Construction of vaccine from *Lactococcus lactis* bacteria using *Aeromonas hydrophila* virulent Aerolysin gene

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Abstract

In this study the forward and reverse primers were designated to amplify the segments (~250 bps and ~650 bps) of the gene coding domains 1 and 4 of aerolysin of *Aeromonas hydrophila*. These two domains are involved in pathogenesis of the aerolysin gene. Sequences for two restriction enzymes, *Pst* I and *Hind* III, were included in the forward and reverse primers respectively. These restriction enzyme sites were used because they are not present within the genes of interest but are available in the multiple cloning sites of plasmid pNZ8048. Amplified PCR products were analyzed with 1% agarose gel electrophoresis and results showed that amplifications were very specific. In comparison with the DNA marker, the sizes of the amplified PCR products were determined to be approximately ~250 bps and ~650 bps respectively. PCR products were then purified by the DNA purification kit, digested with REs and ligated with linearised pNZ8048 plasmid using T4 DNA ligase. Transformation of *Lactococcus lactis* NZ9000 cells was performed by the electroporation method. Verification for cloning of virulent genes was performed by REs digestion and also DNA sequencing. Since several antigens (bacterial and viral) and cytokines have been efficiently produced in *L. lactis*, constructing and expression and utilization of recombinant *L. lactis* harboring the aerolysin domains (virulent) genes from *A. hydrophila* may induce production of antibodies in fish against this pathogen.

Keywords: Aerolysin, *Aeromonas hydrophila*, *Lactococcus lactis*, Live vaccines

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Introduction

Aeromonas hydrophila is frequently associated with disease in carp, eels, milkfish, channel catfish, tilapia, trout, ayu and a few other animals. This bacterium causes high mortality and great economic losses in freshwater fish farming worldwide. Many of the extracellular products (ECPs) are related to the virulence of this bacterium with cytotoxins, haemolysins (aerolysin) and proteases being the most important. Among them aerolysin is the most common virulence gene in almost all *A. hydrophila* strains. Antibiotic treatment of *A. hydrophila* has some disadvantages such as increase in plasmid-encoding antibiotic resistance and delay in fish sales as they need to be clear of the antibiotics before human consumption. In addition, antibiotic treatment is cost-prohibitive to farmers of many undeveloped and developing countries (Rahman et al., 1997; Vijai et al., 2009; Sasan 2010).

Since several studies have shown that different vaccine formulations may provide protection, the immune prophylaxis against *A. hydrophila* becomes an attractive option. 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 common protective antigens of *A. hydrophila* becomes critical for developing a common vaccine against this bacterium (Leung et al., 1997, 1998). One of the potential candidates of conserved antigens would be some 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 aerolysin of *A. hydrophila* (Vaughan et al., 1993; Zhu et al., 2007).

Bacterial-based systems as live vectors for the delivery of heterologous antigens offer a number of advantages as a vaccination strategy. Using molecular biology, genetics and recombinant DNA techniques has allowed the insertion of genes encoding the antigens to be delivered into non-pathogenic carrier for expression (Liljeqvist and Stahle, 1999; Lee et al., 2001). *Lactococcus lactis* is among the Lactic Acid Bacteria (LAB) that are Gram-positive and designated Generally Recognized as Safe (GRAS) organisms**.** They are non-pathogenic, non-invasive, non-colonising and they bare no threat to human and animal health (Wells et al., 1993; Raha et al., 2005). They also have the capacity to secrete proteins allowing surface expression or extracellular production of heterologous enzymes or proteins. Developments in genetic engineering have given these Gram-positive Lactic Acid Bacteria (LAB) the advantage to be used as a host expression system for antigen delivery to induce immune responses (Robinson et al., 1997; Naima et al., 2007).

In this study, cloning of domains 1 and 4 of aerolysin gene from isolated *A. hydrophila* was carried out into *L. lactis* bacteria for the first time. Such safe, probiotic, genetically engineered and live recombinant bacteria harboring the foreign virulent genes may induce immune responses against pathogens in living things.

