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Secretory delivery of heterologous proteins in attenuated *Vibrio anguillarum* for potential use in vaccine design

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Abstract To synthesize and secrete heterologous proteins in an attenuated *Vibrio anguillarum* strain for potential multivalent live vaccine development, different antigen-delivery systems based on bacterial-originated secretion signal peptides (SPs) were designed and identified in this work. Four SPs were derived from hemolysin of *Escherichia coli*, RTX protein of *V. cholerae*, hemolysin of *V. anguillarum*, zinc-metalloprotease of *V. anguillarum*, respectively, and their abilities to support secretion of green fluorescent protein (GFP) in an attenuated *V. anguillarum* strain MVAV6203 were assayed. Immunodetection of GFP showed that the capability of the tested signal leaders to direct secretion of GFP varied greatly. Although all the four signal peptide-fused GFPs could be expressed correctly and trapped intracellularly in recombinant strains, only the EmpA signal peptide could confer efficient secretion to GFP. For the investigation of its potential application in live bacteria carrier vaccines, a heterologous protein EseB of *Edwardsiella tarda* was fused to the SP_{empA} antigen-delivery system and introduced into the strain MVAV6203. Further analysis of EseB demonstrated that the constructed SP_{empA} antigen-delivery system could be used to secrete foreign protein in attenuated *V. anguillarum* and be available for carrier vaccines development.

Keywords EseB · Green fluorescent protein · Signal peptide · Secretion · Vaccine · *Vibrio anguillarum*

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Introduction

With the steadily growing knowledge of genetic basis of bacterial pathogenicity and the available modern genetic technology, several new concepts for vaccine development have been proposed. One of these rational designs is the multivalent vaccine based on virulence-attenuated pathogens which can be used as carriers for introducing heterologous protective antigens of other pathogenic microorganisms. Compared with the cytoplasmic expression of antigen in live attenuated bacterial vaccines, secretion or surface presentation of antigen seems to be superior in terms of immunogenicity (Georgiou et al. 1997). Thus, many antigen-delivery systems have been explored (Gentschev et al. 1996; Liljeqvist et al. 1997; Russmann et al. 1998) and successfully applied in virulence-attenuated bacterial carriers during the last decade (Gentschev et al. 2000; Hess et al. 1996; Isoda et al. 2007; Lee et al. 2000; Shao et al. 2005; Yang et al. 2008).

Up to date, six pathways (type I to type VI) for extracellular secretion have been identified in the gram-negative bacteria (Burns 1999; Henderson et al. 1998; Jacob et al. 2000; Sandkvist 2001a). Since the type I is considered to be the simplest secretion system with only three components, its secretion elements have been practically used to develop several antigen delivery systems. Among them, the *Escherichia coli* α-hemolysin (HlyA) secretion system, including three basic elements HlyB, HlyD, and TolC, was most widely applied in the delivery of heterologous proteins in bacterial carriers (Gentschev et al. 1996, 2002). In the construction of HlyA secretion expression vector, two specific components HlyB and HlyD, together with the target protein in-framed fused with the HlyA secretion signal, were cloned as a complete gene cluster into a plasmid, and the resultant recombinant

plasmid was transformed into the carrier strain to realize the efficient secretion of the target protein.

Vibrio anguillarum is an important marine fish pathogen responsible for both marine and fresh-water epizootics and causes fatal hemorrhagic septicemia disease (Crosa 1980). In the watershed over north of China, *V. anguillarum* infects many commercial farmed fish and results in a massive death as well as the loss of profits in aquaculture. In our previous work, several attenuated *V. anguillarum* strains derived from a wide-type MVM425 had been constructed. The plasmid-free derivative MVAV6203 with an *aroC* in-framed deletion was attenuated by 10,000 times and was conferred excellent immune protection against *Vibrio* pathogens including *V. anguillarum* and *V. alginolyticus* in animal tests (Ma et al. 2004).

For developing potential multivalent recombinant vaccines based on an attenuated *V. anguillarum* live vaccine, efficient antigen delivery systems are needed to present protective antigens in the live carrier. To achieve this aim, we attempted to screen efficient signal peptides (SPs) from the different secretion pathways and to construct antigen-delivery vectors by only fusing signal peptide with target protein into the plasmid without introducing related secretion elements, since the bacterial secretion systems, except for type I, are composed of many complex functional elements. In this way, the fused protein can be secreted by the aids of the endogenous secretion machinery located on the genome of the carrier bacteria.

