Thesis for the Degree of Master of Science

Recombinant expression and immunological characterization of *Streptococcus iniae* antigens

by

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Feb. 2007

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연쇄구균 (*Streptococcus iniae*) 항원 유전자 재조합 발현 및 면역학적 특성 분석

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by

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A thesis submitted in partial fulfillment of the requirements
for the degree of
Master of science
in the Department Fish Pathology, Graduate School,
Pukyong National University
Feb. 2007

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Abstract

During the last decade, sporadic and epidemic outbreaks of fish diseases due to gram-positive cocci have been reported world-widely, and are responsible for significant economic losses in the fish farm industry. In Korea, warm-water streptococcosis have been a main cause of mortality in cultured fish, and 3 species - *S. iniae, S. parauberis*, and *L. garviae* - have been identified as the causing.

To design potent and universally applicable subunit protein vaccines, it is necessary to identify those antigens that are recognized as nonself by the immune systems of a wide fish population or species during infection. In the present study, 4 genes - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, streptolysin, and ABC iron transporter - were selected as subunit vaccine candidates. The full open reading frames (ORF) of the selected 4 genes of *S. iniae* were cloned and produced as recombinant proteins to characterize their function and to obtain antisera. In the investigation of vaccine efficacy, the organisms immunized with recombinant

proteins showed higher serum bactericidal activity than those of control.

As a nonliving delivery system with the capacity to be loaded with foreign antigens, bacterial ghosts are a safe alternative to live-bacterial delivery system. The display of heterologous antigens on the surfaces of pathogenic bacteria is of considerable value for the development of combination vaccines, and would be advantageous for the induction of antigen specific antibody responses when using attenuated or inactivated recombinant bacteria for immunization.

In this study, to generate combination vaccine system, we have generated *E. coli* and *E. tarda* ghosts displaying the above selected antigens of *S. iniae* on the outer membrane, and expression of *S. iniae* antigens on the outer membrane was confirmed by immunoblot.

Introduction

Streptococci are Gram positive cocci (spherical or ovoid) in the family Streptococcaceae often occurring in pairs and chains. They are facultatively anaerobic and catalase-negative. During the last decade, sporadic and epidemic outbreaks of fish diseases due to gram-positive cocci have been reported world-widely, and are responsible for significant economic losses in the fish farm industry. The main pathogenic species responsible for these streptococcal infections are *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum,* and *Carnobacterium piscicola* (Bercovier *et al.*, 1997; Eldar *et al.*, 1997, 1999; Eldar and Ghittino, 1999). Outbreaks associated with infections by *L. piscium*, *V. salmoninarum,* and *C. piscicola* usually occur at water temperatures below 15°C and are termed cool-water streptococcosis (Muzquiz *et al.*, 1999). On the other hand, outbreaks that occur at water temperatures above 15°C, or warm-water streptococcosis, are produced by *L. garvieae*, *S. iniae*, *S. parauberis*, and *S. difficilis* (Muzquiz *et al.*, 1999). In Korea, warm-water streptococcosis have been a main cause of mortality in cultured fish, and 3 species – *S. iniae, S. parauberis*, and *L. garviae* – have been identified as the causing.

Since first isolation of *Streptococcus iniae* from a subcutaneous abscess of a captive freshwater dolphin (*Inia geoffrensis*) in 1976 (Pier and Madin, 1976; Pier *et al.*, 1978), it has been associated with disease outbreaks in aquaculture farms of different fresh and seawater commercial fish species (Kitao *et al.* 1981; Baya *et al.* 1990; Kusuda 1992; Eldar *et al.* 1994 & 1995; Perera *et al.* 1994), with mortality rates of up to 50% (Eldar *et al.* 1995

Zlotkin *et al.* 1998). Moreover, this pathogen has recently been reported to cause fulminant soft tissue infection in humans (Weinstein *et al.* 1996 & 1997). Traditional classification of streptococci has been based on the serogrouping of the carbohydrate antigens of the cell wall and their haemolytic activities. *S. iniae* is a β-haemolytic streptococcus, which does not react to Lancefield A-V grouping antisera (Low *et al.*, 1999 Shoemaker *et al.*, 2000). The most significant clinical signs of *S. iniae* infections in fish are septicemia and meningoencephalitis (Eldar *et al.*, 1994, 1995), which are very similar to those produced by other bacterial fish pathogens, mainly *Streptococcus parauberis*, *Streptococcus difficilis* or *Lactococcus garvieae* (Doménech *et al.*, 1993, 1996 Eldar *et al.*, 1994, 1995 Bercovier *et al.*, 1997 Eldar and Ghittino, 1999). Currently, *S. iniae* is an economically significant cause of death of major cultured marine fish such as olive flounder (*Paralichthys olivaceus*), rockfish (*Sebastes schlegeli*), and rock bream (*Oplegnathus fasciatus*) in Korea.

Since antibiotic therapy of farmed fish infected with streptococci gives unsatisfactory results, the need for development of an effective vaccine is paramount in the control of streptococcal disease. Although formalin-killed vaccines [bacterins or modified bacterins (i.e., containing extracellular products)] have been developed against *S. iniae* (Eldar *et al.*, 1997 Klesius *et al.*, 1999; Klesius *et al.*, 2000; Klesius *et al.*, 2002), their efficacy against different serotypes has not been proved.

Streptococcal surface capsules are generally complex anionic polysaccharides that contribute importantly to virulence by virtue of their antiphagocytic properties, and the capsular polysaccharides are also frequently a principal target of protective immunity. However, polysaccharide antigens are poorly immunogenic and are also induces poor memory immune responses. Concern also exists that non-vaccine serotypes may eventually

replace those contained in the vaccine, and if serotype replacement is proven to occur, the vaccine will need to be reformulated. In fact, recently, it has been shown that following a 5-year routine vaccination program against *S. iniae* in rainbow trout farms of Israel, a novel serotype, capable of producing a generalized bacterial meningitis, has emerged (Bachrach *et al.*, 2001). In this case, *S. iniae* probably gains access to the bloodstream and maintains a high level of bacteremia, leading to the onset of a generalized disease and meningitis (Ferrieri *et al.*, 1980; Lin *et al.*, 2001). The new strains were characterized as Serotype II strains; they differed from 'classical' type I strains by serological, phenotypic and genetic criteria. Rainbow trout antisera to serotype I did not agglutinate serotype II but some cross-reactivity was observed in the opposite direction (Bachrach *et al.*, 2001). Serotype II strains are able to enter phagocytes and multiply within them, causing them subsequently to undergo death through apoptotic processes (Zlotkin *et al.* 2003). *S. iniae* type II strains have also been detected in the USA, indicating the wide distribution of *S. iniae* variants (Barnes *et al.*, 2003). Similarly to *Streptococcus pyogenes* (group A streptococcus) infection in humans, where serotype replacement in a population (Kaplan *et al.*, 2001) is most likely the result of the immune status of the individuals along with the introduction of a highly virulent organism (Dale and Shulman, 2002), the propensity of *S. iniae* to cause an invasive disease in fish is likely related not only to the immune status of the fish but also to the pathogenetic mechanism(s) of virulent strains (Zlotkin *et al.* 2003).

Since vaccines composed of polysaccharides induce serotype-specific immune responses and contribute to serotype redistribution, antigenic proteins that show little sequence variation in diverse clinical isolates will likely be superior antigens for the development of a broadly effective

vaccine against streptococcosis. Protein-based vaccines have the potential advantages of being antigenically conserved across capsular types, comparatively inexpensive to produce by recombinant DNA techniques, and able to induce memory responses which are long-lasting and can be boosted by revaccination.

To design potent and universally applicable subunit protein vaccines, it is necessary to identify those antigens that are recognized as nonself by the immune systems of a wide fish population or species during infection. In the present study, 4 genes - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, streptolysin, and ABC iron transporter - were selected as subunit vaccine candidates. The full open reading frames (ORF) of the selected 4 genes of *S. iniae* were cloned and produced as recombinant proteins to characterize their function and to obtain antisera.

The ground of those genes selections subunit vaccine candidates is as follows. GAPDH has been considered a classical cytosolic glycolytic protein; however, recent evidence demonstrates that some pathogenic prokaryotic and eukaryotic organisms have cell surface-associating GAPDH, which may have differing roles. In group A streptococci and in *Staphylococcus aureus,* a cell-surface GAPDH is involved in the interaction with host cells and ligands (Pancholi and Fischetti, 1992, 1993, 1997 Winram and Lottenberg, 1996, 1998 Modun and Williams, 1999). Gil-Navarro *et al.* (1997) have reported that an immunogenic cell-wall-associated GAPDH is found at the cell surface of *Candida albicans* in an active form. Since *C. albicans* GAPDH is a fibronectin- and laminin-binding protein, the surface GAPDH could participate in the adhesion of fungal cells to host tissues, thus playing a role in virulence (Gozalbo *et al.*, 1998 Villamon *et al.*, 1999). Also the surface GAPDH of *Schistosoma mansoni* has been associated with human resistance to schistosomiasis (Charrier-Ferrara *et al.*, 1992 Goudot-Crozel *et al.*, 1989).

Recently, the presence of a surface-localized α-enolase that binds both plasmin and plasminogen has been reported from *S. pyogenes* (Pancholi and Fischetti, 1998) and from *S. pneumoniae* (Bergmann *et al.*, 2001). The plasminogen system plays an important role in host defense by dissolving fibrin clots and maintaining homeostasis and vascular potency (Plow *et al.*, 1995), and may be subverted by pathogens to allow tissue invasion. It has been known that pathogenic bacteria utilize the proteolytic activity of plasmin to migrate faster through the host extracellular matrix (Eberhard *et al.*, 1999), and this enzymatic activity may facilitate their invasion and dissemination in the infected host (Lottenberg *et al.*, 1994; Lottenberg, 1997).

One of the few distinguishing phenotypes of *S. iniae* is the zone of beta-hemolysis surrounding colonies cultured on blood agar (Pier and Madin, 1976). Hemolysins, or cytolysins, are well-recognized features of many bacterial species, including streptococci, and are generally associated with damage to the membranes of a variety of eukaryotic cell types. Examples of streptococcal cytolysins include streptolysin O (SLO) and streptolysin S (SLS) of group A streptococci (GAS), the beta-hemolysin/cytolysin of group B streptococci (GBS), and the pneumolysin of *Streptococcus pneumoniae*, each of which has been implicated as a virulence factor in animal models of infection (Berry *et al.*, 1989; Betschel *et al.*, 1998; Limbago *et al.*, 2000; Wennerstrom *et al.*, 1985).

Iron is an essential nutrient, as it is required for many cellular metabolic processes, but free ferric iron is scarce in a fish host since it is bound to high-affinity iron binding proteins such as transferrin. Pathogenic bacteria have adapted well to the severe iron-restricted environment encountered in the host; the extremely low availability of iron in host body fluids serves as a major environmental signal to the bacteria to express virulence determinants and they have developed specialized mechanisms for scavenging

iron from the host, thereby enabling them to multiply in host tissue. An effective mechanism for scavenging iron involves the production and secretion of low-molecular-weight ferric iron chelators, siderophores, which scavenge iron from the host and transport it into the cell via specific ABC transporters (Nikaido and Hall, 1998).

The display of heterologous antigens on the surfaces of pathogenic bacteria is of considerable value for the development of combination vaccines, and would be advantageous for the induction of antigen specific antibody responses when using attenuated or inactivated recombinant bacteria for immunization. In mammals, avirulent strains of *Salmonella typhimurium* are being widely considered as delivery systems for heterologous antigens because of their ability to induce complex mucosal and systemic immune responses after oral administration.

As a nonliving delivery system with the capacity to be loaded with foreign antigens, bacterial ghosts are a safe alternative to live-bacterial delivery system. Bacterial ghosts are empty cell envelopes. They are produced from Gram-negative bacteria by controlled expression of protein *E* from the cloned PhiX174 lysis gene *E*. Protein *E* forms a tunnel structure spanning the whole cell wall complex, through which the cytoplasmic contents are expelled (Witte *et al.*, 1990a,b, 1992). In contrast to heat or chemical inactivation procedures, all native cell wall components are preserved during the formation of bacterial ghosts (Witte *et al.*, 1990b, 1992). Therefore bacterial ghosts derived from a variety of bacteria provide an alternative to conventionally inactivated vaccines (Eko *et al.*, 1994; Huter *et al.*, 2000; Hensel *et al.*, 2000). In the extended ghost system, foreign proteins can be displayed outside outer membrane by specific membrane anchors prior to lysis of bacteria (Lubitz, 2001). Furthermore, it has been demonstrated that the cell targeting and adjuvant properties of ghosts are superior to Alum and

complete Freund's adjuvant (Eko *et al.*, 1999). Therefore, epitopes from viruses, Gram-positive bacteria or parasites can be inserted in the ghost envelope, and thus, combination vaccines can be developed.

Edwardsiella tarda, a Gram negative bacterium of the family *Enterobacteriaceae*, is the causative agent of edwardsiella septicaemia and leads to extensive losses in a diverse array of commercially important fish, including eels (Wakabayashi and Egusa, 1973), chinook salmon (Amandi *et al.*, 1982), olive flounder (Nakatsugawa, 1983), tilapia (Kubota *et al.*, 1981), carp (Sae-Oui *et al.*, 1984), channel catfish (Meyer and Bullock, 1973), and mullet (Kusuda *et al.*, 1976). We have recently generated *Edwardsiella tarda* ghosts (ETG) by gene E mediated lysis (Kwon *et al.*, 2005), and have demonstrated its high vaccine potential by in vivo immunization experiment (Kwon *et al.*, 2006). In this study, to generate combination vaccine system, we have generated ETG displaying the above selected antigens of *S. iniae* on the outer membrane, and expression of *S. iniae* antigens on the outer membrane was confirmed by immunoblot.

Materials and methods

1. Bacterial strain and culture conditions

Streptococcus iniae JSL0208 was kindly provided by Dr. J. S. Lee, National Fisheries Research & Development Institute, Korea. *Lactococcus garvieae* was provided by the American Type Culture Collection (ATTC). *Streptococcus parauberis* was provided by the Korean collection for type cultures (KCTC). They were cultured at 37℃ overnight in brain heart infusion broth (BHI, Difco) supplemented with 1.0% NaCl.

Escherichia coli BL21 (DE3) was used as the host strain for recombinant pET 28a (Novagen, USA) and pGEX-4T-1 expression plasmid and cultured at 37℃ on Luria-Bertani (LB, Difco) agar containing kanamycin and ampicillin. Expression of the His-tagged fusion protein and Glutathione S-transferase (GST) fusion were induced with 1 mM IPTG after reaching an OD_{600} of 1.0, and growth continued at 27℃ or 37℃.

Plasmid pInaN-UreA-ghost provided by Dr. Nam, Pukyoung National University were used as a template for construction of surface expression vector. High constitutive expression vector, pHCEⅡB was purchased from Bioleaders, corp. The constructed vector was transformed into *E. coli* DH5α (Invitrogen) and *E. tarda* FSW910410 strain. Transformed bacteria was inoculated in Luria Broth (LB, Difco) containing 50 ㎍/㎖ ampicillin (Sigma) at 27℃ or 37℃. Growth and lysis of bacterial cultures were monitored by measuring the optical density at 600 nm (OD_{600}).

2. Preparation of genomic DNA from S. *iniae*

Cultured bacteria was pelleted by centrifugation at 2,500 × g for 10 min and washed three times with Phosphate buffered saline (PBS, pH 7.0). Total genomic DNA of *S. iniae* was extracted using *AccuPrep*® genomic DNA extraction kit according to the manufacturer's instructions (Bioneer, Taejon, Korea). The quality and quantity of DNA was determined by ultraviolet(UV) spectrophotometer (*Ultraspec*® 3100 *pro*, Amersham Pharmacia Biotech).

3. Polymerase chain reaction (PCR) amplication of 4-genes

PCR was utilized to amplify the open reading frame (ORF) of the 4-genes.

3.1. PCR amplification for full sequencing of GAPDH and α-enolase

Degenerate PCR primers were designed based on the conserved sequences found at either N- or C-terminal region of genes reported previously from various streptococci (Fig. 1 and Fig. 2).

The sequences of the GAPDH primers are: forward primer; 5'-ATGGTAGTTAAAGTTGGTATTAACGG-3', reverse primer; 5'-TTATTTAGCRATTTTTGCRAAG-3'. The sequences of the α-enolase primers are: forward primer; 5'-ATGTCAATTATTACTGATGTWTACGC -3', reverse primer; 5'-YTATTTTTTHARGTTRTAGAATG-3'.

The extracted DNA (100 ng) was used in a 20 ㎕ of PCR reaction containing 10 pmoles of each primer and 0.5 U of Taq DNA polymerase (Takara, Shiga, Japan). The PCR reaction was carried out 30 cycles at 95°C for 30 sec, 50°C or 42°C for 30 sec and 72°C for 40 sec, with an initial denaturation at 95°C for 30 sec using an automated thermal cycler (iCycler, BioRad). The PCR product was separated on 0.7% agarose gel and stained

with ethidium bromide.

3.2. PCR amplification for full sequencing of streptolysin S, SagA

The SagA gene of *S. iniae* was registered in NCBI and the primers were designed from Genbank (NCBI; AF465842). The sequences of the SagA primers are: forward primer; 5'-ATGTTACAATTTACTTCAAATATC TTAGC-3', reverse primer; 5'-TTACTTTGGAGCTGGTGCTG-3').

The extracted DNA (100 ng) was used in a 20 ㎕ of PCR reaction containing 10 pmoles of each primer and 0.5 U of Taq DNA polymerase (Takara, Shiga, Japan). The PCR reaction was carried out 30 cycles at 95°C for 30 sec, 50°C for 30 sec and 72°C for 30 sec, with an initial denaturation at 95°C for 30 sec using an automated thermal cycler (iCycler, BioRad). The PCR product was separated on 0.7% agarose gel and stained with ethidium bromide.

3.3. PCR amplification for full sequencing of iron uptake ABC transporter, piaA

3.3.1. PCR cloning of partial genomic DNA using degenerated primer

Due to unknown full nucleotide sequence of piaA, degenerate PCR primers were designed based on the conserved sequences found at either N- or C-terminal region of genes reported previously from various Gram positive bacteria (Fig. 3). The sequences of the primers are: forward primer; 5'-ACYCGYAATCCAWTWGCAGATCCT-3', reverse primer; 5'-TAAACCDACAAARCCAATWGGTCC-3'.

For 20 ㎕ of PCR reaction volume, it was composed genomic DNA (100 ng), 10 pmols of each primer, TE buffer, and 0.5 U of Taq DNA polymerase (TaKaRa, Shiga, Japan). PCR was performed with 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for

30 sec, with an initial denaturation at 95°C for 30 sec using an automated thermal cycler (iCycler, BioRad). The PCR product was separated on 0.7% agarose gel and stained with ethidium bromide.

3.3.2. Primer walking of genomic DNA

Genomic DNA of *S. iniae* were used as template. Primer walking was conducted using DNA Walking *SpeedUp*™ Primix Kit (Seegene) following the manufacturer's protocol. The three nested primer sets for DNA walking in downstream and upstream region were designed on the basis of the partial sequences, respectively (Table 1). It was performed on ice in downstream and upstream of pia A. Before the first PCR, PCR master mix prepared. For each 50 ㎕ PCR reaction, it was composed 44 ㎕ of 19 ㎕ of distilled water and 25 ㎕ of 2× SeeAmp™ ACP™ Master Mix II per reaction tube. 1 ㎕ of genomic DNA, 4 ㎕ of 2.5 μM DW-ACP (one of DW-ACP 1, 2, 3 and 4) primers and 1 ㎕ of primer walking TSP 1 primer (10μM) were added to each tube. The first PCR was performed using the following three-step cycle parameters : 1 cycle at 94℃ for 5 min, 42℃ for 1 min, 72℃ for 2 min, 30cycles at 94℃ for 40 sec, 55℃ for 40 sec, 72℃ for 1.5 min and 1 cycle at 72℃ for 7 min. The first PCR products were purified using *AccuPrep*® PCR Purification Kit (Bioneer).

Before the second PCR, PCR master mix prepared. For each 20 ㎕ PCR reaction, it was composed 15 ㎕ of 5 ㎕ of distilled water and 10 ㎕ of 2× SeeAmp™ ACP™ Master Mix II per reaction tube. 3 ㎕ of purified first PCR products, 1 ㎕ of DW-ACPN primer (10 μM) and 1㎕ of primer walking TSP 2 primer (10 μM) were added to each tube. The second PCR was performed using the following two-step cycle parameters : 1 cycle at 94℃ for 3 min and 35 cycles at 94℃ for 40 sec, 60℃ for 40 sec, 72℃ for 1.5 min.

Before the third PCR, PCR Master Mix prepared. For each 20 ㎕ PCR

reaction, it was composed 17 ㎕ of 7 ㎕ of distilled water and 10 ㎕ of 2× SeeAmp™ Master Mix II per reaction tube. 1 ㎕ second PCR products, 1 ㎕ of universal primer (10 μM) and 1 ㎕ of primer walking TSP 3 primer (10 μM) were added to each tube. The third PCR was performed using the following two-step cycle parameters : 1cycle at 94℃ for 3 min and 35 cycles at 94 ℃ for 40 sec, 60℃ for 40 sec, 72℃ for 1.5 min. The PCR products analyzed on a 1.5% agarose gel stained with ethidium bromide.

