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# Identification of vaccine candidates from differentially expressed outer membrane proteins of *Vibrio alginolyticus* in response to NaCl and iron limitation

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ABSTRACT

*Vibrio alginolyticus* is the etiological agent that causes great losses in aquacultures and clinical emanating cases in humans. Identification of highly efficient vaccine candidates to control *V. alginolyticus* infection has been highly concerned since vaccines offer a powerful approach to provide efficient protection from bacterial infections. In the present study, we firstly investigated the altered outer membrane proteins (OM proteins) of *V. alginolyticus* in response to NaCl concentrations and iron limitation using Western blotting, and then identified the protective activity of these altered OM proteins by bacterial challenge post immunization. Ten OM proteins were differentially expressed in response to the osmolarity changing or/and iron limitation, in which VA2212, OmpV, VPA1186, OmpU, VPA1644, VA1061, VA1631 and VPA0860 were markedly altered in response to osmolarity, and VPA1186, OmpU, OmpV, VA0449, VPA0860, VPA1435 and VA1631 were determined to be iron-limited responsive proteins. Out of the ten OM proteins, VA1061, OmpU, VPA1435 and VPA0860 could be effective vaccine candidates against infection by *V. alginolyticus* in vivo. Further results indicated that VA1061 and VPA0860 were dominant antigens and could stimulate hosts to produce stronger antibody response than other two in live or inactivated whole-cell vaccines. These results not only expand knowledge on osmolarity-, iron-responsive proteins, but also provide a valuable strategy for identify protective proteins suitable for use in vaccine development.

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## 1. Introduction

Increased seafood consumption throughout the world has led to more attention being paid to the control of bacterial infections than before. *Vibrio alginolyticus* is a Gram-negative halophilic marine bacterium that causes vibriosis of fishes, crustaceans and bivalve mollusks [1,2]. The bacterium always encounters different environment conditions, such as iron limitation, salinity concentrations and temperature alteration, between its native and host environments. Reports have indicated that these conditions have a significant action on the virulence and growth of *V. alginolyticus* [3–6]. The iron uptake mechanisms contribute to bacterial virulence directly. Marked attenuation in virulence of *V. alginolyticus* was observed when mutants of the TonB systems were inoculated intraperitoneally into the fish *Brachydanio rerio* [6]. At a low salinity level, shrimp reduced immune ability and decreased resistance against *V. alginolyticus* infection [3]. *V. alginolyticus* adhesion was influenced remarkably by environmental factors such as temperature, pH and salinity [7]. Comparatively, higher salinity increased Vibrio virulence toward shrimp more than higher temperature did [8]. In summary, the bacterium may alter protein expression and metabolism for adaptation to these changing environments when it evades hosts. Thus, these altered proteins are related to bacterial infections.

Outer membrane proteins (OM proteins), between the outmost of the cell and its external natural environment, are playing a critical role for the response to environmental changes in osmolarity, iron stress, and antibiotics [9–11]. On the other hand, OM proteins as potential vaccine candidates have been highly concerned recently. Some OM protein-original vaccine candidates have been reported, including OmpU in *Vibrio parahaemolyticus* [12], PorB in *Neisseria* [13], and OmpF in *Pseudomonas aeruginosa* [14,15]. Our recent report has indicated that *V. alginolyticus* OM proteins VA0764 (OmpA) and VA1061 (Pal), and *V. parahaemolyticus* VP1061 and VP2850 show broad cross-protective activity [16, 17]. However, information regarding association between the stress OM proteins to environmental factors and vaccine candidates is not available. We hypothesized that protective immunogens might be identified in these altered OM proteins since these proteins are associated with bacterial invading.

In the present study, Western blotting analysis was applied to identify altered OM proteins of *V. alginolyticus* in response to

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different osmotic concentrations and iron limitation. Then, immune protective ability was detected in these OM proteins varied with the sodium chloride concentration and/or under depleted iron growth conditions using actively immune protection test in carp (*Cyprinus carpio*). At last, whether these altered proteins were dominant antigens in live or inactivated whole-cell vaccines was investigated. These results may have significant implications not only in developing vaccines against this marine *Vibrio* gastrointestinal pathogen, but also quick identification of vaccine candidates in other microbes.

## 2. Materials and methods

### 2.1. Bacterial strain and culture conditions

*V. alginolyticus* was the same strain as being reported in our previous publications, which was isolated from sick abalone [9]. The bacterium was grown at 28 °C in Luria–Bertani (LB) medium with 3.5% NaCl and harvested at an $OD_{600}$ of 1.0. For preparation of different concentrations of NaCl and depleted iron cultures, the bacterium was cultured in 0.5, 3.5 and 10% concentrations of NaCl, or in the presence of 200 μM ferrous iron chelator 2, 2-dipyridyl (DIP) (Sinopharm Chemical Reagent CO., Ltd, China).

### 2.2. Fish

Carp (mean weight about 10 g), *C. carpio*, were purchased from a commercial fish farm (Huangsha Aquarium, Guangzhou, China). The ornamental fish were acclimatized to laboratory conditions for 3 weeks prior to initiating experiments. They were maintained in well aerated dechlorinated water at room temperature throughout the holding and experimental period. They were fed with commercial food pellets (2% body weight daily).

