Thesis for the Degree of Master of Engineering

Cloning of phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene of *Vibrio anguillarum* related to synthesis of LPS and construction of knock out mutant

by

Ryunkyoung Oh

Department of Biotechnology

The Graduate School

Pukyong National University

August 2012

Cloning of phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene of *Vibrio anguillarum* related to synthesis of LPS and construction of knock out mutant

*Vibrio anguillarum*로부터 LPS 생합성에 관여하는 phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene 의 클로닝과 돌연변이 균주 제작

Advisor: Prof. In-Soo Kong

by

Ryunkyoung Oh

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Engineering

in Department of Biotechnology, The Graduate School,
Pukyong National University

August 2012

Cloning of phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene of *Vibrio anguillarum* related to synthesis of LPS and construction of knock out mutant

A dissertation
by
Ryunkyoung Oh

Approved by:

_____
(Chairman) Joong Kyun Kim Ph. D.

_____ _____
(Member) Gwi-Taek Jeong Ph. D. (Member) In-Soo Kong Ph. D.

August 24, 2012

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Cloning of phosphomannomutase/phosphoglucomutase gene (*pmm/pgm*) of *Vibrio anguillarum* related to synthesis of LPS and construction of knock out mutant

Ryunkyoung Oh

Department of Biotechnology, The Graduate School,

Pukyong National University

Abstract

We sequenced 1341bp ORF of phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene from *Vibrio anguillarum,* the causative agent of fish vibriosis catalyzes the interconversion of mannose 1- phosphate and mannose 6- phosphate, and glucose 1- phosphate and glucose 6- phosphate. We found that *pmm/pgm* gene was up regulated under cold stress by Reverse Trancriptase-Polymerase Chain Reaction (RT-PCR). We constructed knock out mutant by homologous recombination to identify relation between PMM/PGM protein and synthesis of lipopolysaccharide (LPS). LPS from *V. anguillarum* wild type and mutant was isolated. We checked high molecular weight (HMW) of LPS was immigrated and several bands smeared by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Growth and viability of mutant was lower than *V. anguillarum* wild type on stationary phase. In this study, we identified *pmm/pgm* gene has a important role for biosynthesis of LPS.

I. INTRODUCTION

Vibrio anguillarum, the causative agent of fish vibriosis, is a fatal hemorrhagic septicemic disease of fish and causes huge economic losses in aquaculture industry (1). It causes vibriosis in about 50 species of fish, including cultured and wild fish, mollusks, and crustaceans, in marine, and fresh water. About 23 serotypes of *V. anguillarum* have been reported, and among them the O1 and O2 serotypes are the most significant pathogens of fish vibriosis (9, 19).

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria which makes up approximately 75% bacterial membrane (17). Structure of LPS is divided into three parts: lipid A, core oligosaccharide, and O-antigen (3). LPS is contributed greatly to the structural integrity of the bacteria, and responsible for permeability barrier (14), resistance to serum, antibiotics (17), and naturally occurring antimicrobial peptides (2). LPS acts as an important adhesion molecule (13) and as endotoxin (17). In various environments, the bacterium utilizes LPS to protect its intracellular contents from certain kinds of damaging or conditions (18). Bacteria communicate with, react and adapt to their environment thorough the

outer membrane (4).

Lipid A is highly conserved and exerts the endotoxic activity, while the O-antigen carbohydrate chain is a polymer of repeating oligosaccharides, which differs between species and is responsible for the serological specificity of bacteria (6).

Phosphomonnomutase (PMM)/Phosphoglucomutase (PGM) belongs to α -D-phosphohexomutase enzyme superfamily and is an essential enzyme for biosynthesis of LPS (5). PMM/PGM encoded by the gene *pmm/pgm* catalyzes the interconversion of mannose 1-phosphate and mannose 6-phosphate, and glucose 1-phosphate and glucose 6-phosphate via the enzyme-bound intermediate, glucose 1,6-bisphosphate. Glucose 1,6-bisphosphate acts as cofactor to remain in an active phosphorylated form (5) (Fig. 1).

In this study, we sequenced *pmm/pgm* gene and constructed knock out mutation of *pmm/pgm* gene from *V. anguillarum* to check the role of *pmm/pgm* gene related to biosynthesis of LPS, and the protein encoding by this gene from *V. anguillarum* was cloned and overexpressed in *E. coli*.

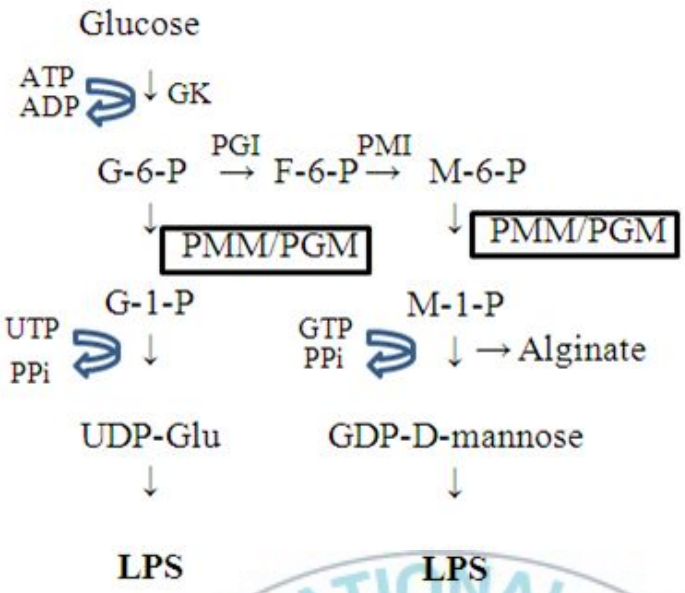

Fig. 1. Biosynthetic pathway of the formation of LPS

GK: glucose kinase; G-6-P: Glucose-6-phosphate; G-1-P: Glucose-1-phosphate; UDP-Glc: UDP-glucose; PGI: Phosphoglucose isomerase; PMI: Phosphomannose isomerase; F-6-P: Fructose6-phosphate; M-6-P: Mannose-6-phosphate; M-1-P: Mannose-1-phosphate; GDP-man: GDP-manose

