Pang H, Qiu M, Zhao J, Hoare R, Monaghan S, Song D, Chang Y & Jian J (2018) Construction of a Vibrio alginolyticus hopPmaJ (hop) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (Epinephelus coioides), *Fish and Shellfish Immunology*, 76, pp. 93-100.

DOI: 10.1016/j.fsi.2018.02.012

Accepted Manuscript

Construction of a *Vibrio alginolyticus hopPmaJ* (*hop*) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (*Epinephelus coioides*)

Huanying Pang, Mingsheng Qiu, Jingmin Zhao, Rowena Hoare, Sean J. Monaghan, Dawei Song, Yunsheng Chang, Jichang Jian

PII: S1050-4648(18)30074-3

DOI: 10.1016/j.fsi.2018.02.012

Reference: YFSIM 5119

To appear in: *Fish and Shellfish Immunology*

Received Date: 15 November 2017

Revised Date: 31 January 2018

Accepted Date: 6 February 2018

Please cite this article as: Pang H, Qiu M, Zhao J, Hoare R, Monaghan SJ, Song D, Chang Y, Jian J, Construction of a *Vibrio alginolyticus hopPmaJ* (*hop*) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (*Epinephelus coioides*), *Fish and Shellfish Immunology* (2018), doi: 10.1016/j.fsi.2018.02.012.

Construction of a *Vibrio alginolyticus hopPmaJ* (*hop*) mutant and evaluation of its potential as a live attenuated vaccine in orange-spotted grouper (*Epinephelus coioides*)

Huanying Pang [ab, 1], Mingsheng Qiu[a, 1], Jingmin Zhao[a, 1],Rowena Hoare[c], Sean J. Monaghan[c], Dawei Song[ab], Yunsheng Chang[ab], Jichang Jian[ab*]

[a]College of Fishery, Guangdong Ocean University, Zhanjiang 524025, China

[b]Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang 524025, China; Guangdong Key Laboratory of Control for Diseases of Aquatic Economic Animals, Zhanjiang 524025, China

[c]Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK

[*]Corresponding author: Ji-Chang Jian

Tel./fax: +86-759-2339319;

Address: College of Fishery, Guangdong Ocean University, No. 40 of East Jiefang Road, Xiashan District, Zhanjiang, Guangdong Province, 524025, China;

E-mail:jianjic@gmail.com

1 These authors contributed to the work equally and should be regarded as co-first authors.

ABSTRACT

Vibrio alginolyticus, a bacterial pathogen in fish and humans, expresses a type III secretion system (T3SS) that is critical for pathogen virulence and disease development. However, little is known about the associated effectors (T3SEs) and their physiological role. In this study, the T3SE gene *hopPmaJ* (*hop*) was cloned from *V. alginolyticus* wild-type strain HY9901 and the mutant strain HY9901Δ*hop* was constructed by the in-frame deletion method. The results showed that the deduced amino acid sequence of *V .alginolyticus* HopPmaJ shared 78-98% homology with other *Vibrio* spp. In addition, the HY9901Δ*hop* mutant showed an attenuated swarming phenotype and a 2600-fold decrease in the virulence to grouper. However, the HY9901Δ*hop* mutant showed no difference in morphology, growth, biofilm formation and ECPase activity. Finally, grouper vaccinated via intraperitoneal (IP) injection with HY9901Δ*hop* induced a high antibody titer with a relative percent survival (RPS) value of 84% after challenging with the wild-type HY9901.Real-time PCR assays showed that vaccination with HY9901Δ*hop* enhanced the expression of immune-related genes, including MHC-Iα, MHC-IIα, IgM, and IL-1 β after vaccination, indicating that it is able to induce humoral and cell-mediated immune response in grouper. These results demonstrate that the HY9901Δ*hop* mutant could be used as an effective live vaccine to combat *V. alginolyticus* in grouper.

Key words: *Vibrio alginolyticus;* T3SS; *hopPmaJ*; live attenuated vaccine; *Epinephelus coioides*

1. Introduction

Vibrio alginolyticus，a Gram-negative motile rod bacterium, is the causative agent of Vibriosis which is a devastating fish disease prevailing in worldwide aquaculture industries and leads to extensive losses in a diverse array of commercially important fish including orange-spotted grouper (*Epinephelus coioides*), large yellow croaker (*Larimichthys crocea*), sea bream (*Sparus aurata* L), Kuruma prawn (*Penaeus japonicus*) and causes symptoms of septicemia, hemorrhaging, dark skin, and ulcers on the skin surface [1-4].Moreover, this pathogen has also been reported to cause diarrhea, otitis, and wound infections in humans [5-6]. Therefore, it is important to understand the pathogenesis of *V. alginolyticus* and to develop an efficacious vaccine to prevent Vibriosis.

The type III secretion system (T3SS) is a highly conserved apparatus among several Gram-negative bacteria，such as *Yersinia* spp., *Salmonella* spp. and *Shigella* spp [7-9], which delivers bacterial proteins, known as effectors, directly into host cells [10]. Many of these effectors are virulence factors that can trigger host-cell death and manipulate the innate and adaptive immune system [11-12].Although the T3SS machinery is often conserved among Gram-negative pathogens, the effectors differ widely in their function. Comparative genome analysis has demonstrated that T3SS of *V. alginolyticus* is similar to T3SS1 of *V. parahaemolyticus* [13], but little is known about the effectors of *V. alginolyticus.* Therefore functional characterization of T3SS effectors is necessary.

In a previous study, we identified a *V. alginolyticus* effector HopPmaJ [14],

which was homologue to the T3SEs HopPmaJ of *Chryseobacterium gleum* [15].However, its role in *V. alginolyticus* is still unknown. To better understand the function of HopPmaJ in the T3SS from *V. alginolyticus*, we first constructed a *hop* gene mutant, then investigated the physiology and pathogenicity of the Δ*hop* strain. Furthermore, we evaluated the immunoprotective potential of Δ*hop*, and found that the Δ*hop* mutant could be used as an effective live vaccine to combat *V.alginolyticus* in grouper.

2. Materials and methods

2.1 Bacterial strains and culture conditions

The bacterial strains, plasmids and cell line used in this work are listed in Table 1.*V. alginolyticus* wild-type strain HY9901 was isolated from *Lutjanus erythopterus*[16] and was utilized as the parent strain for constructing the deletion mutant Δ*hop*. *V. alginolyticus* was cultured on trypticase soy broth (TSB, Huankai Co Ltd., Guangzhou, China) or on 1.5% TSB agar plates (TSA) at 28℃. *Escherichia coli* strains were cultured in Luria-Bertani (LB, Huankai Co Ltd., Guangzhou, China) or on LB agar at 37℃. When required, the appropriate antibiotics were added: ampicillin (Amp, 100μgmL^{-1}); kanamycin (Km, 50μg mL^{-1}); chloramphenicol (Cm, 25μg mL^{-1}).

2.2 Orange-spotted Grouper

E. coioides (average weight 20.0 ±2.0g) were obtained from a commercial fish farm in Zhanjiang, China, and kept in seawater in a circulation system at 26-27℃ for two weeks before experiment. Prior to the experiment, sera were taken randomly from

three fish and tested by slide agglutination against formalin-inactivated *V. alginolyticus*. Internal organs (spleen, liver, and kidney) of grouper were also collected and tested by bacteriological recovery tests. Fish that were negative in the sera agglutination and bacterial analysis were used in this study.