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Materials and methods

Bacterial Strains, Plasmids, Primers, and Growth Media

The bacterial cells and plasmids used in this study are listed (Table 1). *Aeromonas hyrophila* AHMP was isolated from diseased fish and cells were grown on Luria Bertani (LB) broth or LB agar solidified with 1.2% (w/v) bacteriological agar at 37º C. *Lactococcus lactis* NZ9000 (host cells) and *L. lactis* pNZ8048 (harboring pNZ8048 expression plasmids) cells were grown on SGM17 broth and SGM17 agar containing glucose (0.5 % w/v) and 0.5 M sucrose at 30º C. Chloramphenicol was used at a concentration of 7.5 µg/ml (Sasan et al., 2007). Primers to amplify the full length segment of the gene coding for domains 1 and 4 of aerolysin of *A. hydrophila* AHMP were designated according to the published sequences (Rossjohn et al., 1997, Wang et al., 2003). Sequences for two restriction enzymes, *Pst*I and *Hind* III, were included in the forward and reverse primers respectively (Table 2). These restriction enzyme sites (REs) were used because they are not present within the genes of interest but are available in the multiple cloning sites (MCS) of plasmid pNZ8048.

Table 1: Bacterial strains and plasmids

Bacterial strains and Plasmids	Features	References
Bacteria		
L. lactis NZ9000	MG1363 *pepN*::*nisRK*	Kuipers, 1998
L. lactis NZ8048	*L. lactis* NZ9000 cells harboring pNZ8048 vector	Kuipers, 1998
A. hydrophila AHMP	Isolated from diseased fish	Sasan, 2007
Plasmids		
pNZ 8048	Promoter: nisA, Antibiotic:Chlor., Expression vectorfor *Lactococcus*	Kuipers, 1998
pNZ 8048D1Aer	*L. lactis* plasmid carrying the Domain1 of Aerolysin gene from *A. hydrophila*	Current work
pNZ 8048D4Aer	*L. lactis* plasmid carrying the Domain 4 of Aerolysin gene from *A. hydrophila*	Current work

Table 2: Primers used for amplification of two domains of aerolysin genes of *A. hydrophila* AHMP

Primers	TM (°C)	Sequences
D1AerF	60	5'-ATG***CTGCAG***AAATGATGAATAGAATA ATTACCGC-3'
D1AerR	64	5'-ATGC***AAGCTT***GCCCCATAATCTCCCA GCGAT-3
D4AerF	64	5'-ATG***CTGCAG***AAAACTCGCCATCGAGA TCGCT-3'
D4AerR	60	5'-ATGC***AAGCTT***GTTATTGAACCGGAAC TATGGTC-3'

* Underlined sequences: RE sites, ***CTGCAG***; *Pst*I and ***AAGCTT***; *Hind*III

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DNA Extraction and PCR Amplification

Genomic DNA of *A. hydrophila* AHMP was extracted using the modified mini preparation method (Ausbel et al., 1987) with minor modification. PCR amplifications were performed using a pair of primers: D1AerF (5'- AT GCT……..3') and D1AerR (5'-AT GCA……. -3') to amplify a segment DNA, ~250-bp coding for domain1, a pair of primers: D4AerF (5'-AT GCT -3') and D4AerR (5'- AT GCA…….-3') to amplify another segment, ~650-bp coding for domain 4 of aerolysin gene, from *A. hydrophila* AHMP (Table 2).

PCR assay was performed using a PCR mixture containing 3 µl of template DNA (100 nM) and 2 µl of 10 mM deoxynucleoside triphosphate (dNTP), 3 µl of the 25 mM $MgCl_2$ solution, 2 µl of the 20 mM solution of each primer, 0.3 µl of *Taq* polymerase (5u/µl), 5 µl of 10 × PCR buffer and 32.70 µl of dH_2O water, to make the final volume of 50 µl. The amplification program consisted of 27 cycles of amplification under the following condition: an initial denaturation of 5 minutes at 95° C followed by 27 cycles each of 1 minute at 95 °C, 1 minute at 60° C and 1 minute at 72 °C and additional final extension at 72° C for 10 minutes. Finally, 10 µl of PCR product was loaded on electrophoresis gel.