In this work, four signal peptides derived from HlyA (Felmlee et al. 1985), RTX protein of *V. cholerae* (RtxA; Lin et al. 1999), hemolysin of *V. anguillarum* (Vah3; Hirono et al. 1996), and zinc-metalloprotease of *V. anguillarum* (EmpA; Zhang et al. 2006) were selected to assay their capabilities to direct the secretion of green fluorescent protein (GFP) in an attenuated *V. anguillarum* MVAV6203, and a heterologous protein EseB of *Edwardsiella tarda* (Zheng et al. 2007) was also successfully expressed and secreted via the best functional candidate SP_{empA} in MVAV6203, indicating that the constructed SP_{empA} antigen-delivery system had a great potential use in the development of the *V. anguillarum* carrier vaccines.

Materials and methods

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown at 37 °C in lysogeny broth (LB) medium (tryptone 1%, yeast extract

Table 1 Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics	Source
Strains		
E. coli		
Top10F'	F' mcrAΔ(*mrr-hsd*RMS-*mcr*BC) *ϕ80 lac*Z ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^r) *end*A1 *nup*G, transformation host	Invitrogen
V. anguillarum		
MVM425	Wide-type, Pacific Ocean prototype (O1 serotype)	Our lab
MVAV6203	Attenuated strain derived from MVM425	Our lab
E. tarda		
ATCC15947	Isolated from human feces (serotype O1483: H1)	ATCC
Plasmids		
pUC18	Cloning/expression vector, Ap^r *lacZ-α*, *Ori*: pMB1	TaKaRa
pG	pUC18 derivative containing 0.7 kb *gfp*, Ap^r	This work
pG-hlyA	pUC18 derivative containing 0.7 kb *gfp* overlapped with 240 bp signal sequence of *hlyA*, Ap^r	This work
pG-rtxA	pUC18 derivative containing 0.7 kb *gfp* overlapped with 240 bp signal sequence of *rtxA*, Ap^r	This work
pG-vah3	pUC18 derivative containing 0.7 kb *gfp* overlapped with 120 bp signal sequence of *vah3*, Ap^r	This work
pG-empA	pUC18 derivative containing 0.7 kb *gfp* overlapped with 120 bp signal sequence of *empA*, Ap^r	This work
pE	pUC18 derivative containing 0.6 kb *eseB*, Ap^r	This work
pE-empA	pUC18 derivative containing 0.6 kb *eseB* overlapped with 120 bp signal sequence of *empA*, Ap^r	This work
pANN-202–812	pBR322 derivative containing operon *hlyCABD*, Ap^r	Gentschev et al. (1996)
mTn5*gusA-pgfp21*	Mini-Tn5 transposon derivative containing *gfp*	Xi et al. (1999)

0.5%, NaCl 0.5%). *V. anguillarum* strains were cultured at 30 °C in LB medium supplemented with 2% NaCl (LBS). When required, ampicillin (100 μg/ml for *E. coli* and 200 μg/ml for *V. anguillarum*) and/or isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM) were added.

General DNA procedure

General DNA operations were carried out following the standard protocols (Ausubel and Kingston 1995). Automated DNA sequencing and primer synthesis were carried out by Invitrogen (Shanghai, China).

Plasmids construction

In the construction of secretion vectors, pUC18, which was demonstrated to be stable and duplicable in *V. anguillarum* strain, was used as the initial plasmid. SP_{hlyA} fragment was amplified from plasmid pANN-202–812 (provided by Dr. Andersen, Technical University of Denmark) (Gentschev et al. 1996), and SP_{rtxA} was amplified from the genomic DNA of *V. cholerae* (provided by Dr. Biao Kan, Chinese Academy of Preventive Medicine, Beijing, China). Both SP_{vah3} and SP_{empA} were cloned from *V. anguillarum* MVM425 genomic DNA. The *gfp* fragment was amplified from plasmid mTn5*gusA-pgfp* (Xi et al. 1999) and then from C-terminal fused with SP_{hlyA} and SP_{rtxA} and N-terminal fused with SP_{vah3} and SP_{empA} by overlap polymerase chain reaction (PCR), respectively. The four resulting overlap fragments were digested with the corresponding enzymes and cloned into the same sites of pUC18, generating four secretion plasmids pG-hlyA, pG-rtxA, pG-vah3, and pG-empA. The GFP cytosolic expression plasmid was also constructed by fusing the *gfp* gene into the multiple cloning site of pUC18, resulting in pG as a control plasmid for either the expression or secretion assays. The gene of *eseB* was cloned from the chromosome of *E. tarda* ATCC15947, and the EseB secretion plasmid pE-empA and cytosolic expression plasmid pE were constructed based on SP_{empA} and pUC18.

