Vol. 81: 249–254, 2008
doi: 10.3354/dao01964

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 24

NOTE

Generation of safety enhanced *Edwardsiella tarda* ghost vaccine

Dong Jin Lee[1], Se Ryun Kwon[1], Kosuke Zenke[1], Eun Hye Lee[1], Yoon Kwon Nam[2], Sung Koo Kim[3], Ki Hong Kim[1,*]

[1]Department of Aquatic Life Medicine, [2]Department of Aquaculture, [3]Faculty of Food Science and Biotechnology, Pukyong National University, Pusan 608-737, Korea

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

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,

*Corresponding author. Email: khkim@pknu.ac.kr

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 *Nde*I F (5′-CATATGATGATGGCAACTTCAACTAA AAAATTAC-3′), and SNA *Sac*I R (5′-GAGCTCTTA TTGACCTGAATCAGCGTTG-3′), containing *Nde*I and *Sac*I restriction sites (underlined), respectively. The λP_R-cI_{857} regulatory system was also obtained by PCR using pLDR20 (American Type Culture Cell) as the template and the primers LPR-*Sac*II F (5′-CC GCGGACAACCTCCTTAGTACATGCAAC-3′) and LcI-*Aat*II 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 *Sal*I and *Nde*I digestion of both plasmids, the λP_R-cI system was inserted into the plasmid containing the nuclease A gene, and the resulting plasmid was designated as pλP_R-cI-SNA. The plasmid pλP_R-cI-Cro-SNA was created by replacing λP_R promoter upstream of SNA in the pλP_R-cI-SNA plasmid, with the λP_R promoter fused with the N-terminal 26 aa of the λ phage *Cro* gene. The λP_R-*Cro* region was PCR-amplified by forward primer PR *Sal*I F: GTCGACACGTTAAATCTATCACCGCAAG and reverse primer PRcro *Nde*I 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 pλP_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 pDJPRPRC, 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 *Spe*I and *Pst*I-digested pλP_R-cI-Elysis plasmid. Subsequently, the staphylococcal nuclease A (SNA) fragment was ligated into the *Nde*I and *Sac*I-digested plasmid, and finally *Sal*I and *Nde*I-digested λPR 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* DH5α (Invitrogen), and plasmids prepared from the DH5α 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 μg 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 (OD_{600}) 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 pλP_R-cI-Cro-SNA were converted to CGC and CTG, respectively

Primers	Sequence
SDM-1F	5′-ACCAATGACATTC**CGC**CTA**CTG**TTGGTTGATACAC-3′
SDM-1R	5′-GTGTATCAACCAA**CAG**TAG**GCG**GAATGTCATTGGT-3′
SDM-2F	5′-ACTTCAACTAAAAAA**CTG**CATAAAGAACCTGCG-3′
SDM-2R	5′-CGCAGGTTCTTTATG**CAG**TTTTTTAGTTGAAGT-3′
SDM-3F	5′-GAACCTGCGACA**CTG**ATTAAAGCGATTGATGG-3′
SDM-3R	5′-CCATCAATCGCTTTAAT**CAG**TGTCGCAGGTTC-3′
SDM-4F	5′-GGTGATACGGTTAAA**CTG**ATGTACAAAGGTC-3′
SDM-4R	5′-GACCTTTGTACAT**CAG**TTTAACCGTATCACC-3′
SDM-5F	5′-GTTTGACAAAGGTCAA**CGC**ACTGATAAATATGG-3′
SDM-5R	5′-CCATATTTATCAGT**GCG**TTGACCTTTGTCAAAC-3′
SDM-6F	5′-GGACGTGGC**CTG**GCGTATATTTATGCTGATGG-3′
SDM-6R	5′-CCATCAGCATAAATATACGC**CAG**GCCACGTCC-3′
SDM-7F	5′-GGTAAACGAAGCT**CTG**GTTCGTCAAGGCTTGGC-3′
SDM-7R	5′-GCCAAGCCTTGACGAAC**CAG**AGCTTCGTTTACC-3′
SDM-8F	5′-CACATGAACAACTT**CTGCGC**AAAAGTGAAGC-3′
SDM-8R	5′-GCTTCACTTTT**GCGCAG**AAGTTGTTCATGTG-3′
SDM-9F	5′-GCAAAAAAAGAGAAA**CTG**AATATTTGGAGCGAAG-3′
SDM-9R	5′-CTTCGCTCCAAATATT**CAG**TTTCTCTTTTTTTGC-3′

