Thesis for the Degree of Master of Fisheries Science

Analysis of Recombinant Gas Vesicle Protein Expressed in *Escherichia coli* and *Halobacterium halobium*

by

Kyung Myo Kang

Department of Fisheries Biology

The Graduate School

Pukyong National University

February 2012

Analysis of Recombinant Gas Vesicle Protein Expressed in *Escherichia coli* and *Halobacterium halobium*

*Escherichia coli*과 *Halobacterium halobium*에서 재조합 gas vesicle 단백질 발현 분석

Advisor: Prof. Jong-Myoung Kim

By

Kyung Myo Kang

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

Master of Fisheries Science

in the Department of Fisheries Biology, The Graduate School, Pukyong National University

February 2012

강경묘의 수산학석사 학위논문을 인준함.

2012년 2월 24일

주 심 농학박사 김 창 훈

위 원 농학박사 공 승 표

위 원 이학박사 김 종 명

Analysis of Recombinant Gas Vesicle Protein Expressed in *Escherichia coli* and *Halobacterium halobium*

A dissertation
by
Kyung Myo Kang

Approved by:

(Chairman) Prof. Chang-Hoon Kim

(Member) Prof. Seung Pyo Gong

(Member) Prof. Jong-Myoung Kim

February 24, 2012

Contents

Abstract iii

Figure legends iv

Table legends vii

Ⅰ. Introduction **1**

Ⅱ. Materials and Methods **3**

1. Materials 3
2. Construction of recombinant gvp gene 4
   2-1. Isolation of genomic DNA from *Halobacterium halobium* 4
   2-2. Transformation into *E. coli* 4
      2-2-1. Preparation of competent cells 4
      2-2-2. Transformation of recombinant vector into *E. coli* 5
   2-3. Purification and identification of recombinant plasmid DNA 5
      2-3-1. Purification of recombinant plasmid DNA 5
      2-3-2. Identification of recombinant plasmid DNA 6
   2-4. Expression of gvp genes 6
      2-4-1. Expression of recombinant gvp 7
      2-4-2. SDS-PAGE and western blot 7
      2-4-3. Purification of recombinant protein fused with Histiding-tag 8
3. Cloning of recombinant gvp gene 10
   3-1. Construction of recombinant gas vesicle genes 10
      3-1-1. DNA fragments encoding lipase and heavy metal binding motif 10
      3-1-2. Plasmid isolation and restriction digestion 11
   3-2. Activity assay 11

4. Isolation of recombinant gvp in *Halobacterium halobium* 13
4-1. Construction of recombinant gvp *E. coli* 13
4-2. Transformation in *Halobacterium halobium* 13
4-3. Isolation of gvp in *Halobacterium halobium* 14

Ⅲ. Results and Discussion **17**
1. Amplification of genes encoding gas vesicle proteins 17
2. Cloning of gvp genes into expression vector 20
3. Construction of recombinant gas vesicle genes fused with binding motif for silica and mercury 21
4. Expression of recombinant proteins 27
5. Purification of gvp 33
6. Activity assay of recombinant gvpC containing lipase 40
7. Isolation of gas vesicle protein from *Halobacterium* sp. 41

Abstract (Korean) **44**

Ⅳ. Acknowledgement **45**

Ⅴ. References 46

Analysis of Recombinant Gas Vesicle Protein Expressed in *Escherichia coli* and *Halobacterium halobium*

Kyung Myo Kang

Department of Fisheries Biology, The Graduate School,
Pukyong National University

Abstract

Halophiles are extremophile organisms that thrive in environments with high concentration of salt. Halobacterium is a good model organism for the study of Living Modified Organisms (LMO) as the group cannot survive in most aquatic environment due to difference in osmotic pressure. Recombinant gas vesicle protein fused with lipase and heavy-metal binding motif were constructed for exploring its application on marine ecology. Gas vesicle gene was amplified by PCR using genomic DNA template isolated from *Halobacterium halobium* sp. NRC-1. Recombinant gvp gene was designed to combine with binding sites for mercury and silica as well as histidine-tag for its detection and purification. Expression of recombinant gvp in *E. coli* was identified by SDS-PAGE and western blot. Functional expression of recombinant fusion gvp were also identified in *Halobacterium halobium.*

Figure legends

Fig 1. Identification of genomic DNA (A) and gvp C gene amplified by PCR (B) in 0.8 % agarose gel electrophoresis 18

Fig 2. Comparison of gvpC gene obtained by PCR (15m611, 15m911, and 10m911) together with gvpC gene reported in NCBI by using Clustal W. 19

Fig 3. Identification of recombinant pET19b plasmids containing gvpC gene 20

Fig 4. Identification of recombinant DNA containing gvpC genes fused with silica binding motif 22

Fig 5. Identification of recombinant DNA containing gvpC genes fused with mercury binding motif 23

Fig 6. Restriction digestion of pFM101D containing gvpA and gvpC gene 24

Fig 7. Identification of recombinant DNA containing gvpA and gvpC genes fused with silica binding motif in pFM101d 25

Fig 8. Identification of recombinant DNA containing gvpA and gvpC genes fused with mercury binding motif in pFM101d 26

Fig 9. SDS-PAGE analysis of proteins prepared from *E. coli* 27

Fig 10. SDS-PAGE analysis of total cell lysate prepared with or without IPTG induction 29

Fig 11. SDS-PAGE analysis of recombinant gvpC fused with lipase 30

Fig 12. Identification of gvpC protein fused with metal binding motif by western blot 31

Fig 13. Identification of gvpC protein containing lipase by western blot 32

Fig 14. SDS-PAGE analysis of purified gvpC fused with (His)6-tag protein 34

Fig 15. Identification of recombinant gvpC fused with (His)6-tag by western blot 35

Fig 16. SDS-PAGE analysis of purified gvpC containing binding motif for silica (A) and mercury (B) fused with (His)6-tag 36

Fig 17. Identification of purified recombinant gvpC containing metal binding motif fused with (His)6-tag by western blot 37

Fig 18. SDS-PAGE analysis of purified recombinant gvpC containing lipase fused with (His)6-tag 38

Fig 19. Identification of recombinant gvpC containing lipase and (His)6-tag by western blot 39

Fig 20. Activity assay of recombinant gvpC containing lipase 40

Fig 21. Analysis of morphology *Halobacterium* sp. SD109 by light microscopy 41

Fig 22. Functional identification of gas vesicle protein in *Halobacterium* sp. .. 42

Fig 23. Identification of recombinant gvpC protein in *Halobacterium* by SDS-PAGE followed by western blot 43

Table legends

Table 1. List of oligonucleotides used for the experiment 16

Analysis of Recombinant Gas Vesicle Protein Expressed in *Escherichia coli* and *Halobacterium halobium*

Ⅰ. Introduction

Halophiles are extremophile organisms that thrive in environments with high concentrations of salt. While most of halophiles are classified as Archaea, bacterial halophiles and some Ekaryota are also known. They are categorized as the slight, moderate or extreme halophiles by the extents of halotolerance. High salinity represents an extreme environment in which relatively few organisms are able to adapt and occupy the niches. Most halophiles are unable to survive outside their high-salt environment as they are immediately lysed upon exposure to low osmotic condition.

Halobacterium is a group of Archaea that have a high tolerance for elevated levels of salinity such as a water nearly saturated with salt and known to grow aerobically or anaerobically. Some well-known species give off a red color from carotenoid compounds. Parts of the membranes of *Halobacterium* is purplish in color, mainly due to the presence of bacteriorhodopsin pigment. Some species of halobacteria have acidic proteins that resist the denaturing effects of salts. Some halophiles are also known to be found in the fermented salty foods as either essential ingredients or accidental contaminants.