Fusion protein expression and cellular fractions preparation

The recombinant plasmids pG, pG-hlyA, pG-rtxA, pG-vah3, pG-empA, pE, and pE-empA were introduced into an attenuated *V. anguillarum* MVAV6203 by electroporation, yielding AV(pG), AV(pG-hlyA), AV(pG-rtxA), AV(pG-vah3), AV(pG-empA), AV(pE), and AV(pE-empA). All the recombinant strains were grown in LBS supplemented with ampicillin. For expression, overnight cultures were subcultured with 1:100 and cultured at 30 °C for 9 h. IPTG was added at 3 h after inoculation when needed. One milliliter cell culture was harvested by centrifugation at 10,000 *g* for 10 min, and the supernatants were collected as 'supernatant fractions' for further assays. The pellets were then resuspended in 1 ml phosphate-buffered saline (PBS, pH 7.0) and treated with an ultrasound sonication for 5 min on the ice, and the supernatants were harvested as 'intracellular fractions' for further analysis.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed in a 96-well flat-bottom polyvinyl microtiter plate (Costar, USA). The wells were coated with 100 μl of each supernatant fraction and intracellular fraction by overnight incubation at 4 °C. Unbound protein was removed by washing with PBS containing 0.05% Tween-20 (PBST) and the wells were blocked with 200 μl of PBST containing 1% bovine serum albumin (BSA; PBST-BSA) for 1 h at 37 °C. After removing the blocking solution, the plate was washed for three times by PBST and then incubated for 2 h with a rabbit anti-GFP (Proteintech, USA) antibody at a dilution of 1:3,000 (*v*/*v*) or with a rabbit anti-EseB antibody (YingJi Technology, Shanghai, China) at a dilution of 1:500,000 (*v*/*v*). After three washes, the plate was incubated for another 1 h at 37 °C with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Jackson Immuno Research Laboratories, USA) at a dilution of 1:5,000 (*v*/*v*). Finally, the wells were washed three times with PBST, and 3,3′,5,5′-tetramethylbenzidine (TMB) solution (TIANGEN Biotech, Beijing, China) was added for color development. After the addition of 2 M H_2SO_4 for termination of the reaction, the A_{450} in each well was measured with a microplate reader (Bio-Rad model 550, Hercules, CA, USA).

Western blot analysis

The cell fraction (supernatant and intracellular) was mixed with sample loading buffer, boiled for 5 min, and subjected to the 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. For Western blot analysis, proteins were transferred to polyvinylidene-fluoride membrane (Millipore) by using a Mini-Protean 3 Cell (Bio-Rad, Hercules, CA, USA) at 100 V for 3 h. The membrane was blocked with PBST-BSA buffer at 37 °C for 2 h. For immunodetection, the membrane was then incubated for 2 h at 37 °C in an antibody solution (PBST-BSA buffer) containing polyclonal rabbit anti-GFP antibody (1:3,000 *v*/*v*; Proteintech) or rabbit anti-EseB antibody (1:500,000 *v*/*v*; YingJi Technology) and then probed with a secondary antibody horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000 *v*/*v*; Jackson Immuno Research Laboratories). After successive washing with PBST, the TMB solution (TIANGEN Biotech) was added and the reaction was quenched with 2 M H_2SO_4.

