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RESEARCH ARTICLE

Antigenicity analysis of *Vibrio harveyi* TS-628 strain

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Abstract *Vibrio harveyi*, the major causative agent of vibriosis, affects a diverse range of marine cultured organisms over a wide geographical area. However, reports about screening the effective antigen and research on vaccines of *V. harveyi* are scarce. Flagellin, lipopolysaccharide (LPS) and outer membrane proteins (OMP) are major immunogenic antigens in many Gram-negative bacteria. In this study, the flagellin, OMP and LPS of the *V. harveyi* TS-628 strain isolated from infected groupers were extracted and Western blot analysis was used to detect the antigenicity of these extractions. Results of the Western blot assay reveal that there are four positive flagellin bands: 35 kDa, 38 kDa, 43 kDa, and 52 kDa, of which the 43 kDa and 52 kDa bands displayed the strongest positive reaction. There are five positive OMP bands about 35 kDa, 38 kDa, 43 kDa, 47 kDa, and 52 kDa, of which the 43 kDa appeared to have the strongest positive reaction although the other four proteins also displayed strong reactions. However, LPS is Western blot-negative. These results indicate that the 43 kDa and 52 kDa flagellin and OMP of size 43 kDa, 52 kDa can be candidates for developing vaccines against *V. harveyi*.

Keywords *Vibrio harveyi*, flagellin, LPS, OMP, antigenicity

1 Introduction

Flagellin, outer membrane proteins (OMP) and lipopolysaccharide (LPS) are major immunogenic antigens in many Gram-negative bacteria. Flagellins, known as Hauch antigens, are the primary chemical components of flagella (Shen, 2000). OMP are important components of the outer membrane of bacteria. According to research on bacteria cells, OMP can be divided into two categories, major protein and microprotein. Major OMP are excellent components for the development of vaccines because it is relevant to the pathogenicity and immunoprotection of bacteria (Suzuki et al., 1996; Tu and Kawai, 1999). LPS is the characteristic component of bacterial outer membrane. On the one hand, LPS is regarded as pathogenic factors, which play an important role in the pathogenicity of Gram-negative bacteria. On the other hand, LPS may benefit the infected hosts by reinforcing non-specific immunoprotections, resisting tumors and even triggering antibody production (Chen and Chen, 2002; Su and Nie, 2001). However, studies on the surface antigen of *Vibrio* are limited, and information about the surface antigen of *Vibrio harveyi* is scarce.

V. harveyi, a marine Gram-negative organism with a single polar flagellum, has been recognized as a significant pathogen of marine vertebrates and invertebrates. The disease attributed to *V. harveyi* is initially distributed to the seawater aquaculture of China, Australia, India, Indonesia, Thailand, Philippines, Taiwan and other countries and regions and brought large financial losses (Saeed, 1995; Liu et al., 1997; Wu and Pan, 2001). In China, researches on *V. harveyi* are limited to isolating their pathogenic strains and describing their biological characteristics (Chen et al., 2004; Wang and Jin, 2000; Lin et al., 1999). Abroad, studies on V. *harveyi* have gone further (Lowery et al., 2005; Ulrich et al., 2005; Tendencia et al., 2004), however, studies on effective antigens and vaccines of *V. harveyi* are rare. Until now there is no available measure to prevent and cure the disease caused by *V. harveyi*. For the reasons above, further research on the pathogenicity and immunogenicity of *V. harveyi* are necessary, in order to make a foundation for the establishment of a credible serology detection method and the development of effective vaccines. Therefore, in this study, the flagellin, OMP and LPS of the pathogenic *V. harveyi* TS-628 strain isolated from infected *Epinephelus awoara* were extracted and their antigenicity analyzed by SDS-PAGE and Western-blot.

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2 Materials and methods

2.1 Bacterial strains

V. harveyi TS-628 isolated from infected grouper (*E. awoara*) have been identified and stored at −82°C in the laboratory (Qin et al., 2006). Other bacterial strains used for cross absorption test including *V. alginolyticus*, *V. parahaemolyticus*, *V.fluvialis*, *V. campbellii* and *Aeromonas hydrophila* were donated by Dr. Yan.

