Fish & Shellfish Immunology 39 (2014) 8–16

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Cell-penetrating peptides mediated protein cross-membrane delivery and its use in bacterial vector vaccine

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ARTICLE INFO

Article history:
Received 6 December 2013
Received in revised form
15 March 2014
Accepted 2 April 2014
Available online 16 April 2014

Keywords:
Cell-penetrating peptides
Antigen delivery
Edwardsiella tarda
Bacterial vector vaccine

ABSTRACT

It is an attractive strategy to develop a recombinant bacterial vector vaccine by expressing exogenous protective antigen to induce the immune response, and the main concern is how to enhance the cellular internalization of antigen produced by bacterial vector. Cell-penetrating peptides (CPPs) are short cationic/amphipathic peptides which facilitate cellular uptake of various molecular cargoes and therefore have great potentials in vector vaccine design. In this work, eleven different CPPs were fused to the C-terminus of EGFP respectively, and the resultant EGFP-CPP fusion proteins were expressed and purified to assay their cross-membrane transport in macrophage J774 A.1 cells. Among the tested CPPs, TAT showed an excellent capability to deliver the cargo protein EGFP into cytoplasm. In order to establish an efficient antigen delivery system in *Escherichia coli*, the EGFP-TAT synthesis circuit was combined with an *in vivo* inducible lysis circuit P_{viuA}-E in *E. coli* to form an integrated antigen delivery system, the resultant *E. coli* was proved to be able to lyse upon the induction of a mimic *in vivo* signal and thus release intracellular EGFP-TAT intensively, which were assumed to undergo a more efficient intracellular delivery by CPP to evoke protective immune responses. Based on the established antigen delivery system, the protective antigen gene *flgD* from an invasive intracellular fish pathogen *Edwardsiella tarda* EIB202, was applied to establish an *E. coli* recombinant vector vaccine. This *E. coli* vector vaccine presented superior immune protection (RPS = 63%) under the challenge with *E. tarda* EIB202, suggesting that the novel antigen delivery system had great potential in bacterial vector vaccine applications.

1. Introduction

Generally there are several ways to develop vaccines based on protective antigens, such as subunit vaccine, DNA vaccine and bacterial vector vaccine. Compared with the previous two vaccines, the strategy of bacterial vector vaccine is highly desirable, representing significant advantages of low-cost and adjuvant-non-dependent [1,2]. The efficacy of a bacterial live-vector vaccine lies on its ability to present sufficient foreign antigens to the host immune system to initiate the desired protective immune response [3,4]. In order to establish an efficient antigen-delivery system, two major barriers need to be overcome. The first barrier is how to transport antigens from bacterial vector into host tissues effectively, which will increase the chance of antigen to contact with the host immune system. The second barrier is how to transport antigens from host tissues into professional antigen-presenting cells (APCs), which will evoke more significant host immune response.

In our previous work, we have designed a novel antigen delivery system in *Escherichia coli* [5]. A strict iron-regulated promoter, P_{viuB}, was identified and applied to control the expression of lysis gene *E*, thus establishing an *in vivo*-inducible lysis system. An IPTG-inducible P_{T7}-controlled antigen expression circuit was integrated into the lysis system to build a novel *E. coli*-based antigen delivery system. A protective antigen, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), from the important fish pathogen *Aeromonas hydrophila* was introduced into the delivery system and the recombinant strain BL21(DE3)/pUTaBE + pETGA was used as the vaccine candidate for further evaluation of immune protection efficacy in turbot (*Scophtalmus maximus*). The recombinant *E. coli* could produce a large amount of antigen *in vitro*, and when administrated into animal host via injection or mucosal routes, they lyse themselves efficiently in response to the specific signal *in vivo* in host to implement mass release of antigens, and thus enhance the protective immune response in turbot. Based on the antigen

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http://dx.doi.org/10.1016/j.fsi.2014.04.003
1050-4648/

system, it is of great interest to further modify the system to promote antigen internalization by APCs.

Cell-penetrating peptides (CPPs), as short cationic peptide sequences, have been demonstrated to mediate intracellular delivery of a range of biological cargos, such as antigenic peptides, full-length proteins, DNA and nanoparticles [6–8]. The cellular entry mechanisms of CPPs are categorized into two groups: energy-dependent endocytosis and energy-independent direct translocation across the membrane bilayer [9,10]. There is now a wealth of evidence demonstrating the superior cellular delivery of cargos through conjugation to CPPs, ultimately leading to the generation of potent immune responses [11–14]. Therefore, CPP could offer significant potential to enhance antigen uptake by APCs and thus could be used in the design and development of bacterial vector vaccine.

Iron is often bound to metal-chelating proteins *in vivo*, and the availability of free iron is extremely limited in hosts [15]. Therefore, a low free iron concentration serves as an *in vivo* environmental signal [16]. To adapt to the iron starvation conditions present in the host, bacteria have evolved various iron uptake, storage, and metabolism systems to acquire and utilize iron in this environment. Among them, a typical iron uptake regulon has been well described. This iron-dependent regulation is carried out by the ferric uptake regulator (Fur), which coordinates the level of intracellular iron with expression of iron-related genes. When iron is abundant, Fur complexes with ferrous iron and blocks transcription of target genes by binding to conserved promoter regions termed Fur boxes [17]. In our previous work, the iron-regulated promoter P_{viuB} was identified and its origin gene *viuB* encodes a cytoplasmic protein necessary for ferric vibriobactin utilization in *Vibrio cholerae*. In this work, P_{viuA}, another strict iron-regulated promoter encoding vibriobactin outer membrane receptor in *V. cholerae*, was introduced to control the lysis gene *E* transcription.

