Short Communication

Cloning of *EprA1* gene of *Aeromonas hydrophila* in *Lactococcus lactis*

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Abstract
Bacterial-based systems as live vectors for the delivery of heterologous antigens offer a number of advantages as vaccination strategies. Developments in genetic engineering have given Gram-positive lactic acid bacteria (LAB) the advantage of being used as a host expression system for antigen delivery to induce the immune response. A fragment containing the full length of the "*eprA1*" gene, encoding a temperature-stable metalloprotease of *Aeromonas hydrophila* isolated from fish was amplified by polymerase chain reaction (PCR) using the genomic DNA of this bacterium as template. The amplified 1038 bp fragment was digested by *Pst*I and *Hind*III, followed by ligation into the corresponding site on the pNZ8048 plasmid. The ligated DNA was then transformed into *Lactococcus lactis* NZ9000 cells by electroporation method. Verification of cloning was carried out using restriction enzyme digestion and DNA sequencing. Gel electrpophoresis technique also detected the expression of the recombinant protease protein. The successful cloning and expression of the *eprA1* gene into *L. lactis* can be developed as a useful and safe system to control *A. hydrophila* infections in fish.
***Keywords*:** *Aeromonas hydrophila*; Protease; *Lactococcus lactis*; Live vaccine; Fish

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Many of *Aeromonas hydrophila*'s extracellular products (ECPs) are virulent factors. Among these, cytotoxins, haemolysins, and proteases are the most important (John, 2000). Serine protease and metalloprotease produced by *A. hydrophila* have been implicated in the spoilage of meat and fish. They cause tissue damage, aid invasiveness and establishment of infection by overcoming host defenses, provide nutrients for cell proliferation and activate other bacterial toxins such as aerolysin (Vivas *et al*., 2004; Pemberton *et al*., 1997). Induced mutations in protease gene of A. hydrophila have shown that mutant proteolytic activities are 90% lower than in the wild type strains. Fish that are injected with the parental strain die more rapidly than those injected with the isogenic protease mutant (Cascon *et al*., 2000). It has been also reported that *A. hydrophila* Tn5-induced protease deficient mutants are less virulent than the wild type strains, thus indicating that proteases play an important role in pathogenicity of this bacterium. In addition, it has been found that proteases and other ECP components of *A. hydrophila* are major antigenic components of a vaccine against hemorrhagic septicemia in fish (Hernanz *et al*., 1998). Infectious pathogens are the most important problems in aquaculture; as approximately 10% of the fish population in this sector is lost due to microbial diseases. Chemical drugs are used against these diseases, but there are some undesirable side effects such as accumulation in the body and contamination of the aquatic environment (Heppel and Davis, 2000).

The health-promoting (probiotic) properties of certain lactic acid bacteria (LAB) in humans and animals have recently been extensively studied. These features

include antagonistic effects against invading pathogens and cancer, production of antimicrobial substances, an anticholesteremic effect and immunomodulation. Several products containing probiotic LAB strains are already commercially available for human use or for the prevention of intestinal infections in domestic animals. Some other advantages of LAB bacteria include non-pathogenicity, non-invasiveness, non-colonization, acid tolerance (surviving the passage through the stomach) and the absence of lipopolysaccharides in their cell walls. Such factors make LAB attractive candidates, as carriers of antigens for vaccination (Chatel *et al*., 2003; Steidler *et al*., 2003; Jos and Seeger, 2002; Norton *et al*., 1995).

The *eprA1* gene that encodes the heat-labile protease of *A. hyrophila*, was studied by Chang *et al.* (1997) for the first time. It was detected as an extracellular temperature-stable protease and also as an extracellular temperature-labile serine protease gene. The predicted molecular mass of *eprA1* approximately agrees with the major 37 kDa band, but a 29 kDa band has been associated with caseinolytic activity (Chang *et al*., 1997). Many proteases are initially synthesized as inactive form and then changed to lower molecular mass active enzymes. In the case of EprA1 protease the 29 kDa enzyme with proteolytic activity, is the active form of EprA1 protease with the initial molecular mass of 37 kDa (Wandersman, 1989).

In the present work, cloning and expression of the *eprA1* gene, a temperature-stable metalloprotease of *A. hydrophila* isolated from fish, in *L. lactis* system were successfully carried out for the first time. Since LAB bacteria are probiotic organisms, so such useful and safe carriers can be developed as useful and safe systems to control microbial infections in different living organisms.

