ORIGINAL ARTICLE

Aquaculture

# Microbiological characteristics of *Vibrio scophthalmi* isolates from diseased olive flounder *Paralichthys olivaceus*

**Guo Qiao · Deok Chan Lee · Sung Ho Woo · Hua Li · De-Hai Xu · Soo Il Park**

**Abstract** In 2005, massive mortality occurred in olive flounder *Paralichthys olivaceus* farms in Korea, and five isolates were collected from diseased fish. In this study, microbiological and pathogenic characteristics of these isolates were studied. The isolates gave negative results in lysine and ornithine decarboxylase, *ortho*-nitrophenyl-$\beta$-galactoside, and citrate tests, and positive results in urease, esculinase, and nitrate reduction tests. The isolates produced acid from adipate, fructose, D-glucose, and maltose, and gave positive results in alkaline phosphatase, esterase lipase, leucine arylamidase, and naphthol-AS-BI-phosphohydrolase. According to genetic analysis, 16S rRNA gene sequences showed 98–100 % identity with both *Vibrio scophthalmi* and *V. ichthyoenteri*. The *dnaJ* gene sequences presented a higher identity with *V. scophthalmi* than with *V. ichthyoenteri*. Thus, the isolates were identified as *V. scophthalmi*. Pathogenicity of the five isolates in olive flounder was different and $LD_{50}$ values were from $10^6$ to $10^8$ CFU/g fish. Symptoms included darkening of skin, hemorrhage of liver and intestine, ascites, and distended abdomen. Histopathological changes included hemopoiesis dilatation and epithelial hyaline droplets in kidney, macrophage infiltration and ellipsoid dilatation in spleen, vascular dilatation, submucosal edema, and serosa inflammation of intestine. Cumulative mortality was 25 % for fish singly infected by isolate A19008 or *Streptococcus parauberis*, and increased to 87.5 % in super-infection group with these two pathogens.

**Keywords** *Vibrio scophthalmi* · Microbiological characteristics · Olive flounder · Mortality · Diseases

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## Introduction

Olive flounder *Paralichthys olivaceus* is one of the most important commercial food fishes, and it is commonly cultured through flatfish aquaculture in tanks located in inland Korea. According to statistics issued by the National Fisheries Research and Development Institute (NFRDI), the production of olive flounder accounts for 48.1 % of the fish cultured in Korea [1]. However, diseases associated with viruses, bacteria, and parasites have become the primary constraint on sustainable aquaculture [2]. In Korea, some new bacterial isolates were discovered from the cultured olive flounder. Kang [3] investigated the diversity of the bacteria associated with diseased olive flounder in the Jeju area from 2001 to 2002. *Vibrio scophthalmi* (26 %) was found to be the second most common species after *V. harveyi* (32 %). Jo et al. [4] studied the *Vibrio* population isolated from diseased olive flounder with

symptoms of vibriosis, and found *V. scophthalmi* (60.1 %), *V. harveyi* (20.73 %), *V. anguillarum* (4.15 %), *V. fischeri* (2.59 %), *V. alginolyticus* (0.52 %), and *P. damselae* (0.52 %). Among these *Vibrio* species, *V. harveyi* and *V. anguillarum* have been widely reported to be pathogenic and virulent towards olive flounder [5–7]. However, the significance and role of *V. scophthalmi* in the disease process are not clear, even though the bacterium is known to be the dominant *Vibrio* species associated with the disease. *V. scophthalmi* was first isolated from the intestine of juvenile turbot *Scophthalmus maximus* in Spain [8]. Since then, it has been isolated from healthy turbot larvae, diseased turbot, reared clams, intestine of normal olive flounder in Japan, diseased olive flounder in Korea, diseased summer flounder *Paralichthys dentatus*, and common dentex *Dentex dentex* L. [4, 8–15]. It is difficult to distinguish *V. scophthalmi* from the closely related species *V. ichthyoenteri*, *V. aestuarianus*, and *V. splendidus* due to their very similar biochemical characteristics and 16S rRNA gene sequences. In the present study, five new isolates obtained from diseased olive flounder were identified through phenotypic and genetic characteristics. Some key characteristics were developed to distinguish the new isolates from the related species, and challenge tests were conducted to evaluate if they were novel pathogens to olive flounder.

## Materials and methods

### Bacterial isolates and reference strains

Five isolates (A19003, A19006, A19008, A19010, and A19011) were collected from cultured olive flounder (body length 14.5–43.0 cm) at 17–23 °C in 2005 (Table 1) and preserved below −80 °C at the Fish Disease and Prevention Laboratory, Pukyong National University, Korea. Symptoms of the naturally diseased olive flounder included darkening of skin, hypertrophy of kidney and spleen, and light hyperemia of liver, muscle, and enteron. Closely related reference strains were used for biochemical tests (Table 1): *Vibrio scophthalmi* CECT4638 (strain A089, CAIM75), *V. scophthalmi* CAIM1797, *V. ichthyoenteri* ATCC700023, *V. splendidus* ATCC33125. All strains were stored at −80 °C using tryptic soy broth (TSB) supplemented with 2 % NaCl and 10 % glycerol.

