Effect of sypQ gene on poly-N-acetylglucosamine biosynthesis in Vibrio parahaemolyticus and its role in infection process

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The syp locus includes four genes encoding putative regulators, six genes encoding glycosyltransferases, two encoding export proteins, and six other genes encoding unidentified functional proteins associated with biofilm formation and symbiotic colonization. However, the individual functions of the respective genes remain unclear. Amino acid alignment indicates that sypQ is presumably involved in biosynthesizing poly-N-acetylglucosamine (PNAG), which is proposed to be a critical virulence factor in pathogen infection and is regarded as a target for protective immunity against a variety of Gram-negative/positive pathogens. However, no evidence showing that Vibrio parahaemolyticus also produces PNAG has been reported. Herein, the V. parahaemolyticus sypQ gene is confirmed to possess potential for producing PNAG for the first time. Our results indicated that gene sypQ is associated with PNAG biosynthesis and PNAG is involved in pathogen colonization. We propose that the function of pgaC in Escherichia coli could be taken over by sypQ from V. parahaemolyticus. We also tested whether PNAG can be used as a target against V. parahaemolyticus when it infects Pseudosciaena crocea. Our results showed that PNAG isolated from V. parahaemolyticus is an effective agent for decreasing V. parahaemolyticus invasion, implying that PNAG could be used to develop an effective vaccine against V. parahaemolyticus infection.

Keywords: colonization / P. crocea / PNAG / sypQ

V. parahaemolyticus

Introduction

Vibrio parahaemolyticus, a Gram-negative and halophilic bacterium, extensively inhabits natural and farmed aquatic environments around the world as a well-known food-borne pathogen causing gastrointestinal disease due to consumption of raw or undercooked seafood (Jiang and Chai 1996; Li et al. 2013). It has as well become a primary infectious disease for Pseudosciaena crocea cultivation and would lead to a catastrophic economic loss for coastal farmers if the disease outbreaks. Although dramatic progress has been made in the elucidation of infection mechanism for V. parahaemolyticus in the past decades, including full-length gene sequencing (Makino et al. 2003; Jun et al. 2013), discovery of a series of virulence factors exotoxin (Huntley and Hall 1996), serine protease (Lee et al. 2002), outer membrane protein (Inoue et al. 1995) and the roles of these virulence factors in the infection process are still unclear, and it is not even known how V. parahaemolyticus adheres to the host mucosal epithelial cells and avoids the host immune defenses.

Adhesion to the mucosal epithelial cell surface is the first step for infectious bacteria surviving in host (Acord et al. 2005), and is intermediated in essence by the binding interaction between the special ligand of pathogen surface and the host surface receptor. This selective binding activity leads to pathogenic bacteria accumulation in high density and takes a chance to break through the first immunobarrier-mucosal epithelial cell surface (Burgel and Nadel 2004; Guang et al. 2010). To investigate whether surface components are responsible for the intestinal adhesion, a capsular polysaccharide (CPS) was isolated from V. parahaemolyticus O4:K8 and it was found that it plays an important role in the adherence to epithelial cells (Hsieh et al. 2003). It was indicated that this polysaccharide component might be considered a potential target for the development of pathogenic vaccine (Nagayama et al. 1995; Hsieh et al. 2003); however, no biochemical characterization and structural information were further reported. It has also been reported that the opaque strain of V. parahaemolyticus produces abundant levels of extracellular polysaccharides (EPSs), in contrast to the translucent strains, and glucose, galactose, fructose and N-acetylgalactosamine were identified as four major sugars in EPS (Enos-Berlage and McCarter 2000). Furthermore, it has been confirmed that NtrC-regulated EPSs are crucial in biofilm formation of Vibrio vulnificus. It has also been proposed that some EPS components are important in interacting with the hosts (Kim et al. 2009).

