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# 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*)

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

4  
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23 **ABSTRACT**

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

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

## 45 1. Introduction

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

56 The type III secretion system (T3SS) is a highly conserved apparatus among  
57 several Gram-negative bacteria, such as *Yersinia* spp., *Salmonella* spp. and *Shigella*  
58 spp [7-9], which delivers bacterial proteins, known as effectors, directly into host cells  
59 [10]. Many of these effectors are virulence factors that can trigger host-cell death and  
60 manipulate the innate and adaptive immune system [11-12]. Although the T3SS  
61 machinery is often conserved among Gram-negative pathogens, the effectors differ  
62 widely in their function. Comparative genome analysis has demonstrated that T3SS of  
63 *V. alginolyticus* is similar to T3SS1 of *V. parahaemolyticus* [13], but little is known  
64 about the effectors of *V. alginolyticus*. Therefore functional characterization of T3SS  
65 effectors is necessary.

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

67 which was homologue to the T3SEs HopPmaJ of *Chryseobacterium gleum*  
68 [15]. However, its role in *V. alginolyticus* is still unknown. To better understand the  
69 function of HopPmaJ in the T3SS from *V. alginolyticus*, we first constructed a *hop*  
70 gene mutant, then investigated the physiology and pathogenicity of the  $\Delta hop$  strain.  
71 Furthermore, we evaluated the immunoprotective potential of  $\Delta hop$ , and found that  
72 the  $\Delta hop$  mutant could be used as an effective live vaccine to combat *V. alginolyticus*  
73 in grouper.

74

## 75 2. Materials and methods

### 76 2.1 Bacterial strains and culture conditions

77 The bacterial strains, plasmids and cell line used in this work are listed in Table  
78 1. *V. alginolyticus* wild-type strain HY9901 was isolated from *Lutjanus*  
79 *erythropterus* [16] and was utilized as the parent strain for constructing the deletion  
80 mutant  $\Delta hop$ . *V. alginolyticus* was cultured on trypticase soy broth (TSB, Huankai Co  
81 Ltd., Guangzhou, China) or on 1.5% TSB agar plates (TSA) at 28°C. *Escherichia coli*  
82 strains were cultured in Luria-Bertani (LB, Huankai Co Ltd., Guangzhou, China) or  
83 on LB agar at 37°C. When required, the appropriate antibiotics were added: ampicillin  
84 (Amp, 100 µg mL<sup>-1</sup>); kanamycin (Km, 50 µg mL<sup>-1</sup>); chloramphenicol (Cm, 25 µg mL<sup>-1</sup>).

### 85 2.2 Orange-spotted Grouper

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

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

### 93 2.3 Cloning and sequencing of the *hop* gene from *V. alginolyticus* HY9901

94 A pair of primers hop1 and hop2 was designed as showed in Table 2 according to  
95 the *V. alginolyticus* gene sequence (GenBank Number: NZ\_AAPS00000000). PCR  
96 was performed in a Thermocycler (Bio-Rad, CA, USA) under the following  
97 optimized amplification conditions: an initial denaturation at 94 °C for 4 min, followed  
98 by 35 cycles of 94 °C for 30 s, 41°C for 30 s and 72 °C for 30 s. 5 µL of each  
99 amplicon was examined on 1% agarose gels, stained with ethidium bromide. The PCR  
100 product was recovered from the agarose gel to ligate into the pMD18-T vector and  
101 transformed into *E. coli* DH5α (Table 1). The inserted fragment was sequenced by  
102 Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).  
103 Similarity analyses of the determined nucleotide sequences and deduced amino acid  
104 sequences were performed by BLAST programs ([http://blast.ncbi.nlm.nih.gov/  
105 Blast.cgi](http://blast.ncbi.nlm.nih.gov/Blast.cgi)) and aligned using the program Clustal-X (version 1.81). Protein analysis  
106 was conducted with ExPASy tools (<http://expasy.org/tools/>). Location of the domain  
107 was predicted using the InterProScan program ([http://www.ebi.ac.uk/Tools  
108 /pfa/iprscan/](http://www.ebi.ac.uk/Tools/pfa/iprscan/)).

### 109 2.4 Construction of in-frame deletion mutant of *hop* gene

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

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

## 126 2.5 Characterization of the $\Delta hop$

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



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

142 LD<sub>50</sub> of the wild-type and  $\Delta$ *hop* were evaluated in *E. coioides*. Briefly, twenty  
143 grouper were injected intraperitoneally with 100 $\mu$ L HY9901 or  $\Delta$ *hop* suspended in  
144 sterile phosphate buffered saline (PBS) containing 10<sup>4</sup>-10<sup>9</sup>cfu mL<sup>-1</sup> with an injection  
145 of 100 $\mu$ L sterile PBS serving as a negative control, respectively. The fish were  
146 monitored for 14 days, and any fish that died were removed for bacteriological  
147 examination. The experiment was performed twice, and the LD<sub>50</sub> values were  
148 calculated by the statistical approach of Reed and Muench [27].