Gas vesicle is a hollow structure made of protein conferring a buouancy to the cell. Gas vesicles were found in five phyla of the Bacteria and two groups of the Archaea although mostly restricted to planktonic

microorganisms. Aquatic microbes are able to perform vertical migrations by regulating their relative gas vesicle content. Gas vesicle is normally filled with air and impermeable to liquid water but highly permeable to gases. Up to 14 genes were known to be implicated in gas vesicle production *Halobacterium*. Among these, GvpA makes the ribs that form the structure, and GvpC binds to the outside of the ribs and stiffens the structure against collapse (Walsby et al, 1994).

Heavy metals and oil are important ingredients for life on the earth. Oil is one of the important energy sources but sometimes become one of pollutants in the environment. This can be exemplified by oil spillage in the ocean as it blocks and covers on the top of the water resulting in mass mortality of marine organisms. On the other hans, heavy metal is an essential ingredient constructing biomolecular in living organism. However, excess heavy metals in marine environment spoil ecosystem in the water as heavy metals absorbed into the animals results in many diseases in humans. Therefore, it is important to remove excess heavy metals flooded into the watery environment.

Recombinant genes encoding gas vesicle gene fused with binding motif for heavy metal and lipase were constructed to explore the possibility of using gas vesicle for removals of heavy metal and oil spill. Recombinants gas vesicle were expressed in *E. coli* were identified by SDS-PAGE and western blotting.

Ⅱ. Materials and Methods

1. Materials

TOPcloner™ TA core kit and TOPspeed™ DNA Ligation kit were purchased from Enzynomics (Daejeon, Korea). Plasmid purification and gel Extraction kits were purchased from Nucleogen (Seoul, Korea). Various Restriction endonuclease enzymes were purchased from Bioneer (Daejeon, Korea), New England Biolabs (Beverly, MA), and Enzynomics (Daejeon, Korea). pET-19b and pET-Duet vectors were obtained from Novagen (Darmstadt, Germany). pNRC100, pFM101D, and pFM104D were obtained from Dr. DasSarma Laboratory, University of Maryland, Baltimore, USA. Oligonucleotides and 5 x HiQ-PCRmix were purchased from Genotech (Daejeon, Korea). PCR primers are listed in the Table 1. Kits used for the purification of PolyHistidine-Tagged protein was purchased from MACHEREY-NAGEL (Düren, Germany). Anti-His6 antibody was purchased from IG Therapy Co. (Chuncheon, Korea). Immunnopure Goat Anti-Mouse IgG and Supersignal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Rockford, USA). Nickel-NTA Conjugates used for the direct detection of His-tagged proteins was purchased from KPL (Gaithersburg, USA).

2. Construction of recombinant gvp genes

2-1. Isolation of genomic DNA from *Halobacterium halobium*

Genomic DNA was isolated from *Halobacterium halobium* sp. NRC-1 and used as a template in the PCR using 5 x HiQ-PCR Mix containing buffers [10 mM Tris (pH 9.0), 1.5 mM $MgCl_2$, 40 mM KCl], 1 mM dNTPs [250 μM each] and Taq DNA polymerase. Primers used for PCR were listed in Table 1.

For PCR reaction, the mixture (30 μL) contained 3 μL of genomic DNA, 3 μL of gvp-F and gvp-R primers (10 μM), 6 μL of 5 x HiQ-PCRMix and 15 μL of distilled water. PCR was carried out with an initial denaturation at 95℃ for 3 min, 30 cycles of 30 sec at 95℃, 30 sec at 55 ℃ and 1 min at 72℃, and final extension for 5 min at 72℃ in a MyCycler™ Thermal Cycler (BioRad Laboratories). PCR product corresponding to the expected size was isolated from agarose gel and ligated into a pTOP TA V2 using TOPcloner™ TA core kit. The ligation mixture containing 4 μL of isolated PCR product, 1 μL of pTOP TA V2 (10 ng/μL plasmid DNA), and 1 μL of 6 x TOPcloner™ buffer was incubated at 25℃ for 30 min. Ligated DNA was transformed into *E. coli* DH5α.

2-2. Transformation into *E. coli*

2-2-1. Preparation of competent cells

Competent cells were prepared by using *E. coli* BL21 for expression. Competent *E. coli* cell was prepared by using Inoue method (Inoue, et. al., 1990). For this, 1 mL of *E. coli* seed culture fully growth in Luria-Bertani

(LB) medium was inoculated into 200 mL of LB for growth at 37℃ with moderate shaking (160 rpm). When OD_{600} of *E. coli* culture reached 0.6, the culture was chilled on ice for 10 min and transferred into the centrifuge tube. Cells were collected by centrifugation at 2,500 x g for 10 min at 4℃. Cells were resuspended in 20 mL of ice-cold transformation buffer (TB, 10 mM Hepes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7) and transferred into 50 mL conical tube followed by centrifugation at 2,500g for 1 min. Cell pellets were resuspended in 20 mL of ice-cold TB, and incubated on ice for 10 min and then harvested by centrifugation at 7,000 rpm for 10 min at 4℃. Cells were resuspended in 10 mL of ice-cold TB together with 7% DMSO (1.5 mL), and aliquoted in the microcentrifuge tube. Competent cells were frozen in liquid nitrogen and then stored at -75℃ until use.

2-2-2. Transformation into *E. coli*

Ligation mixture was mixed with 100 μL competent cells and incubated on ice for 30 min. After heat-shock at 42℃ for 1 min, the mixture was rapidly chilled on ice for 3 min. Upon addition of 1 mL of LB medium, the mixture was incubated at 37℃ for 1 hr. Transformed *E. coli* was plated onto the LB agar medium containing the Ampicillin (100 μg/mL) and incubated overnight at 37℃.

2-3. Purification and identification of recombinant plasmid DNA

2-3-1. Purification of recombinant plasmid DNA

A single bacterial colony selected from the plate was inoculated into LB medium containing Ampicillin (100 μg/mL) followed by overnight incubation

at 37℃, with shaking. Plasmids were isolated by using plasmid purification mini kit according to the protocol provided by manufacturer. For this, 3 mL of cells were collected by centrifugation twice at 14,000 rpm for 1 min. Collected cells were resuspended with 250 μL resuspension solution containing RNase A (100 μg/mL) by vortexing. After the addition of 250 μL of lysis solution followed by several times inversion and incubation for 5 min until cell lysate became clear, the lysate was mixed with 350 μL of Neutralization Solution by inversion. Upon centrifugation at 14,000 rpm for 10 min, supernatant containing recombinant plasmid was transferred into the spin column. The column was washed with 750 μL of Washing A solution followed by centrifugation at 14,000 rpm for 1 min. After removal of flow-through, the column was centrifuged for 2 min, and then transferred onto a new microcentrifuge tube. Membranes were added onto 50 μL of distilled water or elution buffer and then recombinant plasmids were eluted by centrifugation at 14,000 rpm for 1 min.

2-3-2. Identification of recombinant plasmid DNA

To identify the constructs, cloned into Topo TA vector, recombinant plasmid DNA were digested with EcoR I restriction endonuclease. For digestion reaction, the mixture included 5 μL of plasmid DNA, 1 μL of 10 x reaction EcoR I buffer, 0.6 μL of enzyme (10 units/L), and 3.5 μL of distilled water and incubated at 37℃ for 2 hrs in water bath. Digested DNA was identified in 1 % agarose gel electrophoresis. DNA fragment corresponding to the size was purified for further experiments.