4. Cloning and sequences analysis

Amplified PCR products were purified with Gel Extraction Kit (Nuclogen) from 1.0% agarose gel and cloned into the pGEM-T easy vector (Promega, WI, USA). Recombinant plasmid containing insert of correct size was screened and the selected clones were purified using an *Accuprep*® Plasmid Extraction Kit (GENE ALL) for sequencing analysis. Sequencing reaction was carried out using BigDye Terminator Ready Reaction Mix (Applied Biosystems Inc, Foster City, CA, USA) and the sequences were analyzed with an Automated DNA Sequencer (ABI Prism 377, Applied Biosystems Inc, Foster City, CA, USA). The resulting sequences were aligned with GenBank database through the BLAST search (http://www.ncbi.nlm,nih.gov/BLAST/).

5. Recombinant protein production and purification

5.1. Polymerase chain reaction (PCR) amplification assay and sequencing

The reaction primer sets were shown in Table 2. The primer sets contained restriction enzyme sites (underlined). The extracted DNA (100 ng) was used in a 20 ㎕ of PCR reaction containing 10 pmoles of each primer and 0.5 U of Taq DNA polymerase (TaKaRa, Shiga, Japan). PCR was

performed with 30 cycles of denaturation at 95℃ for 30 sec, annealing at 42~55℃ for 30 sec and extension at 72℃ for 30~40 sec using an automated thermal cycler (iCycler, BioRad Inc., USA). The PCR products were separated on 0.7% agarose gel and stained with ethidium bromide. The amplified product was purified using a PCR Purification Kit (Nucleogen) and cloned into pGEM-T easy vector (Promega). Recombinant plasmid containing insert of correct size was screened and the selected clones were purified using an *Accuprep*® Plasmid Extraction Kit (GENE ALL) for sequencing analysis. Sequencing reaction was carried out using BigDye Terminator Ready Reaction Mix (Applied Biosystems Inc, Foster City, CA, USA) and the sequences were analyzed with an Automated DNA Sequencer (ABI Prism 377, Applied Biosystems Inc, Foster City, CA, USA).

5.2. Construction of recombinant protein expression vector

5.2.1. Construction of recombinant protein expression vector, pET28a

Sequenced plasmids of GAPDH, α-enolase were digested with appropriate restriction enzymes and inserted into the pET 28a vector (Novagen, USA). The His-tagged fusion recombinant plasmids were prepared by transforming *Escherichia coli* BL21 (DE3) competent cells.

5.2.2. Construction of recombinant protein expression vector, pGEX-4T-1

Sequenced plasmids of SagA-toxic and piaA were digested with appropriate restriction enzymes and inserted into the pGEX-4T-1 vector (Amersham Biosciences). The Glutathione S-transferase (GST) fusion recombinant plasmids were prepared by transforming *Escherichia coli* BL21 (DE3) competent cells.

5.3. Expression and purification of recombinant proteins

5.3.1. Expression and purification of His-tagged fusion protein

The 10 ㎖ of overnight culture of *E. coli* BL21 (DE3) harboring pET 28a/GAPDH and pET 28a/α-enolase were diluted in 1ℓ of fresh LB medium containing 30 ㎍/㎖ kanamycin and grown 37℃ with vigorous shaking. Isopropyl 1-thio-β-$_D$-galactoside (IPTG) was added to a final concentration of 1 mM when the OD_{600} reached about 1.0. Bacteria were harvested 4 h after the addition of IPTG by centrifugation at 2000 × g at 4℃ for 10 min and resuspended in 100 ㎖ of binding buffer(5 mM imidazole, 500 mM NaCl, 20 mM Tris/pH7.9). Lysozyme was added to a final concentration of 200 ㎍/㎖ and the cells were lysed by sonication using Sonic Dismembrator (Fisher Scientific) for 5 min at intervals of 20 sec at output power 7. The lysate was centrifuged at 10,000 × g at 4℃ for 40 min and the supernatant was collected by 0.45 ㎛ syringe filter and the pellet was resuspended in binding buffer. The His-tagged fusion protein was purified by chromatography under native conditions on Ni-nitrilotriacetic acid resin according to the manufacturer's protocols (Novagen). The integrity of insert DNA was verified by sequence analysis. Protein purity was monitored by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Brilliant Blue, and the protein concentration was determined using the BCA protein assay (Sigma, MO, USA).

5.3.2. Expression and purification of GST fusion protein

The 10 ㎖ of overnight culture of *E. coli* BL21 (DE3) harboring pGEX-4T-1/SagA-toxic and pGEX 4T-1/pia A were diluted in 1 ℓ of fresh LB medium containing 50 ㎍/㎖ ampicillin and grown 37℃ with vigorous shaking. Isopropyl 1-thio-β-$_D$-galactoside (IPTG) was added to a final concentration of 1 mM when the OD_{600} reached about 1.0. Bacteria were harvested 4 h after the addition of IPTG by centrifugation at 2000 × g at 4℃ for 10 min

and resuspended in 100 ㎖ of binding buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4). Lysozyme was added to a final concentration of 200 ㎍/㎖ and the cells were lysed by sonication using Sonic Dismembrator (Fisher Scientific) for 5 min at intervals of 20 sec at output power 7. The lysate was centrifuged at 10,000 × g at 4℃ for 40 min and the supernatant was collected by 0.45 ㎛ syringe filter and the pellet was resuspended in binding buffer. The GST fusion protein was purified by affinity chromatography using gluthatione-sepharose 4 FF columns (Amersham Biosciences) and eluted with 10 mM reduced glutathione (Sigma). The integrity of insert DNA was verified by sequence analysis. Protein purity was monitored by Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), stained with Coomassie Brilliant Blue, and the protein concentration was determined using the BCA protein assay (Sigma, MO, USA).

6. Antibody production

6.1. Production of Ab (Antibody) from rat against recombinant proteins

Specific-pathogen-free male Wistar rats (4 weeks old) were used for the immunization experiment. At the first immunization, three rats were immunized by intraperitoneal injection of 0.15 ㎖ of purified recombinant proteins emulsified with an equal volume of complete Freund's adjuvant (FCA, Sigma), and boosted with the injection of a same dose of the protein emulsified with incomplete Freund's adjuvant (FIA, Sigma) two weeks later. Three rats of the control group were intraperitoneally injected with PBS and FCA mixture at the first injection, and boosted with PBS and FIA mixture. On 2 weeks post-boost immunization, all rats were bled to obtain serum. These sera were stored at -80℃ until analysis.

6.2. Bactericidal activity of serum

The serum bactericidal activity was determined according Barnes AC *et al.* (2003) with some modifications. Pellets were washed and resuspended in PBS to an OD_{540} of 1.0 (5.32×10^7 cfu ml^{-1}). This was diluted 50-fold in PBS and 25 ㎕ was incubated with an equal volume of heat-inactivated normal serum or antiserum for 1 h at room temperature. Complement activity was provided by the addition of fresh normal serum (1:4; bacterial suspension/serum : fresh normal serum). Suspensions were incubated in a 'U'-shaped microtitre plates for 1.5 h at room temperature. Following incubation, suspensions were serially diluted in PBS and spotted (25 ㎕) onto THA plates, which were incubated at 27℃ for 48 h. Viable colonies were counted.

7. Extraction of cell wall proteins

Cell-wall protein was extracted by using a slightly modified version of the method of Gatti *et al.* (1997). *S. iniae* cultivated in BHI broth for 48 h were harvested, washed twice in 0.05 mol l^{-1} Tris-HCl, pH 7.5, containing 0.1 mol l^{-1} $CaCl_2$, and resuspended in 1 ㎖ of the same. After centrifugation at 8,700 × g for 5 min, cell wall proteins were extracted from the pellets with 1 ㎖ of extraction buffer, pH 8.0, containing 0.01 mol l^{-1} EDTA, 0.01 mol l^{-1} NaCl and 2% (wt/vol) SDS. Suspensions were stored at room temperature for 60 min, heated at 100℃ for 5 min and centrifuged at 10,000 × g for 10 min at 4℃. The supernatants were analyzed by SDS-PAGE and stained with Coomassie blue.

7.1. Western blot

Cell wall proteins were solublized in SDS-PAGE loading buffer (2% SDS,

14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue, 60 mM Tris-HCl, pH 6.8), boiled for 5 min and fractionated on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. The membrane was blocked with blocking solution (3% bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with diluted rat sera (1:500) for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phophatase conjugated goat anti-rat IgG (1:2000, Santa Cruz Biotechnology) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indoly phosphate (NBT-BCIP)substrate buffer (Sigma).

8. Immunoblot analysis for plasmin(ogen) binding

Purified human plasmin, plasminogen (Sigma) and human immunoglobulin M (IgM, as a control) were transferred to nitrocellulose membrane after SDS-PAGE as described above. After blocking with bovine serum albumin, the membrane was incubated with the purified His-GAPDH and His-Enolase (1:100 dilution) at RT for 2 h and then it was incubated at 4℃ for overnight. The membranes washed 3 times with TTBS. His-anti rabbit IgG (1:1000) was added, incubated for 2 h at RT and washed 3times with TTBS. The membranes were incubated with alkaline phophatase conjugated goat anti-rat IgG (1:2000) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with NBT-BCIP substrate buffer (Sigma).

9. Display the 4 genes on the surface of *E. tarda* ghost

9.1. PCR amplification for pHCE-InaN-4genes-ghost 37 SDM

The reaction primer sets were shown in Table 3. The primer sets contained restriction enzyme sites (underlined). The extracted DNA (100 ng) was used in a 20 ㎕ of PCR reaction containing 10 pmoles of each primer and 0.5 U of Taq DNA polymerase (TaKaRa, Shiga, Japan). PCR was performed with 30 cycles of denaturation at 95℃ for 30 sec, annealing at 50℃ for 30 sec and extension at 72℃ for 30 sec using an automated thermal cycler (iCycler, BioRad Inc., USA). Each amplified PCR product was visualized on 0.7% agarose gels stained with ethidium bromide, purified with gel extraction kit (Nucleogen).

9.2. Construction of surface expression vector

Ice nucleation protein, InaN combined with UreA was obtained by Nco I -Sac I digestion of pInaN-UreA-ghost and cloned into pHCEⅡB. This plasmid was designated as pHCE-InaN-UreA.

The PCR products of 4-genes were cloned into pGEM-T easy vector (Promega) and designated as pGEM/GAPDH, pGEM/α-enolase, pGEM/SagA and pGEM/piaA, respectively. The 4-plasmids DNA were digested with Spe I and Sac I, purified and subcloned into the Spe I -Sac I cleaved pHCE-InaN-UreA. Thus UreA was replaced with 4-genes, respectively and the resulting plasmid was designated as pHCE-InaN-target gene.

For ligation pHCE-InaN-target gene with ghost cassette, pλP_R-c I -Elysis 37 SDM was digested with Apa I and Sal I , and then cloned into pBluescript Ⅱ KS (Stratagene). This plasmid was designated as pBS-λP_R-c I -Elysis 37 SDM. pHCE-InaN-target gene and pBS-λP_R-c I -Elysis 37 SDM were digested with Sac I and Sal I and ligated with each other. The resultant plasmid was

pHCE-InaN-target gene-ghost 37 SDM (Fig. 33).

9.3. Transformation of bacteria

9.3.1. Transformation of *E. coli* DH5α by chemical method

E. coli DH5α was inoculated into 100 ㎖ LB and grown at 37℃ to an OD_{600} of 0.4 with vigorous agitation (200 rpm). The cultures were prepared to a chemically competent cell with 0.1M $CaCl_2$. pHCE-InaN-target gene-ghost 37 SDM were added to the competent cells. Mixtures of cell and DNA were stored on ice for 30 min and transferred in a preheated 42℃ circulating water bath for 90 s. After cooling the tube, 800 ㎕ of SOC medium was added to cells and incubated for 1.5 h at 37℃. After incubation, cells were plated onto LB agar plate containing 50 ㎍/㎖ ampicillin.

9.3.2. Transformation of *E. tarda* FSW910410 by electroporation

E. tarda FSW910410 was inoculated into 10 ㎖ LB and grown at 27℃ to an OD_{600} of 0.4 with vigorous agitation (200 rpm). The cultures were prepared to a electrocompetent cell with ice-cold 10% glycerol solution.

pHCE-InaN-target gene-ghost 37 SDM were added to the competent cells. Mixtures of cell and DNA were stored on ice for 50 sec, dried off any moisture from cuvette outside and transferred in a chilled cuvette. It was flicked to settle cell and DNA mixture into bottom of cuvette and gave pulse with a field strength of 12.5 kV/cm for 4-5 milliseconds. Rapidly 1 ㎖ of SOC medium was added to cells and incubated for 1.5 h at 37℃. After incubation, cells were plated onto LB agar plate containing 50 ㎍/㎖ ampicillin.

10. Induction of target genes expression and preparation of bacterial ghosts

E. coli and *E. tarda* carrying pHCE-InaN-target gene-ghost 37 SDM were inoculated 100 ㎖ LB broth containing 50 ㎍/㎖ ampicillin at 27℃ or 37℃. When the cultures were reached an OD_{600} of 0.2~0.3, the expression of *E* gene was induced by a temperature upshift from 27℃ to 42℃. At different time points after expression of *E* gene, an optical density was measured until no further decrease in optical density was detected. After the lysis has been completed, ghost bacterial cells were washed 3 times with a phosphate buffered saline (pH 7.0) by centrifugation (5000 × g, 4℃, 10 min). The harvested cell was lyophilized and stored at 4℃ until further use.

11. Isolation of outer membrane

Cell fractionation was performed according to the method described in Shi and Su (2001). After induction of heterologous proteins, harvested cells were diluted to set as unit cell density (OD_{600} = 1.0), washed, and resuspended in PBS buffer containing 1 mM EDTA and lysozyme at 100 ㎍/㎖. After 2 h incubation, cell suspension was treated with an ultrasound sonication at 30 sec 2 cycles. To obtain total membrane fraction, whole cell lysate was pelleted by centrifugation at 100,000 x g for 1 h using an ultracentrifuge (Himac CS-150GX, Hitachi). For further outer membrane fractionation, the pellet was resuspended with PBS containing 0.01 mM $MgCl_2$ and 2% Triton X-100 for solubilizing inner membrane and incubated at room temperature for 30 min, and then the outer membrane fraction was collected by ultracentrifugation. These samples were saved for further analyses.

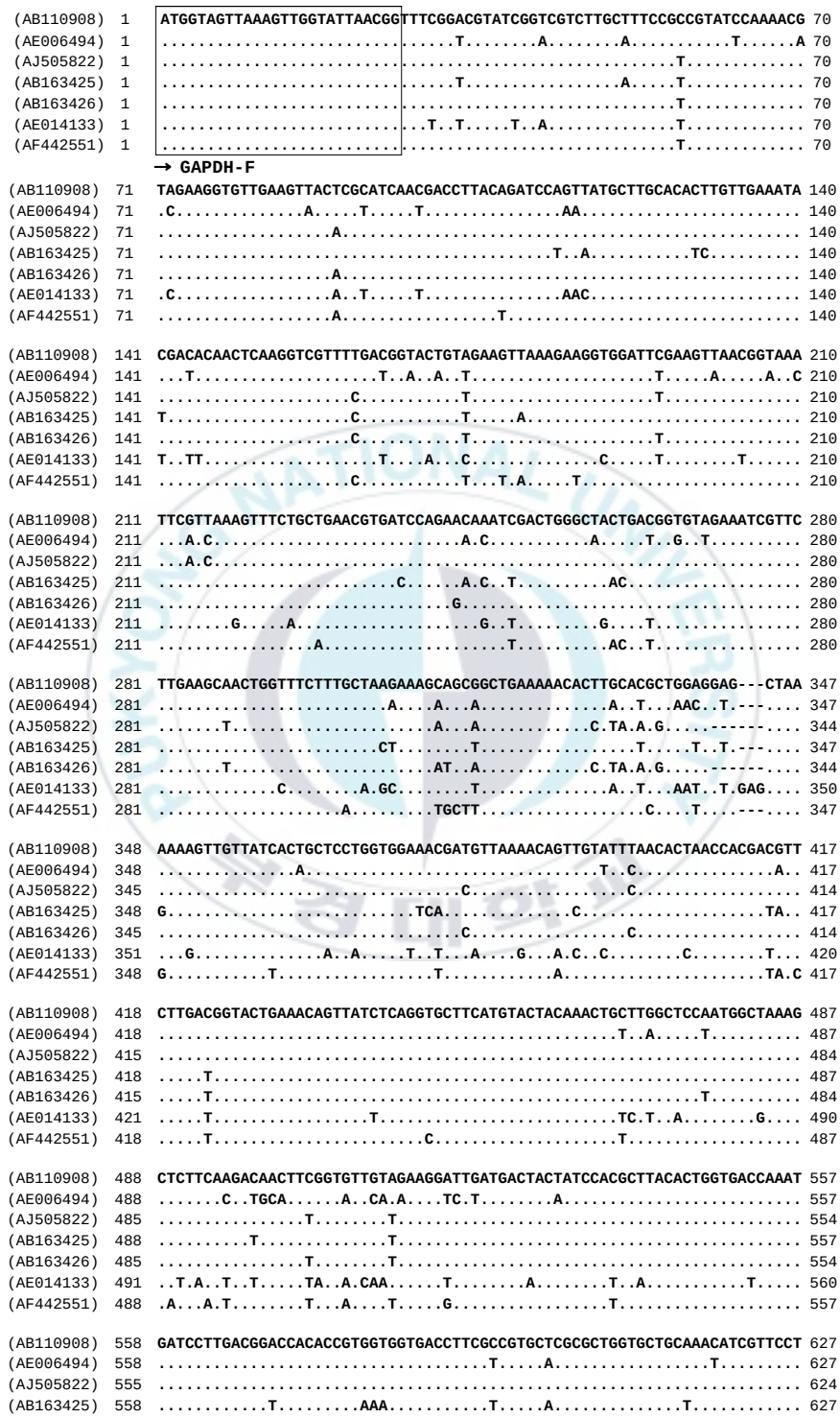
11.1. Western blot analysis

An equal volume of outer membrane fraction of the cells and target gene induced cells were mixed with SDS sample buffer (10% sodium dodecyl sulfate

(SDS), 10% β-mercaptanol, 0.3 M Tris-HCl (pH 6.8), 0.05% bromophenol blue, 50% glycerol), boiled for 10 min at 95℃, and resolved by 10% (wt/vol) SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrophoretic transfer to nitrocellulose membrane with transfer buffer (48 mM Tris-HCl, 39 mM glycine, 20% methanol, pH 9.2) by using a Trans-Blot SD Cell (Novex) at 25V for 90 m. The membrane was blocked with blocking solution (3% Bovine serum albumin in TBS; 150 mM NaCl, 10 mM Tris-HCl, pH 7.5) for 2 h at room temperature (RT), washed with TTBS (0.05% Tween 20 in TBS, pH 7.5) and incubated with diluted rat sera (1:500) for 2 h at RT. The membranes were washed three times with TTBS and incubated with alkaline phosphatase conjugated goat anti-rat IgG (1:2000, Santa Cruz, Biotechnology) for 2 h at RT. After washing off unbound secondary antibody, the specific antigen-bound antibody was visualized with NBT-BCIP substrate buffer (Sigma).