2.2 Antiserum preparation

Antiserum was prepared according to the method described by Zhu (Zhu and Chen, 2002).

2.3 Extraction of flagellar proteins

Crude flagellar proteins were isolated following the modification procedure of that described by Milton (Milton et al., 1996).

2.4 Extraction of OMPs

A modification of the protocol of Nikaido (1997) was employed for the separation of outer membrane proteins (OMPs). The bacterial cells were harvested by centrifugation at 6,000 g for 20 min at room temperature, washed in 20 mmol/L sterile Tris-HCl buffer (pH 7.2) twice and resuspended in the Tris-HCl buffer containing 1 μg/mL DNase and RNase. Then the cells were disrupted using an Ultrasonic cell disrupter system. Unbroken cells and cellular debris were removed by centrifugation at 1,000 rpm for 30 min at 4°C. Supernatant was collected and centrifuged for pellet at 40,000 rpm for 1 h at 4°C. The pellet was resuspended in 0.5% Sarksyl at room temperature for 30 min and was further centrifuged at 40,000 rpm for 1 h at 4°C. Then the pellet was resuspended in sterile saline and stored at −20°C after determined the concentration.

2.5 Extraction of LPS

LPS of *V. harveyi* TS-628 was prepared according to the method described by Westphal (Westphal and Jann, 1965).

2.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The flagellar proteins and OMP were analyzed following the procedure of that described in Molecular Cloning (Sambrook and Russel, 2002). The samples were separated electrophoretically on a nondenaturing PAGE with a mini-PROTEAN II™ apparatus (Bio-Rad) using 1-mm-thick slab gels containing 5% stacking and 15% resolving gels. After electrophoresis, the gels were stained with coomassie brilliant blue-R250. Low-molecular-weight protein standard (MBI) was used to calculate sample molecular weights.

LPS electrophoresis and silver staining were performed using the method described by Ming (Ming and Frasch, 1982).

2.7 Western-blotting

Western-blotting was carried out following the procedure of that described in Electrophoresis a Experimental Technology for Protein (Gou, 2001). After being separated by SDS-PAGE, the proteins and LPS were transferred to nitrocellulose membranes using the Mini-Protein II cell system (Bio-rad), which was carried out at a constant voltage of 100 mA overnight at 4°C. The membranes were blocked with 3% BSA/TBS for 60 min. After being removed from the blocked solution, the membranes were washed with TBS three times and antiserum of rabbit was added as primary antibody and incubated overnight at room temperature. Subsequently, primary antibody was discarded and the membranes were washed with TBS three times. Horseradish peroxidase conjugated goat anti-rabbit IgG was then added as a secondary antibody to incubate the membranes overnight at room temperature. The secondary antibody was then removed and the membranes washed with TBS three times. Finally, the immunoblots were detected by incubation in 0.2 mg/mL DAB/0.015 H_2O_2 at room temperature.

3 Results

3.1 SDS-PAGE and western blotting of flagellar proteins and OMP

The results of SDS-PAGE of flagellar proteins and OMP revealed that only two flagellar protein bands, about 35 kDa and 43 dDa, respectively, were clearly visible and there were seven OMP bands that were obvious, the molecular weight of which are about 21 kDa, 27 kDa, 35 kDa, 37 kDa, 40 kDa, 42 kDa and 44 kDa, respectively. Among them the 35 kDa protein was the most obvious (Figs. 1 and 2).

The western blotting results showed that there were four positive flagellin bands, about 35 kDa, 38 kDa, 43 kDa, 52 kDa, of which the 43 kDa and 52 kDa bands displayed the strongest positive reaction. There were five positive OMP bands, about 35 kDa, 38 kDa, 43 kDa, 47 kDa, 52 kDa, of which the 43 kDa showed the strongest positive reaction, although the other four proteins also displayed strong reactions (Fig. 3).

3.2 LPS electrophoresis and western blotting

LPS was not separated to thin bands after electrophoresis, but converged to a patch just following the bromophenol blue band (Fig. 4). LPS was detected negative by Western blotting.

