

NOTE

Generation of safety enhanced *Edwardsiella tarda* ghost vaccine

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ABSTRACT: A dual vector expressing the ghost-inducing PhiX174 lysis *E* gene and the bacterial DNA degrading staphylococcal nuclease A (SNA) gene was constructed to solve the problem of remnant antibiotic resistance genes and genomic DNA with intact pathogenic islands in the final product of *Edwardsiella tarda* ghosts (ETG). The SNA (devoid of secretion signal sequence and the nuclease B amino terminus sequence), fused with the 26 amino acid N-terminal sequence of the λ phage *Cro* gene, showed successful degradation of bacterial nucleic acids. Furthermore, the nuclease activity of SNA in *E. tarda* was enhanced by codon optimization of the SNA gene using site-directed mutagenesis. ETG were generated via coexpression of the SNA gene and lysis gene *E* under the control of each λP_R promoter. The ghost bacteria generation system we describe is advantageous as it allows the use of a single plasmid, improves safety and vaccine purity by limiting residual genetic content from the ghost bacteria, and reduces production costs through cheap means of induction that use only temperature shifts.

KEY WORDS: *Edwardsiella tarda* · Ghost bacteria · Staphylococcal nuclease A · Dual vector · Safety enhancement

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INTRODUCTION

Edwardsiellosis, caused by the Gram-negative, motile, flagellated and rod-shaped bacterium *Edwardsiella tarda*, leads to extensive losses in many commercially important freshwater and marine fish such as channel catfish, eels, mullet, chinook salmon, flounder, carp, tilapia, and striped bass (Thune et al. 1993, Plumb 1999). Wide distribution of antibiotic resistant strains (Waltman & Shotts 1986, Aoki & Takahashi 1987, Aoki et al. 1989) and existence of diverse O-serotypes (Tamura et al. 1988) in *E. tarda* have hindered effective treatment with antibiotics or the development of practical vaccines.

Most of the vaccines used commercially for cultured fish are inactivated (killed) disease agents. However,

traditional inactivation of bacteria by heat or formalin influences the physico-chemical characteristics of surface antigens, especially protein antigens; and immune responses against the modified antigens may not be protective against live pathogenic bacteria. Bacterial ghosts, an alternative method for inactivation of bacteria without chemical or physical stress, are produced by the controlled expression of bacteriophage PhiX174 lysis gene *E*. With bacterial ghosts, surface protein antigens remain intact, which is advantageous as it cross-protects hosts against different serotypes of bacteria (Szostak et al. 1990, 1993, 1996, Szostak & Lubitz 1991, Witte et al. 1992, Hensel et al. 1996, Huter et al. 2000). The potential usefulness of bacterial ghost technology has been reported in various mammalian pathogenic Gram-negative bacteria (Eko et al. 1994,

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Katinger et al. 1999, Panthel et al. 2002, Marchart et al. 2003).

Recently, we have generated *Edwardsiella tarda* ghosts (ETG) by gene *E* mediated lysis (Kwon et al. 2005), and have demonstrated significantly higher protection against infection of *E. tarda* in tilapia and olive flounder immunized with ETG than in fish immunized with formalin-killed *E. tarda* (Kwon et al. 2006, 2007). In these studies, although the bacteria were inactivated by induction of *E* gene expression, large-sized genomic DNA or plasmid DNA was detected from the produced ETG, suggesting the presence of nonlysed or partially lysed inactivated cells within the ghost preparation. The presence of genomic DNA with intact pathogenic islands and/or antibiotic resistance genes in the ETG preparation would be problematic to use as a practical vaccine for cultured fish. In this study, we constructed a dual vector expressing both the ghost inducing PhiX174 *E* gene and the bacterial DNA degrading staphylococcal nuclease A (SNA) gene to minimize the presence of antibiotic resistance genes and genomic DNA with pathogenic islands in the ETG vaccine. We also examined the potential utility of the vector in producing safety-improved ETG.

MATERIALS AND METHODS

Bacterial strains. *Edwardsiella tarda* FSW910410, isolated in 1991 from moribund olive flounder in a natural outbreak of edwardsiellosis on a commercial farm in Korea (Bang et al. 1992), was used. This strain is used in a commercial edwardsiellosis vaccine in Korea, and was kindly provided by the National Fisheries Research & Development Institute, Korea. For cloning of nuclease gene, *Staphylococcus aureus* KCCM 11335 was purchased from the Korean Culture Center of Microorganisms.