Microorganisms and plasmids: *Aeromonas hydrophila* AHMP strains were isolated from diseased tilapia fish using selective medium, glutamate starch phenol agar (GSP agar), a range of biochemical tests and biolog diagnostic kits (Sasan *et al*., 2007). Genomic DNA was extracted from *A. hydrophila* to amplify the protease gene (Sambrook *et al*., 1989). The *L. lactis* NZ9000 strain was used as cloning host cells. The pNZ8048 plasmids (carrying the *nisA* promoter and a chloramphenicol selectable marker) isolated from *L. lactis* NZ8048 (MG1363: *pepN*::*nisRK*) cells were used as vector for cloning of the protease gene in Gram-positive bacteria (Kuipers, 1998). The pNZ8048*eprA1 L. lactis* plasmid carrying the protease gene from *A. hydrophila* was constructed in this work.

Media, growth conditions, primers and amplification: *A. hydrophila* strains were grown in tryptone soya broth (TSB) at 37°C with agitation at 150 rpm for 18 to 24 h. Lactococcus cells were grown at 30°C in M17 broth (Oxoid) or on M17 agar with 0.5% w/v glucose as a standing culture (Raha *et al*., 2005). Whenever required, chloramphenicol (7.5 µg/ml) was used for the selection of recombinant *L.* strains. Stock cultures of the bacterial strains were stored at -80°C in appropriate medium containing 15% (v/v) glycerol. Both forward and reverse primers were designed and synthesized to amplify the full length of the protease fragment gene, according to published sequences (Leung *et al*., 1997). The ProForward is a 35-mer forward primer (5´-ACTGCAGCAAATGATGATGAAAGCGACTCCAATTG-3´) in which the first 10 nucleotides include the *Pst*I restriction endonuclease site and the last 25 nucleotides of the primer are complementary to the N-terminal sequence of the *A. hydrophila* protease structural gene. The reverse primer, ProReverse, is also a 31-mer oligonucleotide (5´-ATGCAAGCTTGTCCAA CTCAATTTCGCTCGG-3´) the first 10 nucleotides of which includes the *Hind*III restriction endonuclease site and the last 21 nucleotides are complementary to the carboxyl-terminus of the gene of interest. The forward and reverse primers were such designed to facilitate an in-frame cloning into the pNZ8048 expression vector.

The protease gene was amplified in a 50 µl reaction mixture by using a Mastercycler (Eppendrof, Germany). The reaction mixture contained 5 µl of 10X PCR buffer, 3 µl of $MgCl_2$ (25 mM), 1 µl of dNTPs (each at 10 mM), 2 µl of each primer (20 mM), 0.2 µl of *Pfu* polymerase (5 U/ml), 5 µl of *A. hydrophila* genomic DNA (100 nM) and 33.8 µl of sterile double distilled water (ddH_2O). Samples were denatured at 95ºC for 6 min and amplification was then performed in 33 cycles of 95°C for 1 min, 60°C for 1 min and 72°C for 1.5 min; and after the last cycle the reaction was held at 72ºC for 7 min. The amplified products were then resolved on 1% (w/v) agarose gels followed by staining with ethidium bromide.

Construction of the pNZ8048*eprA1* plasmid and cloning: The amplified 1038-bps fragment was digested by *Pst*I and *Hin*dIII, followed by ligation into the corresponding site on the pNZ8048 plasmid. The ligated DNA was transformed into *L. lactis* NZ9000 cells by the electroporation method (Holo and Nes, 1989;

Sambrook *et al.*,1989) with minor modifications. Chloramphenicol-resistant transformants were then selected and followed by plasmid extraction and restriction digestion analyses. Analysis of plasmid stability was carried out by plating the transformants on media without the antibiotic.

