Fish Sci (2013) 79:99–109
DOI 10.1007/s12562-012-0567-4

ORIGINAL ARTICLE

Aquaculture

Pathogenicity comparison of high- and low-virulence strains of *Vibrio scophthalmi* in olive flounder *Paralichthys olivaceus*

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Received: 24 July 2012 / Accepted: 11 October 2012 / Published online: 27 November 2012

Abstract *Vibrio scophthalmi*, a bacterial pathogen of olive flounder *Paralichthys olivaceus*, exhibits strain-dependent virulence. No information is available on the comparative pathogenicity of different strains of *V. scophthalmi* toward olive flounder. In this study, high- and low-virulence strains (HVS and LVS, respectively) were compared in terms of their pathogenic characteristics, including adhesion and survival, superoxide dismutase (SOD) activity, and extracellular products (ECP) of bacterial cells. The cell-mediated defense of macrophages from olive flounder against *V. scophthalmi* infection in vitro was also investigated. The results demonstrated that the SOD activity of the HVS was higher than that of the LVS. The number of viable cells of the HVS in serum increased by two log units after 18 h, whereas that of the LVS decreased. The number of cells of the HVS in skin mucus increased significantly while that of the LVS remained constant. The LD_{50} values of the HVS and LVS ECP toward olive flounder were 10.14 and 15.99 μg protein/g fish, respectively. The ECP were positive for naphthol-AS-BI-phosphohydrolase, lipase, gelatinase, and leucine arylamidase. The extracellular O_2^- overflow and intracellular O_2^- concentration of macrophages induced by the HVS were lower than those induced by the LVS. Significantly more nitric oxide was produced by the HVS than by the LVS.

Keywords *Vibrio scophthalmi* · Virulence factors · Olive flounder · Macrophages · Biophylaxis

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Introduction

Vibrio scophthalmi was first reported as a species commonly found in the bacterial population from the intestine of juvenile turbot *Scophthalmimus maximus* in Spain, and was characterized as a novel species of genus *Vibrio* based on its phenotypic characteristics, DNA G+C content, 16S rRNA gene sequence, and DNA–DNA hybridization similarity [1]. *V. scophthalmi* has subsequently been isolated from olive flounder *Paralichthys olivaceus* and diseased turbot in Asia [2, 3]. It was reported to be one of the main bacterial species present in diseased olive flounder [2], and it exhibited strain-dependent virulence [4]. Under stressed conditions, olive flounder were found to become more sensitive to *V. scophthalmi*. Infected olive flounder showed symptoms such as skin darkening, distended abdomen, hemorrhage of liver and intestine, ascites, and hypertrophy of spleen and kidney [4]. However, little information is

available on the pathogenic characteristics of this species. The enzymatic properties and virulence of proteases from *Vibrio* species that are pathogenic to fish and shellfish have been studied in depth [5, 6]. Montero and Austin [7] reported that virulence factors of extracellular proteins (ECP) such as hemolysin, proteases, acid phosphatase (ACP), and cytotoxins played significant roles in the progress of the disease. Remaley et al. [8] noted that parasite-derived ACP inhibited the production of host-derived reactive oxygen species (ROS) by stimulated hemocytes during a respiratory burst. Proteolytic activity by leucine arylamidase has been observed in *Vibrio* and others [9]. There is currently no information available on the comparative pathogenicity of different strains of *V. scophthalmi* toward olive flounder. In the present study, we compared two strains of *V. scophthalmi* with different virulences in terms of their pathogenic characteristics, including cell surface hydrophobicity, slime layer and biofilm formation, SOD and catalase activities, survival activities with respect to the host immune mechanism, and characteristics of ECP. In addition, we evaluated the host immune defense against *V. scophthalmi* infection using olive flounder macrophages.

Materials and methods

Bacterial strains

In this study, two strains of *V. scophthalmi* with different virulences (a high-virulence strain, HVS, A19008; and a low-virulence strain, LVS, A19010) were used. The HVS was isolated from kidney and the LVS was obtained from spleen of olive flounder. The HVS was highly virulent and the LVS showed relatively low virulence toward olive flounder [4]. These strains were stored at −80 °C using tryptic soy broth (TSB) supplemented with 2 % NaCl and 10 % glycerol. Prior to the experiments, the bacterial strains were subcultured on ST (tryptic soy agar supplemented with a final 2 % NaCl) at 27 °C for 24 h.

Virulence factors of *V. scophthalmi*

Adhesion ability of V. scophthalmi

The cell surface hydrophobicity of *V. scophthalmi* was assessed using both a salt aggregation test (SAT) [10, 11] and a microbial adhesion to hydrocarbons (MATH) assay. The MATH assay was performed with the hydrophobic hydrocarbon *n*-hexadecane (Sigma, purity >99 %). The adherence was measured by counting colony forming units in a modified CFU MATH assay [12]. The fraction that partitioned to the hydrocarbon phase (%Adh) was calculated as:

$$\%\mathrm{Adh} = \left[1 - C_{\text{aqueous phase}} / C_{\text{original bacterial suspension}} \times 100\right],$$

where C refers to the number of colony forming units per ml in the bacterial suspension. Strains with %Adh ≥70 %, 50–70 %, and <50 % were arbitrarily classified as having high, moderate, and low hydrophobicity, respectively. Slime layer production was detected qualitatively by culturing on congo red agar plates (CRA). Slime layer producing bacteria appeared as black colonies, whereas bacteria that did not produce a slime layer remained non-pigmented [13]. Biofilm production was assessed on a polystyrene microtiter plate using a method described by Stepanovic et al. [14]. The biomass of biofilm was quantified by solubilizing the dye in 95 % ethanol and then measuring the absorbance of the resulting solution at 570 nm. The optical density (ODs) was obtained by calculating the mean absorbance for nine wells in triplicate, and this value was compared with the absorbance of negative controls (ODnc) [12]. ODnc was calculated for each microtiter plate separately. Biofilm production was determined according to the following criteria: ODs ≤ ODnc, no biofilm production; ODnc < ODs ≤ 2ODnc, weak biofilm production; 2ODnc < ODs ≤ 4ODnc, moderate biofilm production; and ODs > 4ODnc, strong biofilm production.