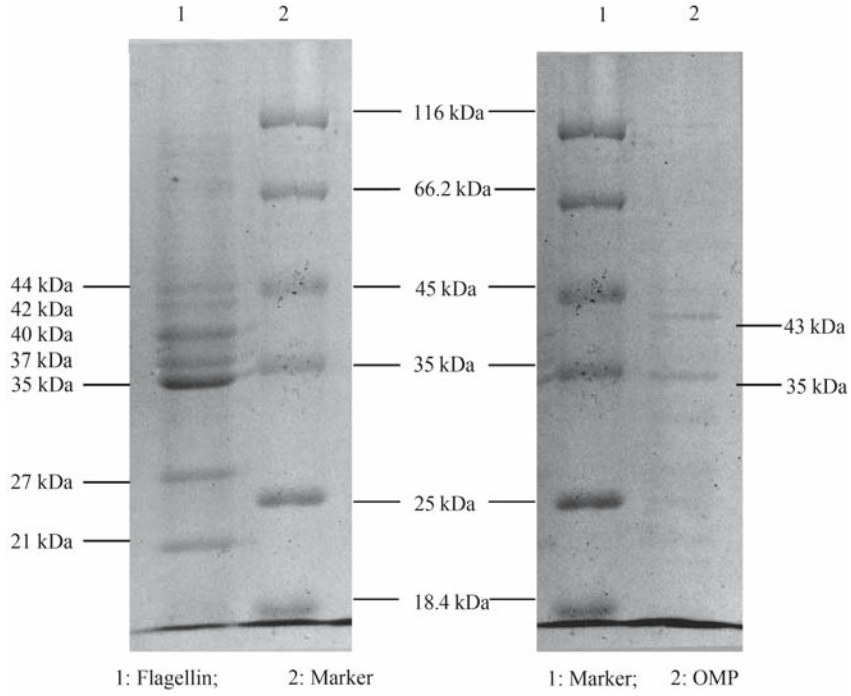

Fig. 1 SDS-PAGE of Flagellin

Fig. 2 SDS-PAGE of OMP

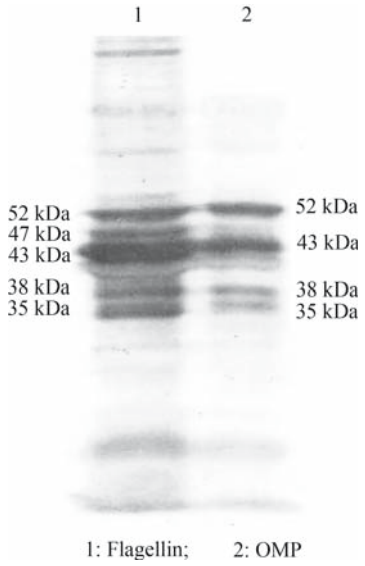

Fig. 3 Western blotting profiles of Flagellin and OMP

Fig. 4 Detection of LPS by silver stain

4 Discussion

The flagellum is a complex structure made up of three functional domains, the basal body, the hook and the filament, which are in turn are composed of many different proteins. These proteins are major antigenic determinants in many bacteria. McDonough and Smith (1976) found that molecular masses of bacterial flagellins were 28.6–8.2 kDa. Yang once purified the flagellar cores (FC) of *V. cholerae* and found this FC was composed of a single protein subunit with an estimated molecular weight of 45 kDa. The study of Yang et al. (1977) also indicated antibody prepared in rabbits against purified FC reacted with the flagellum of intact *V. cholerae* or purified FC. Later, Das et al. (1998) cloned, sequenced and expressed the flagellin core protein of *V. cholera*, and the result showed that the complete *flaA* gene would encode the flagellin core protein, which was approximately 45 kDa in size. The flagellin core protein reacted with both individual serum and pooled sera from convalescent cholera patients

in a Western blot. Others who studied the flagellin core protein of *Pseudomonas aeruginosa* (Totten and Lory, 1990), *V. parahaemolyticus* (McCarter, 1995) and *V. anguillarum* (McGee et al., 1996) also found that these proteins were 40–45 kDa. When computer analysis was used to determine the homology between the deduced amino acid sequence of the flagellin core protein from *V. cholerae* O395, *Pseudomonas aeruginosa*, *V. parahaemolyticus* and *V. anguillarum*, the flagellin core protein exhibited econservation, especially in the N-and C-termini (Das et al., 1998). According to the researches mentioned above, a conclusion that the flagellin core protein of *V. harveyi* should be 40–45 kDa in size could be made. In this study, a protein of 43 kDa was detected and further research should be carried out to determine whether this protein was the flagellin core protein of *V. harveyi* or not.