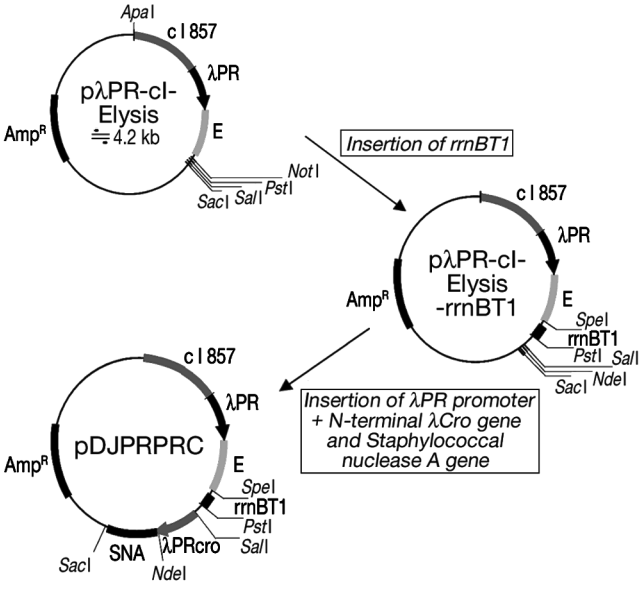

Fig. 1. Construction of dual vector, pDJPRPRC, expressing both lysis gene *E* and staphylococcal nuclease A (SNA). Amp^R: ampicillin resistance gene; rrnBT1: ribosomal RNA operon T1 terminator

1 mM $MgCl_2$ and 10 mM $CaCl_2$ 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 µl of cultures was inoculated onto LB agar plates to examine the presence of surviving cells. Efficiency of ghost induction 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 × *g*). Bacterial genomic DNA was prepared using Labo-Pass™ 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 pλP_R-cI-SNA vector. SNA gene expression was induced by temperature upshift and addition of $MgCl_2$/$CaCl_2$. 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, pλP_R-cI-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 pλP_R-cI-Cro-SNA was inactivated by temperature upshift with $MgCl_2$/$CaCl_2$ supplementation, and degradation of the genomic DNA was confirmed by electrophoretic analysis (Fig. 2a).

Edwardsiella tarda transformed with pλP_R-cI-Cro-SNA also showed successful degradation of intact genomic DNA by temperature upshift and $MgCl_2$/$CaCl_2$ 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 $MgCl_2$ and $CaCl_2$ 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 pλP_R-cI-Elysis (Fig. 4). However, in both bacteria carrying plasmid

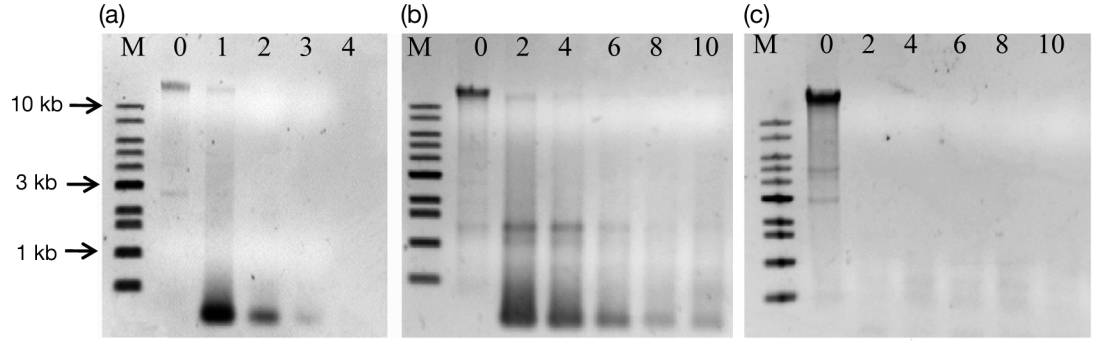

Fig. 2. Electrophoretic analysis. (a) *Escherichia coli* DH5α, and (b,c) *Edwardsiella tarda* total DNA. Each bacterial species was transformed with pλP_R-Cro-cI-SNA vector (a,b) or codon-optimized SNA gene expressing pλP_R-Cro-cI-SNA vector (c). Time points of sampling given in h, and cultures shifted from 27 to 42°C at t_0. Mg^{2+} and Ca^{2+} ions were added 1 h before temperature elevation

