RESEARCH LETTER

Bacillus subtilis spores expressing the VP28 antigen: a potential oral treatment to protect *Litopenaeus vannamei* against white spot syndrome

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Abstract

The envelope protein VP28 of white spot syndrome virus (WSSV) is considered a candidate antigen for use in a potential vaccine to this important shrimp pathogen (the cause of white spot syndrome, WSS). Here, we used spores of *Bacillus subtilis* to display VP28 on the spore surface. Trials were conducted to evaluate their ability to protect shrimps against WSSV infection. The gene *cotB-vp28* was integrated into the chromosome of the laboratory strain *B. subtilis* PY79, and expression of CotB-VP28 was detected by Western blotting and immunofluorescence. Expression of CotB-VP28 was equivalent to 1000 molecules per spore. PY79 and CotB-VP28 spores were mixed with pellets for feeding of whiteleg shrimps (*Litopenaeus vannamei*), followed by WSSV challenge. Superoxidase dismutase (SOD), phenoloxidase activities and mortality rates of the two shrimp groups were evaluated. Groups fed with PY79 and CotB-VP28 spores at day 7 had increased SOD activities of 29% and increased phenoloxidase activities of 15% and 33%, respectively, compared to those of the control group. Fourteen days postchallenge, 35% of vaccinated shrimps had died compared to 49% of those fed naked spores (PY79) and 66% untreated, unchallenged animals. These data suggest that spores expressing VP28 have potential as a prophylactic treatment of WSS.

Introduction

White spot syndrome virus (WSSV) was identified in Taiwan in 1992 and widely accepted as the most important pathogen of cultivated shrimps having caused substantial losses in the shrimp industry over the last two decades (Sanchez-Martinez *et al.*, 2007). Although, the mechanism of interaction between WSSV and host cells is not well defined, protein VP28 (27.5 kDa) is a major capsid protein of WSSV (Van Hulten *et al.*, 2001; Witteveldt *et al.*, 2004), which plays an important role in the infection process as an attachment protein, binding the virus to shrimp cells and helping it enter the cytoplasm (Yi *et al.*, 2004). This is the primary reason why VP28 has been chosen as a potential 'vaccine candidate' and is being used a target for diagnostic studies (Van Hulten *et al.*, 2000; Rout *et al.*, 2007).

With regard to 'vaccination', it is important to realise that the immune system of shrimps is far less developed than in fish and other vertebrates. More specifically, shrimps have no adaptive memory, or in other words, they have no ability to produce immunoglobulins. This means that they depend on innate defence systems to protect against pathogens (Sarathi *et al.*, 2007). Parameters reflecting the innate immune response are total hemocyte counts and enzyme activities such as superoxide dismutase (SOD) and phenoloxidase. Hemocytes including nongranular cells (hyaline cells) play important roles in phagocytosis and in the elimination of all foreign organisms potentially hazardous for the host. During

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phagocytosis, hyaline cells produce oxygen free radicals of superoxide (O^-), hydroxyl free radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) that are necessary to kill pathogens. These result in increased levels of SOD that reduce levels of these free radicals and subsequent host toxicity. On the other hand, granular cells can contribute to an innate immune defence by activating the conversion of prophenoloxidase (pro-PO) to an active form phenoloxidase that can then catalyse the oxidation of tyrosine to produce toxic quinones and other short-lived reaction intermediates leading to the formation of melanin. It has been reported that melanin binds to the surface of bacteria and increases the adhesion of hemocytes to bacteria, thus accelerating their removal by formation of small nodes (Cerenius & Soderhall, 2004).

