Study of Capsular Polysaccharide from *Vibrio parahaemolyticus*

Yu-Chi Hsieh,1 Shu-Mei Liang,2 Wan-Ling Tsai,3 Yee-Hsiung Chen,1 Teh-Yung Liu,1 and Chi-Ming Liang1*

Institute of Biological Chemistry,1 Institute of Bioagricultural Sciences,2 and Office of Public Affairs,3 Academia Sinica, Taipei, Taiwan 11529

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The leading cause of food poisoning in both Taiwan and Japan is *Vibrio parahaemolyticus* infection, whose mechanism of enteropathogenesis is still unclear. To evaluate whether surface components are responsible for the intestinal adhesion of *V. parahaemolyticus*, we have developed a novel method for isolating the capsular polysaccharide (CPS) from *V. parahaemolyticus* (serotype O4:K8). We found that culturing of *V. parahaemolyticus* in broth for 1 week or more changed the colony form of the bacteria on an agar plate from opaque to translucent. The translucent colonies of *V. parahaemolyticus* contained little CPS and exhibited a much lower level of adherence to epithelial cells (Int-407) than the opaque colonies of the bacteria. Incubation of *V. parahaemolyticus* in medium supplemented with bile increased the levels of CPS and adherence. Treatment of *V. parahaemolyticus* with anti-CPS but not anti-LPS serum decreased the level of bacterial adherence. In addition, purified CPS bound to epithelial cells in a dose-dependent manner. Intranasal administration of CPS to mice in the presence of adjuvants such as immunostimulatory sequence oligodeoxynucleotides or cholera toxin elicited CPS-specific mucosal and systemic immune responses. These results indicate that CPS plays an important role in the adherence of *V. parahaemolyticus* to its target cells and may be considered a potential target for the development of a vaccine against this pathogen.

*Vibrio parahaemolyticus*, a widely distributed gram-negative facultative anaerobic bacillus that causes acute gastroenteritis, was first isolated from its victims in 1950 by Fujino et al. (14). Under favorable conditions, the doubling time of *V. parahaemolyticus* can be as short as 9 min (26), enabling the organism to multiply rapidly. The onset of the illness usually takes place 3 to 24 h after consumption of *V. parahaemolyticus*-contaminated food. The symptoms include diarrhea, abdominal cramps, nausea, vomiting, headache, and low-grade fever (19). Exotoxins of the organism, such as thermostable direct hemolysin and its related toxin, have been intensively studied. It is generally believed that these exotoxins alone are not sufficient to generate the pathogenicity of the organism (19, 36).

Before enteric pathogens can cause disease, they usually adhere to, replicate in, and produce virulence factors on or in epithelial cells of the enteric tract. The adherence of *V. parahaemolyticus* to epithelial cell lines has been evaluated in several in vitro studies (6, 7, 16, 43). The association of pili of a *V. parahaemolyticus* strain with bacterial colonization of epithelial cells has been documented (30, 44). However, this result has not yet been generally verified for other strains. Whether there is a mechanism of bacterial colonization other than pili needs further elucidation.

*V. parahaemolyticus* synthesizes three major surface antigens: heat-stable somatic O antigens (lipopolysaccharide [LPS]), heat-labile capsular K antigens (capsular polysaccharide [CPS]), and H antigens (flagellar antigens). Although H antigens of all types are serologically identical, 13 different O antigens and 71 different K antigens have been identified (8, 22, 41). Recently, culturing of *V. parahaemolyticus* with bile or deoxycholate was shown to increase not only the bacterial capsule size but also the production of a uronic acid-containing capsule and to enhance the level of adherence to epithelial cells (32). For other enteric bacteria, it has been shown that capsule size can play a major role in virulence (8, 45) and that the production of a uronic acid-containing extracellular capsule enables the bacteria to aggregate and adhere to intestinal cells (32). Therefore, it is possible that the adherence ability and pathogenicity of *V. parahaemolyticus* are related to its capsule. However, which major surface components are responsible for adherence remains to be determined.

In this study, we have purified and characterized a CPS from *V. parahaemolyticus* and evaluated the inhibitory effect of anti-CPS antibodies on the adherence of the bacterium to an intestinal cell line (Int-407). In addition, we have used CPS as an antigen together with either cholera toxin (CT) or immunostimulatory sequence oligodeoxynucleotides (ODN) to immunize mice (18, 20). CPS-specific IgA in the feces and IgG in the plasma of these immunized mice have been analyzed to determine the ability of CPS to elicit mucosal and systemic immunity.