Plasmid Extraction, *Lactocccus lactis* NZ9000 Competent Cell Preparation, RE Digestion and Ligation

Plasmids pNZ8048 from *L. lactis* pNZ8048 and recombinant plasmids, pNZ8048D1Aer and pNZ8048D4Aer from *L. lactis* transformants (Table 1) were extracted according to Leenhouts and Venema 1993 with minor modification. Preparation of *L. lactis* NZ9000 competent cells was done according to the published method (Holo and Nes, 1989). A 24 hours old *L. lactis* NZ9000 colony was cultured in SGM17 (M17 with 0.5 M sucrose and 0.5% glucose) supplemented with 1.5% glycine and incubated at 30º C for 24 hours. One ml of an overnight culture was added into 100 ml fresh SGM17 + 1.5% glycine and incubated at 30º C, a few hours until the OD_{600} of 0.3-0.6 was achieved.

The cells were then collected by centrifugation at 3500 rpm for 10 min at 4º C and washed twice with half volume ice-cold 0.5 M sucrose with 15% glycerol. Centrifugation was done again before the cells were resuspended in 1/200 culture volume of 0.5 M sucrose with 15% glycerol. While preparing competent cells, it is very important that the cells are kept cold at all times. A volume of 40 µl of cell suspensions were transferred to fresh 1.5 ml tube and kept in -80°C until usage for electro-transformation (electroporation).

Restriction enzyme (RE) digestion mixture was included of 1 µg of substrate DNA (PCR product or plasmid), 2 µl of 10X RE reaction buffer, 1 µl of enzyme 1 , 1 µl of enzyme 2 and an appropriate volume of sterile water to make up a total volume of 20 µl. The reaction mixtures were incubated according to the RE's optimum temperature, 37° C for 3 hours. Ligation of double stranded DNA contained 1 unit of T4 DNA Ligase (Fermentas), 2 µl of 10X ligase buffer, 1000 ng of vector DNA, 5 folds excess of insert and sterile distilled water to make up a final volume of 20 µl. The reaction mixture was incubated at 16° C for 8

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hours. The recombinant plasmids were excised from the agarose gel (1% (w/v)) and purified with Gel Purification Kit (Qiagen, Germany) according to the protocol.

Transformation of Recombinant pNZ8048 Plasmids into *Lactococcus lactis* NZ9000 and Verification

A total of 3–5 µl of the overnight ligation mixture was then used to transform the *L. lactis* NZ9000 competent cells by electroporation. Different recombinant *L. lactis* pNZ8048 plasmids produced in this work (pNZ8048D1Aer and pNZ8048D4Aer) which carried virulence genes of *A. hydrophila* were transformed into *L. lactis* according to the proposed protocol (Holo and Nes 1989) with minor modification. A volume of 10 µl of ligation mixture (about 1 µg of DNA plasmid) was mixed with 40 µl of aliquoted competent cells.

The mixture was transferred into an ice-cooled electroporation cuvette (2mm electrode gap) and exposed to a single electrical pulse at 2.3 kV (capacitance of 25 µF and resistance of 200 Ω). The electrical pulse was delivered using BioRad, the USA Gene Pulser Electroporation System equipped with a pulse controller unit. Immediately after the discharge, electroporated cells were mixed with 960 µl of ice-cold SGM17MC broth (SGM17 + 20 mM $MgCl_2$ + 2mM $CaCl_2$) and left on ice for 10 mins.