12. Statistical analysis

Statistical analysis was performed using the SPSS/PC+ statistical package (SPSS Inc, Chicago, USA). Significant differences between groups were analyzed by the Student's *t*-test. The level of significance was established at $P<0.05$.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(AJ505822) 1 .....T..... 70
(AB163425) 1 .....T.....A.....T..... 70
(AB163426) 1 .....T..... 70
(AE014133) 1 ...T..T.....T..A.....T..... 70
(AF442551) 1 .....T..... 70
→ GAPDH-F
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(AE006494) 71 .C.....A.....T.....T.....AA..... 140
(AJ505822) 71 .....A..... 140
(AB163425) 71 .....T..A.....TC..... 140
(AB163426) 71 .....A..... 140
(AE014133) 71 .C.....A..T.....T.....AAC..... 140
(AF442551) 71 .....A.....T..... 140
(AB110908) 141 CGACACAACTCAAGGTCGTTTTGACGGTACTGTAGAAGTTAAAGAAGGTGGATTCGAAGTTAACGGTAAA 210
(AE006494) 141 ...T.....T..A..A..T.....T.....A.....A..C 210
(AJ505822) 141 .....C.....T.....T..... 210
(AB163425) 141 T.....C.....T.....A..... 210
(AB163426) 141 .....C.....T.....T..... 210
(AE014133) 141 T..TT.....T...A..C.....C.....T.....T..... 210
(AF442551) 141 .....C.....T...T.A.....T..... 210
(AB110908) 211 TTCGTTAAAGTTTCTGCTGAACGTGATCCAGAACAAATCGACTGGGCTACTGACGGTGTAGAAATCGTTC 280
(AE006494) 211 ...A.C.....A.C.....A.....T..G..T..... 280
(AJ505822) 211 ...A.C..... 280
(AB163425) 211 .....C.....A.C..T.....AC..... 280
(AB163426) 211 .....G..... 280
(AE014133) 211 .....G.....A.....G..T.....G....T..... 280
(AF442551) 211 .....A.....T.....AC..T..... 280
(AB110908) 281 TTGAAGCAACTGGTTTCTTTGCTAAGAAAGCAGCGGCTGAAAAACACTTGCACGCTGGAGGAG---CTAA 347
(AE006494) 281 .....A....A...A.....A..T...AAC..T.---.... 347
(AJ505822) 281 .....T.....A...A.....C.TA.A.G.....---------.... 344
(AB163425) 281 .....CT.....T.....T.....T..T.---.... 347
(AB163426) 281 .....T.....AT..A.....C.TA.A.G.....---------.... 344
(AE014133) 281 .....C.....A.GC.....T.....A..T...AAT..T.GAG.... 350
(AF442551) 281 .....A.....TGCTT.....C....T.---.... 347
(AB110908) 348 AAAAGTTGTTATCACTGCTCCTGGTGGAAACGATGTTAAAACAGTTGTATTTAACACTAACCACGACGTT 417
(AE006494) 348 .....A.....T..C.....A.. 417
(AJ505822) 345 .....C.....C..... 414
(AB163425) 348 G.....TCA.....C.....TA.. 417
(AB163426) 345 .....C.....C..... 414
(AE014133) 351 ...G.....A..A.....T..T...A...G...A.C..C.....C.....T... 420
(AF442551) 348 G.....T.....T.....A.....TA.C 417
(AB110908) 418 CTTGACGGTACTGAAACAGTTATCTCAGGTGCTTCATGTACTACAAACTGCTTGGCTCCAATGGCTAAAG 487
(AE006494) 418 .....T..A.....T..... 487
(AJ505822) 415 484
(AB163425) 418 .....T..... 487
(AB163426) 415 .....T.....T..... 484
(AE014133) 421 .....T.....T.....TC.T..A.....G.... 490
(AF442551) 418 .....T.....C.....T..... 487
(AB110908) 488 CTCTTCAAGACAACTTCGGTGTTGTAGAAGGATTGATGACTACTATCCACGCTTACACTGGTGACCAAAT 557
(AE006494) 488 .....C..TGCA.....A..CA.A....TC.T.....A..... 557
(AJ505822) 485 .....T.....T..... 554
(AB163425) 488 .....T.....T..... 557
(AB163426) 485 .....T.....T..... 554
(AE014133) 491 ..T.A..T..T.....TA..A.CAA.....T.....A.....T..A.....T..... 560
(AF442551) 488 .A...A.T.....T...A....T.....G.....T..... 557
(AB110908) 558 GATCCTTGACGGACCACACCGTGGTGGTGACCTTCGCCGTGCTCGCGCTGGTGCTGCAAACATCGTTCCT 627
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(AJ505822) 555 624
(AB163425) 558 .....T.....AAA.....T.....A.....T..... 627

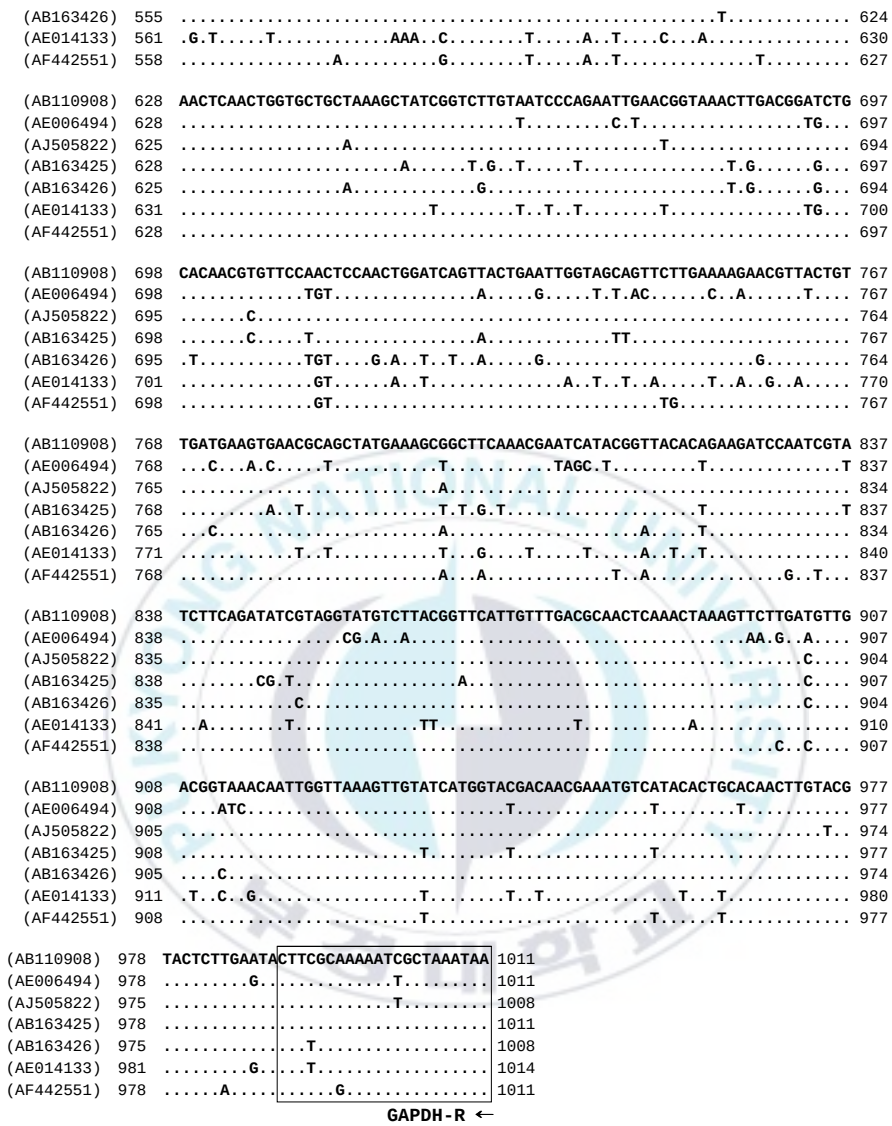
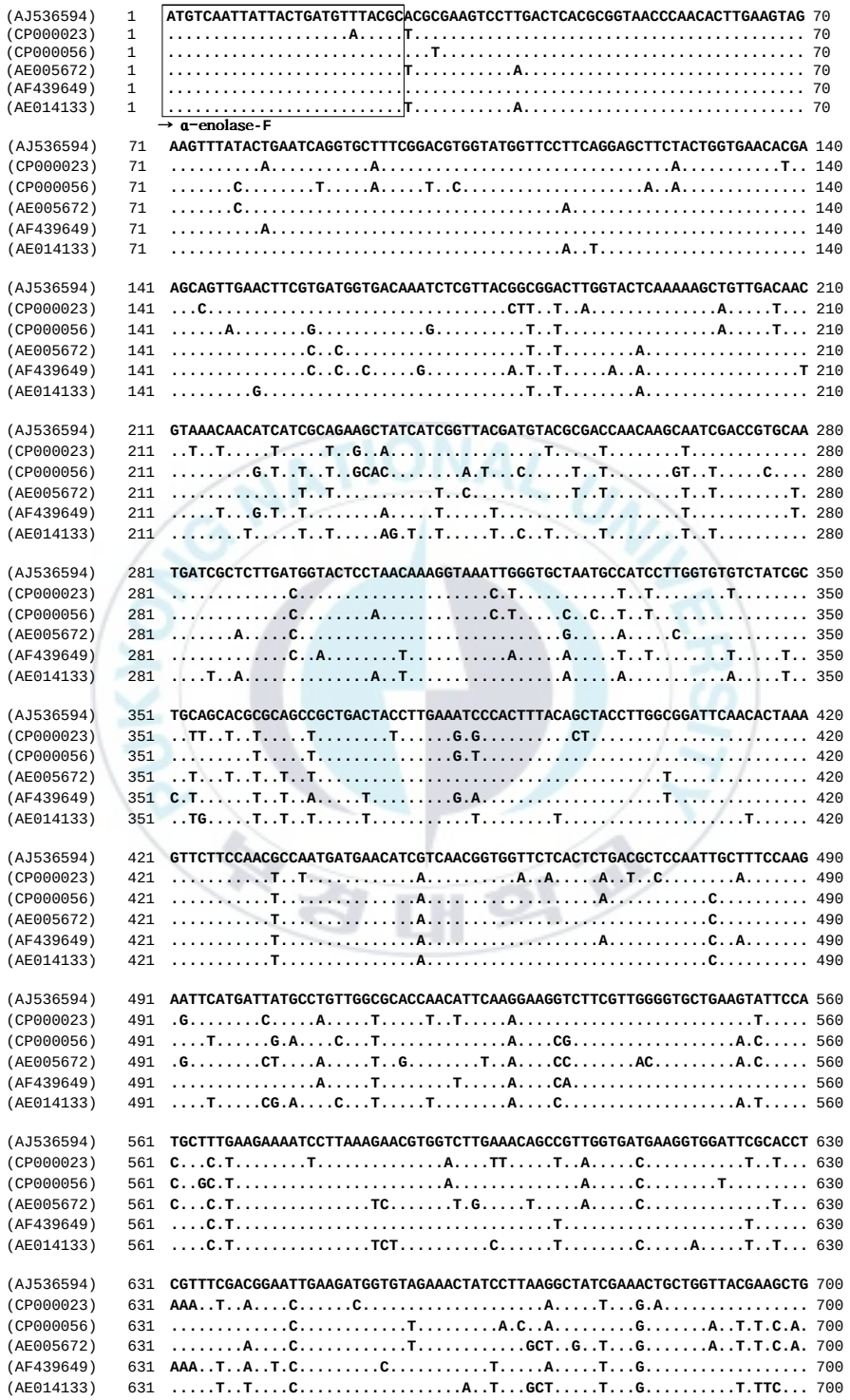


Fig. 1. Nucleotide sequence alignment of GAPDH from different Streptococci.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(AE005672) 1 .T..A.. 70
(AF439649) 1 .. 70
(AE014133) 1 .T..A.. 70
→ α-enolase-F
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(CP000023) 71 ..A..A..A..T.. 140
(CP000056) 71 .C..T..A..T..C..A..A.. 140
(AE005672) 71 .C..A.. 140
(AF439649) 71 ..A.. 140
(AE014133) 71 ..A..T.. 140
(AJ536594) 141 AGCAGTTGAACTTCGTGATGGTGACAAATCTCGTTACGGCGGACTTGGTACTCAAAAAGCTGTTGACAAC 210
(CP000023) 141 ...C..CTT..T..A..A.....T... 210
(CP000056) 141 ......A.....G.....G.....T..T.....A.....T... 210
(AE005672) 141 .....C..C.....T..T.....A.. 210
(AF439649) 141 .....C..C..C.....G.....A.T..T.....A..A.....T 210
(AE014133) 141 .....G.....T..T.....A.. 210
(AJ536594) 211 GTAAACAACATCATCGCAGAAGCTATCATCGGTTACGATGTACGCGACCAACAAGCAATCGACCGTGCAA 280
(CP000023) 211 ..T..T.....T.....T..G..A.....T.....T.....T..... 280
(CP000056) 211 .....G.T..T..T..GCAC.....A.T...C.....T..T.....GT..T.....C.... 280
(AE005672) 211 .....T..T.....T..C.....T..T.....T..T.....T. 280
(AF439649) 211 .....T...G.T..T.....A.....T.....T.....T.....T. 280
(AE014133) 211 .....T.....T..T.....AG.T..T.....T..C..T.....T.....T..T..... 280
(AJ536594) 281 TGATCGCTCTTGATGGTACTCCTAACAAAGGTAAATTGGGTGCTAATGCCATCCTTGGTGTGTCTATCGC 350
(CP000023) 281 .....C.....C.T.....T..T.....T..... 350
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(AE014133) 281 ....T..A.....A..T.....A.....A.....A.....T.. 350
(AJ536594) 351 TGCAGCACGCGCAGCCGCTGACTACCTTGAAATCCCACTTTACAGCTACCTTGGCGGATTCAACACTAAA 420
(CP000023) 351 ..TT..T..T.....T.....T.....G.G.....CT..... 420
(CP000056) 351 .....T.....T.....G.T..... 420
(AE005672) 351 ..T...T..T..T..T.....T..... 420
(AF439649) 351 C.T.....T..T..A.....T.....G.A.....T..... 420
(AE014133) 351 ..TG.....T..T..T.....T.....T.....T.....T..... 420
(AJ536594) 421 GTTCTTCCAACGCCAATGATGAACATCGTCAACGGTGGTTCTCACTCTGACGCTCCAATTGCTTTCCAAG 490
(CP000023) 421 .....T..T.....A.....A..A.....A..T..C.....A..... 490
(CP000056) 421 .....T.....A.....A.....C..... 490
(AE005672) 421 .....T.....A.....C..... 490
(AF439649) 421 .....T.....A.....A.....C..A..... 490
(AE014133) 421 .....T.....A.....C..... 490
(AJ536594) 491 AATTCATGATTATGCCTGTTGGCGCACCAACATTCAAGGAAGGTCTTCGTTGGGGTGCTGAAGTATTCCA 560
(CP000023) 491 .G.....C.....A.....T.....T..T.....A.....T..... 560
(CP000056) 491 ....T.....G.A....C...T.....A....CG.....A.C..... 560
(AE005672) 491 .G.....CT....A.....T..G.....T..A....CC.....AC.....A.C..... 560
(AF439649) 491 .....A.....T.....T.....A....CA..... 560
(AE014133) 491 ....T.....CG.A....C...T.....T.....A....C.....A.T..... 560
(AJ536594) 561 TGCTTTGAAGAAAATCCTTAAAGAACGTGGTCTTGAAACAGCCGTTGGTGATGAAGGTGGATTCGCACCT 630
(CP000023) 561 C...C.T.....T.....A....TT.....T..A.....C.....T..T... 630
(CP000056) 561 C..GC.T.....A.....A.....C.....T..... 630
(AE005672) 561 C...C.T.....TC.....T.G.....T.....A.....C.....T... 630
(AF439649) 561 ....C.T.....T..... 630
(AE014133) 561 ....C.T.....TCT.....C.....T.....C.....A.....T..T... 630
(AJ536594) 631 CGTTTCGACGGAATTGAAGATGGTGTAGAAACTATCCTTAAGGCTATCGAAACTGCTGGTTACGAAGCTG 700
(CP000023) 631 AAA..T..A....C.....C.....A.....T...G.A..... 700
(CP000056) 631 .....C.....T.....A.C..A.....G.....A..T.T.C.A. 700
(AE005672) 631 .....A....C.....T.....GCT..G..T...G.....A..T.T.C.A. 700
(AF439649) 631 AAA..T..A..T.C.....C.....T.....A.....T...G..... 700
(AE014133) 631 .....T..T....C.....A..T...GCT.....T...G.....T.TTC... 700

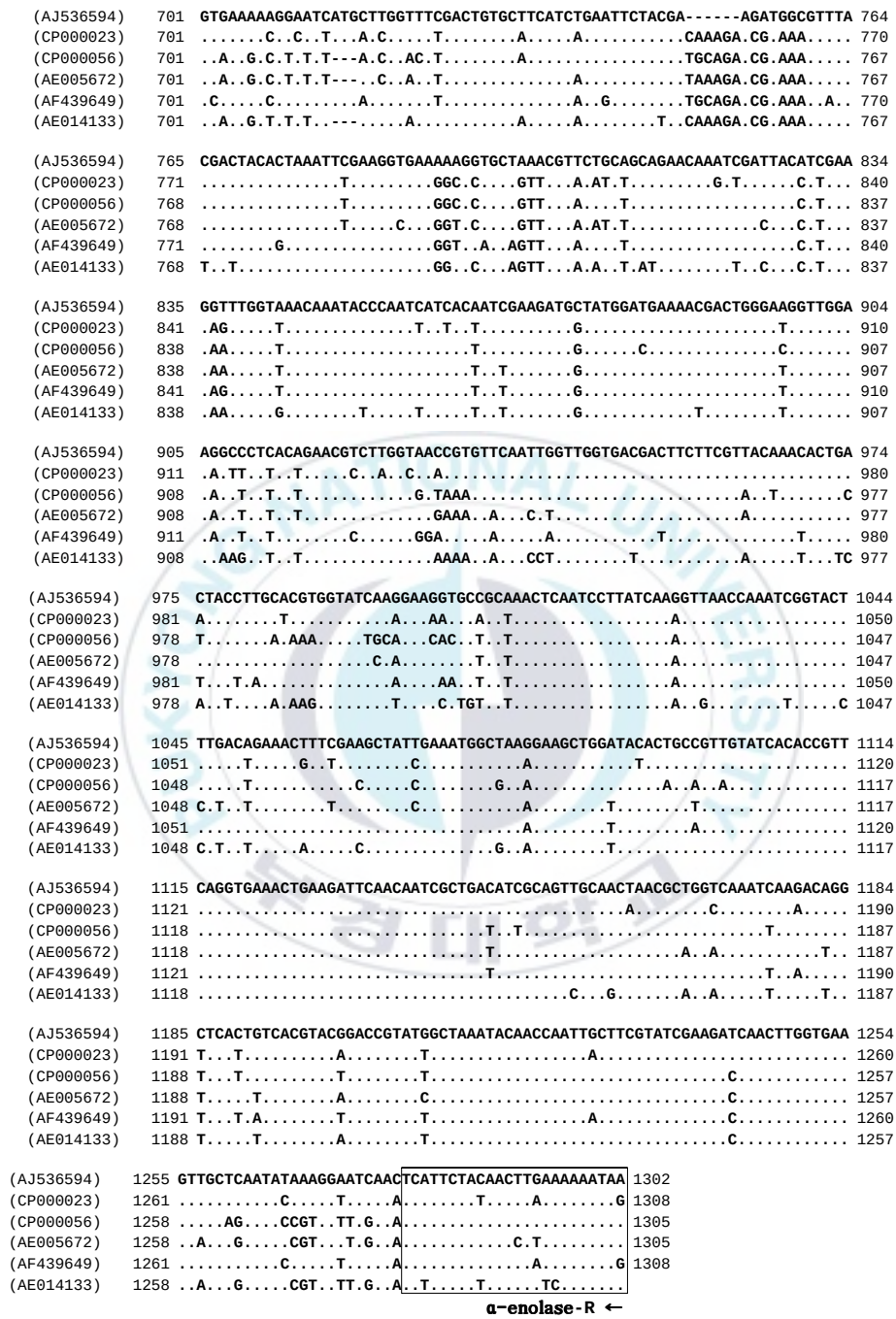
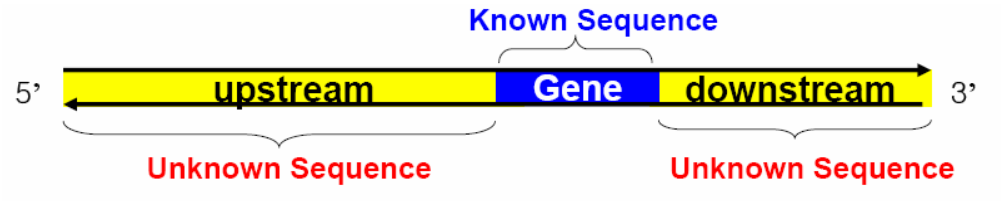


Fig. 2. Nucleotide sequence alignment of α-enolase from different Streptococci.

Fig. 3. Amino acid sequence alignment of iron uptake ABC transporter, piaA from gram positive bacteria.

Table 1. Target-Specific Primers (TSPs), DNA Walking-Annealing Control Primers (DW-ACPs) and Universal Primer used for DNA walking

Name of primer		Sequence (5' to 3')
up stream	TSP 1	GGTCCGGCAATAGCAACAGAG
	TSP 2	CCAGCTTGCCAGCCAATGAC
	TSP 3	GTGGTAATCCCTTGCCCTATGGC
down stream	TSP 1	GCAGATCCTGGACTTTTGGGG
	TSP 2	GGCTACCAGCAATTGCGTCTGG
	TSP 3	TCATTGGCTGGCAAGCTGGG
DW-ACP	1	AGGTC
	2	TGGTC
	3	GGGTC
	4	CGGTC
	N	GGTC
Universal primer		TCACAGAAGTATGCCAAGCGA



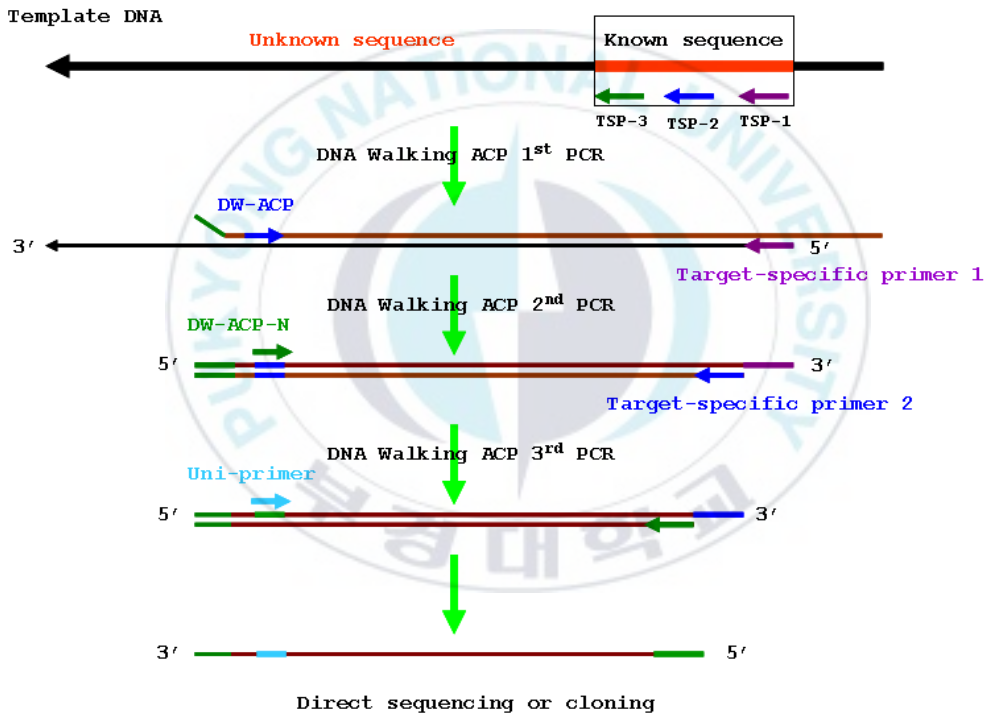


Fig. 4. Amplification of unknown sequences from upstream or downstream of the piaA known sequence

Table 2. Primers used for construction of recombinant protein expression vector

Name of primer		Sequence (5' to 3')	Restriction enzyme
GAPDH	F	CGCGAATTCATGGTAGTTAAAGTTGG	EcoR I
	R	CGCGTCGACTTATTTAGCRATTTTTG	Sal I
α-enolase	F	CGCGGATCCATGTCAATTATTACTGATG	BamH I
	R	CGCAAGCTTCTATTTTTTTAGGTTGTAG	Hind III
Sag A-toxic	F	GAATTCGTGGCGGTAAATGTTGGAAG	EcoR I
	R	GTCGACTTACTTTGGAGCTGGTGCTG	Sal I
pia A (partial)	F	GAATTCATGGCTTGGAATTTTTCACATCAAG	EcoR I
	R	GTCGACTTATTCCCAAAAAATACTGGATTGTCG	Sal I

(Restriction enzyme sites are underlined.)