Biofilm formation has been shown to play a key role in host colonization by V. fisheri (Nyholm et al. 2000; Yip et al. 2006), which is found to colonize shellfish (Nair et al. 2007).
However, this area of research remains mostly unexplored (Yildiz and Visick 2009). Studies have also revealed that biofilm formation depends on specific structural genes including genes related to polysaccharide biosynthesis, which regulate EPS or CPS expression (Yildiz and Visick 2009). A syr locus includes four genes encoding putative regulators, six genes encoding glycosyltransferases, two encoding export proteins and six other genes encoding unidentified functional proteins identified to be related to biofilm formation and symbiotic colonization (Shibata et al. 2012). However, the functions of individual genes remain elusive. Previous studies have also shown a kind of specific polysaccharide, designated polysaccharide intercellular adhesion (PIA) (Vuong et al. 2004; Izano et al. 2007) involved in the adhesion process. The research also confirmed that PIA is a poly-N-acetylglucosamine (PNAG), which is produced accompanying the biofilm formation in extensively pathogenic bacteria such as Staphylococcus aureus, Escherichia coli, Bordetella pertussis, Bordetella parapertussis, Yersinia pestis, Aggregatibacter actinomycetemcomitans and Staphylococcus epidermidis (Kaplan et al. 2004; Erickson et al. 2008; Hinnebusch and Erickson 2008; Itoh et al. 2008; Choi et al. 2009). However, the characterization of PNAG varied with different glycosyl residues and acetylation degree (Cerca et al. 2007). Studies have shown that purified PNAG can elicit protective immunity against coagulase-negative staphylococci and S. aureus, suggesting its potential as a broadly protective vaccine for staphylococci (Maira-Litran et al. 2004). Immunization-induced polyclonal animal antisera and monoclonal antibodies specific to either CPS or PNAG antigens have excellent in vitro opsonic killing activity in human blood (Skurnik et al. 2010). Some PNAG-synthesized genes including icaABDC, fbe, atIE, mecA and IS256 have been identified recently. Interestingly, the strain does not possess pathogenicity if it lacks these genes. To test our hypothesis that syrQ gene is related to PNAG production in pathogen V. parahaemolyticus, as well as PNAG function in the V. parahaemolyticus colonization process, related experiments were designed and performed herein.

Results and discussion

Bioinformatic analyses and gene identification

Potential function for individual gene of Vibrio parahaemolyticus was annotated by RAST server (Aziz et al. 2008; Meyer et al. 2008), and comparison with similar organisms by Clustal X2 method in the Seed Viewer revealing the contig from 151,913 to 1,570,720 should belong to putative glycosyltransferase syrQ gene (Supplementary data, Figure S1), which share identifications of 86.8, 85.9 and 75.8% with those genes in V. sp. Ex25, V. alginolyticus 12G01 and V. harveyi American Type Culture Collection (ATCC) BAA-1116, respectively, suggesting we designate it as syrQ gene in V. parahaemolyticus. Although a variety of studies have put their focuses on the function of structural symbiosis polysaccharide (syr) gene cluster in host colonization, biofilm formation, polysaccharide biosynthesis and the specific function of individual gene in syr locus is still uncovered (Shibata et al. 2012). Furthermore, BlastP alignment analysis (Altschul et al. 1997) of the genome sequences by PNAG biosynthesis related gene pgaC of E. coli O157:H7 (accession number NC_013008.1) showed the contig also sharing 25% amino acid identity, 42% positive with the pgaC gene, which encodes N-glycosyltransferase involving in a specific glycan PNAG biosynthesis (Kulasekara et al. 2009) in E. coli. To further understand the specific role of syrQ and test the hypothesis from bioinformatics deduction, a series of experiments were performed addressing PNAG-related function.