II. MATERIALS AND METHODS

1. Bacterial strains, plasmid, and culture condition

The strains of bacteria and plasmids used in this study are listed in Table 1. *Vibrio anguillarum* O1 wild type as parent strain and *E. coli* strain SM10λ*pir* was used as host strain for mutagenesis experiments. Wild strain and mutant was grown at 25℃ in brain heart infusion (BHI) broth (Difco) or on BHI agar plates.

E. coli strains for cloning host strain were grown at 37℃ in Luria-Bertani (LB) broth (Difco) or on LB agar plates. pET-28a(+) was used as cloning vector and pNQ705 was used as suicide vector for knock out mutation. Appropriate antibiotics (Kanamycin at 100 μg/ml and Chloramphenicol at 10 μg/ml) were added to the media when required.

Table 1. Bacterial strains and plasmids used in this study

Strains and plasmid	Relevant properties	Reference or source
Strains		
V. anguillarum O1	wild type	Holmstrøm, and Gram (2003)
E. coli		
DH5a	cloning host strain	Promega
BL21(DE3)	cloning host strain	Novagen
SM10λ*pir*	Donor strain for conjugation Km^r	Miller and Mekalanos (1988)
Plasmids		
pNQ705	suicide vector Cm^r Mob. Ori R	Milton et al. (1992)
pET-28a(+)	His tag fusion expression vector; Km^r	Novagen

2. Sequencing

ABI 3730xl 96-capillary DNA analyzer (Applied Biosystems, USA) was used for sequencing the Polymerase chain reaction (PCR) product. Upstream and downstream of open reading frame (ORF) were identified by DNA Walking SpeedUP Premix Kit (Seegene, Korea).

3. RNA isolation and cDNA synthesis

Total RNA was isolated by TRIzol Reagent (Invitrogen, USA) using protocol of Invitrogen. After isolation, DNase I (TaKaRa, Japan) was added to get pure RNA. cDNA was synthesis using First strand cDNA synthesis kit for RT-PCR (Promega, USA). Synthesized cDNA was stored at -20℃.

4. RT-PCR

PCR amplification reactions were carried out in a final volume of 20 μl containing 1 μl of cDNA as template, 1X PCR buffer containing $MgCl_2$, 0.2 mmol/l of dNTP, 0.6 U of Taq polymerase (TaKaRa, Japan) and 1pmol of each primer. The PCR conditions were denaturation at 94℃ for 5 min followed by 25 cycles of denaturation at 94℃ for 30 s, annealing at 55℃ for 30 s, and extension at 72℃ for followed by final extension at 72℃ for 7 min using a thermal cycler (2720 Thermal cycler; Applied Biosystems, Carlsbad, CA, USA).

5. Overexpression and purification of recombinant protein

Overexpression plasmid was constructed with primers based on the *pmm/pgm* gene sequence (Forward: 5′- GGCC CATATG TCT GAC AAA AGA

CGT TAC -3', the *Nde*I site is underlined; Reverse: 5'- GGCC GGATCC GCA ATT ATC TTT AAC CGC TTG-3', the *Bam*HI site is underlined). Polymerase chain reaction (PCR) was performed with primers to amplify the 1341 bp open reading frame (ORF) of the *pmm/pgm* gene. PCR amplification reactions were carried out in a final volume of 50 μl containing 1 μl of DNA template, 1 X PCR buffer containing $MgCl_2$, 0.2 mmol/l of dNTP, 0.6 U of Taq polymerase and 1 pmol of each primer. The PCR conditions were denaturation at 94℃ for 5 min followed by 25 cycles of denaturation at 94℃ for 30 s, annealing at 57℃ for 30 s, and extension at 72℃ for followed by final extension at 72℃ for 7min using a thermal cycler (2720 Thermal cycler; Applied Biosystems, Carlsbad, CA, USA).

The 1341 bp *Nde*I-*Bam*HI fragment was ligated to pET-28a(+). The recombinant plasmid was introduced into the *E. coli* BL21. Cell was cultivated in 300 ml of LB liquid medium containing kanamycin 100 μg/ml and grown at 37℃ until the culture optical density at 600nm reached up to 0.4. IPTG (Isopropyl-D-1- thiogalactopyranoside) was added at a final concentration of 1 mM, and growth was continued for 4 h. Cells were harvested and suspended with 40 ml of 50 mM Tris-HCl buffer (pH 8.0). Suspended cell was disrupted by sonication and centrifuged at 12000 rpm for 10 min at 4℃. Supernatant

was purified with Ni-NTA his-tag affinity chromatography. After purification, collected fraction was dialyzed in 20 mM Tris-HCl buffer (pH 8.0).

