50 ng μl⁻¹. The 16S rRNA gene was amplified with universal primers (27F, AGAGTTTGATCMTTGCTCAG; 1491R, TACGGYTACCTTGTATTGAATT). The amplification program was one cycle at 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min; and one final extension cycle at 72 °C for 5 min. Sanger sequencing was done by Macrogen (Korea). 16S rRNA sequences were cleaned and aligned with Geneious ver. 7.1.9 (BioMatters Ltd., New Zealand) and phylogeny reconstruction was done with Mega ver. 6.0 (Tamura et al. 2013). Identification was done following Kim et al. (2012) using the EzTaxon server (http://www.ezbiocloud.net/eztaxon). GenBank Accession numbers are presented in Table 1.

Genome sequencing of isolates ME9 and ME15

The whole genome of the isolates ME9 and ME15 was sequenced with an Ion Torrent platform as described earlier (González-Castillo et al. 2015). Briefly, the library was prepared with an Ion Plus Fragment Library Kit (Applied Biosystems, USA) and fragmented with a BioRuptor Sonication System following the manufacturers’ instructions. The libraries were barcoded (NEXTflex DNA Barcodes, Bioo Scientific, USA) and quantification was calculated with an Ion Library Quantization kit using TaqMan on a CFX96 Real-Time PCR System (Bio-Rad, USA). Emulsion PCR and enrichment steps were performed using an Ion OneTouch2 200 Template Kit v2, and sequencing was carried out in a 318 chip. Reads were processed with the Torrent Suite 1.5, cleaned with proprietary scripts and assembled de novo into contigs with Newbler (RunAssembly ver. 2.3). Genes were annotated with the RAST server (http://rast.nmpdr.org/) (Aziz et al. 2008). The genome sequences have been submitted to GenBank under the Accession numbers MK1W00000000.1 and NZ_LNGM0000000.1 for ME9 and ME15, respectively.

Rearing of blue mussel D-larvae

Wild-caught mature blue mussels were stimulated to spawn by thermal shocking in sterile sea water for 20–30 min at 5 °C and 20 °C, respectively, until gametes were released. Spawning males and females were transferred to sterile plastic cups containing 50 ml sterile sea water and allowed to spawn for 15 min. Sperm and eggs were collected and gently mixed at a 10:1 ratio in a beaker containing 1 l of sterile sea water. After the appearance of polar bodies, the eggs were gently rinsed with sterile sea water using a sterile 30 μm sieve to remove excess sperm. Fertilised eggs were incubated in 2 l of sterile sea water (max 100 eggs ml⁻¹) containing chloramphenicol, nitrofurazone and enrofloxacin (each at 10 mg l⁻¹). After 2 days of incubation, D-larvae were harvested on a sterile 60 μm sieve. The larvae were washed gently with sterile sea water to remove the antibiotics. Rinsed D-larvae were transferred to a beaker containing 1 l of sterile sea water and distributed uniformly using a plunger. Subsamples were taken to calculate the larval density, and the density was corrected in order to obtain a final concentration of...
250 larvae ml⁻¹. All manipulations were performed under a laminar flow hood.

To evaluate the microbial load of the larvae, a subsample of ~2000 larvae was homogenised in 1 ml sterile sea water and plated on MA at the start of the challenge test (after washing) and during the challenge test. Aliquots (100 μl) of the culture water of the different control treatments were plated using a spiral plater (L.E.D. TechnoAB, Belgium) on MA and TCBS. Plates were incubated at 18 °C for 48 h.

**Challenge tests**

One millilitre of aliquots of the larval suspension were transferred to 24-well plates. Rifampicin was added at 10 mg l⁻¹ to avoid contamination. Tryptone (Biokar diagnostics, Belgium) and yeast extract (Thermo Fisher Scientific, Belgium) were added at 10 and 5 mg l⁻¹, respectively. The final larval density was 200 larvae ml⁻¹. Natural rifampicin-resistant mutants of the isolates were inoculated into the rearing water at 10⁵ cells ml⁻¹. Larvae to which no bacteria were added and that were otherwise treated in the same way as the challenged larvae were used as controls. Each treatment was performed in 24 replicates. The plates were incubated at 18 °C. Each day, four replicates per treatment were stained with lugol (5% (v/v)), and stained larvae were counted under a binocular microscope (Nikon Eclipse E 200, Nikon Instruments Europe). Larvae were considered alive when stained black by lugol, death if only parts of the larvae were stained or if shells were empty. The challenge test was validated using a known bivalve pathogen, *Vibrio tasmaniensis* LGP32 (Le Roux et al. 2009) and *Vibrio anguillarum* NB10 (Norqvist, Norman and Wolf-Watz 1990).

