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Bacillus subtilis expressing dsVP28 improved shrimp survival from WSSV challenge

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ABSTRACT: White spot syndrome virus (WSSV) is a potent shrimp viral pathogen responsible for significant economic losses to shrimp aquaculture all over the world. Several studies have demonstrated efficient RNAi-based approaches for suppressing viral genes and reducing shrimp mortality. However, the application has been difficult in real practical use. The present work aimed to develop an efficient approach for the delivery of VP28 specific-siRNA using *Bacillus subtilis*, a novel live oral vaccine vehicle, to protect shrimp against WSSV infection. A plasmid that can function in both bacteria and eukaryotic cells was developed by combining pBE-sDNA and the pCMV promoter from the pcDNA4 plasmid to obtain pBE:cDNA4. Subsequently, to evaluate the efficacy of pBE:cDNA4 *in vivo*, dsGFP-expressing pBE:cDNA4 (pBE:cDNA4-dsGFP) and dsVP28-expressing pBE:cDNA4 (pBE:cDNA4-dsVP28) were used to knockdown target transcripts in WSSV-infected shrimp. A high survival rate was shown for WSSV-infected shrimp injected with pBE:cDNA4-dsVP28 at 7–14 days post-infection (dpi). The pBE:cDNA4-dsVP28 plasmid was transformed into *B. subtilis*, and *B. subtilis* was used as a delivery vehicle during oral feeding. WSSV-infected shrimp fed with *B. subtilis* carrying pBE:cDNA4-dsVP28 showed 91.67% survival compared to the control, which showed only 28.57% survival. The results here demonstrate the efficient delivery of specific siRNAs by using bactofection with *B. subtilis*.

KEYWORDS: white spot syndrome virus, RNA interference, VP28, bactofection, *B. subtilis*

INTRODUCTION

White spot syndrome virus (WSSV) is the most serious cause of disease in *Penaeid* shrimp, generating huge economic losses. The control of viral infection is currently one of the most important challenges regarding shrimp culture [1]. Thus, applications of modern technology in shrimp farming are needed to boost production and prevent economic loss.

RNA interference (RNAi) technology is based on recent discoveries of molecular processes used by host cells to prevent the expression of foreign genetic material [2]. Major proteins involved in the RNAi pathway, including dicer and argonaute, have been identified in *Penaeus monodon*, confirming the existence of the RNAi mechanism in shrimp [3, 4]. To date, RNAi is rapidly becoming a powerful tool for gene silencing applications, and these particular RNAi techniques would be very useful for developing viral vaccines to use in shrimp culture.

A number of studies performed in shrimp have demonstrated efficient RNAi-based approaches for controlling shrimp virus. Exogenous double-stranded RNAs (dsRNAs) targeting the viral gene [5] or endogenous shrimp genes essential for viral infection/replication are injected or administered into shrimp prior to viral challenge [6, 7]. As a result, the replication of the particular virus is suppressed, significantly reducing shrimp mortality.

Among WSSV proteins, VP28 is a popular protein target for controlling WSSV spread due to its location in the viral envelope and the role in WSSV invasion into shrimp [8, 9]. To restrict WSSV infectivity, several studies have demonstrated that injection or administration of dsRNA homologous to VP28 mRNA can induce protection against WSSV in shrimp through RNA interference [10, 11]. To ease the use of RNAi in shrimp farming, a labour-saving, cost-effective and easy to perform siRNA delivery method has been developed. For example,

VP28-specific siRNAs were transferred into shrimp via oral administration by using *E. coli* as a vehicle. The other method was feeding the animal with VP28dsRNA-chitosan nanoparticles [12, 13]. However, the delivery of dsRNA by ingestion is less effective for inducing the RNAi pathway than injection. To improve the efficiency of this process, *B. subtilis*, a probiotic bacterium, was selected as a vehicle to transport recombinant plasmid encoding VP28-specific siRNA into shrimp.