Preparation of ECP from V. scophthalmi

Extracellular products (ECP) were prepared according to the cellophane overlay method [15]. In brief, sterilized cellophane membrane sheets were placed onto the surface of a ST plate, the bacterial suspension was streaked and incubated, and then the cellophane overlay was transferred to a new Petri dish and washed with ice-cold sterilized phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Following centrifugation, the supernatant (comprising ECP) was filtered successively through 0.22 μm pore-size Millipore filters (Advantec MFS, Inc., Tokyo, Japan) and stored at −80 °C. None of the samples were frozen more than once. The protein concentration of the ECP was determined by the Bradford method [16] using bovine serum albumin (BSA) as standard.

Fish challenge test using ECP

Olive flounder with an average body weight of 8.7 ± 0.4 g were obtained from a fish farm in Jeju Island, maintained in static aquaria at 23 °C, and fed with commercial fish pellets (National Federation of Fisheries Cooperatives feed, Seoul, Korea) until they were acclimatized. The fish were injected intraperitoneally (IP) with 0.1 ml of ECP at concentrations of 3.89, 5.85, 8.71, 13.06, 19.59, and 29.38 μg protein/g fish,

Table 1 Comparison of the characteristics of high- and low-virulence strains of *Vibrio scophthalmi* (HVS A19008 and LVS A19010, respectively)

Characteristic	*V. scophthalmi*	
	HVS A19008	LVS A19010
Adhesion		
Slime layer	+	+
Biofilm production (OD_{570})	1.23 ± 0.54 (S)	1.20 ± 0.54 (S)
SAT (M)	0.2 (S)	0.9 (S)
CFU MATH (%Adh)	56.7* ± 3.2 (M)	81.9 ± 2.3 (S)
Motility		
Swimming (mm)	9.0** ± 0.3 (+)	5.6 ± 1.0 (+)
Swarming	−	−
Twitching	++	+
SOD activity (U/mg protein)	42.8* ± 0.4	35.8 ± 0.2
Catalase inhibition zone (mm)		
1.76 mmol[a]	27.8 ± 1.2	29.3 ± 0.5
0.88 mmol[a]	27.4 ± 1.0	28.7 ± 1.1
0.44 mmol[a]	26.7 ± 0.4	25.4 ± 1.2
0.22 mmol[a]	21.7 ± 1.2	22.9 ± 0.9
3% H_2O_2[b]	+	+
ECP		
Test method(s)		
APIzym kit		
Alkaline phosphatase	+	+[w]
Esterase (C4)	−	−
Esterase lipase (C8)	−	+[w]
Lipase (C14)	−	−
Leucine arylamidase	+	+[w]
Valine arylamidase	−	−
Crystine arylamidase	−	−
Trypsin	−	−
α-Chymotrypsin	−	−
Acid phosphatase	−	−
Naphthol-AS-BI-phosphohydrolase	+	+
α-Galactosidase	−	−
β-Galactosidase	−	−
β-Glucuronidase	−	−
α-Glucosidase	−	−
β-Glucosidase	−	−
N-acetyl-β-glucosaminidase	+	+
α-Mannosidase	−	−
α-Fucosidase	−	−
Standard method		
Phospholipase[#] against egg yolk	−	−
Lipase[#] against Tween 80	++++	+++
Caseinase	−	−
Gelatinase[##] (mm)	+ (368)	+ (94)
Hemolysis	−	−

Data are expressed as the mean ± SD

SAT salt aggregation test, *CFU MATH* colony-forming unit microbial adhesion to hydrocarbons, *M* moderate, *S* strong

+, positive; ++, strongly positive; −, negative; [w] weak reaction

[#] −, absence of degradative activity

[#] ++++, very pronounced activity with a diameter of the zone of clearing or opalescence of ≥9.5 mm

[##] Diameter of opalescence

* Significant difference between HVS and LVS (* $p < 0.05$, ** $p < 0.01$)

[a] H_2O_2 concentration per disc

[b] Effervescence in the presence of 3 % H_2O_2

respectively. Controls were inoculated with the same volume of sterilized PBS. Each group included eight fish. The fish were examined daily for seven days post-injection (dpi), and their external and internal clinical signs were recorded. 50 % lethal dose (LD_{50}) values were calculated as described by Muhammad [17].

Enzymatic activity of ECP

The caseinase, phospholipase, lipase activities of ECP were examined as previously described [15]. Hemolysis for olive flounder, starry flounder *Platichthys stellatus*, and sheep erythrocytes was detected as previously described [4]. Gelatinase activity was measured as follows: 50 µl of ECP were incubated with 450 µl of 0.8 % gelatin in PBS at 27 °C for 30 min. The reaction was stopped by adding 30 % TCA (w:v). The absorbance at 280 nm was determined on a microtiter plate, and an increase of 0.001 in A_{280} against a blank represented one unit of gelatinase. The other 19 enzymatic activities were determined using the APIzym kit (bioMérieux, Inc., Durham, NC, USA) according to the manufacturer's instructions (Table 1). Briefly, 60 µl of ECP (0.2 mg protein/ml) were inoculated into each well, which was then incubated at 30 °C for 4 h.

