Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

ORIGINAL ARTICLE

Investigation of the roles of T6SS genes in motility, biofilm formation, and extracellular protease Asp production in *Vibrio alginolyticus* with modified Gateway-compatible plasmids

H. Liu, D. Gu, L. Sheng, Q. Wang and Y. Zhang

State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai, China

Keywords
Asp, biofilm, gateway, T6SS, *Vibrio alginolyticus*.

Correspondence
Q. Wang, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China.
E-mail: oaiwqiyao@ecust.edu.cn

2012/0513: received 21 March 2012, revised 15 April 2012 and accepted 23 April 2012

doi:10.1111/j.1472-765X.2012.03263.x

Abstract

Aims: The aims of this study were to create and evaluate the Gateway-compatible plasmids for investigating the function of genes in *Vibrio alginolyticus* and other Gram-negative bacteria.
Methods and Results: In this study, Gateway-compatible plasmids were successfully constructed for rapid and comprehensive function analysis of genes. Taking advantage of these plasmids, the in-frame deletion mutant strains and their complemented strains of five T6SS genes, including *dotU1*, VEPGS_0008, VEPGS_0011, *hcp2* and *ppkA2*, were obtained. The results illustrated that all the mutant strains showed no significant effects on extracellular protease production, expression of Hcp1, and biofilm formation when compared to the wild-type strain, but in-frame deletion of VEPGS_0008 resulted in obvious biofilm reduction and the complemented strain restored to the level of the wild-type strain. Besides, in-frame deletion of *dotU1*, VEPGS_0008 and *ppkA2* abolished the swarming ability.
Conclusions: A set of Gateway-compatible vectors for internal insertion, in-frame deletion and complementation of the target genes is constructed to facilitate the general and rapid function analysis of genes involved in T6SS in *Vibrio alginolyticus*.
Significance and Impact of the Study: The modified Gateway-compatible plasmids greatly facilitate the high-throughput and convenient function analysis of the unidentified genes.

Introduction

Vibriosis, an infection caused by *Vibrio* species, is a widespread threat to the marine culture and brings about huge economic loss worldwide. There have been more than 10 kinds of vibrios reported to be the causative agents in the universal marine culture at present, including *Vibrio anguillarum*, *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi* and *V. alginolyticus* (Austin 2010). The genomes of all these species have been sequenced and the information is available on GenBank. Yet only a small portion of these genes has been experimentally assigned a function.

Genetic tools are essential for gene function analysis including the ectopic expression, the site-directed in-frame deletion, and the complementation. The discovery of restriction enzymes in the early 1970s led to the revolutionary progress in the analysis of nucleic acids. However, this traditional method takes much time for the restriction enzyme digestion and ligation, meanwhile the process may be hampered by the limited restriction enzyme sites located on the plasmid and versatile sites contained in the target DNA sequence. Furthermore, the efficiency of obtaining positive clones is closely correlated to the effectiveness of the restriction enzyme digestion. It is therefore a burning need to expand the tools for high-throughput screening and gene function analysis.

Gateway technology (Invitrogen, Carlsbad, CA, USA) is a cloning technique based on site-specific recombination

characteristics of the lambda bacteriophage (Landy 1989) that encompasses two opposite recombination reactions (BP and LR). The BP recombination reaction catalysed by BP clonase refers to the recombination reaction between *attB* sites in the target gene and *attP* sites carried on the donor vector, resulting in the *attL*-containing entry plasmid. Similarly, the *attL*-flanked target gene carried on entry plasmid can be transferred to various destination vectors that contain homologous *attR* sites by the LR reaction catalysed by the LR clonase, generating the target plasmid carrying the *attB*-flanked sequences. Gateway technology avoids the labour-intensive restriction enzyme digestion and ligation process and enhances the efficiency of getting positive strains for the presence of the counter-selective marker, CcdB. At present, Gateway cloning system has been successfully used for high-throughput gene function analysis in various microorganisms (Dupuy *et al.* 2004; Hope *et al.* 2004; Busso *et al.* 2005; Yahata *et al.* 2005).

In this study, we have described the construction of a set of modified Gateway-compatible vectors and their application in the rapid investigation of roles of type VI secretion system (T6SS) genes in fish pathogen *V. alginolyticus*. The Gateway-compatible vector derivatives are also useful in gene function analysis in other vibrios and Gram-negative bacteria.

Materials and methods

Vectors, bacterial strains, and culture conditions

The vectors and strains utilized in the study are listed in Table 1. *Escherichia coli* DH5α (Tiangen, Shanghai, China) was used for routine propagation of the entry plasmids, *E. coli* SM10 *λpir* (Liang *et al.* 2003) for the modified Gateway-compatible destination plasmids, and One Shot® *ccdB* Survival™ 2T1R competent *E. coli* (Invitrogen) for pDONR221 and pDEST 17. All the *E. coli* strains were