B. subtilis is a gram-positive bacterium generally recognized as safe (GRAS) status and classified as a novel food being used as a probiotic for both human and animal consumption [14]. *B. subtilis* can deliver antigens and cytokines to the systemic and mucosal immune systems via mucosal routes. Therefore, its use as a vaccine delivery system using different antigens and cytokines has been widely studied [15, 16]. Moreover, studies have developed *B. subtilis* into a new tool for use as a food source and as a shuttle organism to express dsRNA in RNAi experiments [17].

Here, we developed a new bacterial plasmid that can propagate in bacterial cells and transcribe a gene of interest in eukaryotic cells, namely, pBE:cDNA4. To test whether this plasmid was suitable for targeted gene expression *in vitro*, we constructed pBE:cDNA4 encoding GFP- or VP28-specific siRNA and investigated whether these plasmids could reduce their target transcripts *in vivo*, which would result in the reduction of the GFP intensity or increase shrimp survival rate after WSSV infection.

For application in shrimp aquaculture, *B. subtilis* was subsequently used as the carrier for carrying pBE:cDNA4 encoding VP28-specific siRNA (pBE:cDNA4-dsVP28) against WSSV infection. The results showed that shrimp orally fed with *B. subtilis* carrying pBE:cDNA4-dsVP28 showed a higher survival rate than controls. Overall, the novel pBE:cDNA4 plasmid and *B. subtilis* were efficient strategic tools for practical use in shrimp aquaculture against WSSV infection.

MATERIALS AND METHODS

Experimental shrimp

Healthy *Litopenaeus vannamei* (bodyweight 7–10 g) were collected from a farm in Songkhla, Thailand. Shrimp have been maintained in a laboratory aquaria at a salinity of 15 ppt for at least 1 week before use in each experiment.

WSSV preparation

Shrimp were intramuscularly injected with 100 μl of various dilutions of WSSV virus in phosphate-buffered saline (PBS). The dose of WSSV used in the immunization experiments was 1.08×10^6 copies; this dose resulted in the death of 50% of shrimp within 5–7 days.

Construction of recombinant pBE:cDNA4 plasmid

The pBE:cDNA4 plasmid was developed by fusion of part of a eukaryotic expression cassette derived from pcDNA4 B His/Max and the replication origin of *B. subtilis* from pBE-sDNA. Both plasmids were digested by the same two restriction enzymes, *Mlu*I and *Sca*I, generating 2 fragments with the sizes of 4654 bp and 3118 bp, which were from pcDNA4 B His/Max and pBE-sDNA, respectively (Fig. 1). After ligation, the fusion plasmids were transformed into *E. coli* Top10 and plated onto LB agar containing 100 μg/ml ampicillin and 10 μg/ml kanamycin. Subsequently, pBE:cDNA4 was transformed into the *B. subtilis* strain CU1065 using a two-step transformation described in the product manual for the Takara *B. subtilis* secretory protein expression system (Takara, USA). The selective medium for screening *B. subtilis* harbouring pBE:cDNA4 was LB agar plates containing 10 μg/ml of kanamycin.

Construction of long hairpin-specific dsRNA-expressing pBE:cDNA4 plasmid

A 2-step cloning strategy was developed to construct a long-strand dsRNA hairpin expression vector [18]. The plasmid map and cloning strategy are depicted in Fig. 2A. The sizes of the cloned forward and reverse VP28-specific long-hairpin dsRNA fragments are 403 bp (base number 15–417) and 510 bp (base number 15–524), respectively. These sizes almost cover the full length of VP28 (Accession number: AY422228.1, 615 bp). The forward VP28 fragment was cloned into the pGEM-T easy vector (Promega, USA) and digested by *Hin*dIII and *Bam*HI for the subsequent cloning step into the pBE:cDNA4 vector. For the reverse VP28 fragment, the reverse sequence was cloned into the pGEM-T easy vector, which was then cut by *Bam*HI and *Eco*RI to release a reverse repeat flanked by the restriction sites. Finally, the reverse repeat was subcloned into the pBE:cDNA4 plasmid containing the forward VP28 fragment using T4 DNA ligase (Promega, USA). The recombinant plasmid was transformed into *E. coli* Top10 and digested by restriction enzymes to obtain