Construction of staphylococcal nuclease A (SNA) expressing vector. The gene fragment encoding staphylococcal nuclease A (SNA) was amplified by PCR using *Staphylococcus aureus* genomic DNA as the template and a pair of oligonucleotide primers, SNA *NdeI* F (5'-CATATGATGATGGCAACTTCAACTAA AAAATTAC-3'), and SNA *SacI* R (5'-GAGCTCTTA TTGACCTGAATCAGCGTTG-3'), containing *NdeI* and *SacI* restriction sites (underlined), respectively. The λ P_R-cI₈₅₇ regulatory system was also obtained by PCR using pLDR20 (American Type Culture Cell) as the template and the primers LPR-*SacII* F (5'-CCGCGACAACCTCCTTAGTACATGCAAC-3') and LcI-*AatII* R (5'-GACGTCAGCCAAACGTCTCTTCA GG-3'). PCR amplifications were performed for 1 cycle of 3 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C; and a final extension step of 7 min

at 72°C. PCR reactions were conducted using the iCycler thermal cycler (Bio-Rad). Each amplified PCR product was visualized on 0.7% agarose gel stained with ethidium bromide, purified with a gel extraction kit (Nucleogen) and cloned into pGEM-T easy vector (Promega). After *SalI* and *NdeI* digestion of both plasmids, the λ P_R-cI system was inserted into the plasmid containing the nuclease A gene, and the resulting plasmid was designated as p λ P_R-cI-SNA. The plasmid p λ P_R-cI-Cro-SNA was created by replacing λ P_R promoter upstream of SNA in the p λ P_R-cI-SNA plasmid, with the λ P_R promoter fused with the N-terminal 26 aa of the λ phage *Cro* gene. The λ P_R-*Cro* region was PCR-amplified by forward primer PR *SalI* F: GTCGACACGTTAAATCTATCACCGCAAG and reverse primer PRcro *NdeI* R: CATATGGGCCCTT-TAGCTGTCTTGG, using pLDR20 plasmid as the template.

Codon optimization of staphylococcal nuclease A (SNA) gene using site-directed mutagenesis. Three AGA triplets coding for arginine (R) and 8 TTA triplets coding for leucine (L) in the SNA gene of the plasmid p λ P_R-cI-Cro-SNA were converted to CGC and CTG, respectively, by site-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Stratagene). Mutagenic oligonucleotide primer pairs were designed for mutagenesis (Table 1), and the site-directed mutagenesis was conducted according to the manufacturer's instructions.

Construction of a dual vector expressing PhiX174 lysis *E* gene and staphylococcal nuclease A gene. The plasmid pDJPRPC, which was used in coexpression of both lysis gene *E* and staphylococcal nuclease A gene, was created as in Fig. 1. Briefly, the ribosomal RNA operon T1 terminator (*rrnBT1*) sequence was ligated into the *SpeI* and *PstI*-digested p λ P_R-cI-Elysis plasmid. Subsequently, the staphylococcal nuclease A (SNA) fragment was ligated into the *NdeI* and *SacI*-digested plasmid, and finally *SalI* and *NdeI*-digested λ PR promoter plus N-terminal *Cro* gene was ligated into the plasmid.

Induction of lysis *E* gene and/or SNA gene. All constructed vectors were transformed into *Escherichia coli* DH5 α (Invitrogen), and plasmids prepared from the DH5 α transformant were used for transformation of *Edwardsiella tarda* by electroporation (Gene Pulser, BioRad). Transformed *E. tarda* was grown in Luria Broth (LB, Difco) containing 50 μ g ml⁻¹ ampicillin (Sigma) at 27°C. Incubation temperatures for repression and expression of the lysis gene and/or nuclease gene in transformants were 27 and 42°C, respectively. When the cultures reached an optical density at 600 nm (OD₆₀₀) of 0.2 to 0.3, the expression of the lysis gene *E* and/or SNA gene was induced by a temperature elevation. For full nuclease activity of the SNA,

Table 1. Primer pairs used in the site-directed mutagenesis of staphylococcal nuclease A gene. Underlined nucleotides are mutagenic points. Three AGA triplets coding for arginine (R) and 8 TTA triplets coding for leucine (L) in the SNA gene of the plasmid p λ P_R-cl-Cro-SNA were converted to CGC and CTG, respectively