### Biochemical characteristics

Physiological and biochemical characteristics were determined following the approach described in [16, 17] and *Bergey's Manual of Determinative Bacteriology* [18]. The following tests were conducted: growth in TSB under different NaCl concentrations, TSB with 2 % NaCl at different pH values and 25 °C, TSB with 2 % NaCl at different temperatures for three days; three types of motilities, including flagellum-mediated swimming, swarming [19], and type IV pilus-mediated twitching [20]; Gram stain; susceptibility to vibriostatic agent O/129 (2,4-diamino-6,7-diisopropylpteridine); growth on thiosulfate-citrate-bile-sucrose (TCBS); cytochrome-oxidase; O–F test; production of $H_2S$ on TSI; ONPG; gas production from glucose; indole and VP tests; arginine dihydrolase; lysine and ornithine decarboxylase; nitrate reduction; acid production from 20 sugars. The following activities were also determined: catalase (3 % $H_2O_2$), urease, gelatinase, and hemolysis on erythrocytes of sheep, olive flounder, and starry flounder *Platichthys stellatus* [21]. Hemolysis was recorded from sheep blood agar (Asanpharm, Korea) and CBA medium (Columbia Blood Agar Base, Difco) supplemented with 1.5 % (w:v) NaCl and 1 % (v:v) olive flounder or starry flounder erythrocytes, respectively, for

**Table 1** The bacterial isolates and reference strains used in this study

| Strains | Origin | |
|---|---|---|
| **Reference strains** | | |
| *V. scophthalmi* CECT4638 | Intestine of healthy turbot (*Scophthalmus maximus*) | 1997, Spain |
| *V. scophthalmi* CAIM1797 | Common dentex (*Dentex dentex* L.) | 2005, Spain |
| *V. ichthyoenteri* ATCC700023 | Intestine of diseased Japanese flounder larvae (*Paralichthys olivaceus*) | 1996, Japan |
| *V. splendidus* ATCC33125 | Marine fish | 1970, USA |
| **Tested isolates** | | |
| *Vibrio* sp. A19003 | Spleen, diseased olive flounder | 2005, Jeju, Korea |
| *Vibrio* sp. A19006 | Kidney, diseased olive flounder | 2005, Jeju, Korea |
| *Vibrio* sp. A19008 | Kidney, diseased olive flounder | 2005, Jeju, Korea |
| *Vibrio* sp. A19010 | Spleen, diseased olive flounder | 2005, Pohang, Korea |
| *Vibrio* sp. A19011 | Kidney, diseased olive flounder | 2005, Busan, Korea |

*CECT* Colecció Española de Cultivos Tipo, *CAIM* Collection of Aquatic Important Microorganisms, Spain, *ATCC* American Type Culture Collection

three days at 25 °C. Fish erythrocytes were collected freshly from healthy fish maintained in an aquarium following centrifugation for 10 min at 3,000×*g* and 4 °C. The tests for the acidification of glucose, urease, *β*-glucosidase, gelatinase, assimilation of *N*-acetyl-glucosamine and glucose, potassium gluconate, capric acid, adipic acid, malic acid, phenylacetic acid were performed using the API 20NE strip (bioMèrieux® SA, Marcy, íÉtoile, France). All of the test media were supplemented with 1.5 % NaCl. The test kits were inoculated and incubated using the modifications described by Austin et al. [6]. The test preparations were examined 24 or 48 h postincubation at 25 °C.

Enzymatic activity of the bacterial culture

Caseinase activity was observed on skimmed milk agar plates [22]. Phospholipase and lipase activities were examined as described by Liu et al. [23]. The other 19 enzyme activities of bacterial cells were detected using the Api Zym kit (bioMérieux, Inc., USA), according to the manufacturer's instructions.

Whole-cell protein analysis

The whole-cell protein was prepared and SDS-PAGE was carried out on a Mini GE 200 gel electrophoresis unit (Bio-Rad) [24].

Genetic characteristics

*DNA extraction and PCR amplification*

DNA extraction and purification were carried out using a High Pure PCR template preparation kit (Roche Diagnostics GmbH, Germany) following the manufacturer's instructions, and it was examined as previously described by Li et al. [24]. Two universal bacterial primers [25], Eubac27F (5′-AGA GTI TGA TC(C/A) TGG CTC AG-3′) and Eubac1492R (5′-TAC GG(C/T) TAC CTT GTT ACG ACT T-3′), were synthesized by Bioneer (Korea) and used to amplify the bacterial 16S rRNA gene. Each PCR sample included 20 μl of premix (Accupower™ PCR Premix, Bioneer), 1 μl of each primer (10 μmol), 1 μl of template (about 20 ng), and 17 μl of triple-distilled water. The thermal cycle was run in a MyCycler™ thermal cycler (Bio-Rad, USA) at 95 °C initially for 5 min, then 25 cycles were performed of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and finally the system was held at 72 °C for 7 min. PCR products were subjected to electrophoresis for 35 min at 60 V in 0.8 % agarose gel with 0.5× TAE buffer (Sigma–Aldrich) and visualized under UV light after ethidium bromide staining (EtBr). The *dnaJ* gene was amplified using the degenerate primers 805-VibrioMF

(5′-TTT TAY GAA GTD YTD GGY GT-3′) and 806-VibrioMR (5′-GAC AVG TWG GAC AGG YYT GY T G-3′) [26]. PCR premix was prepared as above, and PCR amplification was carried out as follows: 3 min for initial denaturation, followed by 40 cycles of 94 °C for 30 s, 56 °C for 30 s, and 74 °C for 1 min, with a final extension of 7 min at 72 °C. Amplified products of approximately 558 bp were examined in 1.2 % agarose gel and visualized as described above.

*Cloning and sequencing of PCR products*

The PCR products of 16S rRNA and the *dnaJ* gene were gel purified using a MEGA-spin™ agarose gel extraction kit (iNtRON Biotechnology, Inc.). Subsequently, the purified PCR products were cloned in *Eschelichia coli* DH5α using the pGEM®-T easy vector kit (Promega, USA). The recombinant plasmids were purified using the Exprep geneAll plasmid SV mini kit (GeneAll Biotechnology, Korea) according to the manufacturer's recommended protocol. The recombinant plasmids containing the inserted DNA were sequenced using the primers SP6 and T7 in an ABI 377 automatic sequencer (Genotech, Korea).