### 2.3. Extraction of membrane proteins

Membrane proteins of *V. alginolyticus* were prepared according to a procedure described previously [18]. Briefly, the bacterial cells were harvested by centrifugation at 4000 *g* for 15 min at 4 °C. The cells were then washed in 40 mL sterile saline (0.15 M NaCl) for three times, and then resuspended in 5 mL sterile saline. Cells were disrupted by intermittent ultrasonic treatment. Unbroken cells and cellular debris were removed by centrifugation at 5000 *g* for 20 min and the supernatants were collected and were further centrifuged at 100,000 *g* for 40 min at 4 °C in a Beckman Coulter L-100XP centrifuge using a SW 41Ti Rotor. The collecting pellets were resuspended in 50 mM Tris–Cl and stored at −80 °C. Concentrations of the proteins in the final preparation were determined using the Bradford method.

### 2.4. SDS-PAGE and Western blotting analyses

SDS-PAGE and Western blotting assays were prepared according to a procedure described previously [18]. In brief, discontinuous buffer system of Laemmli with 12% resolving gels and 4% stack gels was used to resolve membrane proteins. Aliquot of 5 and 50 μg proteins were loaded to each lane of a gel and a gel, respectively, for SDS/PAGE and Western blotting analyses. All samples were heated for 10 min in boiling water and electrophoresed with constant voltage of 120 V until the dye-front reached the bottom of these gels. For SDS analysis, these protein bands were visualized by Coomassie Brilliant blue-R250 (Sigma) staining. The Western blotting detection was performed in the same three or two NC papers of samples, respectively, from bacterial cultures with three NaCl concentrations or under depleted iron conditions. Proteins from the gels were transferred to nitrocellulose (NC) membranes for 1 h at 70 V in transfer buffer at 4 °C. These NC membranes were blocked for 1 h with 5% skim milk in TNT buffer at 24 °C and cut into strips and separately incubated with 24 types of rabbit anti-sera (available in our laboratory as reference 18) against *V. alginolyticus* and *V. parahaemolyticus* OM proteins. These OM proteins are classified into OmpA family (two), transport proteins (three), lipoproteins (three), porins (five), TolC family (three), iron-regulated proteins (four), receptor proteins (one) and unknown (three) (Table 1). After rinsing three times for 10 min with TNT buffer, the NC membranes were separately incubated with these rabbit anti-sera to OM proteins at

**Table 1**
Anti-sera to OM proteins in this study.

| No. | Protein | Definition | Classification | MW | NCBI No. |
|---|---|---|---|---|---|
| ***V. alginolyticus*** | | | | | |
| 1 | VA0764 | Outer membrane protein OmpA | OmpA family | 34,073 | EU625283 |
| 2 | VPA0860 | Long-chain fatty acid transport protein | Transport protein | 47,749 | FJ176401 |
| 3 | VA1192 | Putative outer membrane lipoprotein Pcp | Lipoprotein | 16,121 | FJ177528 |
| 4 | VA0760 | Putative chitoporin | Porin | 40,790 | EU927695 |
| 5 | VA2467 | Outer membrane protein OmpU | Porin | 36,285 | FJ176404 |
| 6 | VA0154 | Transcriptional regulator OmpR | Unknown | 27,362 | FJ177525 |
| 7 | VA1631 | Agglutination protein | TolC family | 50,705 | EU625280 |
| 8 | VA1061 | Peptidoglycan-associated lipoprotein | Lipoprotein | 18,713 | EU643509 |
| 9 | VPA1644 | Maltose-inducible porin | Porin | 46,963 | EU625279 |
| 10 | VA0802 | Putative exported protein | Transport protein | 52,780 | FJ176402 |
| 11 | VA0449 | Putative lipoprotein | Lipoprotein | 67,557 | FJ176403 |
| ***V. parahaemolyticus*** | | | | | |
| 12 | VPA0096 | Outer membrane protein OmpW | Iron-regulated proteins | 23,468 | NP_799606 |
| 13 | VP2212 | Putative long-chain fatty acid transport protein | Transport protein | 49,139 | NP_798591 |
| 14 | VP2362 | Outer membrane protein OmpK precusor | Receptor protein | 29,877 | NP_798741 |
| 15 | VPA0318 | Putative outer membrane protein OmpV | Iron-regulated proteins | 28,148 | NP_799828 |
| 16 | VPA0527 | Outer membrane protein OmpN | Porin | 37,778 | NP_800037 |
| 17 | VPA1186 | Outer membrane protein OmpA | OmpA family | 36,014 | NP_800696 |
| 18 | VP2850 | Conserved hypothetical protein | Unknown | 20,227 | NP_799229 |
| 19 | VPA0526 | Putative OmpU | Porin | 34,941 | NP_800036 |
| 20 | VP0425 | Outer membrane protein TolC | TolC family | 47,983 | NP_796804 |
| 21 | VP1998 | putative outer membrane protein TolC | TolC family | 46,573 | NP_798377 |
| 22 | VP2602 | Iron-regulated outer membrane virulence protein homolog | Iron-regulated protein | 71,948 | NP_798981 |
| 23 | VPA1435 | Putative sodium/glutamate symporter | Iron-regulated protein | 77,059 | NP_800945 |
| 24 | VP1475 | Hypothetical protein | Unknown | 32,929 | NP_797854 |

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