6. PMM and PGM assay

PMM and PGM activity was assayed following the method of Köplin et al. (1992). Reaction buffer (1 ml) contained MOPS (Morpholine propanesulfonic acid) buffer (pH 7.6), 1mM mannose-1-phosphate, 20 mM $MgCl_2$, 1.0 mM NADP, 0.075 mM glucose 1,6-bisphosphate, 0.7 unit glucose-6-phoshphate dehydrogenase, 1 unit phosphoglucose isomerase, 1 unit phosphomannose isomerase and mixed with enzyme (100 µl) for PMM assay. Reaction buffer (1 ml) contained MOPS (morpholine propanesulfonic acid) buffer (pH 7.6), 1 mM glucose-1-phosphate, 20 mM $MgCl_2$, 1.0 mM NADP, 0.075 mM glucose 1,6-bisphosphate, 0.7 unit glucose-6-phoshphate dehydrogenase with enzyme (100 µl) for PGM assay. Reaction mixture was incubated at 25 ℃ for 15 min, and the formation of NADPH was monitored at 340 nm with UV-spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 µmol of NADP per min under the assay conditions.

7. Construction of *pmm/pgm*::pNQ705 mutant

The 370 bp fragment of *pmm/pgm* gene from *V. anguillarum* was amplified by PCR in order to construction of *pmm/pgm* mutant using primers (Forward: 5′- GGCC GCTGAC GCT GAA AAT CGT CGT TGA -3′, the *Sal*I site is underlined; Reverse: 5′- GGCC TCTAGA ACC TAA CTG CTT TAA GC -3′, the *Xba*I site is underlined). The 370 bp SalI-XbaI fragment was ligated to suicide vector pNQ705. The recombinant plasmid was introduced into the conjugal donor *E. coli* SM10λ*pir*. Conjugation between recipient *V. anguillarum* wild type and the donor *E. coli* SM10λ*pir* containing the suicide plasmid (pNQ705) was perfomed. The mixture of cells was dropped, incubated for at least 8 h on LB agar plate. Grown cells were taken with sterile cotton swabs, suspended in 1 ml of BHI liqud medium, and spreaded on TCBS (Thiolshlfate cutrate bile salts) agar plate containing chloramphenicol 10 μg/ml to select mutants.

8. PCR for knock out mutant

To confirm knock out mutant, we amplified PCR by using primers listed in Table 2.

Table 2. Primers used in this study for knock out mutant

primers	sequence
VA-KO-check up	5′- GGA TAA ACC GAT TGA GTG TGT CGA G -3′
pNQ705 rp-I	5′- GTT GTG GAC AAC AAG CCA GGG ATG -3′
pNQ705 rp-II	5′- CGA ACT AAA CCC TCA TGG CTA ACG -3′

9. Isolation of Crude LPS and SDS-PAGE

Crude LPS was isolated by hot phenol/water method (Westphal et al. 1965). Wild type and mutant were incubated in BHI liquid medium for 12 h. Cells were suspended with 50 mM sodium phosphate pH 7.0 containing 5 mM EDTA, mixed with lysozyme (100 mg) and incubated at 4℃ for 12 h. The cell suspension was incubated at 37℃ for 20 min, mixed with 20 mM $MgCl_2$ and incubated at 37℃ for 2 h. The cell suspension was mixed with equal volume of 90% hot phenol, incubated at 68℃ for 30 min. After cooling in ice water bath for 15 min, the phase were separated by centrifugation (12000 rpm, 20 min, 4℃). Aqueous phase was carried out and dialyzed for 48 h until the residual phenol was totally eliminated. Dialyed sample was dissolved with distilled water. Sample was suspendned in 5 X sample buffer (2% sodium dodecyl sulfate, 14.4 mM mercaptoethanol, 1 M Tris-HCl (pH 6.8), 25% glycerol, 0.1% bromophenol blue) and the samples were boiled for 10 min. LPS profiles were determined by sodium dodecyl sulfate-polyacrylamaid gel electrophoresis (SDS-PAGE). LPS was visualized using a silver staining kit. (Biosesang, Korea)

III. RESULTS and DISCUSSION

1. Sequencing and analysis of amino acid sequences

We sequenced 1341 bp ORF and 447 amino acid residues by DNA Walking SpeedUp Premix Kit (Seegene, Korea). The amino acid sequence encoded by *pmm/pgm* was compared with data available in the GenBank database using the BLAST network service at the National Center for Biotechnology Information. Alignments to determine protein similarities was performed with the ClustalW program. A 1341 bp ORF and 447 amino acid residue of *pmm/pgm* gene was compared with other *Vibrio* species (Fig. 2). The homology was high level 86.5% - 92.3%.

The three highly conserved site of phosphohexomutase were found in ORF among eight species. Active site, the catalytic phosphoserine residue, which is located between 102-106 and necessary for phosphoryl transfer to and from the bisphosphorylated reaction intermediate. Metal binding site, is located between 242-247, coordinates Mg2+ ion required for enzyme activity. Sugar binding site, is located between 225-230, has key residues that enable the enzyme to recognize the two different binding orientations of its 1-and 6-phospho sugar substrates (16). The six invariant residues in the

phosphohexomutase family, Asp 242, Asp 244, Asp 246, Arg 247, Gly 308 and Ser 413, were found in the sequence. The two additional residues, Arg 14 and Arg 411, were also conserved among all eight species.