SDS-PAGE analysis of ECP

SDS-PAGE was carried out on a Mini GE 200 gel eluter apparatus (Bio-Rad, Hercules, CA, USA), as described by Li et al. [18].

SOD activity of V. scophthalmi

Superoxide dismutase activity was detected by a SOD assay kit (Dojindo, Kumamoto, Japan). One unit of SOD activity was defined as the amount of lysate that inhibited the rate of xanthine/xanthine oxidase-dependent cytochrome *c* reduction at 25 °C by 50 %. The specific activity was expressed in units per milligram of protein (U/mg protein).

H_2O_2 inhibition zone test of V. scophthalmi

A drop of 3 % H_2O_2 solution was added to 24 h cultured bacterial colonies on a ST plate in order to measure catalase activity based on the degree of brisk effervescence associated with the breakdown of H_2O_2 by endogenous catalase [19]. An H_2O_2 inhibition zone test was performed using a modified version of the method of Xu and Pan [20]. Briefly, 200 µl of bacterial suspension (10^7 CFU/ml) were spread evenly across the top of a ST plate. Three sterilized Whatman discs (6 mm in diameter) containing 20 µl of H_2O_2 solution at various concentrations [1.76 mmol (3 %), 0.88 mmol (1.5 %), 0.44 mmol (0.75 %), or 0.22 mmol (0.375 %)] were placed on the bacterial lawn, and inhibitory zones were recorded after incubation at 27 °C for 18 h.

Survival of *V. scophthalmi* in fish serum and skin mucus

Serum and skin mucus of olive flounder with an average body weight of 47.28 g were prepared according to the techniques described by Bordas et al. [21]. The bacterial cells were suspended in fresh serum or skin mucus (1:1, v:v) and adjusted to 1×10^6 CFU/ml of bacterial cells. One hundred microliters of each sample were removed at 0, 1, 3, 6, and 18 h after incubation at 25 °C, and ten-fold serial dilutions in PBS were spread onto ST plates. The survival rate of each strain in serum or skin mucus was defined as the number of viable bacteria after co-culture divided by the initial bacterial count, as described previously [22]. The experiments were conducted in triplicate and results are presented as the mean ± SD.

Macrophage defense against *V. scophthalmi* infection

Macrophage monolayer preparation

A monolayer of head kidney macrophages in olive flounder was prepared as described by Secombes [23] with slight modifications. Briefly, the head kidney was removed and pushed through a sterile nylon mesh with L-15 medium (Gibco, Carlsbad, CA, USA) containing 2 % fetal bovine serum (FBS, Gibco), penicillin (100 IU/ml), streptomycin (100 µg/ml), and heparin (10 U/ml) (Sigma, St. Louis, MO, USA). The cell suspension was layered onto a 34–51 % Percoll density gradient in siliconized tubes on ice and centrifuged at 400×*g* and 4 °C for 30 min. The band of macrophages lying above the Percoll interface was harvested and washed with L-15 medium containing 0.1 % FBS. The cell pellet was suspended in L-15 medium supplemented with 0.1 % FBS, penicillin (100 IU/ml), and streptomycin (100 µg/ml), and the cell viability was determined by staining with 0.5 % trypan blue [24]. The macrophages could be used if the survival rate was more than 95 % after separation. Nonadherent cells were washed off after 3–5 h at 18 °C, and the remaining monolayer was fed with L-15 medium (containing 5 % FBS, 100 IU/ml penicillin, and 100 µg/ml streptomycin) and maintained at 18 °C for 1–3 days before use.

Phagocytosis assay

The phagocytic activity of the macrophages was evaluated according to the procedure described in [25] with a slight modification. Each strain of bacteria was added at 5×10^4 cells/well to the macrophage suspension (1×10^5 cells/well) in L-15 medium supplemented with 10 % FBS. After incubation at 18 °C for 1 h, norfloxacin (16.9 µg/ml) was added to the cell mixture and incubated at 18 °C for 1 h to kill any remaining extracellular bacteria. The cells were washed with PBS to remove norfloxacin, and 20 µl of the ice-cold 0.1 % Triton X-100 was added to each well to lyse cells. The number of viable intracellular bacteria was then quantified on a ST plate by the colony-forming unit method. The phagocytosis assay for each bacterial strain was conducted in triplicate, and the number of viable intracellular bacteria for each assay was quantified in triplicate. To visualize the internalized bacteria, the macrophages incubated with each strain of bacteria and subsequently treated with norfloxacin were stained using Giemsa solution. The average number of bacteria per macrophage was determined by counting the number of bacteria associated with macrophages. Ten macrophages were observed for each assay.

Bactericidal assay

The bactericidal assay was performed according to the description of Graham et al. [26]. The difference in the optical density readings at any one bacterial concentration between times T_o and T_x during incubation represented the degree of killing. The bactericidal assay for each bacterial strain was conducted in triplicate.