VP28 has been used as a vaccine candidate originating from seminal studies conducted by Witteveldt *et al.* (2004) where purified recombinant VP28 (rVP28) was coated onto feed pellets and administered orally to *Penaeus monodon* resulting in approximately 70% protection. In other studies, feeding *Penaeus japonicus* (Kuruma shrimp) for 2 weeks using pellets coated with purified rVP28 provided protection ranging from 30% to 70% (Caipang *et al.*, 2008). A major drawback with using a recombinant protein is its stability and the cost of production. Alternative approaches have used expression of VP28 in *Bacillus subtilis*. Fu *et al.* (2010) have expressed rVP28 in vegetative cells of *B. subtilis* and obtained 83% protection in *Fenneropenaeus chinensis* (Chinese white shrimp) when feeding recombinant vegetative cells for 14 days before challenging. In more recent work, Ning *et al.* (2011) have expressed VP28 as protein chimeras on the spore coat of *B. subtilis* spores. Crayfish (*Cambarus clarkia*) administered orally with pellets mixed with recombinant spores expressing VP28 fused to the spore coat proteins CotB-VP28 and CotC-VP28 exhibited significant levels of protection (46–50%) when challenged with WSSV (Ning *et al.*, 2011). Clearly, the use of spores is more attractive than the use of live bacteria as spores are inherently heat stable and desiccation resistant making them suitable for inclusion in feed products. In this study, we have evaluated the use of *B. subtilis* spores expressing a chimeric protein, CotB-VP28, on the spore surface for vaccination to WSSV in *Litopenaeus vannamei* (Whiteleg shrimp). The *vp28* gene has been amplified from the genome of the most common WSSV variant (*vp28-ST*) isolated in Vietnam, for development of a vaccine specifically against WSSV infection in Vietnam. This shrimp species was chosen because it is the number one export species in Vietnam. Our results show that oral administration of recombinant CotB-VP28 spores had potential in protecting shrimps from WSSV infection.

Materials and methods

Bacterial strains and general methods

Bacillus subtilis wild-type strain PY79 (Spo^+) was used for strain constructions, this strain is a laboratory strain (Youngman *et al.*, 1984). Basic methods for *B. subtilis* were those described in Harwood & Cutting (1990). Basic molecular biology methods followed Sambrook & Russell (2001).

Construction of gene fusions

The *vp28* encoding gene was amplified by PCR from WSSV isolated from shrimp samples collected in Soc Trang province, Vietnam. The *vp28-ST* (*vp28 Soc Trang*) gene was cloned into plasmid pGEMT (to create recombinant plasmid designated pGEMT-vp28-ST) and confirmed by DNA sequencing. For cloning the gene coding VP28-His tag, PCR primers had flanking sequences at their 5′-ends, which were recognised by BamHI (VP28-Fw1 primer) and HindIII (VP28-Rv1 primer). The sequences were as follows: VP28-Fw1: 5′-CGCGGATCCGATCTTTCTTTCACTCTTTCGG-3′; VP28-Rv1: 5′-CCCAAGCTTTTACTCGGTCTCAGTGCCAGAG-3′. The *vp28-ST* gene was ligated into pET28b via BamHI and HindIII cohesive ends and transformed into *Escherichia coli* BL21-RIL, creating recombinant plasmid designated pET28b-*vp28-ST*.

The method for cloning CotB-*VP28* in *B. subtilis* is similar to what described previously with CotB-streptavidin (Nguyen *et al.*, 2013). The primers carried flanking sequences at their 5′-ends, which were recognised by HindIII (for VP28-Fw2 primer) and EcoRI (for VP28-Rv2 primer) were used in the PCR amplifying *vp28-ST*. The sequences were as follows: VP28-Fw2: 5′-CCCAAGCTTGATCTTTCTTTCACTCTTTCGG-3′ and VP28-Rv2: 5′-AAAGAATTCTTACTCGGTCTCAGTGCCAGAG-3′. The *vp28-ST* gene was ligated into pDG364 *cotB* plasmid, which already contains the natural promoter region (P_{cotB}) and the 825-bp DNA fragment coding for truncated CotB, via HindIII and EcoRI cohesive ends, creating recombinant plasmid designated pDG364 *cotB-vp28-ST*. Then, the recombinant plasmids were linearised by XhoI and transferred into competent cells of the *B. subtilis* PY79 strain. Chloramphenicol-resistant (Cm^R) clones were the result of a double crossover recombination, resulting in the interruption of the nonessential *amyE* gene on the *B. subtilis* chromosome. The created clone was named CotB-VP28.