**Growth rates of the isolates**

Growth rates of the isolates were calculated as described by Hall et al. (2013). Freshly grown cultures (OD₆⁰₀ = 1) were diluted in fresh LB₅₅ broth. Aliquots (200 μl) were loaded into a 96-well plate in triplicate. Absorbance was measured every hour till stationary phase with infinite M200 reader (TECAN) at 18 °C. Data were analysed with the Growth Rates software, as described previously (Hall et al. 2013).

**Virulence factor assays**

Caseinase, gelatinase and haemolytic activities were determined according to Natrah et al. (2011). For each assay, overnight grown cultures were diluted to an OD₆₅₀ of 0.5, and 5 μl of the diluted cultures were inoculated in the centre of the test plates. All assays were done at least in triplicate. Caseinase activity was assessed on skim milk agar plates. Colony and clearing zone diameters were measured after 24 h incubation at 18 °C.

Gelatinase assay plates were prepared by mixing 0.5% gelatin (Sigma–Aldrich) into the agar. After incubation for 7 days, saturated ammonium sulphate (80% solution in distilled water) was poured over the plates and after 2 min, the diameters of the clearing zones around the colonies were measured.

Haemolytic assay plates were prepared by supplementing Marine Agar with 5% defibrinated sheep blood (Thermo Fisher Scientific), and clearing zones were measured after 2 days of incubation at 18°C.

Swimming motility was assessed as described previously by Yang and Defoirdt (2015). Briefly, 5 μl aliquots of the isolates (OD₆₅₀ = 1) were inoculated in the centre of LB₅₅ soft agar (0.2% agar) plates in triplicate. Plates were incubated upright at 18 °C, and motility halos were measured after 24 h.

**Testing of antibiotic susceptibility**

Susceptibility to a set of 12 antibiotics was screened by a disc diffusion assay. Fifty microlitres of overnight grown cultures of the isolates were spread over LB₅₅ agar plates, after which antibiotic discs (Oxoid) were placed on top of the agar. The discs contained 30 mg of antibiotic, except for enrofloxacin (5), ampicillin (10), streptomycin and trimetoprim/sulphamethoxazole (25) and nitrofurantoin (50). The plates were incubated for 2 days at 18 °C, after which the diameters of the growth inhibition zones were measured. Each isolate was assessed in triplicate.

**Statistics**

All statistical analyses (Spearman’s rank correlation coefficient, independent samples t-tests) were performed using the SPSS software, version 24. A significance level of 1% was used in all analyses, except for the virulence of the isolates towards blue mussel larvae where both 1% and 0.1% significance level was calculated.

**RESULTS**

**Isolation of *Vibionaceae* from mass mortality events in wild-caught blue mussel adults**

The growth of heterotrophic bacteria associated with wild-caught blue mussel adults was stimulated by adding easily degradable dissolvable organic matter to the rearing water to simulate high organic load of densely stocked rearing systems. Complete mortality was observed within 1 week, and this could be prevented by the addition of rifampicin, resulting in at least 75% survival after one week. Bacteria were isolated from mussel rearing water, mussel haemolymph and mussel tissue at the onset of mass mortality. After purification and excluding duplicate strains by ERIC-PCR fingerprinting, 17 isolates were retained for further testing (Table 1).

**Virulence of the isolates towards blue mussel larvae**

In a further experiment, we aimed at investigating the virulence of the selected isolates towards blue mussel larvae in an immersion challenge test using a highly controlled experimental setup. Due to the mussel egg’s fragile physiology (no egg shell), it was not possible to completely disinfect the eggs with strong disinfectants. Therefore, in order to reduce the microbial load of the larvae as much as possible, the spawning was performed in filtered autoclaved seawater, and a mixture of antibiotics was administered during the development to D-larvae. Moreover, rifampicin was administered to the rearing water throughout the challenge and natural rifampicin-resistant mutants of the different isolates were used. The challenge test protocol was validated with two strains: *Vibrio tasmaniensis* LGP32 (a known bivalve pathogen) and *Vibrio anguillarum* NB10 (Norqvist, Norman and Wolf-Watz 1990).
challenged larvae ranging from 2% to 83% after 5 days. Eight strains (i.e. ME3A, ME4A, ME5, ME6, ME7, ME9, ME14A and ME15) caused more than 50% mortality after 5 days of challenge (Fig. 2). Isolate ME9 was the most virulent one, causing 69% mortality within 2 days and more than 98% mortality after 5 days. Almost no mortality (<1%) was observed in the control treatment, indicating good larval quality and absence of pathogens associated with the larvae.