*Phylogenetic data analysis*

The 16S rRNA and *dnaJ* gene sequences of the isolates were deposited in the National Center for Biotechnology Information GenBank (NCBI) database (accession numbers are shown in Figs. 2 and 3). The DNA sequences obtained were aligned with representative sequences from the GenBank NCBI database. Multiple sequences were aligned with the CLUSTAL-W program in the BioEdit package [27]. Distance matrices were created with the MEGA4 package [28]. Unrooted evolutionary trees were constructed via the neighbor-joining (N-J) tree algorithm [29]. Distance estimates were obtained according to the maximum composite likelihood model for 16S rRNA [30] and the Kimura two-parameter model for the *dnaJ* gene [31], respectively. The resulted tree topologies were evaluated by bootstrap analysis of the N-J method [29] based on 1000 replications.

Challenge test

*Olive flounder maintenance*

Normal olive flounder with an average body weight of 8.7 g were obtained from a fish farm on Jeju Island and maintained in static aquaria at approximately 30 practical salinity units (psu), 23–25 °C and pH of 8.0 ± 0.2 for two weeks prior to the challenge test. The fish were fed with commercial fish pellets (National Federation of

Fisheries Cooperatives feed, Korea) until they were used for experiments three days later.

### Challenge test (lethal dose 50 % test)

The pathogenicity of the isolates was determined in vivo [32]. Bacterial suspensions were prepared by culturing the isolates in TSB at 25 °C for 24 h, washing them, and then adjusting them to the appropriate concentrations of $10^5$–$10^{10}$ CFU/ml with sterilized physiological saline (PS). The fish were injected with 0.1 ml of each bacterial suspension intraperitoneally (IP). The control group was inoculated with the same volume of PS. The fish were observed daily for 14 days following the bacterial challenge. All mortalities and clinical signs were recorded daily. The moribund or freshly dead fish were collected in order to isolate the inoculated bacteria and identify the pathogen responsible. The 50 % lethal dose ($LD_{50}$ value) was calculated as described by Muhammad [33].

### Superinfection protocols

The isolates were moderately or weakly virulent towards olive flounder based on the $LD_{50}$ values obtained from the challenge test. In order to investigate differences in the virulence of the isolates, superinfection tests were conducted in this study with *Streptococcus parauberis* PH0710 [34] and two representative isolates, a highly virulent strain (HVS A19008) and a low-virulence strain (LVS A19010). Briefly, bacterial suspensions of three strains were prepared to the appropriate concentrations (1.26 × $10^7$ and 1.26 × $10^6$ CFU/fish) with PS as mentioned above. The infection groups included single infection with one of the three strains (PH0710, HVS A19008, and LVS A19010) and superinfection with either PH0710/HVS A19008 or PH0710/LVS A19010. In the superinfection groups, the fish were first infected with PH0710 by IP injection. Three days later, the fish were infected secondly with HVS A19008 or LVS A19010. Meanwhile, the fish that were first injected with sterilized PS were infected with HVS A19008 or LVS A19010, and then the fish injected with PH0710 were inoculated with PS again as controls. All of the fish were injected with 0.1 ml of the bacterial suspension or PS each time by IP. The superinfected fish were observed daily for 14 days. Mortalities and clinical signs were recorded. Moribund or freshly dead fish were tested for bacteria to determine the pathogen responsible.

### Histopathology

The histopathological changes in the main organs of olive flounder were observed after staining by the H&E method [35].

## Results

### Microbiological characteristics

#### Biochemical characteristics

All five isolates grew in TSB from 10 to 30 °C, but not below 4 °C or above 37 °C. The optimal growth temperature was 25–30 °C. The isolates grew in TSB containing 0.5–6 % NaCl, but did not grow when NaCl was above 6 % or in the absence of NaCl in alkaline peptone water. The optimal concentration range of NaCl was 1–6 %. The isolates were able to grow at pH 5–10, but they did not grow below pH 5 or above pH 10, with optimal growth occurring at pH 7–9. The isolates were facultatively anaerobic short rods, G⁻, motile with swimming and twisting, did not swarm, were unpigmented, sensitive to O/129, did not produce $H_2S$, were fermentative from glucose but did not produce gas, were catalase and oxidase positive, and could form green or yellow colonies on TCBS. The isolates gave negative results in lysine and ornithine decarboxylase, indole production, MR, VP, and citrate tests, and positive results in urease, esculinase, and nitrate reduction tests. The isolates produced acid from D-glucose, fructose, D-maltose, trehalose, adipate, N-acetyl-glucosamine (NAG), potassium gluconate and adipic acid, but no acid from melibiose, salicin, sorbitol, xylose, capric acid, malic acid, phenylacetate, adonitol, arabinose, dulcitol, inositol, and rhamnose.

#### Enzymatic activity of the bacterial cells

No hemolysis was detected in sheep, olive flounder, and starry flounder erythrocytes for all five isolates. All isolates were able to produce alkaline phosphatase, esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and N-acetyl-β-glucosaminidase, but did not produce lipase (C14), crystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosidase, α-mannosidase, α-fucosidase, phospholipase against egg yolk, and caseinase. These isolates (except for A19010) were positive for esterase (C4) and valine arylamidase. The production of lipase against Tween-80 and gelatinase among the isolates varied. The isolates A19008 and A19010 produced lipase against Tween-80 very well, while the others could not. The isolates A19008, A19010, and A19011 produced gelatinase weakly, but the others could not.