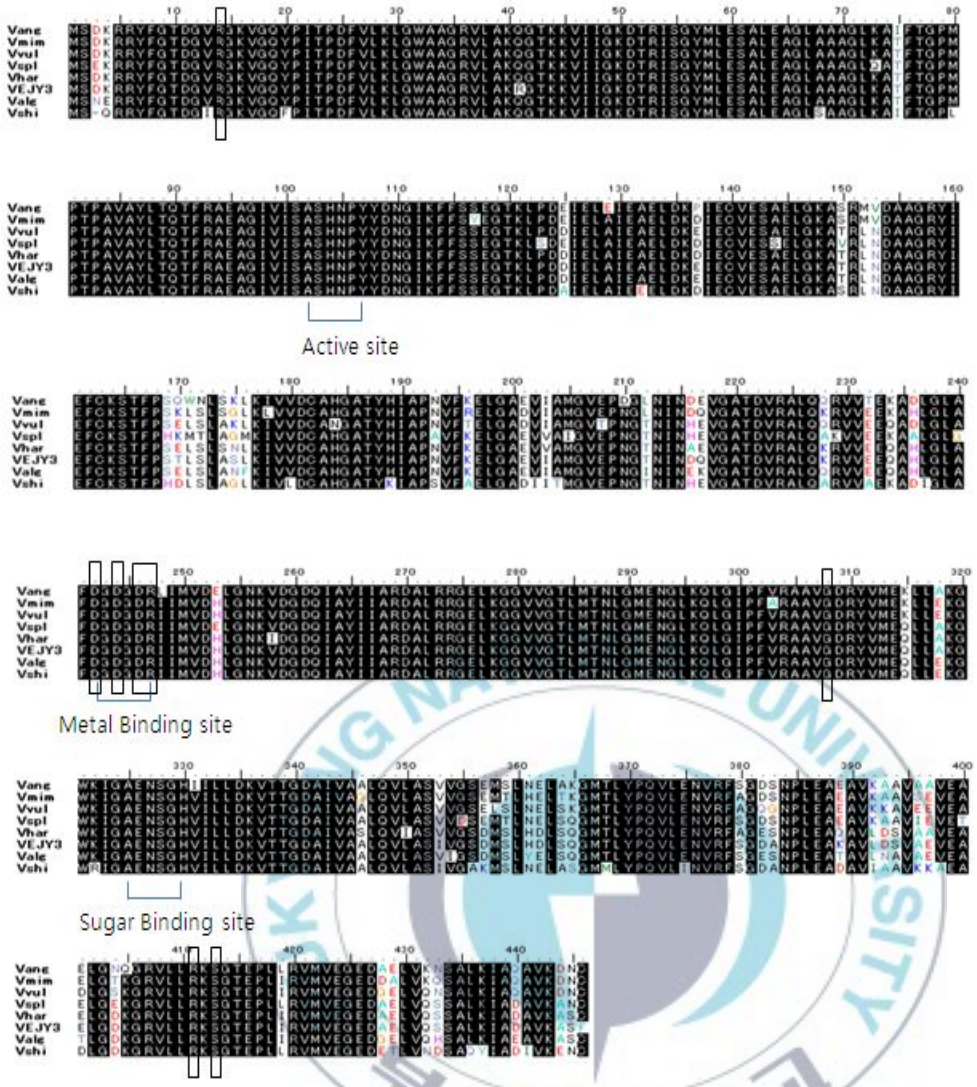

Fig. 2. Comparison of amino acid sequences of PMM/PGM of *Vibrio species*. The sequences were aligned with the program BioEdit. *Vibrio anguillarum* (Vang), *Vibrio mimicus* (Vmim), *Vibrio vulnificus* (Vvul), *Vibrio splendidus* (Vspl), *Vibrio harveyi* (Vhar), *Vibrio* sp. EJY3 (VEJY3), *Vibrio alginolyticus* (Valg), *Vibrio shilonii* (Vshi).

2. Cloning of *pmm/pgm* from *V. anguillarum*

We amplified *pmm/pgm* gene by PCR using *V. anguillarum* genomic DNA as a template and a 1341 bp PCR fragment was obtained. It contained an ORF corresponding to 447 amino acid residues.

The target gene fragment was cloned in pET28-a(+) vector, transformed to *E. coli* DH5α followed by *E. coli* BL21, and finally the recombinant protein was expressed. Overexpression of rPMM/rPGM was induced by adding 1 mM IPTG. It was recovered in the soluble fraction after a cell fractionation. The purified enzyme was easily achieved by Ni-NTA his-tag affinity chromatography. About 1.35 mg of protein was routinely obtained from 300 ml of culture. The purest fraction eluted from column migrated as a single band with a molecular mass of about 50 kDa under denaturing conditions by SDS-PAGE (Fig. 3).

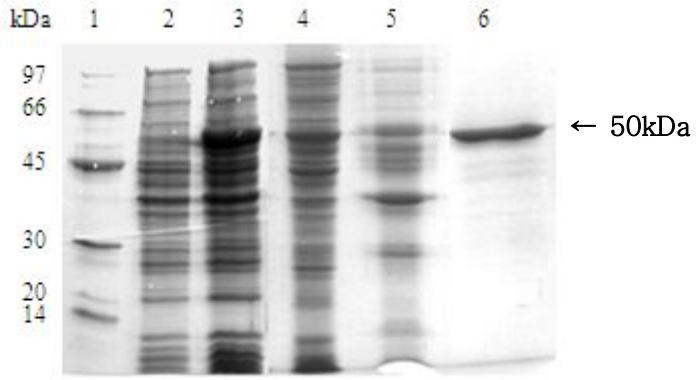

Fig. 3. SDS-PAGE of recombinant PMM/PGM during purification. Lane1, standard molecular weight proteins; Lane2, crude extract of cells transformed with pET-28a(+) vector; Lane3, cell harboring for 4h after induction; Lane4, soluble proteins; Lane5, insoluble proteins; Lane6, eluted fraction from a Ni-NTA his-tag affinity chromatography.