Table 3. Primers used for construction of surface expression vector

Name of primer		Sequence (5' to 3')	Restriction enzyme
GAPDH	F	ACTAGTATGGTAGTTAAAGTTGGTATTAACGG	Spe I
	R	GAGCTCTTATTTAGCGATTTTTGCGAAGTAC	Sac I
α-enolase	F	ACTAGTATGTCAATTATTACTGATGTTTACG	Spe I
	R	GAGCTCCTATTTTTTTAGGTTGTAGAATG	Sac I
Sag A-toxic	F	ACTAGTGTGGCGGTAAATGTTGGAAG	Spe I
	R	GAGCTCTTACTTTGGAGCTGGTGCTG	Sac I
pia A (partial)	F	ACTAGTATGGCTTGGAATTTTTCACATCAAG	Spe I
	R	GAGCTCTTATTCCCAAAAAATACTGGATTGTCG	Sac I

(Restriction enzyme sites are underlined.)

Results

1. Cloning and sequence analysis

1.1 Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

The PCR amplification of GAPDH gene of *S. iniae, S. parauberis* and *L. garvieae* was conducted using a series of degenerate primers, which were based on the conserved amino acid regions in different species of streptococci. The coding sequence of GAPDH comprised 1,011 nucleotides with a G + C content of 40.06%. A protein of 336 amino acid residues (calculated M = 35,887 Da) was encoded by the open reading frame of GAPDH. Nucleotides sequence of *S. iniae* GAPDH showed 89.9% and 82.7% identity with that of *S. parauberis* and *L. garviae*, respectively (Fig. 5). Deduced amino acid sequence of *S. iniae* GAPDH showed 92.6% and 83.4% identity with that of *S. parauberis* and *L. garviae*, respectively (Fig. 6).

```
(  S. iniae    ) 1   ATGGTAGTTAAAGTTGGTATTAACGGTTTCGGACGTATCGGTCGTCTTGCATTCCGTCGTATTCAAAATG 70
( S. parauberis ) 1   ..............................T..C........A........T................... 70
( L. garvieae  ) 1   ..................................................T.................C. 70

(  S. iniae    ) 71  TTGAAGGTGTTGAAGTAACTCGTATCAATGACCTTACAGATCCTAACATGCTTGCACACTTGTT-GAAAT 139
( S. parauberis ) 71  .A..............T.....C.....C..............A..T.................-A.... 139
( L. garvieae  ) 71  .................GTAGCA.....C..............AGCA........T........-..... 139

(  S. iniae    ) 140 ATGATACAACTCAAGG-TCGTTTTGACGGTACAGTTGAAGTTAAAGATGGTGGATTCGAAGTTAACGGAA 208
( S. parauberis ) 140 .C..............-..............T..A...................T..C.......... 208
( L. garvieae  ) 140 ....C...........-......C......GA.....................T..............T. 208

(  S. iniae    ) 209 GCTTTGTTAAAGTTTCTGCAGAACGCGAACCAGCAAACATTGACTGGGCTACTGATGGTGTAGACATCGT 278
( S. parauberis ) 209 AA..CA.............T...AAA..T....A.C.A...........A.....C.....T..A..... 278
(  L.garvieae  ) 209 AA.....A......A........GCTA.C.....T.....CA........GAA.......C...A..... 278

(  S. iniae    ) 279 TCTTGAAGCAACAGGTTTCTTCGCTTCTAAAGCAGCTGCTGAACAACACATTCACGCTAACGGTGCGAAA 348
( S. parauberis ) 279 ............T........T...AAA..............A....TT.A..T.AA..T.....T... 348
( L. garvieae  ) 279 ............T...........AA......A.AAA............T.G..............T... 348

(  S. iniae    ) 349 AAAGTTGTTATCACAGCTCCTGGTGGAAATGACGTTAAAACAGTTGTTTACAACACTAACCATGATATTC 418
( S. parauberis ) 349 ..............T............G.......G...........A.TT.................C. 418
( L. garvieae  ) 349 .................A...........C..T........TA.C....T............C..A..CT 418

(  S. iniae    ) 419 TTGATGGAACTGAAACAGTTATCTCAGGTGCTTCATGTACTACAAACTGTTTAGCTCCAATGGCTAAAGC 488
( S. parauberis ) 419 ......................T..................................................... 488
( L. garvieae  ) 419 .GAC...TGAA..............T.....A...........T......C.C..A.........G.T.. 488

(  S. iniae    ) 489 ATTACAAGATAACTTTGGTGTAAAACAAGGTTTAATGACTACTATCCATGGTTACACTGGTGACCAAATG 558
( S. parauberis ) 489 T.................C.......................A.....C.C............T...... 558
( L. garvieae  ) 489 T..GA.CA.A.....C...C.T...GTT...AC......A........C..................... 558

(  S. iniae    ) 559 GTTCTTGACGGACCACACCGTGGTGGTGATCTTCGTCGTGCTCGTGCAGCTGCAGCAAACATCGTTCCTA 628
( S. parauberis ) 559 C.......T.....T..............CT.A........C.....T.G...TAAC..T..T....... 628
( L. garvieae  ) 559 AC......T..C.................CT.C........A.....T..A..T.A.........A.... 628

(  S. iniae    ) 629 ACTCAACTGGTGCTGCTAAAGCAATCGGTCTTGTTATCCCAGAATTAAATGGTAAACTTGACGGTGCTGC 698
( S. parauberis ) 629 .........................................T............................. 698
( L. garvieae  ) 629 .......A..............T............AT.G........G............C.A...CAC.. 698

(  S. iniae    ) 699 ACAACGTGTTCCTGTTCCAACTGGATCAGTAACTGAATTAGTAGCAGTTCTTGAAAAAGATACTTCAGTA 768
( S. parauberis ) 699 .........A..A........A..T........A..................A.T.....A......... 768
( L. garvieae  ) 699 .........A..A...........T...T.G..A...C.T...T..........T.....AGT.A.T..T 768

(  S. iniae    ) 769 GAAGAAATCAATGCAGCTATGAAAGCAGCAGCTAACGATTCATACGGTTACACTGAAGATGCTATCGTAT 838
( S. parauberis ) 769 ........T..CT...TA........T........T.....G..T..............C.A....... 838
( L. garvieae  ) 769 ......G.A.....................T.......A..T..........AC.......AA.....T. 838

(  S. iniae    ) 839 CATCAGATATCGTAGGTATTTCTTACGGTTCATTATTTGATGCTACTCAAACTAAAGTACAAACTGTTGA 908
( S. parauberis ) 839 ....T........T.....G....T............C.............................. 908
( L. garvieae  ) 839 ....T.............C...A..TC....C.C.....C............G....TACT..A.C... 908

(  S. iniae    ) 909 TGGAAATCAATTGGTTAAAGTTGTTTCATGGTATGACAATGAAATGTCTTACACTGCTCAACTTGTTCGT 978
( S. parauberis ) 909 ............A.............................................................. 978
( L. garvieae  ) 909 C..CGT.............ACA...G.T.....C..T..C..............AT..A.C..C...... 978

(  S. iniae    ) 979 ACTCTTGAGTACTTCGCAAAAATCGCTAAATAA 1011
( S. parauberis ) 979 ..A............................. 1011
( L. garvieae  ) 979 ..G.....A....................... 1011
```

Fig. 5. The comparison of the GAPDH nucleotide sequence of *S. iniae*, *S. parauberis* and *L. garvieae*.

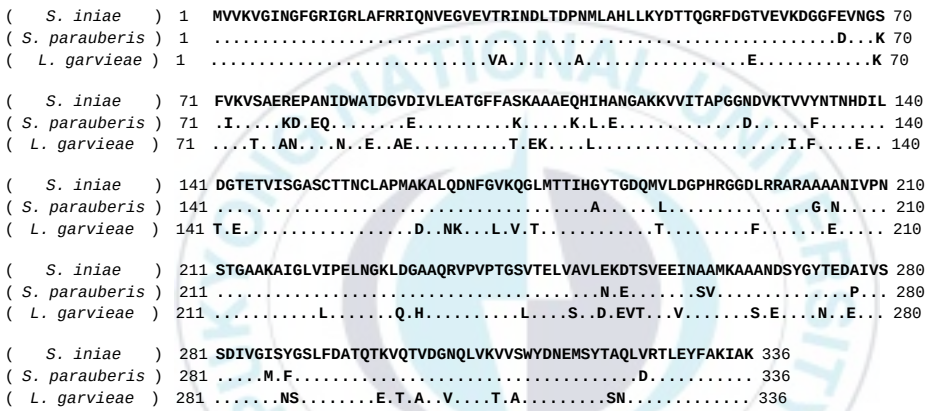


Fig. 6. The comparison of the GAPDH amino acid sequence of *S. iniae*, *S. parauberis* and *L. garvieae.*

1.2 α-enolase

The PCR amplification of α-enolase gene of *S. iniae, S. parauberis* and *L. garvieae* was conducted using a series of degenerate primers, which were based on the conserved amino acid regions in different species of streptococci. The coding sequence of α-enolase comprised 1,302 nucleotides with a G + C content of 40.67%. A protein of 435 amino acid residues (calculated M = 47,183 Da) was encoded by the open reading frame of α-enolase. Nucleotides sequence of *S. iniae* α-enolase showed 92.7% and 99.8% identity with that of *S. parauberis* and *L. garviae*, respectively (Fig. 7). Deduced amino acid sequence of *S. iniae* α-enolase showed 97.7% and 99.8% identity with that of *S. parauberis* and *L. garviae*, respectively (Fig. 8).

```
(  S. iniae    ) 1    ATGTCAATTATTACTGATGTTTACGCTCGCGAAGTCTTAGACTCACGCGGTAACCCAACACTTGAAGTAG 70
( S. parauberis ) 1    ..................................................................... 70
(  L. garvieae ) 1    ..................................................................... 70

(  S. iniae    ) 71   AAGTTTATACTGAATCAGGTGCTTTCGGACGTGGAATGGTTCCTTCAGGAGCTTCTACTGGTGAGCATGA 140
( S. parauberis ) 71   ..................................................................... 140
(  L. garvieae ) 71   ..................................................................... 140

(  S. iniae    ) 141  AGCAGTTGAATTACGTGATGGCGACAAATCTCGTTACTTAGGTTTAGGTACAGAAAAAGCAGTTGACAAC 210
( S. parauberis ) 141  ..................................................................... 210
(  L. garvieae ) 141  ..................................................................... 210

(  S. iniae    ) 211  GTTAACAACATTATCGCTGAAGCAATCATCGGTTTTGATGTTCGTGATCAACAAGCTATTGACCGTGCTA 280
( S. parauberis ) 211  ..................................................................... 280
(  L. garvieae ) 211  ..................................................................... 280

(  S. iniae    ) 281  TGATTGCACTTGATGGTACTCCTAACAAAGGTAAACTTGGTGCTAATGCTATTCTTGGTGTTTCTATTGC 350
( S. parauberis ) 281  ..................................................................... 350
(  L. garvieae ) 281  ..................................................................... 350

(  S. iniae    ) 351  CGTAGCTCGTGCTGCAGCTGACTACCTTGAAGTGCCACTTTATAACTACTTAGGTGGTTTTAACGCTAAA 420
( S. parauberis ) 351  ..................................................................... 420
(  L. garvieae ) 351  .....................................................................G 420

(  S. iniae    ) 421  GTTCTTCCAACTCCAATGATGAACATCATCAACGGTGGATCTCACTCAGATGCTCCAATTGCATTCCAAG 490
( S. parauberis ) 421  ..................................................................... 490
(  L. garvieae ) 421  ..................................................................... 490

(  S. iniae    ) 491  AGTTCATGATTATGCCTGTTGGTGCACCAACATTTAAAGAAGCACTTCGTTGGGGTGCTGAAGTATTCCA 560
( S. parauberis ) 491  ..............T...................................................... 560
(  L. garvieae ) 491  ..................................................................... 560

(  S. iniae    ) 561  CGCTCTTAAGAAAATCCTTAAAGAACGCGGACTTGTTACTGCTGTTGGTGACGAAGGTGGATTTGCTCCT 630
( S. parauberis ) 561  ..................................................................... 630
(  L. garvieae ) 561  ..................................................................... 630

(  S. iniae    ) 631  AAGTTTGAAGGAACTGAAGACGGTGTAGAAACTATCCTTAAAGCTATCGAAGCTGCTGGATATGAAGCAG 700
( S. parauberis ) 631  ..................................................................... 700
(  L. garvieae ) 631  ..................................................................... 700

(  S. iniae    ) 701  GCGAAAACGGCATCATGATTGGTTTTGACTGTGCTTCATCAGAATTCTACGACAAAGAACGTGGTGTATA 770
( S. parauberis ) 701  ..................................................................... 770
(  L. garvieae ) 701  ..................................................................... 770

(  S. iniae    ) 771  TGACTACACTAAATTTGAAGGTGAAGGAGCAGCTGTTCGTACTTCAGCTGAACAAATTGATTACCTTGAA 840
( S. parauberis ) 771  ..................................................................... 840
(  L. garvieae ) 771  ..................................................................... 840

(  S. iniae    ) 841  GAATTGGTTAACAAATACCCAATCATCACTATCGAAGATGGTATGGATGAAAATGACTGGGATGGTTGG- 909
( S. parauberis ) 841  .....................................................................- 909
(  L. garvieae ) 841  .....................................................................- 909

(  S. iniae    ) 910  AAAGCACTTACTGAACGTTTAGGCGGACGTGTTCAATTAGTTGGTGACGACTTCTTCGTTACAAACACTG 979
( S. parauberis ) 910  .....................................................G..... 979
(  L. garvieae ) 910  .....................................................G..... 979

(  S. iniae    ) 980  ATTACTTAGCACGTGGTATCAAAGAAGAAGCTGCTAACTCAATCCTTATCAAAGTTAACCAAATCGGTAC 1049
( S. parauberis ) 980  ..................................................................... 1049
(  L. garvieae ) 980  ..................................................................... 1049

(  S. iniae    ) 1050 TTTGACTGAAACTTTTGAAGCTATTGAAATGGCTAAAGAAGCTGGTTACACTGCAGTAGTATCTCACCGT 1119
( S. parauberis ) 1050 ..................................................................... 1119
(  L. garvieae ) 1050 ..................................................................... 1119

(  S. iniae    ) 1120 TCAGGTGAAACTGAAGATTCAACAATTGCTGATATCGCAGTTGCTACAAATGCAGGACAAATCAAAACAG 1189
( S. parauberis ) 1120 ..................................................................... 1189
(  L. garvieae ) 1120 ..................................................................... 1189

(  S. iniae    ) 1190 GTTCATTGTCACGTACAGACCGTATTGCTAAATACAACCAATTACTTCGTATCGAAGATCAACTTGGTGA 1259
```

```
( S. parauberis )  1190 ..................................................................... 1259
(   L. garvieae )  1190 ..................................................................... 1259

(   S. iniae    )  1260 AGTTGCTCAATACAAAGGAATCAAATCATTCTACAACCTAAAAAAATAG 1308
( S. parauberis )  1260 ..................................................... 1308
(   L. garvieae )  1260 ..................................................... 1308
```

Fig. 7. The comparison of the α-enolase nucleotide sequence of *S. iniae, S. parauberis* and *L. garvieae*.

```
( S. iniae     ) 1   MSIITDVYAREVLDSRGNPTLEVEVYTESGAFGRGMVPSGASTGEHEAVELRDGDKSRYLGLGTEKAVDN 70
( S. parauberis ) 1   ..................................................................... 70
( L. garvieae  ) 1   ..................................................................... 70

( S. iniae     ) 71  VNNIIAEAIIGFDVRDQQAIDRAMIALDGTPNKGKLGANAILGVSIAVARAAADYLEVPLYNYLGGFNAK 140
( S. parauberis ) 71  ..................................................................... 140
( L. garvieae  ) 71  ..................................................................... 140

( S. iniae     ) 141 VLPTPMMNIINGGSHSDAPIAFQEFMIMPVGAPTFKEALRWGAEVFHALKKILKERGLVTAVGDEGGFAP 210
( S. parauberis ) 141 ..........................S..................................... 210
( L. garvieae  ) 141 ..................................................................... 210

( S. iniae     ) 211 KFEGTEDGVETILKAIEAAGYEAGENGIMIGFDCASSEFYDKERGVYDYTKFEGEGAAVRTSAEQIDYLE 280
( S. parauberis ) 211 ..................................................................... 280
( L. garvieae  ) 211 ..................................................................... 280

( S. iniae     ) 281 ELVNKYPIITIEDGMDENDWDGW-KALTERLGGRVQLVGDDFFVTNTDYLARGIKEEAANSILIKVNQIG 349
( S. parauberis ) 281 ......................XSTY.F.RTCSISWR----LLRYKHL.STWYQRRSC------L.PYQ 340
( L. garvieae  ) 281 ......................-.....................S..................... 349

( S. iniae     ) 350 TLTETFEAIEMAKEAG-YTAVVSHRSGETEDSTIADIAVATNAG-QIKTGSLSRTDRIAKYNQLLRIEDQ 417
( S. parauberis ) 341 SPNRY.DNFSYNGRSWLHCSSI.PFRNRFNNCYRSCYKCR..QNRF.V.YRPYCIQP.TS.-----.RSTW 406
( L. garvieae  ) 350 ..............-.........................-..................... 417

( S. iniae     ) 418 LGEVAQYKGIKSFYNLKK 435
( S. parauberis ) 407 SCSIQRNQI.LQPKKI-- 422
( L. garvieae  ) 418 ................. 435
```

Fig. 8. The comparison of the α-enolase amino acid sequence of *S. iniae, S. parauberis* and *L. garvieae*.

Table 4. Similarities of nucleotide and deduced amino acid sequences of *Streptococcus iniae* α-enolase to those of other *Streptococcus* spp.

Bacteria	Genebank Accession number	Similarity (%)	
		Nucleotide	Amino acid
Streptococcus intermedius	AB029313	84	90
Streptococcus sobrinus	AJ536594	87	93
Streptococcus thermophilus	CP000023	86	91
Streptococcus pyogenes	CP000056	91	97
Streptococcus pneumoniae	AE005672	87	93
Streptococcus agalactiae	AAJP01000047	90	97
Streptococcus mutans	AE014133	87	91

1.3 Streptolysin S-SagA

The SagA gene was amplified from chromosomal *S. iniae* JSL0208 DNA by PCR employing primers designed from SagA gene sequence recorded in Genbank (Accession number; AF465842).

The N terminus of the propeptide of SagA (Fig. 9) contains eight cysteine and one threonine residues, which is involved in its cytolytic activity.

The SagA gene encodes a 53-aa prepropeptide with a Gly-Gly proteolytic cleavage site that has been predicted to release a 30-aa propeptide from the 23-aa leader sequence. A synthetic peptide corresponding to the propeptide sequence evoked antibodies that neutralized the hemolytic activity of SLS, a finding that strongly supports identification of SagA as the structural gene for SLS. The remaining genes in the operon have features consistent with export functions, posttranslational modification of the SLS peptide, and a possible immunity protein; this gene organization is typical of that for the bacteriocin family of antimicrobial peptides. The molecular size of the SLS peptide and its amino acid composition also resemble those of bacteriocins produced by other bacterial species.

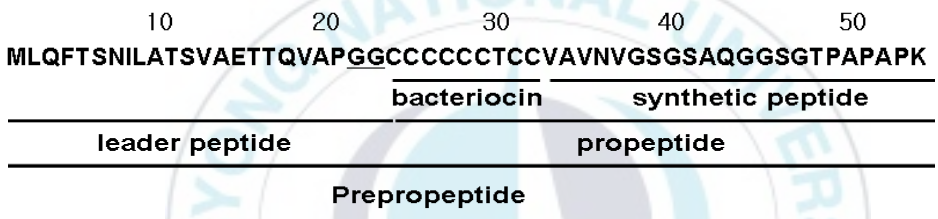


Fig. 9. Amino acid sequence similarity between SagA proteins of *S. iniae* and GAS. Putative Gly-Gly cleavage sites are underlined, and residue thought to undergo posttranslational modification are shaded.