PNAG-positive strain screening

To investigate whether pathogenic V. parahaemolyticus also produce PNAG like other pathogenic Gram-negative/positive bacteria such as E. coli (Tagliabue et al. 2010) and S. aureus (Cerca et al. 2007), nine V. parahaemolyticus strains from different sources were used for evaluation and screening of PNAG production, as well as biofilm formation. Name strain and some characteristics including colony surface morphology are listed in Supplementary data Table S1. As shown in Figure 1, five strains including reference strain of V. parahaemolyticus RIMD2210633 (Makino et al. 2003) from ATCC exhibited mild abilities of biofilm formation in a variety of pathogens (Cerca et al. 2007), nine V. parahaemolyticus strains from different sources were used for evaluation and screening of PNAG production, as well as biofilm formation. Name strain and some characteristics including colony surface morphology are listed in Supplementary data Table S1. As shown in Figure 1, five strains including reference strain of V. parahaemolyticus RIMD2210633 (Makino et al. 2003) from ATCC exhibited mild abilities of biofilm formation in which strain V. parahaemolyticus ZIGS2010003 was demonstrated to be the strongest one of forming biofilm as well as PNAG synthesis by immune-blotting assay. Interestingly, this strain morphology is also the most wrinkle one among these tested strains (not shown). The strains exhibited defects in the formation of wrinkled colonies resulting in no PNAG detected, whose biofilm formation abilities were also limited. This is consistent with the previous report that PNAG appears to play an important role in biofilm formation in a variety of pathogens (Cerca and Jefferson 2008).

Polysaccharide homogeneity and relative molecular weight

The purified PNAG was acquired by a series of enzyme digestions followed by ion-exchanged chromatography and Sephacryl S-200 High-Resolution chromatography (Supplementary data, Figure S2A) in sequence, and the objective fractions were monitored by OD490 and immunoblot assay. As well, the relative molecular weight of PNAG was measured by high pressure (or high performance) liquid chromatography (HPLC) (Supplementary data, Figure S2B) equipped with serially linked TSK PWXL 4000 and 3000 gel filtration columns and signals were detected by an RI detector. The result indicated the purified PNAG with a relative molecular weight of ~1.7 × 10^4 Da calibrated with...
Dextran standards, which is consistent with the observation from western blotting assay (Supplementary data, Figure S3A). It appears that only a symmetrical peak was found in HPLC profile, indicating that the isolated polysaccharide should be homogenous (Supplementary data, Figure S2B). However, one small band detected in western blotting assay besides the main band could be due to contaminated polysaccharide, and their molecular weights are very close resulting in only one peak detected in HPLC.

**Structural characteristics of PNAG**

$^1$H NMR spectrum was obtained by 600 M NMR spectrometer (Supplementary data, Figure S4) and a signal at δ2.08 was observed, which was assigned to proton chemical shift of acetate group (CH$_3$–C–O). Meanwhile, two anomic proton signals observed at δ4.65 and δ4.37 were identified to be from residues of N-acetylglucosamine (GlcNAc) and GlcNH$_2$, respectively. A signal of δ2.74 was assigned to the H-2 chemical shift of GlcNH$_2$ (Choi et al. 2009). Interestingly, a peak at δ1.28 should be caused by CH$_3$ group from a small amount of deoxyhexose such as Fucose or Rhamnose (Ye et al. 2008), implying that the deoxyhexose residue contained in PNAG could be due to contamination or intrinsic constituent of PANG from *V. parahaemolyticus*. The SDS-PAGE in Supplementary data, Figure S3A also exhibited a small band below the main band of PNAG, which cannot exclude contamination of the sample and cross-immunity phenomenon happened. Of course, it also could be due to degradation of PNAG. The NMR analysis also revealed that almost 48% glucosamine residues are acetylated, as determined by comparing the integration of the area under the curves for the GlcNac-HI peak with all H-1 peaks (assuming that deoxyhexose is an inherent component of PNAG) from glycosyl residues of sample.