**MATERIALS AND METHODS**

**Materials.** Sera against O4:K8 *V. parahaemolyticus* (anti-O4:K8 whole-bacterium sera) were obtained from the Institute of Biological Chemistry, Academia Sinica. Sera containing antibodies against O4 antigens (LPS) and K8 antigens (CPS) were purchased from Denka (Seiken, Japan).

**Culturing of bacteria and intestinal cell line.** *V. parahaemolyticus* isolates (13,023 clinical isolates, serotype O4:K8) obtained from the Culture Collection and Research Center in Taiwan (CCRC) were grown at 37°C in brain heart infusion (BHI) broth (Difco, Detroit, Mich.) supplemented with 3% NaCl. Inoculation of the bacterial cell line Int-407, purchased from CCRC, was routinely cultured as monolayers in basal Eagle medium (GIBCO BRL, Gaithersburg, Md.) containing 15% calf serum at 37°C in a humidified atmosphere of 5% CO₂ in air.
Isolation of CPS. A single bacterial colony isolated from BHI agar (1.5% agar in BHI broth supplemented with 3% NaCl) was inoculated into a 2-ml glass flask containing 500 ml of BHI culture broth and 3% NaCl. The mixture was shaken at 200 rpm overnight at 37°C. Bacterial cell particles and their debris were removed by centrifugation at 16,000 × g and 4°C for 20 min, and the supernatant was concentrated 10-fold by ultrafiltration (Labscale TFF system; 300,000-molecular-weight-cutoff membrane [Millipore, Bedford, Mass.]). The concentrated supernatant was centrifuged at 48,000 × g and 4°C for 2 h and subjected to enzymatic digestion with RNase (100 μg/ml), DNase I (50 μg/ml plus 1 mM MgCl₂), and pronase (250 μg/ml) at 37°C for 1 h. The enzyme-treated sample was dialyzed (100 native molecular-weight-cutoff membrane [Millipore]), and the resultant solution was lyophilized.

The lyophilized material was subsequently mixed with sample buffer solution (0.05 M glycine–0.001 M EDTA–10% sodium deoxycholate [pH 9.6]) and adjusted to a concentration of 10 mg of lyophilized powder per ml of buffer. The sample was passed through a 0.45-μm-pore-size nitrocellulose membrane (Amersham Biosciences, Uppsala, Sweden) to remove large particles. Thirty milliliters of the filtered sample was subsequently applied to a column of Sephacryl S-400 (5.0 by 90 cm; Amersham) that had been preequilibrated with running buffer solution (0.05 M glycine–0.001 M EDTA–3% sodium deoxycholate [pH 9.6]) (24). The effluent was monitored for the presence of CPS by a Western blot assay as described below. Fractions containing CPS were pooled, dialyzed with multiple changes of distilled water, and lyophilized. The lyophilized sample was mixed with 85% (vol/vol) ethanol and centrifuged at 16,000 × g and 4°C for 30 min. The pellet was collected, washed with 85% ethanol twice, dissolved in distilled water, and adjusted to a concentration of 10 mg of dry pellet per ml of distilled water.

Western blot analysis. Samples were subjected to electrophoresis on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham). The membrane was blocked by treatment with phosphate-buffered saline (PBS) containing 0.1% nonfat dry milk for 1 h at room temperature. After the blocking procedure, the membrane was incubated for 1 h with anti-O4:K8 whole-bacterium, anti-K8 CPS, or anti-O4 LPS serum (diluted 1/10,000 in PBS) and then washed with PBST containing 0.1% Tween 20 (PBST) and 5% nonfat dry milk for 1 h at room temperature. After the blocking procedure, the membrane was incubated for 1 h with anti-O4:K8 whole-bacterium, anti-K8 CPS, or anti-O4 LPS serum (diluted 1/10,000 in PBS) and then washed with PBST containing 0.1% Tween 20 (PBST) and 5% nonfat dry milk for 1 h at room temperature. After the blocking procedure, the membrane was incubated with anti-O4:K8 whole-bacterium, anti-K8 CPS, or anti-O4 LPS serum (diluted 1/10,000 in PBS) and then washed with PBST containing 0.1% Tween 20 (PBST) and 5% nonfat dry milk for 1 h at room temperature. After the blocking procedure, the membrane was incubated with anti-O4:K8 whole-bacterium, anti-K8 CPS, or anti-O4 LPS serum (diluted 1/10,000 in PBS) and then washed with PBST containing 0.1% Tween 20 (PBST) and 5% nonfat dry milk for 1 h at room temperature.