Fig. 10. Nucleotide and deduced amino acid sequence of SagA and SagA-toxic of *Streptococcus iniae* in outside region. The top lines show the nucleotide sequence, the bottom lines the deduced amino acid sequence in the single-letter code.

1.4 piaA

For the cloning of the gene piaA in *S. iniae*, firstly, a partial 569 bp fragment was amplified using degenerate primers derived from highly conserved consensus sequences among gram positive bacteria. As a result, the nucleotide sequence of the amplified fragment showed high homology with the sequences of Gram positive bacterial piaA gene, which is known to iron uptake ABC transporter. From this initial sequence, the full open reading frame (ORF) of *S. iniae* piaA gene was amplified by primer walking. The predictive sequence of the piaA gene is shown in Fig. 13. The ORF contains 945 nucleotides and encodes a protein of 314 amino acid residues.

Fig. 11. Partial sequence of piaA gene using primer walking.

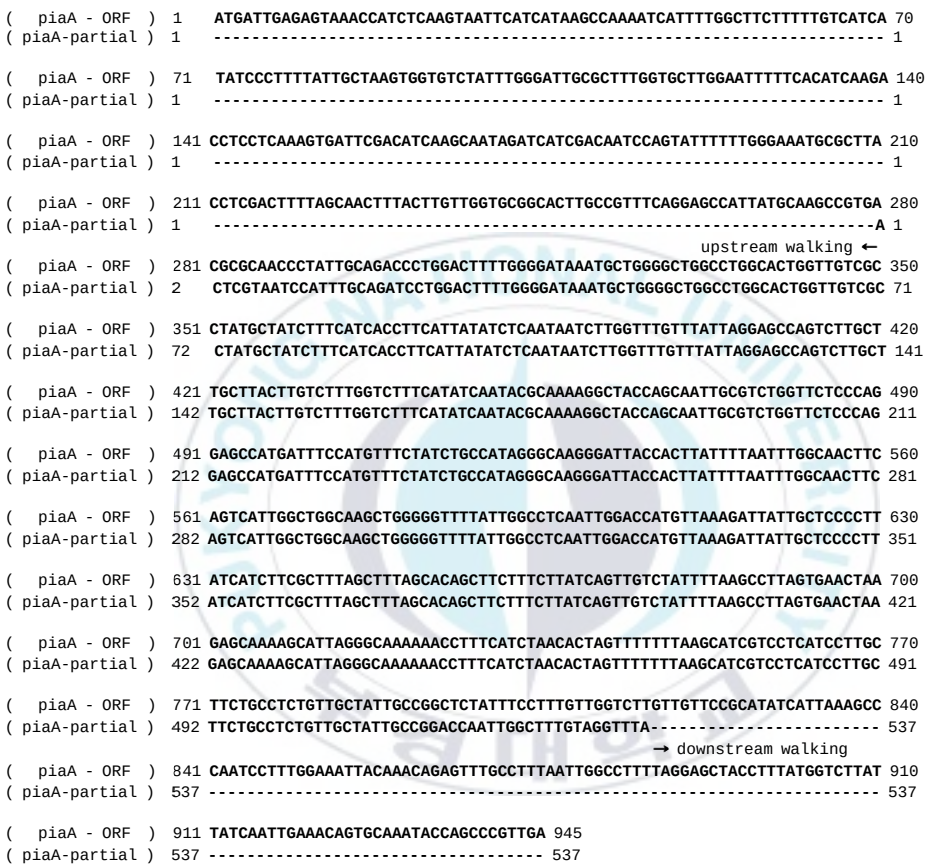


Fig. 12. piaA gene sequence by primer walking.

```
1   ATGATTGAGAGTAAACCATCTCAAGTAATTCATCATAAGCCAAAATCATTTTGGCTTCTTTTTGTCATCA 70
     M  I  E  S  K  P  S  Q  V  I  H  H  K  P  K  S  F  W  L  L  F  V  I

71  TATCCCTTTTATTGCTAAGTGGTGTCTATTTGGGATTGCGCTTTGGTGCTTGGAATTTTTCACATCAAGA 140
    I  S  L  L  L  L  S  G  V  Y  L  G  L  R  F  G  A  W  N  F  S  H  Q  D

141 CCTCCTCAAAGTGATTCGACATCAAGCAATAGATCATCGACAATCCAGTATTTTTTGGGAAATGCGCTTA 210
     L  L  K  V  I  R  H  Q  A  I  D  H  R  Q  S  S  I  F  W  E  M  R  L

211 CCTCGACTTTTAGCAACTTTACTTGTTGGTGCGGCACTTGCCGTTTCAGGAGCCATTATGCAAGCCGTGA 280
     P  R  L  L  A  T  L  L  V  G  A  A  L  A  V  S  G  A  I  M  Q  A  V

281 CGCGCAACCCTATTGCAGACCCTGGACTTTTGGGGATAAATGCTGGGGCTGGCCTGGCACTGGTTGTCGC 350
    T  R  N  P  I  A  D  P  G  L  L  G  I  N  A  G  A  G  L  A  L  V  V  A

351 CTATGCTATCTTTCATCACCTTCATTATATCTCAATAATCTTGGTTTGTTTATTAGGAGCCAGTCTTGCT 420
     Y  A  I  F  H  H  L  H  Y  I  S  I  I  L  V  C  L  L  G  A  S  L  A

421 TGCTTACTTGTCTTTGGTCTTTCATATCAATACGCAAAAGGCTACCAGCAATTGCGTCTGGTTCTCCCAG 490
    C  L  L  V  F  G  L  S  Y  Q  Y  A  K  G  Y  Q  Q  L  R  L  V  L  P

491 GAGCCATGATTTCCATGTTTCTATCTGCCATAGGGCAAGGGATTACCACTTATTTTAATTTGGCAACTTC 560
    G  A  M  I  S  M  F  L  S  A  I  G  Q  G  I  T  T  Y  F  N  L  A  T  S

561 AGTCATTGGCTGGCAAGCTGGGGGTTTTATTGGCCTCAATTGGACCATGTTAAAGATTATTGCTCCCCTT 630
     V  I  G  W  Q  A  G  G  F  I  G  L  N  W  T  M  L  K  I  I  A  P  L

631 ATCATCTTCGCTTTAGCTTTAGCACAGCTTCTTTCTTATCAGTTGTCTATTTTAAGCCTTAGTGAACTAA 700
    I  I  F  A  L  A  L  A  Q  L  L  S  Y  Q  L  S  I  L  S  L  S  E  L

701 GAGCAAAAGCATTAGGGCAAAAAACCTTTCATCTAACACTAGTTTTTTTAAGCATCGTCCTCATCCTTGC 770
    R  A  K  A  L  G  Q  K  T  F  H  L  T  L  V  F  L  S  I  V  L  I  L  A

771 TTCTGCCTCTGTTGCTATTGCCGGCTCTATTTCCTTTGTTGGTCTTGTTGTTCCGCATATCATTAAAGCC 840
     S  A  S  V  A  I  A  G  S  I  S  F  V  G  L  V  V  P  H  I  I  K  A

841 CAATCCTTTGGAAATTACAAACAGAGTTTGCCTTTAATTGGCCTTTTAGGAGCTACCTTTATGGTCTTAT 910
    Q  S  F  G  N  Y  K  Q  S  L  P  L  I  G  L  L  G  A  T  F  M  V  L

911 TATCAATTGAAACAGTGCAAATACCAGCCCGTTGA 945
    L  S  I  E  T  V  Q  I  P  A  R  *
```

Fig. 13. Nucleotide and deduced amino acid sequence of piaA of *Streptococcus iniae*. The top lines show the nucleotide sequence, the bottom lines the deduced amino acid sequence in the single-letter code.

Table 5. Similarities of nucleotide and deduced amino acid sequences of *Streptococcus iniae* iron uptake ABC transporter, piaA to those of other gram positive bacteria.

Bacteria	Genebank Accession number	Similarity (%)	
		Nucleotide	Amino acid
Streptococcus suis	ZP_00875973	57	76
Streptococcus pyogenes	YP_059666	60	76
Streptococcus agalactiae	ZP_00782854	56	72
Bacillus cereus	NP_830221	45	66
Exiguobacterium sibiricum	ZP_00540351	42	66
Bacillus weihenstephanensis	ZP_01183328	41	66
Clostridium acetobutylicum	NP_347425	38	65
Desulfitobacterium hafniense	YP_519805	37	64
Listeria monocytogenes	YP_014581	22	59

2. Recombinant protein purification and Western blot analysis

2.1 GAPDH

2.1.1 Recombinant protein production and purification

GAPDH gene of *S. iniae* was amplified from genomic DNA by PCR and inserted into the pET 28a vector. The recombinant protein was successfully expressed using *E. coli* BL21(DE3). The purified recombinant His-tagged GAPDH protein (Fig. 14) was used for immunization of rat to get antiserum, and the specificity of the produced rat antiserum against *S. iniae* GAPDH was confirmed by Western blot analysis.

2.1.2 Production of Ab against recombinant protein in the immunized rat

A. Western blot

The His-tagged GAPDH fusion protein and bacteria lysate were separated by SDS-PAGE. The band of approximately 40 kDa in His-tagged GAPDH fusion protein and the band of approximately 37 kDa in the lysate of *S. iniae*, *S. parauberis* and *L. garvieae* was observed. The protein size of the GAPDH was approximately 37 kDa, thus the band in Fig. 15. A lane 1 (*L. garvieae* lysate), lane 2 (*S. parauberis* lysate) and lane 3 (*S. iniae* lysate) was presumed GAPDH protein.

B. Bactericidal activity

In serum bactericidal activity test, antisera obtained from rats immunized with the recombinant GAPDH showed significantly higher bactericidal activities than sera obtained from rats injected with PBS (Fig. 16).

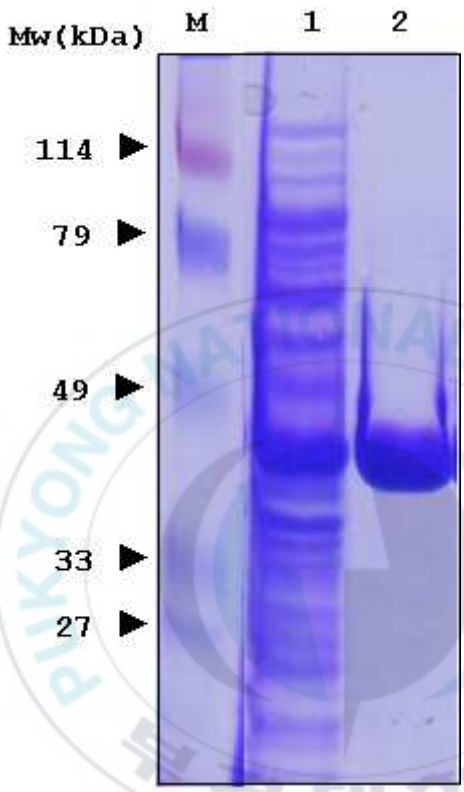


Fig. 14. Coomassie blue stained SDS-PAGE gel of His-tagged GAPDH of *Streptococcus iniae*. M : prestained protein marker (Pierce), Lane 1; Total lysate of bacteria with IPTG induction: Lane 2; Recombinant GAPDH protein purified by a Ni-NTA His-Bind® Resin (Novagen) open column.

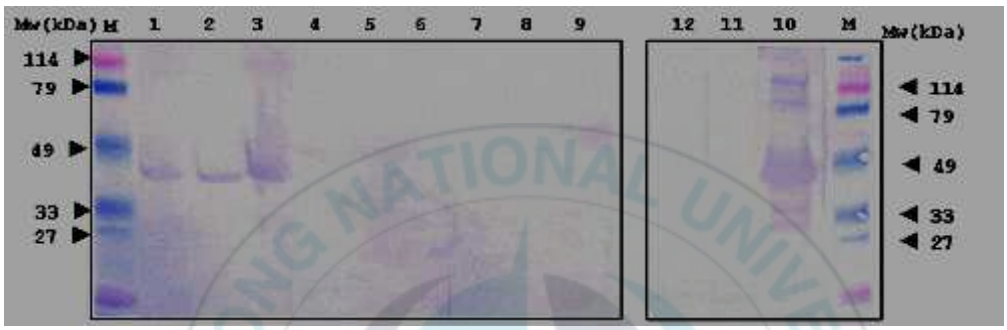


Fig. 15. Western blot analysis of recombinant GAPDH protein immunized-rat serum against purified GAPDH or *Streptococcus* lysate. M : prestained protein marker (Pierce), Lane 1, 4, 7; *L. garvieae* lysate: Lane 2, 5, 8; *S. parauberis* lysate: Lane 3, 6, 9; *S. iniae* lysate: Lane 10, 11, 12; recombinant GAPDH protein. The primary antibody in Lane 1, 2, 3, 10 was recombinant GAPDH injected immune serum, in Lane 4, 5, 6, 11 was PBS injected control serum and in Lane 7, 8, 9, 12 was adjuvant alone injected control serum.

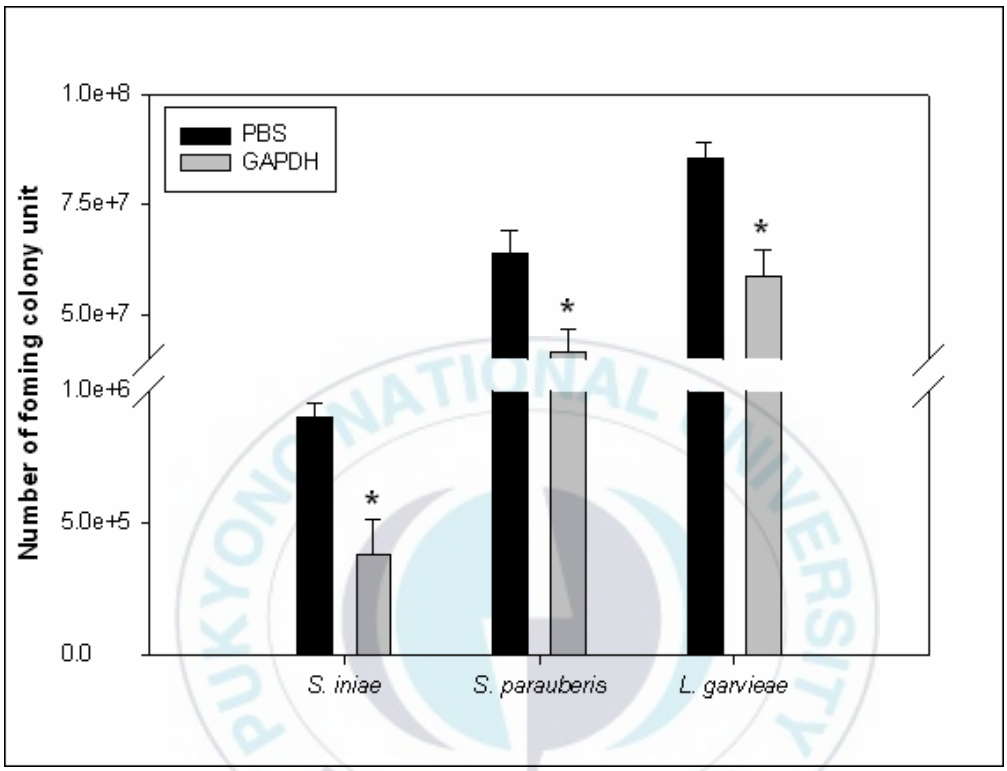


Fig. 16. Serum bactericidal activity of rat immunized intraperitoneally with Recombination GAPDH protein, PBS injected control and adjuvant alone injected control at 2 weeks post boost immunization. The bactericidal activity of the serum was expressed as the number of colony forming unit (CFU)/ml of three species of *Streptococcus* with serum. Values are means and T-bars indicate standard error. Bars with different letters indicate statistically differences at $P < 0.05$.

2.2 α-enolase

2.2.1 Recombinant protein production and purification

α-enolase gene of *S. iniae* was amplified from genomic DNA by PCR and inserted into the pET 28a vector. The recombinant proteins were successfully expressed using *E. coli* BL21 (DE3). The purified recombinant His-tagged α-enolase protein (Fig. 17) was used for immunization of rat to get antiserum, and the specificity of the produced rat antiserum against *S. iniae* α-enolase was confirmed by Western blot analysis.

2.2.2 Production of Ab against recombinant protein in the immunized rat

A. Western blot

The His-tagged α-enolase fusion protein and bacteria lysate were separated by SDS-PAGE. The band of approximately 50 kDa in His-tagged α-enolase fusion protein and the band of approximately 47 kDa in the lysate of *S. iniae*, *S. parauberis* and *L. garvieae* was observed. The protein size of the α-enolase was approximately 47 kDa, thus the band in Fig. 18. A lane 1 (*L. garvieae* lysate), lane 2 (*S. parauberis* lysate) and lane 3 (*S. iniae* lysate) was presumed α-enolase protein.

B. Bactericidal activity

In serum bactericidal activity test, antisera obtained from rats immunized with the recombinant α-enolase showed significantly higher bactericidal activities than sera obtained from rats injected with PBS (Fig. 19).

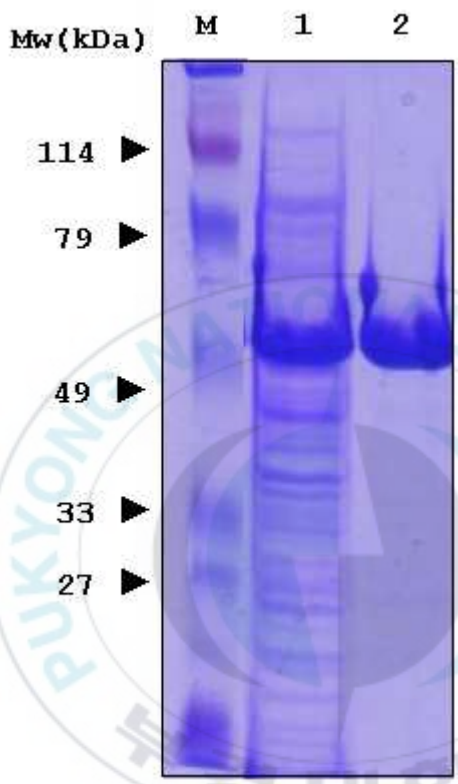


Fig. 17. Coomassie blue stained SDS-PAGE gel of His-tagged α-enolase of *Streptococcus iniae*. M : prestained protein marker (Pierce), Lane 1; Total lysate of bacteria with IPTG induction: Lane 2; Recombinant α-enolase protein purified by a Ni-NTA His-Bind® Resin (Novagen) open column.

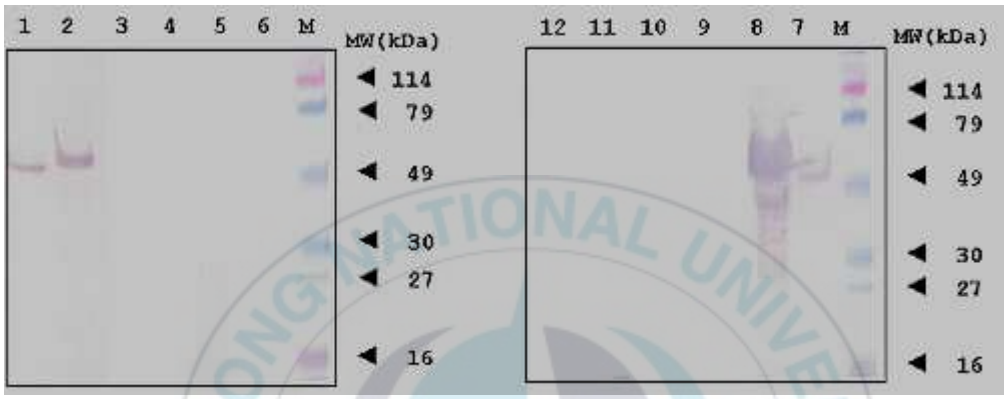


Fig. 18. Western blot analysis of recombinant α-enolase protein immunized-rat serum against purified α-enolase or *Streptococcus* lysate. M : prestained protein marker (Pierce), Lane 1, 3, 5; *S. parauberis* lysate : Lane 2, 4, 6; *L. garvieae* lysate: Lane 7, 9, 11; *S. iniae* lysate: Lane 8, 10, 12; recombinant α-enolase protein. The primary antibody in Lane 1, 2, 7, 8 was recombinant α-enolase injected immune serum, in Lane 3, 4, 9, 10 was PBS injected control serum and in Lane 5, 6, 11, 12 was adjuvant alone injected control serum.

