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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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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 HY9901Δhop 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 HY9901Δhop mutant showed an attenuated swarming phenotype and a 2600-fold decrease in the virulence to grouper. However, the HY9901Δhop mutant showed no difference in morphology, growth, biofilm formation and ECPase activity. Finally, grouper vaccinated via intraperitoneal (IP) injection with HY9901Δhop 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 HY9901Δhop enhanced the expression of immune-related genes, including MHC-Iα, MHC-IIα, IgM, and IL-1β after vaccination, indicating that it is able to induce humoral and cell-mediated immune response in grouper. These results demonstrate that the HY9901Δhop 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*), 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 Δhop strain. Furthermore, we evaluated the immunoprotective potential of Δhop, and found that the Δhop mutant could be used as an effective live vaccine to combat *Valginolyticus* 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 Δhop. *V. alginolyticus* was cultured on trypticase soy broth (TSB, Huankai Co Ltd., Guangzhou, China) or on 1.5% TSB agar plates (TSA) at 28°C. *Escherichia coli* strains were cultured in Luria-Bertani (LB, Huankai Co Ltd., Guangzhou, China) or on LB agar at 37°C. When required, the appropriate antibiotics were added: ampicillin (Amp, 100µg mL⁻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°C 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 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 41°C for 30 s and 72 °C 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 KpnI site at the 5'-end) and hop-int-rev; whereas primers hop-int-for and hop-rev (contains a SmaI 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<sup>r</sup>) to generate pRE-Δ*hop*. This recombinant suicide plasmid was transformed into *E. coli* MC1061λpir [18] and subsequently S17-1λpir[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<sub>50</sub>). 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 HY9901Δhop and HY9901, respectively.

LD$_{50}$ of the wild-type and Δhop were evaluated in *E. colioides*. Briefly, twenty grouper were injected intraperitoneally with 100µL HY9901 or Δhop suspended in sterile phosphate buffered saline (PBS) containing 10$^{4}$-10$^{9}$cfu 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$^{8}$cfu 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 $1 \times 10^8$ cfu mL$^{-1}$. Confirmation of bacterial death was confirmed by incubating a culture for 48 h at 28 °C on TSA, and stored at -4°C until use.

2.7 *E. colioides* vaccination

*E. colioides* 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 HY9901Δhop group were injected intraperitoneally with 100 µL $1 \times 10^5$ cfu mL$^{-1}$Δhop. Fish in the FKC group were injected intraperitoneally with 100 µL $1 \times 10^8$ 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°C. The experiment was repeated three times.

2.8 Investigation of the livability of HY9901Δhop in vivo post vaccination

The fish injected intraperitoneally with 100 µL $1 \times 10^5$ cfu mL$^{-1}$HY9901Δhop 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°C 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. colioides* 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°C. Excess
cells were discarded, and wells were blocked with 100 µL of PBS containing 2% bovine serum albumin (BSA) for 3 h at 22°C. After removing the blocking solution and washing three times with PBS added with 0.05% Tween-20 (PBST), the wells were incubated for 3 h at 22 °C with 100 µL of serially diluted *E. colioides* serum. Antibody binding to the antigen was detected using *E. colioides* IgM monoclonal antibody (1:10000) which was produced according to Li *et al.* [30], followed by rabbit anti-mouse IgG-HRP (Wuhan Boster, Wuhan, China) at 1:20000 dilution, and colour was developed with a chromogenic reagent TMB (tetramethylbenzidine) (Amresco, Ltd, MA, USA) for 20 min with the reaction being stopped by the addition of 2.0 M H₂SO₄. The plates were then read at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Sera were considered positive for anti-*V. alginolyticus* specific antibodies if the absorbance was at least double of the control sera, and antibody titers were scored as the highest positive dilution.

2.10 Immune-related gene expression analysis

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

2.11 Challenge experiment.

Four weeks post immunization, *E. colioides* (n=30) were anesthetized and
challenged separately by IP inoculation of 100µL 1×10⁸ cfu mL⁻¹ of *V. alginolyticus* HY9901 [28]. The relative percent survival (RPS) of post-challenged 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 HY9901$\Delta$hop

Morphology was assessed by scanning electron microscopy. HY9901$\Delta$hop showed no discernible morphological difference from HY9901 when cultured in TSB (Fig. 3). HY9901$\Delta$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 HY9901$\Delta$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 HY9901$\Delta$hop and the wild-type strain ($p$>0.05). HY9901$\Delta$hop showed a smaller swarming diameter than HY9901 ($p$<0.01) (Table 3).

The adherence rate (0.88%) of the HY9901$\Delta$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).
LD$_{50}$ levels of HY9901$\Delta$hop were 3 logs higher than that of HY9901 ($p<0.01$).

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

3.3 Investigation of the livability of HY9901$\Delta$hop in vivo

HY9901$\Delta$hop was able to disseminate into but survive transiently in fish head-kidney and spleen then was gradually eliminated from the host body (Fig. 5). The highest bacterial number was detected in spleen on day 3, followed by the head-kidney.