Primers	Sequence
SDM-1F	5'-ACCAATGACATTCC <u>CGC</u> CTA <u>CTG</u> TTGGTTGATACAC-3'
SDM-1R	5'-GTGTATCAACCAA <u>CAG</u> TAG <u>GCG</u> GAATGTCATTGGT-3'
SDM-2F	5'-ACTTCAACTAAAAAA <u>CTG</u> CATAAAGAACCTGCG-3'
SDM-2R	5'-CGCAGGTTCTTTATG <u>CAG</u> TTTTTTTAGTTGAAGT-3'
SDM-3F	5'-GAACCTGCGACACT <u>CTG</u> ATTAAAGCGATTGATGG-3'
SDM-3R	5'-CCATCAATCGCTTTAAT <u>CAG</u> TGTCGCGAGGTTTC-3'
SDM-4F	5'-GGTGATACGGTTAAAA <u>CTG</u> ATGTACAAAAGGTC-3'
SDM-4R	5'-GACCTTTGTACAT <u>CAG</u> TTTAACCGTATCACC-3'
SDM-5F	5'-GTTTGACAAAAGGTCAA <u>CGC</u> ACTGATAAATATGG-3'
SDM-5R	5'-CCATATTTATCAGT <u>GCG</u> TTGACCTTTGTCAAAC-3'
SDM-6F	5'-GGACGTGGC <u>CTG</u> GCGTATATTATGCTGATGG-3'
SDM-6R	5'-CCATCAGCATAAATATACGC <u>CAG</u> GCCACGTCC-3'
SDM-7F	5'-GGTAAACGAAGCT <u>CTG</u> GTTCTGTCAGGCTTGGC-3'
SDM-7R	5'-GCCAAGCCTTGACGAAC <u>CAG</u> AGCTTCGTTTACC-3'
SDM-8F	5'-CACATGAACAACCTT <u>CTG</u> CGCAAAAAGTGAAGC-3'
SDM-8R	5'-GCTTCACTTTT <u>GCG</u> CAGAAAGTTGTTTCATGTG-3'
SDM-9F	5'-GCAAAAAAAGAGAAA <u>CTG</u> AATATTTGGAGCGAAG-3'
SDM-9R	5'-CTTCGCTCCAAATATT <u>CAG</u> TTTCTCTTTTTTGC-3'

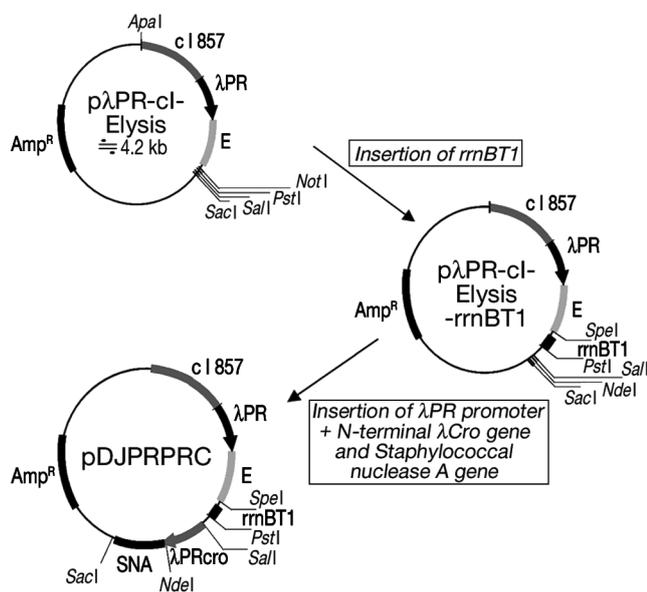


Fig. 1. Construction of dual vector, pDJPRPRC, expressing both lysis gene *E* and staphylococcal nuclease A (SNA). Amp^R: ampicillin resistance gene; rrnBT1: ribosomal RNA operon T1 terminator

1 mM MgCl₂ and 10 mM CaCl₂ were added before or after induction. At different post-induction time points, the optical density was monitored until no further decrease in optical density was noted, and total nucleic acids were isolated to confirm degradation by nuclease activity. At the end of the lysis process, 1 μ l of cultures was inoculated onto LB agar plates to examine the presence of surviving cells. Efficiency of ghost induc-

tion was expressed as the percentage of viable cells over total cell number at the point of inoculation.

Preparation of bacterial genomic DNA and electrophoretic analysis. To extract the genomic DNA of *Edwardsiella tarda*, 1 ml of the bacterial culture was harvested by centrifugation (10 min, 4°C, 10 000 \times *g*). Bacterial genomic DNA was prepared using LaboPass™ mini kit (Cosmo Genetech) according to the instruction manual. Degradation of genomic DNA by nuclease activity was analyzed on 0.7% agarose gel.