Preparation of spores and extraction of spore coat proteins

Sporulation of PY79 and the CotB-VP28 were made in DSM (Difco Sporulation Media) at 37 °C in a fermenter

using the exhaustion method. Sporulating cultures were harvested 24 h after the initiation of sporulation. Purified suspensions of spores were made using lysozyme treatment, followed by washing in 1 M NaCl, 1 M KCl and dH_20 water (Nicholson & Setlow, 1990).

VP28 purification

VP28-His tag production was induced in *E. coli* BL21-RIL (Novagen) containing the pET28b-vp28 recombinant plasmid with 1 mM IPTG in LB medium. The induced protein was purified by Ni-NTA affinity chromatography using Ni^{2+}-NTA His-Bind resins (Novagen) under denaturing conditions (8 M urea). The purified protein was renatured by dialysis (three times) in PBS pH 7.4.

Western and Immunofluorescence analysis

Spore coat proteins were extracted from spore suspensions as described elsewhere (Nicholson & Setlow, 1990). Colour was developed using the NBT/BCIP substrate, and the intensity of specific bands was analysed by Scion Image® software of NIH, USA (Nguyen *et al.*, 2013). Immunofluorescence labelling and observation were similar to what described previously (Nguyen *et al.*, 2013), except that the primary antibody used was an anti-VP28 rabbit polyclonal (1 : 2000 dilution). Fluorescent images of all samples were acquired by confocal fluorescence microscope LSM5 (Carl-Zeiss) under the same excitation condition of green laser 525 nm and detection condition of camera.

Preparation of feed pellets coated with spores

A concentrated suspension of either PY79 or CotB-VP28 spores was repeatedly sprayed onto shrimp feed pellets and mixed, then followed by coating with cod liver oil (Merck). The mixing ratio of spores and pellet was optimised so that final concentration of spores was 1×10^9 CFU g^{-1} pellet. The ratio of cod liver oil to pellets was 2 mL 100 g^{-1}. The feed pellets were then stocked for a maximum of 5 days at 4 °C before use.

Oral feeding of whiteleg shrimps

Whiteleg shrimps (*c.* 5 g) were divided into four experimental groups ($n = 60$ per group), and each group housed in two separate composite 275-L, round-shape tanks ($n = 30$ per tank) containing 100 L of artificial sea water and equipped with an air supply system. Shrimps were maintained under the following conditions: 26–28 °C, pH 7.5–8.5, DO ≥4 mg L^{-1}, 25 ppt salinity. For oral administration of *B. subtilis* spores, pellets coated or uncoated were used at amounts equivalent to 0.1–0.2% of shrimp body weight. Shrimps were fed for different periods of time (7, 14 and 21 days) depending on further experiments.

Persistence of spores in the intestinal track of shrimp

Whiteleg shrimps of the control, PY79 and CotB-VP28 groups before (day 0) and after 7, 14 and 21 days of feeding were collected for dissection of the intestinal track and enumeration of intestinal spore counts. Individual intestinal tracks of shrimps ($n = 3$) of each group collected at each time point were homogenised in 0.9% NaCl and spore heat counts (65 °C for 20 min) determined by serial dilution and plating on LB agar (for PY79) and LB agar + chloramphenicol (5 μg mL^{-1}) for CotB-VP28. Three colonies from representative plates were randomly screened for the presence of the *vp28* gene by PCR.

SOD activity

Ten whiteleg shrimps from each experimental group (negative control, PY79, CotB-VP28) were collected at each time point (days 0, 7 and 14), and the chitin shell was removed. Approximately 100 mg of muscle tissue in 400 μL of phosphate buffer was homogenised. After centrifugation (7000 ***g*** for 10 min at 4 °C), the supernatant was analysed for SOD activity using the method of McCord & Fridovich (1969).