#### Whole-cell protein analysis

One-dimensional SDS-PAGE of whole-cell protein of five isolates and two reference strains of *V. scophthalmi* is
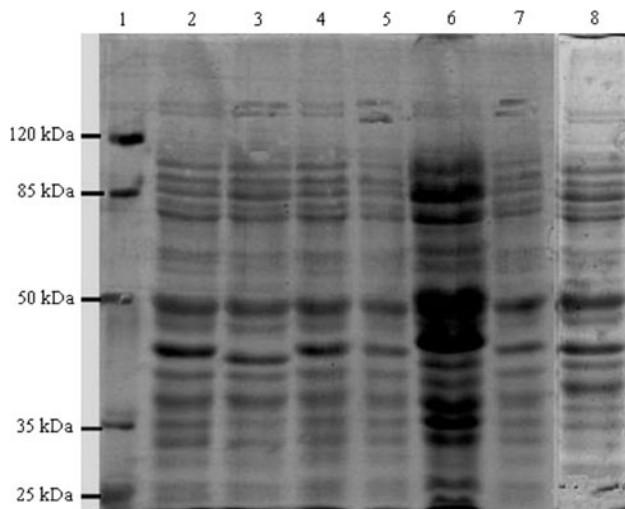
**Fig. 1** SDS-PAGE of whole-cell protein profiles of the isolates and *Vibrio scophthalmi* reference strains in 12 % polyacrylamide gel. *1* Marker; *2–5* isolates A19003–11; *7* and *8* references CECT4638 and CAIM1797

shown in Fig. 1. The calculated regression equation was as follows:

$$y = -1.0769\,x + 2.2654\,\left(R^2 = 0.9893\right),$$

where $y$ represents the log of the molecular weight of standard proteins, $x$ represents the mobility of the protein band, and $R^2$ is the correlation coefficient.

All strains generated 11 discrete bands at $M_r$ values of 102, 95, 87, 83, 81, 50, 43 or 45, 39, 36, 34 and 31 kDa (Fig. 1). Overall, most of the whole-cell protein profiles had a protein band at an $M_r$ of 43 kDa, although the isolate A19008 contained a large protein band at an $M_r$ of 45 kDa.

### 16S rRNA gene analysis

Phylogenetic analysis of the 16S rRNA gene was performed for the five isolates, and the accession numbers are listed in Fig. 2. The sequences of the isolates (A19003, A19006, A19008, A19010, and A19011) showed 99.67, 98.77, 98.56, 97.2, and 99.39 % identities with *V. scophthalmi*, and 98.88, 98.50, 98.29, 97.24, and 99.18 % identities with *V. ichthyoenteri*, respectively.

### dnaJ gene analysis

The sequences of the five isolates showed identities of 83–99 % with *Vibrio* species, and all 100 blast hits belonged to *Vibrio* spp. The accession numbers of the *dnaJ* gene sequences of the isolates (A19003, A19006, A19008, A19010, and A19011) in NCBI are listed in Fig. 3. The sequences of the isolates showed identities of 98.85, 99.04, 99.03, 98.85 and 98.85 % with *V. scophthalmi*

(AB263066), and 92.88, 92.68, 91.04, 92.50 and 92.88 % with *V. ichthyoenteri* (AB263043), respectively.

### Pathogenicity assays

#### Challenge test (LD$_{50}$)

Clinical signs of olive flounder were similar for fish infected experimentally and naturally, including darkened skin, distended abdomen, hemorrhage of muscle, liver, and intestine, ascites, and hypertrophy of spleen and kidney. The 14-day LD$_{50}$ values of five isolates (A19003, A19006, A19008, A19010, and A19011) were $3.87 \times 10^7$, $9.72 \times 10^8$, $4.81 \times 10^6$, $1.19 \times 10^8$, and $1.79 \times 10^8$ CFU/g fish, respectively. Thus, the isolate A19008 was considered to be moderate, isolates A19003, A19010 and A19011 to be weak, and isolate A19006 to be nonvirulent towards olive flounder according to the criteria established by Mittal et al. [36] and Santos et al. [37]. The challenged strains were reisolated from liver, spleen, kidney, and ascites of dead olive flounder.

#### Superinfection test

The results of the superinfection test are shown in Fig. 4. For the group superinfected with PH0710/HVS A19008, one out of eight fish died at 3 dpi, one fish died at 6, another at 7, and another at 10 dpi, and then three fish at 9 dpi. The cumulative mortalities were 87.5 % in fish challenged by the bacteria at $10^7$ CFU/fish and 50 % at $10^6$ CFU/fish. For the superinfection groups of PH0710/LVS A19010, the cumulative mortalities were 75 % in fish challenged by the bacteria at $10^7$ CFU/fish and 50 % at $10^6$ CFU/fish, respectively (Fig. 4). For the control groups challenged by a single strain of bacteria, the cumulative mortality was 25 % in fish challenged with PH0710 or HVS A19008 at a bacterial concentration of $10^7$ CFU/fish. In the LVS A19010 single-infection groups, the cumulative mortality was 12.5 % at a bacterial concentration of $10^7$ and 0 % at $10^6$ CFU/fish (Fig. 4). The cumulative mortalities in the superinfection groups were much higher than those in single-infection groups. The HVS A19008, LVS A19010, and PH0710 were successfully reisolated from ascites, liver, kidney, and spleen of olive flounder. Except for those organs, PH0710 was also reisolated from heart and brain of fish.