Results

Determination of secretion signal peptides

For screening signal peptide, four secretory proteins including *E. coli* HlyA, *V. cholerae* RtxA, *V. anguillarum* Vah3, and *V. anguillarum* EmpA were selected. Among them, HlyA and RtxA are classified as repeats in toxin (RTX) proteins exported by type I secretion pathway (Delepelaire 2004), while Vah3 and EmpA are important virulence factors secreted by *V. anguillarum* via type II secretion system. The putative secretion SPs and signal peptidase cleavage sites were analyzed by using the SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP/) (Bendtsen et al. 2004). The output data from the SignalP 3.0 comprised three scores, *Ymax*, *Smean*, and *Dscore*. *Ymax* represents peptidase cleavage site, while *Smean* and *Dscore* mean the discrimination of SP and non-SP. As shown in Table 2, positive *Smean* and *Dscore* were actually obtained in SP_{vah3} and SP_{empA}. Since the C-terminal signal sequence could not be predicted well using SignalP, SP_{hlyA} and SP_{rtxA} were determined based on the knowledge of functional domains of HlyA and RtxA (Felmlee et al. 1985; Lin et al. 1999). Because the N-terminal charge status of the chosen SP-containing proteins, as suggested by SignalP, is essential for their translocation across the membrane, the length of each SP fragment to be cloned was designed to contain the whole putative SP and even a stretch of its downstream polypeptide to keep the charge status as original as possible. Accordingly, SP_{hlyA}(240 bp), SP_{rtxA} (240 bp), SP_{vah3} (120 bp), and SP_{empA} (120 bp) were amplified, respectively, and in-framed fused with *gfp* (720 bp) by overlap PCR. These PCR products were cloned into pUC18 respectively to result in the recombinant plasmids pG-hlyA, pG-rtxA, pG-vah3 and pG-empA.

Signal peptides screening in *V. anguillarum*

All the GFP expression plasmids were transformed into an attenuated *V. anguillarum* MVAV6203 to result in AV(pG), AV(pG-hlyA), AV(pG-rtxA), AV(pG-vah3), and AV(pG-empA) for further study. The strains were grown in LBS medium at 30 °C with IPTG adding at 3 h after inoculation. Considering the aged cells in the later stationary phase might release intracellular GFP to result in a background contamination, we harvested the cell cultures in later exponential phase to assure 100% cell viability. In order to examine the expression and secretion of fusion proteins, the collected samples were divided into intracellular fractions and supernatant fractions and then subjected to ELISA and Western blot assays.

For each recombinant strain, both intracellular fraction and supernatant fraction were prepared from the same volume of cultures so that the optical density $(OD)_{450}$ values of ELISA revealed the relative amount of GFP fusions in each fraction. As shown in Fig. 1a and b, the cytosolic expression strain AV(pG) produced large amount of GFP only in intracellular fraction but not in supernatant fraction, confirming the negative background contamination of the intracellular GFP fusion under our experimental conditions. Compared with AV(pG), the secretion expression strain AV(pG-empA) not only synthesized GFP inside the cell but also secreted it efficiently, while the other three secretion expression strains only produced intracellular GFP in low amount, indicating that EmpA signal peptide functions well in the marine bacterial host *V. anguillarum*.

For detailed determination of expression and secretion, equal volumes (15 μl) of different samples were subjected to Western blot analysis. Coinciding with the results in ELISA analysis, the GFP fusions were detected in the intracellular fractions of all the four secretion strains (Fig. 1c, lane 5, 7, 9, 11), while both the GFP-hlyA and GFP-rtxA fusions were a bit smaller in size than the estimated protein weight (about 35 kDa without the cleavage of the SP). Only the GFP-empA fusion could be detected in the supernatant fractions (lane 12), suggesting that EmpA signal peptide could guide the fusions to the extracellular environment in attenuated *V. anguillarum*. However, as showed in Fig. 1c, the GFP-empA fusion

Table 2 Amino acid sequences of signal peptides used in this study

Origin of signal peptide		SignalP 3.0	
		Smean	*Dscore*
E. coli HlyA	ITHGMDELYKHSAGQSTKDALAYGSQGDLN PLINEISKIISAAGSFDVKEERTAASLLQLSGNASDFSYGRNSITLTTSA	NT	NT
V. cholerae RtxA	SDYFNGNRAQVVIGMSEKDLSGEREYTMLS DSAIDALVQAMSGFEPQAGDNGFIDSLESKSQAAISMAWSDVVHKKGLMV	NT	NT
V. anguillarum Vah3	MTSSKFSLCAVGLLSISSIAVSTIATA ↓ SNPS EINTQLKWS	0.844	0.735
V. anguillarum EmpA	MKKVQRQMKWLFLAASISAALPVSA ↓ AKM VQVDDPSLLEQA	0.901	0.735

As suggested by SignalP 3.0, the *Dscore* values above 0.5 are classified as signal peptides. '↓' Stands for the signal peptidase cleavage sites
NT No determination (since C-terminus SP could not be finely predicted using SignalP 3.0)