Monosaccharide and fatty acid composition analyses. Purified CPS was hydrolyzed with 18% (wt/wt) hydrochloric acid (2°C, 3 h) and dried with nitrogen gas at 2°C. The lipid portion and dephosphorylated chain fragments were separated twice by phase partitioning in CHCl₃–methanol–0.05 M NaH₂PO₄ (1:1:0.9). Water-soluble and organic-soluble fractions were dried by using nitrogen gas. The water-soluble fraction was used for monosaccharide composition analysis, while the organic-soluble fraction was used for fatty acid composition analysis. In brief, bacteria were cultured in BHI culture broth over-night, subcultured in BHI culture broth with or without bile (1% [vol/vol] preheated at 100°C for 30 min and then cooled to room temperature; Sigma, St. Louis, Mo.) for 3 h, and then cultured for another 30 min in freshly prepared, prewarmed BHI medium (without bile) at 37°C. The bacteria (about 10⁷ bacteria per 10 μl) were added to the Int-407 cell monolayer (10⁵ epithelial cells per well in a 24-well tissue culture plate) overlaid with antibiotic-free Hanks’ balanced salt solution (HBSS [GIBCO BRL]). After incubation for 30 min, the plate was washed with HBBS five times to remove loose bacteria, and cultured cells were lysed in 0.5 ml of HBSS containing 0.1% sodium deoxycholate. The solution was diluted 100-fold, and 50 μl was plated on BHI culture agar. Bacterial colonies were counted after overnight incubation at 37°C. The end-point titer was defined as the reciprocal of the highest dilution that resulted in an absorbance value two times greater than that of the negative controls. A nonpaired t test was used to analyze the significance of the difference in the antibody titers.

RESULTS

Extraction and purification of CPS. To isolate capsular materials, V. parahaemolyticus cell cultures were shaken overnight. To separate CPS from cellular DNA, RNA, and protein contaminants, culture media were digested with DNase, RNase, and pronase and then subjected to ultrafiltration. Western blot analysis of treated media revealed two major bands (Fig. 1A, lane 2) reactive with anti-O4:K8 whole-bacterium serum. One of them (the upper band) also reacted with anti-K8 CPS serum (Fig. 1B), while the lower band reacted with anti-O4 LPS serum (Fig. 1C).

To separate the components contained in the upper and lower bands, Sephacryl S-400 gel filtration was used. After elution of the column with 3% sodium deoxycholate buffer, the fractions that contained K8 CPS (fractions 44 to 46, shown in Fig. 2, lanes 2 and 3) and O4 LPS (fractions 52 to 56, shown in Fig. 2, lanes 6, 7, and 8) were collected and lyophilized. The yield of purified CPS (pooled fractions 44, 45, and 46) was about 120 mg per liter of culture medium.

Composition analysis of K8 CPS. UV spectral analysis of the isolated K8 CPS revealed the absence of absorption at 260 and 280 nm (data not shown), indicating that it contained little, if

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any, nucleic acid or protein. The sugar content in K8 CPS, as measured by the phenol-sulfuric acid method, was about 70% (wt/wt). The monosaccharide composition analysis showed that this CPS contained glucose, galactose, fucose, and N-acetylgalatosamine in a ratio of 2:2:5:16. Fatty acid composition analysis showed that it also contained palmitic acid and stearic acid in a ratio of 1:4.