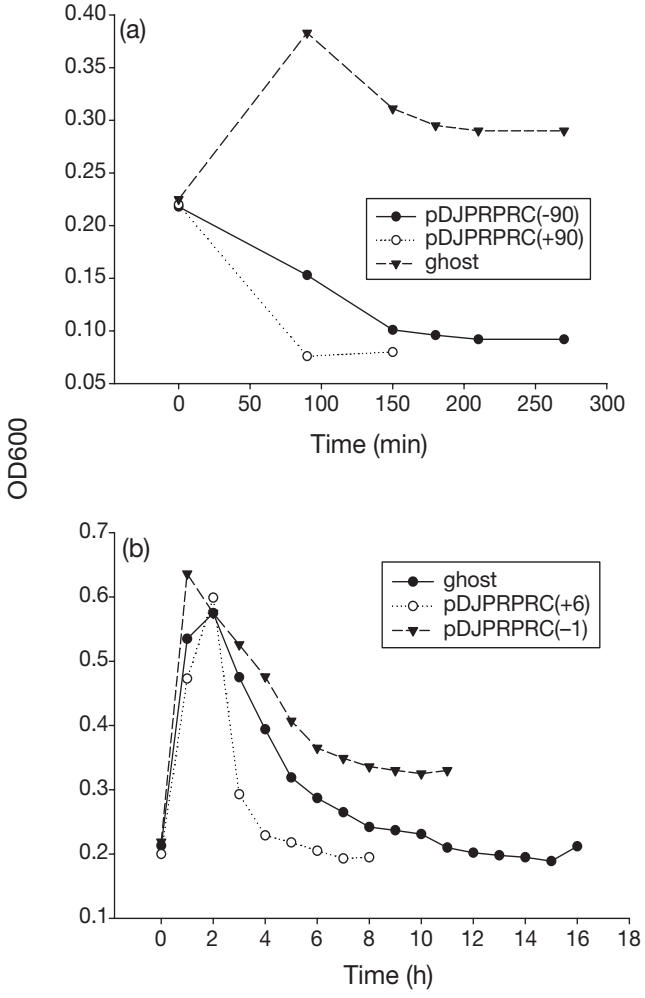

Fig. 3. Growth and lysis curves. (a) *Escherichia coli* DH5α, and (b) *Edwardsiella tarda* harboring plasmid pλP_R-cI-Elysis or pDJPRPRC by induction of lysis gene *E* and staphylococcal nuclease A. $MgCl_2$ and $CaCl_2$ were added before 90 min (–90) or after 90 min (+90) of temperature upshift in (a); 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

pDJPRPRC, 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 ± 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