3.4 Analysis of antibody levels

Grouper (*E. coioides*) were immunized with two different types of *V. alginolyticus* vaccines, HY9901$\Delta$hop and FKC. The immune response of grouper was assessed by ELISA at week 1, 2, 3, 4, 5, 6, 7 and 8 after vaccination (Fig. 6). The result indicated that the specific antibody titers of fish immunized with FKC and HY9901$\Delta$hop were markedly higher than those of in the control group ($p < 0.05$). In the immunized group the antibody titer reached the highest level at week 4 ($p < 0.01$). Compared to the FKC group, HY9901$\Delta$hop vaccinated group had significantly greater
titer of *E. coioides* specific serum antibodies from week 3 pv (*p* < 0.01).

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

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

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

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

4. Discussion

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

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

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

The first step of the bacterial infection is the adherence of bacteria to the surface of host epithelial cells, which facilitates colonization on or penetration of the cells [38]. In the current study, we tested if *V. alginolyticus* *hop* contributes to bacterial adhesion to FHM cells. The data from this work showed that the adherence rate (0.88%) of the 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 HY9901Δhop was 3 logs higher than that of wild-type HY9901 and showed low or no lethality virulence in E. colioides when administered via i.p. injection (Table 3 and data not shown). Moreover our findings also show that HY9901Δhop has almost no side effects in terms of growth performance in E. colioides. We evaluated the efficacy of HY9901Δhop as a live attenuated vaccine (LAV) by injection route in an E. colioides model, resulting in a RPS of 84% 4-week post vaccination. The significantly enhanced specific antibody confirmed the immune responses in E. colioides.

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 HY9901Δhop in *E. coioides* or other fish.

In conclusion, we have successfully constructed an in-frame deletion strain of HY9901Δhop and investigated its physiology and pathogenicity. HY9901Δhop 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**

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**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...


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


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

<table>
<thead>
<tr>
<th>Strains , plasmids , cell line</th>
<th>Relevant characteristics</th>
<th>Source or references</th>
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<tr>
<td><strong>V. alginolyticus HY9901</strong></td>
<td>Wild type, isolated from diseased <em>Lutjanus sanguineus</em> off the Southern China coast</td>
<td>[16]</td>
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<tr>
<td><strong>Δhop</strong></td>
<td>HY9901 carrying an in-frame deletion of <em>hop</em>&lt;sub&gt;46-342&lt;/sub&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td><em>supE44 ΔlacU169</em> (φ80 <em>lacZDM15</em>) <em>hsdR17</em></td>
<td>Sangon</td>
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<td></td>
<td><em>recA1 gyrA96 thi-1 relA1</em></td>
<td></td>
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<tr>
<td><strong>MC1061 (λpir)</strong></td>
<td><em>lacY1 galK2 ara-14 xyl-5 supE44 λpir</em></td>
<td>[18]</td>
</tr>
<tr>
<td><strong>pRE112</strong></td>
<td>pGP704 suicide plasmid, <em>pir</em> dependent, <em>oriT</em>, <em>oriV</em>, <em>sacB</em>, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[19]</td>
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<td><strong>S17-1 (λpir)</strong></td>
<td>Tp&lt;sup&gt;r&lt;/sup&gt; Sm&lt;sup&gt;r&lt;/sup&gt; *recA pro hsdR&lt;sup&gt;−&lt;/sup&gt; M&lt;sup&gt;r&lt;/sup&gt; RP4 :2-Tc : Mu&lt;sup&gt;r&lt;/sup&gt;</td>
<td>[20]</td>
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<td><strong>MC1061-pRE-Δhop</strong></td>
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<td>pRE-Δhop</td>
<td>pRE112 containing <em>hop</em> gene in-frame deletion of codons 46-342, Cm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>This study</td>
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<td><strong>FHM</strong></td>
<td>fathead minnow epithelial cell; Pen&lt;sup&gt;r&lt;/sup&gt;; Strep&lt;sup&gt;r&lt;/sup&gt;</td>
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Table 2 Sequences of primers used in this study.