Superoxide anion (O_2^-) detection assay

Extracellular O_2^- was quantified by reduction of ferricytochrome *c* (Sigma). Briefly, the macrophage suspension was

covered with 100 µl of a 160 µmol cytochrome *c* solution dissolved in phenol red-free Hank's balanced salt solution (HBSS) with phorbol 12-myristate-13-acetate (PMA, Sigma) at 1 µg/ml. To test the specificity, the macrophage suspension and 100 µl of cytochrome *c* solution containing PMA and superoxide dismutase (SOD, Sigma; 300 U/ml) were prepared in triplicate. The samples were mixed, incubated at 18 °C for 90 min, and the optical density was read at 550 nm against cytochrome *c* as a blank. Optical densities were converted into nmol O_2^- by subtracting the OD of the PMA/SOD-treated supernatant from that treated with PMA alone for each fish and then multiplying by 15.87 [27]. The final results were adjusted to nmol O_2^- produced per 10^5 macrophages. To detect the intracellular O_2^-, nitroblue tetrazolium (NBT) was dissolved at 1 mg/ml in L-15 medium containing PMA (1 µg/ml) and SOD (300 U/ml). Briefly, the infected macrophage monolayer was covered with NBT solution at 18 °C for 30 min. During this reaction, NBT is reduced by O_2^- into an insoluble blue formazan. The reduced formazan within macrophages was solubilized in 120 µl of KOH (2 M) and 140 µl of DMSO (100 %) after mixing and washing with 100 and 70 % methanol, respectively. The optical density of the turquoise-blue colored solution was read at 620 nm using KOH/DMSO without bacteria as a blank. The extracellular O_2^- and intracellular O_2^- detection assays for each bacterial strain were conducted in triplicate separately.

Nitric oxide (NO) assay

NO production was determined by the Griess method [28]. Briefly, 100 µl of adherent macrophages (2×10^4 cells/well) in serum-free L-15 medium were treated with 20 µl of bacterial suspension (6.5×10^6 cells/well) at 18 °C. After 24 h of incubation, 50 µl of macrophage supernatant were transferred to one well of a 96-well plate, and the NO level was determined by the Griess method. One hundred microliters of 1 % sulfanilamide in 2.5 % phosphoric acid were added to each well following 100 µl of 0.1 % *N*-1-naphthlenediamine dihydrochloride (Sigma) in 2.5 % phosphoric acid. After incubating with the Griess reagent at room temperature for 20 min, the absorbance was measured at 540 nm. The experiments were conducted in triplicate. The molar concentration of nitrite in the sample was determined from standard curves generated using known concentrations of sodium nitrite.

Statistical analysis

All of the results are expressed as the mean ± SD. The characteristics of the high- and low-virulence strains of *V. scophthalmi* were compared using Student's *t* test and Tukey's multiple comparison post hoc analysis following one-way ANOVA. Probabilities of 0.05 or less were considered statistically significant.

Results

Virulence factors of *V. scophthalmi*

Adherence ability of V. scophthalmi

The results for the SAT values, cell partitioned to hydrocarbon (%Adh), biofilm production, and motility of *V. scophthalmi* are shown in Table 1. The SAT values of the HVS A19008 and the LVS A19010 were 0.2 and 0.9 M, respectively. The %Adh was 56.7 % for the HVS and 81.9 % for the LVS according to CFU MATH assay, respectively. The cell surface hydrophobicity was moderate for the HVS and strong for LVS based on the criteria published by Lee and Yii [29]. Both the HVS and the LVS formed black colonies with a slime layer on the CRA plate. Strong biofilm production was seen for both the HVS and the LVS, which showed absorbances of 1.23 and 1.20, respectively. The strains were motile, with swimming and twisting but no swarming.

Toxicity of ECP

For the HVS ECP infection groups, fish began to die at 2 dpi, and all of them died at 10 dpi while injected with 19.59 µg protein/g fish group. For 5.84 µg protein/g fish group, the cumulative mortality was 25 % within 14 dpi. For the LVS ECP infection groups, death occurred at 4 dpi, and the cumulative mortality was 50 % while injected with 19.59 µg protein/g fish group at the end of the experiment. No death was noted at 5.84 µg protein/g fish group. The LD_{50} values of the HVS and the LVS ECP were 10.14 and 15.99 µg protein/g fish, respectively. All of the dead fish showed similar symptoms to those challenged with live bacteria, including skin darkening, hemorrhage, and ascites [4].

Enzymatic activity of ECP

The results for enzymatic activity are shown in Table 1. Both the HVS and the LVS ECP demonstrated alkaline phosphatase, leucine arylamidase, naphtol-AS-BI-phosphohydrolase, *N*-acetyl-β-glucosaminidase, lipase against Tween 80, and gelatinase activities. Negative reactions were observed for esterase (C4), crystine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucosidase, α-mannosidase, α-fucosidase, lipase (C14), phospholipase against egg yolk, caseinase, and hemolysis on sheep, olive flounder, and starry flounder erythrocytes.

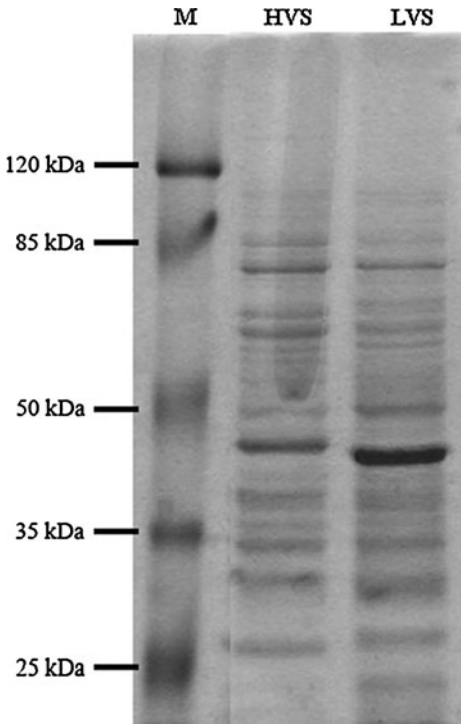

Fig. 1 SDS-PAGE of extracellular product proteins (ECP) extracted from high- and low-virulence strains of *Vibrio scophthalmi* (HVS A19008 and LVS A19010, respectively) in 12 % polyacrylamide gel