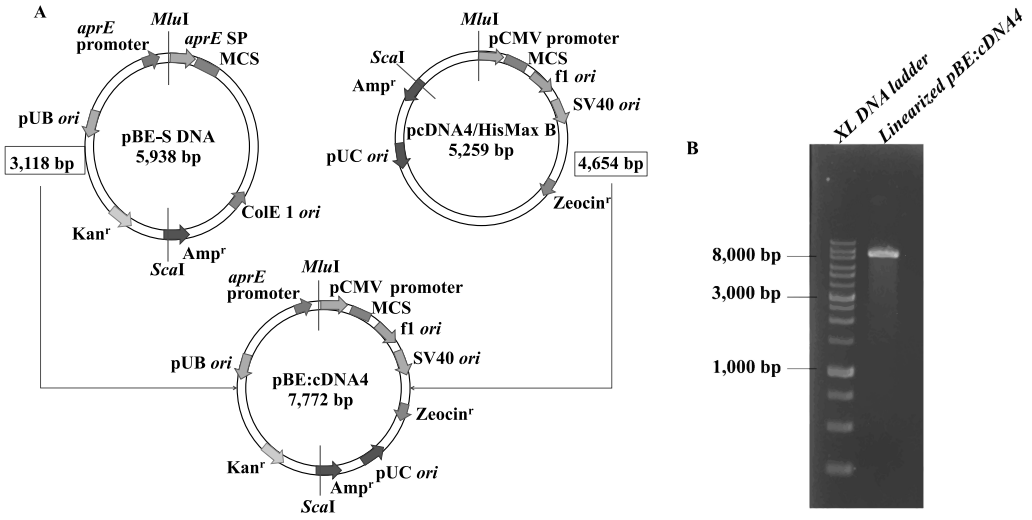

Fig. 1 Schematic representation of the pBE:cDNA4 construction strategy. (A) Ligation of *Mlu*I- and *Sca*I-digested pBE-sDNA and pcDNA4/HisMax B plasmid products resulting in the creation of the new DNA vaccine reporter plasmid pBE:cDNA4. (B) The linearized pBE:cDNA4 shuttle plasmid (7.772 Kb).

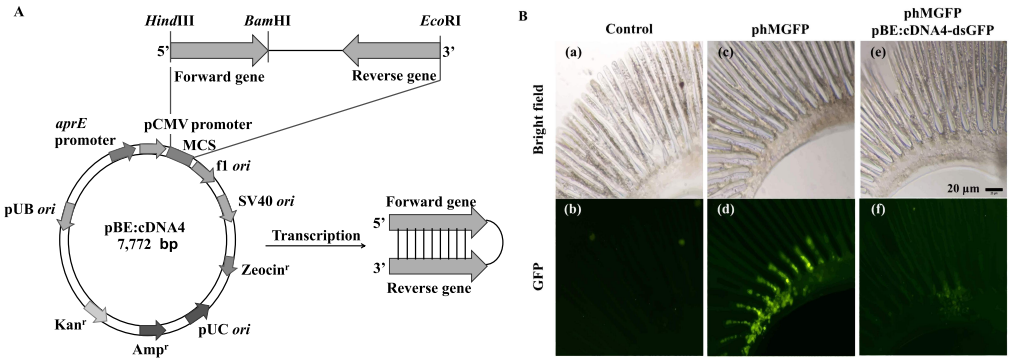

Fig. 2 Plasmid features for transcribing hairpin dsRNAs *in vivo*. (A) A plasmid containing the target gene configured as a forward repeat with a reverse fragment flanked by a single promoter (pCMV) can be generated in two steps. First, a forward fragment is inserted behind the promoter using the *Hin*dIII and *Bam*HI restriction sites. Second, a reverse DNA fragment is then inserted behind the first fragment using *Bam*HI and *Eco*RI restriction sites. (The *Hin*dIII, *Bam*HI, and *Eco*RI restriction sites can be created using appropriately designed PCR primers.) (B) Effective inhibition of GFP production by RNAi in shrimp gills, including normal shrimp (a–b), shrimp injected with phMGFP (c–d) and shrimp injected with phMGFP and pBE:cDNA-dsGFP (e–f).