The electroporated cells were then incubated at 30° C without shaking for 2-3 hours to allow for phenotypic expression. A volume of 100 µl of the cells was then spread on SGM17 agar containing chloramphenicol at a final concentration of 7.5 $\mu g.ml^{-1}$ and incubated at 30 ºC for 2-3 days. A few well-isolated colonies were sub-cultured onto fresh SGM17 agar plates containing 7.5$\mu g.ml^{-1}$ chloramphenicol. Transformants were then cultured in SGM17 broth for plasmid extraction and analysis. Verification of cloning of the genes of interest into *L. lactis* was carried out using REs digestion analysis and DNA sequencing.

Results

PCR Amplification of the genes of interest

Primers were designated (Table 2) according to the published sequences by (Wang et al., 2003; Rossjohn et al., 1997) to amplify two domains, domain 1 and 4 of aerolysin gene from *A. hydrophila* AHMP isolated from infected fish. The sizes of the genes of interest, domain 1 and domain 4 of aerolysin from *A. hydrophila* were around 250 bps and 650 bps respectively. In addition, specific RE sites, *Pst*I and *Hind*III had been placed in all forward and reverse primers respectively, for directional cloning into *L. lactis* expression vector, pNZ8048. Amplified PCR products were analysed with 1% agarose gel electrophoresis and results showed that amplifications were very specific (Fig. 1). In comparison with DNA markers, the sizes of the amplified PCR products were determined to be approximately 250 bps and 650 bps respectively. These bands, ~250 and ~650 bps (Fig. 1, Lines 1, 2, 3) completely agreed with the expected values. The specific bands relevant to the expected sizes indicated that primers for amplification of domain 1 and domain 4 of aerolysin gene have high efficiency and specificity.

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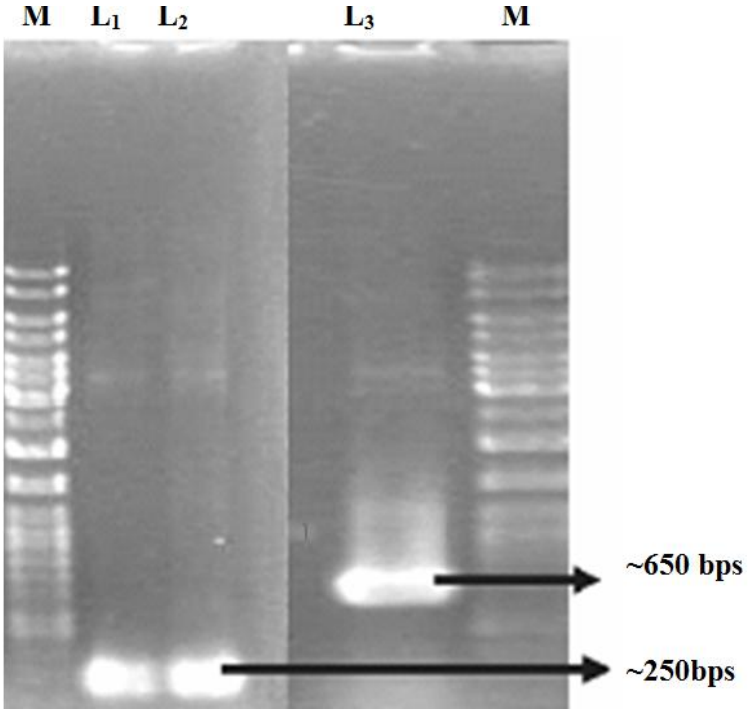

Figure 1: Agarose gel electrophoresis of PCR amplification products of the virulent genes, two domains of aerolysin from *A. hydrophila* AHMP

M: Marker, 1 kb DNA ladder., Lanes 1& 2: PCR products of domain1 of aerolysin gene from *A. hydrophila*., Lane 3: PCR amplification of domain4of aerolysin gene from *A. hydrophila*

Cloning and transformation of the genes of interest into gram-positive bacteria, *L. lactis* NZ9000