#### 149 2.6 Preparation of formalin-killed cell (FKC) suspensions

150 The *V. alginolyticus* FKC suspensions were produced as described by Zhou *et al.*  
151 [28]. Briefly, *V. alginolyticus* strain HY9901 was grown in TSB for 18 h. The cells  
152 were harvested by centrifugation at 5000 $\times$ g for 10 min and suspended in 0.85% saline  
153 solution to 1 $\times$ 10<sup>8</sup>cfu mL<sup>-1</sup>. Formaldehyde was then added at a final concentration of 1%  
154 to inactivate the bacteria for 3 days. The killed bacteria were washed three times and

155 resuspended in PBS to  $1 \times 10^8$  cfu mL<sup>-1</sup>. Confirmation of bacterial death was confirmed  
156 by incubating a culture for 48 h at 28 °C on TSA, and stored at -4 °C until use.

### 157 2.7 *E. coioides* vaccination

158 *E. coioides* were randomly divided into three groups with 80 fish per group.  
159 Prior to vaccination, the fish were anaesthetized by immersion in a 20 mg L<sup>-1</sup> solution  
160 of tricainemethanesulfonate (MS-222, Sigma). Fish in the HY9901 $\Delta$ hop group were  
161 injected intraperitoneally with 100 $\mu$ L  $1 \times 10^5$  cfu mL<sup>-1</sup>  $\Delta$ hop. Fish in the FKC group  
162 were injected intraperitoneally with 100 $\mu$ L  $1 \times 10^8$  cfu mL<sup>-1</sup> FKC as previously  
163 described[28]. Control fish were injected intraperitoneally with 100  $\mu$ L sterile PBS.  
164 All of fish were maintained at 26-28 °C. The experiment was repeated three times.

### 165 2.8 Investigation of the livability of HY9901 $\Delta$ hop in vivo post vaccination

166 The fish injected intraperitoneally with 100 $\mu$ L  $1 \times 10^5$  cfu mL<sup>-1</sup> HY9901 $\Delta$ hop  
167 extended to 7 days post vaccination. The organs including spleen and head-kidney  
168 were aseptically collected from day 1 to day 7. All the samples were weighed and  
169 homogenized in 1 ml PBS. The homogenates were serially diluted and plated in  
170 triplicate onto TCBS plates and incubated at 28 °C for 18 h. The bacteria counts were  
171 calculated by dividing the weights of the tissues and from the mean of three samples.

### 172 2.9 Analysis of antibody levels

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

177 cells were discarded, and wells were blocked with 100  $\mu$ L of PBS containing 2%  
178 bovine serum albumin (BSA) for 3 h at 22°C. 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 °C with 100  $\mu$ L 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<sub>2</sub>SO<sub>4</sub>. 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 2.10 Immune-related gene expression analysis

191 Kidney and spleen samples were taken from three fish from each group  
192 respectively at 1 day before challenge. Immune-related genes expression levels were  
193 detected with real-time PCR. Primers for MHC-I $\alpha$ , MHC-II $\alpha$ , IgM, and IL-1 $\beta$  are  
194 shown in Table 2.  $\beta$ -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

199 challenged separately by IP inoculation of 100 $\mu$ L 1 $\times$ 10<sup>8</sup>cfu mL<sup>-1</sup> of *V. alginolyticus*  
200 HY9901 [28]. The relative percent survival (RPS) of post-challenged fishes were  
201 measured per day in a 14-days time frame as previously described [32].

## 202 2.12 Statistical Analysis

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

## 209 3. Results

### 210 3.1 Cloning and mutagenesis of *hop* in *V. alginolyticus* HY9901

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

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

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

224 3.2 Morphology, growth, activity of ECPase, biofilm formation, swarming motility,  
225 and the LD<sub>50</sub> of HY9901Δ*hop*

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

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

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

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

243 LD<sub>50</sub> levels of HY9901 $\Delta$ *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  $10^5$  cfu mL<sup>-1</sup> of  
249 HY9901 $\Delta$ *hop*. HY9901 $\Delta$ *hop* has almost no side effects in terms of growth  
250 performance in *E. coioides*, when doses less than  $10^5$  cfu mL<sup>-1</sup>. 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 HY9901 $\Delta$ *hop* in vivo