2-4. Expression of gvp genes

Topo TA vector was used for the cloning of DNA fragments amplified by PCR. Plasmid pET-19b was one of the vectors used for expression of recombinant proteins in *E. coli*. To combine the vector with insert DNA at suitable site, pET-19b vector was digested by restriction endonuclease included in the primers and Topo vector. Digested DNA was identified in 0.7 % agarose gel electrophoresis and extracted by Gel Extraction kit. Ligation mixture was incubated at 25℃ for 30 min by TOPspeed™ DNA Ligation kit. Reaction mixture consisted of 1 μL of designed vector DNA, 8 μL of extracted gas vesicle gene, 10 μL of 2 x Top speed ligation buffer and 1 μL of TOPspeed™ DNA ligase. Transformation was carried out as described above.

2-4-1. Expression of recombinant gvp

Recombinant plasmids were transformed into *E. coli* BL21 for expression. Transformants were inoculated at 37℃ and 200 rpm overnight. Cells were inoculated into 20 mL of fresh LB medium containing Amp and grown further until reached at OD_{600} 0.6. IPTG was added for inducing expression of the proteins followed by incubation for 3 hrs at 37℃ with shaking at 200 rpm. Cells were harvested by centrifugation at 14,000 rpm for 2 min. Cells were resuspended in 200 μL of 0.1 M Tris (pH8.0), and total proteins were mixed with 150 μL of 2.5 x SDS loading dye with β-mercaptoethanol. Samples were heated in boiling water for 5 min, and then, centrifuged at 14,000 rpm for 3 min. Supernatant was transferred into a new microcentrifuge tube and loaded onto SDS-PAGE.

2-4-2. SDS-PAGE and western blot

Proteins were analysed by 10 % SDS-PAGE according to the procedure as described. Proteins were identified by Coomassie Blue (CB) staining as well as by silver staining. For CB staining, gel was immersed into staining solution [methanol 50 %, acetic acid 10 % and 0.25 g of Coomassie Brilliant Blue R-250 in 100 mL of solution], heated in microwave for 20 sec, and destained as destaining solution [methanol 50 %, acetic acid 10 %] by changing destaining solution 3 times.

For detecting recombinant proteins, western blot was carried out by using Anti-His6 antibody. Proteins were transferred onto nitrocellurose membrane by Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell at 15 V for 2 hrs. Membranes were incubated in 10 ml of 1 x TBS-T [Tris-HCl 50 mM, NaCl 150mM and 0.1%(v/v) Tween-20] with 5 % non-fat dry milk at 4℃ overnight washed in 20 mL of 1 x TBS-T for 5 min 3 times, and then incubated with 0.5 μL of Anti-His6 antibody (1 mg/mL) in 20 ml of 1 x TBS-T for 1 hr at room temperature (RT). After three times washing in 20 mL of 1 x TBS-T for 5 min, 2 μL of Immunnopure Goat Anti-Mouse IgG antibody (0.8 mg/mL) were added into 20 mL of 1 x TBS-T for 2 hrs at RT. After washing of the membrane as described above, antibody on the membrane were detected by chemiluminescent solutions and upon exposure onto X-ray film.

2-4-3. Purification of recombinant protein fused with Histidine-tag

Recombinant protein was purified by using Ni-IDA Protein. At first, cells were lysed with LEW (Lysis-Equilibraion-Wash) buffer [50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0] by pipetting on ice. Lysozyme (1 mg/mL) was added to help lysis on ice by agitating for 30 min at 4℃ together with repeated freezing and thawing. When viscosity of the lysate was observed, DNase I (5 μg/mL) was added to the lysate and incubated by agitating on ice for 15

min. The crude lysate was centrifuged at 20,000 x g for 30 min at 4℃ and transferred onto a column equilibrated with 320 μL of 1 x LEW. Upon three times washing of the column with 500 μL of 1 x LEW, proteins were eluted by 240 μL of 1 x elution buffer [50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole , pH 8.0] 3 times. All fractions were collected and stored at -20℃ for further identification of the recombinant protein.

3. Cloning of recombinant gvp gene

3-1. Construction of recombinant gas vesicle genes

3-1-1. DNA fragments encoding lipase and heavy metal binding motif

Gene encoding lipase was obtained by PCR reaction, using pES01029D12 as a template, together with forward primer 5′- GGA TCC ATG ACT TCA ACG GCA AA - 3′ and reverse primer 5′- AAG CTT CCT TAT GAG CAA ATT CCA - 3′ (Jeon et al, 2008)

Oligonucleotides encoding structual motif for binding with mercury and silica were such motifs were designed according to reports (Kirschke, A. 2006) Mercury binding site (MBS) has been known to contain Cys-Gly motifs with a high affinity for Hg2+ (Pazirandeh et al, 1998). The end of the oligonucleotide was designed to contain Nco I restriction endonuclease recognition sequence to facilitate the cloning. One of oligonucleotides was MBS-1 5′- CAT GGC ATG CGG TTG CTG TGG CAA AGG TCA TTG TGG CTG TTG CGG CAA AGG TCA CTG CGG TTG CTG TGG -3′ and the other of oligonucleotides was MBS-2 5′- CAT GCC ACA GCA ACC GCA GTG ACC TTT GCC GCA ACA GCC ACA ATG ACC TTT GCC ACA GCA ACC GCA TGC -3′. Silica bind motif were designed from amino acid sequence (Kirschke, A. 2006). The end of the oligonucleotide also contains Nco I restrcition endonuclease site for easier identification. One of oligonucleotides was SBS-1 5′- CAT GGC CCT TCC GGA TTG GTG GCC ACC GCC TCA GCT TTA TCA -3′ and the other of oligonucleotide was SBS-2 5′- CAT GTG ATA AAG CTG AGG CGG TGG CCA CCA ATC CGG AAG GGC -3′. Two oligonucleotides contain restriction enzyme site Sph I in MBS and BspE I in SBS for easy identification of recombinant DNA. Double stranded oligonucleotides were prepared from

incubation of the mixture consisted of 4 pmole/μL of each DNA, 10 x NEBuffer #4 [20 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol].

3-1-2. Plasmid isolation and restriction digestion

Plasmid isolation was carried out by using alkaline lysis method as described above.

Restriction digestion of recombinant DNA was carried out by using a single or double digestion of plasmid followed by agarose gel electrophoresis. Direction of the insertion sequence were identified by Sph I digestion for MBS and by Nco I and Sph I for SBS. For this, the mixture consisted of 7 μL of plasmid DNA with MBS, 5.5 μL of distilled water, 1.5 μL of 10 x NEBuffer #2 and 1 μL of restriction endonuclease enzyme. In case of SBS, the mixture consisted of 10 μL of plasmid DNA, 2.3 μL of distilled water, 1.5 μL of 10 x NEBuffer #2 and 0.6 μL of restriction endonuclease enzyme. The reaction was carried out at 37℃ for 2 hrs in water bath and identified by 1.5 % agarose gel electrophoresis.

3-2. Activity assay

Rhodamine plate assay was used to confirm lipase activity. Rhodamine plate medium was prepared with LB together with 1 % arabic gum followed by autoclaving. Upon cooling down to 60℃, 1 % olive oil and 0.001% rhodamine B were added, and emulsified by mixing with a stirring bar at max speed. Upon addition of antibiotics and IPTG, media were poured onto plate. On the rhodamine plate, several samples were streaked to compare the activity and incubated at 37℃ for 3 hrs. Activity was examined by ultraviolet

rays illumination of the rhodamine plate.