**Deletion of putative sypQ leads to loss of PNAG production**

To understand whether gene *sypQ* plays a critical role in the PNAG biosynthesis of *V. parahaemolyticus*, we firstly generated an in-frame deletion strain of *sypQ* gene as well as complementation strains. Immunoblot assay was then applied for evaluating the impact of *sypQ* on PNAG production. As shown in Supplementary data, Figure S3A, PNAG fractions were demonstrated in wild-type (WT) and complementation strains were shown to be of similar molecular weights detected by immublot assay. In contrast, the *sypQ*-deleted strain totally lost the ability of producing PNAG. These results may imply that *sypQ* play an important role in PNAG biosynthesis in *V. parahaemolyticus*. It is also found that ∆*sypQ* strain became a smooth colony in morphology compared with WT and complementation strains, which simultaneously indicates that *sypQ* could be associated with biofilm formation, as it was reported in other Vibrio bacteria (Shibata et al. 2012). According to the model proposed for PNAG secretion of *E. coli* (Whitney and Howell 2013), we also preliminarily draw a schematic showing the *sypQ* role in PNAG biosynthesis (Supplementary data, Figure S3B). In this model, the role of *pgaC* in *E. coli* is replaced by *sypQ* in *V. parahaemolyticus*, which acts to catalyze the polymerization of β-1,6-linked N-acetyl-D-glucosamine and facilitate its export across the inner membrane. In the process of polymerization, it is thought the *pgaD* play an auxiliary role for producing PNAG with possibility of binding c-di-GMP (Little et al. 2012). Once the PNAG polymer is partially de-acetylated by carbohydrate esterase regulated by gene *pgaB* (Little et al. 2012), *pgaA* is assumed to export matured de-N-acetylated PNAG to out-membrane (Itoh et al. 2008; Whitney and Howell 2013).

**Biofilm formation and eDNA release**

Even if the major constituents of biofilms are thought to consist of polysaccharides (including PNAG), cell surface and secreted bacterial proteins and extracellular DNA (eDNA), the exact composition of biofilms often varies (Cue et al. 2012). Therefore, a question is raised “Does PNAG defection lead to impair biofilm formation or totally lose the ability of biofilm?” As you have noticed in previous PNAG-positive strain screening (Figure 1), it seems that a linear relationship exists between PNAG production and biofilm formation in *V. parahaemolyticus*. To answer the question, biofilm production assay for WT, *sypQ* deletion mutant (PNAG deficient strain) and complementation were conducted, and the results were shown in Figure 2A. Consistent with previous research report (Shibata et al. 2012), deletion of the *sypQ* gene resulted in sharp decrease in the productivity of biofilm. However, complementation by knock-in of the *sypQ* gene restored the productivity of biofilm in comparison with the WT. However, the result was supposed to be based on the *sypQ* gene regulating PNAG production. In fact, the evidence herein can not set up the direct connection between PNAG and biofilm formation yet, owing to *sypQ* could regulate other molecular associated with biofilm formation except for PNAG.

As mentioned, eDNA also belongs to one of important biofilm matrices and is thought of as playing an important role in stabilizing the matrix that holds bacterial biofilm together (Jermy 2010), and it was recently established as a critical structural component in other pathogen biofilm matrices. Therefore, the hypothesis is that eDNA resulting from cell lysis would be more abundant in culture supernatants of WT and complemented strains than mutant deficient in PNAG production, which might result in cell lysis due to failure in forming integrated biofilm (Thomas et al. 2008). Consistent with research reports in other pathogen bacteria, deletion of *sypQ* gene obviously resulted in increase in the amount of released eDNA in supernatant (Figure 2B), which means that the biofilm formation was partially blocked under the condition of *sypQ* gene deletion.

**Gene deletion and PNAG-competitive inhibition revealing PNAG involved in adhesion process**

PNAG has been identified in a number of Gram-negative bacteria as an important component of the biofilm matrix contributing to overall persistence during infection (Whitney and Howell 2013). Besides, although a number of virulence factors (Huntley and Hall 1996; Lee et al. 2002; Yildiz and Visick 2009) have been identified in *V. parahaemolyticus*, the exact mechanism of the pathogen invading into host has not yet been clarified. Even which component intermediates pathogen adhesion to host is not known. As we described, more and more evidences indicated that polysaccharides could play a vital role in the invasion process. WT and its derivative adherence to *P. croceae* intestinal epithelial cells and the effects of PNAG on *V. parahaemolyticus* adhesion were herein performed to test
whether PNAG is a critical actor in pathogen adhesion. As shown in Figure 3A, deletion of the sypQ gene resulted in obvious loss of the adhesion ability of V. parahaemolyticus to P. crocea IEC cells. Conversely, the adhesion ability can almost be restored by complemented strain construction. Interestingly, inhibiting ability exhibited PNAG concentration dependence in PNAG-competitive experiments (Figure 3B), and the inhibiting rate can reach 70% when the concentration of PNAG was 0.1 mg/mL. However, the inhibiting rate cannot increase even using a higher PNAG concentration. These results indicated that PNAG could be involved in intermediating adhesion between pathogen and host, but this intermediation could be partial.