Table 1 Strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference or source
Vibrio alginolyticus		
EPGS	Pathogenic isolate from aquaculture farm. Amp^r	CCTCC No. M 2010277
asp$^-$	EPGS carrying the null mutation of *asp* gene	(Rui *et al.* 2009)
Δ*dotU1*	EPGS carrying in-frame deletion of *dotU1* gene	This study
dotU1$^+$	EPGS, Δ*dotU1* complemented with intact *dotU1* gene	This study
Δ*hcp2*	EPGS carrying in-frame deletion of *hcp2* gene	This study
hcp2$^+$	EPGS, Δ*hcp2* complemented with intact *hcp2* gene	This study
DN08	EPGS carrying in-frame deletion of VEPGS_0008 gene	This study
DN08$^+$	EPGS, DN08 complemented with intact VEPGS_0008 gene	This study
DN11	EPGS carrying in-frame deletion of VEPGS_0011 gene	This study
DN11$^+$	EPGS, DN11 complemented with intact VEPGS_0011 gene	This study
Δ *ppkA2*	EPGS carrying in-frame deletion of *ppkA2* gene	This study
ppkA2$^+$	EPGS, Δ*ppkA2* complemented with intact *ppkA2* gene	This study
Δ*hcp1*	EPGS carrying in-frame deletion of *hcp1* gene	(Sheng *et al.* 2012)
hcp1$^+$	EPGS, Δ*hcp1* complemented with intact *hcp1* gene	(Sheng *et al.* 2012)
Δ *pppA*	EPGS carrying in-frame deletion of *pppA* gene	(Sheng *et al.* 2012)
Escherichia coli		
CC118 *λpir*	*λpir* lysogen of CC118 Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1*	(Dennis and Zylstra 1998)
SM10 *λpir*	*thi thir leu tonA lecY supE recA*::*RP4-2-Tc*::*Mu* Km^r (*pirR6K*)	(Liang *et al.* 2003)
BL21 (DE3)	F^- *dcm ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3)	Invitrogen
DH5α	F^- φ80 *lacZ*Δ*m15*Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (rk−,mk+) *supE44* λ− *thi-1gyrA96 relA1 phoA*	Tiangen
One Shot® *ccdB* Survival™ 2T1R	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80 *lacZ*Δ*m15* Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^r) *end*A1 *nup*G *fhuA::IS2*	Invitrogen
Plasmids		
pDM4	Cm^r, *sacBR*, suicide vector containing an R6K origin of replication (*pir* requiring)	(Milton *et al.* 1996)
pNQ705-1	Cm^r; suicide vector that contains an R6K origin (*pir* requiring)	(Wang *et al.* 2002)
pMMB206	Cm^r, IncQ *lacI*^{q} Δ*bla* $P_{tac\text{-}lac}$ *lacZa*	(Morales *et al.* 1991)
pDONR221	Km^r, Cm^r, the donor vector used in Gateway system	Invitrogen
pDEST17	Amp^r, Cm^r, the destination vector used in Gateway system	Invitrogen
pDM4.DEST	Cm^r, pDM4 carrying the *attR1-ccdB*/Cm^r-*attR2* cassette in *Sal*I sites	This study
pNQ705-1.DEST	Cm^r, pNQ705-1 carrying the *attR1-ccdB*/Cm^r-*attR2* cassette in *Sal*I sites	This study
pMMB206.DEST	Cm^r, pMMB206 carrying the *attR1-ccdB*/Cm^r-*attR2* cassette in *Eco*RI sites	This study

grown in Luria-Bertani (LB) medium containing 1% (w/v) NaCl supplemented with appropriate antibiotics at 37°C. *Vibrio alginolytics* strains were grown in LB medium containing 3% (w/v) NaCl at 30°C. The antibiotics were used at the following concentrations: 100 μg ml^{-1} ampicillin (Amp) and 7 μg ml^{-1} chloramphenicol (Cm) for *V. alginolaticus*, 100 μg ml^{-1} Amp, 100 μg ml^{-1} kanamycin (Km), and 25 μg ml^{-1} Cm for *E. coli*.

Construction of the entry vectors

To transfer the target genes to different destination vectors for diverse applications, a series of entry vectors carrying the target genes on the donor vector, pDONR221 (Invitrogen), were constructed. To generate the Hcp1 expression strains, we amplified its open reading frame (ORF) using the forward primer Ghcp1F carrying the *attB1* sites on the upstream of the 5′ end and the reverse primer Ghcp1R carrying the *attB2* sites (Table 2) from the genomic DNA of *V. alginolyticus* EPGS. Then the fragment was cloned into pDONR221 by BP reaction according to the instruction of the manufacturer (Invitrogen).

To generate the in-frame deletion mutant strains for gene function analysis, primers containing the *attB* sites were designed for the indicated genes. Here, three T6SSVA1 genes (including *dotU1*, VEPGS_0008 and VEPGS_0011) located on the chromosome I and two T6SSVA2 genes (*ppkA2* and *hcp2*) on chromosome II in *V. alginolyticus* were selected for in-frame deletion mutant construction based on the Gateway cloning technology. Taking *dotU1* as an example, the genomic DNA of *V. alginolyticus* EPGS was used as a template with PCR primers