4. Isolation of recombinant gvp in *Halobacterium halobium*

4-1. Construction of recombinant gvp in *E. coli*

pFM101D is shuttle plasmid vector for amplification in *E. coli* and *Halobacterium halobium.* GvpC fused with his-tag was cloned into pFM101D. Inserting the target DNA, restriction endonuclease digestion of the plasmid was carried out. Upon preparation of DNA fragments for vector and gvp genes fused with, silica and mercury binding motif, recombinant DNA were transformed into *E. coli*. Recombinant plasmids were purified as described above and identified by double digestion endonucleases Sph I and Xho I digestion for MBS and Nco I and Xho I digestion for SBS.

4-2. Transformation in *Halobacterium halobium*

H. halobium sp. SD109 and S9 which do not have functional gvp were cultured for transformation at 42℃ for 3-4 days. Upon chilled cells on ice for 10 min, cell was centrifuged at 7,000 rpm and 4℃ for 10 min. Collected cell was resuspended by pipetting in 1/10 volume of medium Spheroplasting solution [2.0 M NaCl, 27.0 mM KCl, 50.0 mM Tris-HCl (pH 8.75), 15 % sucrose] on ice. Ice-colded 0.5 M EDTA was added as 1/200 volume and the mixture was incubated at RT for 10 min. Upon confirming coccus morphology of transformed cells examined by microscopy, 200 μL transformed cells were aliquoted into microcentrifuge tube, added with 1 μg of recombinant DNA and then incubated at RT for 5min. PEG-600 solution [40 % spheroplasting solution and 60 % PEG 600] was added to mixture as equal volume and mixed by rotating and tapping the tube. The mixture was incubated at RT for 20 min. To remove PEG-600 solution in the mixture, 1 mL of spheroplast

dilution medium [4.3 M NaCl, 27.0 mM KCl, 50.0 mM Tris-HCl (pH 7.4), 80.0 mM $MgSO_4$, 10.0 mM Na-citrate, 1.4 mM $CaCl_2$, 15 % Sucrose , 1 % Peptone] was added and mixed by inversion. Centrifugation was carried out 6,000 rpm for 4 min and the supernatant was discarded. Cell pellet was resuspended in 1 mL of spheroplast dilution medium by pipetting into test tube, and then incubated at 180 rpm and 42℃ for 24 hrs. One mL of CM+ medium [250 g Sodium Chloride, 20 g Magnesium Sulfate, 3 g (Tri) Sodium Citrate, 2g Potassium Chloride , 10 g Oxoid Neutralized Bacteriological pepton in 1 L (adjust pH 7.2) with trace metal] was added and incubated for 12 hrs. Transformed cell was plated out on CM+ plate with mevinolin (50 μM) and incubated at 42℃ for 1 week. The plate was stored in a container which can avoid evaporation from plate during incubation.

4-3. Isolation of gvp in *Halobacterium halobium*

The pink colony was picked up from the plate. Inoculation was carried out in CM+ medium with mevinolin (50 μM) at 180 rpm and 42℃ for 3-4 days. To produce gvp, 1 ml of cell was plated out on CM+ plate or CM+ medium containing mevinolin (50 μM).

For this, *H. halobium* sp. NRC-1 and transformed cell containing gvp C gene fused with his-tag was plated out and incubated in 42℃ incubator for more than 5 days. When cell grew up on the plate, 15 mL of 10 mM $MgSO_4$ with DNase I (10 μg/mL) was added on the plate for 15 min. Floated cell was collected and incubated at 37℃ for 2 hrs in water bath. After then, centrifugation was carried out at 60 x g overnight with tubes covered with parafilm. On the next day, floated gas vesicles were collected in 10 mL of 1.5 M NaCl and the centrifugation was carried out at same condition repeatedly.

To analyze protein, collected gas vesicle was diluted by adding distilled

water to 1/3 volume of protein and added 2.5 x SDS loading buffer with β -mercaptoethanol. The mixture was heated boiling water for 3 min followed by centrifugation at 14,000 rpm for 4 min. Supernatant was collected into a new microcentrifuge tube and loaded onto SDS-PAGE.

oligonucleotide name	sequence (5′→3′)	comment
gvp-F	CTT CGG CCG GAT GAT AAA ACA CAC CAT CAC CAT	Primers for gvp C
gvp-R	TCA GGC CAT GGT TTT ATC ATC CGG CCG AAG GGG GA	
MBS-1	CAT GGC ATG CGG TTG CTG TGG CAA AGG TCA TTG TGG CTG TTG CGG CAA AGG TCA CTG CGG TTG CTG TGG	MBS (mercury binding site)
MBS-2	CAT GCC ACA GCA ACC GCA GTG ACC TTT GCC GCA ACA GCC ACA ATG ACC TTT GCC ACA GCA ACC GCA TGC	
SBS-1	CAT GGC CCT TCC GGA TTG GTG GCC ACC GCC TCA GCT TTA TCA	SBS (silica binding site)
SBS-2	CAT GTG ATA AAG CTG AGG CGG TGG CCA CCA ATC CGG AAG GGC	
Lipase-F	CCA TGG CAA GTA CTA CAT ATC CAA TTG TTT	Primers for lipase
Lipase-R	TCA TGA GTG GTG ATG GTG ATG GTG	

Table 1. List of oligonucleotides used for the experiment.

Ⅲ. Results and Discussion

1. Amplication of genes encoding gas vesicle proteins

Genomic DNA was isolated from *Halobacterium halobium* sp. NRC-1. Genes encoding gas vesicle genes A, C and N were amplified by PCR reaction. DNA fragments amplified by PCR were ensured by agarose gel electrophoresis. About 1 Kb DNA fragment encoding gvp C gene on the agarose gel was extracted from gel and cloned into Topo TA vector. Amplification of gvp genes using primers specific to gvpA and gvpC resulted in 0.3 Kb and 1.2 Kb fragments, respectively. Recombinant plasmid DNA were isolated from transformants and subject to restriction endonucleases digestion. DNA sequence analysed was compared to confirm gas vesicle gene. Figure 3 showed a complete match with gvp C sequence.

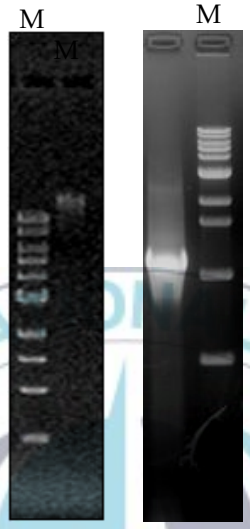


Figure 1. Identification of genomic DNA (A) and gvp C gene amplified by PCR (B) in 0.8 % agarose gel electrophoresis.