Expression studies and protein gel electrophoresis: A well-grown colony of recombinant *L. lactis* NZ9000 cells (carrying the protease gene of *A. hydrophila*) was inoculated in 3 ml of SGM17 broth (0.5 M Sucrose 0.5%(w/v) Glucose and 37.25 g M17 broth (Oxid, USA)) containing 7.5 µg/ml of chloramphenicol, followed by incubation at 30°C, overnight. Two ml of bacterial culture was then sub-cultured in 10 ml of SGM17 broth and induced with 5 ng/ml of nisin for a few hours (McAuliffe *et al.*, 2000). SDS-PAGE of proteins was performed (Laemmli, 1970) using a 12.0% (w/v) polyacrylamide gel. The *L. lactis* NZ9000 recombinant cells (2 ml) were harvested by centrifugation at 2,000 *g* for 10 min. The pellet was resuspended in 100 µl of 2X sample buffer (3.5 ml of dH_2O, 1.25 ml of 0.5 M Tris-HCl, pH 6.8, 2.5 ml of glycerol, 2 ml of 10% w/v SDS, 0.2 ml of 0.5% bromophenol blue, 0.5 ml of β-mercaptoethanol) prior to boiling at 95°C for 5 min. After boiling, the supernatant was used as a sample for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Transformation of recombinant plasmids into host cells: The protease gene was amplified using DNA *Pfu* polymerase with primers incorporating specific recognition sites for the two restriction enzymes (RE), *Pst*I and *Hind*III. These REs were selected in order to achieve a directional cloning of the genes of interest into the expression vector, pNZ8048, at its multiple cloning sites (MCS). The directional cloning was to ensure that the open reading frames (ORFs) of such genes are in frame with the ORF of the *nis* promoter of the vector, since the transcription and translation of the genes are dependent on the promoter present upstream of the MCS. In addition, such primers were designed to amplify the full length of the protease virulence gene.

PCR amplification (Fig. 1) showed that the size of the amplified protease gene was approximately 1000 bps, which was in agreement with observations by other researchers (Song *et al.*, 2004; Chang *et al.*, 1997; Chopra *et al.*, 1993; Hirono and Aoki, 1993). Since the result obtained by PCR amplification was completely similar to the expected published sizes, therefore, the synthesized primers in this study could be used as specific primers for the detection of the protease gene from *A. hydrophila*. Figure 2 shows the map of the pNZ8048 construct carrying the *eprA1* gene vector. This map was made by the clone manager computer software (version 9, 2008). Ten colonies of each culture were selected and sub-cultured in medium sup-

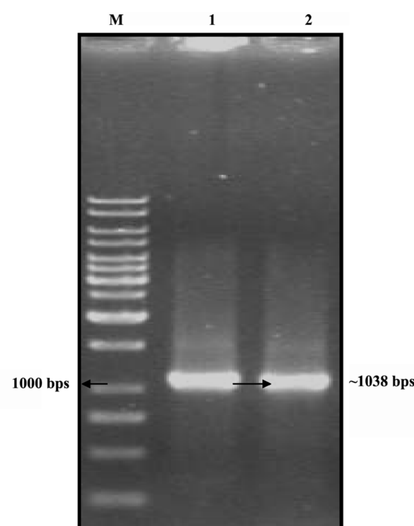

Figure 1. PCR amplification of the protease gene of *A. hydrophila* AHMP isolated from fish. Lanes 1 and 2: PCR products of the protease gene of *A. hydrophila* AHMP isolated from fish M: Marker, GeneRuler ™ DNA ladder Mix (Promega).

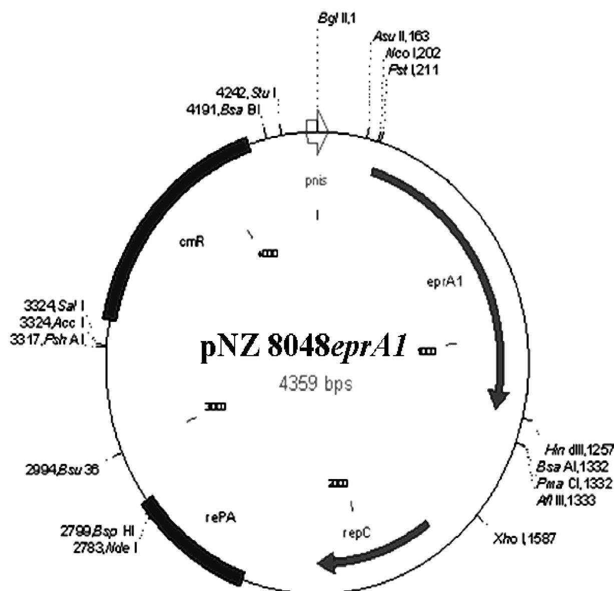

Figure 2. Map of pNZ8048*eprA1* construct indicating the site of the insertion of the protease (*eprA1*) gene from *Aeromonas hydrophila* and the orientation of this gene. The vector contains the *nis* promoter, start of an open reading frame, Multiple cloning site (MCS), cmr; chloramphenicol resistance gene.

plemented with 7.5 µg/ml of chloramphenicol antibiotic. The extracted plasmids, pNZ8048*eprA1* were analyzed by RE digestion and agarose gel electrophoresis. Figure 3 shows the digestion profile of recombinant pNZ8048*eprA1*and non-recombinant plasmids. Restriction enzyme (RE) digestion of recombinant and non-recombinant plasmids with single and double enzymes showed that cloning of the gene of interest (protease) into a Gram-positive and probiotic system was successful. Cloning was further verified by DNA sequencing and results showed 95% similarity with previously published sequences (Sasan *et al*., 2007; Chang *et al*., 1997).