#### Histopathology

Histopathological changes were observed in liver, kidney, spleen, and intestine of olive flounder infected experimentally (Fig. 5). No obvious histopathological changes were noted in liver (Fig. 5b). Hemopoiesis dilatation and epithelial hyaline droplets were noted in kidney (Fig. 5d). Macrophage
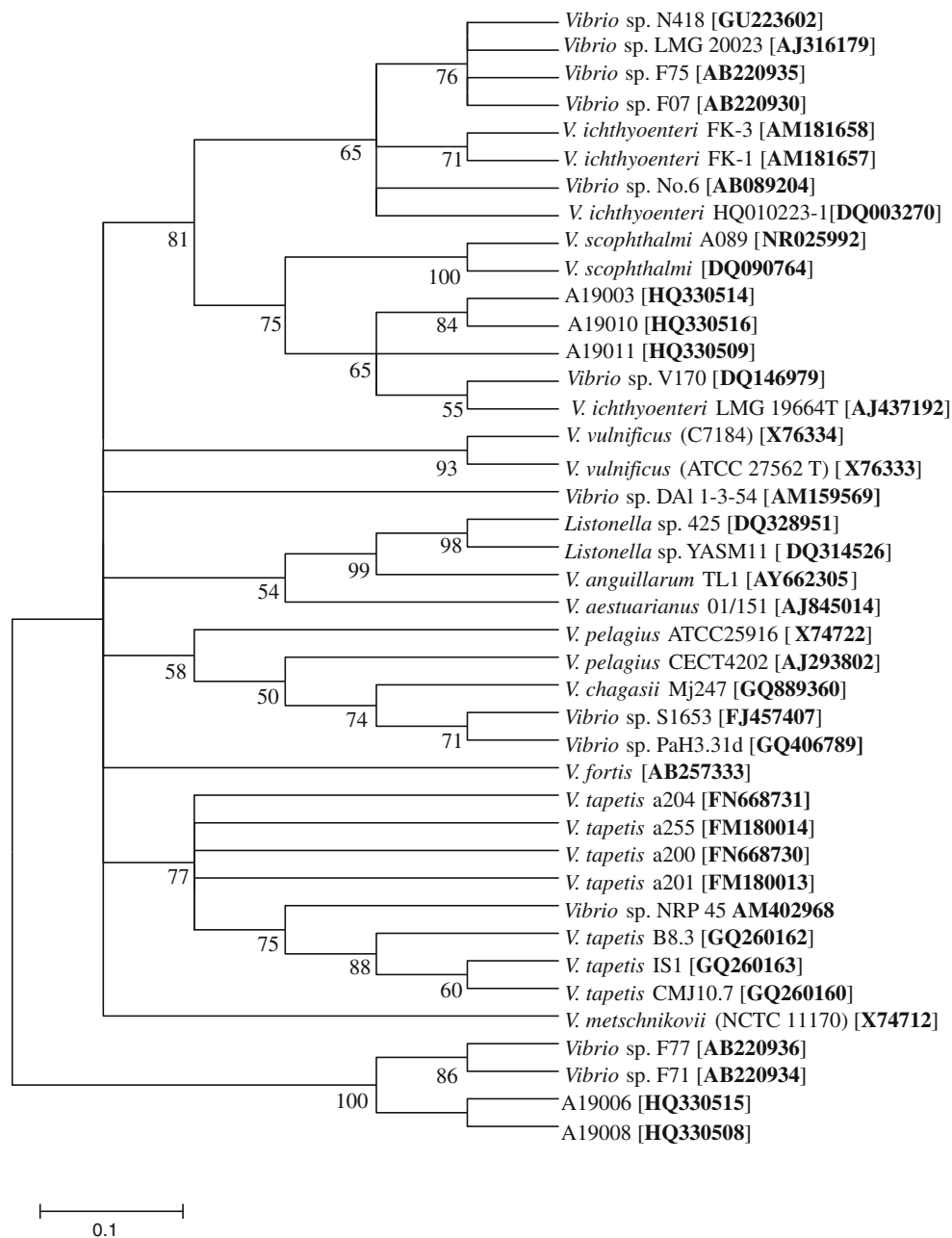
**Fig. 2** Phylogenetic tree of the five isolates, based on partial 16S rRNA gene sequences. The dendrogram was constructed by the neighbor-joining method, and estimated distances were obtained using the maximum likelihood model in the Mega 4.0 program.

Bootstrap analysis was performed with 1000 replications. Only values above 50 % are shown. The *scale bar* represents 0.1 substitutions per nucleotide site

infiltration and ellipsoid dilatation were observed in spleen (Fig. 5f). Vascular dilatation, submucosal edema, and serosal inflammation were seen in intestines (Fig. 5h).

## Discussion

The biochemical characteristics of these five new isolates were generally similar to those of the reference strains