3. Transcriptional analysis of *pmm/pgm* gene

To compare gene expression at mRNA level, *V. anguillarum* was cultured at 15℃ and 25℃, and total RNA was isolated from the cultured cells using TRIzol reagent. The expression of 16S rRNA was also checked as control under same conditions. The expression of *pmm/pgm* gene was relatively weaker than control gene, but the expression of *pmm/pgm* gene was stronger at 15℃ compare to 25℃ (Fig. 4.). This indicates that the *pmm/pgm* gene of *V. anguillarum* is expressed during stress condition.

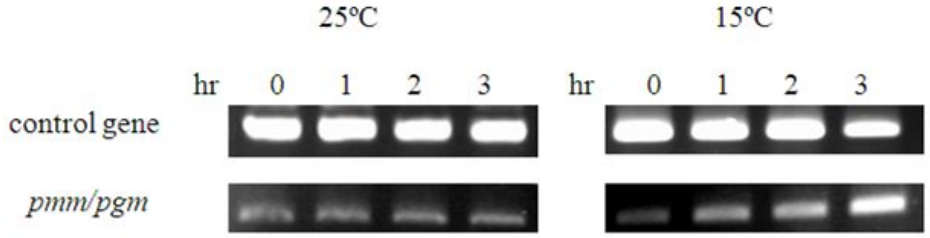

Fig. 4. *Vibrio anguillarum* **gene expression profiles:** *pmm/pgm* **gene and 16S rRNA as internal control gene**

3. PMM and PGM activity

The specific activities of PMM/PGM was assayed with the purest fraction eluted from column. The increase in absorbance at 340 nm due to the reduction of NADP at 25℃. Previous works on the characterization of PGM and PMM activities in other strains indicated that the metal ions Mg2+ or Mn2+ are necessary as cofactors (20). PMM/PGM from *V. anguillarum* had no activity without Mg2+.

One unit of enzyme activity was defined as the amount of enzyme needed to reduce 1 μmol of NADP per min. The specific activities were detected that PMM (8.22 U/mg) and PGM (4.64 U/mg).

Table 3. PMM and PGM activity

Substrate	Total protein (mg)	Total activity (U)	rPMM/rPGM Sp act (U/mg)
Mannose 1-phosphate (PMM)	1.80	14.80	8.22
Glucose 1-phosphate (PGM)		8.35	4.64

4. Knock out mutation

The 370 bp fragment which is a part of *pmm* gene was cloned into a suicide vector pNQ705 to inactivated *pmm/pgm* gene of *V. anguillarum* wild type, and transformed into *E. coli* SM10λ*pir*. We selected only mutant on the TCBS (Cm,10μg) plate after conjugation. The mutant was constructed between transformant and *V. anguillarum* wild type by allelic exchange (Fig. 4). To confirm knock out mutation, PCR was conducted with three designed primers (Table. 2). Primer VA-KO-check up, was designed based on ORF region for upstream primer, but not exist on the sequence of knock out part. Reverse primers, pNQ705 rp-I and pNQ705 rp-II, were designed based on the downstream sequence of sucide vector pNQ705. In Fig. 6, PCR fragments were present in the *pmm/pgm* mutant, while the wild type did not amplify as negative control.

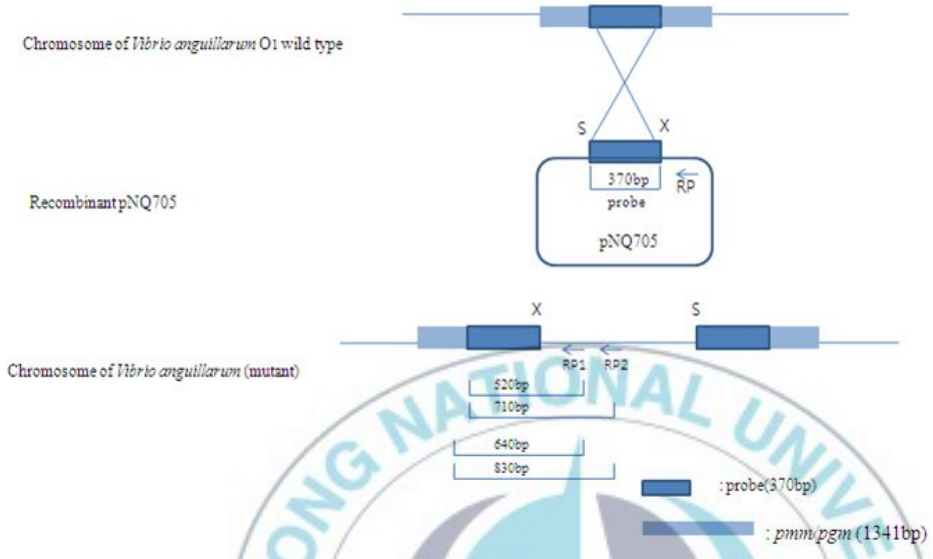

Fig. 5. Construction of *V. anguillarum* mutant by insertional mutagenesis

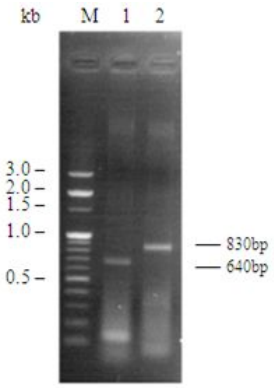

Fig. 6. Agarose gel eletrophoresis of PCR products from *pmm/pgm* knock out mutant by PCR using the primers VA-KO-check up, pNQ705 rp-I and pNQ705 rp-II. M, molecular weight marker; Lane 1, PCR products of the mutant using the primers VA-KO-check up and pNQ705 rp-I; Lane 2, PCR products of the mutant using the primers VA-KO-check up and pNQ705 rp-II.