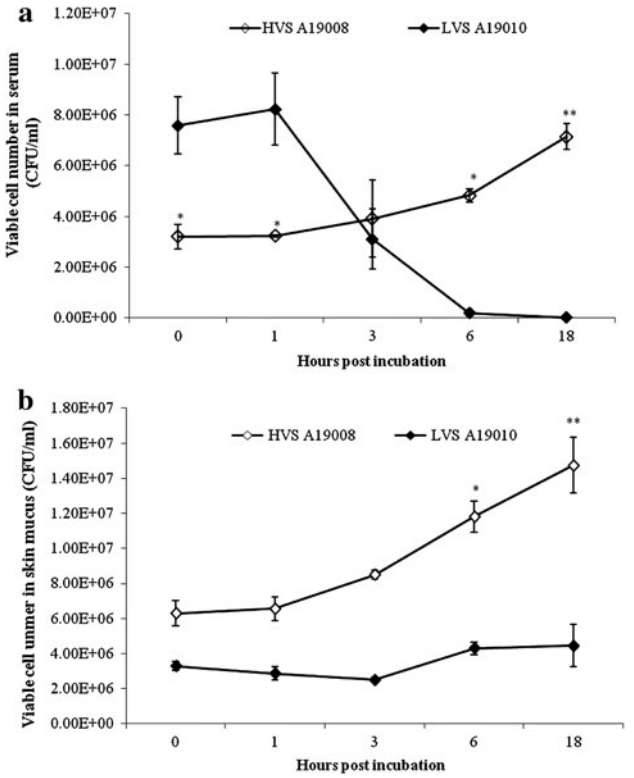

Fig. 2 Survival of the *Vibrio scophthalmi* HVS A19008 and LVS A19010 in olive flounder *Paralichthys olivaceus* serum (**a**) and skin mucus (**b**). * Indicates a significant difference in the number of viable cells between HVS A19008 and LVS A19010 ($p < 0.01$)

The HVS ECP was negative and the LVS ECP was positive for esterase lipase (C8).

SDS-PAGE analysis of ECP

The one-dimensional SDS-PAGE of ECP is listed in Fig. 1. The calculated regression equation was as follows:

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

where y represents the log of the molecular weight of standard proteins, x is the mobility of the protein band, and R^2 is the correlation coefficient. The HVS and the LVS ECP shared eight discrete banding profiles at M_r values of 82, 66, 63, 59, 50, 39, 34, and 26 kDa. The HVS ECP showed another band with a higher M_r of 45 kDa compared with that of the LVS ECP (43 kDa) (Fig. 1).

SOD activity of V. scophthalmi

The superoxide dismutase activities of the HVS and the LVS were 42.8 and 35.8 U/mg protein, respectively, and were thus significantly different (Table 1).

H_2O_2 inhibition zone test of V. scophthalmi

Both the HVS and the LVS were inhibited by discs containing different concentrations of H_2O_2, and they also showed effervescence in the presence of 3 % H_2O_2 (Table 1). The H_2O_2 inhibition zones against the HVS and the LVS were not significantly different.

Survival of *V. scophthalmi* in fish serum and skin mucus

The results for the survival of the strains in serum and skin mucus at 18 h are shown in Fig. 2. The HVS survived in fresh serum and increased its number of viable cells by approximately two log units. However, the LVS showed serum sensitivity and decreased its number of viable cells to below the limit of detection. The HVS was able to survive and replicate in skin mucus, with its number of viable cells increasing from 7.33×10^5 to 1.59×10^6 CFU/ml, whereas the number of viable cells of the LVS stayed at their original levels in skin mucus.

Macrophage defense against *V. scophthalmi* infection

Phagocytic activity

The phagocytic activity of olive flounder macrophages toward the HVS and the LVS was estimated by direct