RESULTS

To test the effects of the SNA on the degradation of nucleic acids and inactivation of the host bacteria, *Escherichia coli* was transformed with the constructed p λ P_R-cl-SNA vector. SNA gene

expression was induced by temperature upshift and addition of MgCl₂/CaCl₂. However, the transformed *E. coli* growth was not different from control *E. coli* and the genomic DNA of the transformed *E. coli* was not degraded. To solve this problem, we constructed another vector, p λ P_R-cl-Cro-SNA, in which the N-terminal of the SNA gene was fused with the nucleotides encoding N-terminal 26 amino acids of the λ phage Cro gene. *Escherichia coli* transformed with p λ P_R-cl-Cro-SNA was inactivated by temperature upshift with MgCl₂/CaCl₂ supplementation, and degradation of the genomic DNA was confirmed by electrophoretic analysis (Fig. 2a).

Edwardsiella tarda transformed with p λ P_R-cl-Cro-SNA also showed successful degradation of intact genomic DNA by temperature upshift and MgCl₂/CaCl₂ supplementation (Fig. 2b). Furthermore, the ability of SNA to degrade *E. tarda* nucleotides was clearly enhanced by optimization of codons, which was confirmed by electrophoretic analysis (Fig. 2c).

Generation of ghosts in the transformants of *Escherichia coli* and *Edwardsiella tarda* carrying plasmid pDJPRPRC was performed successfully by upshifting incubation temperature to 42°C. Although the addition of MgCl₂ and CaCl₂ before temperature upshift interfered with *E* protein mediated lysis of both bacterial species, *E. tarda* was less affected than *E. coli* (Fig. 3).

In electrophoretic analysis, the intact genomic DNA and plasmids were observed in *Escherichia coli* and *Edwardsiella tarda* to harbor plasmid p λ P_R-cl-Elysis (Fig. 4). However, in both bacteria carrying plasmid

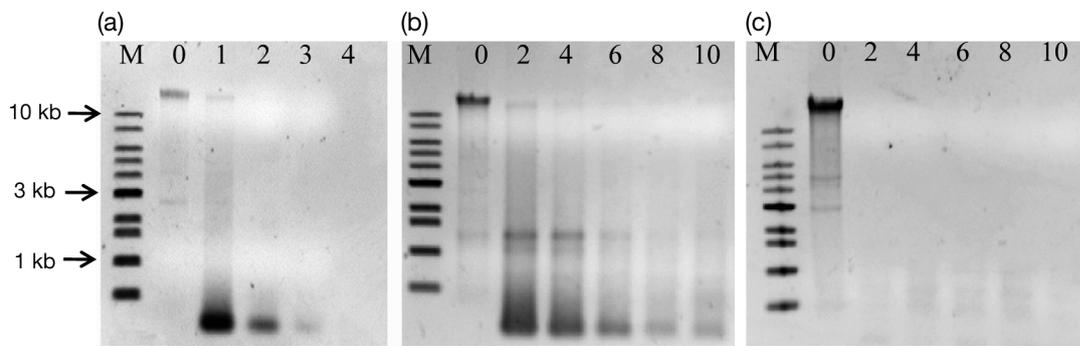


Fig. 2. Electrophoretic analysis. (a) *Escherichia coli* DH5 α , and (b,c) *Edwardsiella tarda* total DNA. Each bacterial species was transformed with p λ P_R-Cro-cl-SNA vector (a,b) or codon-optimized SNA gene expressing p λ P_R-Cro-cl-SNA vector (c). Time points of sampling given in h, and cultures shifted from 27 to 42°C at t_0 . Mg²⁺ and Ca²⁺ ions were added 1 h before temperature elevation