the correctly sized hairpin product. For the GFP-derived hairpin, nonspecific control, the template of full-length GFP gene is 684 bp (Accession number: AY218848.1). The forward and reverse fragments are 392 bp (base number 5–396) and 583 bp (base number 5–587), respectively. The primers for constructing the VP28 and GFP plasmids are listed in Table S1. The purified pBE:cDNA4-dsVP28 and pBE:cDNA4-dsGFP plasmids were transformed into the *B. subtilis* strain CU1065.

Evaluation of the dsGFP encoded from pBE:cDNA4-dsGFP in shrimp

The efficacy of dsRNA expression from the pBE:cDNA4 plasmid to inhibit the target transcript was determined. In this step, exotic GFP was knocked down with GFP specific-dsRNA transcribed from pBE:cDNA4-dsGFP plasmid as a model to confirm that dsGFP could inhibit the GFP protein. Shrimp were divided into the following three groups, each group containing 3 shrimp: (1) PBS-injected shrimp (negative control), (2) phMGFP with pBE:cDNA4-dsGFP plasmid-

injected shrimp, and (3) empty phMGFP plasmid-injected shrimp (positive control for the appearance of the green fluorescence signal), by injecting the individual shrimp with 40 μg of each plasmid. The effectiveness of the GFP-specific dsRNA was monitored by measuring the intensity of GFP in shrimp gills at 48 h post-injection using a fluorescence microscope. To measure the GFP intensity, the shrimp gills from each group were collected, fixed with 4% paraformaldehyde for 15 min, and then washed three times with PBS. The GFP intensity in the samples was visualized using a fluorescence microscope.

Protection of shrimp by injection of pBE:cDNA-dsVP28 plasmid against WSSV infection

Shrimp were divided into four groups with each group containing 10 shrimp. Shrimp in the first and second groups were injected with 40 μg of pBE:cDNA4-dsGFP and pBE:cDNA4-dsVP8 plasmid into the third abdominal segment, respectively. The third and fourth shrimp groups were injected with PBS alone. On the 3rd day after plasmid injection, shrimp in the first, second, and third groups were challenged by WSSV. In this experiment, the third and fourth groups were used as a positive and negative control, respectively. The mortality was recorded for 14 days and the shrimp survival rate was calculated. Additionally, VP28 transcript expression was also randomly investigated from gills in both dead and surviving shrimp by RT-PCR at the end of the experiment. The VP28 specific primers are shown in Table S1. The *EF-1α* gene was used as an internal control. The experiments were performed in triplicate.

Protection of shrimp against WSSV infection by oral administration with *B. subtilis* containing pBE:cDNA4-dsRNA

Coating of feed diets

To prepare the diet incorporating *B. subtilis*, *B. subtilis* suspensions containing pBE:cDNA4-dsVP28 or pBE:cDNA-dsGFP (10^6 CFU/ml) were mixed per 0.1 g of commercial diet. The diet incorporating *B. subtilis* was incubated on ice for 15 min to allow absorption of the bacterial suspension into the diets. The diet was pelleted by pressing through a syringe (10 ml). PBS- and empty *B. subtilis*-mixed diets were used as controls. The diets were dried at room temperature for 2–3 days and stored at 4 °C until use.

WSSV challenge test

Shrimp were divided into 5 groups with each group containing 10 shrimp. Shrimp in the first and second groups were fed with *B. subtilis* containing pBE:cDNA4-dsGFP and pBE:cDNA4-dsVP28, respectively. The third group was fed with empty *B. subtilis*. The fourth and fifth groups were fed with PBS-mixed diets. The diets were fed for 7 days before challenging with WSSV, and feeding continued throughout the experiment. After that, shrimp in the first through fourth groups were challenged with WSSV. The mortality was recorded for 14 days and the shrimp survival rate was calculated. Additionally, VP28 transcript expression was also investigated as described above. The experiments were performed in triplicate.