Table 2 Primers used for cloning in this study

Primers	Sequences (5′–3′)*
Ghcp1F	ACAAGTTTGTACAAAAAAGCAGGCTATGCCAACTCCAGCATATAT
Ghcp1R	CCACTTTGTACAAGAAAGCTGGGTTTAAGCTTCGCGTGGAGCA
dotU11	ACAAGTTTGTACAAAAAAGCAGGCTTGAGTTACGCAGAAACCG
dotU12	AGATGTCTCAAGGGATCGAAGACATACAA
dotU13	TCGATCCCTTGAGACATCTGGTGGTGACA
dotU14	CCACTTTGTACAAGAAAGCTGGGTAGGTTGCCGCATACATAA
hcp21	ACAAGTTTGTACAAAAAAGCAGGCTATAATTGGGAGTTGGTTC
hcp22	GAAGTGTTGTTTGTATTGCTCGGCTGTT
hcp23	AGCAATACAAACAACACTTCTGCTCCATTG
hcp24	CCACTTTGTACAAGAAAGCTGGGTAGCTTTAGCACGCAATGA
DN081	ACAAGTTTGTACAAAAAAGCAGGCTTTACTACCACGGTCCAAG
DN082	CTTCTTACCCTAACCAATGTTTCGGGATA
DN083	AAACATTGGTTAGGGTAAGAAGAATGATGT
DN084	CCACTTTGTACAAGAAAGCTGGGTGGTAATGGAGAAGGGAAC
DN111	ACAAGTTTGTACAAAAAAGCAGGCTTGTTCATTATCAACCGTTTAGC
DN112	GAGTGAATGGACTTCAACCTTCGCAGCAC
DN113	GAAGGTTGAAGTCCATTCACTCGCATCTC
DN114	CCACTTTGTACAAGAAAGCTGGGT CGAATACCAAAGCCGAGT
ppkA21	ACAAGTTTGTACAAAAAAGCAGGCTGTTATTGGGCATGGTGGG
ppkA22	AATGCCGCTAATTAGCGGGCTTTAGGTC
ppkA23	GCCCGCTAATTAGCGGCATTGTATTTGGTT
ppkA24	CCACTTTGTACAAGAAAGCTGGGTCGGTAGAGGGCTGTTGGA
dotU1comF	ACAAGTTTGTACAAAAAAGCAGGCTAGAAGTGAAGCCTGATGG
dotU1R	CCACTTTGTACAAGAAAGCTGGGTCGCAAACTGCAATCTCTC
hcp2comF	ACAAGTTTGTACAAAAAAGCAGGCTGGTTATGGAGAAGGAGGTG
hcp2comR	CCACTTTGTACAAGAAAGCTGGGT TATCGAATGGCGAAGAAA
DN08comF	ACAAGTTTGTACAAAAAAGCAGGCTCCAATGGTACAGCGAGAT
DN08comR	CCACTTTGTACAAGAAAGCTGGGTACCCAGGTGATAAAGACG
DN11comF	ACAAGTTTGTACAAAAAAGCAGGCTTCTTTGGCCTAGACAGCT
DN11comR	CCACTTTGTACAAGAAAGCTGGGTTAACTTCGGATGATATTTGG
ppkA2comF	ACAAGTTTGTACAAAAAAGCAGGCTAAAAGCCGCAAGTTCACC
ppkA2comR	CCACTTTGTACAAGAAAGCTGGGTTACATACGGACGCCAGAC
pMDF	CC TGCAGCCAAGCTTGAGACCACAACGGTTTCCCTCT
pMDR	GCCAGTGCCAAGCTTTTTCGGGCTTTGTTAGCAGCCT
pDDF	GCATGCGGGTAACCTGAGCTCGAGACCACAACGGTTTCCCTCT
pDDR	GTGGAATTCCCGGGAGAGCTCTTTCGGGCTTTGTTAGCAGCCT

*Nucleotides underlined represent *attB* sites added to the 5′ end of the primers.

dotU11 and dotU12 (Table 2) to create the 198-bp upstream fragment of *dotU1* and primers dotU13 and dotU14 (Table 2) to get 334-bp downstream fragment of *dotU1*. The fragment with 210-bp in-frame deletion of the *dotU1* gene generated by overlap PCR was cloned into the donor vector, pDONR221 with the BP reaction to get the entry plasmid. Besides, for the construction of corresponding complemented strains, the sequences containing the intact promoter regions and ORFs of the indicated genes were obtained by PCR with the designed primers harbouring *attB* sites and then transferred to the pDONR221 with BP reaction. The primers used for gene cloning and plasmid construction are listed in Table 2.

Construction of the destination vectors

To create the Gateway-compatible plasmids, first, the *attR1-ccdB/*Cm^r-*attR2* cassette was amplified from pDEST17 (Invitrogen) with primers pDDF and pDDR, which covered 15-bp sequence homologous to either side of the *Sal*I site located on pNQ705-1 (Wang *et al.* 2002) and pDM4 (Milton *et al.* 1996) at the 5′ end, *Sal*I site in the middle, and the specific sequence of the *attR1-ccdB/*Cm^r-*attR2* cassette at the 3′ end. Then the fragment was cloned into *Sal*I digested linearized pNQ705-1 and pDM4 respectively, to generate the destination plasmids pNQ705-1.DEST and pDM4.DEST for the mutant construction using the seamless cloning kit according to the manufacturer's instruction (Genescript, Shanghai, China). Likewise, the pMMB206.DEST was produced by inserting *attR1-ccdB/*Cm^r-*attR2* cassette amplified from pDEST17 using primers pMDF and pMDR into the *Eco*RI digested pMMB206 (Morales *et al.* 1991). All destination plasmids were transformed into SM10 *λpir* strain that was resistant to the CcdB toxicity and also supported replication of the π protein required R6K plasmids such as pNQ705-1 and pDM4 for positive screening.

Transfer of target genes into the destination vectors

All the entry vectors and corresponding destination plasmids were harvested from various hosts (Table 1). LR recombination reaction was carried out according to the manufacturer's instruction (Invitrogen) and the reaction mixture was then transformed into *E. coli* CC118 *λpir* (Dennis and Zylstra 1998). The destination vectors carrying the target sequences were confirmed by PCR and sequencing.

