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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 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-1β 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.

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

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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 (λ*pir*)	*lacY*1 *galK*2 *ara*-14 *xyl*-5 *sup*E44 λ*pir*	[18]
pRE112	pGP704 suicide plasmid, *pir* dependent, *oriT*, *oriV*, *sacB*, Cm^r	[19]
S17-1 (λ*pir*)	Tp^r Sm^r *recA thi pro hsdR-M*+RP4:2-*Tc*: *Mu*: Km Tn*7* λ*pir*	[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]

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 °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, 100μgmL^{-1}); kanamycin (Km, 50 μg mL^{-1}); chloramphenicol (*Cm*, 25 μg mL^{-1}).

2.2. Orange-spotted grouper

E. coioides (average weight 20.0 ± 2.0 g) 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 °Cfor 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 *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-△*hop*. This recombinant suicide plasmid was transformed into *E. coli* MC1061λ*pir* [18] and subsequently S17-1λ*pir* [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 △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 24 h 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 10^4-10^9 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].