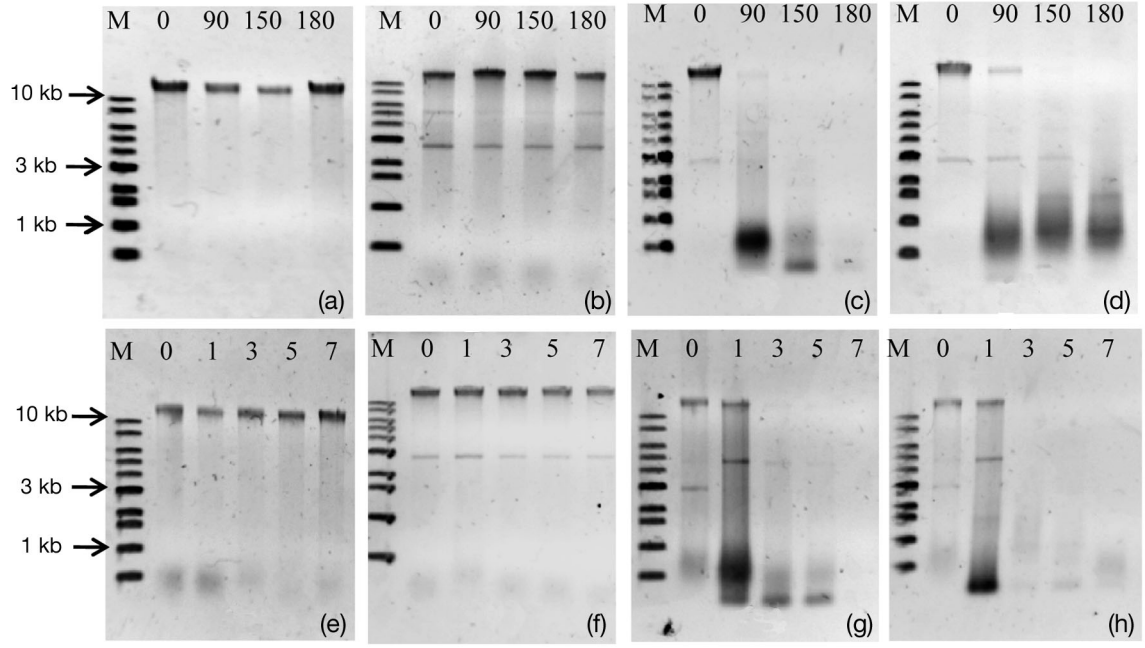

Fig. 4. *Escherichia coli*, and *Edwardsiella tarda*. Electrophoretic analysis of DH5α and total DNA, respectively. Time points of sampling in min for *E. coli* or h for *E. tarda*. Cultures were shifted from 27 to 42°C at t_0. (a) *E. coli* DH5α (positive control), (b) *E. coli* DH5α harboring pλP_R-cI-Elysis, (c,d) *E. coli* DH5α harboring pDJPRPRC, (e) *E. tarda* (positive control), (f) *E. tarda* harboring pλPR-cI-Elysis, (g,h) *E. tarda* harboring pDJPRPRC. (c,g) Addition of Mg^{2+} and Ca^{2+} at 90 min and at 6 h after induction of lysis, respectively, (d,h) addition of Mg^{2+} and Ca^{2+} 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 λP_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, pλP_R-cI-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 pDJPRPRC. 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.

Acknowledgements. This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2006-311-F00090).

LITERATURE CITED

Aoki T, Takahashi A (1987) Class D tetracycline resistance determinants of R plasmids from the fish pathogens *Aeromonas hydrophila, Edwardsiella tarda*, and *Pasteurella piscicida*. Antimicrob Agents Chemother 31:1278–1280

Aoki T, Kitao T, Fukudome M (1989) Chemotherapy against infection with multiple drug resistance strains of *Edwardsiella tarda* in cultured eels. Fish Pathol 24:161–168

Bang JD, Chun SK, Park SI, Choi YJ (1992) Studies on the biochemical and serological characteristics of *Edwardsiella tarda* isolated from cultured flounder, *Paralichthys olivaceus.* J Fish Pathol 5:29–35

Eko FO, Szostak MP, Wanner G, Lubitz W (1994) Production of *Vibrio cholera*-ghosts (VCG) by expression of a cloned phage lysis gene: potential for vaccine development. Vaccine 12:1231–1237

Haidinger W, Mayr UB, Szostak MP, Resch S, Lubitz W (2003) *Escherichia coli* ghost production by expression of lysis gene *E* and staphylococcal nuclease. Appl Environ Microbiol 69:6106–6113

Hensel A, van Leengoed LAMG, Szostak M, Windt H and 8 others (1996) Induction of protective immunity by aerosol or oral application of candidate vaccines in a dose-controlled pig aerosol infection model. J Biotechnol 44: 171–181

Huter V, Hensel A, Brand E, Lubitz W (2000) Improved protection against lung colonization by *Actinobacillus pleuropneumoniae* ghosts: characterization of a genetically inactivated vaccine. J Biotechnol 83:161–172