253 HY9901 $\Delta$ *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, HY9901 $\Delta$ *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 HY9901 $\Delta$ *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, HY9901 $\Delta$ *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 HY9901 $\Delta$ hop

267 qRT-PCR was carried out to analyze the transcription levels of genes encoding  
268 MHC-I $\alpha$ , MHC-II $\alpha$ , IgM and IL-1 $\beta$ . The results showed that compared to FKC  
269 injection, vaccination with HY9901 $\Delta$ hop significantly increased the expression of  
270 IL-1 $\beta$ , MHC I $\alpha$ , MHC II $\alpha$  and IgM genes in the spleen and head kidney ( $p < 0.01$ )  
271 (Fig. 7). MHC-I $\alpha$  and MHC-II $\alpha$  are respectively responsible for humoral and cellular  
272 mediated immunity. As proinflammatory factor, IL-1 $\beta$  can induce the inflammatory  
273 response. Taken together, all of the above results suggested that HY9901 $\Delta$ hop can  
274 effectively elicit protective immune responses in *E. coioides*.

275 3.6 Immune protective effects of HY9901 $\Delta$ hop in *E. coioides*

276 *E. coioides* were vaccinated with FKC and HY9901 $\Delta$ 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 HY9901 $\Delta$ 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 HY9901 $\Delta$ *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 HY9901 $\Delta$ *hop* was significantly lower than that of the HY9901 (1.77%) ( $p<0.01$ ),



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

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

322 It has already been confirmed that live attenuated vaccines can induce a more  
323 robust humoral and cell-mediated immune response than killed bacteria [42]. The  
324 increase of MHC I expression in the spleens was also found in golden pompano  
325 vaccinated with a *Streptococcus agalactiae* *phoB* mutant[43].MHC II is displayed on  
326 surface of antigen presenting cells (APC) to activate T-help cells to regulate immune  
327 network[44]. IL-1  $\beta$ , an important pro-inflammatory cytokine, can induce the  
328 inflammatory response by regulating the expression of other cytokines. Xiao *et al.*  
329 [40]. IgM gene expression can be induced by intraperitoneal injection with *Yersinia*  
330 *ruckeri* in rainbow trout [45].In this study, the elevated expression of immune-related

331 genes (MHC-I $\alpha$ , MHC-II $\alpha$ , IgM, and IL-1 $\beta$ ), confirmed the stimulation of innate and  
332 acquired immune responses in *E. coioides*. Future work using immunohistochemical  
333 methods or flow cytometry sorting rather than qRT-PCR will further provide a deeper  
334 understanding of the protective immune mechanisms of HY9901 $\Delta$ hop in *E. coioides*  
335 or other fish.

336 In conclusion, we have successfully constructed an in-frame deletion strain of  
337 HY9901 $\Delta$ hop and investigated its physiology and pathogenicity. HY9901 $\Delta$ hop  
338 exhibited a high level of protection against virulent *V. alginolyticus* challenge, and  
339 could elicit both humoral and cell-mediated immune responses in *E. coioides*. These  
340 results may provide further evidence for the importance of T3SE in *V. alginolyticus*  
341 and serve as a reference for further investigation on this virulence factor

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

Strains , plasmids , cell line	Relevant characteristics	Source or references
<i>V. alginolyticus</i> HY9901	Wild type, isolated from diseased <i>Lutjanus sanguineus</i> off the Southern China coast	[16]
$\Delta hop$	HY9901 carrying an in-frame deletion of <i>hop</i> <sub>46-342</sub>	This study
<i>E. coli</i> DH5 $\alpha$	<i>supE44</i> $\Delta lacU169$ ( $\phi 80 lacZDM15$ ) <i>hsdR17 recA1 gyrA96 thi-1 relA1</i>	Sangon
MC1061 ( $\lambda pir$ )	<i>lacY1 galK2 ara-14 xyl-5 supE44</i> $\lambda pir$	[18]
pRE112	pGP704 suicide plasmid, <i>pir</i> dependent, <i>oriT</i> , <i>oriV</i> , <i>sacB</i> , Cm <sup>r</sup>	[19]
S17-1 ( $\lambda pir$ )	Tp <sup>r</sup> Sm <sup>r</sup> <i>recA thi pro hsdR<sup>-</sup>M<sup>+</sup></i> RP4 :2-Tc : Mu: Km Tn7 $\lambda pir$	[20]
MC1061-pRE- $\Delta hop$	MC1061 containing plasmid of pRE- $\Delta hop$ , Cm <sup>r</sup>	This study
S17-1-pRE- $\Delta hop$	S17-1 containing plasmid of pRE- $\Delta hop$ , Cm <sup>r</sup>	This study
pMD18-T	Cloning vector, Amp <sup>r</sup>	TakaRa
pRE- $\Delta hop$	pRE112 containing <i>hop</i> gene in-frame deletion of codons 46-342, Cm <sup>r</sup>	This study
FHM	fathead minnow epithelial cell; Pen <sup>R</sup> ; Strep <sup>R</sup>	[26]

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512 Table 2 Sequences of primers used in this study.