2.3 Cloning and sequencing of the *hop* gene from *V. alginolyticus* HY9901

A pair of primers hop1 and hop2 was designed as showed in Table 2 according to the *V. alginolyticus* gene sequence (GenBank Number: NZ_AAPS00000000). PCR was performed in a Thermocycler (Bio-Rad, CA, USA) under the following optimized amplification conditions: an initial denaturation at 94 ℃for 4 min, followed by 35 cycles of 94 ℃ for 30 s, 41℃ for 30 s and 72 ℃ for 30 s. 5 μL of each amplicon was examined on 1% agarose gels, stained with ethidium bromide. The PCR product was recovered from the agarose gel to ligate into the pMD18-T vector and transformed into *E. coli* DH5α (Table 1). The inserted fragment was sequenced by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Similarity analyses of the determined nucleotide sequences and deduced amino acid sequences were performed by BLAST programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and aligned using the program Clustal-X (version 1.81). Protein analysis was conducted with ExPASy tools (http://expasy.org/tools/). Location of the domain was predicted using the InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/).

2.4 Construction of in-frame deletion mutant of *hop* gene

Overlap extension PCR was applied to generate an in-frame deletion of the *hop*

gene on the *V. alginolyticus* wild-type HY9901 chromosome [17]. The in-frame deletion of *hop* in the *V. alginolyticus* was generated according to the method of Rubires *et.al* [18].For the construction of Δ*hop,* two PCR fragments were generated from HY9901 genomic DNA. The first fragment was amplified using primers hop-for （contains a *Kpn*I site at the 5'-end） and hop-int-rev；whereas primers hop-int-for and hop-rev（contains a *Sma*I site at the 5'-end ）were used to amplify the second fragment. Both fragments containing a 20bp overlapping sequence and used as templates for the subsequent PCR procedure, which used primers hop-for and hop-rev. The resulting PCR product, containing a deletion from amino acid (aa) 46-342 of *hop,* was ligated into suicide vector pRE112[19] (Cm^r) to generate pRE-Δ*hop*. This recombinant suicide plasmid was transformed into *E. coli* MC1061*λpir* [18] and subsequently S17-1*λpir*[20]. The single crossover mutants were obtained by conjugal transfer of the resulting plasmid into *V. alginolyticus* HY9901. Deletion mutants were screened on 10% sucrose TSA plates. Its presence was subsequently confirmed by PCR and sequencing using primers hop-up and hop-down.

2.5 Characterization of the Δ*hop*

The Δ*hop* phenotype was characterized by cell morphology, growth ability, extracellular protease (ECPase) activity, biofilm formation, swarming motility, and fifty percent lethal dose (LD_{50}). Briefly, the wild-type HY9901 strain and the Δ*hop* were cultured in TSB for 18 h, and cell morphology was observed by scanning electron microscopy. To measure the growth level of bacteria in TSB, overnight cultures of the wild-type HY9901 strain and Δ*hop* mutant were inoculated into TSB

with an initial OD_{600} of 0.01, respectively. Samples were removed every 1 h and the optical density was measured at 600 nm. Extracellular protease (ECPase) activity was performed according to the method of Windle and Kelleher [21]. Biofilm formation was assayed using the crystal violet stain method described previously [22]. Swarming motility was assayed using the method described by Mathew *et al*. [23]; swarming diameter was measured after 24h incubation. The cell adherence was performed as previously described [24-25]. Confluent monolayers of fathead minnow epithelial cell line (FHM) (Table 1) [26] grown in 24-well plates were infected with HY9901Δ*hop* and HY9901, respectively.

LD_{50} of the wild-type and Δ*hop* were evaluated in *E. coioides*. Briefly, twenty grouper were injected intraperitoneally with 100μL HY9901 or Δ*hop* suspended in sterile phosphate buffered saline (PBS) containing 10^4-10^9cfu mL^{-1} with an injection of 100μL sterile PBS serving as a negative control, respectively. The fish were monitored for 14 days, and any fish that died were removed for bacteriological examination. The experiment was performed twice, and the LD_{50} values were calculated by the statistical approach of Reed and Muench [27].

2.6 Preparation of formalin-killed cell (FKC) suspensions

The *V. alginolyticus* FKC suspensions were produced as described by Zhou *et al*. [28]. Briefly, *V. alginolyticus* strain HY9901 was grown in TSB for 18 h. The cells were harvested by centrifugation at 5000×g for 10 min and suspended in 0.85% saline solution to 1×10^8cfu mL^{-1}. Formaldehyde was then added at a final concentration of 1% to inactivate the bacteria for 3 days. The killed bacteria were washed three times and

resuspended in PBS to 1×10^{8}cfu mL^{-1}. Confirmation of bacterial death was confirmed by incubating a culture for 48 h at 28 ℃ on TSA, and stored at -4℃ until use.

2.7 *E. coioides* vaccination

E. coioides were randomly divided into three groups with 80 fish per group. Prior to vaccination, the fish were anaesthetized by immersion in a 20 mg L^{-1} solution of tricainemethanesulfonate (MS-222, Sigma). Fish in the HY9901Δ*hop* group were injected intraperitoneally with 100μL 1×10^{5}cfu mL^{-1}Δ*hop*. Fish in the FKC group were injected intraperitoneally with 100μL 1×10^{8}cfu mL^{-1} FKC as previously described[28] .Control fish were injected intraperitoneally with 100 μL sterile PBS. All of fish were maintained at 26-28℃. The experiment was repeated three times.

2.8 Investigation of the livability of HY9901Δ*hop* in vivo post vaccination

The fish injected intraperitoneally with 100μL 1×10^{5}cfu mL^{-1}HY9901Δ*hop* extended to 7 days post vaccination. The organs including spleen and head-kidney were aseptically collected from day 1 to day 7. All the samples were weighed and homogenized in 1 ml PBS. The homogenates were serially diluted and plated in triplicate onto TCBS plates and incubated at 28℃ for 18 h. The bacteria counts were calculated by dividing the weights of the tissues and from the mean of three samples.

2.9 Analysis of antibody levels

During the experimental period from one to eight weeks post-vaccination, *E. coioides* serum-pools (from 3 fish) of each group were collected in order to measure antibody levels using ELISA as previously described [29].Microtiter plate wells were coated with 100 μL of *V. alginolyticus* FKC by overnight incubation at 4℃.Excess

177 cells were discarded, and wells were blocked with 100 μL of PBS containing 2%
178 bovine serum albumin (BSA) for 3 h at 22℃. After removing the blocking solution
179 and washing three times with PBS added with 0.05% Tween-20 (PBST), the wells
180 were incubated for 3 h at 22 ℃ with 100 μL of serially diluted *E. coioides* serum.
181 Antibody binding to the antigen was detected using *E. coioides* IgM monoclonal
182 antibody (1:10000) which was produced according to Li *et al.* [30], followed by rabbit
183 anti-mouse IgG-HRP (Wuhan Boster, Wuhan, China) at 1:20000 dilution, and colour
184 was developed with a chromogenic reagent TMB (tetrame-thylbenzidine) (Amresco,
185 Ltd,MA, USA) for 20 min with the reaction being stopped by the addition of 2.0 M
186 H_2SO_4. The plates were then read at 450 nm with a microplate reader (Bio-Rad,
187 Hercules, CA, USA). Sera were considered positive for anti-*V. alginolyticus* specific
188 antibodies if the absorbance was at least double of the control sera, and antibody titers
189 were scored as the highest positive dilution. .

190 2.10Immune-related gene expression analysis

191 Kidney and spleen samples were taken from three fish from each group
192 respectivetly at 1 day before challenge. Immune-related genes expression levels were
193 detected with real-time PCR. Primers for MHC-Iα, MHC-IIα, IgM, and IL-1β are
194 shown in Table 2. β-actin was used as internal reference. The procedures of RNA
195 extraction, cDNA synthesis, real-time PCR for analysis of immune gene expression
196 were described by Li *et al*. [31].

197 2.11 Challenge experiment.

198 Four weeks post immunization, *E. coioides* (n=30) were anesthetized and

challenged separately by IP inoculation of 100μL 1×10^{8}cfu mL^{-1} of *V. alginolyticus* HY9901 [28]. The relative percent survival (RPS) of post-challgenged fishes were measured per day in a 14-days time frame as previously described [32].

