Journal of Invertebrate Pathology 106 (2011) 242–249

Contents lists available at ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Isolation and characterization of a virulent *Vibrio* sp. bacterium from clams (*Meretrix meretrix*) with mass mortality

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ARTICLE INFO

Article history:
Received 12 April 2010
Accepted 24 October 2010
Available online 3 November 2010

Keywords:
Meretrix meretrix
Vibrio
MLSA
TEM

ABSTRACT

MM5 was a bacterial strain isolated from moribund clam (*Meretrix meretrix*) collected from a farm with mass mortality outbreak. Primary genotypic and phenotypic identification including 16S rDNA sequence analysis, multilocus sequence analysis (MLSA) of four housekeeping genes (*gapA*, *ftsZ*, *mreB* and *topA*) and biochemical tests suggested that strain MM5 was a *Vibrio* species closest to but different from *Vibrio furnissii*. Our previous study indicated that MM5 could induce a high mortality of *M. meretrix* (Yue et al., 2010). Quantitative challenge test was performed in this study to further evaluate the pathogenic potential of MM5, which showed that at 84 h post-inoculation, the cumulative mortalities of the MM5-injected group were significantly higher than those of control groups ($P < 0.05$). Cytopathological and histopathological features of the clam infected by MM5 were carried out by transmission electron microscopy (TEM) and Hematoxylin and Eosin (H&E) staining, respectively. Cytopathologically, foci of MM5 were found in hepatocytes of the clam infected by MM5. In addition, cytopathological lesion was detected in foot of infected clam. Histopathologically, MM5 was detected in different tissues of infected clam, including hepatopancreas, mantle and gill. Challenge test combined with pathological features indicated that MM5 was virulent to *M. meretrix*.

1. Introduction

Clam (*Meretrix meretrix*) is an important marine bivalve for commercial fisheries along the coastal and estuarine areas in China, Korea, Japan, Vietnam and India (Tang et al., 2006). In recent years, mass mortalities of *M. meretrix* have been reported and caused heavy economic losses. It is important to investigate the pathogens causing the mortalities and the virulence mechanisms of these pathogens.

Among the causative agents of mass mortalities of marine animals, *Vibrio* is a common finding (Cheng et al., 2004). In juvenile even adult bivalves, many bacterial diseases are provoked by Gram-negative bacteria, mostly the genus *Vibrio* (Paillard et al., 2004). Beaz-Hidalgo et al. (2010) have provided an update overview focusing specifically on pathogenic *Vibrio* species associated with bivalves. In the past decades, many *Vibrio* species have been reported to be associated with shellfish diseases, including *Vibrio tapetis* (Paillard et al., 1989, 1994; Paillard and Maes, 1995; Paillard, 2004; Allam et al., 2000; Park et al., 2006), *Vibrio splendidus* (Lacoste et al., 2001; Le-Roux et al., 2002; Waechter et al., 2002; Gómez-León et al., 2005), *Vibrio tubiashii* (Tubiash et al., 1965; Hada et al., 1984; Elston et al., 2008), *Vibrio alginolyticus* (Liu et al., 2001; Gómez-León et al., 2005), *Vibrio anguillarum* (Riquelme et al., 1995), *Vibrio pectenicida* (Lambert et al., 1998), *Vibrio parahaemolyticus* (Cai et al., 2006; Yue et al., 2010), *Vibrio aestuarianus* (Garnier et al., 2007) and *Vibrio furnissii* (Wang et al., 1992).

Vibrios are widespread in the aquatic environment. Sawabe et al. (2007) have divided 78 *Vibrio* species into different clades by the means of multilocus sequence analysis. *Cholerae* clade is one of them, which contains *Vibrio fluvialis*, *V. furnissii*, *Vibrio cholerae*, *Vibrio mimicus*, *Vibrio cincinnatiensis* and *Vibrio metschnikovii*. *V. fluvialis* is a bacterial pathogen isolated from river and estuarine water, mollusks, crustaceans, fishes and humans with diarrhea (Fruzzo et al., 2005). *V. furnissii* is a Gram-negative bacterium which has been associated with acute diarrhoeal illness after consumption of contaminated seafood (Brenner et al., 1983), and it has been reported to be associated with the disease of clam (Wang et al., 1992). *V. fluvialis, V. cincinnatiensis*, *V. mimicus*, and *V. metschnikovii* have also been associated with bivalves (Montilla et al., 1994; Maugeri et al., 2000; Cavallo and Stabili, 2002; Sutton and Garrick, 1993). Although most of the species within the *Cholerae* clade can cause diarrhea (Sawabe et al., 2007), there are limited reports on the pathogenicity of this clade to bivalve-related species.

In late September 2007, a heavy mortality of clam *M. meretrix* occurred in Jiangsu province of China. This mortality was similar

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0022-2011/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.jip.2010.10.006

to those induced by *Vibrios* reported previously (Lacoste et al., 2001; Travers et al., 2008). In a previous study, we have examined the pathogenicity of MM21, a bacterium with the highest virulence to clam among the total 21 bacterial strains isolated from moribund clams. In addition, MM5 also evoked a relatively high mortality of *M. meretrix* in the preliminary virulence screening experiment (Yue et al., 2010). The aim of this study was to further analyze the pathogenicity of MM5 to *M. meretrix*.

2. Materials and methods

2.1. Bacterial isolation

Moribund clams were collected from Rudong of Jiangsu Province, China, in late September 2007, when high-level mortality occurred. The operation of bacterial isolation from those clams was described in detail previously (Yue et al., 2010). MM5, which was one of the total 21 stains isolated, was applied to the followed study.