Statistical analysis

Data were expressed as the mean ± SD. The significance of the percentage survival observed in the different experimental groups was analyzed by one-way ANOVA at 95% confidence level ($p < 0.05$).

RESULTS AND DISCUSSION

Construction of pBE:cDNA4

The pBE:cDNA4 shuttle plasmid (7.772 Kb) was constructed using a pBE-S DNA fragment (3.118 Kb) that was ligated to a pcDNA4/HisMax B plasmid fragment (4.654 Kb). As illustrated in Fig. 1, the new plasmid harbours a eukaryotic region containing pCMV, a multiple cloning site (MCS), a T7 primer binding site for sequencing and the BGH polyadenylation signal polyA necessary for correct mRNA maturation. The prokaryotic region contains a pUB origin of replication, kanamycin resistance gene (Kan^r) for *B. subtilis* selection, a pUC origin of replication, and an ampicillin-resistant (Amp^r) gene for *E. coli* selection. The new plasmid was successfully stabilized in *E. coli* Top10 and in *B. subtilis* CU1065.

Construction of the pBE:cDNA4-dsGFP vector and its inhibitory efficacy on exotic GFP protein in shrimp

A 2-step cloning strategy was developed to construct a long-strand dsRNA hairpin-expression vector. The plasmid map and cloning strategy are depicted in Fig. 2A. For the VP28-specific hairpin, the sizes of the cloned forward and reverse fragments were 403 and 510 bp, respectively. For the GFP-derived hairpin nonspecific control, the forward and reverse fragments were 392 and 583 bp, respectively.

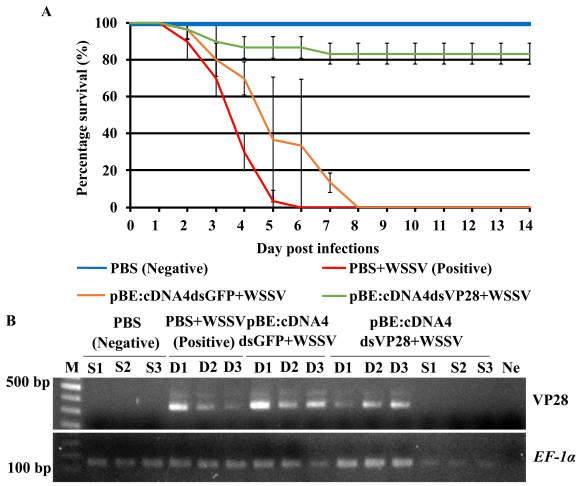

Fig. 3 Percentage survival of *L. vannamei* that was injected with PBS, pBE:cDNA4-dsGFP and pBE:cDNA4-dsVP28 3 days prior to WSSV injection. (A) Shrimp injected with PBS and the PBS and WSSV mixture were used as negative and positive controls, respectively. The test groups were pBE:cDNA-dsGFP-dsVP28- and pBE:cDNA-dsGFP-injected shrimp. (B) Determination of VP28 mRNA expression by RT-PCR. RNA was isolated from gill tissues of moribund (D1–D3) and surviving (S1–S3) shrimp. *EF-1α* was used as an internal control.