Colonization evaluation
To further understand the role of the sypQ gene and its proposal synthesized derivative PNAG in host colonization, we used constructed mutants strain as well as WT to inoculate uncolonized P. crocea and assessed colonization levels. The results indicated that mutant ΔsypQ exhibited severe defect in its ability to colonize P. crocea. Conversely, the complemented mutant almost recovered the ability of colonization from...
deletion mutant (Figure 4). This result implied that the gene of V. parahaemolyticus is associated with colonization, and its function could be exerted by PNAG intermediation. However, no direct evidence was documented so far concerning the relationship between sypQ gene and PNAG due to PNAG biosynthesis in organisms is a complicate process, and the synthesis mechanism is still elusive.

**Conclusion**

The previous studies have shown that deletion of the sypQ gene in another vibrio bacterium, V. fisheri, results in impairment of glass attachment ability, loss of matrix production and production of syp-polysaccharide with an antigenicity associated with PNAG biosynthesis. It also indicated that PNAG is involved in intermediating adhesion and colonization of V. parahaemolyticus to its host of P. crocea, which provides new insight into V. parahaemolyticus pathogen mechanism.

**Materials and methods**

**Strains, plasmids, reagents and growth conditions**

The bacterial strains and plasmids used in this research are listed in Supplementary data, Table S1. The culture medium for V. parahaemolyticus WT strain is as follows tryptic soy broth with sodium chloride (TSBS medium): TSB medium containing 1% glucose, 1% yeast extract, 1% glycerol and 3.5% NaCl. Antibiotics such as ampicillin (100 µg/mL), erythromycin (100 µg/mL) and kanamycin (1000 µg/mL) were added respectively, according to different screening and growth conditions for mutants. Escherichia coli strains were cultured under vigorous shaking of 250 rpm at 37°C in lysogeny broth (LB) medium (1 L medium containing 10 g tryptone, 5 g yeast extract, 5 g NaCl) with different antibiotics required. All antibiotics were purchased from Sigma chemical. Antibody to purified PNAG was raised in New Zealand White rabbits by the first immunization with 500 µg of polysaccharide in complete Freund adjuvant, and another three immunizations were followed in incomplete Freund adjuvant every three weeks, and the Rabbits were bled in 10 days of each immunization. The procedure was completed by Hangzhou Biosea Biotechnological Company and sera tested by enzyme-linked immunosorbent assay (ELISA) assay.

**Construction of V. parahaemolyticus ΔsypQ and ΔsypQ_C mutants**

The primers used for constructing deletion and complemented mutants are listed in Supplementary data, Table S2. For deletion mutant construction, PCR amplicon was prepared using overlap extension polymerase chain reaction (PCR) as described in the previous report (Heckman and Pease 2007). In brief, the upstream fragment (0.73 kb) and downstream fragment (0.65 kb) were amplified in reactions with corresponding primer pairs PsypQ-1 and PsypQ-2, PsypQ-3 and PsypQ-4, respectively. The upstream and downstream amplicons were then used as templates in a second PCR using Primers PsypQ-1 and PsypQ-2 to construct the gene-deletion fragments. The fragment of lacking sypQ gene was then ligated into suicide vector pXAC623 (Kuroda et al. 2005). The constructed plasmid subsequently transformed into E. coli strain π3813 and mobilized into an appropriate V. parahaemolyticus strain by filter mating. (Ditta et al. 1980; Visick and Skoufos 2001). The resultant successful colonies were selected on LB plates containing 10 µg/mL of chloramphenicol, and further screened by the PCR method (Le Roux et al. 2007), and the mutant strain was designated V. parahaemolyticus ΔsypQ. To construct complemented strain, PCR was performed containing the full-length sypQ gene and ligated into pGEM-T vector with restrict sites XbaI and SacI. The pGEM-T vector containing target gene was digested with XbaI and SacI, then second ligated into plasmid pRK415 (Keen et al. 1988) resulting in plasmid pRK415-sypQ. Then, the plasmid was mobilized into V. parahaemolyticus ΔsypQ to construct the V. parahaemolyticus ΔsypQ_C mutant.