Screening of target gene deletion mutants

In-frame deletion mutants were constructed in the selected genes by double-crossover allelic exchanges according to previous protocols (Liu *et al.* 2011).

Western-blot analysis of Hcp1 and Asp

To detect the production of Hcp1 and Asp, Western-blot was carried according to the previous work (Sheng *et al.* 2012).

Extracellular protease activity, biofilm formation, motility assay, and LD_{50} determination

The extracellular product (ECP) production assays determined with hide powder azure (HPA) digestion assay and milk plate assay, biofilm formation, motility assay, and LD_{50} determination with healthy zebrafish were carried out according to the previously described methods (Liu *et al.* 2011).

Aggregation assay

Overnight cultures of all the tested strains were harvested and normalized to the same OD_{600} values in 5 ml LBS broth in glass test tubes. All the tubes were shaken vigorously first and then cultured statically at room temperature for 10 min. Afterwards, OD_{600} of each strain was assayed to determine the aggregation.

Statistical analysis

Statistical significance was determined using Student's *t*-test (SPSS 11.5; SPSS Inc., Chicago, IL, USA).

Results

Construction of the Gateway-compatible destination vectors

The plasmid pNQ705-1 (Wang *et al.* 2002) used for insertion mutation construction, pDM4 (Milton *et al.* 1996) for in-frame deletion mutant construction, and pMMB206 (Morales *et al.* 1991) for complementation construction are widely used for the construction of mutant and complemented strains. They carried defined restriction enzyme sites, and thus the flexibility was limited. Besides, all the above plasmids are recalcitrant to be definitely digested or observed to show smearing on agarose gel (H. Liu, D. Gu and Q.Y. Wang, unpublished data). Thus, it is difficult and time-consuming for gene cloning using these plasmids with the conventional method of restriction enzyme digestion and ligation. To circumvent these limitations, we set to modify the plasmids to make them compatible for using with the Gateway system (Invitrogen). The *attR1-ccdB/*Cm^r-*attR2* cassette was amplified from pDEST17 (Invitrogen) using the primers with the addition of the 21-bp consensus sequence to the defined sequences

of pDM4, pNQ705-1, and pMMB206 plasmids respectively (Fig. 1) and then moved onto these plasmids by seamless cloning method (Genescript). The desired destination plasmids maintained in *E. coli* SM10 *λpir* strain that was resistant to the toxic effect of CcdB and meanwhile supplied the *pir*-encoded π protein for replication of R6K plasmids (e.g. pNQ705-1 and pDM4) (Table 1) were selected on the plates containing the indicated antibiotics. The sequencing results confirmed the presence and the validity of the *attR1-ccdB/*Cm^r*-attR2* cassette in all tested strains. Therefore a set of Gateway-compatible plasmids for convenient and systemic gene function analysis, including pNQ705-1.DEST, pDM4.DEST, and pMMB206.DEST, was successfully constructed.

Expression of Hcp1 based on Gateway destination plasmid pDEST17

To evaluate the Gateway-based cloning technology, we cloned the ORF sequences of *hcp1* (the hallmark of the T6SS in *V. alginolyticus*) into the donor vector pDONR221 (Invitrogen) to generate the entry plasmid. Then the ORF of *hcp1* was transferred from the entry vector into the destination vector, pDEST17 (Invitrogen), which contains the strong bacteriophage T7 promoter for the controlled and inducible expression of the proteins of interest and 6 × His at the N-terminal of the specific sequence for the convenient purification, by LR reaction to obtain the expression plasmid. The efficiency of recovering desired clones was high (49/50 positive, data not shown). However, 25-bp *attB* sites remained flanking the ORF of *hcp1* (Fig. 2a). To verify the expression of Hcp1 based on Gateway system, the Hcp1 expression was induced by adding isopropyl-*β*-D-thiogalactopyranoside (IPTG) when the OD_{600} reached 0·4. Then the total proteins were harvested and loaded onto 12% SDS-PAGE. The result demonstrated that Hcp1 was expressed successfully by using the Gateway-based plasmids and the presence of the *attB* sites seems to exert no effect on the normal expression of Hcp1 (Fig. 2b). The expression of Hcp1 utilizing pDEST17 was also verified by Western-blot using Hcp1-specific antibody (Sheng *et al.* 2012) (Fig. 2b). Taken together, the protein expression can be achieved rapidly and efficiently with Gateway technology.

Functional analysis of T6SS genes using Gateway-compatible plasmids

To validate and evaluate the Gateway-compatible plasmids for mutant construction, five genes involved in T6SS in *V. alginolyticus* were selected to generate the in-frame deletion mutants. The process avoided the restriction enzyme selection, and the transfer efficiency of the target genes into the destination plasmids reached 90–100%. All the five in-frame deletion mutant strains were obtained successfully following the double-crossover allelic exchanges according to previous protocols (Liu *et al.* 2011). Meanwhile, all the complemented strains were also generated successfully using Gateway-compatible plasmid pMMB206.DEST. Thus the Gateway-compatible plasmids apparently facilitate the genetic analysis (Fig. 3a). Furthermore, the pNQ705-1.DEST for site-directed insertion