Expression of K8 CPS in medium containing bile or surfactants. The size and composition of the capsule may play an important role in the pathogenicity of enteric bacteria (8, 17, 43). It has been reported that the addition of 1.5% bile or 0.1% deoxycholate to V. parahaemolyticus cultures enhances not only bacterial capsule size on agar plates but also adherence to epithelial cells (32). To determine whether the amount of K8 CPS or O4 LPS was increased by the addition of bile, V. parahaemolyticus was incubated overnight in tubes containing medium with serially increasing concentrations of bile. After incubation, all tubes were adjusted to contain the same concentration of bile and were then rocked at 4°C for 3 h to minimize any differences due to the potential detergent effect of bile. The culture media were subsequently collected and analyzed by Western blotting. Figure 3 shows that the amount of CPS but not LPS increased as the bacteria were cultured with increasing concentrations of bile (from 0.1% up to 1.5%).

Deoxycholate, one of the major active components of bile (4, 32), had a similar enhancing effect on CPS of V. parahaemolyticus (Fig. 4). Since deoxycholate is a surfactant, we examined...
other surfactants to determine whether they have a similar effect (Fig. 4). Our results indicated that in terms of potency for increasing the expression of CPS, the order of these surfactants was as follows: deoxycholate > SDS > Triton X-100 > NP-40 > Tween 20.

Relationship between K8 CPS content and adherence of opaque and translucent *V. parahaemolyticus* colonies. Colonies of *V. parahaemolyticus* CCRC strain 13023 on BHI agar plates were usually in the opaque form. However, after 1 week or more of culturing in BHI culture broth, translucent colonies appeared upon plating on BHI agar plates again. Western blot analysis of the capsular antigen compositions in the translucent and opaque colonies showed that the translucent-colony bacteria had a much smaller amount of K8 CPS (Fig. 5A). Although the rates of growth of translucent and opaque colonies were equivalent (both exhibited similar doubling times of 15 min under our experiment conditions), we found that the adherence ability of the translucent colonies was at least 10-fold lower than that of the opaque colonies (Fig. 5B). Culturing of the translucent-colony bacteria in broth containing bile converted them to opaque-colony bacteria and increased their CPS content as well as their adherence to Int-407 cells (Fig. 5B).

Relationship among CPS, anti-CPS antibodies, and adherence. To evaluate whether the adherence of *V. parahaemolyticus* to Int-407 cells can be inhibited by any specific antibodies, the interactions of anti-O4:K8 whole-bacterium, anti-O4 LPS, and anti-K8 CPS sera with the bacteria and their effects were investigated. By mixing serum with *V. parahaemolyticus* for 1 h at room temperature, filtering the mixture through a 0.22-μm-pore-size membrane to isolate antibody-bacterium complexes, and using enzyme-labeled secondary antibodies to identify the complexes, we found that anti-O4 LPS serum, like anti-K8 CPS and anti-O4:K8 whole-bacterium sera, contained antibodies that bound to the bacteria, while preimmune and normal sera did not (data not shown). Although antibodies in all three kinds of sera bound to the bacteria, the adherence of bacteria to Int-407 cells was inhibited by anti-O4:K8 whole-bacterium and anti-K8 CPS sera but not by anti-O4 LPS serum (Fig. 6). Moreover, the inhibitory effect of the anti-O4:K8 whole-bacterium serum was affected by preincubation of the serum with CPS in a dose-dependent manner (Fig. 7). Preincubation of the anti-O4:K8 whole-bacterium serum with LPS (at a concentration even as high as 400 μg/well), on the other hand, had no effect (data not shown). In addition, we found that purified CPS bound to Int-407 cells in a dose-dependent manner (Fig. 8). These results suggest that K8 CPS may play an important role in the adherence of *V. parahaemolyticus* to enteric cells.
Elicitation of immune responses by K8 CPS. To evaluate whether CPS is a potent immunogen suitable for vaccination purposes, mice were immunized intranasally with CPS of *V. parahaemolyticus* alone or in combination with adjuvants. To monitor the induction of mucosal and systemic immune responses, an ELISA was used to detect CPS-specific IgA in feces and IgG in plasma. The top panel of Fig. 9 shows IgA titers in animal feces after CPS vaccination. The administration of CPS together with either ODN or CT as an adjuvant resulted in much higher responses (*P* < 0.05). In the ODN group, fecal IgA could be detected in almost every mouse (8 out of 9), compared to 0 out of 10 in the negative control group and 4 out of 10 in the group receiving CPS alone. The average IgA titer in the ODN group was three times higher than that in the group given CPS alone. When CT was used as an adjuvant, 10 out of 10 mice had CPS-specific IgA in their feces, and the average titer was eight times higher than that obtained with CPS alone. The trend for CPS-specific IgG in plasma was similar to that for IgA in feces. Higher CPS-specific IgG titers were detected in the plasma of mice immunized with adjuvants and CPS (Fig. 9, bottom panel) (*P* < 0.05). These results demonstrated clearly that in the presence of adjuvants, such as ODN and CT, *V. parahaemolyticus* CPS can elicit both mucosal and systemic immune responses.