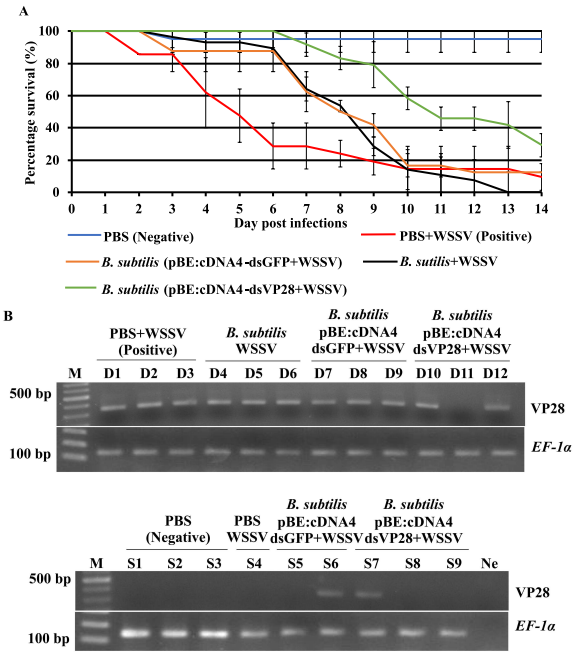

Fig. 4 Percentage of survival rate of *L. vannamei* fed with diets containing empty *B. subtilis* and *B. subtilis* carrying pBE:cDNA4 expressing either dsVP28 or dsGFP for 7 days before WSSV challenge. The shrimp survival rate was recorded every day for 14 days after WSSV challenge. (A) Determination of VP28 mRNA expression by RT-PCR from the gills of moribund (D1–12) and surviving (S1–S9) shrimp. *EF-1α* was used as an internal control.

To evaluate the effectiveness of dsRNA expression, green fluorescent protein (GFP) expression was compared between the shrimp injected with phMGFP+pBE:cDNA4-dsGFP and phMGFP alone. GFP protein expression in shrimp gills was investigated by fluorescence microscopy at 48 h after injection. As shown in Fig. 2B, GFP protein expression was evident in shrimp gills after injection with phMGFP plasmid (middle). The shrimp injected with phMGFP+pBE:cDNA4-dsGFP showed lower GFP protein expression levels than the shrimp injected with phMGFP alone. GFP protein was not present in the gills of normal shrimp, which was served as a negative control. These results suggested that dsGFP against GFP was produced from pBE:cDNA4 and is capable of specifically knocking down the GFP expression in shrimp cells.

Protection of shrimp by pBE:cDNA-dsVP28 immunization

Shrimp survival in each treatment group after WSSV infection was monitored daily to examine the efficiency of antiviral immunity elicited by dsRNA corresponding to VP28 gene expressed from pBE:cDNA4. The transcription of specific dsRNAs within eukaryotic cells can be investigated by injecting shrimp with DNA constructs designed to express dsRNA. Shrimp were challenged by WSSV on the 3rd day after immunization. The groups of shrimp injected with pBE:cNDA4-dsVP8, pBE:cNDA4-dsGFP and PBS (positive control) were challenged with WSSV. In total, 83% survival was observed in the shrimp injected with pBEcDNA4-dsVP28, while 100% mortality was observed in the shrimp injected with pBEcDNA4-dsGFP and the positive control shrimp (Fig. 3A).

VP28 expression was investigated in the dead and surviving shrimp by RT-PCR to confirm WSSV infection. The survivors showed no sign of disease and VP28 expression was not detected (Fig. 3B).

Delivery of pBE:cDNA4-dsVP28 expressing dsVP28 into shrimp using *B. subtilis* as a delivery vehicle

Based on the above results, shrimp injected with pBE:cDNA4-dsVP28 were resistant to WSSV infection. In an attempt to obtain an efficient approach

for the delivery of the antiviral dsVP28 expressed from pBE:cDNA4-dsVP28 into shrimp, *B. subtilis* was selected as a delivery vehicle. Shrimp were fed with food diets containing empty *B. subtilis* or *B. subtilis* containing pBE:cDNA4 expressing either dsVP28 or dsGFP for 7 days before WSSV challenge. After the WSSV challenge, the percentage survival of shrimp was observed for 14 days post-infection. At 7 days post-infection, shrimp in the negative control showed the highest survival rate of 95.2%, and its survival rate was stable throughout the experiment. Shrimp fed with a diet containing *B. subtilis* carrying pBE:cDNA4-dsVP28 showed the highest survival rate of 91.7% while shrimp fed with a diet containing *B. subtilis* carrying pBE:cDNA4-dsGFP and empty *B. subtilis* showed survival rates of 62.5% and 64.1%, respectively. The WSSV-infected shrimp fed with a commercial diet (positive control) showed the lowest survival rate of 28.6%. At 14 dpi, WSSV-infected shrimp fed with a diet containing *B. subtilis* carrying pBE:cDNA4-dsVP28 had a higher survival rate than those fed with a diet containing *B. subtilis* carrying pBE:cDNA4-dsGFP and the positive control, with survival rates of 29.2%, 12.5% and 9.5%, respectively. In contrast, no shrimp survived among the WSSV-infected shrimp fed with a diet containing empty *B. subtilis* (Fig. 4A). Taken together, the novel pBE:cDNA4 plasmids could be used *in vivo*, and bactofection of a DNA vaccine using *B. subtilis* was an efficient strategy for the delivery of pBE:cDNA4 expressing specific siRNAs to protect shrimp against WSSV infection. Additionally, we also investigated VP28 mRNA expression in moribund and surviving shrimp (Fig. 4B).