Phenoloxidase activity

Ten whiteleg shrimps from each experimental group (negative control, PY79, CotB-VP28) were collected at each time point (days 0, 7 and 14), and hearts removed. Cacodylate buffer (0.01 M sodium cacodylate pH 7.0, 0.45 M NaCl, 0.01 M $CaCl_2$, 0.26 M $MgCl_2$) was added at a ratio of 200 μL buffer to 10 mg of shrimp heart and homogenised. The supernatant was removed after centrifugation (7000 ***g*** for 10 min at 4 °C) and phenoloxidase activity determined using the method described previously (Luciane & Margherita, 1997).

WSSV challenge

The titre of virus required for the desired challenge of 60–100% mortality in 14 days was determined by *in vivo* infection experiments to be a 10^{-4} dilution from a WSSV stock aliquot at concentration of 2×10^7 copies mL^{-1}. Four experimental groups ($n = 60$ per group) were used: Group 1, 'unchallenged', animals fed with uncoated pel-

lets; Group 2, 'PY79', pellets coated with PY79 spores (10^9 CFU g^{-1} of pellets); Group 3, 'CotB-VP28', pellets coated CotB-VP28 spores (10^9 CFU g^{-1} of pellets); and Group 4, 'untreated', animals fed with uncoated pellets and challenged with PBS (100 μL). After 7 days of feeding, individual shrimps in each experimental group were intramuscularly injected with 100 μL of WSSV at 2×10^3 copies mL^{-1}. For the untreated group, shrimps were injected with 100 μL of PBS buffer. The shrimps which died immediately after injection (within 24 h) were excluded and not taken into counting of the mortality rate due to WSSV. Cumulative mortality of shrimps in each group was recorded after 14 days. Dead shrimps were randomly selected to test for the presence of WSSV using specific primers for the *vp28* gene by PCR method. SD was calculated based on data collected from two experiments. The relative survival rate was calculated as (1 − mortality of immunised shrimp/mortality of the untreated group) × 100.

Results and discussion

Construction of spores expressing VP28 displayed on the spore surface

The WSSV antigen VP28 was expressed as a fusion protein on the surface of *B. subtilis* spores by fusion of the *vp28* gene with *cotB* that encodes a major protein of the outer spore coat. The *cotB-vp28* chimeric gene was inserted ectopically into the *amyE* sequence of *B. subtilis* genome such that a partial diploid carried one intact gene of *cotB* together with a chimera, *cotB-vp28*. Previous studies have shown that expression of CotB chimeras is optimal in the presence of a full length CotB protein (Isticato *et al.*, 2001). Four isolates of *B. subtilis* were identified that expressed CotB-VP28 and proteins extracted from the spore coats of one isolate which we refer to henceforth as CotB-VP28, were probed with anti-VP28 polyclonals. Western analysis identified a single species of 63 kDa (Fig. 1a, lane 1) that corresponded in size to the CotB-VP28 chimera consisting of CotB (35 kDa) and VP28 (27.5 kDa). This band was absent in extracts of proteins obtained from spores of PY79 which was used in the construction of CotB-VP28 (Fig. 1a, lane 2). As a further control, we demonstrated that the anti-VP28 recognised rVP28 (*c.* 28 kDa; Fig. 1a, lane 3). Using analysis by SCION IMAGE® software, we estimated the amount of VP28 extracted from 5×10^9 CotB-VP28 spores to be about 225 ng. Based on the molecular weight of VP28 (27.5 kDa), we could therefore calculate that about 1000 molecules of CotB-VP28 were expressed on a single CotB-VP28 spore. As a second approach to confirm expression of CotB-VP28, we used immunofluorescence microscopy. The twofold- higher intensity for CotB-VP28 (Fig. 1b; lower panels) compared to PY79 (Fig. 1b; upper panels) confirmed stable expression of VP28 on the surface of CotB-VP28 spores.