Primer name	Primer sequence(5'-3')	references
Cloning		
primers		
hop <sub>1</sub>	TTA TTT AGC GGT TAA A	This study
hop <sub>2</sub>	ATG GAA TTA AAA TCG	This study
Mutant		
construction		
hop-for	GGGGTACCATGAACACGCGATGG( <i>Kpn</i> I)	This study
hop-int-rev	CTCTGGTGACGCTGCCAATACATCGTTTTCTGACTGGTGTTTA	This study
hop-int-for	TATTGGCAGCGTCACCAGAGTAAACACCAGTCAGAAAACGATG	This study
hop-rev	CCCCCGGG TCGAGCAGCATGTA( <i>Sma</i> I)	This study
hop -up	TAAACTTCGTTGCTACCGCC	This study
hop -down	AAACTTAATGCCTTCCCACC	This study
qPCR		
primers		
MHC-I $\alpha$ F	GCCGCCACGCTACAGGTTTCTA	This study
MHC-I $\alpha$ R	TCCATCGTGGTTGGGGATGATC	This study
MHC-II $\alpha$ F	GGAGCCTCAGCCCAGCTTCA	This study
MHC-II $\alpha$ R	CCAGTGGGAGGTCCTTCATG	This study
IgM F	TACAGCCTCTGGATTAGACATTAG	This study
IgM R	CTGCTGTCTGCTGTTGTCTGTGGAG	This study
IL-1 $\beta$ - F	ACACGGCTTTGTCGTCTTTC	This study
IL-1 $\beta$ - R	ACGCTGCTGGACCTTTATCG	This study
$\beta$ -actin F	AAATCGCCGCACTGGTTG	This study
$\beta$ -actin R	TCAGGATACCCCTCTTGCTCT	This study

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528 Table 3 Characteristics of HY9901 $\Delta$ hop

Characteristics	HY9901	HY9901 $\Delta$ hop
Activity of ECPase (A <sub>442</sub> ) <sup>a</sup>	0.08±0.01	0.11±0.01
Biofilm formation <sup>b</sup>	0.32±0.06	0.36±0.15
Swarming (mm) <sup>c</sup>	45±0.15	23±0.5**
Adherence rate (%) <sup>d</sup>	1.77±0.11	0.88±0.25**
LD <sub>50</sub> (cfu mL <sup>-1</sup> ) <sup>e</sup>	2.5×10 <sup>5</sup>	6.5×10 <sup>8</sup> **

529 Values are mean ± standard deviation for three trials. Significant differences between

530 HY9901 and HY9901 $\Delta$ hop indicated by asterisk. \*\* $p < 0.01$ .

531 a Bacteria were incubated in TSB for 18 h at 28°C.

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

533 c Swarming diameters were measured after 24 h incubation on TSA containing 0.3%  
534 agar plates.535 d Adherence rate were expressed as percentage of observed CFU relative to the total  
536 input bacteria.537 e LD<sub>50</sub> were evaluated in healthy *E. colioides* with an average weight of 20.0 ±2g.

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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. orientalis* T3SS  
556 Effector Protein HopPmaJ, UniRef90\_C9QFQ5; *Flavobacterium* T3SS Effector Protein,  
557 UniRef90\_UPI00047A9F35

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559 Figure 2 Construction and confirmation of the knockout mutant strain HY9901 $\Delta$ hop  
560 M: DL2000 marker; Lane 1. The 313bp upstream fragment amplified from genomic DNAs of the  
561 wild-type strain HY9901 using primer pairs of hop-for / hop-int-rev. Lane 2. The 342bp downstream  
562 fragment amplified from genomic DNAs of the wild-type strain HY9901 using primer pairs of  
563 hop-int-for / hop-rev. Lane 3. The 655 bp fragment amplified from genomic DNAs of HY9901 $\Delta$ hop  
564 using primer pairs of hop-for / hop-rev. Lane 4. The 952 bp fragment amplified from genomic DNAs of  
565 the wild-type strain HY9901 using primer pairs of hop-for / hop-rev.