Expression of the protease gene from *A. hydrophila* in the *L. lactis* system: Total protein was extracted from *L. lactis* NZ9000 transformants carrying the pNZ8048*eprA1* plasmid using both lysozyme and glass beads methods (Laemmli, 1970 and Raha *et al*., 2005). SDS-PAGE of the extracted proteins (from transformed *L. lactis* NZ9000 strains containing the recombinant pNZ8048*eprA1* plasmid and non-transformant strains) was performed on 12.5% (w/v) polyacrylamide gel (Fig. 4). In this Figure, lanes 2, 3 and 4 show the protein profile of the strain containing the recombinant plasmid. As shown, an extra protein band was detected around 28 kDa compared to the control strains (strains containing non-recombinant plasmids) (lanes 1 and 5). The size of the observed protease protein on SDS-PAGE, ~28 kDa, was close to the expected size, 29 kDa (Chang *et al*., 1997; Wandersman *et al*., 1989).

A. hydrophila is frequently associated with disease in carp, eel, milkfish, channel catfish, tilapia, trout and ayu and a few other animals (Rahman *et al*., 1997). However, a commercial vaccine for this pathogen has not yet been introduced. At present, generally controlling of the diseases caused by A. hydrophila is done by antibiotics. The extensive use of antibiotics has led to an increase in plasmid-encoding antibiotic resistance in *A. hydrophila* (Ansary *et al*., 1992; Aoki and Egusa, 1971). In addition, the use of antibiotics may delay the sale of fish as they need to be clear of the antibiotics before human consumption. Furthermore, antibiotic treatment is cost-prohibitive to farmers of many undeveloped and developing countries. Therefore, the immune prophylaxis against *A. hydrophila* becomes an attractive option (Leung *et al*., 1998b). However, the antigenic diversity of *A. hydrophila* has posed a great difficulty in developing a vaccine, and at present, no vaccine for protection against *A. hydrophila* is commercially available. In this situation, the search for

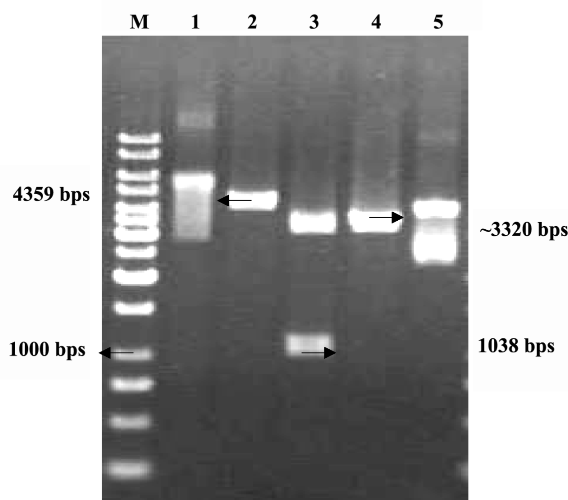

Figure 3. Cloning of protease gene from *A. hydrophila* AHMP into *L lactis* NZ9000 probiotic cells. Lane 5: Undigested non-recombinant pNZ8048 plasmid. Lane 4: Single digestion of non-recombinant pNZ8048 plasmid with *Hind*III. Lane 1: Undigested recombinant pNZ8048*eprA1* plasmid. Lane 2: Single digestion of the recombinant pNZ8048*eprA1* plasmid with *Hind*III containing the protease gene. Lane 3: Double digestion of recombinant pNZ8048*eprA1* plasmid with *Hind*III and *Pst*I. Lane M: GeneRuler ™ 1 kb DNA Ladder #SM0311 Fermentas.