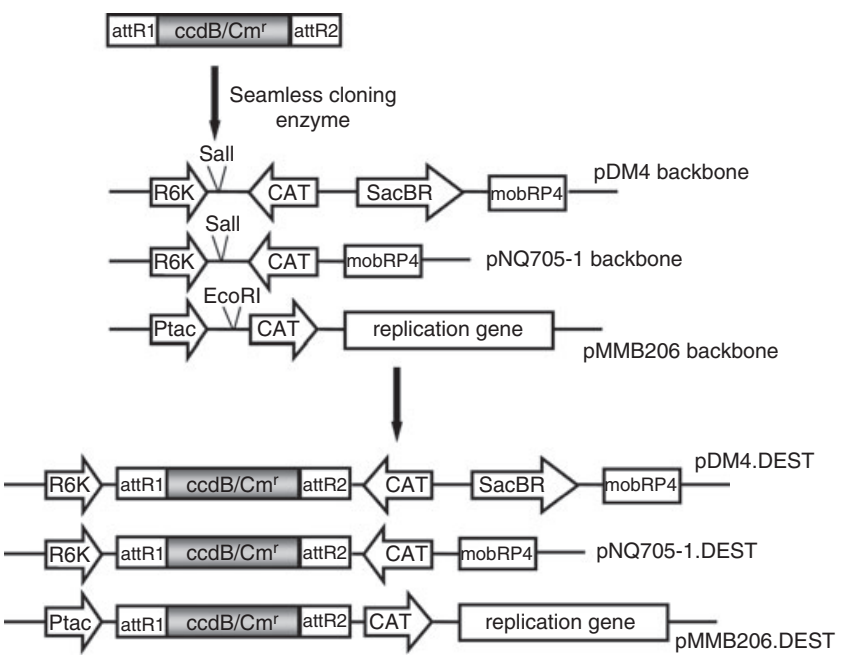

Figure 1 Construction of the destination vectors. Schematic flowchart of the Gateway cassettes transferred into the plasmids, pDM4, pNQ705-1, and pMMB206 was indicated. The *attR*-flanked *ccdB* cassette was amplified by PCR with pDEST17 as the template and the product was then inserted into the indicated linearized plasmids to obtain the three destination plasmids for different functional analysis.

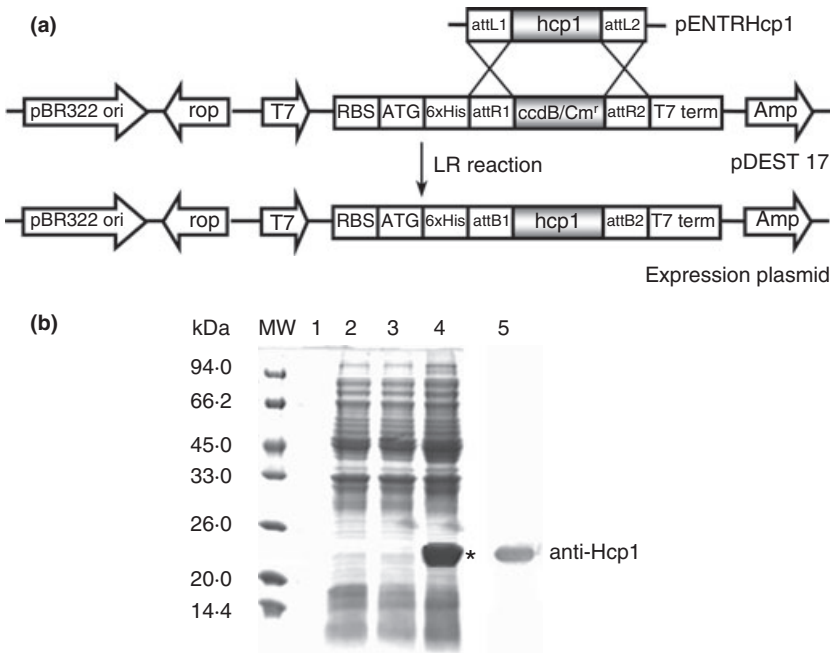

Figure 2 Evaluation and validation of the Gateway-based expression vector pDEST17. (a) Flow chart of the expression vector construction based on Gateway system. (b) The expression of Hcp1. Lane 1: LB broth without the bacteria; Lane 2: the total protein sample of the *Escherichia coli* BL21 (DE3); Lane 3: the total protein sample of the *E. coli* BL21 (DE3) with *hcp*1 carried on the pDEST17 vector but without the IPTG induction; Lane 4: the total protein sample of the *E. coli* BL21 (DE3) with *hcp*1 carried on the pDEST17 vector but with the IPTG (100 μmol l^{-1}) induction; Lane 5: Western-blot against Hcp1 with the total protein sample of the *E. coli* BL21 (DE3) with *hcp*1 carried on the pDEST17 vector under induction. MW means the protein molecular weight and the asterisk represents the expected protein.

mutation of the target genes was also achieved in *V. alginolyticus* (data not shown).

The T6SS has been linked to a myriad of processes, including biofilm formation (Aschtgen *et al.* 2008), motility (Jana *et al.* 2011), extracellular protease production (Weber *et al.* 2009), and promoting or limiting virulence (Filloux 2009). Here in, all the five obtained mutant strains based on Gateway-compatible plasmids were analysed for their roles in regulating the virulence-related elements, including motility, extracellular protease production, biofilm formation, and T6SS hallmark Hcp1 secretion in *V. alginolyticus*. As shown in Fig. 3 and Table 3, there were no significant differences in swimming and aggregation in all tested strains, but Δ*dotU1*, DN08 harbouring in-frame deletion of VEPGS_0008 gene, and Δ*ppkA2* lost the swarming ability. The extracellular protease production in the mutant strains displayed no significant difference in comparison with the wild-type strain. However, DN11 strain, which carried in-frame deletion of VEPGS_0011 gene, exhibited almost 50% reduction in extracellular protease production. Unfortunately, the complemented strain did not fully restore to the level of the wild-type strain (Fig. 3b).