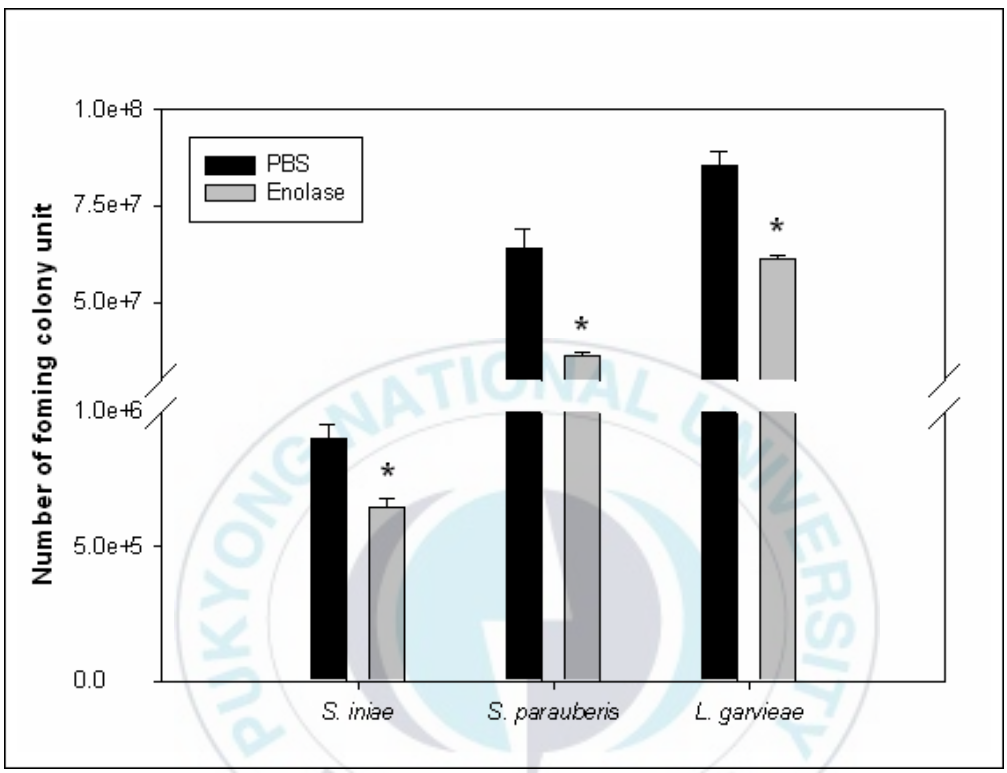


Fig. 19. Serum bactericidal activity of rat immunized intraperitoneally with Recombination α-enolase protein, PBS injected control and adjuvant alone injected control at 2 weeks post boost immunization. The bactericidal activity of the serum was expressed as the number of colony forming unit (CFU)/ml of three species of *Streptococcus* with serum. Values are means and T-bars indicate standard error. Bars with different letters indicate statistically differences at $P < 0.05$.

2.3 Streptolysin S-SagA

2.3.1 Comparison of sagA & sagA-toxic recombinant protein expression and purification

SagA & sagA-toxic gene of *S. iniae* was amplified from genomic DNA by PCR and inserted into the pGEX-4T-1 vector. The sagA-toxic recombinant proteins were successfully expressed using *E. coli* BL21 but, sagA recombinant proteins containing bacteriocin-like toxin sequence were not expressed. The purified recombinant GST fusion sagA-toxic protein (Fig. 21) was used for immunization of rat to get antiserum, and the specificity of the produced rat antiserum against *S. iniae* sagA-toxic was confirmed by Western blot analysis.

2.3.2 Production of Ab against recombinant protein in the immunized rat

A. Western blot

The GST fusion sagA-toxic protein and bacteria lysate were separated by SDS-PAGE. The band of approximately 28 kDa in GST fusion SagA-toxic protein and the band of approximately 2 kDa in the lysate of *S. iniae*, *S. parauberis* and *L. garvieae* was not observed. The protein size of the GST fusion sagA-toxic was approximately 28 kDa, thus the band in Fig. 22.

B. Bacterial activity of serum

In serum bactericidal activity test, antisera obtained from rats immunized with the recombinant sagA-toxic showed significantly higher bactericidal activities than sera obtained from rats injected with PBS (Fig. 23).

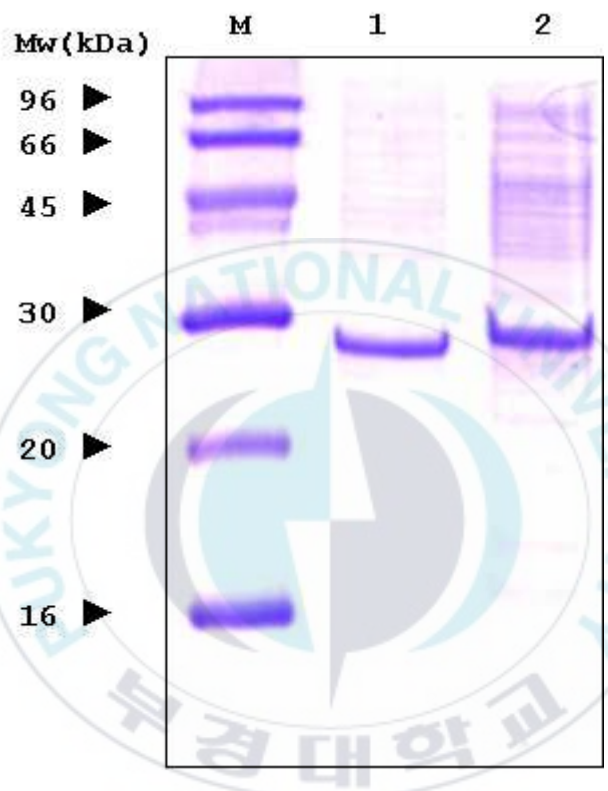


Fig. 20. Coomassie blue stained SDS-PAGE gel of GST protein and GST fusion sagA-toxic protein of *Streptococcus iniae* with IPTG induction. M : prestained protein marker (Pierce), Lane 1; GST protein is approximately 26 kDa; Lane 2; Recombinant sagA-toxic protein is approximately 28 kDa.

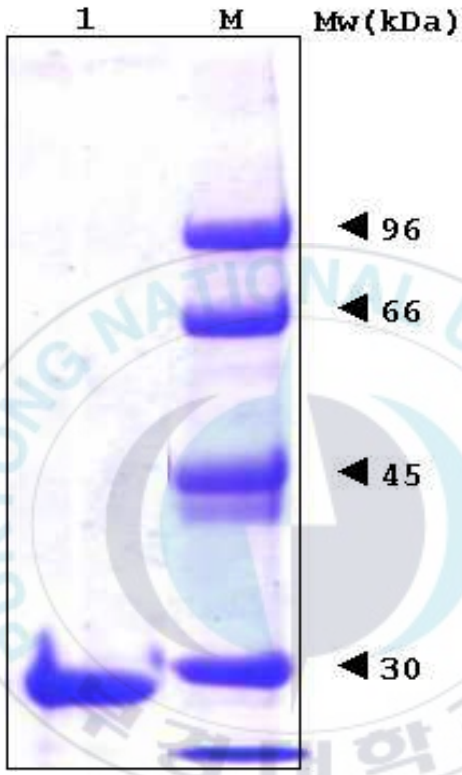


Fig. 21. Coomassie blue stained SDS-PAGE gel of GST fusion sagA-toxic of *Streptococcus iniae*. M : prestained protein marker (Pierce), Lane 1; Recombinant sagA-toxic protein purified by affinity chromatography using gluthatione-sepharose 4 FF columns (Amersham Biosciences)

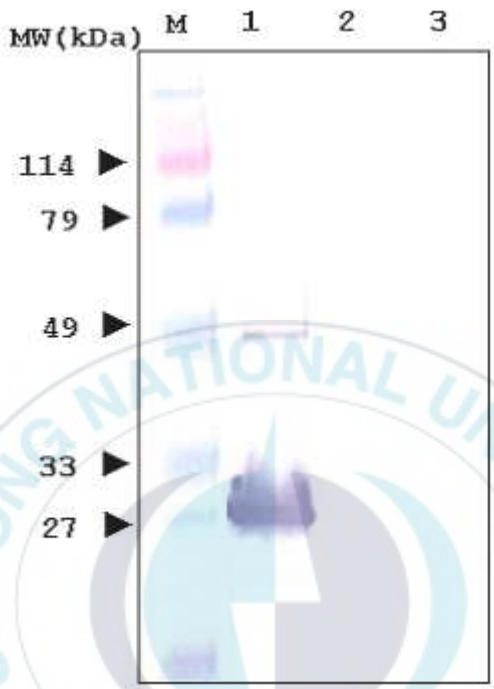


Fig. 22. Western blot analysis of recombinant sagA-toxic protein immunized-rat serum against purified SagA-toxic or *Streptococcus iniae* lysate. M : prestained protein marker (Pierce), Lane 1, 2, 3; recombinant SagA-toxic protein. The primary antibody in Lane 1 was recombinant SagA-toxic injected immune serum, in Lane 2 was PBS injected control serum and in Lane 3 was adjuvant alone injected control serum.

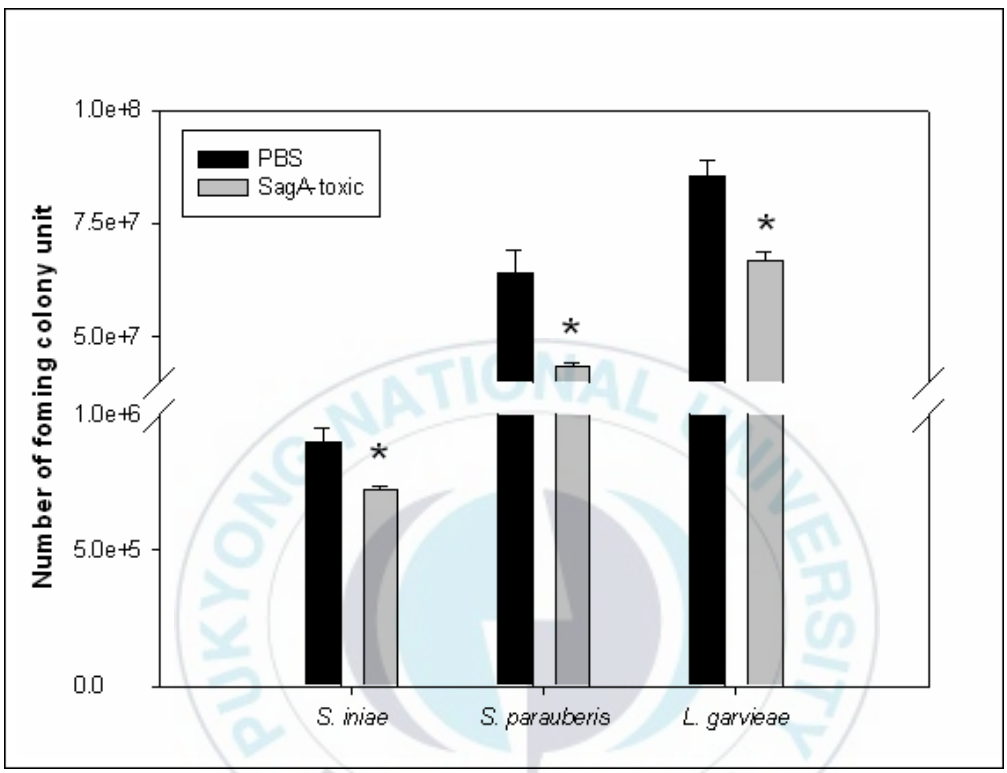


Fig. 23. Serum bactericidal activity of rat immunized intraperitoneally with Recombination sagA-toxic protein, PBS injected control and adjuvant alone injected control at 2 weeks post boost immunization. The bactericidal activity of the serum was expressed as the number of colony forming unit (CFU)/ml of three species of *Streptococcus* with serum. Values are means and T-bars indicate standard error. Bars with different letters indicate statistically differences at $P < 0.05$.

2.4 piaA

2.4.1 Recombinant protein production and purification

piaA gene of *S. iniae* was amplified by chromosome walking PCR using specific primers and inserted into the pGEX-4T-1 vector. For piaA recombinant protein was induced expression in cytoplasm, it was investigated transmembrane helices structure, previously. The piaA recombinat outer membrane protein successfully expressed using *E. coli* BL21 in cytoplasm. The purified recombinant GST fusion piaA protein (Fig. 26) was used for immunization of rat to get antiserum, and the specificity of the produced rat antiserum against *S. iniae* piaA was confirmed by Western blot analysis.

2.4.2 Production of Ab against recombinant protein in the immunized rat

A. Western blot

The GST fusion piaA protein and bacteria lysate were separated by SDS-PAGE. The band of approximately 28 kDa in GST fusion piaA protein and the band of approximately 34 kDa in the lysate of *S. iniae*. The protein size of the GST fusion piaA was approximately 28 kDa, thus the band in Fig. 27.

B. Bacterial activity of serum

In serum bactericidal activity test, antisera obtained from rats immunized with the recombinant piaA showed significantly higher bactericidal activities than sera obtained from rats injected with PBS (Fig. 28).

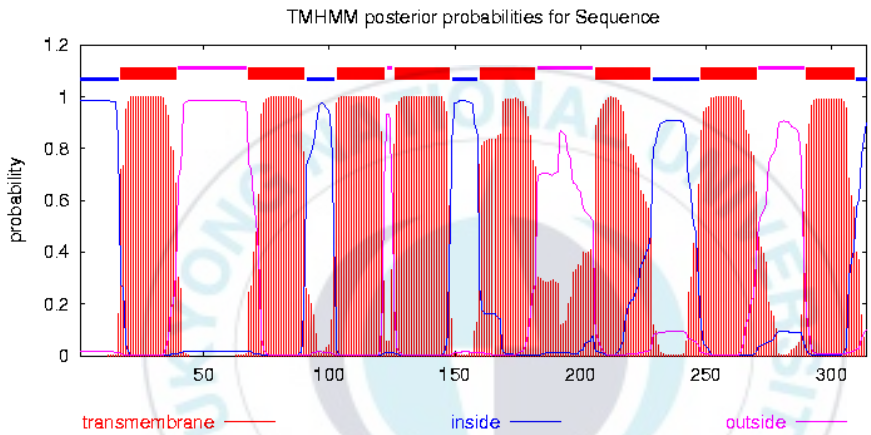


Fig. 24. Prediction of transmembrane helices in piaA protein.

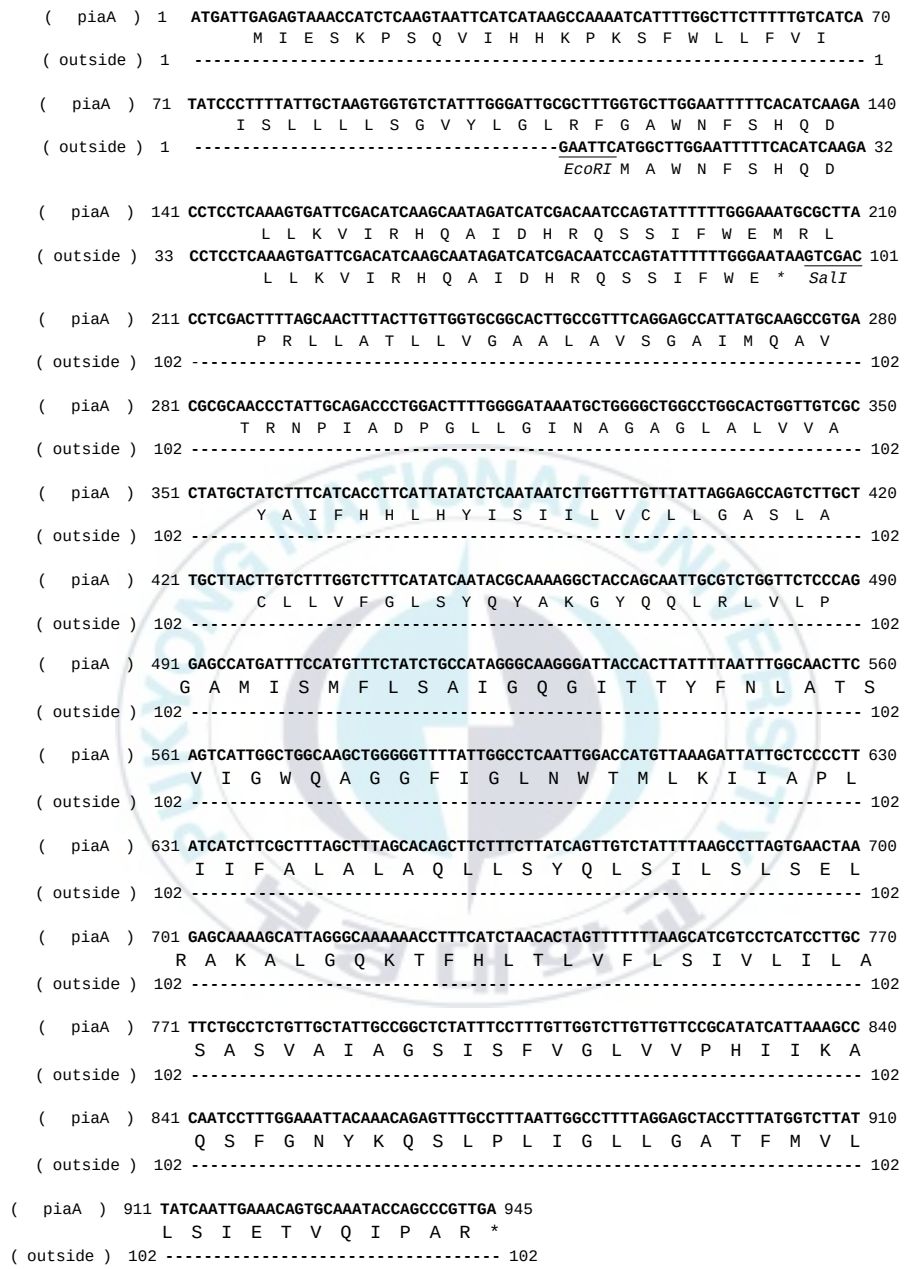


Fig. 25. Nucleotide and deduced amino acid sequence of piaA of *S. iniae* in outside region. The top lines show the nucleotide sequence, the bottom lines the deduced amino acid sequence in the single-letter code.

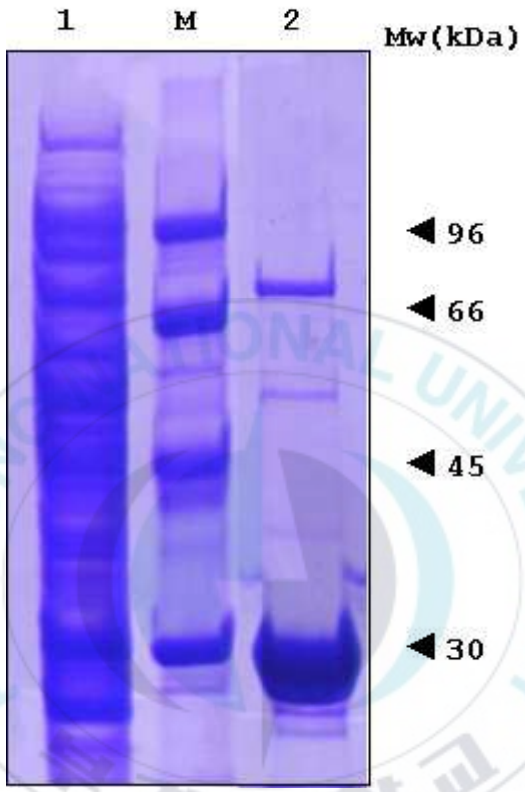


Fig. 26. Coomassie blue stained SDS-PAGE gel of GST fusion piaA protein of *Streptococcus iniae.* M : prestained protein marker (Pierce), Lane 1; Total lysate of bacteria with IPTG induction: Lane 2; Recombinant piaA protein purified by affinity chromatography using gluthatione-sepharose 4 FF columns (Amersham Biosciences).

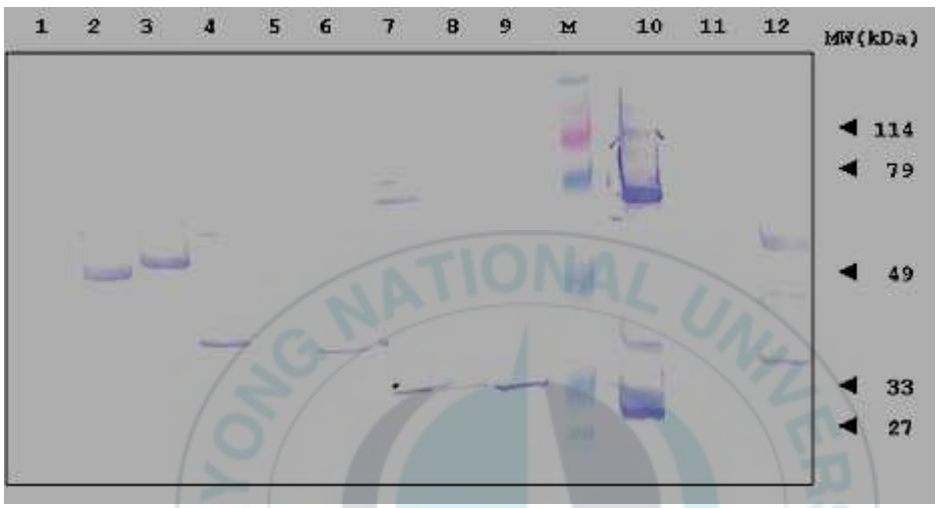


Fig. 27. Western blot analysis of recombinant piaA protein immunized-rat serum against purified piaA or *Streptococcus* lysate. M : prestained protein marker (Pierce), Lane 1, 4, 7; *S. parauberis* lysate : Lane 2, 5, 8; *L. garvieae* lysate : Lane 3, 7, 9; *S. iniae* lysate : Lane 10, 11 ,12; recombinant piaA protein. The primary antibody in Lane 1, 2, 3, 12 was adjuvant alone injected control serum, in Lane 4, 5, 6, 11 was PBS injected control serum and in Lane 7, 8, 9, 10 was recombinant piaA injected immune serum.