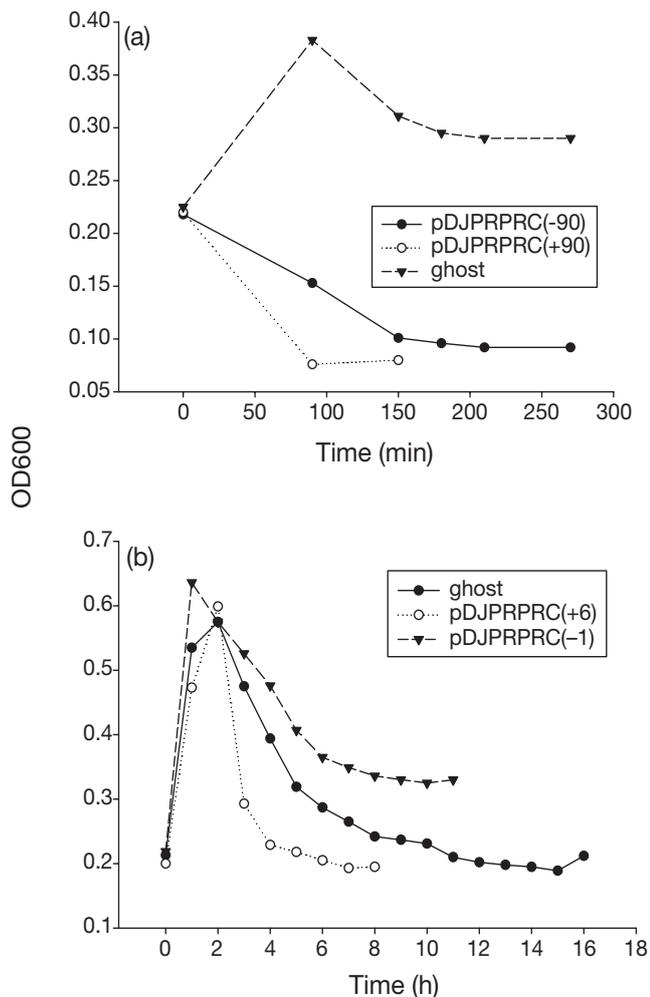


Fig. 3. Growth and lysis curves. (a) *Escherichia coli* DH5 α , and (b) *Edwardsiella tarda* harboring plasmid p λ P_R-cl-Elysis or pDJPRRC by induction of lysis gene *E* and staphylococcal nuclease A. MgCl₂ and CaCl₂ were added before 90 min (-90) or after 90 min (+90) of temperature upshift in (a); before 1 h (-1) or after 6 h (+6) of temperature upshift in (b). At t_0 , cultures were shifted from 27 to 42°C

pDJPRRC, the intact genomic DNA and plasmids were gradually degraded and were not observed 3 h after induction (Fig. 4). The differences in *E. tarda* genomic DNA degradation between wild and codon-optimized SNA genes could not be observed by electrophoretic analysis (data not shown). At the end of the lysis process, the efficiency of ghost induction in *E. coli* was 99.99 \pm 0.01 %. No bacterial growth was detected in *E. tarda*.

DISCUSSION

In the present study, we used coexpression of SNA gene and lysis gene *E* to generate *Edwardsiella tarda* ghosts (ETG) that lacked intact pathogenic islands and plasmids containing genes for antibiotic resistance. These changes improve the safety of ETG when used in vaccines. The production of bacterial ghosts by expression of both *E* and SNA genes have recently been described in *Escherichia coli* K12 strain NM522 (Haidinger et al. 2003) and a pathogenic strain, *E. coli* O157:H7 (Mayr et al. 2005). These studies used co-transformation with 2 plasmids: one expressing *E* gene (induced by a temperature shift to 42°C), and the other expressing the SNA gene (induced by addition of IPTG). In this study, instead of using 2 compatible plasmids, we constructed a new dual vector containing both the ghost inducing cassette and the bacterial DNA degrading cassette. Using our dual vector, we were able to induce the expression of both *E* and SNA genes by simply increasing the incubation temperature. This is important in vaccine development as it avoids the use of expensive chemical inducers like IPTG.

Staphylococcal nuclease (SNase) is an extracellular secreting enzyme, and the mature form, nuclease A (SNA), is a processed form of the initially exported nuclease B. Therefore, to express functional SNase in