DISCUSSION

The RNAi pathway is thought to be an ancient mechanism for protecting the host genome against virus genetic elements through mRNA degradation induced by double-stranded RNA (dsRNA) in a sequence-specific manner [19, 20]. Several studies have reported that siRNAs or dsRNA serves as potential therapeutic agents for treating viral disease [21, 22]. In shrimp, dsRNA is a potent trigger of an innate immune response against viruses, and dsRNA induces antiviral protection as described in *L. vannamei* infected with viruses including white spot syndrome virus [23], Taura syndrome virus [24], yellow head virus [25], *Penaeus stylirostris* densovirus [26]. The VP28 has been identified as a viral surface protein that plays a key role in the initial steps of systemic WSSV infection in shrimp [27]. Several reports have experimentally tested dsRNAs targeting the structural gene VP28 for their efficacy to confer prevention of WSSV infection in *P. monodon* and *L. vannamei* [28, 29]. Thus, RNAi technology is now applicable as a prevention strategy against WSSV infection in shrimp. Recombinant VP28 protein is also effective in the prevention of WSSV infection when added to shrimp feed [30, 31]. This work demonstrates a new strategy using a bacterium gene delivery system for the production of VP28 dsRNA in shrimp cells. A number of pathogens having invasive properties, such as *Shigella*, *Salmonella*, *Yersinia*, and *Listeria*, have been exploited for their use in DNA delivery to mammalian cells [32]. However, the risk of reverting to a virulent phenotype cannot be completely mitigated, and there remains a need to search for safer alternatives [33, 34]. *B. subtilis* was exploited as a safer alternative compared with invasive pathogens. Several studies have demonstrated that oral administration of *B. subtilis* expressing VP28 protein can induce protection in shrimp against WSSV [35–37].

In the present study, noninvasive *B. subtilis* was explored as a DNA carrier for the delivery of a newly constructed plasmid, pBE:cDNA4 (7.772 Kb in size) Fig. 1, to eukaryotic cells. The pBE:cDNA4 offers several attractive features as a DNA vaccine candidate, especially as it is capable of replicating in both prokaryotic cells such as *E. coli* and *B. subtilis* and eukaryotic cells. We evaluated the efficacy of dsGFP expressed from the pBE:cDNA4-dsGFP vector to interfere with green fluorescent protein (GFP) transgene expression. The results showed that the GFP intensity in gills of pBE:cDNA4-dsGFP-injected shrimp was decreased (Fig. 2B). This implied that dsRNA could be produced in-frame from the pBE:cDNA4 plasmid and was capable of effective gene-specific knockdown of target gene expression in shrimp. The pBE:cDNA4-dsVP28 expressing dsVP28 targeting WSSV VP28 was also constructed. The shrimp survival rate in WSSV-infected shrimp injected with pBE:cDNA4-dsVP28 was higher than in WSSV-infected shrimp injected with pBE:cDNA4-dsGFP (Fig. 3A). After checking the VP28 transcript, the results showed a reduction in VP28 transcriptional levels in WSSV-infected shrimp injected with pBE:cDNA4-dsVP28 compared to WSSV-infected shrimp injected with pBE:cNDA4-dsGFP and the control group, indicating that the protective effects against WSSV infection depended on dsRNA specific to VP28 expression in shrimp. It is clear that VP28-specific dsRNA expression regulated by the pCMV promoter in pBE:cDNA4 promoted protective effects against WSSV infection.