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567 Figure 3 Observation the morphological feature of HY9901(A) and HY9901 $\Delta$ hop (B) by SEM.

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569 Figure 4 Growth features of HY9901 $\Delta$ hop and HY9901. Aliquots of cell culture were taken at various  
570 time points and measured for cell density at OD<sub>600</sub>.

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572 Figure 5 Propagation of HY9901 $\Delta$ hop in grouper kidney (A) and spleen (B) following i.p. injection  
573 with 100 $\mu$ L 1 $\times$ 10<sup>5</sup>cfu mL<sup>-1</sup> $\Delta$ hop. Control fish were i.p. injection with 100  $\mu$ L sterile PBS The number  
574 of viable bacteria was shown as the mean  $\pm$  standard of three samples.

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576 Figure 6 Antibody titers in sera of grouper injected IP with HY9901 $\Delta$ hop, FKC and PBS. Sera  
577 collected at week 1 to 8 post-vaccination were assayed by ELISA. Each column represents the mean of  
578 log<sub>2</sub> antibody titer with standard deviation bar. Groups that do not share a letter are significantly  
579 different (p < 0.01).

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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  $\beta$ -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).

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

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	1	10	20	30	40	
<i>V. alginolyticus</i> HY9901	MELKSFLDLLAASPEQ	VEFEATMAVIEDNYTFEPTAFVN				
<i>Vibrio alginolyticus</i> NBRC15630	MELKSFLDLLAASPEQ	VEFEATMAVIEDNYTFEPTAFVN				
<i>V. parahemolyticus</i>	MELKSFLDLLAASPEQ	VEFEATMAVIEDNYTFEPTAFVN				
<i>V. harveyi</i>	MELKEFLDALAASPETVEFETTMAAIEANYAFTPAAFVN					
<i>Vibrio genomosp.</i>	MDLNTFISQLKREPELIEFEQTMSVIDENFSFTPTFTFN					
<i>Vibrio coralliilyticus</i>	MELSVFIEQLNQSPATVQFEQSMVIDANYEFTPTAFIN					
<i>Vibrio vulnificus</i>	MSLKDLLAKLAETPEKVEFEQVIDVIDSHYVFPAAAFQN					
<i>Vibrio orientalis</i>	MELNNFLATLSETPTEIQFEDTMAVIEANYEFVPTAFVN					
<i>Flavobacterium</i>	MNIQTFLEKQKQTPEAITFPETIEVIEANYDFTPTAFQN					
		50	60	70	80	
<i>V. alginolyticus</i> HY9901	GETQNNAGENN	GSKIFAFGLLNLDKEATLACFGRFYREDVL				
<i>V. alginolyticus</i> NBRC15630	GETQNNAGENN	GSKIFAFGLLNLDKEATLACFGRFYREDVL				
<i>V. parahemolyticus</i>	GETQNNAGENN	GSKIFAFGLLNLDKEATLACFGRFYREDVL				
<i>V. harveyi</i>	GETQNNAGENN	GSKIFAFGLLNLDKEATLACFGRFYREDVL				
<i>V. genomosp.</i>	GKTLNQAGQNN	GSKIFALGALQQLSIEETLACFGRFYREDVL				
<i>V. coralliilyticus</i>	GETKNEANQNN	GSKIFAFQNLQLTEQDTLACFGRFYREDVL				
<i>V. vulnificus</i>	GDTNEANQNN	GSKIFAFQNLNELNEEQTLACFGRFYREDVL				
<i>V. orientalis</i>	GDTSEANQNN	GSKIFAFARLKELEQASTLACFGRFYREDVL				
<i>Flavobacterium</i>	GNTNNAAGTNS	GSKLFAFAQLQNLSDQDETLACFGSFRYDEVL				
	90	100	110			Identity
<i>V. alginolyticus</i> HY9901	QHPENSDHQNIRNFMVTGWEGIKFEASALTAK:	114				100%
<i>V. alginolyticus</i> NBRC15630	QHPENSDHQNIRNFMVTGWEGIKFEASALTAK:	114				100%
<i>V. parahemolyticus</i>	QHPENNDHQNIRNFMVTGWEGIKFEAPALTAK:	114				98%
<i>V. harveyi</i>	QHPENNDHQNIRNFMVTGWEGIKFEAPALTAK:	114				96%
<i>V. genomosp.</i>	KHPEGDDHQNIRNFMVTGWEGVEFEAVLVKK:	114				78%
<i>V. coralliilyticus</i>	QNPDGDDHANIRNFIFGWQGIQFESDALVSK:	114				76%
<i>V. vulnificus</i>	LHPENDDHQNIRNFIRFGWSGVQFDTAALTEK:	114				82%
<i>V. orientalis</i>	GNPDGDDHANIRNFIFGWQGIKFEADALVAK:	114				78%
<i>Flavobacterium</i>	GEPEGTNHQNIRNFMVHGWSGIQFEGTALTELK:	114				74%