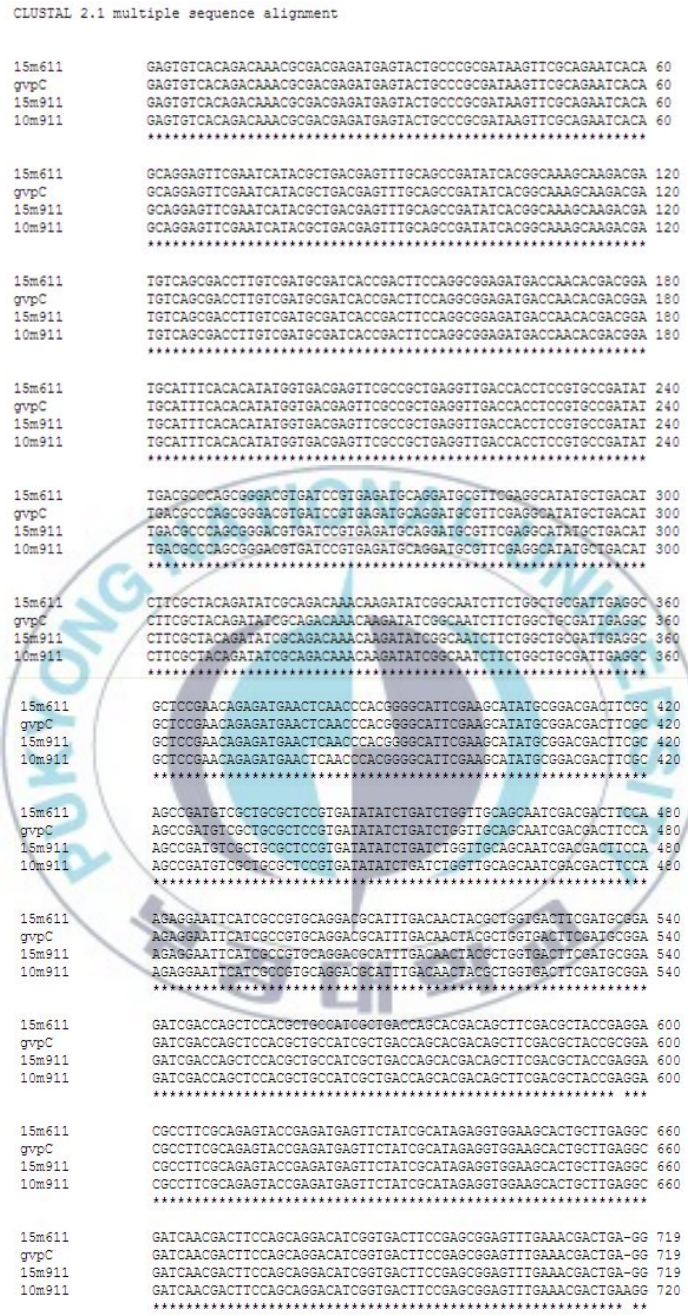


Figure 2. Comparison of gvpC gene obtained by PCR (15m611, 15m911, and 10m911) together with gvpC gene reported in NCBI by using Clustal W.

2. Cloning of gvp genes into expression vector

In order to express gvp genes in *E. coli*, gvp genes were cloned into expression vector, pET19b and pETDuet-1. For this, gvp genes in Topo vector was digested with Nco I and Xho I and then cloned into the corresponding site of pET19b vector. Ligated DNA were first transformed into *E. coli* DH5α. Recombinant plasmids were isolated from transformants and subject to restriction endonuclease digestion followed by agarose gel electrophoresis. Upon confirming DNA sequence, recombinant plasmid was transformed into *E. coli* BL21 for expression analysis.

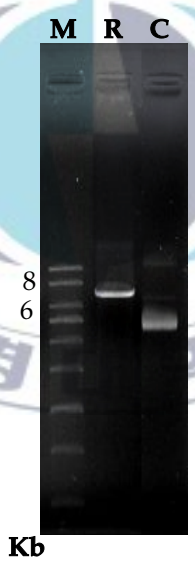


Figure 3. Identification of recombinant pET19b plasmids containing gvpC gene : Tested samples were digested with Nco I resulted in 6.7 Kb (R) as compare to the recombinant gas vesicle gene undigested control (C). M includes 1 Kb ladder (10, 8, 6 from top to 3rd band) and the size of the fragments are indicated on the left.

3. Construction of recombinant gas vesicle genes with binding motif for silica and mercury

Oligonucleotides containing binding sites for mercury and silica were annealed to be cloned into recombinant vector containing gvp gene. Direction of the regions encoding for the binding site were restriction digestion and sequencing analysis.

Recombinant fusion protein expressed in *E. coli* was identified by SDS-PAGE.

Gas vesicle genes fused with mercury and silica binding motif were constructed as described above. For this, plasmid containing gvpA genes were digested with Nco I followed by ligation with annealed oligonucleotides. For effective ligation, different ratio of vector to insert DNA was tested as the concentration and ratio of vector and insert DNA also affect the ligation efficiency. The higher the ratio of the vectors to the insert was used for ligation, the more colonies grew on plate because of self-ligation. Recombinant DNA isolated from transformant were subject to digestion with restriction endonuclease to confirm the direction of the oligonucleotides.

In order to produce gvp genes in *H. halobium*, recombinant gvp genes were cloned into shuttle vector, pFM101D in *E. coli*. For this, gvp genes with binding motif were cloned into the corresponding site of pFM101D vector as describe above. Ligated DNA were first transformed into *E. coli* DH5α. Recombinant plasmids were isolated from transformants and subject to restriction endonuclease digestion followed by agarose gel electrophoresis. Upon confirming DNA sequence, the second cloning into corresponding site of an expression vector, pFM104d, was carried out for expressing gas vesicle protein in *H. halobium*.

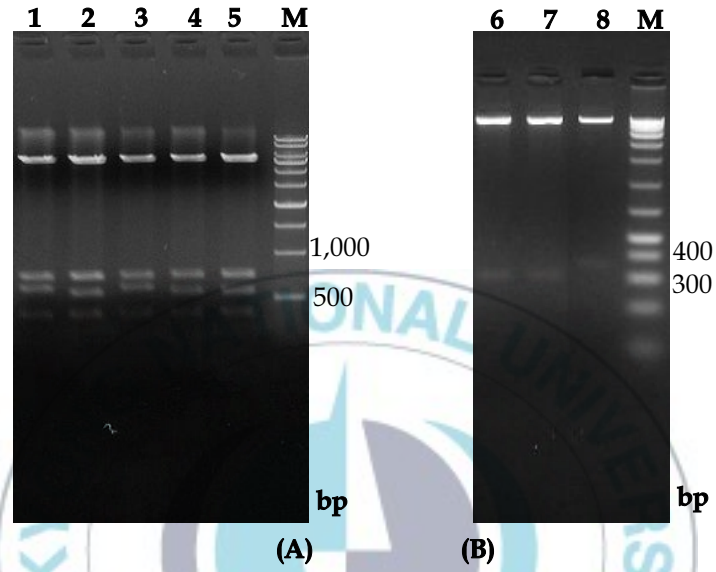


Figure 4. Identification of recombinant DNA containing gvpC genes fused with silica binding motif : DNAs were double digested with EcoR I and Xba I (A) and Sph I (B) followed by 1% and 1.5%, respectively, agarose gel electrophoresis. Tested samples were recombinant DNA with (1 and 3) without (2, 4 and 5) sequences corresponding to silica binding motif. Direction of the inserted sequence was identified by Sph I digestion followed by 1.5% agarose gel electrophoresis. Included DNAs were recombinant without insert (6) together with binding motif inserted on the right (7) or opposite (8) direction. DNA ladders included were 1Kb (A) and 1Kb plus (B) ladder.