In order to obtain an indication of the bacterial load of the larvae, at the onset of the challenge test, 2000 larvae were homogenised in triplicate in 1 ml sterile sea water, and the suspensions were plated on Marine Agar. After 72 h of incubation, no growth was observed on any of the plates. Furthermore, the bacterial load of the rearing water of the control treatment was monitored daily during the 5 days of the experiment by plate counting on Marine Agar and TCBS Agar. No growth was observed in any of the samples.

Growth rates of the isolates

All isolates grew well at 18°C in LB<sub>35</sub> broth, with a relatively low variability in growth rate amongst the isolates (coefficient of variation of 9%) (Table S1, Supporting Information). No significant correlation was found between the growth rate of the isolates and the mortality rate of mussel larvae challenged with the respective isolates (Spearman’s $\rho = 0.118; P = 0.629$).

Identification of the isolates

The isolates were identified based on their 16S rRNA gene sequences (Table 1). Eight isolates were assigned to the genus *Vibrio* and nine to the genus *Photobacterium*. All isolates identified as *Vibrio* belonged to the Splendidus clade (Fig. 3); seven were closest to *V. hemicentroti* and one to *V. lentus*. The isolates identified as *Photobacterium* (Fig. 4) could not be identified below genus.
The genomes of the most virulent Vibrio sp. isolate and the most virulent Photobacterium sp. isolate, i.e. ME9 and ME15, respectively, were sequenced (Tables S2–S5 and Fig. S1, Supporting Information). Average nucleotide analyses (Richter and Rosselló-Móra 2009) of the ME9 and ME15 genomes against their respectively type strains permitted to clearly identify the strains; ME9 belongs to the species V. hemicentroti (ANI 96.7), whereas isolate ME15 belongs to Photobacterium sanguinicancri (Gomez-Gil et al. 2016).

Production of virulence factors and sensitivity to antibiotics

We screened the genome sequences of the most virulent isolates (ME9 and ME15) for genes that are involved in processes that have been associated with virulence towards bivalves, such as extracellular protease (gelatinase and caseinase) production and haemolytic activity (Labreuche et al. 2006, 2010), and flagellar motility (Yang and Defoirdt 2015). Several genes related to these processes were indeed present in the genomes of these two isolates (Tables S4 and S5, Supporting Information). We subsequently tested all isolates for the production of these virulence factors, and all isolates tested positive for all of them (Table 2). Overall, there was a low variability between the different strains with respect to production of the lytic enzymes (variation coefficients of 8%, 12% and 5% for gelatinase, caseinase and haemolysin, respectively). Swimming motility, however, showed a relatively high variability between the strains (variation coefficient of 52%). Finally, no correlation was observed between the levels of these (putative) virulence factors and the mussel larval mortality induced by the respective isolates (Spearman’s rho: < 0.139; P > 0.561).
We also noticed that the genome sequences of ME9 and ME15 contained a surprisingly high number of genes that are related to (multiple) antibiotic resistance (Tables S4 and S5) and therefore, we assessed the sensitivity of all the isolates towards a broad range of antibiotics using a disc diffusion test. Isolates ME3A and ME13A showed resistance to cefadroxyl and streptomycin, respectively, as no growth inhibition was observed (Table S6, Supporting Information). Given the fact that many of the genes in the genomes of ME9 and ME15 were linked to multiple antibiotic resistance, we reasoned that there might be a relation between the sensitivity to different antibiotics (e.g. the expression of multidrug efflux pumps would confer resistance to antibiotics from different classes). In order to test this hypothesis, we calculated correlations between the sensitivities of the isolates for the different combinations of antibiotics. For many of the combinations, we indeed found a significant ($P < 0.01$) and strong (Spearman's rho $> 0.6$) correlation (Table 3).