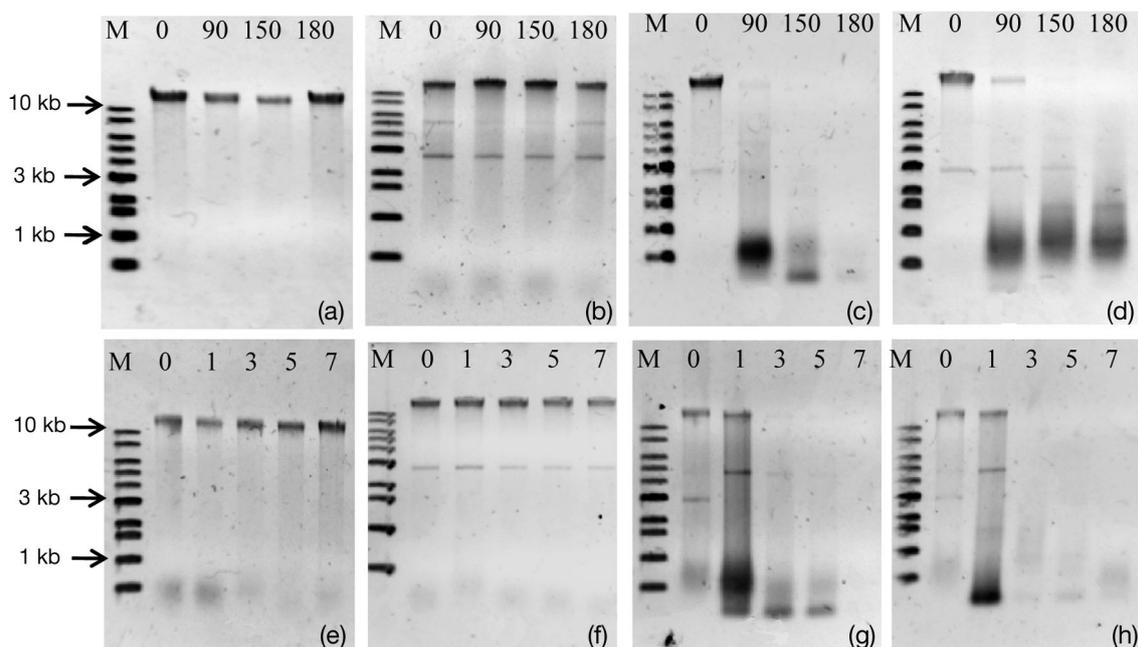


Fig. 4. *Escherichia coli*, and *Edwardsiella tarda*. Electrophoretic analysis of DH5 α and total DNA, respectively. Time points of sampling in min for *E. coli* or h for *E. tarda*. Cultures were shifted from 27 to 42°C at t_0 . (a) *E. coli* DH5 α (positive control), (b) *E. coli* DH5 α harboring p λ P_R-cl-Elysis, (c,d) *E. coli* DH5 α harboring pDJPRPC, (e) *E. tarda* (positive control), (f) *E. tarda* harboring p λ P_R-cl-Elysis, (g,h) *E. tarda* harboring pDJPRPC. (c,g) Addition of Mg²⁺ and Ca²⁺ at 90 min and at 6 h after induction of lysis, respectively, (d,h) addition of Mg²⁺ and Ca²⁺ at 90 min and 1 h before induction of lysis, respectively. M: 1 kb ladder (Bioneer)

the bacterial cytoplasm, the secreting signal sequence and the amino terminus of nuclease B should be excluded. In this study, we first constructed a vector expressing SNA under the control of the λ P_R promoter, and transformed *Escherichia coli* with the vector. However, we could not observe any degradation of genomic DNA or inactivation of the transformed *E. coli*. Recchi et al. (2002) reported that the staphylococcal nuclease expressed recombinantly in *Mycobacterium smegmatis* was extracellularly secreted in spite of the signal sequence deletion. They could express the SNase in the cytoplasm of *M. smegmatis* by fusion of the SNA gene with the N-terminal 26 amino acids of the *M. tuberculosis* urease gene, a cytoplasmic enzyme. In the current study, we rebuilt the SNA vector by fusion of the SNA with the N-terminal 26 aa of the λ phage *Cro* gene, and confirmed the degradation of genomic DNA from *E. coli* transformed with the newly constructed vector, p λ P_R-cl-Cro-SNA. In *Edwardsiella tarda*, the cytoplasmic nuclease activity of SNA was shown by fusion with the N-terminal 26 aa of the λ *Cro* gene. These results suggest that fusion of the secretion signal-deleted SNA with amino acids, which can stabilize cytoplasmic location of SNA, might be needed to induce cytoplasmic nuclease activity of SNA. Difference in codon usage is one of the obstacles in efficiently expressing heterologous genes. In the present

study, the nuclease activity of SNA in *E. tarda* was enhanced by substitution of the biased codons in SNA with optimum codons for *E. tarda*.

In this study, generation of ghost bacteria devoid of intact genomic DNA and plasmids was achieved by the newly constructed dual vector pDJPRPC. In the development of fish vaccines, safety enhancement and reduction of production costs are both important. The ghost bacteria generation system we describe is advantageous as it allows the use of a single plasmid, improves safety and vaccine purity by limiting residual genetic content from the ghost bacteria, and reduces production costs through cheap means of induction that use only temperature shifts.

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