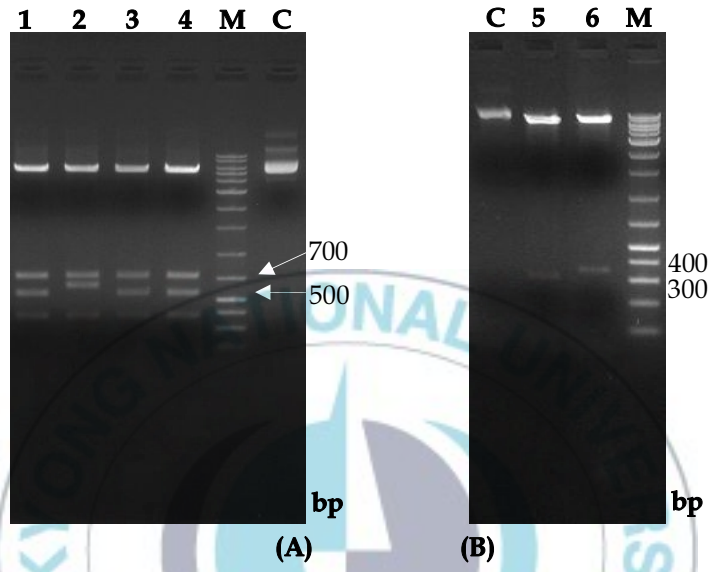


Figure 5. Identification of recombinant DNA containing gvpC genes fused with mercury binding motif : DNAs were double digested with EcoR I and Xba I (A) and Sph I (B) followed by 1% and 1.5% agarose gel electrophoresis. Lanes included were recombinant DNA with no insertion element (1, 3 and 4), recombinant DNA with insertion element of about 550 bp (2), recombinant DNA containing MBS with right direction (5) or reverse (6) direction. Lanes C and M include control DNA without digestion and 1 Kb plus ladder, respectively.

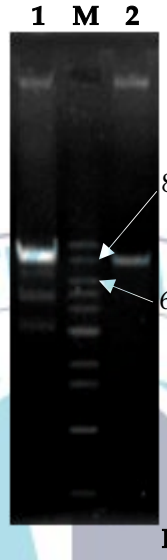


Figure 6. Restiriction digestion of pFM101D containing gvpA and gvpC gene. Recombinant plasmid digested with NcoI resulted in about 7.6 Kb fragment : Tested samples were recombinant plasmids with gvpA gene (1) and gvpC gene (2).

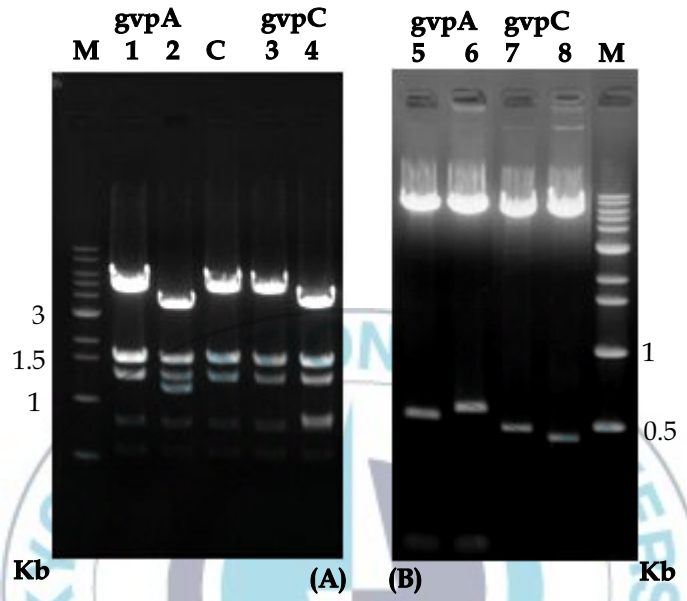


Figure 7. Identification of recombinant DNA containing gvpA and gvpC genes fused with silica binding motif in pFM101d. DNAs were double digested with EcoR I and Xba I (A) and Msc I (B) followed by 1% agarose gel electrophoresis : Tested were recombinant DNA with (2 and 4) or without (1 and 3) sequences corresponding to silica binding motif. Lanes C and M include control plasmid without digestion and 1 Kb ladder, respectively. Direction of the inserted sequence was identified by Sph I digestion followed by 1.5% agarose gel electrophoresis. Included DNAs were recombinant with binding motif inserted on the right (6 and 8) or opposite direction (5 and 7).

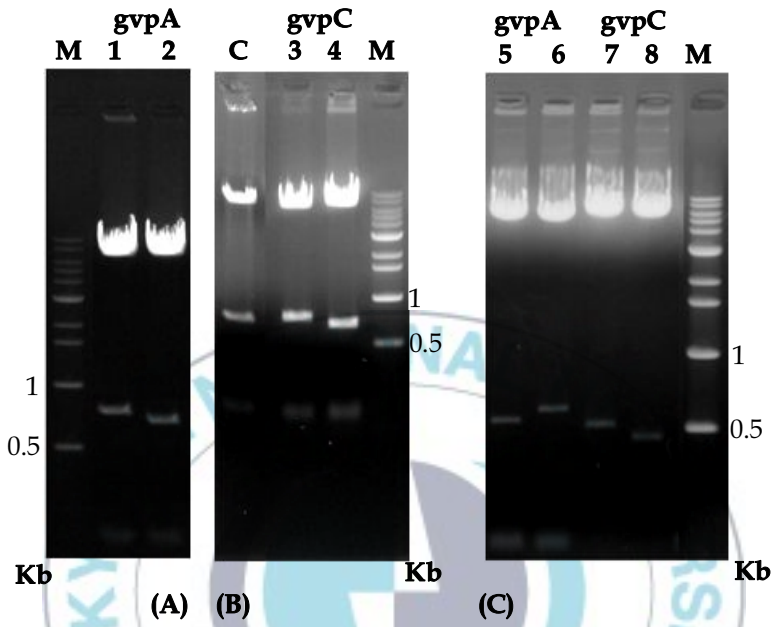


Figure 8. Identification of recombinant DNA containing gvpA and gvpC genes fused with mercury binding motif in pFM101d. DNAs were double digested with EcoR I and Xba I (A and B) and Sph I (C) followed by agarose gel electrophoresis : Tested were recombinant DNA with (2 and 4) or without (1 and 3) sequences corresponding to mercury binding motif. Lanes C and M include control plasmid without digestion and 1 Kb ladder, respectively. Direction of the inserted sequence was identified by Sph I digestion followed by 1.5% agarose gel electrophoresis. Included DNAs were recombinant with binding motif inserted on the right (5 and 7) or opposite direction (6 and 8).

4. Expression of recombinant proteins

Recombinant plasmids were transformed into *E. coli* BL21 for expression analysis. Cells were grown overnight and transferred into fresh media for induction experiment. Upon IPTG induction followed by growth for 3 hrs at 37℃, total proteins were extracted from the cells and analysed by SDS-PAGE as described in Method. Proteins were also prepared from the cells transformed with vector only as a control. Cells transformed with vector showed no differences in the profile of total cellular proteins regardless of IPTG induction (lane 1 and 2). However, an induction of proteins were clearly seen in the cells treated with IPTG addition (lane 3 and 4). The result confirmed the expression of gvpC in *E. coli*.

Recombinant proteins were analyzed by SDS-PAGE and western blot. Induced cell was identified by 10 % SDS-PAGE and compared to other samples prepared from cells transformed with only vector and recombinant DNA containing gas vesicle gene fused with his-tag. Upon induction with IPTG, extracts prepared from cells transformed with recombinant DNA containing gvp gene fused with His-tag were compared with other samples prepared from cells transformed with vector only.

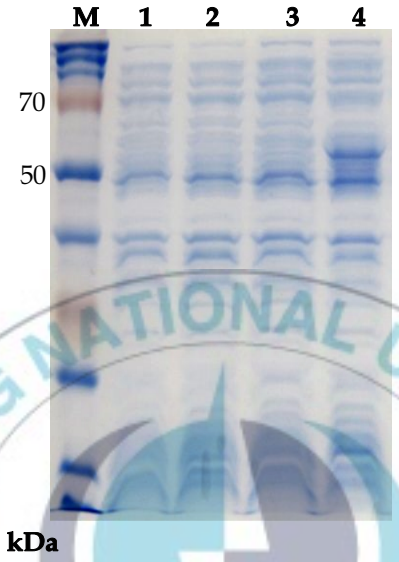


Figure 9. SDS-PAGE analysis of proteins prepared from *E. coli* : Tested were cells transformed with pET19b plasmid (1 and 2), and with recombinant pET19b containing gvpC gene (3 and 4). Lanes 1 and 3 were prepared without IPTG induction, but lanes 2 and 4 were prepared upon IPTG induction. Lane M includes protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom).