2.12 Statistical Analysis

Statistical analyses were performed using SPSS 17.0 (SPSS Inc., USA). The data obtained from analyses of bacterial counts, swarming diameter, biofilm formation, cell adherence, and agglutination titers were shown as X±SD, and the statistical significance of differences between the wild-type strain and Δ*hop* mutant, were determined using the Student's t-test. Group differences were determined by Duncan's test. Data was considered statistically significant when $p<0.05$.

3. Results

3.1 Cloning and mutagenesis of *hop* in *V. alginolyticus* HY9901

The *hop* gene consisted of an open reading frame of 345 bp encoding 114 amino acids with a predicted molecular mass of 12.78 kDa and a theoretical isoelectric point of 4.45. The nucleotide sequence of HY9901 *hopPmaJ* was deposited in the GenBank database under the accession number KX245315. Blast of deduced amino acid of HopPmaJ indicated that it has 78-98% identity with other *Vibrio* spp. And it shared the highest homology to HopPmaJ of *Vibrio parahemolyticus* (98%), which located in T3SS2. However, the role of HopPmaJ in *V. parahaemolyticus* has not been reported (Fig. 1).

To understand possible roles of HopPmaJ in *V. alginolyticus*, an unmarked *hop* deletion mutant was constructed by using overlap PCR and a double-selection strategy.

The mutant was confirmed by inability to grow on TSA supplemented with chloramphenicol, and verified by PCR by generating a fragment of approximately 655 bp (Fig.2).

3.2 Morphology, growth, activity of ECPase, biofilm formation, swarming motility, and the LD_{50} of HY9901Δ*hop*

Morphology was assessed by scanning electron microscopy. HY9901Δ*hop* showed no discernible morphological difference from HY9901 when cultured in TSB (Fig.3). HY9901Δ*hop* showed similar growth as the wild-type strain when cultured in TSB medium (Fig.4).

Biofilm formation is a multicellular behavior by which bacteria colonize surface of host tissue, leading to resistance to antibiotics and host immune-killing [33-34]. However, in the investigation of biofilm formation, we found there was no difference between the HY9901Δ*hop* and wild-type strain HY9901 during the incubation. The results indicated that *hop* gene may not have a role in the biofilm development of *V. alginolyticus* (Table 3).

ECP is a crucial virulence factor, and the activity of ECPase showed no difference between HY9901Δ*hop* and the wild-type strain ($p>0.05$). HY9901Δ*hop* showed a smaller swarming diameter than HY9901 ($p<0.01$) (Table 3).

The adherence rate (0.88%) of the HY9901Δ*hop* was 2 fold lower than that of the HY9901 (1.77%)($p<0.01$).This result indicates that the *hopPmaJ* gene may regulate the transcription of genes encoding cell surface components involved in the adhesion of *V. alginolyticus* to epithelial cells (Table 3).

243 LD_{50} levels of HY9901Δ*hop* were 3 logs higher than that of HY9901 ($p< 0.01$).
244 All of the dead fish exhibited the clinical symptoms of Vibriosis such as ulcers on the
245 skin, hemorrhagic and swelling in the liver and kidney. Bacteria were re-isolated from
246 the ulcers of the skin, liver and kidney of the grouper and identified as *V. alginolyticus*
247 by 16S rDNA sequencing. No disease signs or mortalities were detected within 2
248 weeks following challenge of the fish with doses less than 10^5cfu mL^{-1} of
249 HY9901Δ*hop*. HY9901Δ*hop* has almost no side effects in terms of growth
250 performance in *E. coioides*, when doses less than 10^5cfu mL^{-1}. These results indicated
251 that the *hop* gene contributes to the pathogenesis of *V. alginolyticus* (Table 3).

252 3.3 Investigation of the livability of HY9901Δ*hop* in vivo

253 HY9901Δ*hop* was able to disseminate into but survive transiently in fish
254 head-kidney and spleen then was gradually eliminated from the host body (Fig. 5).
255 The highest bacterial number was detected in spleen on day 3, followed by the
256 head-kidney.

257 3.4 Analysis of antibody levels

258 Grouper (*E. coioides*) were immunized with two different types of *V.*
259 *alginolyticus* vaccines, HY9901Δ*hop* and FKC. The immune response of grouper was
260 assessed by ELISA at week 1, 2, 3, 4, 5, 6, 7 and 8 after vaccination (Fig. 6). The
261 result indicated that the specific antibody titers of fish immunized with FKC and
262 HY9901Δ*hop* were markedly higher than those of in the control group ($p < 0.05$). In
263 the immunized group the antibody titer reached the highest level at week 4 ($p< 0.01$).
264 Compared to the FKC group, HY9901Δ*hop* vaccinated group had significantly greater

265 titer of *E. coioides* specific serum antibodies from week 3 pv ($p< 0.01$).

266 3.5 Immune gene expression in *E. coioides* following vaccination with HY9901Δ*hop*

267 qRT-PCR was carried out to analyze the transcription levels of genes encoding
268 MHC-Iα, MHC-IIα, IgM and IL-1β. The results showed that compared to FKC
269 injection, vaccination with HY9901Δ*hop* significantly increased the expression of
270 IL-1β, MHC Iα, MHC IIα and IgM genes in the spleen and head kidney ($p < 0.01$)
271 (Fig. 7). MHC-Iα and MHC-IIα are respectively responsible for humoral and cellular
272 mediated immunity. As proinflammatory factor, IL-1βcan induce the inflammatory
273 response. Taken together, all of the above results suggested that HY9901Δ*hop* can
274 effectively elicit protective immune responses in *E. coioides*.

275 3.6 Immune protective effects of HY9901Δ*hop* in *E. coioides*

276 *E. coioides* were vaccinated with FKC and HY9901Δ*hop* by intraperitoneal
277 injection, and challenged with the wild type HY9901 30 days pv. As shown in Fig. 8,
278 mortality in the control group administered PBS was 77.5%; whereas grouper
279 vaccinated with the HY9901Δ*hop* had low cumulative mortality of 12.5 %with a RPS
280 of 84 % ($p < 0.05$), and fish vaccinated with FKC had a RPS of 71%.

281

282 4. Discussion

283 Although the T3SS of *V. alginolyticus* is similar to T3SS1 of *V. parahaemolyticus*
284 with respect to gene synteny [13], it is unclear if the same regulatory mechanism is
285 employed by *V. alginolyticus*. As one of the T3SE, the *hopPmaJ* could play a crucial
286 role required for efficient attack in the host. This study included a characterization of

287 the physiology and pathogenicity of the T3SE gene *hopPmaJ* in *V. alginolyticus*.

288 Production of extracellular products (ECP) mainly including protease, hemolysin
289 and siderophore, are thought to be characteristics of the virulent strain of *V.*
290 *alginolyticus*[16,35]. Biofilm formation is a multicellular behavior by which bacteria
291 colonize the surface of host tissue, leading to resistance to antibiotics and host
292 immune responses [33-34]. Nevertheless, our results indicated that there was no
293 significant difference between HY9901 and HY9901Δ*hop* in morphology, growth,
294 biofilm, and ECP. Therefore, *hop* may not be responsible for these characteristics in
295 *V.alginolyticus*.

296 The flagella contributing to the swarming motilities could help bacteria access an
297 appropriate niche inside the host after *Vibrio* infection [36]. Quite a few studies have
298 shown that flagellin is essential for virulence, flagellum forming, normal motility and
299 symbiotic competence during initial squid light organ colonization of *Vibrio* [37]. In
300 the present study, the *hop* mutant of *V. alginolyticus* had suppressed swarming motility.
301 The results suggested that *hop* is a positive contributor to swarming motility in *V.*
302 *alginolyticus*, and might function indirectly through regulating the expression level of
303 *fla*, however this needs further investigation.