*V. scophthalmi* CECT4638 and CAIM1797. There were some differences in arginine dihydrolase and acid production from cellobiose, galactose, mannitol, mannose, and sucrose. The phenotypic characteristics of the five isolates were different from the other three closely related strains. Five isolates could not grow in alkaline peptone water supplemented with 6 % NaCl or at 37 °C, but *V. aestuarianus* grew. *V. aestuarianus* did not produce gelatinase and VP was negative [16], but some isolates could use gelatin
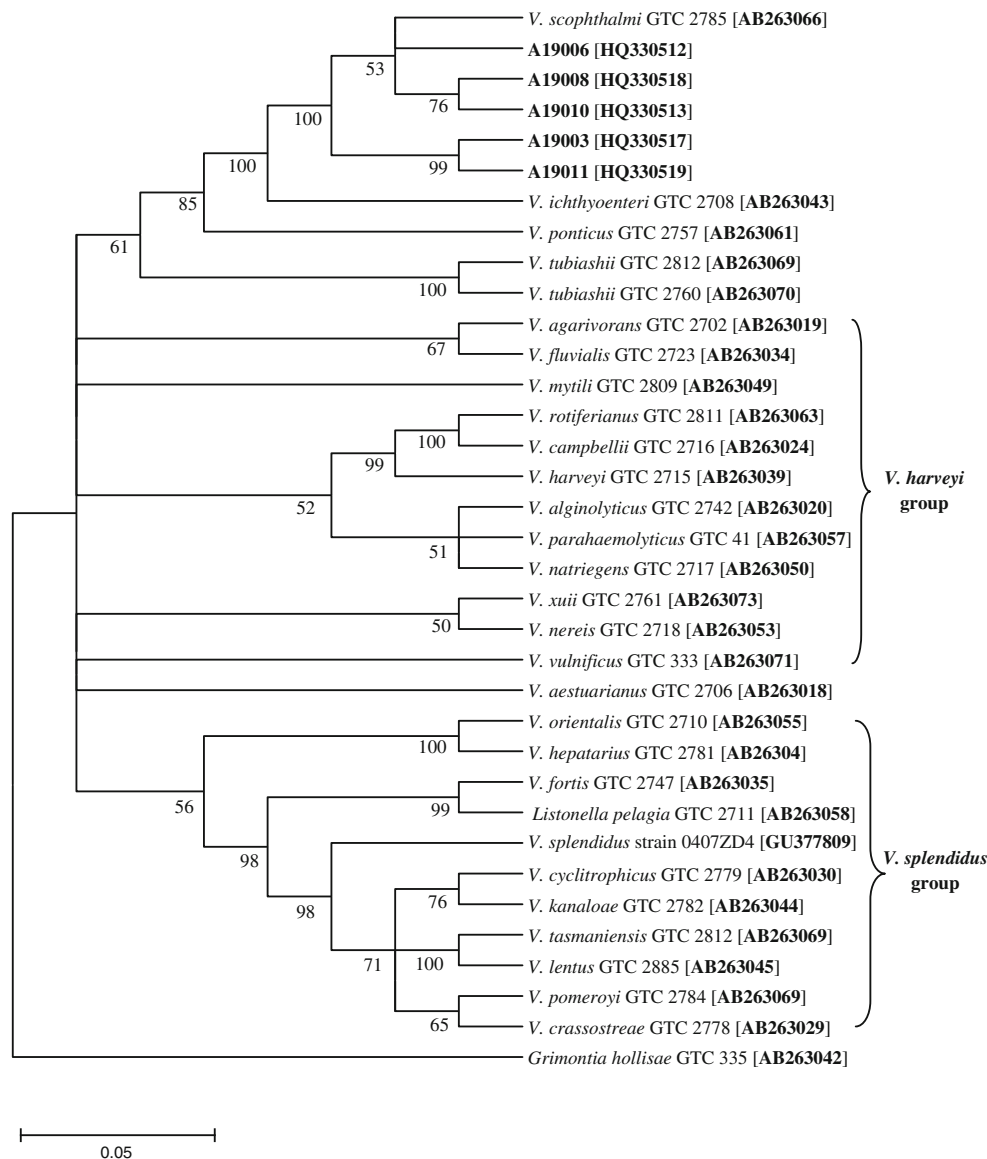
**Fig. 3** Phylogenetic tree for the five isolates based on *dnaJ* gene sequences. Estimated distances were obtained using the Kimura two-parameter model. The *scale bar* represents 0.05 substitutions per nucleotide site

and were VP positive. The differences in the biochemical characteristics of the isolates and *V. ichthyoenteri* were related to gelatin utilization. Some of the isolates could use gelatin, whereas *V. ichthyoenteri* could not use gelatin or perform chitin hydrolysis [13, 38]. According to Sugita and Ito [13], gelatin and chitin hydrolysis are important criteria for differentiating these two closely related species. The biochemical characteristics of the isolates showed some differences from *V. splendidus* in growth at 37 °C, 6 % NaCl, ornithine decarboxylase, gelatinase, indole production, MR, citrate utilization, ONPG and acid production from cellobiose, salicin, and xylose. Among these, ONPG appears to be the phenotypic test that most strongly differentiates between the isolates and *V. splendidus*. All

isolates were negative for ONPG, whereas *V. splendidus* was positive for ONPG [11, 24, 39]. The biochemical characteristics of all of the isolates were very similar to those of *V. scophthalmi* and *V. ichthyoenteri* [9]. Meanwhile, the 16S rRNA gene sequences of all isolates exhibited 98–100% identity with *V. scophthalmi* and *V. ichthyoenteri* simultaneously. According to Montes et al. [40], the 16S rRNA gene sequences of *V. scophthalmi* and *V. ichthyoenteri* are very closely related.

In the past, bacterial phylogeny has relied exclusively on 16S rRNA gene sequence analysis. Recently, multilocus sequence analysis (MLSA) has been used to distinguish similar species and to correctly identify species [41, 42]. Furthermore, housekeeping genes encode products that are
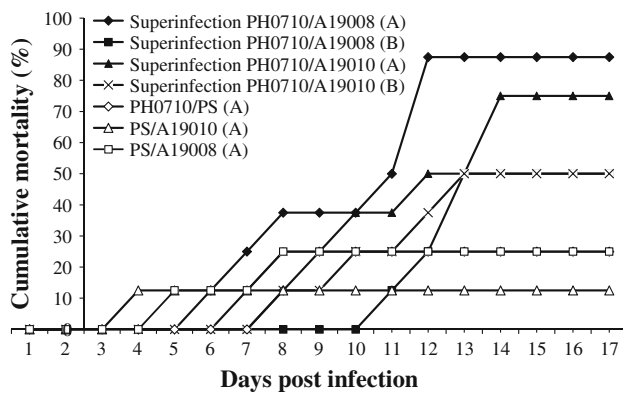
**Fig. 4** Cumulative mortality in superinfection groups of *Streptococcus parauberis* and two tested isolates. *PH0710 S. parauberis* PH0710, *A19008* HVS A19008, *A19010* LVS A19010, *PS* physiological saline. Groups *A* and *B* corresponded to bacterial concentrations of $1.26 \times 10^7$ and $1.26 \times 10^6$ CFU fish$^{-1}$, respectively