Katinger A, Lubitz W, Szostak MP, Stadler M, Klein R, Indra A, Huter V, Hensel A (1999) Pigs aerogenously immunized with genetically inactivated (ghosts) of irradiated *Actinobacillus pleuropneumoniae* are protected against a homologous aerosol challenge despite differing in pulmonary cellular and antibody responses. J Biotechnol 73:251–260

Kwon SR, Nam YK, Kim SK, Kim DS, Kim KH (2005) Generation of *Edwardsiella tarda* ghosts by bacteriophage PhiX174 lysis gene *E.* Aquaculture 250:16–21

Kwon SR, Nam YK, Kim SK, Kim KH (2006) Protection of tilapia (*Oreochromis mosambicus*) from edwardsiellosis by vaccination with *Edwardsiella tarda* ghosts. Fish Shellfish Immunol 20:621–626

Kwon SR, Lee EH, Nam YK, Kim SK, Kim KH (2007) Efficacy of oral immunization with *Edwardsiella tarda* ghosts against edwardsiellosis in olive flounder (*Paralichthys olivaceus*). Aquaculture 269:84–88

Marchart J, Dropmann G, Lechleitner S, Schlapp T, Wanner G, Szostak MP, Lubitz W (2003) *Pasteurella multocida*- and *Pasteurella haemolytica*-ghosts: new vaccine candidates. Vaccine 21:3988–3997

Mayr UB, Haller C, Haidinger W, Atrasheuskaya A, Bukin E, Lubitz W, Ignatyev G (2005) Bacterial ghosts as an oral vaccine: A single dose of *Escherichia coli* O157:H7 bacterial ghosts protects mice against lethal challenge. Infect Immun 73:4810–4817

Panthel K, Jechlinger W, Matis A, Rohde M, Szostak M, Lubitz W, Haas R (2003) Generation of *Helicobacter pylori* ghosts by PhiX protein E-mediated inactivation and their evaluation as vaccine candidates. Infect Immun 71:109–116

Plumb JA (1999) *Edwardsiella* septicaemias. In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Vol 3. Viral, bacterial and fungal infections. CAB International, p 479–521

Recchi C, Rauzier J, Gicquel B, Reyrat JM (2002) Signal-sequence-independent secretion of the staphylococcal nuclease in *Mycobacterium smegmatis.* Microbiology 148: 529–536

Szostak MP, Lubitz W (1991) Recombinant ghosts as multivaccine vehicles. In: Ginsberg HS, Brown F, Chanock RM, Lerner RA (eds) Vaccines 91: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p 409–414

Szostak M, Wanner G, Lubitz W (1990) Recombinant bacterial ghosts as vaccines. Res Microbiol 141:1005–1007

Szostak MP, Auer T, Lubitz W (1993) Immune response against recombinant bacterial ghosts carrying HIV-1 reverse transcriptase. In: Ginsberg HS, Brown F, Chanock RM, Lerner RA (eds) Vaccines 93: modern approaches to new vaccines including prevention of AIDS. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, p 419–425

Szostak MP, Hensel A, Eko FO, Klein R and 6 others (1996) Bacterial ghosts: non living candidate vaccines. J Biotechnol 44:161–170

Tamura K, Sakazaki R, McWhorter AC, Kosako Y (1988) *Edwardsiella tarda* serotyping scheme for international use. J Clin Microbiol 26:2343–2346

Thune RL, Stanley LA, Cooper RK (1993) Pathogenesis of gram-negative bacterial infections in warmwater fish. Annu Rev Fish Dis 3:37–68

Waltman WD, Shotts EB (1986) Antimicrobial susceptibility of *Edwardsiella tarda* from the United States and Taiwan. Vet Microbiol 12:277–282

Witte A, Wanner G, Lubitz W (1992) Dynamics of PhiX174 protein E-mediated lysis of *Escherichia coli.* Arch Microbiol 157:381–388

Editorial responsibility: Stewart Johnson, Nanaimo, British Columbia, Canada

Submitted: October 16, 2007; Accepted: July 7, 2008
Proofs received from author(s): September 9, 2008