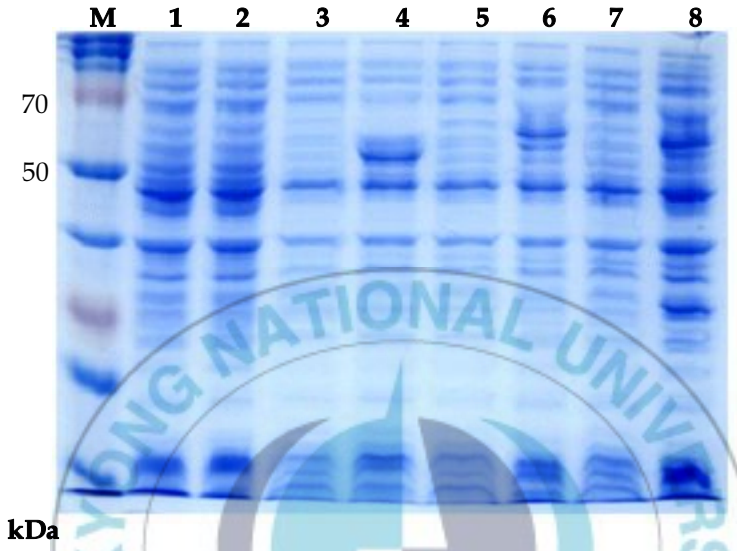


Figure 10. SDS-PAGE analysis of total cell lysate prepared with or without IPTG induction : Tested were cells transformed with vector only (lanes 1 and 2), with plasmids containing recombinant gvpC (lanes 3 and 4), recombinant gvpC fused with silica binding motif (lanes 5 and 6) and recombinant gvpC fused with mercury binding motif (lane 7 and 8). Proteins were extracted from the cells treated with (lanes 2, 4, 6 and 8) and without IPTG induction (lanes 1, 3, 5 and 7). Lane M includes protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom).

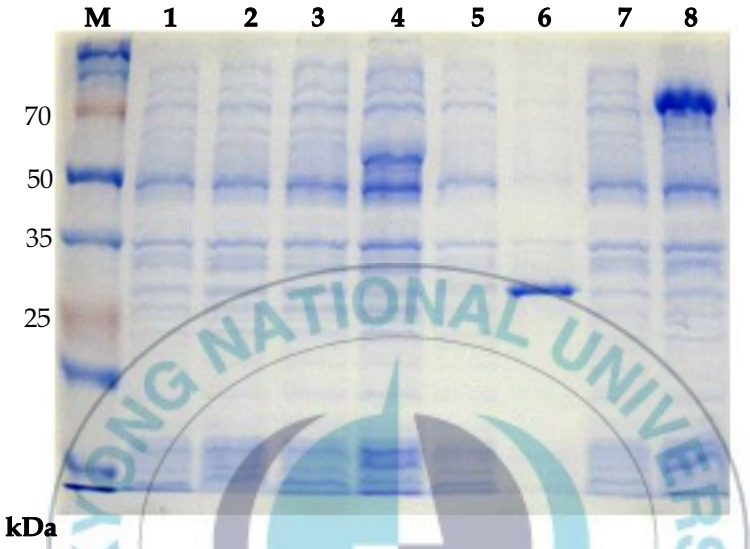


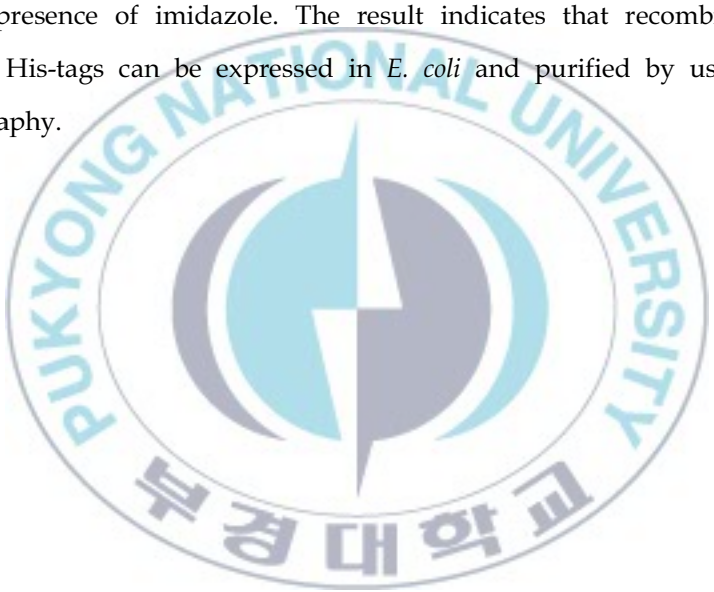
Figure 11. SDS-PAGE analysis of recombinant gvpC fused with lipase : Tested were proteins extracted from cells transformed with vector (lanes 1 and 2), plasmids containing gvpC gene (lanes 3 and 4), plasmids containing lipase (lanes 5 and 6), and plasmids containing gvpC gene fused with lipase (lanes 7 and 8). Proteins were extracted from the cells with (lanes 2, 4, 6 and 8) and without IPTG induction (lanes 1, 3, 5 and 7). Lane M includes protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom).

Figure 12. Identification of gvpC protein fused with metal binding motif by western blot. Proteins were separated on 10% SDS-PAGE : Tested were protein prepared from cells transformed with vector only (lanes 1 and 2), with plasmids containing recombinant gvpC (lanes 3 and 4), recombinant gvpC fused with silica binding motif (lanes 5 and 6) and recombinant gvpC fused with mercury binding motif (lanes 7 and 8). Proteins were extracted from the cells treated with (lanes 2, 4, 6 and 8) or without IPTG induction (lanes 1, 3, 5 and 7).

Figure 13. Identification of gvpC protein containing lipase by western blot. Proteins were separated on 10% SDS-PAGE : Tested samples were proteins extracted from cells transformed with vector (lanes 1 and 2), plasmids containing gvpC gene (lanes 3 and 4), plasmids containing lipase (lanes 5 and 6), and plasmids containing gvpC gene fused with lipase (lanes 7 and 8). Proteins were extracted from the cells with (lanes 2, 4, 6 and 8) or without IPTG induction (lanes 1, 3, 5 and 7).

5. Purification of gvp

Recombinant gvpC was designed to contain His-tag at the either N- or C-termini of gvp genes depending on the primer used (Table 1). Therefore, expression of recombinant gvp not only can be detected by western blotting but also can be purified by using affinity column chromatography. Purification of recombinant gvpC was carried out by Ni-IDA chromatography as described. A distinct protein band in the size of was found in the fraction eluted by presence of imidazole. The result indicates that recombinant gvpC fused with His-tags can be expressed in *E. coli* and purified by using affinity chromatography.