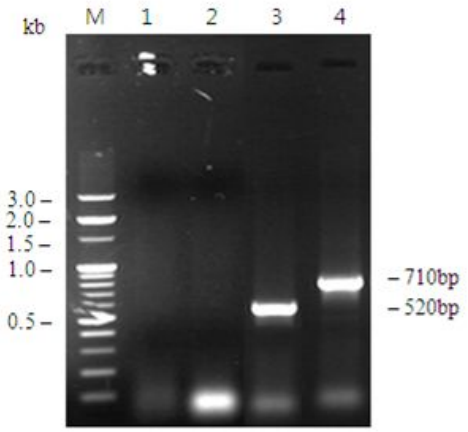

Fig. 7. Agarose gel electrophoresis of PCR products from *pmm/pgm* knock out mutant by PCR using the primers; forward primer for construction knockout mutant, pNQ705 rp-I and pNQ705 rp-II. M, molecular weight marker; Lane 1, negative control 1; Lane2, negative control 2; Lane 3, PCR products of the mutant using primers forward primer for construction knockout mutant and pNQ705 rp-I; Lane 4, PCR products of the mutant using primers forward primer for construction knockout mutant and pNQ705 rp-II.

5. Comparison of LPS profile

The most common method used for the extraction of LPS from Gram-negative bacteria is based on the procedure developed by Westphal and Jahn (1965). Eletrophoretic profiles of isolated LPS demonstrate changed pattern by the mutation of *pmm/pgm*. Isolated LPS from *V. anguillarum* wild type have strong several bands but mutant has smear bands, and the upper strong band immigrated (Fig. 7). Therefore, *pmm/pgm* gene is related to biosynthesis of LPS.

6. Growth and viability

Bacterial growth at 25℃ was followed by measuring optical density. In BHI liquid medium, mutant show a slightly slower growth by stationary. We measured viable bacteria, wild type and mutant at 25℃ by colony-forming unit (CFU) (Fig. 8).

Therefore, LPS is essential for growth and survival of Gram-negative bacteria. The composition of the O-antigenic structure in Gram-negative bacteria, is important with respect to pathogenicity (10). A small change in the sugar composition of the O-antigen has been demonstrated to influence virulence (6). Role of LPS in virulence from *V. anguillarum* can be studied as well.

Fig. 8. Silver stained LPS profiles of *V. anguillarum* O1 wild strain (Lane 1) and *pmm/pgm* mutant (Lane 2).

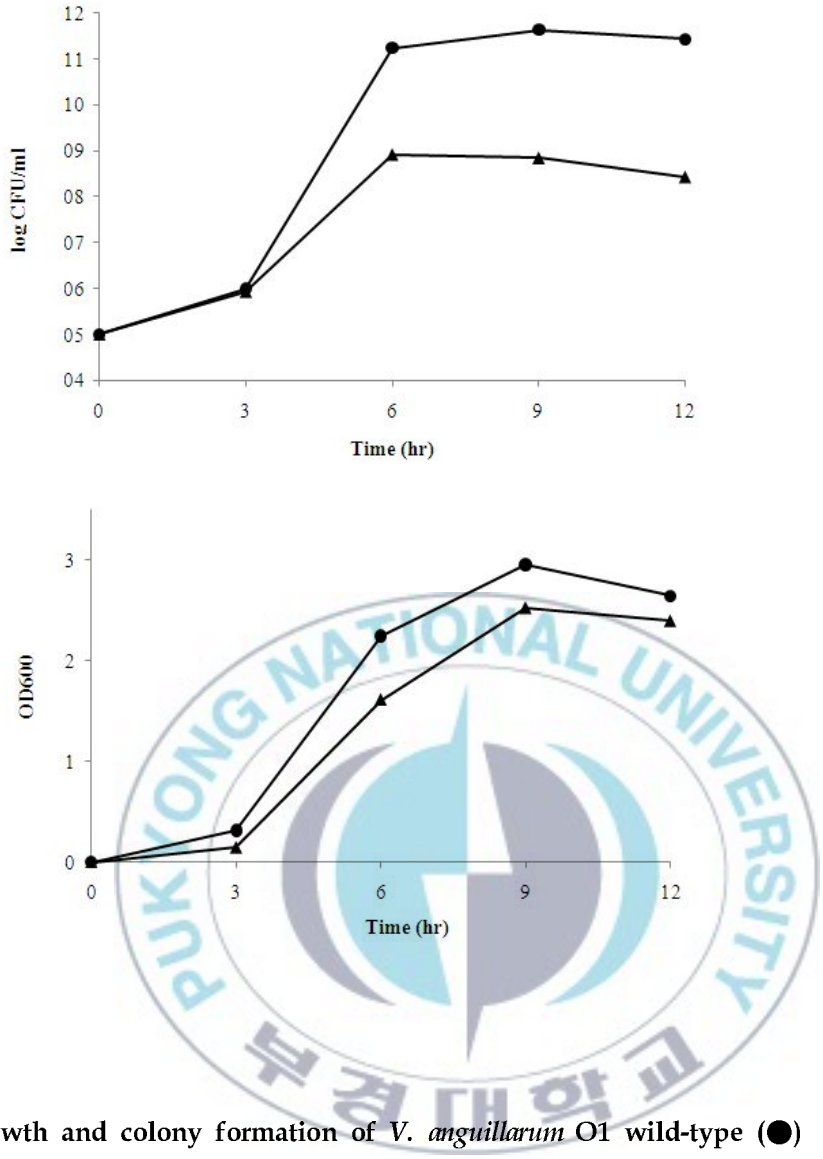

Fig. 9. Growth and colony formation of *V. anguillarum* O1 wild-type (●) and the *pmm/pgm* mutant (▲). Cell growth was determined by measuring optical density at and absorbance of 600 nm.