**Biofilm plate assay**

Biofilm assay was performed based on the method described by Wakiimoto et al. (2004) with some modification. Twenty microliters of a stationary phase culture of different source strains were inoculated into 180 µL TSB supplemented with 1% glucose, 1% yeast extract, 1% glycerol and 3.5% NaCl in a 96-well optical-bottom plate (Thermo Scientific). The plate was incubated overnight (18 h) at 37°C without shaking. The second day, the plate wells were washed thric with phosphate buffered saline (PBS) after the growth medium discarded and
the cells attaching to the plate were dried for 1 h at 60°C, followed by staining with 100 μL Crystal Violet for 5 min. Then, the excessive Crystal Violet solution was washed away by rinsing the plate under tap water at least three times, drying the stained biofilm. OD₉₅₉ was measured by ELISA Reader.

**PNAG preparation**

*Vibrio parahaemolyticus* ZJGS2010003 grew for 3 days in TSB medium containing 1% glucose, 1% yeast extract, 1% glycerol and 3.5% NaCl. The cell pellets were collected by centrifugation at 4°C, 9000 × g, for 15 min, and suspended in 100 mL of 20 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid buffer (pH 8.0), extracting for 30 min at 100°C (The sample can be used for PNAG-positive strains screening at this point). The supernatant was collected by centrifugation at 4°C, 9000 × g, for 15 min for discarding cell pellets and precipitated with four volumes of ethanol. The precipitation was suspended in 40 mL PBS buffer (pH 7.0), and was digested with 0.1 mg/mL DNase I and 0.1 mg/mL RNase A at 37°C for 2 h, followed by digestion with 0.1 mg/mL Proteinase K at 56°C for 2 h. Subsequently, the precipitation was recovered and washed twice with PBS (pH 7.0) and dissolved in 5.0 M HCl. The solution was neutralized with 5.0 M NaOH in ice-bath. The sample was dialyzed and lyophilized and further applied to diethylaminoethanol-Sepharose Fast Flow and Sephacryl S High-Resolution chromatography for purification, and the fractions were detected by immune-blotting, along with the phenol-sulfuric acid method (Dubois et al. 1956), in which reducing groups of polysaccharides will give a sensitive and stable orange-yellow color (absorbance band of 490 nm) when treated with phenol and concentrated sulfuric acid.

**Nuclear magnetic resonance spectroscopy**

A sample of PNAG (10 mg) was dissolved in 50 μL of 5.0 M HCl solution, and then D₂O was added to a total volume of 500 μL. The sample was transferred into a nuclear magnetic resonance (NMR) tube and ¹H NMR spectrum was recorded at a temperature of 50°C using a Varian INOVA 600 NMR spectrometer by water presaturation pulse with 32 scans.

**V. parahaemolyticus adherence to P. crocea intestinal epithelial cells**

*Pseudosciaena crocea* intestinal epithelial cells (IEC) were cultivated in 24-well plates to a density of ~1 × 10⁵ cells/well for 3–5 days. Simultaneously, the *V. parahaemolyticus* bacteria were cultured into logarithmic growth phase and washed three times with Dulbecco’s modified Eagle’s medium (DMEM) medium prior to re-suspended in DMEM medium. To infect the monolayers, bacteria were inoculated at a bacterial cell to IEC in a ratio of 100:1 and incubated for 2 h, and then washed and lysed with 0.25% Triton X-100 for 20 min at 37°C for removal of IECs. The lysates were diluted in PBS and then plated on trypsinase soy agar plates. The number of colony-forming units (designated colony-forming unit (cfu0)) corresponded to the number of bacteria adherence to IECs (Sava et al. 2009).