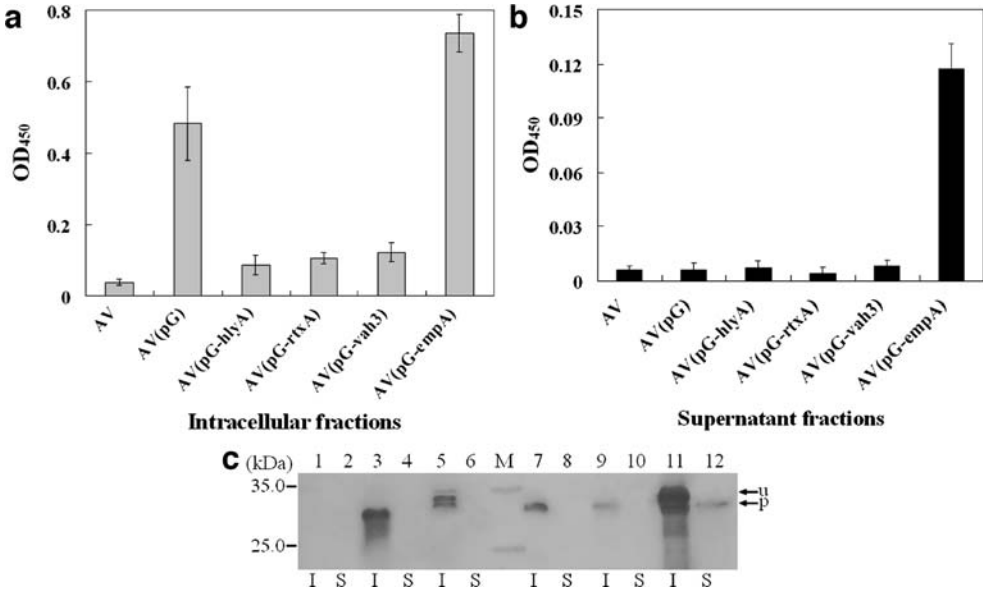

Fig. 1 Screening for functional signal peptides in *V. anguillarum*. Cells were grown in LBS medium at 30 °C and harvested at 9 h after inoculation. GFP fusions in intracellular fractions (**a**) and supernatant fractions (**b**) of different secretion strains were analyzed by ELISA methods. Western blot was used to determine GFP fusions in different fractions of recombinant strains (**c**). Lane 1 and 2, AV; lane 3 and 4, AV(pG); lane 5 and 6, AV(pG-hlyA); lane 7 and 8, AV(pG-rtxA); lane 9 and 10, AV(pG-vah3); lane 11 and 12, AV(pG-empA). *I* intracellular fraction, *S* supernatant fraction, *M* prestained standard marker protein (MBI Fermentas).The positions of the putative unprocessed (u) and processed (p) forms of GFP fusion proteins are indicated by *arrows*

was detected as two bands in the intracellular fraction (lane 11) and one band in the supernatant fraction (lane 12). The upper and lower bands were likely to, respectively, present the unprocessed and processed forms of GFP-empA fusion cleaved by signal peptidase in the periplasm because the difference of the two estimated molecular weight was roughly corresponding to that of the signal peptide.

Effect of inducer on GFP-empA secretion

In our constructs, all the fusion proteins were expressed from IPTG-inducible *lac* promoter of pUC18, and it is interesting to examine whether the expression and secretion of GFP-empA are induced in *V. anguillarum*. As showed in Fig. 2, GFP-empA fusion was detected in intracellular and supernatant fractions and IPTG induction brought about no significant difference in the expression or secretion level. The same results can also be obtained when the recombinant *V. anguillarum* strains were grown on minimal medium (data not shown), which demonstrated that P*lac* worked as a constitutive expression promoter in *V. anguillarum*. The IPTG-independent expression and secretion of GFP-empA in *V. anguillarum* are very important characters in the application of carrier vaccine.