Persistence of CotB-VP28 spores in the shrimp GI-tract and associated immune-related enzyme activities

Shrimps were fed with pellets coated with CotB-VP28 spores for 21 days and the number of CotB-VP28 spores recovered in the shrimp intestines determined by plating heat-treated samples of excised GI-tracts onto selective agar (see Materials and methods). Our data revealed that counts of CotB-VP28 spores were present at a CFU of

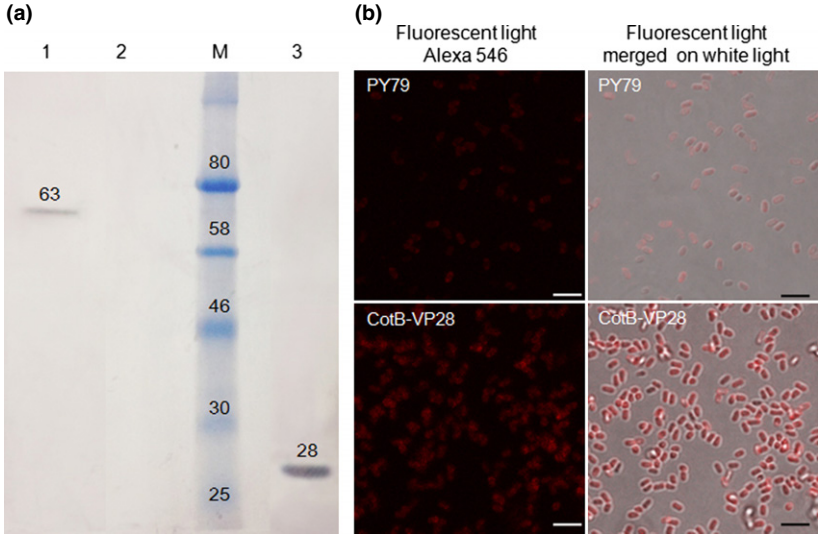

Fig. 1. Expression of CotB-VP28 on the *Bacillus subtilis* outer spore coat. (a) Coat proteins were extracted from spores (2×10^9) of CotB-VP28 (lane 1) and PY79 (lanes 2). Lane M was prestained low-molecular weight bands (25, 30, 46, 58 and 80 kDa). 900 ng of rVP28 was loaded as a control (lane 3). The VP28 chimeric protein was detected using an anti-VP28 polyclonal antibody. (b) Laser scanning confocal micrographs showing individual PY79 (upper images) and CotB-VP28 (lower images) labelled with anti-VP28 rabbit polyclonal antibody and secondary, anti-rabbit antibodies conjugated with Alexa 546 as indicated by red signals (left panel) under excitation by green laser. The right panel shows a fluorescent image merged with a light microscopy image. Bar = 2 μm.

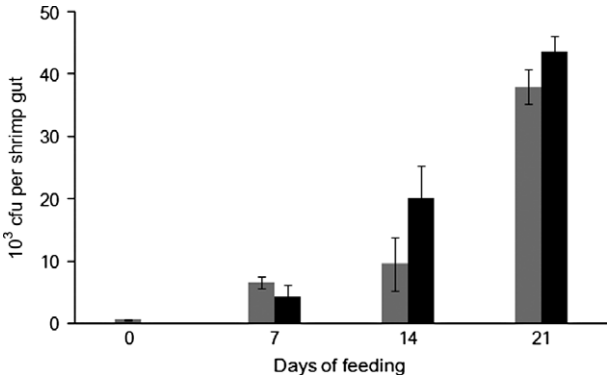

Fig. 2. Counts of PY79 and CotB-VP28 spores in single shrimp's intestine during 21 days of feeding. Whiteleg shrimps were fed with pellets coated with either PY79 (grey bars) or CotB-VP28 spores (black bars) at 1×10^9 CFU g^{-1}. Heat-resistant (65 °C, 25 min) counts were determined from fresh shrimp intestines collected from individual shrimps before (0 day) and after 7, 14 and 21 days of feeding. Counts shown are means of results from three animals. The detection limit for PY79 and CotB-VP28 spores are 5×10^2 and 0 CFU, respectively, as indicated by the counts at day 0. Error bars are standard deviations.