**DISCUSSION**

As the major bacterium causing food-borne diseases in Southeast Asia, *V. parahaemolyticus* has been studied for more than 50 years. The surface antigens CPS (K antigens) and LPS (O antigens) have been used to serotype *V. parahaemolyticus*. For a total of 3,729 pathogenic isolates that were recovered from 1995 to 1999 in Taiwan, 40 serotypes were identified. Among these, O3:K6 was the most prevalent. A total of 2,234 isolates of O3:K6 were identified in 351 of the 542 *V. parahaemolyticus* outbreaks, while O4:K8 and O4:K10 isolates caused 107 outbreaks over the same period (8). In this study, a *V. parahaemolyticus* strain from Taiwan, CCRC strain 13023 (serotype O4:K8) (39, 42), was chosen mainly because sera against CPS, LPS, and whole bacteria of this strain were conveniently available. Once a mechanism of action is identified, other strains will be analyzed to examine its generality.

Enteric pathogens usually adhere to, replicate in, and produce virulence factors on or in epithelial cells of the enteric tract before they cause the syndrome. To evaluate whether major surface components of *V. parahaemolyticus* are responsible for bacterial colonization of enteric epithelium, we have successfully designed a method that easily separates CPS and LPS of *V. parahaemolyticus* CCRC strain 13023 (Fig. 1 and 2). This novel and simple method has helped us to characterize and evaluate CPS in this O4:K8 strain. Since other bacteria, such as *Staphylococcus aureus* and *Salmonella enterica* serovar Typhi, also contain CPS and LPS (3, 9, 25, 38), the purification scheme (with or without minor modifications) may be useful for the isolation of CPS from not only *V. parahaemolyticus* but also such CPS-encapsulated bacteria.

Composition analysis of the fraction containing purified CPS showed that it contained 70% sugar moieties and 30% fatty acids. Some variances, however, were too small to be shown. Normal serum seems to contain some factors that increase colony formation by bacteria.

FIG. 6. Inhibitory effect of antibodies on the adherence ability of *V. parahaemolyticus*. Bacteria were incubated with anti-O4:K8 whole-bacterium serum (O), anti-O4 LPS serum (▲), anti-K8 CPS serum (●), or preimmune serum (◇) before addition to Int-407 cells for the adherence assay. Values are reported as means and standard deviations (n = 3). Some variances, however, were too small to be shown. Normal serum seems to contain some factors that increase colony formation by bacteria.

FIG. 7. Influence of CPS on the inhibitory effect of antibodies. (Top) Bacteria were incubated with anti-O4:K8 whole-bacterium serum (●) or CPS (20 μg/well) plus anti-O4:K8 whole-bacterium serum (▲) before addition to Int-407 cells for the adherence assay. (Bottom) Bacteria were incubated with anti-O4:K8 whole-bacterium serum (1/100 dilution) and CPS at predesignated amounts as indicated. Values are reported as means and standard deviations (n = 3). CPS (100 μg/well) prevented the inhibitory effect of anti-O4:K8 whole-bacterium serum (1/100 dilutions) on the adherence of *V. parahaemolyticus* (*P* < 0.05).
acids, i.e., palmitic acid and stearic acid. Since, during the purification process, we used sodium deoxycholate, a detergent, to separate CPS from LPS, it is unlikely that the fatty acid components are due to the formation of mixed CPS-LPS micelles. However, our results do not conclusively show that CPS is a glycolipid. Using $^{32}$P to intrinsically label polysaccharide, Gotschlich and coworkers (17) showed that there was a lipid material covalently linked to the reducing end of CPS of meningococcal groups A, B, and C. A similar approach is needed to demonstrate that *V. parahaemolyticus* CPS is indeed a glycolipid.