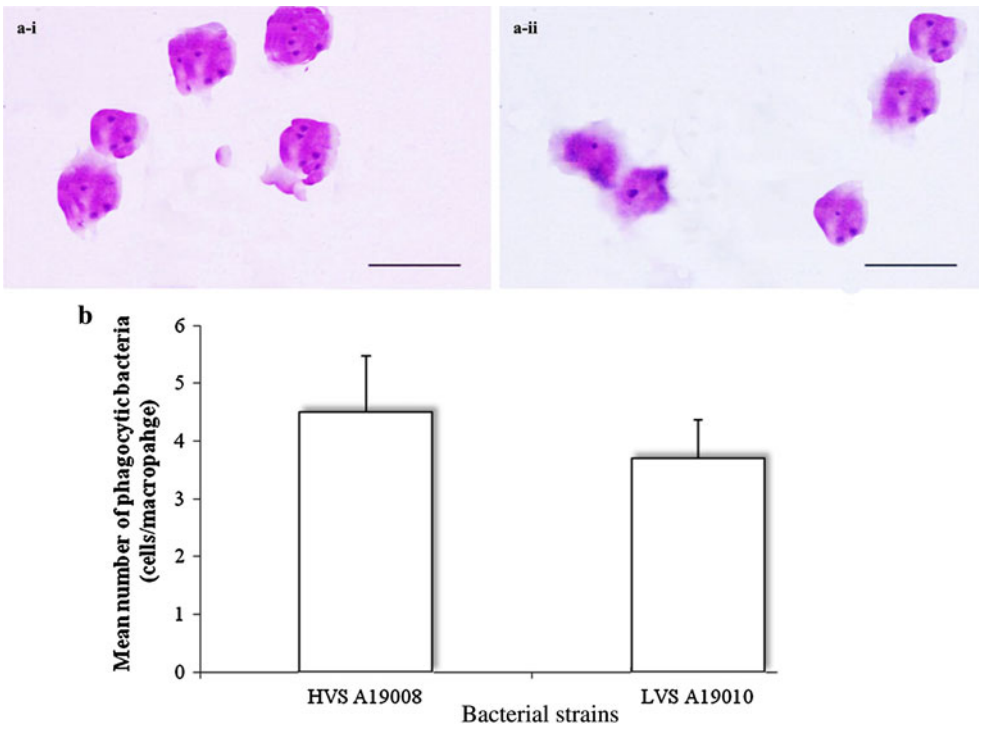

Fig. 3 Phagocytosis of macrophages from olive flounder head kidney after inoculation with bacterial suspensions of *Vibrio scophthalmi* HVS A19008 and LVS A19010. **a** After 1 h of incubation of the macrophages with the opsonized strain: *a-i* HVS A19008, *a-ii* LVS A19010. *Each bar* represents 10 μm. **b** Phagocytic index of macrophages in olive flounder after inoculation with each strain. No significant difference was observed ($p > 0.05$)

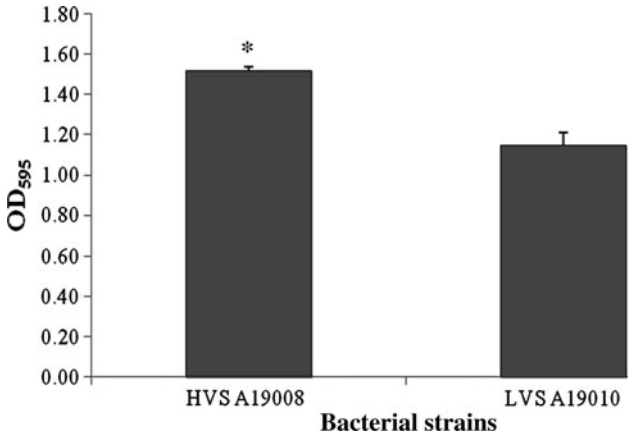

Fig. 4 Bactericidal activities of macrophages against *Vibrio scophthalmi* HVS A19008 and LVS A19010 after 5 h of incubation. Results are presented as the mean ± SD. * Indicates a significant difference between the results for HVS A19008 and LVS A19010 ($p < 0.01$)

microscopic observation. As shown in a-i and a-ii of Fig. 3, both of the bacterial strains were ingested by macrophages. The phagocytic index of the HVS per macrophage was slightly higher than that of the LVS (Fig. 3b).

Bactericidal activity

The bactericidal activities of macrophages stimulated by the HVS and the LVS for 5 h are shown in Fig. 4; they were found to be significantly different ($p < 0.01$).

Respiratory burst activity

The extracellular O_2^- overflow of macrophages (EOOM) stimulated by the *V. scophthalmi* strains was significantly greater than that seen for the control group without bacteria. The EOOM was also higher after stimulation by the LVS than by the HVS ($p < 0.01$; Fig. 5). The intracellular O_2^- concentration of macrophages (IOCM) stimulated by the bacterial strains was significantly higher than that in the control group. The IOCM was greater after stimulation by the LVS than by the HVS ($p < 0.05$; Fig. 5).

Nitric oxide production

The total nitrite production of macrophages stimulated by the bacterial strains increased significantly in comparison with that seen for the control group (0.24 μmol). The nitrite production of macrophages was higher after stimulation by the HVS (11.00 μmol) than by the LVS (8.86 μmol) ($p < 0.05$).

Discussion

Bacterial adhesion to host external surfaces and tissues is an essential initial step in the colonization of host tissues and the subsequent occurrence of disease in pathogenic systems [30]. It is widely considered that hydrophobicity and biofilm production are determining factors in the adhesive process and survival of pathogens in cells [31]. In this study, both the HVS and the LVS of *V. scophthalmi*

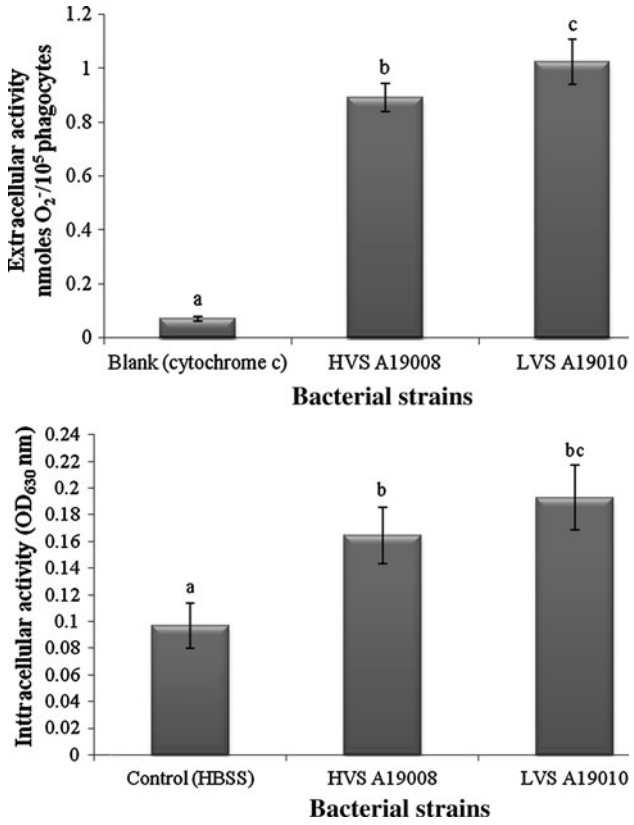

Fig. 5 Extracellular and intracellular superoxide anion production in macrophages stimulated by *Vibrio scophthalmi* HVS A19008 and LVS A19010 for 5 h. Results are presented as the mean ± SD. Means (*bars*) with *different lower case letters* are significantly different ($p < 0.01$)