Therefore, *pmm/pgm* gene from *V. anguillrum* has a important role in the biosynthesis of LPS, which is essential for growth and survival of Gram-negative bacteria. The *pmm* and *pgm* gene have been studied from a variety of bacteria, while study of *pmm/pgm* gene from *V. anguillarum* have not much studied yet.

Futhermore, the functions of LPS can be studied with *pmm/pgm* knock out mutant.

IV. REFERENCES

(1) **Actis, L. A., M. E. Tolmasky, and J. H. Crosa.** 2011. *In* P. T. K. Woo and D. W. Bruno (ed.), Fish diseases and disorders, vol. 3: viral, bacterial, and fungal infections, 2nd ed. Vibriosis, p.570-605.

(2) **Banemann, A., H. Deppisch, and R. Gross.** 1998. The lipopolysaccharide of *Bordetella bronchiseptica* acts as a protective shield against antimicrobial peptides. Infect. Immun. 66: 5607-5612.

(3) **Erridge C, Bennett-Guerrero E, and Poxton IR.** 2004. Structure and function of lipopolysaccharides. Microbes Infect. 4: 837-851.

(4) **Ernst Th. Rietschel, Horst-Werner Wollenweber, Ulrich Zähringer and Otto Lüderitz.** 1982. Lipid A, the lipid component of bacterial lipopolysaccharides: Relation of chemical structure to biological activity. J. Mol. Med, 60: 705-709.

(5) **Grant S. Shackelford, Catherine A. Regni, and Lesa J. Beamer.** 2004. Evolutionary trace analysis of the-D-phosphohexomutase superfamily Protein Science 13 : 2130 - 2138.

(6) **Henrik B. Rasmussen.** 1987. Immunoelectrophoretic study of lipopolysaccharide antigens from *Vibrio anguillarum* strains, serogroup O2. Curr. Microbiol. 15 : 219-222.

(7) **Holmstrøm K, and Gram L.** 2003. Elucidation of the *Vibrio anguillarum* genetic response to the potential fish probiont Pseudomonas fluorescens AH2, using RNA-arbitrarily primed PCR. J. Bacteriol. 185: 831-42.

(8) **Köplin R, Arnold W, Hötte B, Simon R, Wang G, and Pühler A.** 1992. Genetics of xanthan production in *Xanthomonas campestris*: the *xanA* and *xanB* genes are involved in UDP-glucose and GDP-mannose biosynthesis. J. Bacteriol. 174: 191 - 199.

(9) **Larsen, J. L., Pedersen K, and Dalsgaard I.** 1994. *Vibrio anguillarum* serovars associated with vibriosis in fish. J. Fish Dis. 17: 259-267.

(10) **Makela P. H., Valtonen V. V., and Valtonen M.** 1973. Role of O-antigen lipopolysaccharide) factors in the virulence of *Salmonella.* J. Infect Dis. 128: 81-85.

(11) **Miller V. L., and Mekalanos J. J. 1988.** A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR.* J Bacteriol 170: 2575 - 2583.

(12) **Milton D. L., Norqvist A, and Wolf-Watz H.** 1992. Cloning of a metalloprotease gene involved in the virulence mechanism of *Vibrio anguillarum.* J. Bacteriol. 174: 7235 - 7244.

(13) **Nicholas P. West, Heidrun Jungnitz, John T. Fitter, Jason D. MCArthur, Carlos A. Guzma N, and Mark J. Walker.** 2000. Role of phosphoglucomutase of *Bordetella bronchiseptica* in lipopolysaccharide biosynthesis and virulence. Infect. Immun. 68: 4673-4680.

(14) **Nikaido, H**. 1979. Non specific transport through the outer membrane, In M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York. p. 361-405.

(15) **Preston, A., Mandrell, R. E., Gibson, B. W., and Apicella, M. A.** 1996. The lipoligosaccharides of pathogenic Gram-negative bacteria. Crit. Rev. Microbiol. 22: 139-180.

(16) **Ray, W. J., Jr., and E. J. Peck, Jr.** 1972. Phosphomannomutase, In P. D. Boyer (ed.), The enzymes, vol. 6. Academic Press, New York. Evolutionary trace analysis of the α-D-phosphohexomutase superfamily. p.407-477.

(17) **Rietschel E. T., and Brade H.** 1992. Bacterial endotoxins. Sci. Am. 267: 54-61.

(18) **Rietschel, E. T., Kirikae T., Schade F. U., Mamat U., Schmidt G., Loppnow H., Ulmer A. J., Zahringer U., Seydel U., Dipadova F., Schreier M., and Brade H.** 1994. Bacterial endotoxin molecular relationships of structure to activity and function. FASEB J. 8: 217-225.

(19) **Sorensen, U. B., and J. L. Larsen.** 1986. Serotyping of *Vibrio anguillarum.* Appl. Environ. Microbiol. 51: 593-597.

(20) **Su-Yan Wang, Johan Lauritz, Jana Jass, and Debra L. Milton.** 2003. Role for the major outer-membrane protein from *Vibrio anguillarum* in bile resistance and biofilm formation. Microbiology. 149: 1061-1071.