Besides, the biofilm assay results revealed that all the five mutant strains, except DN08, showed comparable level of biofilm production to that of the wild-type strain. The biofilm was almost invisible in DN08 strain and restored to the level observed in the wild-type when the intact gene was introduced into DN08, confirming the crucial roles of VEPGS_0008 gene in biofilm formation in *V. alginolyticus*. For Hcp1 production assay, there are no dissimilarities in all these five mutant strains compared with the wild-type strain when the whole cell lysates were probed with Hcp1-specific antibody. However, no visible band against Hcp1 was present in any of the five mutant strains when the concentrated supernatants were conducted. In contrast, very weak and apparent Hcp1 secretion could be respectively detected in the wild-type strain and the Δ*pppA* mutant strain as previously described (Sheng *et al.* 2012) (Fig. 3d).

The zebrafish infection experiment was further performed to evaluate the impact of the five genes on the virulence of *V. alginolyticus*. The fish infected with a high dose ($3{\cdot}5 \times 10^7$ CFU per fish) of the wild-type and the five mutant strains died within 24 h. At the dose of $3{\cdot}5 \times 10^6$ CFU per fish and $3{\cdot}5 \times 10^5$ CFU per fish, the mortality of the zebrafish reached 90% in 48 h and 60–70% in 96 h respectively. All the dead fish exhibited typical haemorrhagic septicaemia. The LD_{50} values of the mutant strains and the wild-type were calculated and there were no significant attenuation in the five mutant strains compared to the wild-type strain (Table 3).

Discussion

Gateway cloning system has been commonly utilized for high-throughput characterization of ORFs in single standard frame in organisms, including *E. coli*, *Saccharomyces cerevisiae*, plants, and *Caenorhabditis elegans* (Dupuy *et al.* 2004; Hope *et al.* 2004; Busso *et al.* 2005; Earley *et al.* 2006; Alberti *et al.* 2007). Further efforts have been made to clone several ORFs in different frames into the same