2.2. Genotypic identification

16S rDNA sequence analysis and multilocus sequence analysis (MLSA) were applied to genotypic identification of MM5. 16S rDNA and four housekeeping gene loci including *gapA* (glyceraldehyde3-phosphate dehydrogenase), *ftsZ* (a cell division protein), *mreB* (an actin-like cytoskeleton protein), and *topA* (topoisomerase) were amplified by PCR. The sequences of the primers used for amplification the partial sequences of these five loci are listed in Table 1. PCRs were performed essentially as described previously (Lane, 1991; Sawabe et al., 2007). The PCR products were sequenced in Shanghai Sangon Company (Shanghai, China). The 16S rDNA sequence of MM5 was blasted against Genbank sequences. The concatenated sequences of these four housekeeping genes were used to construct a consensus phylogenetic tree. The accession numbers of these housekeeping genes sequences of different strain which were used in this phylogenetic tree referred to the report by Sawabe et al. (2007). The sequences were aligned using ClustalX, and bootstrap consensus trees were constructed by the Neighbor-Joining (NJ) method using Mega 4.0 (Tamura et al., 2007). The robustness of each topology was checked by 1000 bootstrap replications.

2.3. Phenotypic identification

The VITEK test method by the automated microbe identification analyzer (VITEK 2 compact, bioMérieux, France) was used to identify and characterize strain MM5 further (Yue et al., 2010). The GN (Gram-negative) card was employed for the biochemical identification. The test was done as reported by Garcia-Garrote et al. (2000). Conventional plate and tube tests (purchased from Hangzhou Tianhe Microorganism Reagent Co., Ltd., China) are performed according to Berger's Manual of Determinative Bacteriology (Holt et al., 1994) as supplementary to the phenotypic identification. The biochemical tests conducted by conventional method contain: tolerance of NaCl at different concentrations (0%, 1%, 6%, 12%), methyl red (MR), Voges–Proskauer (VP) reaction, citrate utilization, indole production, b-galactosidase (ONPG), H2S production, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, urease activities, sensitivity to the vibrostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine), acid production from sucrose, D-glucose, D-arabitol, D-mannitol, inositol, L-arabinose and maltose. The features selected are mainly the ones used to differentiate *V. furnissii* from other salt-requiring *vibrios* (Brenner et al., 1983).

2.4. Virulence test

The quantitative virulence test was performed according to the description of Yue et al. (2010). Briefly, apparently healthy clams (3.5–4.5 cm in shell length, 1–2 years old) used for the challenge were bought from a market in Qingdao and acclimated for 1–2 week (25 °C, 30‰ salinity and under continuous aeration) in the laboratory. Before challenge, the clams were fed with the algae *Isochrysis galbana*. MM5 was grown overnight in 5 ml TSAYE (tryptic soy agar with 0.6% yeast extract) medium at 28 °C, from which 50 μl was subcultured in 50 ml fresh medium at the same condition for 6.5 h, the cells were harvested by centrifugation (5000 rpm, 10 min), washed and resuspended in PBS to OD_{600} of approximately 0.7, so that the bacterial concentration was $\sim 1 \times 10^9$ CFU ml^{-1} as determined by dilution-plate method. Clams were divided into MM5-infection group, PBS control group and negative control group. Clams of the infection group, PBS control group and negative control group were injected with 100 μl MM5 in PBS ($\sim 5 \times 10^6$ CFU ml^{-1}), 100 μl PBS or 100 μl MM2 in PBS ($\sim 5 \times 10^6$ CFU ml^{-1}) into the pallial cavity of each clam, respectively (Labreuche et al., 2006). MM2 was isolated from the clams in the previous study and identified as an *Acinetobacter baumannii*-related strain, which was used as the negative control bacterium (Yue et al., 2010). There were six replicate tanks in each group (10 clams/tank). Five of these replicates were used to determine mortality rates during the course of infection and one replicate tank was used to select the samples for TEM and H&E staining. Mortalities were recorded every 12 h for a 6-day period. Mortalities were counted when the adductor muscle of the clam was unresponsive (Fouz and Amaro, 2003).

2.5. Cytopathological and histopathological observations

TEM observation and H&E staining were conducted to detect cytopathological and histopathological features of the clam infected by MM5. Live clams were selected from the MM5-infected group and PBS control group in virulence test at 24 h post-injection for TEM and H&E staining. Hepatopancreas, mantle, gill, foot and

Table 1
PCR primers used in the MLSA.

Target gene	Primer sequences (5′–3′)	Predicted product size (bp)	Reference
16S rDNA	27f: AGA GTT TGA TCT TGG CTC AGA 1492r: TAC GGT TAC CTT GTT ACG ACT T	1500	Lane (1991)
topA	VtopA400F: GAGATCATCGGTGGTGATG VtopA1200R: GAAGGACGAATCGCTTCGTG	800	Sawabe et al. (2007)
ftsZ	VftsZ75F: GCTGTTGAACACATGGTACG VftsZ800R: GCACCAGCAAGATCGATATC	750	Sawabe et al. (2007)
gapA	VgapA150F: AACTCACGGTCGTTTCAAC VgapA899R: CGTTGTCGTACCAAGATAC	750	Sawabe et al. (2007)
mreB	VmreB12F: ACTTCGTGGCATGTTTTC VmreB999R: CCGTGCATATCGATCATTTC	1000	Sawabe et al. (2007)