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**Fig. 1**

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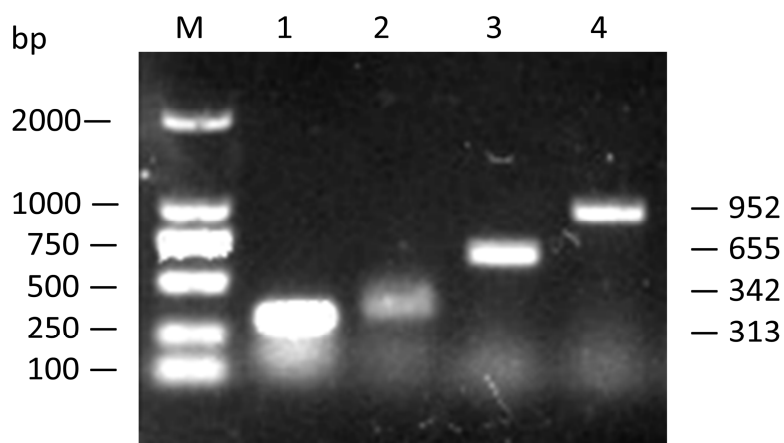
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**Fig. 2**

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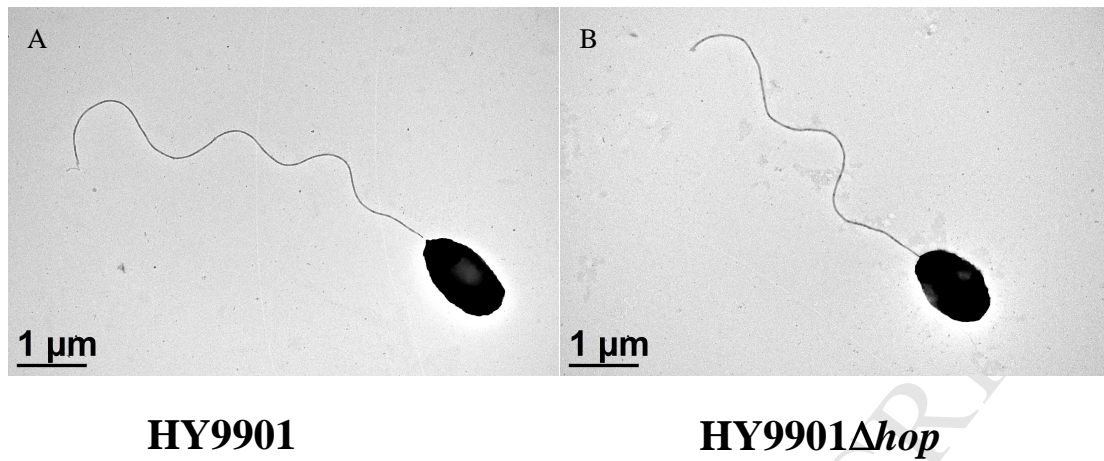
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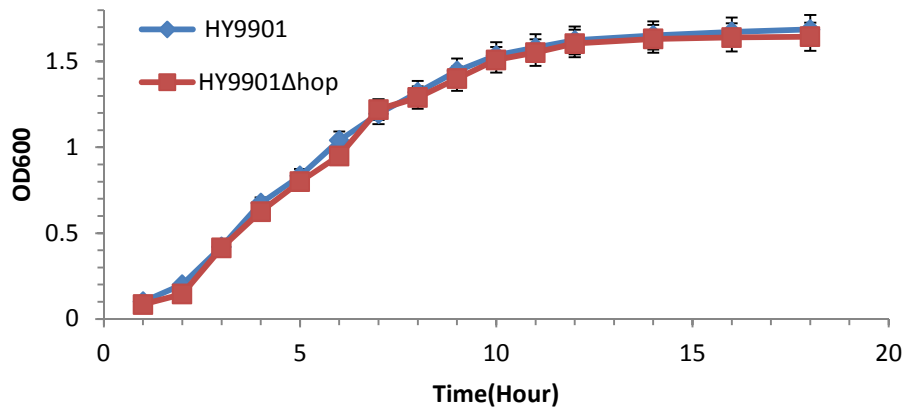
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**Fig. 3**