**DISCUSSION**

Although *Mytilus* species are generally considered to be very robust, we recently reported that stimulation of heterotrophic bacteria associated with wild-caught blue mussel adults by adding dissolved organic matter resulted in mass mortality within 1 week (Eggermont et al. 2014). We hypothesised that the heterotrophic bacteria associated with the mussels might be the cause of mass mortality events in dense larvae cultures. Indeed, many of the major aquaculture pathogens are known to be r-strategists (i.e. capable of quickly increasing their population density under conditions where there is a high level of...
nutrients available per cell; Defoirdt 2016). In this study, we isolated 17 strains from induced mortality events in blue mussels and used a newly developed highly controlled immersion challenge test with mussel larvae to evaluate the virulence of the isolates. Using this challenge test, we demonstrated that several of the isolates are pathogenic to mussel larvae. Indeed, all isolates caused significant (P < 0.01) mortality from 3 days of challenge onwards, and mortality levels ranged between 17 to as high as 98% after 5 days. As far as we know, this is the first documentation of opportunistic pathogens associated with healthy blue mussel adults that are able to cause mass mortality in mussel larvae. Ben Cheikh et al. (2016) very recently reported the isolation of putatively pathogenic strains during episodes of mass mortality in blue mussel farming in France. However, only three of these strains appeared to be pathogenic, which is quite remarkable given the fact that the authors used an injection model of infection, thereby bypassing the physical barrier against infection that is present in real life, and needed a rather high dose (10^6 CFU/mussel). It would be interesting to verify the virulence of these strains using a more natural route of infection (immersion). In addition to the standardised immersion challenge with larvae, we have tried to develop an immersion challenge test with blue mussel adults as well. However, thus far, we have not been successful in obtaining reproducible results. Together, this might indicate that blue mussel adults are indeed quite robust against opportunistic vibrios because mass mortality cannot readily be induced under controlled conditions in the laboratory. Furthermore, biotic or abiotic factors that have not yet been identified might be required for the onset of mass mortality events in mussel adults. Importantly, all isolates used in this study were derived from healthy wild-caught blue mussels, indicating that wild mussels contain a reservoir of pathogenic bacteria that thus might enter hatchery facilities unnoticed. In our previous study (Eggermont et al. 2014), we hypothesised that pathogens can be transferred from the broodstock to their offspring and that this is one of the factors that make mussel larviculture problematic, a reasoning that was shared by Kwan and Bolch (2015). This study further substantiates our

Table 2. Virulence factor production by the different isolates (average ± standard deviation of three replicates).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Swimming motilitya</th>
<th>Caseinase activityb</th>
<th>Gelatinase activityb</th>
<th>Haemolytic activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME2</td>
<td>43 ± 6</td>
<td>1.3 ± 0.0</td>
<td>1.7 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME3A</td>
<td>24 ± 4</td>
<td>1.3 ± 0.0</td>
<td>1.6 ± 0.2</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>ME3B</td>
<td>35 ± 6</td>
<td>1.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>ME4A</td>
<td>41 ± 3</td>
<td>1.4 ± 0.0</td>
<td>1.7 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME4B</td>
<td>49 ± 7</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>ME5</td>
<td>46 ± 7</td>
<td>1.2 ± 0.0</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME6</td>
<td>21 ± 3</td>
<td>1.1 ± 0.0</td>
<td>1.9 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME7</td>
<td>12 ± 4</td>
<td>1.4 ± 0.0</td>
<td>1.9 ± 0.0</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>ME8</td>
<td>26 ± 7</td>
<td>1.2 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME9</td>
<td>14 ± 5</td>
<td>1.1 ± 0.0</td>
<td>1.9 ± 0.2</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>ME10</td>
<td>9 ± 3</td>
<td>1.6 ± 0.1</td>
<td>1.9 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME11</td>
<td>7 ± 2</td>
<td>1.1 ± 0.0</td>
<td>1.9 ± 0.1</td>
<td>1.3 ± 0.0</td>
</tr>
<tr>
<td>ME13A</td>
<td>25 ± 5</td>
<td>1.1 ± 0.1</td>
<td>1.6 ± 0.0</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>ME13B</td>
<td>24 ± 6</td>
<td>1.1 ± 0.0</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>ME14A</td>
<td>33 ± 4</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.1 ± 0.0</td>
</tr>
<tr>
<td>ME14B</td>
<td>17 ± 6</td>
<td>1.1 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>ME15</td>
<td>7 ± 1</td>
<td>1.1 ± 0.0</td>
<td>2.0 ± 0.1</td>
<td>1.2 ± 0.0</td>
</tr>
</tbody>
</table>

aDiameter of motility halo (mm).  
bRatio between clearing/activity zone diameter and colony diameter.