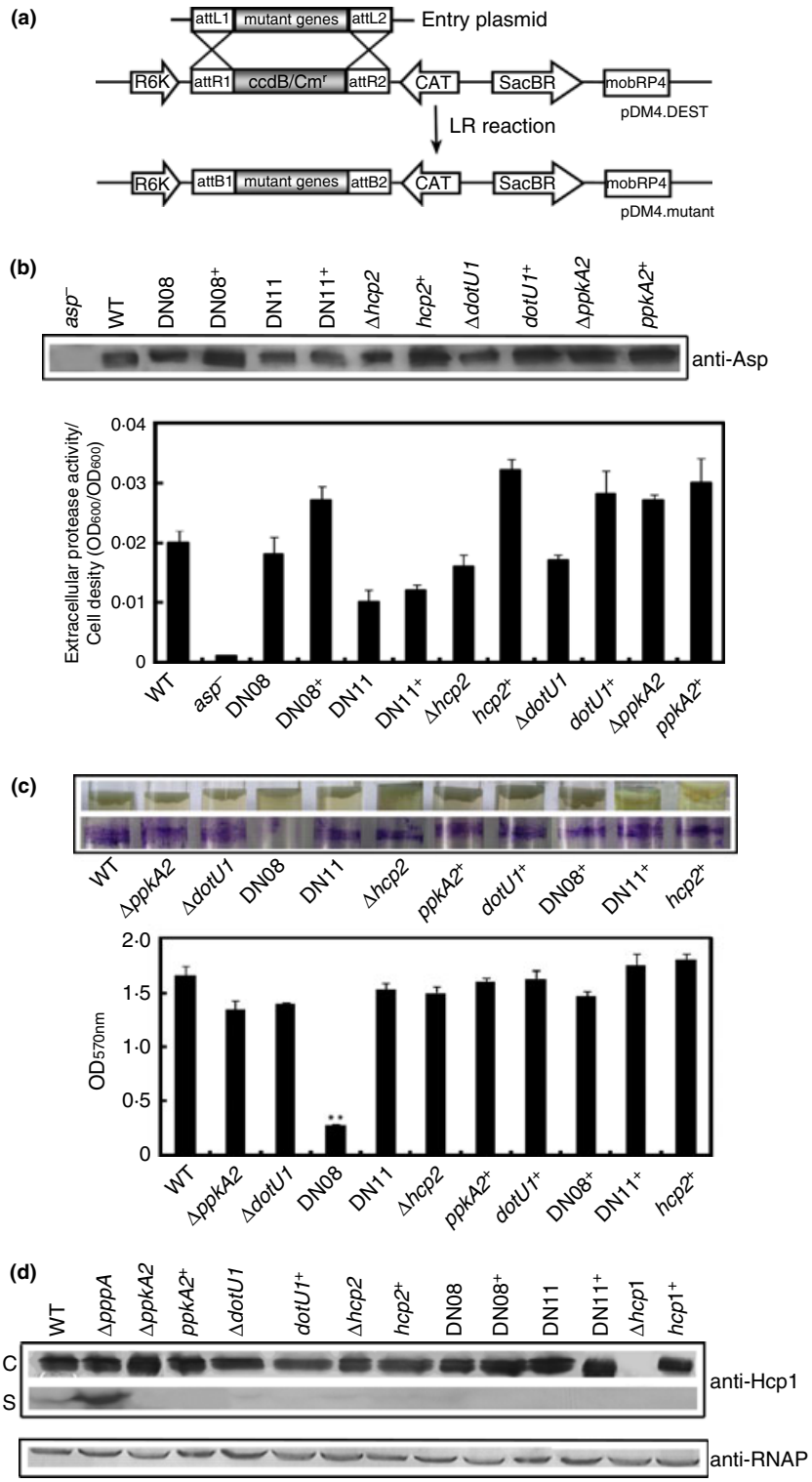

Figure 3 Functional analysis of the selected T6SS genes. (a) Schematic flowchart of the transfer of target genes to the destination vectors. (b) Extracellular protease production. (c) Biofilm formation. The values shown are the average of three independent experiments, and the error bars represent the standard deviations. Double asterisks marked the significant difference at $P < 0{\cdot}01$ from the wild-type strain. (d) Hcp1 production detection. A representative experiment out of three is shown. RNA polymerase (RNAP) was used as a control.

destination plasmid for expression synchronously and conveniently in *Aspergillus aryzae* (Mabashi *et al.* 2006). In this study, we have described the construction of the Gateway-compatible vectors for the systemic and rapid functional analysis of the unidentified genes in *V. alginolyticus*. Here in, five in-frame deletion mutant strains and their complemented strains in *V. alginolyticus* were obtained with high efficiency based on the modified

Table 3 Characterization of the *Vibrio alginolyticus* mutants

Strain	Swarming*	Swimming†	Biofilm‡	Aggregation§	Milk plate assay¶	LD_{50} CFU per fish**
WT	++	+	+	−	++	$9{\cdot}5 \times 10^4$
Δ*dotU1*	−	+	+	−	++	1×10^5
DN08	−	+	−	−	++	$6{\cdot}5 \times 10^4$
DN11	++	+	+	−	+	$1{\cdot}6 \times 10^5$
Δ*hcp2*	++	+	+	−	++	$1{\cdot}2 \times 10^5$
Δ*ppkA2*	−	+	+	−	++	$7{\cdot}2 \times 10^4$
Δ*hcp1*	+++	+	+	−	++	$2{\cdot}8 \times 10^5$
asp−	++	+	−	−	−	$6{\cdot}5 \times 10^6$

*Two microlitre cultures of the wild-type and mutant strains were spotted onto LB plates with 1·5% agar and incubated for 24 h. +++, colony diameter > 3 cm; ++, 2 cm < colony diameter < 3 cm; +, 1 cm < colony diameter < 2 cm; −, colony diameter < 1 cm.

† Two microlitre cultures of the wild-type and mutant strains were spotted onto LB plates with 0·3% agar and incubated for 24 h. +, clone completely covered the plate; −, colony diameter < 1 cm.

‡Biofilms were visualized by crystal violet staining. +, obvious biofilm; −, no biofilm.

§Cell aggregation characteristics and settling from static liquid suspensions. +, visible settling; −, no settling.

¶Wild-type and mutants cultures were spotted onto LBS plates containing 1% skimmed milk incubated for 10 h.++, the width of the clearing zones around the colonies > 1 mm; +, 0 mm < the width of the clearing zones < 1 mm; −, no clearing zones around the colonies.

**The number of mortality is the average of three parallel experiments. LD_{50} is calculated by the method described previously (Liu *et al.* 2011).

Gateway-compatible plasmids, pDM4.DEST and pMMB206. DEST. Meanwhile, the Hcp1 protein was also expressed successfully using the Gateway plasmid, pDEST17. These data illustrated that the Gateway plasmid and the Gateway-compatible plasmids constructed in this study can be used to facilitate systemic and even high-throughput functional study on novel genes. We confirmed that the use of these Gateway-compatible plasmids has greatly facilitated streamlined traditional day-to-day molecular cloning projects in which the restriction enzyme selection, the digestion activity, and the ligation efficiency have to be taken into account and usually become the limiting factors for successful construction and that it made the procedure more rapid, convenient, and efficient in characterization of genes of interest in *V. alginolyticus*. Besides, this Gateway-compatible cloning system was also applied successfully in *V. anguillarum, V. parahaemolyticus* and *Edwardsiella tarda* collected in our laboratory (data not shown).

T6SS is essential for bacterial pathogenesis. Yet the overall virulence regulation architecture of the T6SS is not well known until now. In this study, the roles of five T6SS genes in the virulence regulation of *V. alginolyticus* were determined by the extracellular protease production, biofilm formation, motility, and Hcp1 secretion. The results showed no significant differences in the tested strains in contrast with wild-type strain in Asp production except DN11, which exhibited 50% decrease in extracellular protease production. VEPGS_0011 encodes a putative T6SS lysozyme-related protein and belongs to gp25 family. The DN08 strain was unexpectedly inefficient in the biofilm formation and swarming ability. VEPGS_0008 encodes a putative transcriptional regulator belonging to AsnC family, which regulates the cell metabolism in response to the exogenous amino acid and has not been well characterized so far. Its roles in T6SS and virulence regulation are not clear. Besides, Δ*dotU1* and Δ*ppkA2* displayed no swarming ability. The *dotU1* gene encodes a transmembrane protein while PpkA2 is annotated as a serine/threonine protein kinase. At present, the detailed investigation of the functions of these five genes is underway in our laboratory. The secretion of Hcp1 was absent in all five mutant strains and their complemented strains. This may be caused by the tight post-translational regulation of PpkA/PppA system in which PpkA is established to be required for assembly of the T6SS and secretion of Hcp and its activity is antagonized by PppA (Mougous *et al.* 2007), for the secretion of Hcp1 was enhanced remarkably in Δ*pppA* strain (Fig. 3d). The influence of the T6SS components on Hcp1 secretion should be further assayed in the strain with Δ*pppA* genetic background.