**Inhibition of adherence ability of V. parahaemolyticus by PNAG**

PNAG was diluted into a series of concentration with DMEM medium and mixing with IECs for 30 min prior to addition of the bacteria. The adhesion assay was then performed as described in the section of *Vibrio parahaemolyticus* adherence to *P. crocea* intestinal epithelial cells. The number of colony-forming units corresponded to the number of bacteria adherence to IECs after addition of PNAG, designated cfu1. Therefore, the inhibition efficiency of PNAG was calculated by the following formula: survive rate (%) = [1 - (cfu0 - cfu1)/ cfu0] × 100.

**eDNA assay**

eDNA release was measured by the method described by Thomas et al. (2009) with a minor modification performed briefly as follows: The culture supernatants of overnight cultured *V. parahaemolyticus* were collected by centrifugation, and 200 μL supernatant aliquots were transferred into 96-well microplates in triplicate. SYTOX green (Invitrogen) was supplemented to these aliquots to a final concentration of 1 μM and incubated for 10 min before being spectrofluorometrically measured at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

**Colonization evaluation**

For evaluation of *sypQ* gene effect on colonization, strains *V. parahaemolyticus* WT, *V. parahaemolyticus* Δ*sypQ* and *V. parahaemolyticus* Δ*sypQ* C were used to infect *P. crocea* by the following process. Bacterial cells were inoculated into artificial seawater (ASW) at a concentration of ~1 × 10⁶ cells/mL. The precultured *P. crocea* with the same sizes were then introduced into the culture cages and cultured for 24 h. Subsequently, the ASW of the culture cages were replaced thrice with non-bacterium ASW (100 mM MgSO₄, 20 mM CaCl₂, 0.6 M NaCl, 20 mM KCl), and further cultured 2 for 4 h in ASW without bacteria. The *P. croceas* were then killed and intestines were taken out, washed and homogenized. Serial dilutions of the homogenates were plated onto TSBS agar, and the numbers of CFU were calculated. Statistical significance (P-value) was determined by analysis of variance (ANOVA), and the graphic was performed by the PRISM5 software.

**Bioinformatic and statistical analysis**

*Vibrio parahaemolyticus* genomic sequence was retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/) and was submitted to the RAST server for gene function re-annotation (Aziz et al. 2008; Meyer et al. 2008). An amino acid sequence alignment was performed with ClustalX (http://clustal.org/clustalw2/), and the aligned sequences were analyzed using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html) with a default setting of the fraction of sequences parameter as 0.5. Statistical analyses were performed by the program ANOVA and all values were expressed as mean ± standard deviation (SD). In all statistical analyses, Significance was determined by *P* < 0.05. The graphics were done by PRISM5.

**Supplementary material**

Supplementary material for this article are available online at http://glycob.oxfordjournals.org/.
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Conflict of interest

The authors have declared that there are no confictions of interest, financial or otherwise, associated with the publication of this study.

Abbreviations

ASW, artificial seawater; ATCC, American Type Culture Collection; CFU, colony-forming unit; CPS, capsular polysaccharide; DEAE, diethylaminoethanol; DMEM, Dulbecco’s modified Eagle’s medium; eDNA, extracellular DNA; ELISA, enzyme-linked immunosorbent assay; EPS, extracellular polysaccharide; GlcNAc, N-acetylglucosamine; HPLC, high pressure liquid chromatography; IEC, intestinal enzyme-linked immunosorbent assay; ICA, intestinal carbohydrate; IPA, polysaccharide intercellular adhesion; PNAG, poly-N-acetylglucosamine; syp, symbiosis polysaccharide; TSA, tryptic soy agar; TSBS, tryptic soy broth with sodium chloride; WT, wild-type.

References