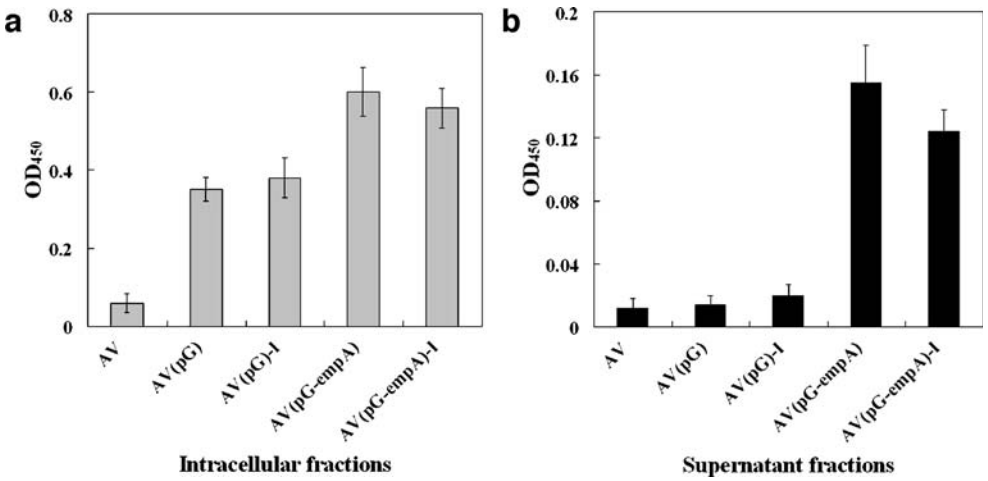

Fig. 2 Effect of inducer on GFP-empA secretion in *V. anguillarum*. Cells were grown in LBS medium at 30 °C and harvested at 9 h after inoculation. GFP fusions in intracellular fractions (**a**) and supernatant fractions (**b**) of the recombinant strains were analyzed by ELISA method. *I* Induced

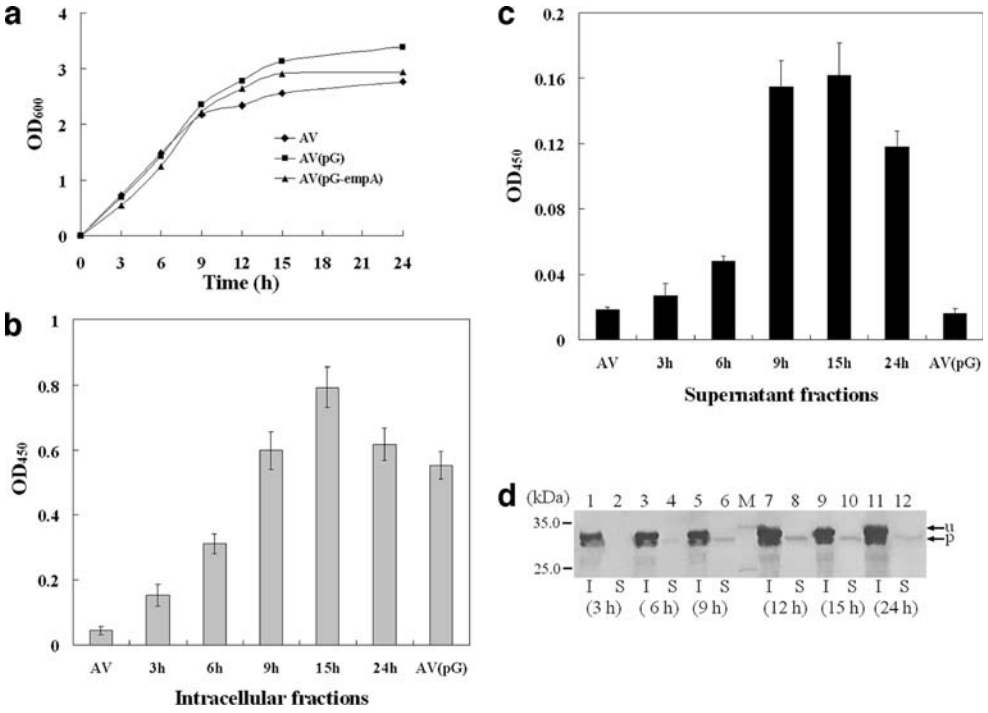

Fig. 3 Time course of GFP-empA secretion in *V. anguillarum*. Cells of the different *V. anguillarum* recombinant strains were grown in LBS medium at 30 °C without induction (**a**). GFP fusions in intracellular fractions (**b**) and supernatant fractions (**c**) of recombinant strains at different culture time point were analyzed by ELISA method. Western blot was used to determine GFP fusions in the different fractions of recombinant strains (**d**). *I* Intracellular fraction, *S* supernatant fraction, *M* prestained standard marker protein (MBI Fermentas).The positions of the putative unprocessed (u) and processed (p) forms of GFP fusion proteins are indicated by *arrows*