OMP is the major component of the outer membrane of bacteria. Recent studies indicated that OMP was an excellent antigen and could inspire both humoral and cellular immunity (Yu and Liu, 2000; Chen and Chen, 2002; Su and Nie, 2001). A study (Zhang et al., 1997) on the OMP profiles of 32 *Vibrio* type strains revealed that the major OMP profiles of different Vibrio species had considerable heterogenecity. Most of the strains had 3–7 major OMPs, with molecular masses ranging between 14 kDa and 91 kDa. Many strains had common major OMPs such as 54 kDa, 43 kDa and 27 kDa. However, no common major OMPs in all *Vibrio* type strains had been found. Another study (Zhou et al., 2003) on major OMPof *V. anguillarum* and *V. alginolyticus* showed that the profiles of major OMP extracted from the same strain by different methods were dissimilar and both of the two strains contained proteins sized 45 kDa, 30 kDa and 27 kDa. In this paper, the OMPs and flagellin of the *V. harveyi* TS-628 strain were found to contain lots of proteins, with molecular masses ranging between 16 kDa and 62 kDa, and most of these proteins were 36–46 kDa in size. However, it has not been easy to compare the results between different studies for the extraction methods were different and there was some errors in estimating their molecular masses.

The SDS-PAGE of flagellins and OMP showed that two flagellin bands and seven OMP bands were obvious and that the positive bands of western blotting were four and five, respectively. Some proteins were not clear or even not present on SDS-PAGE, but exhibited strong reaction on western blotting, which maybe attributed to the fact that western blotting is 20–100 times more sensitive than coomassie brilliant blue. The 43 kDa and 52 kDa flagellins and the 43 kDa OMP displayed strong antigenicity, and this could be ideal components to develop vaccine against *V. harveyi*.

The major flagellins on SDS-PAGE were 35 kDa and 43 kDa and the major OMPs were 35 kDa, while on western blotting the flagellins of 43 kDa and 52 kDa and the OMP of 43 kDa displayed a stronger reaction than other proteins, which revealed that the highest-content protein did not always exhibit the strongest reaction. A study (Zhang and Xu, 1997) comparing the OMP antigenicity of 13 strains of *V. parahaemolyticus* also displayed the same result.

Bacterial outer membrane also contains LPS, which can not only enhance nonspecific immunity but also trigger humoral immunity. In this paper, LPS extracts from the *V. harveyi* TS-628 strain was not separated into thin bands after electrophoresis and silver staining, but converged to a patch just following the bromophenol blue band, which indicated that the LPS was of low molecular masses and that the molecular weight differences between LPS molecules were not obvious. A study (Su and Nie, 2001) on LPS immunogenicity of *Cytophaga columnaris* Reichenbach also showed that there was only one LPS band of 17.5 kDa. And this LPS band was thick on gel and western blotting. This characteristic of LPS possibly connects with the highly variable and inhomogeneous O-polysaccharide chains. O-polysaccharide chains, the most variable component of LPS, are composed of repeated oligosaccharide that three to six monosaccharide units bound together by glycosidic bonds. The length of the O-polysaccharide chain depends on the number of repeated units of oligosaccharide, and the number of repeated units and the length of O-polysaccharide chain rests with the bacteria species. On the other hand, the number of repeated units is between 0 and 50, even the LPS extraction from the same bacteria, which displays remarkable endogenous heterogenicity.

In this study, LPS extraction was detected negative by western blotting, which may be attributed either to the fact that the LPS of the *V. harvey*i TS-628 strain can not trigger antibody production in rabbits or that the LPS displays weak immunogenicity to rabbits because the TS-628 strain is isolated from fish. For the above reasons, further research on LPS immunogenicity should be carried out.

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