The adherence of *V. parahaemolyticus* to epithelial cell lines has been evaluated in several studies (5, 7, 43). Culturing of bacteria with bile or deoxycholate has been found to increase the size of the extracellular capsule and to enhance bacterial adherence to epithelial cells (32). Similar results were observed in our experiments. In addition, we found that the capsule content of CPS but not LPS increased upon supplementation of the bacterial culture with bile or deoxycholate (Fig. 3 and 4). Furthermore, we discovered incidentally that after 1 week or more of culturing of *V. parahaemolyticus* in BHI culture broth, the colonies of bacteria on agar plates had been transformed from opaque to translucent. The ability of the translucent-colony bacteria to adhere to epithelial cells was much lower than that of the opaque-colony bacteria. A comparison of the capsule composition of the translucent-colony bacteria with that of the opaque-colony bacteria showed that they apparently contained small quantities of CPS (Fig. 5). Culturing of translucent colonies of *V. parahaemolyticus* in broth containing bile converted them to opaque colonies and increased their CPS content (Fig. 5B) as well as their ability to adhere to Int-407 cells. We also showed that both anti-O4:K8 whole-bacterium and anti-K8 CPS sera inhibited the adherence of the bacteria to intestinal cells in vitro (Fig. 6). This inhibitory effect of the sera could be blocked by pretreatment of the sera with purified CPS but not LPS (Fig. 7). Moreover, we found that purified CPS bound to Int-407 cells in a dose-dependent manner (Fig. 8). These results clearly demonstrate that CPS rather than LPS is associated with the ability of *V. parahaemolyticus* to adhere to epithelial cells. It must be noted, however, that in other bacteria, such as *Haemophilus influenzae*, unencapsulated bacteria actually adhere better because surface adhesins (13, 35) that promote intimate interactions with epithelial cells and an extracellular matrix are unmasked from CPS. Whether factors other than CPS are also responsible for the adherence of *V. parahaemolyticus* to epithelial cells remains to be elucidated.

The human liver secretes about 1 liter of bile daily, and approximately 50 to 75% of the hepatic bile secreted during fasting enters the gallbladder, which concentrates, stores, and delivers concentrated bile into the duodenum when food leaves the stomach (33, 40). The concentration of bile in the human gut thus may vary not only from time to time but also from one location to another. However, the in vitro effective concentrations of bile (0.3%) and deoxycholate (0.1%) are within the physiological range in the human gut (≥0.1% deoxycholate) (4, 33, 37, 40); therefore, bile also could have an effect on *V. parahaemolyticus* in vivo.

Polysaccharide vaccines based on CPSs of various bacteria linked to a carrier protein have been developed and tested in animals and humans (1–3, 12, 23, 24, 28). However, there is limited information on the ability of such CPS vaccines to induce antibodies on mucosal surfaces (10, 11, 15, 27, 34). In
this study, the CPS of V. parahaemolyticus was administrated intranasally to mice either alone or with an adjuvant (ODN or CT) (20, 29) to evaluate the ability of CPS to induce mucosal and systemic immune response. Since V. parahaemolyticus commonly colonizes the intestinal mucosa, CPS-specific IgA levels in fecal samples from immunized animals were analyzed. We found that fecal IgA titers were threefold higher in sera from both the CPS-ODN and the CPS-CT experimental groups than in sera from the CPS-only group (Fig. 9). Higher CPS-specific IgG titers also were detected in sera from both the CPS-ODN and the CPS-CT experimental groups. These results indicate that with the help of an adjuvant, such as ODN or CT, CPS given intranasally can affect the levels of CPS-specific IgA in the intestine and IgG in plasma. Further studies are needed to determine whether the increased levels of CPS-specific IgA are sufficient to prevent enteric colonization by V. parahaemolyticus in vivo. Once the efficacy of CPS vaccination has been demonstrated, multivalent vaccines based on a cocktail of selected CPS serotypes could be developed for use against V. parahaemolyticus.

In summary, we have successfully isolated the CPS from V. parahaemolyticus and shown that it is positively correlated with the ability of the bacterium to adhere to intestinal cells. In addition, we have shown that the CPS of V. parahaemolyticus can induce mucosal and systemic immune responses. Our results suggest that the CPS of V. parahaemolyticus is a potential target for the development of vaccines against this pathogen.

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REFERENCES