Acknowledgements

This study was supported by National Natural Science Foundation of China (no. 30901113) and Shanghai Leading Academic Discipline Project (no. B505).

References

Alberti, S., Gitler, A.D. and Lindquist, S. (2007) A suite of Gateway cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*. *Yeast* **24**, 913–919.

Aschtgen, M.S., Bernard, C.S., De Bentzmann, S., Lioubès, R. and Cascales, E. (2008) SciN is an outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia coli*. *J Bacteriol* **190**, 7523–7531.

Austin, B. (2010) Vibrios as causal agents of zoonoses. *Vet Microbiol* **140**, 310–317.

Busso, D., Delagoutte-Busso, B. and Moras, D. (2005) Construction of a set Gateway-based destination vectors for high-throughput cloning and expression screening in *Escherichia coli*. *Anal Biochem* **343**, 313–321.

Dennis, J.J. and Zylstra, G.J. (1998) Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. *Appl Environ Microbiol* **64**, 2710–2715.

Dupuy, D., Li, Q.R., Deplancke, B., Boxem, M., Hao, T., Lamesch, P., Sequerra, R., Bosak, S. *et al.* (2004) A first version of the *Caenorhabditis elegans* promoterome. *Genome Res* **14**, 2169–2175.

Earley, K.W., Haag, J.R., Pontes, O., Opper, K., Juehne, T., Song, K. and Pikaard, C.S. (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* **45**, 616–629.

Filloux, A. (2009) The type VI secretion system: a tubular story. *EMBO J* **28**, 309–310.

Hope, I.A., Stevens, J., Garner, A., Hayes, J., Cheo, D.L., Brasch, M.A. and Vidal, M. (2004) Feasibility of genome-scale construction of promoter: reporter gene fusions for expression in *Caenorhabditis elegans* using a multisite gateway recombination system. *Genome Res* **14**, 2070–2075.

Jana, G., Aleš, U., Kamil, H. and Pavel, B. (2011) A eukaryotic-type signalling system of *Pseudomonas aeruginosa* contributes to oxidative stress resistance, intracellular survival and virulence. *BMC Genomics* **12**, 437–457.

Landy, A. (1989) Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annu Rev Biochem* **58**, 913–941.

Liang, W., Wang, S., Yu, F., Zhang, L., Qi, G., Liu, Y., Gao, S. and Kan, B. (2003) Construction and evaluation of a safe, live, oral *Vibrio cholerae* vaccine candidate, IEM108. *Infect Immun* **71**, 5498–5504.

Liu, H., Wang, Q., Liu, Q., Cao, X., Shi, C. and Zhang, Y. (2011) Roles of Hfq in the stress adaptation and virulence in fish pathogen *Vibrio alginolyticus* and its potential application as a target for live attenuated vaccine. *Appl Microbiol Biotechnol* **91**, 353–364.

Mabashi, Y., Kikuma, T., Maruyama, J.I., Arioka, M. and Kitamoto, K. (2006) Development of a versatile expression plasmid construction system for *Aspergillus oryzae* and its application to visualization of mitochondria. *Biosci Biotechnol Biochem* **70**, 1882–1889.

Milton, D.L., O'Toole, R., Horstedt, P. and Wolf-Watz, H. (1996) Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol* **178**, 1310–1319.

Morales, V.M., Bäckman, A. and Bagdasarian, M. (1991) A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. *Gene* **97**, 39–47.

Mougous, J.D., Gifford, C.A., Ramsdell, T.L. and Mekalanos, J.J. (2007) Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. *Nat Cell Biol* **9**, 797–803.

Rui, H., Liu, Q., Wang, Q., Ma, Y., Liu, H., Shi, C. and Zhang, Y. (2009) Role of alkaline serine protease, Asp, in *vibrio alginolyticus* virulence and regulation of its expression by LuxO-LuxR regulatory system. *J Microbiol Biotechnol* **19**, 431.

Sheng, L., Gu, D., Wang, Q., Liu, Q. and Zhang, Y. (2012) Quorum sensing and alternative sigma factor RpoN regulate type VI secretion system I (T6SSVA1) in fish pathogen *Vibrio alginolyticus*. *Arch Microbiol* **194**, 379–390.

Wang, S.Y., Lauritz, J., Jass, J. and Milton, D.L. (2002) A ToxR homologue from *Vibrio anguillarum* serotype O1 regulates its own production, bile resistance, and biofilm formation. *J Bacteriol* **184**, 1630–1639.

Weber, B., Hasic, M., Chen, C., Wai, S.N. and Milton, D.L. (2009) Type VI secretion modulates quorum sensing and stress response in *Vibrio anguillarum*. *Environ Microbiol* **11**, 3018–3028.

Yahata, K., Kishine, H., Sone, T., Sasaki, Y., Hotta, J., Chesnut, J.D., Okabe, M. and Imamoto, F. (2005) Multi-gene gateway clone design for expression of multiple heterologous genes in living cells: conditional gene expression at near physiological levels. *J Biotechnol* **118**, 123–134.