likely to be essential to the bacteria and are therefore expected to be present in all strains of a genus [41]. The housekeeping *dnaJ* gene has already been used successfully to discriminate species and subspecies, even within genera in which the 16S rRNA gene has insufficient resolution, such as *Mycobacterium* [43], *Legionella* [44], *Streptococcus* [45], *Staphylococcus* [46], *Aeromonas*, *Vibrio*, and the Enterobacteriaceae family [26, 47, 48]. In the present study, *dnaJ* gene sequences of the isolates clearly showed higher identities with *V. scophthalmi* (AB263066) than with any other *Vibrio* species. The isolates were closely clustered with known species. The relations among the five isolates and the *Vibrio* species obtained from *dnaJ* gene sequences agreed well with those derived from 16S rRNA gene sequences. The isolates were mostly branched and showed a higher sequence identity with *V. scophthalmi* than with *V. ichthyoenteri*, especially for the *dnaJ* gene sequences. For the genus *Vibrio*, the average substitution rate of the *dnaJ* gene sequence (0.221) is about eight times greater than that of the 16S rRNA gene sequence (0.028). Therefore, the *dnaJ* gene has been considered a phylogenetic marker for identifying *Vibrio* species [26]. In the phylogenetic tree based on *dnaJ* gene sequences, species related to *V. splendidus* or *V. harveyi* formed compact clusters in the present study, which agreed with the results reported by Nhung et al. [26]. The *dnaJ* gene sequence of *V. scophthalmi* showed a >7 % divergence from *V. ichthyoenteri*, although these species exhibited ~100 % sequence similarity in the 16S rRNA gene and 70 % DNA–DNA similarity [26]. To estimate interspecies and intraspecies variations, sequence similarity values were counted, and they were found to range from 68.4 to 92.6 % among species and from 97 to 100 % within species. In the present study, although the *dnaJ* sequences of the five isolates exhibited 98 % identity with *V.*

*scophthalmi*, the closest fit was *V. scophthalmi*, which matched the phenotypic characteristics.