The amplified DNA bands of interest were excised from the gel at ~650 bps and ~250 bps position. These PCR products were then purified using the DNA purification kit, digested with restriction enzymes (*Pst* I and *Hind* III) and ligated with linearised pNZ8048 plasmid using T4 DNA ligsase. Transformation of *L. lactis* NZ9000 cells was performed according to the protocol from Holo and Nes (1989). Figure 2 shows the maps of cloning of domain 1 of aerolysin into pNZ8048 vector, which D1Aer is domain 1 of aerolysin gene and contains from 211 bp to 433 bps and also *Pst* I with *Hind* III RE sites. Similarly, figure 3 shows the maps of cloning of domain 4 of aerolysin into pNZ8048 vector, which D4aer is domain 4 of aerolysin containing from 211 to 855 bps. Maps were made by Clone Manager Computer Software. Ten to fifteen well-grown colonies of each transformant cell types were selected and subjected to plasmid analysis. Figure 4 shows the RE digestion analysis of both recombinant (pNZ8048D4Aer and pNZ8048D1Aer) and non-recombinant pNZ8048 plasmids. Verification for cloning of the virulent genes was performed by REs digestion and DNA sequencing. Figure 4 shows the pattern of RE digestion of recombinant and non-recombinant pNZ8048 plasmids. Line 1 is recombinant pNZ8048D4Aer

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with single digestion. Line 3 is non-recombinant pNZ8048 plasmid with single digestion. Upon double digestion of transformant pNZ8048D1Aer plasmids with *Hind* III and *Pst* I, the molecular sizes of ~3340 bps and ~650 bps were shown by gel electrophoresis (Fig. 4, L_2). These sizes correlated to the estimated and expected data**.** Therefore, the size and orientation of this putative clone obtained was in agreement with the expected recombinant, confirming the presence of domain 4 of aerolysin gene from *A. hydrophila* AHMP insert in pNZ8048 expression vector. Similarly, double digestion of line 7 shows cloning and presence of domain 1 of aerolysin gene from *A. hydrophila* AHMP insert in pNZ8048 expression vector. Further verification was carried out by DNA sequencing using the primers of the genes of interest. Blast analysis showed 97% similarity between sequences of domain 1 and domain 4 of aerolysin gene with the published data respectively (Sasan 2007).

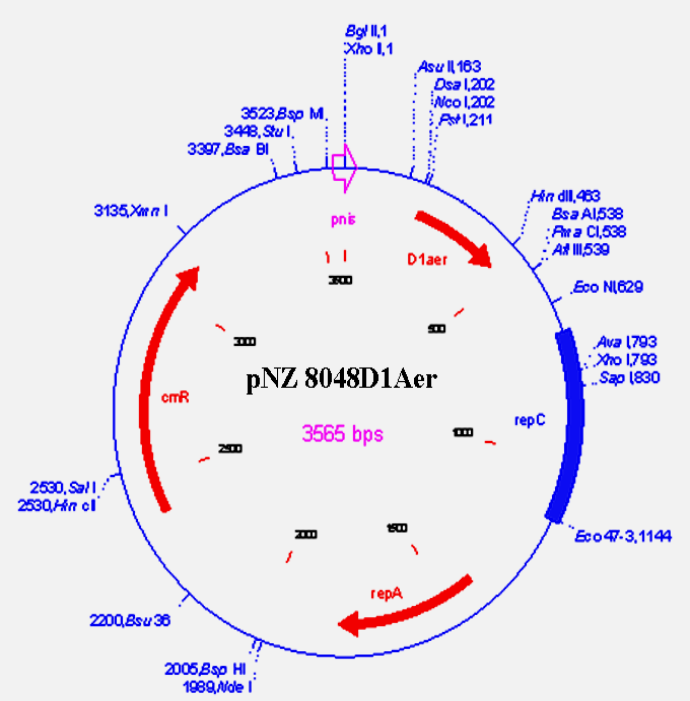

Figure 2: Map of pNZ8048D1Aer construct indicating the site of the insertion of the domain 1 of aerolysin (D1Aer) gene and the orientation of this gene

The vector contains nis promoter, start of an open reading frame, MCS, cmr, chloramphenicol resistance gene (Kuipers, 1998).