<table>
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<th>Primer name</th>
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<td><strong>Cloning primers</strong></td>
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<td>hop&lt;sub&gt;1&lt;/sub&gt;</td>
<td>TTA TTT AGC GGT TAA A</td>
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<td>hop&lt;sub&gt;2&lt;/sub&gt;</td>
<td>ATG GAA TTA AAA TCG</td>
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<td><strong>Mutant construction</strong></td>
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<tr>
<td>hop-for</td>
<td>GGGGTACCATGAACACGCAGATGG(&lt;i&gt;Kpn I&lt;/i&gt;)</td>
<td>This study</td>
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<tr>
<td>hop-int-rev</td>
<td>CTTGTTGAGCTGCAATACATCGTATTTTCGACTGGTGTTTA</td>
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<tr>
<td>hop-int-for</td>
<td>TTATGGACAGGTACACCAGATTTAACACCATTCAAGAAAACGATG</td>
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<tr>
<td>hop-rev</td>
<td>CCCCCGGG TCAGACAGCATGTA(&lt;i&gt;Sma I&lt;/i&gt;)</td>
<td>This study</td>
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<tr>
<td>hop-up</td>
<td>TAAACTTCGGTGTACCCGAC</td>
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</tr>
<tr>
<td>hop-down</td>
<td>AAACCTTAATGCCTTCCCACC</td>
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<td><strong>qPCR primers</strong></td>
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<td>MHC-IαF</td>
<td>GCCGCCACGTACAGGTTTCTA</td>
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<tr>
<td>MHC-IαR</td>
<td>TCCATCGTGTTGGGATGATC</td>
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<tr>
<td>MHC-IIαF</td>
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<td>MHC-IIαR</td>
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<td>IgM F</td>
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<td>IgM R</td>
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<tr>
<td>IL-1β F</td>
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<td>IL-1β R</td>
<td>ACGCTGGTTGACTTATCG</td>
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<td>β-actin F</td>
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<td>β-actin R</td>
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Table 3 Characteristics of HY9901Δhop

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>HY9901</th>
<th>HY9901Δhop</th>
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<tbody>
<tr>
<td>Activity of ECPase (A_{442})^a</td>
<td>0.08±0.01</td>
<td>0.11±0.01</td>
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<tr>
<td>Biofilm formation^b</td>
<td>0.32±0.06</td>
<td>0.36±0.15</td>
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<tr>
<td>Swarming (mm)^c</td>
<td>45±0.15</td>
<td>23±0.5**</td>
</tr>
<tr>
<td>Adherence rate (%)^d</td>
<td>1.77±0.11</td>
<td>0.88±0.25**</td>
</tr>
<tr>
<td>LD_{50} (cfu mL^{-1})^e</td>
<td>2.5×10^5</td>
<td>6.5×10^8**</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation for three trials. Significant differences between HY9901 and HY9901Δhop 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. coliodes* with an average weight of 20.0 ±2g.
Figure legends

Figure 1 Homology comparison of *V. alginolyticus* HY9901 T3SS Effector Protein HopPmaJ

*V. alginolyticus* HY9901 T3SS Effector Protein HopPmaJ; *V. alginolyticus* NBRC 15630 = ATCC T3SS Effector Protein HopPmaJ Accession NC_022359.1 WP_005375560.1; *V. parahaemolyticus* serotype O3:K6 (strain RIMD 2210633) T3SS Effector Protein; *V. harveyi* CMCP6-E0666 T3SS Effector Protein; *V. genomospor* T3SS Effector Protein niRef90_UP1000474712C; *V. coralliilyticus* T3SS Effector Protein niRef90_UP00047A9F35; *V. vulnificus*. T3SS Effector Protein HopPmaJ, UniRef90_E8VUS9; *V. orienta* T3SS Effector Protein HopPmaJ, UniRef90_C9QFQ5; *Flavobacterium* T3SS Effector Protein, UniRef90_UP100047A9F35

Figure 2 Construction and confirmation of the knockout mutant strain HY9901 ∆hop

M: DL2000 marker; Lane 1. The 313bp upstream fragment amplified from genomic DNAs of the wild-type strain HY9901 using primer pairs of hop-for / hop-int-rev. Lane 2. The 342bp downstream fragment amplified from genomic DNAs of the wild-type strain HY9901 using primer pairs of hop-int-for / hop-rev. Lane 3. The 655 bp fragment amplified from genomic DNAs of HY9901 ∆hop using primer pairs of hop-for / hop-rev. Lane 4. The 952 bp fragment amplified from genomic DNAs of the wild-type strain HY9901 using primer pairs of hop-for / hop-rev.

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

Figure 4 Growth features of HY9901 ∆hop and HY9901. Aliquots of cell culture were taken at various time points and measured for cell density at OD600.

Figure 5 Propagation of HY9901 ∆hop in grouper kidney (A) and spleen (B) following i.p. injection with 100µL 1×10^5 cfu mL^-1 ∆hop. Control fish were i.p. injection with 100 µL sterile PBS. The number of viable bacteria was shown as the mean ± standard of three samples.

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

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

Figure 8 Percent survival in groups vaccinated with HY9901 ∆hop, FKC and PBS following challenge with *Vibrio alginolyticus* HY9901.
Fig. 1

V. alginolyticus

Vibrio alginolyticus

V. parahaemolyticus

V. harveyi

Vibrio genomossp.

Vibrio coralliliticus

Vibrio vulnificus

Vibrio orienta

Flavobacterium

V. alginolyticus

Vibrio alginolyticus

V. parahaemolyticus

V. harveyi

V. genomossp.

V. coralliliticus

V. vulnificus

V. orienta

Flavobacterium

Fig. 1
Fig. 2
Fig. 3
Fig. 4
**Fig. 5**

(A) and (B) show the bacterial counts (log10/g) over time (Day). The blue line represents HY9901Δhop, and the red line represents PBS.
Fig. 6
Fig. 7
Fig. 8

Survival rate (%) vs. Time after challenge (day)

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