304 The first step of the bacterial infection is the adherence of bacteria to the surface
305 of host epithelial cells, which facilitates colonization on or penetration of the cells [38]
306 In the current study, we tested if *V. alginolyticus hop* contributes to bacterial adhesion
307 to FHM cells. The data from this work showed that the adherence rate (0.88%) of the
308 HY9901Δ*hop* was significantly lower than that of the HY9901 (1.77%) (p<0.01),

indicating *hop* is required for adhesion to FHM cells.

Several similar studies have demonstrated that mutants with deletion of T3SS effectors encoding genes display decreased virulence in mice, poultry, pigs, and humans [32, 39]. Furthermore a number of studies have shown that mutants deficient in the production of T3SE could induce high levels of long lasting protection against pathogeny [40-41]. In the current study, the LD_{50} of HY9901Δ*hop* was 3 logs higher than that of wild-type HY9901 and showed low or no lethality virulence in *E. coioides* when administered via i.p. injection (Table 3 and data not shown). Moreover our findings also show that HY9901Δ*hop* has almost no side effects in terms of growth performance in *E. coioides*. We evaluated the efficacy of HY9901Δ*hop* as a live attenuated vaccine (LAV) by injection route in an *E. coioides* model, resulting in a RPS of 84% 4-week post vaccination. The significantly enhanced specific antibody confirmed the immune responses in *E. coioides*.

It has already been confirmed that live attenuated vaccines can induce a more robust humoral and cell-mediated immune response than killed bacteria [42]. The increase of MHC I expression in the spleens was also found in golden pompano vaccinated with a *Streptococcus agalactiae* phoB mutant[43].MHC II is displayed on surface of antigen presenting cells (APC) to activate T-help cells to regulate immune network[44]. IL-1β, an important pro-inflammatory cytokine, can induce the inflammatory response by regulating the expression of other cytokines. Xiao *et al*. [40]. IgM gene expression can be induced by intraperitoneal injection with *Yersinia ruckeri* in rainbow trout [45].In this study, the elevated expression of immune-related

genes (MHC-Iα, MHC-IIα, IgM, and IL-1β), confirmed the stimulation of innate and acquired immune responses in *E. coioides*. Future work using immunohistochemical methods or flow cytometry sorting rather than qRT-PCR will further provide a deeper understanding of the protective immune mechanisms of HY9901Δ*hop* in *E. coioides* or other fish.

In conclusion, we have successfully constructed an in-frame deletion strain of HY9901Δ*hop* and investigated its physiology and pathogenicity. HY9901Δ*hop* exhibited a high level of protection against virulent *V. alginolyticus* challenge, and could elicit both humoral and cell-mediated immune responses in *E. coioides*. These results may provide further evidence for the importance of T3SE in *V. alginolyticus* and serve as a reference for further investigation on this virulence factor

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 31402344), Natural Science Foundation of Guangdong Province (No. 2017A030313174), Outstanding Young Backbone Teacher Cultivation Program of Guangdong Ocean University (HDYQ2015005), Project of Enhancing School with innovation of Guangdong Ocean University (GDOU2015050216); Basic research free exploration project of Shenzhen science and technology innovation Committee (57).

References

[1] H. Pang, L. Chen, R. Hoare, Y. Huang, ZaoheWu, J. Jian, Identification of DLD, by immunoproteomic analysis and evaluation as a potential vaccine antigen against

three Vibrio species in *Epinephelus coioides*, Vaccine 34(9) (2016) 1225-1231.

[2] J. Wu, Y.H. Shi, X.H. Zhang, C.H. Li, M.Y. Li, J. Chen, Molecular characterization of an IL-1beta gene from the large yellow croaker (*Larimichthys crocea*) and its effect on fish defense against *Vibrio alginolyticus* infection, Dong wu xue yan jiu = Zoological research 36(3) (2015) 133-141.

[3] M.C. Balebona, M.J. Andreu, M.A. Bordas, I. Zorrilla, M.A. Morinigo, J.J. Borrego, Pathogenicity of *Vibrio alginolyticus* for cultured gilt-head sea bream (*Sparus aurata* L.), Applied and environmental microbiology 64(11) (1998) 4269-4275.

[4] K.K. Lee, S.R. Yu, T.I. Yang, P.C. Liu, F.R. Chen, Isolation and characterization of *Vibrio alginolyticus* isolated from diseased kuruma prawn, *Penaeus japonicus*, Letters in applied microbiology 22(2) (1996) 111-114.

[5] X.C. Li, Z.Y. Xiang, X.M. Xu, W.H. Yan, J.M. Ma, Endophthalmitis caused by *Vibrio alginolyticus*, Journal of clinical microbiology 47(10) (2009) 3379-3381.

[6] G. Sganga, V. Cozza, T. Spanu, P.L. Spada, G. Fadda, Global climate change and wound care: case study of an off-season *Vibrio alginolyticus* infection in a healthy man, Ostomy/wound management 55(4) (2009) 60-62.

[7] K. Pha, L. Navarro, Yersinia type III effectors perturb host innate immune responses, World journal of biological chemistry 7(1) (2016) 1-13.

[8] A. Sukhan, T. Kubori, J.E. Galan, Synthesis and localization of the *Salmonella* SPI-1 type III secretion needle complex proteins PrgI and PrgJ, Journal of bacteriology 185(11) (2003) 3480-3483.

[9] A.D. Roehrich, E. Bordignon, S. Mode, D.K. Shen, X. Liu, M. Pain, I. Murillo, I. Martinez-Argudo, R.B. Sessions, A.J. Blocker, Steps for Shigella Gatekeeper Protein MxiC Function in Hierarchical Type III Secretion Regulation, 292(5) (2017) 1705-1723.

[10] C.J. Hueck, Type III protein secretion systems in bacterial pathogens of animals and plants, Microbiology and molecular biology reviews : MMBR 62(2) (1998) 379-433.

[11] B. Coburn, I. Sekirov, B.B. Finlay, Type III secretion systems and disease, Clinical microbiology reviews 20(4) (2007) 535-549.

[12] W. Salgado-Pabon, C. Konradt, P.J. Sansonetti, A. Phalipon, New insights into the crosstalk between Shigella and T lymphocytes, Trends in microbiology 22(4) (2014) 192-198.

[13] Z. Zhao, C. Chen, C.Q. Hu, C.H. Ren, J.J. Zhao, L.P. Zhang, X. Jiang, P. Luo, Q.B. Wang, The type III secretion system of *Vibrio alginolyticus* induces rapid apoptosis, cell rounding and osmotic lysis of fish cells, Microbiology (Reading, England) 156(Pt 9) (2010) 2864-2872.

[14] J.M. Zhao, M.S. Qiu, H.Y. Pang, D.W. Song, Y.S. Chang, Y.Huang, J.C. Jian, Molecular Cloning and Bioinformatics Analysis of T3SS Effector HopPmaJ from *Vibrio alginolyticu*, Agricultural Biotechnology 6(04)(2017)43-47.

[15] H. Guillon, F. Eb, H. Mammeri, Characterization of CSP-1, a novel extended-spectrum beta-lactamase produced by a clinical isolate of *Capnocytophaga sputigena*, Antimicrobial agents and chemotherapy 54(5) (2010) 2231-2234.

[16] S.H. Cai, Z.H. Wu, J.C. Jian, Y.S. Lu, Cloning and expression of the gene encoding an extracellular alkaline serine protease from *Vibrio alginolyticus* strain HY9901, the causative agent of vibriosis in Lutjanus erythopterus (Bloch), Journal of fish diseases 30(8) (2007) 493-500.

[17] S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77(1) (1989) 51-59.