The infection trials of the five isolates demonstrated that the isolates were pathogenic to olive flounder, and the resulting clinical signs were similar to those found in naturally infected fish in farms. The signs included hyperemia of liver and intestine, hypertrophy of spleen and kidney, and inflammatory ascites. Although no obvious histopathological changes were observed in liver, hepatocytes became concentrated, and this was probably associated with hyperemia of liver. Other clinical signs such as hemopoiesis dilatation and epithelial hyaline droplets in kidney, macrophage infiltration, and ellipsoid dilatation in spleen may be related to the hypertrophy of kidney and spleen. Vascular dilatation, submucosal edema, and serosal inflammation may have caused the hyperemia of intestine and inflammatory ascites. $LD_{50}$ values of the five isolates ($10^6$ to $10^8$ CFU/g fish) were similar to those of *V. harveyi* ($10^5$ to $10^7$ CFU/g fish) [5] and *V. damselae* ($3 \times 10^4$ to more than $10^8$ CFU/g fish) [49], and higher than those of *E. tarda* in olive flounder ($10^{2.5}$–$10^{5.25}$ CFU/fish) [50]. *V. harveyi* can infect a wide variety of marine animals, such as Japanese abalone *Sulculus diversicolor supratexta*, tiger prawn *Penaeus monodon*, summer flounder, sea bass, and turbot, and presents various $LD_{50}$ values, from $10^4$ to more than $10^8$ CFU/g fish [51–53]. These results reveal that the virulence of the bacterium may be host dependent. In the present study, the relatively high $LD_{50}$ values of the isolates may be related to the host olive flounder. Sugita et al. [17] found that the intestinal tracts of larvae, juveniles, and young olive flounder were mainly colonized by members of the genus *Vibrio*. Phylogenetic analysis showed that 82 representative isolates were closely related to three species of marine vibrios: the *V. scophthalmi-V. ichthyoenteri* group (41 isolates), *V. fischeri* (39 isolates), and *V. harveyi* (2 isolates). These findings suggest that olive flounder harbors major bacteria of the *V. scophthalmi–V. ichthyoenteri* group and *V. fischeri* [13], which are similar to those found in turbot [54, 55]. However, *V. ichthyoenteri* has been suggested to be the causative agent of intestinal necrosis and bacterial enteritis in olive flounder larvae [56, 57]. Therefore, the *V. scophthalmi–V. ichthyoenteri* group was considered to be *V. scophthalmi* in addition to the use of chitin [13]. The densities at which the *V. scophthalmi* group is found in specimens of normal olive flounder range from $1.5 \times 10^6$ to $1.5 \times 10^8$ CFU/g fish [13]. *V. scophthalmi* is considered to be a common inhabitant of flatfish [58], irrespective of geographical location [4, 12, 13, 54, 55]. Intestinal vibrios are mostly found in marine animals, and some of them are well-known opportunistic pathogens, such as *V. fischeri* [8] and *V. harveyi* [59]. *Vibrio* species have been isolated from both normal and diseased aquatic animals, and their role depends on many factors, such as
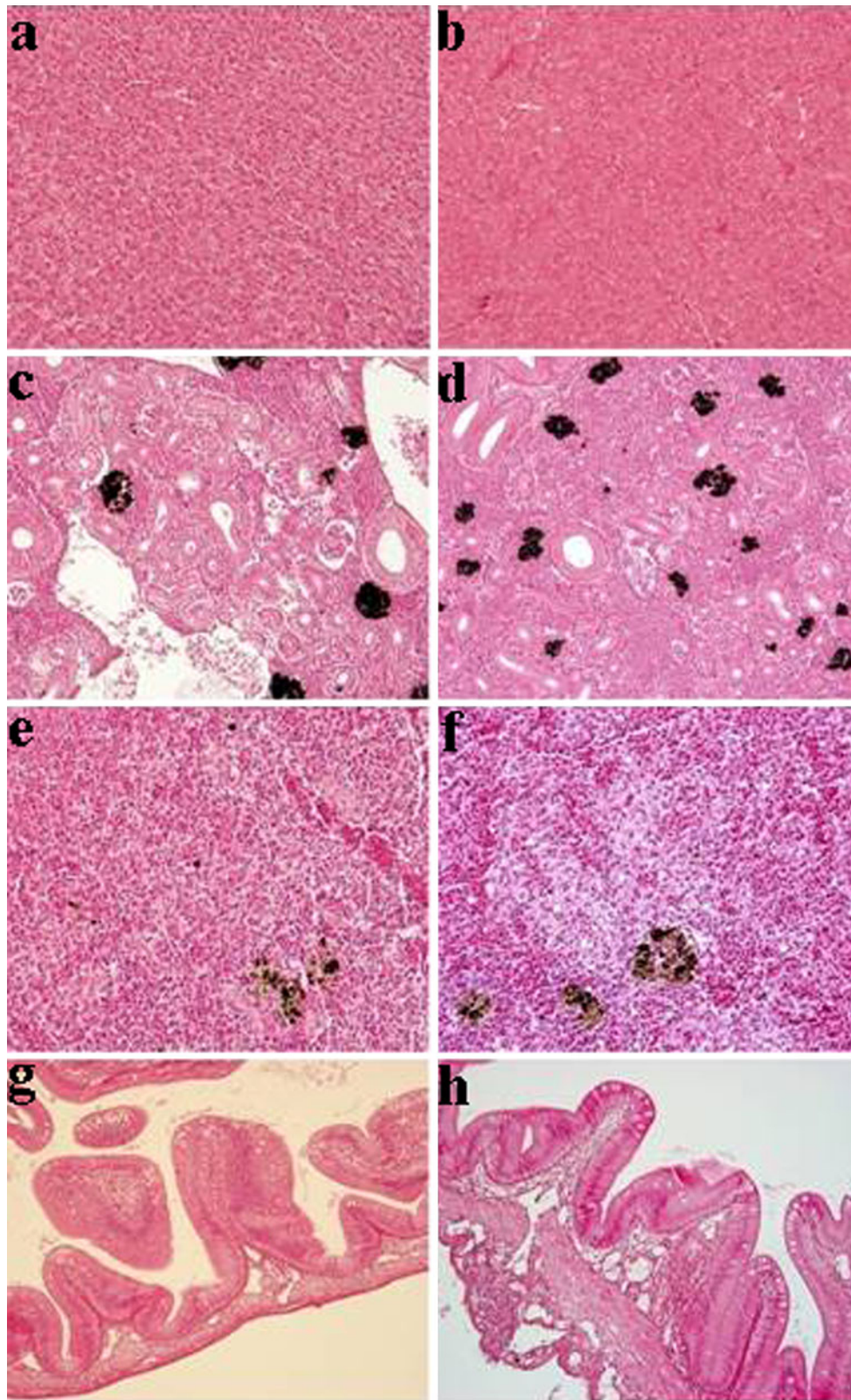
**Fig. 5** Histological sections of liver (**a**, **b**), kidney (**c**, **d**), spleen (**e**, **f**), and intestine (**g**, **h**) of olive flounder challenged with the isolates (H&E, ×400). **a**, **c**, **e**, and **g** are tissues of olive flounder from a control fish injected with physiological saline. **b**, **d**, **f**, and **h** are tissues of olive flounder from a group challenged with the isolates

environmental and fish-related conditions [24]. *V. scophthalmi* was first reported to be the dominant bacterial population in the intestine of a healthy juvenile turbot [8], and was later found to be related to ascites disease in turbot, with symptoms of distended abdomen, hemorrhage of liver, spleen, and even throughout the whole body, as well as inflammatory ascites [10]. *V. scophthalmi* has also reportedly been isolated from intestines of both normal and diseased olive flounder [4, 13]. However, the pathogenicity of *V. scophthalmi* towards olive flounder was not known. The results of this study and other reports indicate that *V. scophthalmi* is an opportunistic pathogen towards olive flounder. The pathogenicity of *V. scophthalmi* is particularly severe if the fish is under stress. Devesa et al. [60] reported that *V. fischeri* and *V. harveyi* were isolated from both normal and diseased turbot, whereas *V. scophthalmi* has been isolated more frequently from diseased turbot infected with multiple pathogens such as slime bacteria and parasites. In the present study, when olive flounder was superinfected with *S. parauberis*/HVS A19008, the cumulative mortality increased greatly from 25 to 87.5 % at a bacterial concentration of $10^7$ CFU/fish group, and from 0 to 50 % at $10^6$ CFU/fish group, compared to that seen with *S. parauberis* single infection (25 % cumulative mortality). During superinfection, the virulent strain can take over a host that is already infected with a weak virulent strain. As a consequence, hosts infected by multiple pathogens transmit only the most virulent strain, and enhance the selection of faster replicators [61, 62]. The results of co-culture of *S. parauberis* and HVS A19008 demonstrated that HVS A19008 multiplied more ($10^{10}$ CFU/ml) under co-culture than in single culture ($10^8$ CFU/ml) after 24 h (personal communication). The high cumulative mortality rates seen may therefore be related to the faster replication that occurs under superinfection conditions. Although *V. scophthalmi* showed moderate or weak virulence towards olive flounder, the exact roles of this bacterium in fish disease merits further study due to its frequent presence in both normal and diseased fish.

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