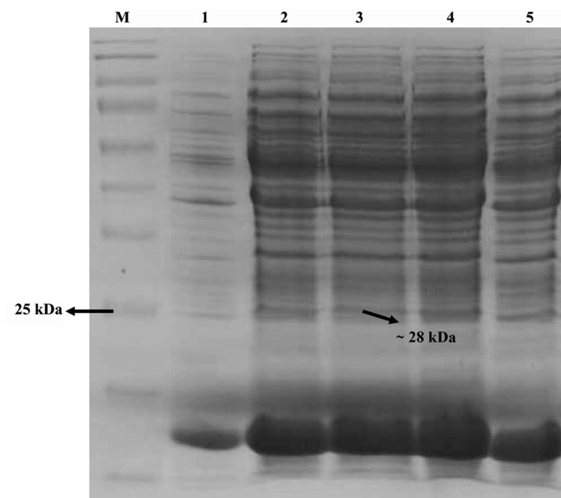

Figure 4. SDS-PAGE analysis of total proteins isolated from transformed *L. lactis* NZ9000 strain carrying recombinant pNZ8048*eprA1* plasmid. The protease gene from *A. hydrophila* AHMP was cloned into pNZ8048, a *L. lactis* expression plasmid. M: Protein marker, pageRuler™ prestained protein ladder #SM0671 Fermentas Lanes 1 & 5: Control; total proteins extracted from untransfomed *L.lactis* NZ9000 strains having the actual pNZ8048 plasmid. Lanes 2, 3 & 4: Total proteins extracted from transformed *L. lactis* strains having the recombinant pNZ8048*eprA1* plasmid, induced with nisin.

common protective antigens of *A. hydrophila* becomes critical for developing a common vaccine against this bacterium. One of the potential candidates of conserved antigens would be some of extracellular products of this bacterium involved in the pathogenicity to fish. It may be possible to prevent *A. hydrophila* infection by blocking bacterial antigens (virulence genes) into fish with antibodies against protease of *A. hydrophila* (Fang *et al*., 2004; Vaughan *et al*., 1993).

Bacterial-based systems as live vectors for the delivery of heterologous antigens offer a number of advantages as a vaccination strategy. Using the molecular biology, genetics and recombinant DNA techniques has allowed the insertion of genes encoding the antigens to be delivered into non-pathogenic carriers for expression (Liljeqvist and Stahle, 1999). *L. lactis* has been shown to deliver heterologous antigens to the systemic and mucosal immune systems *via* mucosal routes (Steidler *et al*., 1998). Several homologous and heterologous genes have been successfully expressed in *L. lactis* (Raha *et al*., 2006; Robinson *et al*., 2004, 1997; Gold *et al*., 1996).

Lactic acid bacteria (LAB) include a large number of Gram-positive cocci or bacilli belonging to a phylogenetically heterogeneous group. Their traditional use in the food industry confirms their lack of pathogenicity; as they are considered to be generally regarded as safe (GRAS) organisms. This group of bacteria has some advantages such as the production of heterologous proteins in bio-reactors, in fermented food products or directly in the digestive tract of humans and other animals. Besides such natural benefits, a new application for LAB, and probably the most promising, is their use as live delivery vectors for antigenic or therapeutic protein delivery to mucosal surfaces (Nouaille *et al*., 2003; Norton *et al*., 1994). Since less caseinolytic and elastolytic activities have been found by mutant deficient protease in *A. hydrophila* (Cascon *et al*., 2000; Leung and Stevenson, 1988a; 1998b); thus such engineered LAB might be useful and is worthy of further investigation for vaccine efficacy. This could control diseases caused by bacterial or viral pathogens (Bahey-El-Din *et al*., 2008; Lee *et al*., 2001).

Some studies have shown and confirmed the efficiency of *L. lactis* for the presentation of antigens to the mucosal immune system, and to elicit a specific response. To date, several antigens (bacterial and viral) and cytokines have been efficiently produced in *L. lactis* (Cortes-Prez *et al*., 2007; Raha *et al*., 2006; Chatel *et al*., 2003; Enouf *et al*., 2001). Furthermore, this group of bacteria is used as probiotic microorganisms in living systems. Finally, development and utilization of recombinant *L. lactis* harboring the protease from *A. hydrophila* can control some diseases by production of antibodies in living organisms such as fish. In this study, PCR amplification of the full length protease gene from A. hydrophila was successfully performed. The gene of interest was then cloned into pNZ8048, as a L. lactis expression vector for the first time. Cloning verification was carried out by RE digestion and also DNA sequencing. SDS-PAGE analysis detected that the expression of the protease protein that was in agreement with the reported and expected size. Results found by this work can be useful in development of live vaccines.

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