[18] X. Rubires, F. Saigi, N. Pique, N. Climent, S. Merino, S. Alberti, J.M. Tomas, M. Regue, A gene (wbbL) from Serratia marcescens N28b (O4) complements the rfb-50 mutation of *Escherichia coli* K-12 derivatives, Journal of bacteriology 179(23) (1997) 7581-7586.

[19] R.A. Edwards, L.H. Keller, D.M. Schifferli, Improved allelic exchange vectors and their use to analyze 987P fimbria gene expression, Gene 207(2) (1998) 149-157.

[20] R. Simon, U. Priefer , A. Pühler. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1 (1983):784-791.

[21] H.J. Windle, D. Kelleher, Identification and characterization of a metalloprotease activity from *Helicobacter pylori*, Infection and immunity 65(8) (1997) 3132-3137.

[22] M.Fletcher, The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can J Microbiol 23(1977) 1-6.

[23] J.A. Mathew, Y.P. Tan, P.S. Srinivasa Rao, T.M. Lim, K.Y. Leung, Edwardsiella tarda mutants defective in siderophore production, motility, serum resistance and

419 catalase activity, Microbiology (Reading, England) 147(Pt 2) (2001) 449-457.

420 [24] P. Sharma, H. Lata, D.K. Arya, A.K. Kashyap, H. Kumar, M. Dua, A. Ali, A.K.
421 Johri, Role of pilus proteins in adherence and invasion of *Streptococcus agalactiae* to
422 the lung and cervical epithelial cells, The Journal of biological chemistry 288(6)
423 (2013) 4023-4034.

424 [25] H. Mikamo, A.K. Johri, L.C. Paoletti, L.C. Madoff, A.B. Onderdonk, Adherence
425 to, invasion by, and cytokine production in response to serotype VIII group B
426 Streptococci, Infection and immunity 72(8) (2004) 4716-4722.

427 [26] M. Gravell, R.G. Malsberger, A permanent cell line from the fathead minnow
428 (Pimephales promelas), Annals of the New York Academy of Sciences 126(1) (1965)
429 555-565.

430 [27] L.J. Reed, H. Muench, A simple method of estimating fifty percent endpoints.Am
431 J Epidemiol 27(1938) 493-497.

432 [28] Z. Zhou, H. Pang, Y. Ding, J. Cai, Y. Huang, J. Jian, Z. Wu, VscO, a putative
433 T3SS chaperone escort of *Vibrio alginolyticus*, contributes to virulence in fish and is a
434 target for vaccine development, Fish & shellfish immunology 35(5) (2013)
435 1523-1531.

436 [29] S.H. Cai, Y.C. Huang, Y.S. Lu, Z.H. Wu, B. Wang, J.F. Tang, J.C. Jian,
437 Expression and immunogenicity analysis of accessory colonization factor A from
438 *Vibrio alginolyticus* strain HY9901, Fish & shellfish immunology 34(2) (2013)
439 454-462.

440 [30] Q. Li, W. Zhan, J. Xing, X. Sheng, Production, characterisation and applicability

441 of monoclonal antibodies to immunoglobulin of Japanese flounder (Paralichthys
442 olivaceus), Fish & shellfish immunology 23(5) (2007) 982-990.
443 [31] W. Li, Z. Yao, L. Sun, W. Hu, J. Cao, W. Lin, X. Lin, Proteomics Analysis
444 Reveals a Potential Antibiotic Cocktail Therapy Strategy for Aeromonas hydrophila
445 Infection in Biofilm, Journal of proteome research 15(6) (2016) 1810-1820.
446 [32] M.J. Marsden, L.M. Vaughan, R.M. Fitzpatrick, T.J. Foster, C.J. Secombes,
447 Potency testing of a live, genetically attenuated vaccine for salmonids, Vaccine
448 16(11-12) (1998) 1087-1094.
449 [33] M.R. Parsek, P.K. Singh, Bacterial biofilms: an emerging link to disease
450 pathogenesis, Annual review of microbiology 57 (2003) 677-701.
451 [34] N. Verstraeten, K. Braeken, B. Debkumari, M. Fauvart, J. Fransaer, J. Vermant, J.
452 Michiels, Living on a surface: swarming and biofilm formation, Trends in
453 microbiology 16(10) (2008) 496-506.
454 [35] K.K. Lee, S.R. Yu, P.C. Liu, Alkaline serine protease is an exotoxin of *Vibrio*
455 *alginolyticus* in kuruma prawn, *Penaeus japonicus*, Current microbiology 34(2) (1997)
456 110-117.
457 [36] P.I. Watnick, C.M. Lauriano, K.E. Klose, L. Croal, R. Kolter, The absence of a
458 flagellum leads to altered colony morphology, biofilm development and virulence in
459 *Vibrio cholerae* O139, Molecular microbiology 39(2) (2001) 223-35.
460 [37] D.S. Millikan, E.G. Ruby, Vibrio fischeri flagellin A is essential for normal
461 motility and for symbiotic competence during initial squid light organ colonization,
462 Journal of bacteriology 186(13) (2004) 4315-4325.

463 [38] K. Rottner, T.E. Stradal, J. Wehland, Bacteria-host-cell interactions at the plasma
464 membrane: stories on actin cytoskeleton subversion, Developmental cell 9(1) (2005)
465 3-17.
466 [39] M. Matulova, H. Havlickova, F. Sisak, I. Rychlik, Vaccination of chickens with
467 *Salmonella* Pathogenicity Island (SPI) 1 and SPI2 defective mutants of Salmonella
468 enterica serovar Enteritidis, Vaccine 30(12) (2012) 2090-2097.
469 [40] J. Xiao, T. Chen, B. Liu, W. Yang, Q. Wang, J. Qu, Y. Zhang, *Edwardsiella tarda*
470 mutant disrupted in type III secretion system and chorismic acid synthesis and cured
471 of a plasmid as a live attenuated vaccine in turbot, Fish & shellfish immunology 35(3)
472 (2013) 632-641.
473 [41] Z. Lin, P. Tang, Y. Jiao, X. Kang, Q. Li, X. Xu, J. Sun, Z. Pan, Immunogenicity
474 and protective efficacy of a *Salmonella enteritidis* sptP mutant as a live attenuated
475 vaccine candidate, 13(1) (2017) 194.
476 [42] K.P. Killeen, V.J. DiRita, Live attenuated bacterial vaccines, New Vaccine
477 Technol. (2001) 151.
478 [43] X. Cai, B. Wang, Y. Peng, Y. Li, Y. Lu, Y. Huang, J. Jian, Z. Wu, Construction of
479 a Streptococcus agalactiae phoB mutant and evaluation of its potential as an
480 attenuated modified live vaccine in golden pompano, Trachinotus ovatus, Fish &
481 shellfish immunology 63 (2017) 405-416.
482 [44] T.J. Xu, S.L. Chen, Y.X. Zhang, MHC class IIa gene polymorphism and its
483 association with resistance/susceptibility to *Vibrio anguillarum* in Japanese flounder
484 (*Paralichthys olivaceus*), Dev. Comp. Immunol. 34 (2010) 1042-1050.

[45] S. Deshmukh, P.W. Kania, J.K. Chettri, J. Skov, A.M. Bojesen, I. Dalsgaard, K. Buchmann, Insight from molecular, pathological, and immunohistochemical studies on cellular and humoral mechanisms responsible for vaccine-induced protection of rainbow trout against *Yersinia ruckeri*, Clinical and vaccine immunology : CVI 20(10) (2013) 1623-41.