Table 3. Spearman’s rank correlation coefficients between the sensitivities of the 17 isolates to the tested antibiotics. A significant positive correlation indicates that the isolates that are most sensitive to antibiotic 1 tend to be the most sensitive to antibiotic 2 as well, and vice versa.

<table>
<thead>
<tr>
<th>Antibiotic 1</th>
<th>Antibiotic 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1</td>
</tr>
<tr>
<td>Cefadroxil</td>
<td>0.896**</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>0.659**</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.659**</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>0.510**</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.844**</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>0.827**</td>
</tr>
<tr>
<td>Trimethoprim/</td>
<td>0.715**</td>
</tr>
<tr>
<td>Sulphametoxazole</td>
<td>0.842**</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>1</td>
</tr>
</tbody>
</table>

**p < 0.01.
hypothesis since we demonstrated that bacteria that were originally associated with healthy wild-caught blue mussel adults were indeed able to cause mass mortality in blue mussel larvae. These findings also indicate that microbiological management will be absolutely essential for a successful blue mussel larviculture. Microbial management could focus both on preventive (e.g. imposing slow growth conditions, which are unfavourable for fast-growing opportunistic pathogens; De Schryver and Vadstein 2014; Defoirdt 2016) and curative approaches. Curative approaches would ideally be compatible with prevention (i.e. they should not harm the non-pathogenic microbiota), and could focus on antivirulence therapy—disarming of pathogens rather than (non-selective) killing by antimicrobials (Defoirdt 2014), or the use of probiotics (Prado, Romalde and Barja 2010).

We identified the isolates based on the sequence of the 16S rRNA gene and found that eight of the isolates belonged to the Splendidus clade of vibrios and nine to the genus Photobacterium. The genomes of the most virulent isolates from each genus, ME9 and ME15, respectively, were sequenced and this revealed that isolate ME9 belongs to the species Vibrio hemicentroti, whereas ME15 was allocated to a new species, Photobacterium sanguinicancri (Gomez-Gil et al. 2016). Vibrios belonging to the Splendidus clade are well-known pathogens of marine bivalves (Travers et al. 2015), and have been associated with mass mortalities in bivalve larvae such as the green shell mussel (Perna canaliculus) (Kesarocodi-Watson et al. 2009), the scallop Argopecten purpuratus (Rojas et al. 2015) and the Japanese oyster (Crassostrea gigas) (Sugumar et al. 1998). Kwan and Bolch (2015) also indicated the association of bacterial communities dominated by the Splendidus clade with mass mortality events in a Mediterranean mussel (Mytilus galloprovincialis) hatchery. Finally, the strains that were recently isolated from mass mortality events in blue mussel farming in France (2010–2014) also belong to the Splendidus clade of vibrios (Ben Cheikh et al. 2016).

Approximately half of the strains we isolated in this study belong to the genus Photobacterium. Photobacterium species have been commonly isolated from different marine environments (Urbanczyk, Ast and Dunlap 2011) and from marine organisms, including bivalves, e.g. P. swingsii from the Pacific oyster (Crassostrea gigas) (Gomez-Gil et al. 2011) and P. galatheae from Mytilus sp. (Machado et al. 2015). Photobacterium sanguinicancri (ME15) was recently isolated from the haemolymph of the spider crab Maja brachydactyla in Spain (Gomez-Gil et al. 2016). Although most Photobacterium species have no described pathogenic activity, some species, e.g. P. damsela, are known pathogens of aquatic animals, mainly fish (Rivas, Lemos and Osorio 2013; Romalde et al. 2013), but also bivalve larvae, e.g. larvae of the giant clam (Tridacna gigas) (Sutton and Garrick 1993). However, this is the first report of Photobacterium sp. being pathogenic towards mussels.