Time-course of GFP-empA secretion

In order to develop the multivalent live vaccine in attenuated *V. anguillarum* based on antigen-delivery system, the most effective candidate SP_{empA} secretion system was selected and the time-course of GFP-empA secretion was further investigated. As showed in Fig. 3a, all the strains had a similar growth curve and reached the stationary phase at approximately 9 h after inoculation. Different subcellular fractions of cell culture at 3, 6, 9, 15, and 24 h were prepared and subjected to ELISA analysis. AV(pG-empA) presented GFP signal after 3 h culture, and the signal increased until 15 h of culture in intracellular fraction (Fig. 3b). However, the GFP signal did not present in supernatant fraction until 6 h of culture, it significantly increased at 9 h of culture and slightly decreased at 24 h of culture (Fig. 3c).

The subcellular fractions of AV(pG-empA) at 3, 6, 9, 12, 15, and 24 h were further analyzed by the Western blot. In all the intracellular fractions of AV(pG-empA), the GFP-empA fusions were also detected in both unprocessed and processed forms, and the processed GFP-empA obviously increased along with time (Fig. 3d). Consistently, GFP-empA fusion in processed form did not appear in the supernatant fraction until 6 h of culture and greatly increase after 9 h culture, indicating that the secretion was obviously lagged behind the protein expression.

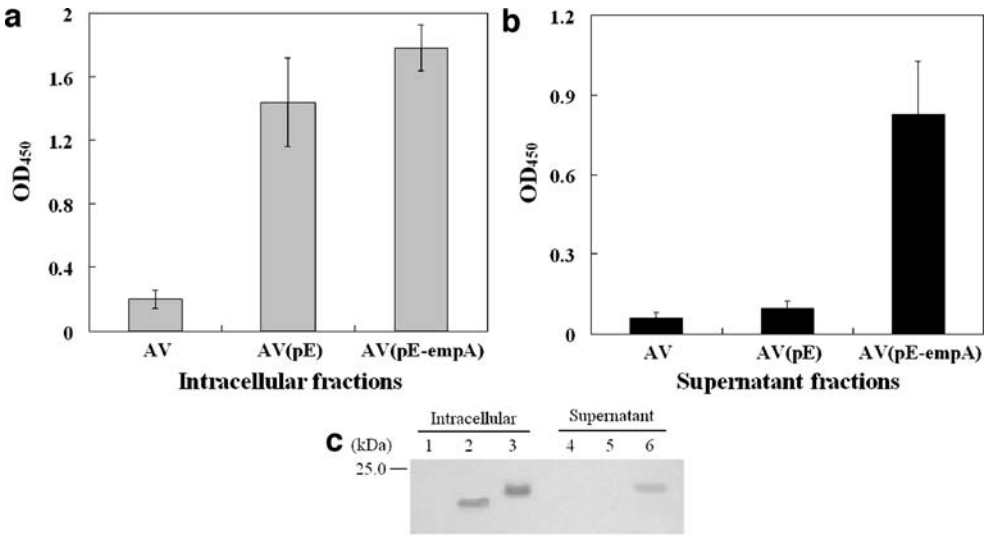

Fig. 4 Secretion of EseB-empA in *V. angullarum*. Cells were cultured in LBS medium at 30 °C for 9 h without induction. EseB fusions in intracellular fractions (**a**) and supernatant fractions (**b**) of recombinant strains were analyzed by ELISA method. Western blot was used to determine EseB fusions from the different samples of recombinant strains (**c**). Lane 1 and 4, AV; lane 2 and 5, AV(pE); lane 3 and 6, AV(pE-empA)

Expression and secretion of EseB-empA

For developing potential multivalent live *V. anguillarum* carrier vaccine, the feasibility of the SP_{empA} delivery system to secrete heterologous antigen in an attenuated *V. anguillarum* strain was preliminarily investigated. EseB was a putative translocon component of the *E. tarda* type III secretion system (Zheng et al. 2007), and it was selected as the assumed antigen for secretion delivery in this work. For EseB intracellular expression strain AV(pE), the EseB band (21.8 kDa) was found only in the intracellular fraction but not in the supernatant (Fig. 4c, lane 2, 5). While for the EseB secretion expression strain AV(pE-empA), the fusion of EseB-empA (23.3 kDa) could be detected in both the intracellular fraction and the supernatant (Fig. 4c, lane 3, 6), indicating that EmpA signal peptide could direct sufficient secretion of EseB in the marine bacterial host *V. anguillarum*, and the secretion efficiency can be roughly estimated at 35% according to the OD_{450} values of ELISA.