507

508 Table 1 Bacterial strains, plasmids and cell line used in this study

Strains , plasmids , cell line	Relevant characteristics	Source or references
V. alginolyticus HY9901	Wild type, isolated from diseased *Lutjanus sanguineus* off the Southern China coast	[16]
Δ*hop*	HY9901 carrying an in-frame deletion of $hop_{46\text{-}342}$	This study
E. coli DH5α	*sup*E44 Δ*lac*U169 (*φ*80 *lac*ZDM15) *hsd*R17 *recA*1 *gyrA*96 *thi*-1 *relA*1	Sangon
MC1061 (*λpir*)	*lacY*1 *galK*2 *ara*-14 *xyl*-5 *sup*E44 *λpir*	[18]
pRE112	pGP704 suicide plasmid, *pir* dependent, *oriT*, *oriV*, *sacB*, Cm^r	[19]
S17-1 (*λpir*)	Tp^r Sm^r *recA thi pro hsdR*$^-$*M*$^+$RP4 :2-*Tc* : *Mu*: Km Tn*7* *λpir*	[20]
MC1061-pRE-Δ*hop*	MC1061 containing plasmid of pRE-Δ*hop*, Cm^r	This study
S17-1-pRE-Δ*hop*	S17-1 containing plasmid of pRE-Δ*hop*, Cm^r	This study
pMD18-T	Cloning vector, Amp^r	TakaRa
pRE-Δ*hop*	pRE112 containing *hop* gene in-frame deletion of codons 46-342, Cm^r	This study
FHM	fathead minnow epithelial cell; Pen^R; $Strep^R$	[26]

509

510

511

512 Table 2 Sequences of primers used in this study.

Primer name	Primer sequence(5'-3')	references
Cloning primers		
hop_1	TTA TTT AGC GGT TAA A	This study
hop_2	ATG GAA TTA AAA TCG	This study
Mutant construction		
hop-for	GGGGTACCATGAACACGCGATGG(*Kpn* I)	This study
hop-int-rev	CTCTGGTGACGCTGCCAATACATCGTTTTCTGACTGGTGTTTA	This study
hop-int-for	TATTGGCAGCGTCACCAGAGTAAACACCAGTCAGAAAACGATG	This study
hop-rev	CCCCCGGG TCGAGCAGCATGTA(*Sma* I)	This study
hop -up	TAAACTTCGTTGCTACCGCC	This study
hop -down	AAACTTAATGCCTTCCCACC	This study
qPCR primers		
MHC-IαF	GCCGCCACGCTACAGGTTTCTA	This study
MHC-IαR	TCCATCGTGGTTGGGGATGATC	This study
MHC-IIαF	GGAGCCTCAGCCCAGCTTCA	This study
MHC-IIα R	CCAGTGGGAGGTCCTTCATG	This study
IgM F	TACAGCCTCTGGATTAGACATTAG	This study
IgM R	CTGCTGTCTGCTGTTGTCTGTGGAG	This study
IL-1β- F	ACACGGCTTTGTCGTCTTTC	This study
IL-1β- R	ACGCTGCTGGACCTTTATCG	This study
β-actin F	AAATCGCCGCACTGGTTG	This study
β-actin R	TCAGGATACCCCTCTTGCTCT	This study

513

514

515

516

517

518

519

520

521

522

523

524

525

526

Table 3 Characteristics of HY9901Δ*hop*

Characteristics	HY9901	HY9901Δ*hop*
Activity of ECPase (A_{442})[a]	0.08±0.01	0.11±0.01
Biofilm formation[b]	0.32±0.06	0.36±0.15
Swarming (mm)[c]	45±0.15	23±0.5**
Adherence rate (%)[d]	1.77±0.11	0.88±0.25**
LD_{50} (cfu mL^{-1})[e]	2.5×10^{5}	6.5×10^{8}**

Values are mean ± standard deviation for three trials. Significant differences between HY9901 and HY9901Δ*hop* indicated by asterisk. **p <0.01.

a Bacteria were incubated in TSB for 18 h at 28□.

b Bacteria were incubated in 96-well polypropylene plates for 48 h at 28□.

c Swarming diameters were measured after 24 h incubation on TSA containing 0.3% agar plates.

d Adherence rate were expressed as percentage of observed CFU relative to the total input bacteria.

e LD_{50} were evaluated in healthy *E. coioides* with an average weight of 20.0 ±2g.

548

549 **Figure legends**

550 Figure 1 Homology comparison of *V.alginolyticus* HY9901 T3SS Effector Protein HopPmaJ
551 *V.alginolyticus* HY9901 T3SS Effector Protein HopPmaJ; *V. alginolyticus* NBRC 15630 = ATCC T3SS
552 Effector Protein HopPmaJ Accession NC_022359.1 WP_005375560.1; *V.parahaemolyticus* serotype
553 O3:K6 (strain RIMD 2210633) T3SS Effector Protein; *V.harveyi* CMCP6-E0666 T3SS Effector Protein;
554 *V. genomosp.*T3SS Effector Protein，niRef90_UPI000474712C; *V.coralliilyticus* T3SS Effector Protein，
555 niRef90_U0ESZ4; *V.vulnificus.* T3SS Effector Protein HopPmaJ, UniRef90_E8VUS9; *V. orienta* T3SS
556 Effector Protein HopPmaJ, UniRef90_C9QFQ5; *Flavobacterium* T3SS Effector Protein,
557 UniRef90_UPI00047A9F35

558

559 Figure 2 Construction and confirmation of the knockout mutant strain HY9901Δ*hop*
560 M: DL2000 marker; Lane 1. The 313bp upstream fragment amplified from genomic DNAs of the
561 wild-type strain HY9901using primer pairs of hop-for / hop-int-rev. Lane 2. The 342bp downstream
562 fragment amplified from genomic DNAs of the wild-type strain HY9901using primer pairs of
563 hop-int-for / hop-rev.Lane 3. The 655 bp fragment amplified from genomic DNAs of HY9901Δ*hop*
564 using primer pairs of hop-for / hop-revLane 4. The 952 bp fragment amplified from genomic DNAs of
565 the wild-type strain HY9901using primer pairs of hop-for / hop-rev.

566

567 Figure 3 Observation the morphological feature of HY9901(A) and HY9901Δ*hop* (B)by SEM.

568

569 Figure 4 Growth features of HY9901Δ*hop* and HY9901. Aliquots of cell culture were taken at various
570 time points and measured for cell density at OD_{600}.

571

572 Figure 5 Propagation of HY9901Δ*hop* in grouper kidney (A) and spleen (B) following i.p. injection
573 with 100μL 1×10^5cfu mL^{-1}Δ*hop*. Control fish were i.p. injection with 100 μL sterile PBS The number
574 of viable bacteria was shown as the mean ± standard of three samples.

575

576 Figure 6 Antibody titers in sera of grouper injected IP with HY9901Δ*hop*, FKC and PBS. Sera
577 collected at week 1to 8 post-vaccination were assayed by ELISA. Each column represents the mean of
578 log 2 antibody titer with standard deviation bar. Groups that do not share a letter are significantly
579 different ($p < 0.01$).