The virulence of vibrios towards bivalves has been associated with the secretion of extracellular products such as proteases and haemolysins (Labeurche et al. 2006, 2010). We indeed found that all isolates tested positive for these phenotypes and could identify several genes that are involved in these processes in the genome sequences of isolates ME9 and ME15. However, no correlation was found between these activities and the virulence of the isolates towards mussel larvae. This might be attributed to several factors. A first possibility is that these phenotypes are not essential for inducing mortality in mussel larvae, or that relatively low levels are sufficient for full virulence and that higher levels do not further increase virulence. Second, in vitro virulence factor production does not necessarily correlate with in vivo production during association with a host, as this will also be affected by sensing of the host environment. Indeed, Ruwan-deepika et al. (2011) also reported that there was no correlation between in vivo expression levels of virulence-related genes in vibrio belonging to the Harveyi clade and their virulence towards brine shrimp. However, in vivo expression levels of these genes during association with brine shrimp were higher in pathogenic isolates than in a non-pathogenic isolate. Furthermore, expression levels of type III secretion system genes in Vibrio harveyi, for example, have been reported to be more than 1000-fold higher in host-associated cells than in in vitro grown cells (Ruwan-deepika et al. 2015). In this regard, it would be interesting to develop a methodology enabling us to measure virulence gene expression of bacteria in association with mussel larvae. However, given the minute size of blue mussel larvae, this will be a highly challenging exercise.

We found a remarkably high number of genes that are linked to antibiotic resistance in the genome sequences of isolates ME9 and ME15, and many of them were linked to multidrug resistance. Based on this observation, we aimed at testing whether there is a correlation between sensitivity to a range of antibiotics in the different isolates because this would indicate that the expression level of multidrug resistance genes determines the antibacterial sensitivity of these bacteria. Indeed, isolates that show a high expression level of multidrug resistance genes would show relatively low sensitivity to a wide range of antibiotics, and vice versa. In case specific genes would be involved (e.g. an antibiotic-degrading enzyme), one would not expect to find correlations between the sensitivities to different compounds. We found significant and strong positive correlations between sensitivity to many of the tested antibiotics, which might suggest that multidrug resistance genes are indeed a major factor determining the antibiotic sensitivity of these bacteria. Further research is needed in order to determine which of the genes are indeed involved in antibiotic resistance and to what extent they confer resistance to different antibiotics. Another intriguing question that deserves further investigation is what has been the driving force for the presence of this high number of multidrug resistance genes in vibrios that are associated with bottom culture mussels in The Netherlands; i.e. have bacteria been regularly exposed to antibiotics in this environment, or is it another feature of these genes that has driven the presence of these high numbers in the genomes of these bacteria? Indeed, multidrug resistance genes (such as multidrug efflux pumps) have been linked to resistance to natural substances produced by host organisms (such as bile, hormones and host defence molecules) (Piddock 2006). Hence, multidrug resistance genes might be an evolutionary adaptation to the association with mussels, which are known to produce a large variety of antimicrobial compounds (Mitta, Vandenbulcke and Rocha 2000).

In conclusion, in this study, we demonstrated that Vibrioaceae isolated from wild-caught adult blue mussels are capable of inducing mass mortality in mussel larvae. Approximately half of the isolates were found to belong to the Splendidus clade of vibrios, a group that is well-known to contain bivalve pathogens, and the other isolates belonged to the genus Photobacterium. The isolates caused between 17% and 98% mortality in blue mussel larvae after 5 days of immersion challenge. These data provide an ecological insight into the possible origin of mass mortality events, which at this moment hit mussel (larviculture). Our data indicate that wild mussels contain a reservoir of pathogenic bacteria that might enter hatchery facilities unnoticed through healthy wild-caught broodstock, and can then be transferred from the broodstock to their offspring (e.g. via the rearing water). These findings confirm that microbiological management will be an absolute requirement for a successful blue mussel
larviculture. Effective microbial management of the hatchery unit could lead to a more sustainable and optimised production of mussel seed. The highly controlled immersion challenge test system developed in this study will be of high value for further studies on host–pathogen interactions in mussels, facilitating a better understanding of the factors causing larval mass mortality and enabling the development of novel tools for disease control in bivalve (larvic)ulture.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

REFERENCES


Norqvist A, Norrman B, Wolf-Watz H. Identification and characterization of a zinc metalloprotease associated with