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**Fig. 4**

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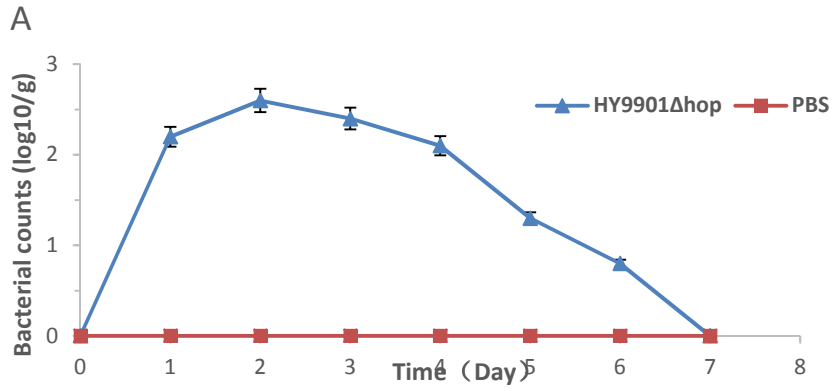
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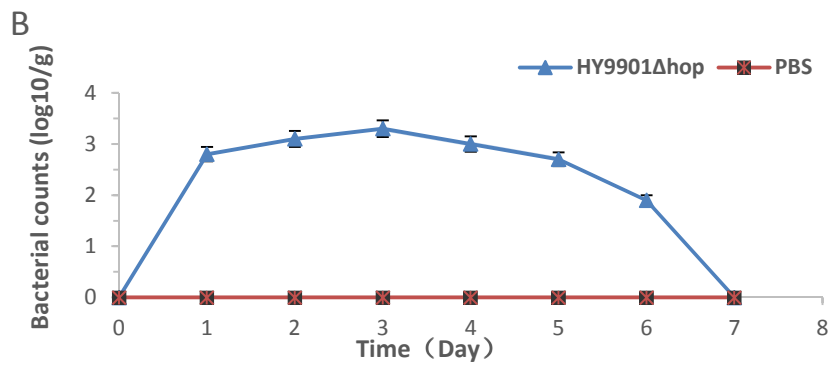
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**Fig. 5**

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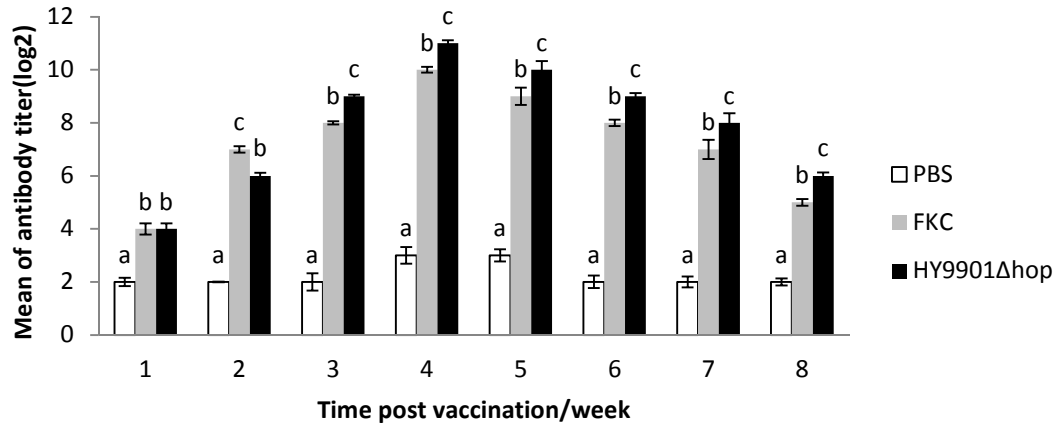
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**Fig. 6**