580

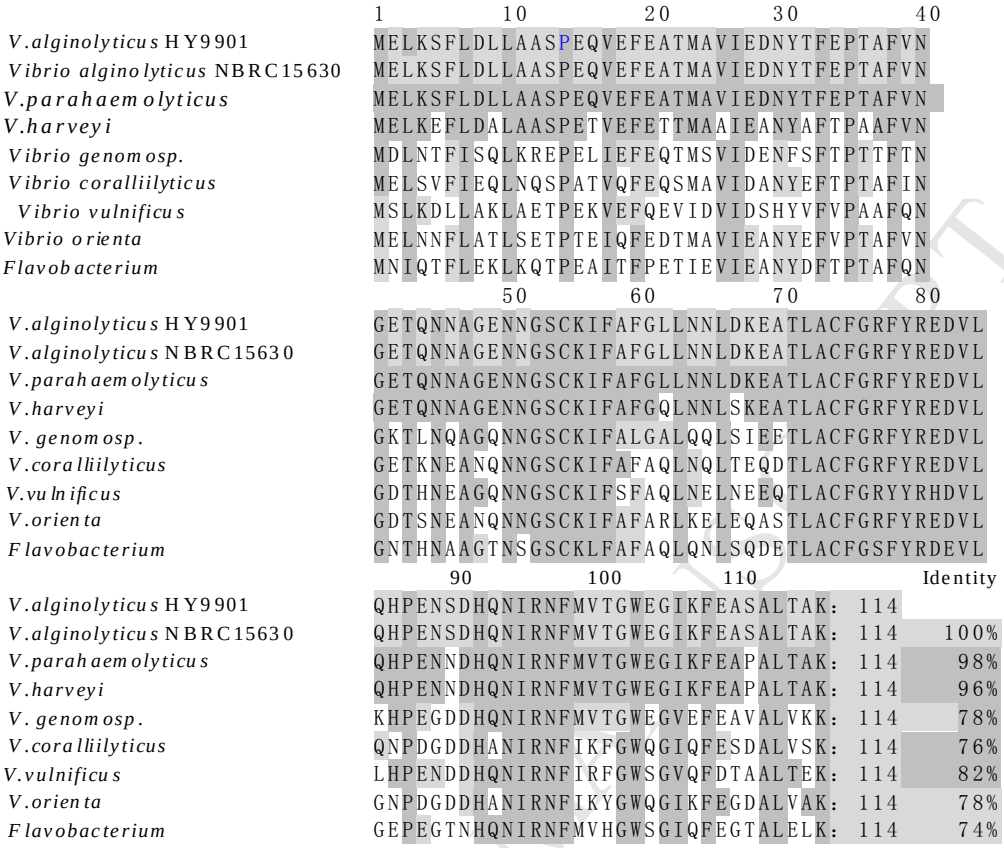
581 Figure 7.The head kidney and spleen of grouper were sampled at 1 day before challenge, and total
582 RNA was extracted for qRT-PCR. The mRNA level of each immune-related gene was normalized to
583 that of β-actin. Bars represent the mean relative expression of three biological replicates and error bars
584 represent standard deviation. Groups that do not share a letter are significantly different ($p < 0.01$).

585

586 Figure 8 Percent survival in groups vaccinated with HY9901Δ*hop*, FKC and PBS following challenge
587 with *Vibrio alginolyticus* HY9901.

588

589



590

591 **Fig. 1**

592

593

594

595

596

597

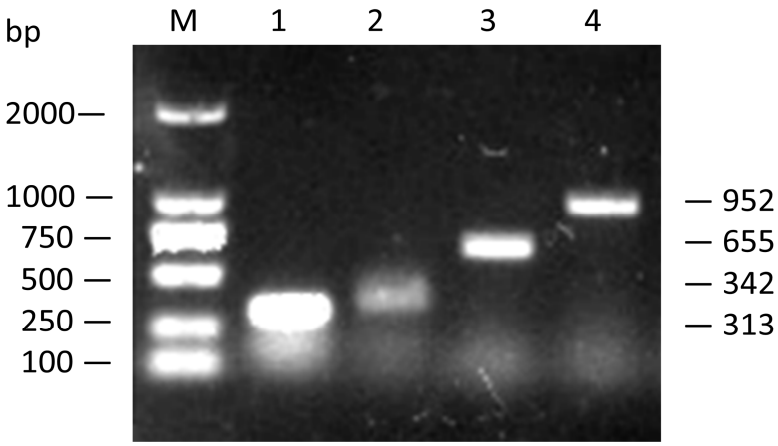
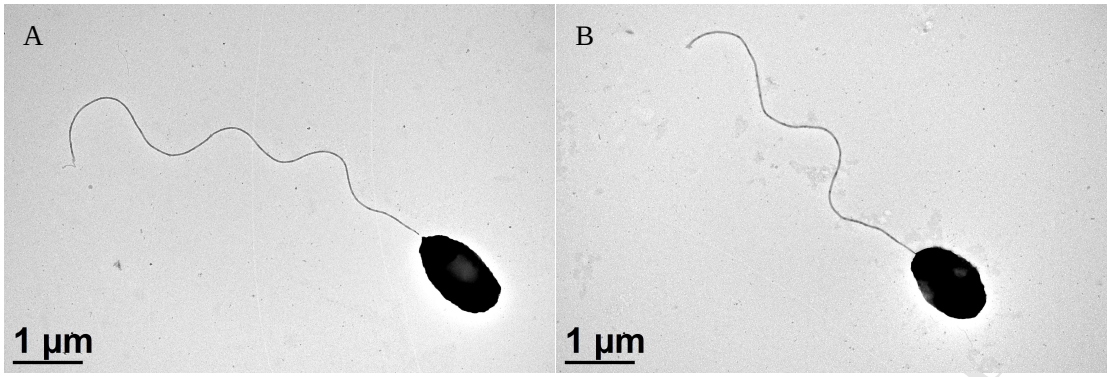