exhibited moderate to strong hydrophobicity as determined by SAT and modified CFU MATH assay. This demonstrated the potential ability of *V. scophthalmi* to adhere to host external surfaces and tissues during the infection process. Won and Park [32] tested eight strains of *V. harveyi* isolated from the diseased olive flounder and found that the most virulent strain, FR-2, exhibited the highest biofilm formation, indicating that biofilm is one of the virulent factors of *V. harveyi*. In this study, the HVS showed greater biofilm formation than the LVS, which demonstrated that biofilm plays a role in the pathogenesis of *V. scophthalmi*. The initial attachment and development of biofilm requires some bacterial cell activities, such as flagella-mediated swimming or swarming motilities, and competition/virulence factors such as type IV pilus-based twitching motility [33]. These activities, mediated by the mechanism of phase variation, are employed by bacteria in order to survive under changing environmental conditions [33]. This study investigated three types of motilities and found that the HVS showed the higher level of motility, suggesting that biofilm formation may be related to competition/virulence factors.

The production of ECP by fish bacterial pathogens has been widely observed [34]. In this study, ECP was demonstrated to be one of the most important determinants of virulence in *V. scophthalmi* pathogenesis after toxicity analysis for olive flounder in challenge tests by ECP and LPS. The LD_{50} value of the HVS ECP toward olive flounder was 10.14 μg protein/g fish, which is a higher virulence than seen in other reports. The LD_{50} values toward olive flounder were reported to be 6.8–12.5 for *Edwardsiella tarda* and 7.98 μg protein/g fish for *V. harveyi* [35, 36]. However, the LVS ECP caused a low number of deaths in the challenge test. This suggests that some toxins were not produced under laboratory conditions or that ECP are not the only virulence factor of *V. scophthalmi*. The ECP probably play a virulent role together with other factors, such as biofilm production, LPS, OMP, etc. The ECP from both the HVS and the LVS of *V. scophthalmi* exhibited several enzymatic activities, such as alkaline phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, and *N*-acetyl-β-glucosaminidase. Compared to the LVS, the HVS ECP was positive for gelatinase and lipase against Tween 80, suggesting that these enzymes may contribute to the pathogenicity of *V. scophthalmi*. The HVS ECP was observed to be negative for esterase lipase (C8), whereas the LVS ECP was positive for this, which indicates that esterase lipase (C8) may not be a key enzyme in pathogenesis. The mechanism of action of lipolytic enzymes in virulence is still not clear and needs further study. The lipases may be mainly involved in energy metabolism, penetration of host cell membranes, or they may be essential for dissolving host-formed capsules [37]. β-glucosaminidase activity was present in both the HVS and the LVS ECP, indicating a high ability to obtain energy from carbohydrates. Gelatinase allows the organisms to break down gelatin into smaller polypeptides, peptides, and amino acids that cross the cell membrane and are utilized by organisms [38]. Leucine arylamidase is a known virulence factor in *Vibrio* and *Aeromonas*, and allows spoilage bacteria to invade and destroy the muscle structure [39]. However, cytotoxic and hemolytic activities were not observed for *V. scophthalmi*, implying that these factors were not main virulence factors, in contrast to their importance in other pathogenic species such as *V. harveyi* and *E. tarda* [32, 40]. Hemolytic activity can cause the lysis of erythrocytes from various animal species, but it still cannot be accepted as an indicator of virulence, since its correlation with pathogenicity is not yet entirely clear [15]. Although this study was unable to precisely determine which enzymes are virulence factors, naphthol-AS-BI-phosphohydrolase, lipase, gelatinase, and leucine arylamidase may play a role in its pathogenicity and need to be addressed in future research.

Bacteria with aerobic metabolism encounter constant risk from toxic ROS, such as superoxide radical (O_2^-),

hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) [41]. Accumulation of the highly toxic superoxide anion causes serious DNA damage, lipid peroxidative injury, and enzyme inactivation [42, 43]. Therefore, superoxide anion must be eliminated for bacterial cells to survive the host immune response. SOD and catalase are generally considered to be antioxidant enzymes with respect to bacterial survival within the host. SOD of *P. damselae* subsp. *piscicida* and *E. tarda* have been reported to greatly increase bacterial resistance to death from oxygen radicals [40, 44]. The present study showed that the SOD activity of the HVS was higher than that of the LVS, suggesting that SOD is an important resistance factor of *V. scophthalmi* against host phagocyte-mediated killing, and aids its survival in olive flounder serum and skin mucus. The HVS was able to survive and replicate in the serum of olive flounder while LVS was unable to do so. This suggests that the survival and reproductive ability of *V. scophthalmi* in the host serum plays a major role in systemic infection. According to Han et al. [40], all virulent strains of *E. tarda* were able to survive and proliferate in serum, whereas the avirulent strain was susceptible to the bactericidal activity of the serum. Similarly, survival ability in fish mucus or serum has been suggested to be a good indicator of virulence in *V. harveyi* and *A. hydrophila* [22, 36].