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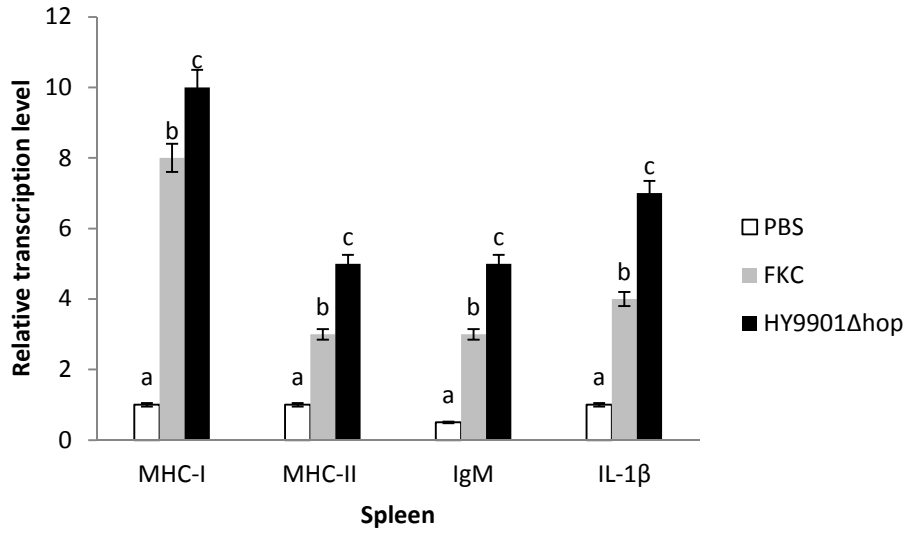
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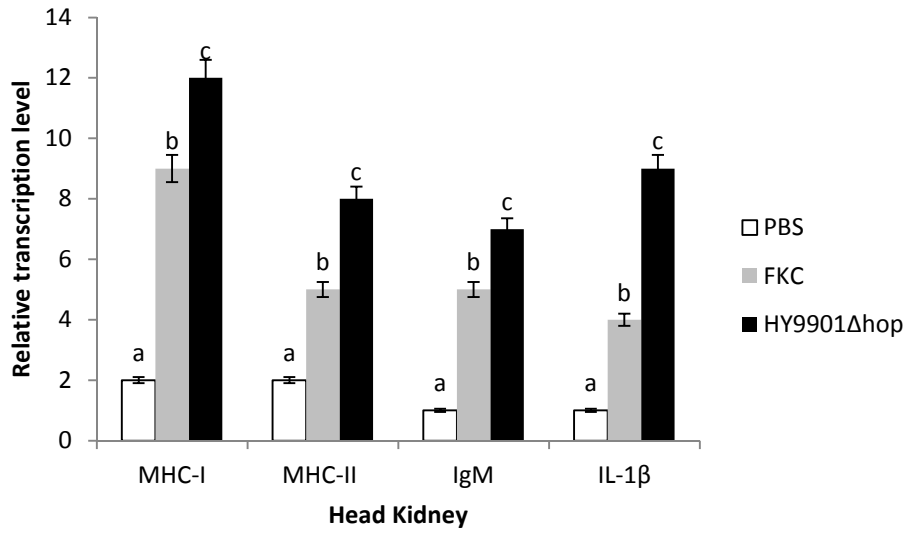
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Fig. 7

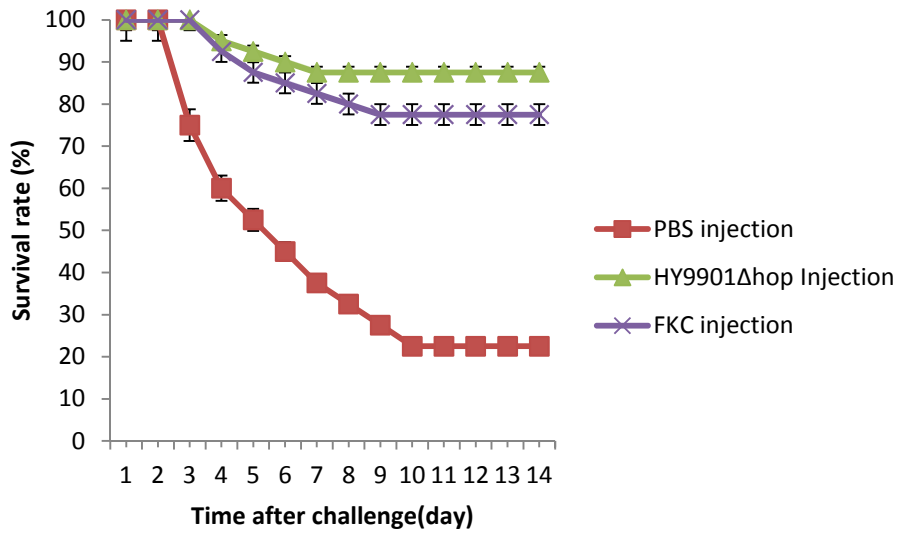
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**Fig. 8**

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The biological functions of HopPmaJ in *alginolyticus* were investigated.  
HY9901 $\Delta$ hop suppressed swarming motility, adhesion and virulence.  
The RPS of grouper vaccinated with HY9901 $\Delta$ hop was 84 %.  
HY9901 $\Delta$ hop could stimulate innate and acquired immune responses in *E. coioides*.

ACCEPTED MANUSCRIPT