Fig. 2



HY9901 **HY9901Δ*hop***

Fig. 3

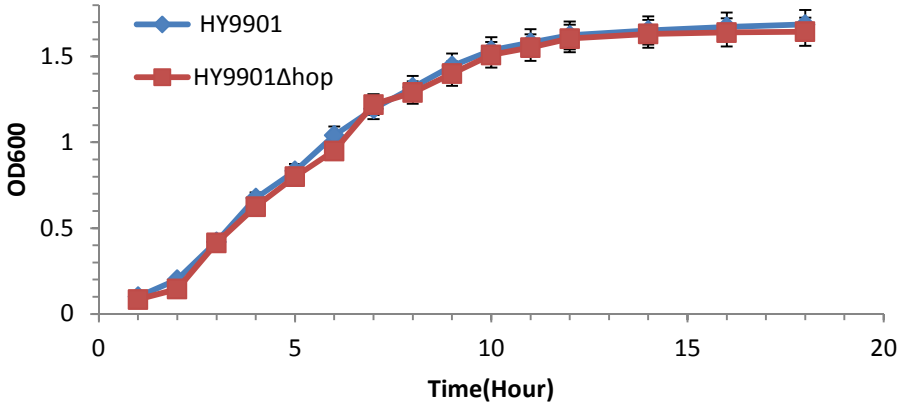


Fig. 4

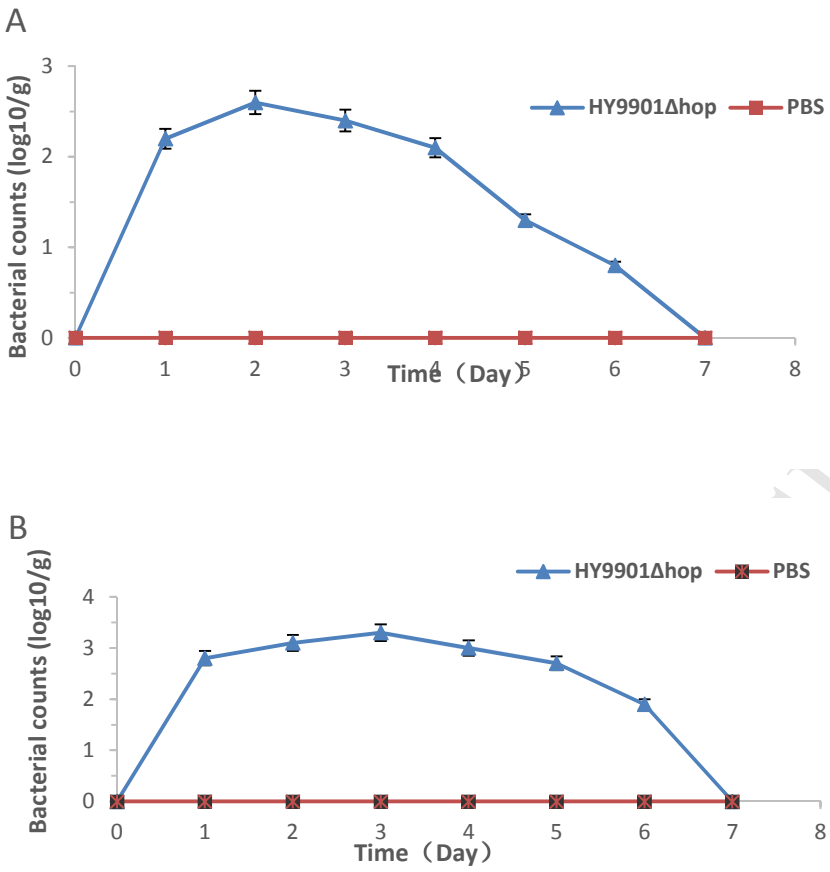


Fig. 5

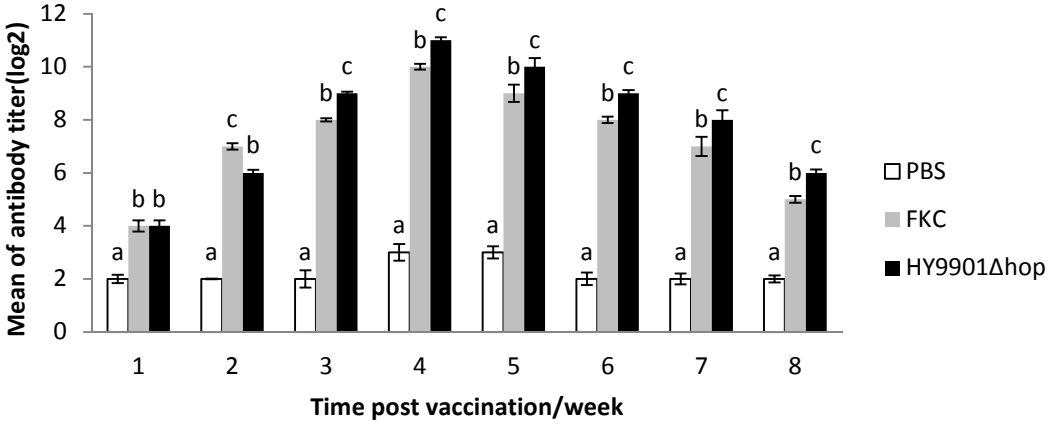
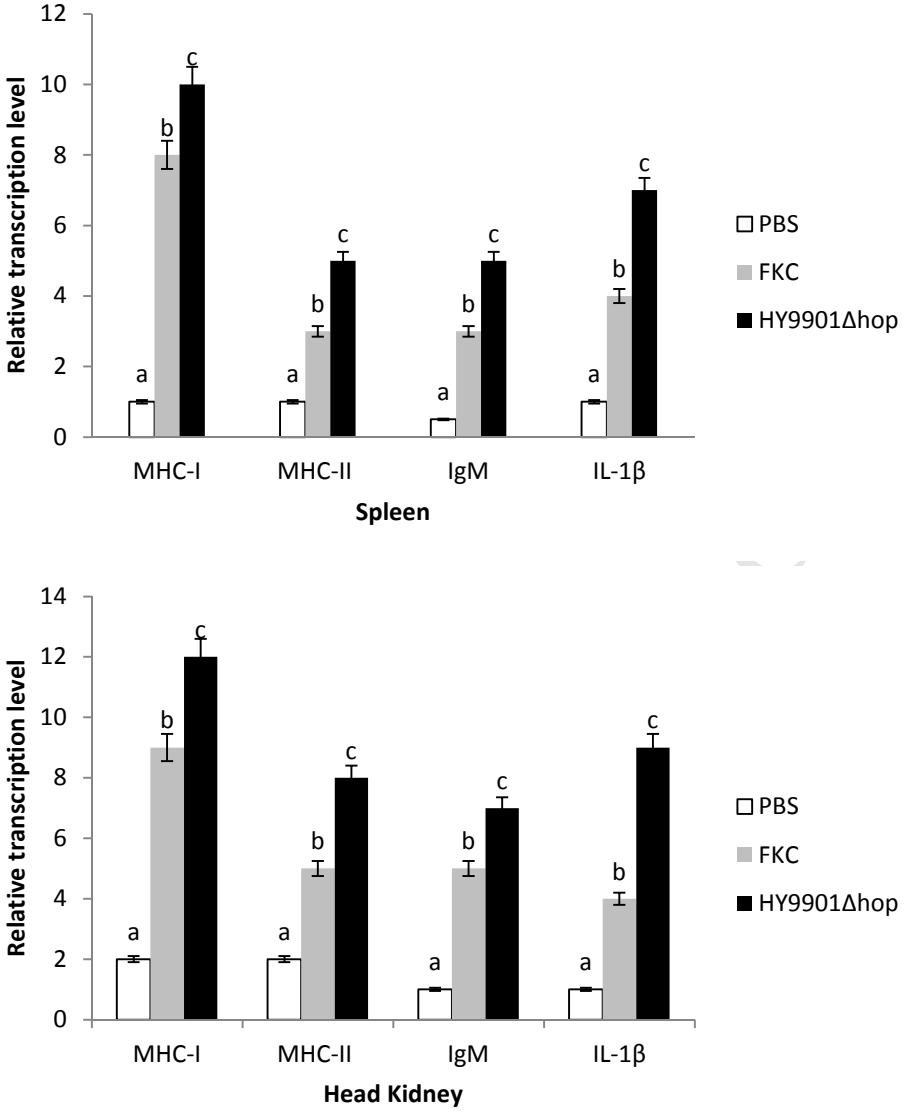


Fig. 6



688

689

690 **Fig. 7**

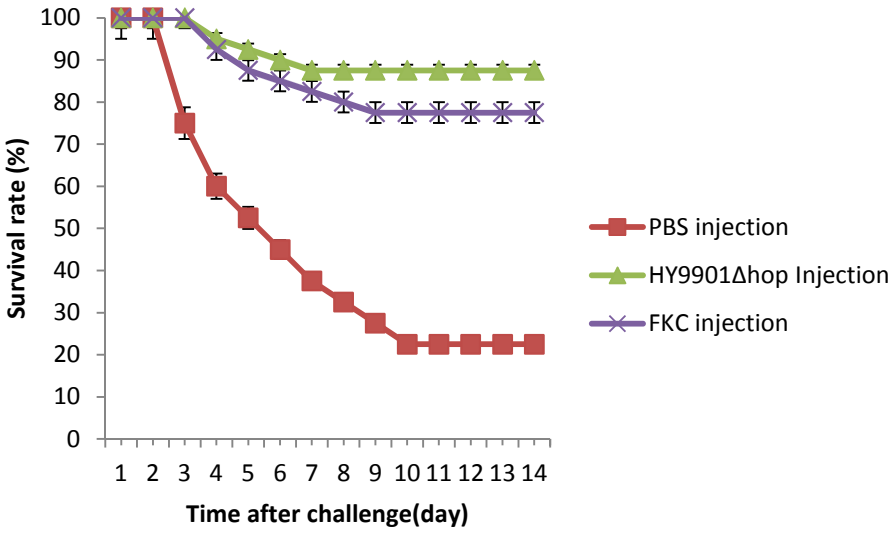
691

692

693

694

695



696

697 **Fig. 8**

698

699

The biological functions of HopPmaJ in *alginolyticus* were investigated.
HY9901Δ*hop* suppressed swarming motility, adhesion and virulence.
The RPS of grouper vaccinated with HY9901Δhop was 84 %.
HY9901Δ*hop* could stimulate innate and acquired immune responses in *E. coioides*.