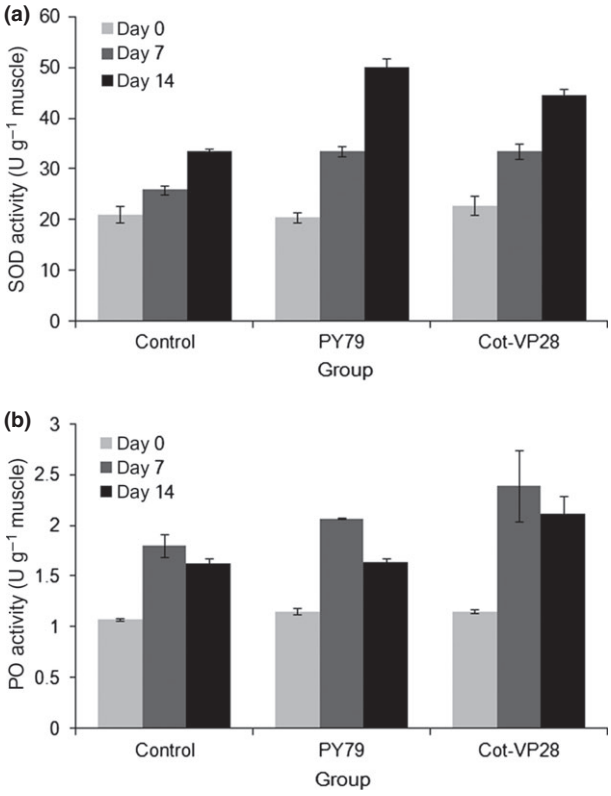

Fig. 3. Enzyme activities of *Litopenaeus vannamei* after feeding with CotB-VP28 spores. SOD (a) and phenoloxidase (b) activities of *L. vannamei* after continuous feeding with pellets coated with 1×10^9 CFU g^{-1} of PY79 or CotB-VP28 spores to shrimps at day 0 (light grey bars), day 7 (dark grey bars) and day 14 (black bars). Groups were negative controls (pellets without coating), PY79 (pellets coated 1×10^9 CFU g^{-1} of PY79 spores), and CotB-VP28 (pellets coated 1×10^9 CFU g^{-1} of CotB-VP28 spores). Data are presented as arithmetic means, and error bars are standard deviations.

4.2×10^3 CFU per shrimp at day 7 but actually increased over time reaching about 4×10^4 CFU per shrimp after 21 days (Fig. 2). We used PCR of the *vp28* gene to verify that heat-resistant spore counts recovered from the shrimp GI-tract were in fact CotB-VP28. In parallel studies using PY79, the congenic strain of *B. subtilis* used to create CotB-VP28, we were able to recognise a similar profile of spore counts in the GI-tract of fed shrimps as Cm-sensitive *B. subtilis* in the shrimp gut increased from 500 CFU per shrimp (background) to 6500 and 9500 CFU per shrimp at days 7 and 14, respectively. If the spores simply transited through the intestine, we might expect the spore CFU to remain relatively constant as the transit time through the GI-tract must be no more than a few hours. We interpret this data then as evidence that spores may be able to accumulate in the GI-tract of shrimps, and presumably this indicates adhesion to the intestinal epithelium.

SOD enzyme activity has been commonly detected in hemocytes of shrimps, for example, in *L. vannamei* that had been induced by β-glucan (Campa-Córdova *et al.*, 2002), and in *F. chinensis* which had been vaccinated with *B. subtilis* vegetative cells expressing VP28 (Fu *et al.*, 2010). We measured SOD and phenoloxidase activities in homogenised tissues in shrimps fed with CotB-VP28 and PY79. As shown in Fig. 3a, SOD activity increased in all groups during shrimp feeding. For groups fed with CotB-VP28 or PY79 spores, the levels of SOD achieved at days 7 and 14 were noticeably higher (increased 29% at day 7 in both groups; increased 49% in PY79 group; and 33% in CotB-VP28 group at day 14) than the control group fed with uncoated pellets. For phenoloxidase activity, levels reached a maximum at day 7 and fell slightly in all groups. Phenoloxidase levels were higher in shrimps fed with PY79 (increased 15%) and CotB-VP28 (increased 33%), but these were not very significant (Fig. 3b). A possible explanation for differences in levels of enzyme activity is that *B. subtilis* spores may have a tendency to stimulate the phagocyte activity of hyaline cells more than to stimulate pro-PO to phenoloxidase converting activity of granular cells in whiteleg shrimps, thereby increasing SOD activity more specifically than increasing phenoloxidase activity. Our data of increased enzyme activities at day 14 was somehow lower than increased 50% phenoloxidase and 120% SOD activities in *F. chinensis* using *B. subtilis* expressing VP28 (Fu *et al.*, 2010). Nevertheless, our study shows that feeding shrimps with spores

has some potential in stimulating immunity-related activities.