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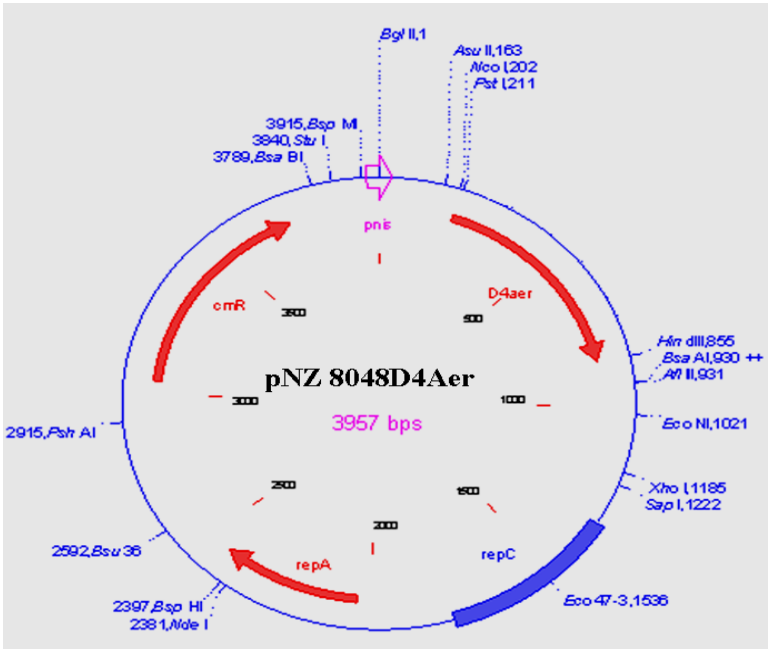

Figure 3: Map of pNZ8048D4Aer construct indicating the site of the insertion of the domain 4 of aerolysin (D4Aer) gene and the orientation of this gene.

The vector contains nis promoter, start of an open reading frame, MCS, cmr, chloramphenicol resistance gene.

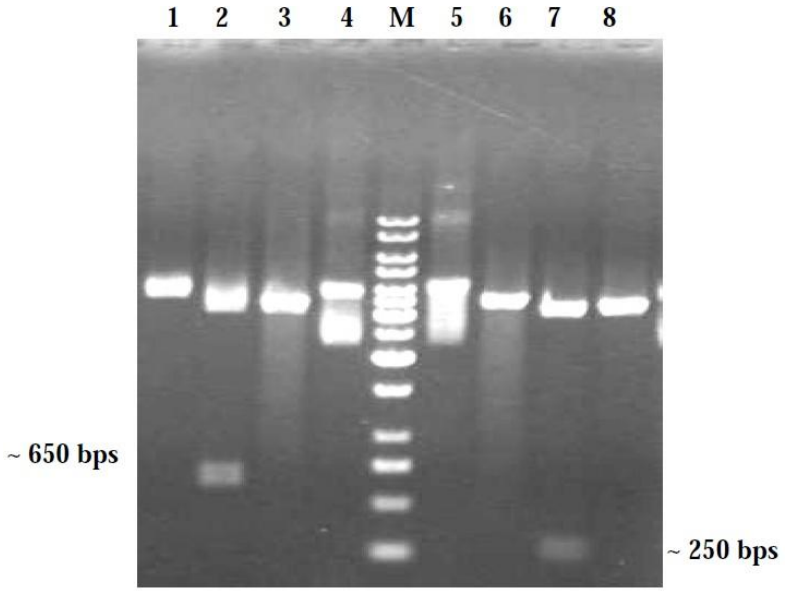

Figure 4: Cloning of virulent genes, aerolysin 1&4 domains genes, of *A. hydrophila* AHMP into *L lactis* NZ9000.