Discussion

In the development of an antigen-delivery system, the direct secretion of antigens by functional signal peptides in host cell is an important routine and the determination of efficient SPs is crucial in the approach. In this study, we selected four bacterial secreted proteins HlyA, RtxA, Vah3, and EmpA to search for the putative SPs. Among them, the HlyA has been generally investigated during the last decades, and its SP has been widely used in the delivery of recombinant proteins, while the RtxA of *V. cholerae* and the Vah3 and EmpA of *V. anguillarum* were also reported to be the typical secreted proteins although so far their SPs have not been used in the antigen delivery. Based on the sequence analysis by SignalP 3.0 software and combined with functional domain analysis, four SPs (SP_{hlyA}, SP_{rtxA}, SP_{vah3}, and SP_{empA}) were determined and subsequently cloned for antigen delivery in attenuated *V. anguillarum*.

The assay for the expression and secretion of GFP fusions indicated that only the EmpA signal peptide could confer efficient secretion in attenuated *V. anguillarum*. For the other three SPs, it was found that once GFP was fused with SP_{hlyA}, SP_{rtxA}, and SP_{vah3}, the expression of the resulting GFP fusions decreased greatly (Fig. 1a). Similar phenomenon also occurred in the heterologous proteins secretion in fission yeast (Kjarulff and Jensen 2005), and the mechanistic basis for this variation is largely unknown. Simultaneously, obvious degradation in intracellular fractions of AV(pG-hlyA) and AV(pG-rtxA; Fig. 1c, lane 5, 7) indicated the instability of the fusion proteins of GFP-hlyA and GFP-rtxA and the possible proteolysis in the cytoplasm. Therefore, the poor expression and protein degradation are assumed to be the important reasons of the failed secretion of GFP by SP_{hlyA}, SP_{rtxA}, and SP_{vah3}, and it is also implied that the efficient secretion of a given antigen in a given host depended not only on suitable signal peptide but also on high protein expression and good protein stability.

It has been identified that the type II pathway involves two steps (Sandkvist 2001b). The proteins containing 0061n N-terminal SP are first translocated across the cytoplasmic membrane via the *sec* machinery and released into the periplasm following with the removal of their SPs, and then the mature proteins crossed the outer membrane in a separate step. In our work, unprocessed and processed forms of the GFP-empA fusion were both detected in the intracellular fraction and only the processed form of fusion was detected in the supernatant The level of protein production greatly exceeded the rate of protein secretion into the growth medium and a large amount of fusion protein was trapped intracellularly (Fig. 1c, lane 11, 12). We believed that a fine match between the production level and secretion rate would be of benefit for the bacterial cells to achieve the efficient secretion.

In the secretions of GFP-vah3 and GFP-empA, both fusion proteins maintained stable structures and were able to be processed to mature forms (Fig. 1c, lane 9, 11). As the probably reason, the endogenous leader sequences derived from Vah3 and EmpA could be easily recognized by the secretory machinery of the *V. anguillarum* host and then directed the foreign proteins to secrete to the extracellular medium. It also gives us a hint that endogenous signal sequence may be more efficient for the secretion in *V. anguillarum* and more host origin SPs should be screened in the further vaccine developments.

To summarize, we constructed and compared the abilities of the four antigen-delivery systems based on signal peptides to support the secretion of green fluorescent protein in an attenuated *V. anguillarum* strain MVAV6203. Among the four candidates, the SP_{empA} system showed the highest expression level and secretion efficiency. Further time-course working of this superior system in *V. anguillarum* revealed that there was a 3–6-h lag phase between the production and secretion of fusion protein and the system exhibited the optimum secretion efficiency at about 9 h of culture. And in this work, we also succeeded to secrete the EseB protein of *E. tarda* via the SP_{empA} in an attenuated *V. anguillarum* MVAV6203, implying that the SP_{empA} antigen-delivery system might be available for the development of carrier vaccines.

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