Protection effect of the CotB-VP28 spores against WSSV in *L. vannamei*

Ning *et al.* (2011) have developed recombinant *B. subtilis* spores expressing VP28 and obtained protection levels of about 46–50% in crayfish against WSSV (Ning *et al.*, 2011). However, they have not evaluated protection study using *L. vannamei* nor evaluated indicators reflecting the immune status of shrimps. Based on the above data of inducing immune responses by PY79 and CotB-VP28, we primary accessed protection potential of the spores in *L. vannamei.* Four groups of *L. vannamei* ($n = 60$ per group) were fed for 7 days before WSSV muscular injection challenge. The number of survival shrimps at 24 h postinjection in untreated, PY79, CotB-VP28 and unchallenged (injected with PBS pH 7.4 only) were 44, 37, 30, and 46, respectively. The resulting mortality of each group at day 14 postchallenge is shown in Fig. 4. The untreated control group (fed with uncoated feed pellets) showed a cumulative mortality rate of 66% postchallenge. For comparison, an unchallenged group showed 18% mortality which reflected the natural mortality of shrimps. The group orally administrated with PY79 spores showed a lower cumulative mortality rate of 49%, and CotB-VP28 spores resulted in lower mortality rates of only 35%. Randomly selected dead shrimps were checked for the presence of WSSV, and all the tested samples were found to be positive in untreated, PY79 and CotB-VP28 groups (data not shown). Based on the mortality rate of the untreated group (66%), PY79 group (49%; $P = 0.0035$) and the CotB-VP28 group (35%; $P = 0.006$), we calculated the relative percentage of survival (RPS) in *L. vannamei* in the PY79 and CotB-VP28 groups to be 26% and 47%, respectively. Similar data regarding low protection against WSSV obtained with wild-type *B. subtilis* vegetative cells (20%) and their spores (13%) have been reported previously (Fu *et al.*, 2010; Ning *et al.*, 2011). The 47% protection in the CotB-VP28 group value obtained with was similar to the 46% protection rate in crayfish (*C. clarkia*) when challenging shrimp at day 7 postoral administration of CotB-VP28 spores obtained by Ning *et al.* (2011). Thus, our data indicate that both PY79 and CotB-VP28 spores can stimulate innate immunity of shrimps, but PY79 spores provided less specific protection against WSSV infection than CotB-VP28 spores. The primary data need to be confirmed repeatedly with longer vaccination time, different time points of challenge postfeeding and different doses of spores to see whether higher protection effect can be obtained.

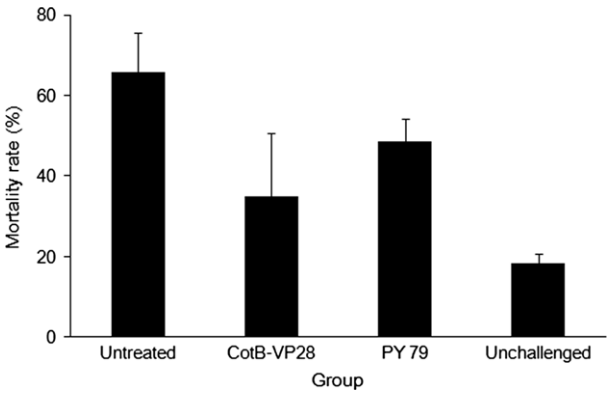

Fig. 4. Protection against WSSV in *Litopenaeus vannamei*. Mortality was assessed at day 14 post-WSSV challenge. Data are presented as arithmetic means, and error bars are standard deviations.

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Authors' contribution

A.T.V.N. and C.K.P. contributed equally to this work.

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