Lane 2: Double digestion of recombinant pNZ8048D4Aer plasmid with *Hind*III and *Pst*I containing the domain 4 of aerolysin gene. Lane 7: Double digestion of recombinant pNZ8048D1Aer plasmid with *Hind*III and *Pst*I containing domain 1 of aerolysin gene. Lanes M: 1 kb DNA Marker, #SM0311.

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Discussions

In this study, primers for the amplification of domain 1 and domain 4 of aerolysin gene from *A. hydrophila* were designated and PCR analysis verified to be very specific. The genes of interest were then cloned into pNZ8048, as *L. lactis* expression vector for the first time using electroporation method. Cloning verification was carried out by restriction enzyme digestion and DNA sequencing. Aerolysin is a well-known pore-forming toxin protein and major virulence factor that was first purified from gram-negative pathogen *A. hydrophila* bacterium, and is capable of killing target cells by forming channels in their membranes. It has been also reported that some domains of aerolysin protein especially domain 1 and 4 have main roles in its pathogenicity (Abrami et al., 2000). In addition, results indicated that this toxin molecule had hemolytic, cytotoxic and enterotoxic activities (Pollard et al., 1990; Chopra et al., 1993). Moreover, recent studies have shown that aerolysin protein is capable of neutralizing human immunodeficiency virus type 1 (HIV-1) in a dose-dependent manner with neutralization dependent upon the presence of the Thy 1 proteins in the viral envelope (Nguyen et al., 1999). Furthermore, it has been shown that mutant strains of *A. hydrophila* deficient in aerolysin production were found to be less virulent than the wild type. Additionally, specific neutralizing antibodies to aerolysin have been detected in animals surviving *Aeromonas* infection (Leung et al., 1997; Leung and Stevenson, 1988).

Lactic acid bacteria (LAB) include a large number of gram-positive cocci or bacilli belonging to a phylogenetically heterogeneous group. Their traditional use in 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 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. Such engineered LABs are able to elicit both mucosal and systemic immune responses. Some studies have shown and confirmed the efficiency of *L. lactis* for the presentation of antigens to the mucosal immune system, to elicit a specific response (Raha et al., 2006; Naima et al., 2007).

Interestingly, several homologous and heterologous genes, a few antigens (bacterial and viral) and cytokines have been successfully expressed in *L. lactis* (Enouf et al., 2001; Chatel et al., 2003). Some of them include several lysozyme genes, the *B. subtilis* neutral protease (van de Guchte et al., 1989, 1990, 1992) tetanus toxin fragments C protein (Wells et al., 1993) protease (Sasan, 2010), manganese superoxide dismutase gene from *E. coli* (Roy et al., 1993), xylanase gene of *B. subtilis* (Raha et al., 2006) and genes encoding for pyruvate ecarboxylase and alcohol dehydrogenase of *Zymomonas mobilis* (Gold et al., 1996). A growing number of studies reported the implication of lactic acid bacteria in fish diseases, while vaccination is not yet fully

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operational. One of the most promising and urgent applications of lactic acid bacteria is therefore to investigate their use both as probiotics against bacterial pathogens, and as sources of immunostimulants (Ringo et al., 1998; Mohammed et al., 2008).

The potential of *L. lactis* acid bacteria as vaccine vectors has been demonstrated in several recent publications, such as *Lactococcus lactis* prototype vaccines against *Brucella abortis,* and *Helicobacter pylori* (Joes and seegers, 2002). Furthermore, *L. lactis* has been extensively engineered for the production of heterologous therapeutic proteins. It has already been used as an antigen delivery vehicle for vaccination against tetanus, virus and more recently, for treatment of murine colitis (Chatel et al., 2003). For this, construction of such genetically engineered safe microorganisms which are made by current work may result in protecting living things against pathogens. Future work should include immunological study of fish response and introduction of multiple epitope recombinant vaccine.

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