To promote the use of RNAi in shrimp farming, *B. subtilis* had been used as a delivery vehicle. Recombinant *B. subtilis* was developed by transformation with pBE:cDNA4-dsGFP. After oral administration of recombinant *B. subtilis* to WSSV-infected shrimp, the highest survival rate was shown for WSSV-infected shrimp fed with a diet containing *B. subtilis* carrying the pBE:cDNA4-dsVP28 plasmid. Moreover, VP28 mRNA in WSSV-infected shrimp fed with a diet containing *B. subtilis* carrying the pBE:cDNA4-dsVP28 plasmid was not present in surviving shrimp. This revealed that oral delivery of the plasmid expressing dsVP28 could activate the RNAi pathway, drastically suppressing VP28 mRNA expression.

The *B. subtilis* bactofection mechanism in shrimp could be similar to the *Lactococcus lactis*, a gram-positive lactic acid bacterium, bactofection mechanism in mice [38]. *B. subtilis* probably adheres to epithelial cells and enters the cell by internalization of a vacuole/phagosome. After vacuole/phagosome lysis, plasmid DNA was released and translocated into the nucleus, resulting in the expression of the gene of interest [39, 40]. This dsRNA delivery system may overcome several limitations associated with the biomolecule application to prevent and protect shrimp from acquiring diseases during aquaculture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2020.S003.

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Appendix A. Supplementary data

Table S1 Bacterial strains, plasmids, and primers used in this study.

Strain, plasmid and primer		Description and sequence (5′–3′)	Source
***B. subtilis* strain**			
CU1065		Wild type (*SPβ-trpC2*)	Loprasert
rB. subtilis (pBE:cDNA4-dsVP28)		Constitutive overexpression of dsVP28; Kan^r (10 μg/ml)	This study
rB. subtilis (pBE:cDNA4-dsGFP)		Constitutive overexpression of dsGFP; Kan^r (10 μg/ml)	This study
Plasmid			
pGEM-T Easy		Cloning vector	Promega
pBE-S DNA		A shuttle vector of *E. coli*/*B. subtilis*; *aprE* promoter; *aprE* SP	Takara
pcDNA4/HisMax B		Eukaryotic expression vector	Promega
phMGFP		Eukaryotic expression vector	Promega
pBE:cDNA4		A shuttle vector of *E. coli*/*B. subtilis*; pCMV promoter	This study
pBE:cDNA4-dsVP28		A shuttle vector of *E. coli*/*B. subtilis*; pCMV promoter for expression dsVP28	This study
pBE:cDNA4-dsGFP		A shuttle vector of *E. coli*/*B. subtilis*; pCMV promoter for expression dsGFP	This study
Primer			
*Hind*III-VP28-F1	Tm 55 °C	AAGCTT CAC TCT TTC GGT CGT GTC G	This study
*Bam*HI-VP28-R1		GGATCC CAT CTG CAT ACC AGT GAT G	GenBank no.
*Eco*RI-VP28-F2	Tm 55 °C	GAATTC CAC TCT TTC GGT CGT GTC G	AY422228.1
*Bam*HI-VP28-R2		GGATCC GTA CCA CAC ACA AAG GTG	
*Hind*III-GFP-F1	Tm 55 °C	AAGCTT GCG TGA TCA AGC CCG ACA T	This study
*Bam*HI-GFP-R1		GGATCC GCC ATT AGC AGG GAA GTT G	GenBank no.
*Eco*RI-GFP-F2	Tm 55 °C	GAATTC GCG TGA TCA AGC CCG ACA T	AY218848.1
*Bam*HI-GFP-R2		GGATCC TGG TCC ACG AAG TGG TAG T	
EF-1α-F	Tm 55 °C	GAACTGCTGACCAAGATCGACAGG	This study
EF-1α-R		GAGCATACTGTTGGAAGGTCTCCA	
VP28-F	Tm 55 °C	GGATCT TTCTTTCACTCTTTC	Nupan et al
VP28-R		TCTGCCCCACAGTCACTTCGA	