The assay to determine the number of viable intracellular cells of the bacterial strains revealed that these strains were capable of invasion and replication within macrophages in vitro, and no significant difference in the number of bacteria ingested per macrophage was observed between the HVS and the LVS. Booth et al. [45] found that the number of bacteria of the virulent species *E. ictaluri* increased 2.6-, 5.1-, and 7.1-fold after 4, 8, and 12 h of incubation within channel catfish macrophages, suggesting *E. ictaluri* can survive and replicate within macrophages in a short time. The replication rate of the HVS within macrophages in vitro was greater than that of the LVS, which helps bacterial pathogens to release more virulence factors and cause host damage in vivo against its immune defense, and this replication ability of the HVS may be related to the stronger resistance to macrophage-mediated killing through its higher SOD activity. Therefore, the ability to survive and replicate within macrophages is a common virulence factor among several pathogenic bacteria. Fish macrophages generate bactericidal ROS during the respiratory burst or during phagocytosis of bacteria, which is an increase in glucose and oxygen consumption [46]. The consequence of the respiratory burst is that a number of oxygen-containing compounds are produced in order to kill the bacteria being phagocytosed, which is referred to as oxygen-dependent intracellular killing. This oxygen-dependent bactericidal mechanism has been reported in phagocytes of many different fish species [47, 48]. ROS of macrophages were significantly induced by *V. scophthalmi*. The measurement of O_2^- has been considered as a direct and accurate way of quantifying the intensity of the respiratory burst, since O_2^- is the first product to be released from the respiratory burst. The extracellular O_2^- overflow and intracellular O_2^- production of macrophages stimulated by the LVS were greater than those stimulated by HVS. Our results are in agreement with some previous studies. The virulent *E. tarda* strain induced only trace-level ROS production of olive flounder peritoneal macrophages in vitro, whereas the low-virulence strain induced ROS production at a significantly higher level, suggesting that the virulent strain failed to induce the oxidative burst and was able to replicate within phagocytes [49]. In contrast, it has been reported that the ROS production of rainbow trout macrophages induced by the high-virulence strain of *F. psychrophilum* was higher than that induced by the low-virulence strain, even though the virulent strain was more resistant to macrophage-mediated killing activity than the low-virulence strain [50]. Therefore, the ability to survive inside macrophages seems to depend on the bacterial species; different mechanisms may be evolved by different bacterial species to overcome the phagocyte-mediated defense system. In principle, there are two effective mechanisms for avoiding ROS-mediated phagocytic microbicidal activity: (1) blocking the elicitation of the respiratory burst or inhibiting the activity itself, or (2) neutralizing the ROS produced during the respiratory burst, as seen for *E. ictaluri* LPS [51]. The virulent strain of *E. tarda* prevented the activation of the ROS generation system in macrophages rather than causing the irreversible inhibition of activity itself [49]. Some bacteria neutralize ROS by producing SOD or catalase [44, 52]. The tested strains of *V. scophthalmi* did not inhibit the ROS activity itself, but neutralized the ROS produced by macrophages by producing SOD or catalase. Such an ability allows *V. scophthalmi* to survive and multiply within macrophages. Hence, resistance to macrophage-mediated bacterial killing plays an important role in the pathogenesis of *V. scophthalmi*. Another bactericidal mechanism is the production of NO, which is a gaseous free radical synthesized during L-arginine metabolism, as catalyzed by nitric oxide synthase (nos) and induced by macrophages when exposed to microbial and nonmicrobial stimuli [53, 54]. In mammals, at least three nos isoforms have been reported: the neural form (n-nos), the endothelial form (e-nos), and the inducible form (i-nos). These isoforms are expressed in different cell types, and a cell type may express two different isoforms. In all cases, NO is produced from arginine to generate citrulline [55]. The production of NO is regulated by the expression of i-nos, which is induced by TNF-α following the binding of bacteria to macrophages. The present study demonstrated that macrophages were capable of producing a high level of NO in response to *V. scophthalmi* infection. The HVS of *V. scophthalmi*

induced higher NO production than the LVS did. Previous studies showed that a higher level of NO was induced by a virulent strain of *E. tarda* in olive flounder and mouse macrophages [49]. Such an ability might be somehow responsible for the virulence of *V. scophthalmi*. *A. hydrophila* induced i-nos-mediated NO production and the secretion of TNF-α and other cytokines from kidney cells of zebra fish *Danio rerio* [56]. NO release was observed in kidney leukocytes of carp *Cyprinus carpio L.* during bacterial infection in vivo, and macrophages of rainbow trout against bacterial infection in vitro [57, 58]. Apart from their defensive roles in the host, excessive production of NO and TNF-α is also known to be toxic and injurious towards host cells and tissues [59, 60]. During sepsis shock or acute inflammation, excessive NO and TNF-α secretion can result in circulatory and multiple organ failure, and even lethal damage [61, 62]. In addition, TNF-α has been identified as a common mediator of hepatocellular apoptosis and liver injury in experimental mouse models [63]. These results suggest that inflammatory mediators such as NO and TNF-α that are produced by macrophages in response to invading bacteria are important in the immune defense system but also cause injury to host cells and tissues. Further studies are required to investigate the characterization and expression of TNF-α following different virulence strains of *V. scophthalmi* infection and the role of LPS in the stimulation of NO production.

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