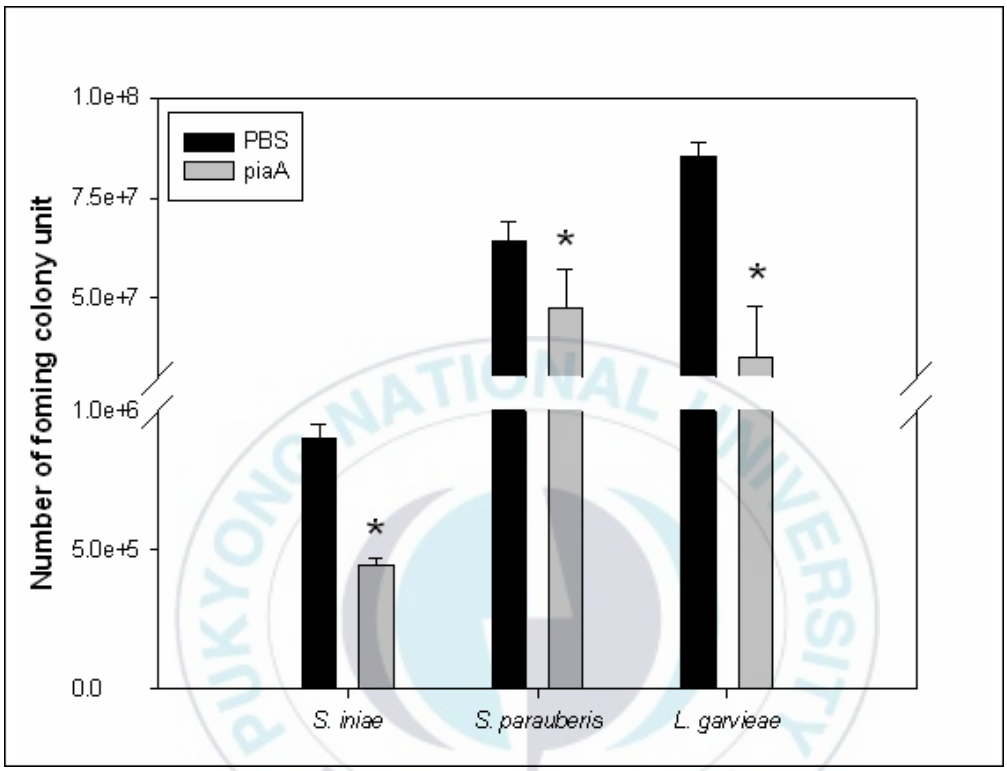


Fig. 28. Serum bactericidal activity of rat immunized intraperitoneally with Recombination piaA protein, PBS injected control and adjuvant alone injected control at 2 weeks post boost immunization. The bactericidal activity of the serum was expressed as the number of colony forming unit (CFU)/ml of three species of *Streptococcus* with serum. Values are means and T-bars indicate standard error. Bars with different letters indicate statistically differences at $P < 0.05$.

3. Cell wall association using immunoblot method

3.1 GAPDH

The cell wall proteins of *S. iniae* were extracted and the presence of GAPDH in the cell wall was analyzed by immunoblot using rat antiserum against the recombinant GAPDH of *S. iniae*. As a result, about a 40 kDa band, which was corresponded to the MW of GAPDH of *S. iniae*, was clearly detected (Fig. 29).

3.2 α-enolase

The cell wall proteins of *S. iniae* were extracted and the presence of α-enolase in the cell wall was analyzed by immunoblot using rat antiserum against the recombinant α-enolase of *S. iniae*. As a result, about a 50 kDa band, which was corresponded to the MW of α-enolase of *S. iniae*, was clearly detected (Fig. 30).

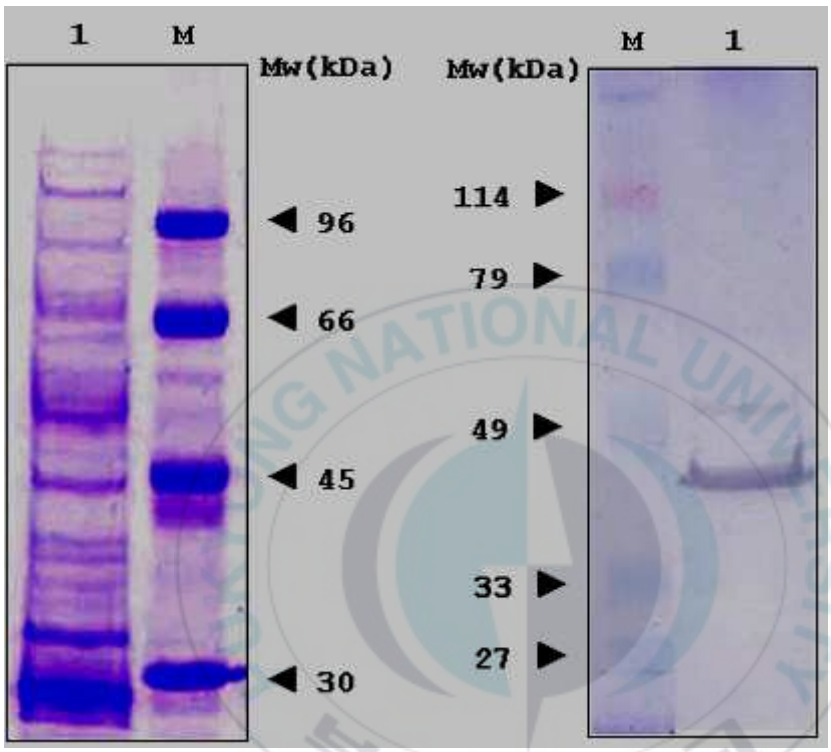


Fig. 29. Cell wall associating GAPDH of *Streptococcus iniae*. Left figure; SDS-PAGE gel of extracted cell-wall proteins of *S. iniae*: Right figure; Western blot of the transferred proteins onto a nitrocellulose membrane. M : prestained protein marker (Pierce), Lane 1 : extracted cell-wall proteins of *S. iniae.*

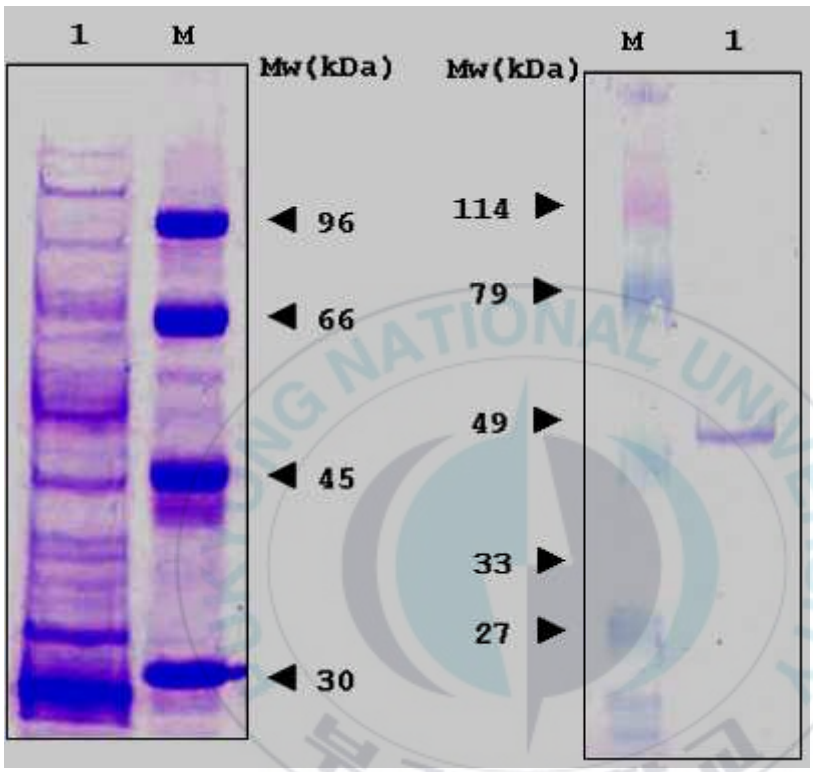


Fig. 30. Cell wall associating α-enolase of *Streptococcus iniae*. Left figure; SDS-PAGE gel of extracted cell-wall proteins of *S. iniae*: Right figure; Western blot of the transferred proteins onto a nitrocellulose membrane. M prestained protein marker (Pierce): Lane 1 extracted cell-wall proteins of *S. iniae*.

4. Plasmin(ogen) binding ability using immunoblot method

4.1 GAPDH

The plasmin-binding activity of the recombinant GAPDH of *S. iniae* was examined by Western blot. Human plasmin, and human immunoglobulin M (IgM as a control) was incubated with the recombinant GAPDH. The results of the immunoblot demonstrate that GAPDH of *S. iniae* has plasmin-binding activity (Fig. 31).

4.2 α-enolase

The plasmin-binding activity of the recombinant α-enolase of *S. iniae* was examined by Western blot. Human plasmin, plasminogen and human immunoglobulin M (IgM as a control) was incubated with the recombinant α-enolase. The results of the immunoblot demonstrate that α-enolase of *S. iniae* has plasmin(ogen)-binding activity (Fig. 32).

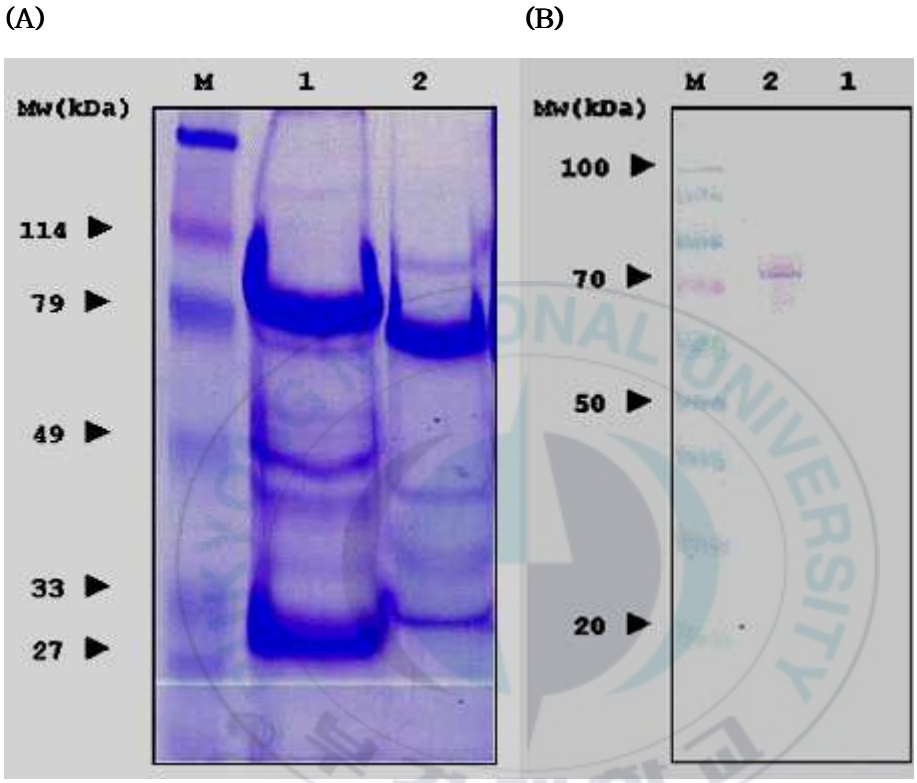


Fig. 31. GAPDH of *Streptococcus iniae* binding to plasmin.
(A) SDS-PAGE gel of immunoglobulin M (Lane 1) and plasmin (Lane 2; as a control). (B) Western blot of the transferred proteins onto a nitrocellulose membrane. M, prestained protein marker (Pierce) Lane 1, immunoglobulin M; Lane 2, plasmin.

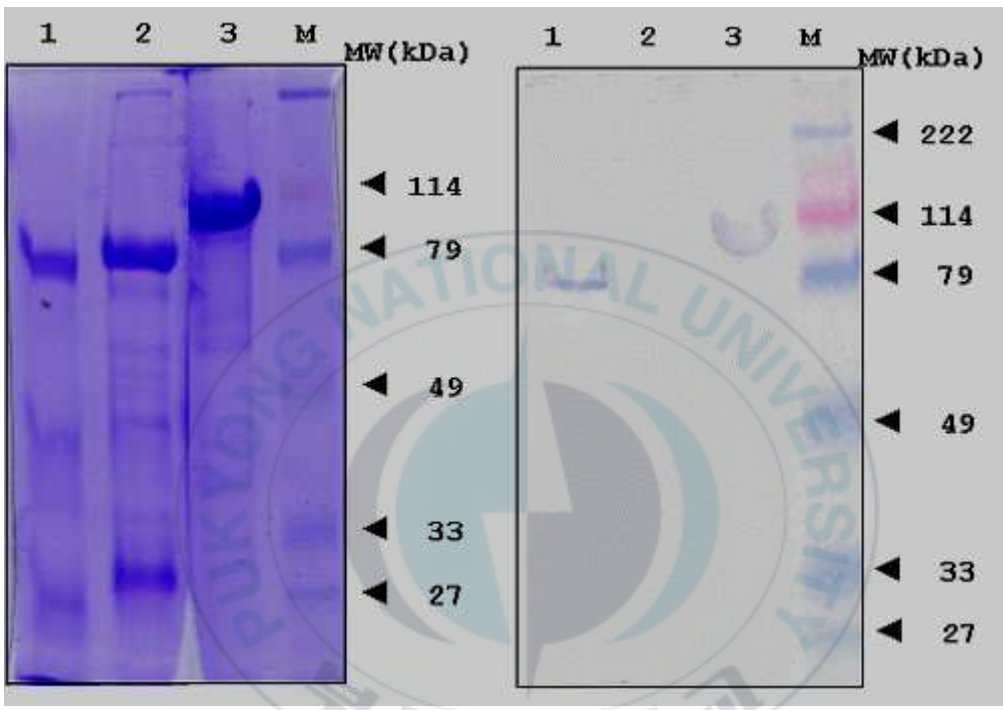


Fig. 32. α-enolase of *Streptococcusiniae* binding to plasmin.

(A) SDS-PAGE gel ofplasmin (Lane 1), immunoglobulin M (Lane 2; as a control) and plasminogen (Lane 3). (B) Western blot of the transferred proteins onto a nitrocellulose membrane. M, prestained protein marker (Pierce) Lane 1, plasmin; Lane 2, immunoglobulin M Lane 3, plasminogen.

5. Display the 4 genes on the surface of *E. tarda* ghost

5.1 Generation of ghost bacteria

Generation of ghosts in the transformants of *E. coli* and *E. tarda* carrying plasmid pHCE-InaN-4 genes-ghost 37 SDM was successfully performed by increasing the incubation temperature up to 42℃. Compared to *E. coli*, in which the lysis was observed within 30 min and completed 2 h after induction of *E* gene expression (Fig. 34), onset of *E. tarda* lysis was delayed until 2 h after temperature elevation and lysis process was completed 16 h after induction (Fig. 35).

5.2 Efficiency of *E. tarda* ghost generation

At the end of lysis process, the efficiency of ghost induction in non-lyophilized *E. tarda* was 99.99±0.01% as results of 10 replicate experiments. However, no bacterial growth was detected in lyophilized *E. tarda*.

5.3 Expression of 4-proteins

The expression of inserted streptococal genes in *E. coli* and *E. tarda* harboring pHCE-InaN-4 genes-ghost 37 SDM vector was confirmed by Western blot analysis (Fig. 36). Extracted outer membranes of *E. tarda* ghosts were subjected to Western blot analysis and successfully detected the antigens with anti-3 genes antibody, respectively (Fig. 37).

(A)

(B)

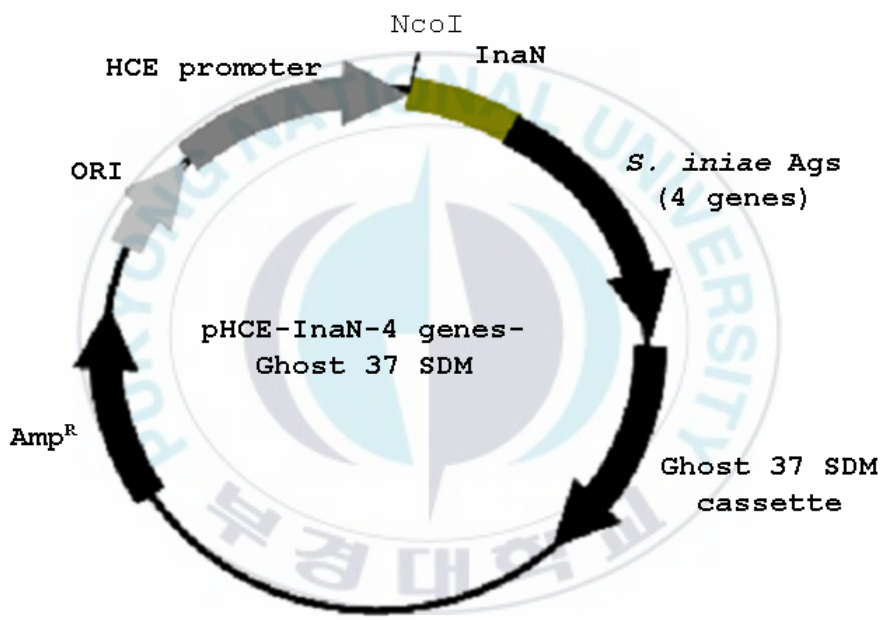


Fig. 33. (A) Partial restriction map of pHCE-InaN-4 genes-ghost 37 SDM. The plasmid harbors ice nucleation gene, 4 genes, the *E* lysis cassette, consisting of the lysis gene E, the leftward Lamda promoter, the temperature sensitive repressor $cI_{857,}$ and ampicillin resistance gene (Amp^R).

(B) Constructed vector map.

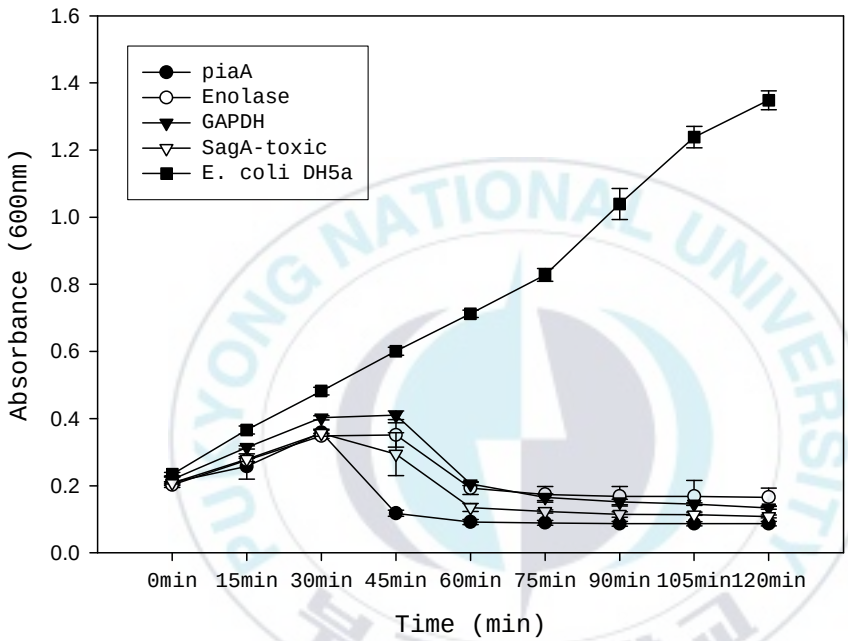


Fig. 34. Spectrophotometric observation for growth and lysis of *E. coli* DH5α harboring plasmid pHCE-InaN-4 genes-ghost 37 SDM by temperature induction of gene *E* expression. At time zero, the cultures were shifted from 37℃ to 42℃. Bars at each detection point indicates standard deviations.

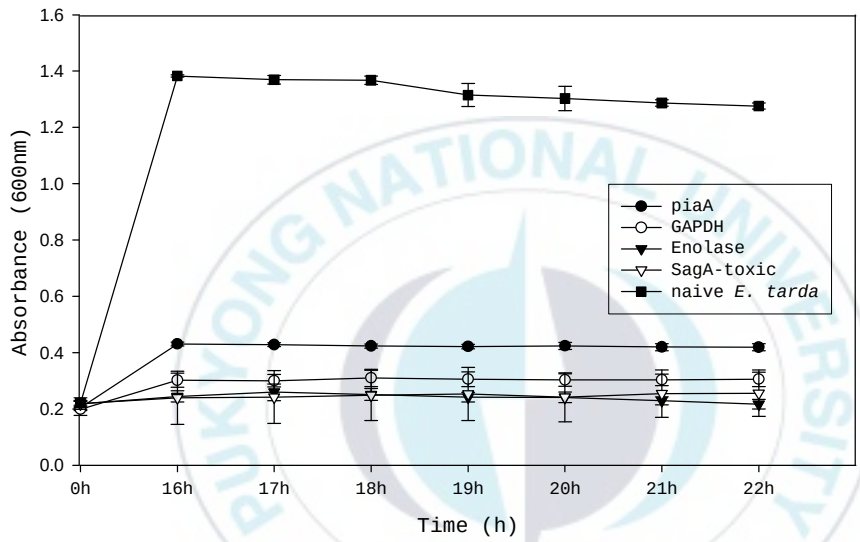


Fig. 35. Spectrophotometric observation for growth and lysis of *E. tarda* harboring plasmid pHCE-InaN-4 genes-ghost 37 SDM by temperature induction of gene *E* expression. At time zero, the cultures were shifted from 27℃ to 42℃. Bars at each detection point indicates standard deviations.