Edwardsiella tarda is an intracellular Gram-negative pathogen that causes enteric septicemia in different fish species and generates severe economic losses in aquaculture [18,19]. Based on the reverse vaccinology and highly pathogenic *E. tarda* EIB202 genome sequence data, flagellar protein FlgD had been identified to be an important protective antigen protein [20]. In this work, eleven candidate CPPs were assayed for their capacity to translocate enhanced green fluorescent protein (EGFP) into macrophage cell line J774 A.1, and the effective CPP (TAT) was engineered into the established antigen delivery system [5] to form an integrated antigen delivery system. The protective antigen FlgD was introduced into this system, and the resultant recombinant bacterial vector vaccine showed a superior immune protection.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study were listed in Table 1. *E. coli* strains were cultured in Luria broth (LB) (Difco, Detroit, MI, USA) at 37 °C and *E. tarda* strains were routinely grown in tryptic soy broth (TSB) (Difco, Detroit, MI, USA) or tryptic soy agar (TSA) (Difco, Detroit, MI, USA) at 28 °C. All stock strains were stored at −80 °C in a suspension of medium containing 20% (v/v) glycerol. Recombinant plasmids were constructed in *E. coli* BL21(DE3). When required, ampicillin (Amp), kanamycin (Kan) and isopropyl-β-D-thiogalactoside (IPTG) were supplemented at final concentrations of 100 μg/ml, 50 μg/ml and 0.5 mmol/L, respectively. To impose different iron concentrations, 2,2′-dipyridyl was added into LB medium at a final concentration of 200 μmol/L for iron-depletion, and $FeSO_4$ was 40 μmol/L for iron-enriched condition.

Table 1
Strains and plasmids used in this study.

Strain or plasmid	Description	Reference of source
Bacteria		
E. coli Top10F′	General cloning strain	Invitrogen
E. coli BL21 (DE3)	F⁻ *ompT hsdS gal*, the expression host, as the vaccine delivery vector	Invitrogen
E. tarda EIB202	Wild type (CCTCC M208068), Col^r, Cm^r, fish pathogen, the gene source of *flgD*	Our lab
Genomes and plasmids		
φX174 genome	The source of *E* gene	NEB
pMD19-T Simple	Cloning vector, Amp^r	TaKaRa
pET28a	Expressing vector, Km^r	Novagen
pUT	pUC18 derivative with lac promoter and MCS deleted, and *rrnBT1T2* terminator and MCS from pBV220 inserted	Our lab
pUTa	pUT derivative, replacement of pBR322 ori. with pACYC ori.	Our lab
pET28a-EGFP	pET28a derivative, containing P_{T7}*egfp*TT (EcoRI/BamHI)	Prof. J. Zhao
pUTa-E	pUTa derivative, containing P_{viuA} *E*TT	This study
pET28a-G	pET28a derivative, containing P_{T7}*egfp*TT (NdeI/XhoI)	This study
pET28a-GC	pET28a derivative, containing P_{T7}*egfp-CPP*TT	This study
pET28a-GT	pET28a derivative, containing P_{T7}*egfp-TAT*TT	This study
pET28a-F	pET28a derivative, containing P_{T7} *flgD*TT	This study
pET28a-FT	pET28a derivative, containing P_{T7} *flgD-TAT*TT	This study

2.2. Plasmids construction

The 720-bp *egfp* gene amplified from plasmid pET28a-EGFP (kindly provided by Prof. J. Zhao, ECUST, China) was inserted into *Nde*I/*Xho*I-digested pET28a (+) vector (Novagen, USA) to yield pET28a-G. According to the amino acid sequence of CPPs (see Table 2) and codon preference in *E. coli*, nucleotide sequence of CPPs were achieved and synthesized into reverse primers. Using *egfp* as template, EG-F and a series of the above primers were designed to amplify the fusion genes *egfp-CPPs* (Supplementary Table 1S). The PCR products of *egfp-CPPs* were inserted into *Nde*I/*Xho*I-digested pET28a to yield the expression plasmid pET28a-GC, which were transformed into *E. coli* BL21 (DE3) for CPPs screening.

The 276-bp *E* gene, amplified from the φX174 genome, was inserted into *BamH*I/*Pst*I-digested pUTa and then iron-regulated promoter P_{viuA} was ligated into the above *EcoR*I/*BamH*I-digested plasmid, resulting in the iron-regulated lysis plasmid pUTa-E. The fusion gene *egfp-TAT* was inserted into *Nde*I/*Xho*I sites of pET28a to yield EGFP-TAT expression plasmid pET28a-GT. Both pUTa-E and pET28a-GT were transformed into *E. coli* BL21(DE3) for evaluating bacterial lysis and protein release *in vitro*. The 699-bp *flgD* gene amplified from the *E. tarda* EIB202 chromosome and the fusion gene *flgD-TAT* was inserted into *Nde*I/*Xho*I sites of pET28a under the control of the T7 promoter, generating pET28a-F and pET28a-FT. Both pUTa-E and pET28a-F or pET28a-FT were transformed into *E. coli* BL21(DE3) for E lysis and antigen FlgD delivery *in vivo*.

2.3. Detection of EGFP synthesis in vitro

Overnight bacterial cultures were inoculated (1:100, v/v) into fresh LB medium containing appropriate antibiotics and cultured in a shaker at 200 rpm and 37 °C. At early log phase, typically an optical density at 600 nm (OD_{600}) of 0.6–1.0, 0.5 mmol/L IPTG was added to induce the expression of enhanced green fluorescent