(21) **Westphal O, and Jann K.** 1965. Bacterial lipopolysaccharides. Methods Carbohydr. Chem. 5: 83-91.

(22) **Ye, R. W., Zielinski, N. A., and Charkrabarty, A. M.** 1994. Purification and characterization of phosphomannomutase/phosphoglucomutase from *Pseudomonas aeruginosa* involved in biosynthesis of both alginate and lipopolysaccharide. J. Bacteriol. 176: 4851-4857.

*Vibrio anguillarum*로부터 LPS 생합성에 관여하는 phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene 의 클로닝과 돌연변이 균주 제작

오 륜 경

부 경 대 학 교 대 학 원 생 물 공 학 과

요 약

수해양성 병원성 미생물로 알려진 *Vibrio anguillarum*으로부터 mannose 1-phosphate를 mannose 6- phosphate, glucose 1- phosphate를 glucose 6-phosphate로 가역적으로 변환시키는 phosphomannomutase/phosphoglucomutase (*pmm/pgm*) gene을 sequencing하여 1341bp의 Open Reading Frame (ORF)을 밝혔다. *pmm/pgm* gene이 cold stress 하에서 up-regulation됨을 Reverse Trancriptase-Polymerase Chain Reaction (RT-PCR)을 통해 확인하였고, PMM/PGM protein과 Lipopolysaccharide (LPS)의 생합성과의 관계를 규명하고자 유전자 상동성 재조합 (homologous recombination)에 의해 knock out mutant를 제작하였다. *V. anguillarum* wild type과 mutant로부터 LPS를 분리하였고 sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)와 silver staining을 통해 LPS의 High molecular weight (HMW) 부분인 O-antigen에서의 변화를 확인하였다. 또한 *V. anguillarum* wild type과 mutant의 growth와 viability를 확인한 결과 mutant가 wild type보다 stationary phase까지 더 낮은 growth를 보였으며 viability가 감소함을 확인하였다. 본 연구를 통하여 *V. anguillarum*의 *pmm/pgm* gene이 bacteria의 growth와 LPS 생합성에 중요한 역할을 함을 밝혔다.

ACKNOWLEDGEMENT

본 논문이 완성되기까지 부족한 저를 아낌없는 관심과 사랑으로 이끌어 주신 공인수 교수님께 고개 숙여 감사드립니다. 또한 저를 학문의 길로 이끌어 주신 공재열 교수님, 홍용기 교수님, 이형호 교수님, 김중균 교수님, 김성구 교수님, 박남규 교수님, 정귀택 교수님께 감사드립니다.

제가 여기까지 올 수 있도록 많은 도움을 주신 윤수철, 하정철, 김구택, 김영옥, 박기재, 박효진, 김대경, 이종희, 이은우, 김현국, 최선영, 이상봉, 강정화, 진철호, 김형섭, 신승렬, 김남현, 한정현, 최윤혁, 박제현, 김미숙, 임준혁, 김선회, 이은미, 안선희, 박은미, 정승하, 홍경은, 민문경, 김동균 선배님들께 진심으로 감사드립니다.

특유의 카리스마로 우리를 이끌어주는 oh my captain! 은영언니, 몸도 마음도 강인한 second captain 유리언니, mind control법을 알려준 삼촌 Hossain, 내가 잘되길 진심으로 (?) 바란다는 깐죽이 종민선배, 우리방 춤꾼 노래꾼 오뚜기맨 원석선배, 여동생삼고 싶은 착한 후배 김소현 양, 당근보다 채찍을 반기는 조건아 양, 묵묵히 성실하게 일 해내는 박혜진 양, 언제나 상큼발랄 상콤이 노규유 양, 차분히 공부만 할 줄 알았지만 놀 줄도 아는 박새봄 양, 우렁찬 목소리가 매력적인 김나경 양, 곧 박사과정에 들어올 멋쟁이 Zzaman, 짧은 시간 이였지만 많은 도움을 줬던 김민주 양, 김민지 양, 특히 여린 이경준 양. 그리고 생물공학과 선후배님들께 감사의 마음을 전합니다.

어느덧 10년지기가 된 사랑하는 내 친구들. Forever 칠공멤버- 지혜, 선영, 성민, 보은, 영진, 희라. 무슨 일이 있어도 항상 내편이 되어 힘이 되어준- 이슬, 수정, 다현, 주영. 자주 못 보지만 항상 서로를 응원하고 있는 근영, 성주, 재석이(재헌), 은주. 죽마고우- 은혜, 나현, 미경, 새 신랑 기주오빠. 미국에서 동고동락하며 이제는 가족 같은- 희영, 하정, 지선언니, 은희. 학부시절 서로 챙겨주며 의지했던 고마운- 미정언니, 늘봄이, 원정이, 선희. 인생은 등가교환이라며 나의 미래를 위해 아낌없이 조언을 해주시는 우리 Eros팀! 특히 석종오빠, 기송오빠. 그리고 든든하게 내 옆을 지켜준 혁사마. 그대들이 있어 큰 힘이 되었습니다.

그리고 가장 소중한 사랑하는 우리 가족 아버지, 어머니, 군대에서 힘든 시간을 보내고 있을 내 동생 륜태. 항상 저를 믿고 아낌없는 지원과 응원해주셔서 너무나 감사하고 사랑합니다.

오늘의 영광이 저의 노력이기에 앞서 많은 분들의 가르침, 배려, 지도, 관심 그리고 애정으로 이루어진 것임을 항상 생각하고, 겸손한 자세로 생활하면서 모든 사람에게 감사하는 마음으로 살겠습니다. 감사합니다.