Fig. 36. Western blot analysis for *E. coli* harboring pHCE-InaN-3 genes-ghost 37 SDM and naive *E. coli*. (A); *E. tarda* harboring pHCE-InaN-3 genes-ghost 37 SDM and naive *E. tarda* (B). M: protein molecular marker (Pierce), lane 1 : *E. coli* or *E. tarda* harboring pHCE-InaN-GAPDH-ghost 37 SDM, lane 2 : *E. coli* or *E. tarda* harboring pHCE-InaN-α-enolase-ghost 37 SDM, lane 3 : *E. coli* or *E. tarda* harboring pHCE-InaN-SagA-ghost 37 SDM, lane 4: naive *E. coli* or *E. tarda*. Respectively, Anti-3 genes antibody was used for this assay. Molecular weight is given in kilodaltons (kDa).

(A) GAPDH (B) α-enolase (C) SagA

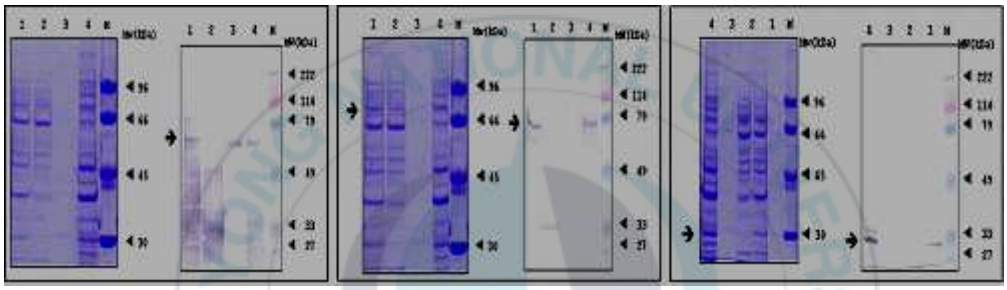


Fig 37. SDS-PAGE and Western blot analysis of outer membrane fractions. M: protein molecular marker, lane 1: total lysate, lane 2: cytoplasm, lane 3: inner membrane, lane 4: outer membrane. Respectively, Anti-3 genes antibody was used for this assay. Molecular weight is given in kilodaltons (kDa).

Discussion

The bacterial surface proteins play an important role in transport of nutrients, cellular metabolism as well as virulence-related functions such as evasion of host defenses, adhesion and invasion. In the present study, 4 genes - glyceraldehyde-3-phosphate dehydrogenase (GAPDH), α-enolase, streptolysin, and ABC iron transporter - were selected as subunit vaccine candidates against *Streptococcus iniae*.

The GAPDH is an enzyme of the glycolytic pathway responsible for the phosphorylation of GAP to generate 1,3-bisphosphoglycerate, besides having a cytoplasmic location, is cell wall-associating protein. The protein sequence homologies of GAPDH confirm that these pathogens possess a highly conserved GAPDH protein implicated in many surface specific activities, including a role as a plasmin receptor, host cytoskeletal protein binding affinities and signal transduction between bacteria and host cells (Winram and Lottenberg, 1996; Gase *et al*., 1996; Pancholi and Fischetti, 1992, 1997).

In the cell surface location of GAPDH, the protein may contribute to the microorganism's invasiveness by its ability to bind to various proteins of host. A prerequisite for the invasiveness of a pathogen is the pathogen's ability to breach epithelial as well as endothelial barriers in order to gain access to the submucosa and blood. A successful strategy used by pathogenic bacteria to degrade the extracellular matrix and to promote invasiveness is the recruitment of proteolytic activity to the bacterial cell surface (Lottenberg, R., 1997, Lottenberg, R. *et al*., 1994). *Streptococcus pneumoniae*, a common etiologic agent of respiratory tract diseases and

life-threatening invasive diseases, is able to capture plasminogen on the bacterial cell surface. Subsequent activation by tissue-type and urokinase-type eukaryotic plasminogen activators allows the bacteria to acquire surface-associated proteolytic activity (Eberhard, T. *et al.*, 1999, Kuusela, P. *et al.*, 1992). Plasminogen, a glycoprotein, is the zymogen of the serine protease plasmin, which is a key enzyme of the fibrinolytic pathway (Collen, D. *et al.*, 1975). The acquired plasmin activity promotes dissemination and transmigration of the pathogen through reconstituted basement membranes (Coleman, J. *et al.*, 1999, Eberhard, T. *et al.*, 1999, Lottenberg, R. *et al.*, 1994). Not only plasmin is binding GAPDH, but α-enolase was identified as the major plasminogen- and plasmin-binding protein of *S. iniae*.

The present results demonstrate that *Streptococcus iniae* α-enolase is also a plasmin(ogen)-binding and cell wall-associating protein similar to the enolase that has been described on the surface of mammalian streptococci (Pancholi and Fischetti, 1998; Bergmann *et al.*, 2001). The plasmin(ogen) binding property of pathogenic bacteria is suggested to be one of the characteristics that may contribute to tissue invasion and the overall pathogenicity of group A streptococci (Lottenberg *et al.*, 1994 Boyle andLottenberg, 1997). Typical examples of bacterial plasminogen activators are the streptokinase of pyogenic streptococci (Tewodros *et al.*, 1995) and the staphylokinase of *Staphylococcus aureus* (Matsuo *et al.*, 1990), and both act as secreted proteins. On the other hand, α-enolase of streptococci including the present *S. iniae* is not secreted but cell-wall associated. The ability of cell-wall associated α-enolase to bind plasmin(ogen) demonstrated here suggests that this protein may be responsible in part for the ability of *S. iniae* to cross tissue barriers thorough plasminogen activation. Recently, α-enolase present in the cell wall of *Candida albicans* has been reported as an abundant immunodominant antigen in invasive candidiasis (van Deventer *et*

al., 1994; Angiolella *et al.*, 1996), and induce a partially protective immune response against systemic candidiasis in mice (van Deventer *et al.*, 1996; Montagnoli *et al.*, 2004). In the case of *S. pyrogenes*, polyclonal antibodies to α-enolase significantly enhanced the phagocytosis of group A streptococci (Pancholi and Fischetti, 1998).

In addition to three species of streptococci share common epitopes of GAPDH and α-enolase, so that are to cross-react with different species of streptococci. In this study, the cross-recognition of GAPDH and α-enolase epitope among *S. iniae*, *S. parauberis* and *L. garvieae* was significantly higher bactericidal activity.

In conclusion, the identification of these proteins located on the outer surface of *S. iniae* may be of use in suggesting potential vaccine candidates.

Streptolysin S (SLS) has been described as the most potent of bacterial cytolytic toxins (Wannamaker, L. W. *et al.*, 1983). The potency of the toxin, the broad range of host cells that it lyses, and the fact that it is always bound to carrier molecules are thought to be the primary reasons that it is not immunogenic. We have shown for the first time that neutralizing antibodies can be evoked by a nontoxic synthetic peptide of SLS (SagA). This observation may have significant implications for our understanding of the role of SLS in the pathogenesis of group A streptococcal infections and also in the development of vaccines designed to prevent these infections and their complications. The characterization of SLS has been an elusive subject of intense interest for many years (Wannamaker, L. W. *et al.*, 1983).

Structural analyses of the translated proteins from the *sag* operon revealed that SagA resembles the family of bacteriocins, with a 23-amino acid leader peptide and a putative enzyme cleavage site following GG, which would result in a 30-amino-acid propeptide.

In addition, those authors predicted that other genes in the *sag* operon may

encode an enzyme responsible for posttranslational modification of the propeptide and integral membrane proteins that could possibly be involved in secretion of the toxin (Nizet, V. *et al*, 2000).

The structural similarities between the SagA propeptide and bacteriocins indicate that posttranslational modifications may lead to the formation of a cyclic structure at the N terminus of SagA resulting from thioester bonds between serine, threonine, or glycine residues and neighboring cysteine residues (Nizet, V. *et al*, 2000, Sahl, H. G. *et al*, 1998). In the present study, we hypothesized that the C-terminal 20-amino-acid peptide of SagA, which does not contain cysteine residues, would be devoid of toxicity and therefore may be immunogenic. When coupled to KLH, the SLS (10-30) peptide evoked antibodies that completely neutralized the hemolytic activity of native SLS in bacterial supernatants and on the bacterial cell surface.

The SLS-neutralizing activity of the synthetic peptide antisera indicates that the antibodies were evoked by epitopes whose conformation was maintained in the native toxin, even after the proposed posttranslational modifications (Nizet, V. *et al.*, 2000, Sahl, H. *et al.*, 1998). And as a result of bactericidal activity was significantly higher by recognizing the SagA epitope among *S. iniae*, *S. parauberis* and *L. garvieae.*

Because of the potency of SLS as a cytolytic toxin for many target cells and its known role in the virulence of GAS, further studies will focus on the potential role of SLS neutralizing antibodies in preventing infections or in modifying the outcomes of these infections. In addition, SLS peptides may be important components of vaccines.

The iron uptake ABC transporters Pia was required for full virulence in mouse models of septicaemia and pneumonia (Brown JS *et al*, 2001) and its lipoprotein components piaA have been investigated as potential vaccine candidates (Brown JS *et al*, 2001). Iron is one such nutrient which is

essential for the growth of most bacteria but whose restricted availability within the host forms a nutritional barrier to infection (Wandersman, C., 2000). As a consequence many bacterial pathogens contain specialized iron uptake mechanisms to acquire iron from iron-containing mammalian proteins such as transferrin, hemin, and ferritin, either by direct binding of the iron source to the bacterial surface or through secreted low-molecular-weight, high-affinity iron scavengers called siderophores (Cornelissen, C. N. *et al.*, 1994, Schryvers, A. B. *et al.*, 1999, Wandersman, C. *et al.*, 2000). Multiple and often partially redundant iron acquisition mechanisms are frequently present within a single pathogen, emphasizing the importance of iron acquisition for bacterial growth (Bearden, S. W. *et al.*, 1997, Bearden, S. W. *et al.*, 1999, Brown, J. S. *et al.*, 2001). Although pathogenic bacteria utilize a variety of environmental iron sources, frequently specific iron uptake ABC transporters transport the iron moiety into the cytosol across the membrane of gram-positive bacteria (Cabrera, G. *et al.*, 2001, Drazek, E. S. *et al.*, 2000).

We can report for the first time the cloning and characterization of the *S. iniae* piaA, iron uptake ABC transporter, which, on the basis of sequence homology, is likely to encode gram-positive bactera. By sequencing, we have confirmed that iron uptake ABC transporters are not highly conserved and varies significantly in amino acid sequence in gram-positive bacteria. However, analysis with anti-recombinant piaA serum of i.p. vaccinated rat indicate that piaA is likely to induce protection against *S. iniae*, *S. parauberis* and *L. garvieae*, and indeed in this paper we have demonstrated that the cross-recognition of piaA epitope among *S. iniae*, *S. parauberis* and *L. garvieae* was significantly higher bactericidal activity.

In the present study, these 4 proteins of *S. iniae* have identified potential vaccine candidates and they are produced recombinant *E. coli* (rECG) and *E.*

tarda (rETG) ghosts. The ghost bacteria is nontoxic, effective delivery vehicles with potent adjuvant properties, and are capable of inducing both T cell and Ab responses in mucosal tissues. We investigated the hypothesis that rECG and rETG could serve as effective delivery vehicles for subunit streptococcal vaccines to induce a high level of protective immunity.

Bacterial ghosts as candidate vaccines and carriers of foreign viral and/or bacterial antigens are under development as multivalent vaccines against diarrheal diseases of humans and might represent new, improved nonliving bacterial vaccines with excellent safety properties and high immunological potential.

Bacterial cell surface display of heterologous proteins can be useful in procedures such as development of live vaccines and multiple antigen antisera (Lee *et al.*, 2000). In the present study, a vector harboring double cassettes, a heterologous 4-genes expression cassette and a ghost cassette was constructed. Various display systems have been created for the expression of a number of heterologous proteins on the surface of gram-negative bacteria. Outer membrane proteins such as LamB (Charbit *et al.*, 1988), PhoE (Agterberg *et al.*, 1987), OmpA (Freudl, 1989), TraT (Harrison *et al.*, 1990), OprI (Cornelis *et al.*, 1996), OprF (Wong *et al.*, 1995) and INP (Jung *et al.*, 1998a) served as anchoring motifs for surface display.

Among them, in this study, we adopt ice nucleation protein (INP), because it include stable expression and outer membrane translocation and modulatable length of internal repeating units (Jung *et al.*, 1998b). INP is a membrane-bound protein which confers on host cells the ability to nucleate crystallization in supercooled water (Mararitis and Bassi, 1991).

Total expression level and surface display efficiency of heterologous proteins were compared following their fusion with either the N-terminal domain of InaK (InaKN), or with the known truncated InaK containing both N- and

C-terminal domains (InaK-NC). Truncated InaK containing only the N-terminal domain can be successfully employed as a cell surface display motif (Lin Li *et al.*, 2003).

In the present study, we used a truncated INP fragment consisting of only the N-terminal domain as an anchoring motif.

Several protein expression systems with various promoters have been developed for the effective expression of foreign proteins in bacteria. Among these, an *E. coli* expression vector including a *trp-lac* fusion promoter (*tac*, *pac*, *rac*, or *trc*), which can be effectively induced by IPTG, is the most frequently used system (Brosius *et al.* 1985; Donovan *et al.* 1996; Yarzábal *et al.* 1997). However, chemical inducers, including IPTG, are costly and can generate a safety problem as the presence of a chemical inducer in the purified recombinant protein product is potentially toxic to humans (Donovan *et al.* 1996). Such problems can be overcome by cloning foreign genes into a constitutive expression vector along with a constitutive promoter, which facilitates the high-level expression of foreign proteins without induction by chemical inducers (Chauhan *et al.* 2001). In the present study, we used a strong constitutive promoter (pHCE) isolated from the D-amino acid aminotransferase gene of *Geobacillus toebii* by Poo *et al.* (2002) as a promoter for foreign protein expression. As a result, the double cassettes vector, which can constitutively express foreign protein during bacterial culture and can also lysis gene *E* expression by temperature elevation, was constructed.

To confirm the ability of InaN to act as a display motif and the production of ghost by E lysis gene, we employed 4 target proteins.

As a result, all of proteins were identified production of rECG and rETG and GAPDH, α-enolase, SagA and piaA proteins were confirmed expression of rECG and ETG on the cell surface. (Fig. 33-34)

These data confirmed that the InaN signal can successfully and efficiently

direct translocation of foreign proteins to the cell surface.

The present recombinant ghost vaccine system coupled with its carrier and targeting functions of heterologous antigens have a great strategic potential for the development of new multivalent vaccines.

연쇄구균 (*Streptococcus iniae*) 항원 유전자 재조합 발현 및 면역학적 특성 분석

김 민 선

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어병학과

요약

현재 국내 양식 어류 특히 우리나라 양식 넙치에 발생하는 연쇄구균증은 *Streptococcus iniae, Streptococcus parauberis* 및 *Lactococcus garvieae*등 3종의 연쇄구균종에 의해 발생한다. 이러한 연쇄구균증은 심각한 폐사를 일으키고 있는데, 이에 대한 뚜렷한 예방 및 치료대책이 없는 실정이다. 연쇄구균증에 의한 질병과 폐사를 예방하고자 이들에 대한 백신 개발이 필요한데, 본 연구에서는 단위항원을 이용한 subunit vaccine을 개발하기 위해 *S. iniae*로 부터 GAPDH, α-enolase, Streptolysin S, iron uptake ABC transporter등의 항원 후보 유전자를 선정하여, 재조합 단백질 발현 및 분자 면역학적 특성을 분석하였다. 분석결과로부터 *S. iniae*의 GAPDH와 α-enolase는 cell wall protein 및 plasmin(ogen) binding protein 이라는 것을 알 수 있었다. hemolysin or cytolysin 작용을 나타내는 Streptolysin S (SagA) 및 세균의 생존 및 독성에 중요한 역할을 하는 철의 수송에 관련된 piaA에 대한 항체는 *S. iniae*에 대한 유의적인 bactericidal activity를 나타내었다. 이러한 분석결과로부터 본 연구에서 선정한 4개의 유전자는 추후 연쇄구균에 대한 subunit vaccine개발에 있어서 주요한 방어항원으로 활용될 수 있을 것으로 여겨진다. 본 연구에서는 또한 위 4개의 연쇄구균 유전자를 항원으로 하여 어류에 효율적으로 delivery 하기 위하여 *Edwardsiellar tarda* ghost bacteria의 표면에 이 항원들을 표면에 발현시키는 vaccine system을 제작함으로써 앞으로 어류 연쇄구균증에 대한 백신개발에 있어서 항원성 및 효율적인 전달을 가능케 하였다.

감사의 글

수산생명의학과와 인연을 맺게 된지 어느덧 5년이란 세월이 흘렀습니다. 이에 관심과 흥미를 가지고 마냥 새로운 맘으로 전공 수업을 들었던 일이 엊그제 같은데 벌써 졸업을 앞두고 있다는 것이 믿어지지가 않습니다. 이렇게 시간이 빠르게 느껴지는 것은 학교생활이 저에게는 무척 소중하고 값진 것이었기 때문일 것입니다.

수산생명의학과에서 공부하고 더불어 연구할 수 있었던 시간은 제게 있어서 앞만 보고 달려와 놓친 것, 잃은 것도 많았지만 그 만큼 제 인생 전체를 볼 때 가장 큰 발전과 발돋움의 기간이었습니다. 비록 제가 타과에서 수산생명의학과로 전과하였지만 뒤처지지 않고 무사히 졸업을 할 수 있게 된 것은 뒤돌아보면 그동안 저를 도와준 많은 고마운 분들이 계셨다는 것을 다시금 생각하게 합니다.

먼저 본 논문의 처음 연구계획에서부터 완성에 이르기까지 학문적 기틀을 잡아 주시고 친절하고 소상한 가르침을 베풀어 주셨던 김기홍 지도교수님께 진심으로 깊은 감사를 드립니다. 여러모로 부족한 제가 논문이라는 작은 결실을 맺을 수 있도록 심사해주시며 아낌없는 격려와 지도를 하여 주신 허민도 교수님, 남윤권 교수님, 그리고 수산생명의학이라는 귀한 학문적 지식을 쌓을 수 있도록 도와주신 박수일 교수님, 정현도 교수님, 정준기 교수님, 강주찬 교수님께도 감사드리며, 항상 자상하신 모습으로 미소를 잃지 않도록 해주신 김성구 교수님께도 머리숙여 감사의 말씀을 드립니다.

아무것도 모르는 철없던 시절에, 어패류기생충학 연구실에 입방하게 된 것은 제겐 큰 행운이었습니다. 어패류기생충학 연구실이 지금까지 발전할 수 있도록 이끌어주신 장명덕 선배님, 황윤정 선배님, 홍주원 선배님, 최은석 선배님, 안경진 선배님께 감사드리며, 함께 실험실 생활을 하며 무지한 저에게 많은 가르침과 조언을 해주신 이선정 선배님, 조재범 선배님, 김천수 선배님, 이찬휘 선배님, 정재혁 선배님, 권세련 선배님, 이은혜 선배님, 김성미 선배님, 김형준 선배님, 코스케 오빠께 감사드립니다. 그리고 자신의 꿈을 위해 한걸음 한걸음 열심히 앞으로 나아가고 있는 이종원 선배님, 서성택 선배님, 성현이 오빠, 실험실에 같이 입방하면서 가장 힘이 많이 되

어준 승혁이, 수경이에게도 고마운 마음을 전합니다. 실험실 방장을 맡아 여러모로 애쓰고 있는 동진이, 실험하는데 있어서 착실하게 많이 도와준 예재 오빠, 기준이, 은숙이, 짧은 시간이지만 함께 하며 큰 힘이 되어준 선영이, 상효에게도 고마움을 전하고 싶습니다. 부족함 없이 실험할 수 있도록 도와주신 우승호 선배님, 정준범 선배님, 전려진선배님, 김재훈 선배님, 김호열 선배님, 신순범 선배님, 박신후 선배님, 이현정 선배님, 류지효 선배님께도 감사드립니다. 석사과정 2년동안 함께 열심히 하여 이번에 같이 학위를 받을 성돈선배, 지영이언니, 정현이, 수진이, 유미, 윤임이에게도 수고하셨다는 말과 감사의 말을 전합니다.

기쁠때나 슬플때나 지치고 힘들 때, 언제나 옆에서 생활의 활력소가 되어준 친구 미지, 재순이, 지연이, 민지, 정순이, 은경이, 은주, 정희, 진희에게도 너무나 고맙고 사랑한다는 말을 전하고 싶습니다.

늘 한결같은 마음으로 큰딸을 곁에서 지켜보고 지지하고 지원해주시는 부모님, 한 살 차이로 많이 다투었지만 정말 사랑하는 하나뿐인 동생 민정이에게도 감사의 마음을 전합니다.

좁은 지면에 그분들을 일일이 열거하면서 감사의 마음을 전하기에는 부족합니다. 하지만, 이 모든 것이 결코 저 혼자의 힘만으로 된 것이 아니었음을 되새기며 이 밖에 저를 도와주신 제 주위 모든 분들께 감사의 말씀을 드립니다.

오늘의 작은 열매에 자만하지 않고 매사에 최선을 다하여 노력하는 자세로 계속 정진할 수 있도록 열심히 공부하고 연구하며 살아가겠습니다.

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