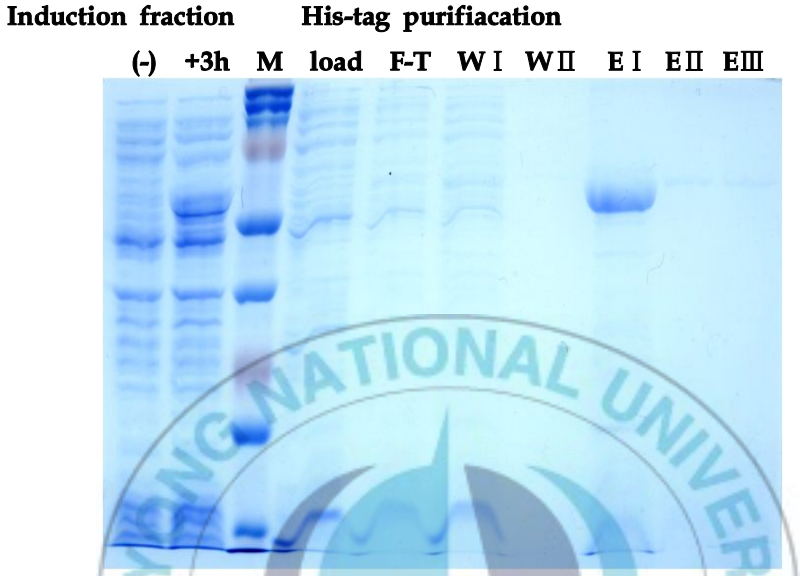


Figure 14. SDS-PAGE analysis of purified gvpC fused with (His)6-tag protein : Tested samples included total proteins extracted from cells transformed by recombinant containing gvpC genes without (-) or with IPTG induction for 3 hrs (+3h). Lane M included protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom) and the rightward lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), washing (W), and elutions (E) of the column.

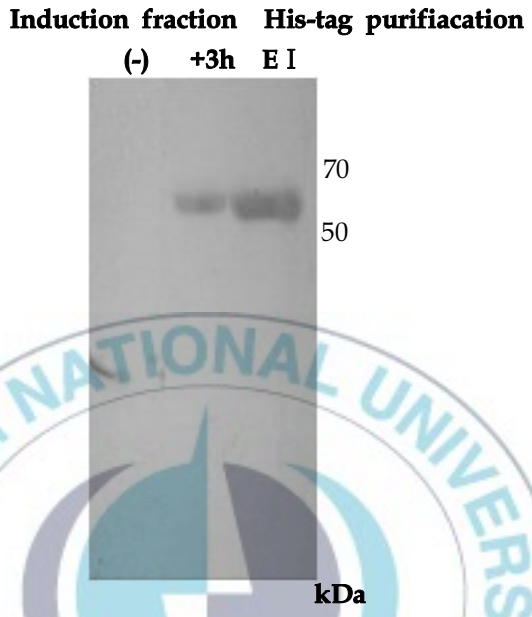


Figure 15. Identification of recombinant gvpC fused with (His)6-tag by western blot. Proteins were separated on 10% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were incubated with an His-Tag-specific antibody and then with secondary antibodies for the detection : Tested samples included total proteins extracted from cells transformed by recombinant containing gvpC genes without (-) or with IPTG induction for 3 hrs (+3h) and the fraction obtained from elution (EI).

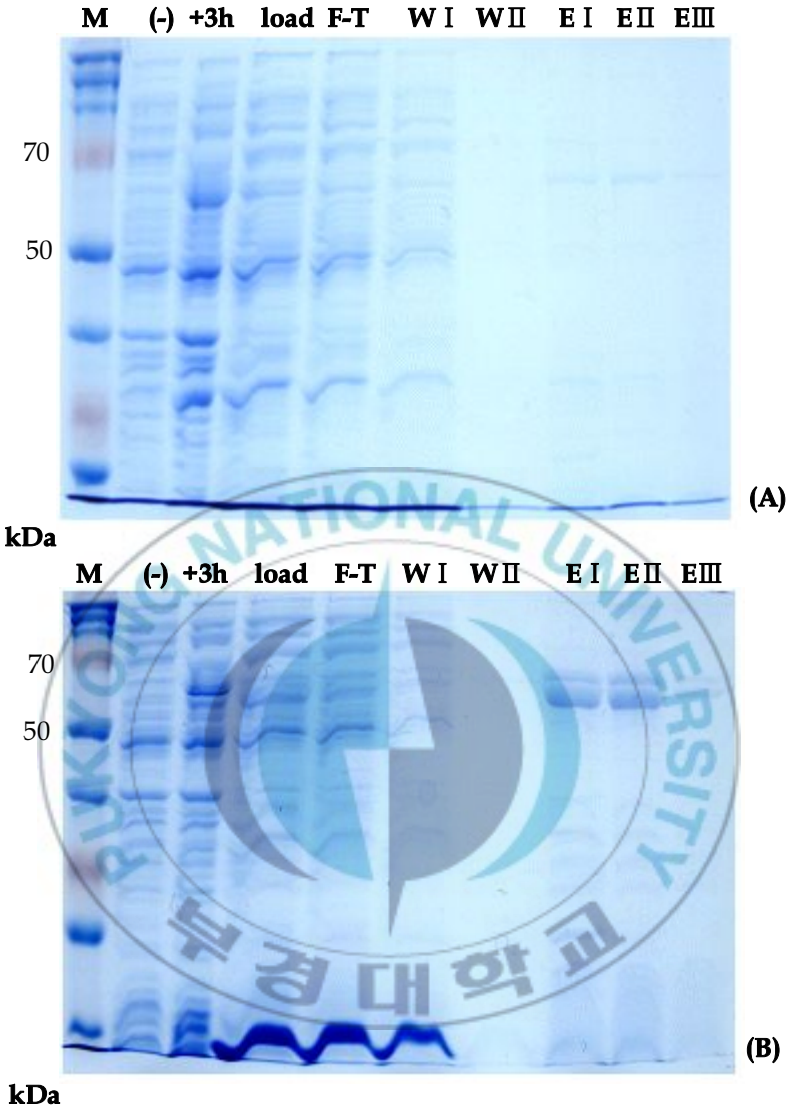


Figure 16. SDS-PAGE analysis of purified gvpC containing binding motif for silica (A) and mercury (B) fused with (His)6-tag : Tested samples included total proteins prepared from cells transformed by recombinant gvpC containing binding motif without (-) or with IPTG induction for 3 hrs (+3h). Lane M includes protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom) and the rightward lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), washing (W), and elutions (E) during affinity chromatograph.

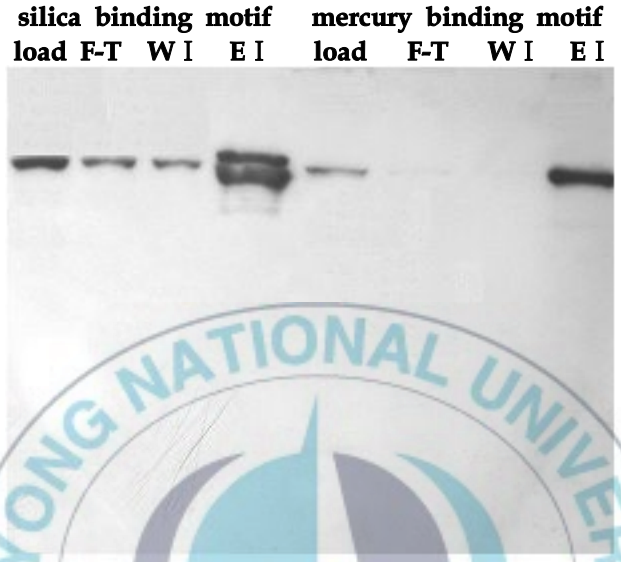


Figure 17. Identification of purified recombinant gvpC containing metal binding motif fused with (His)6-tag by western blot : Tested samples included purified proteins extracted from cells transformed by recombinant gvpC genes containing metal binding motif fused with (His)6-tag. The rightward lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), washing (W), and elutions (E).

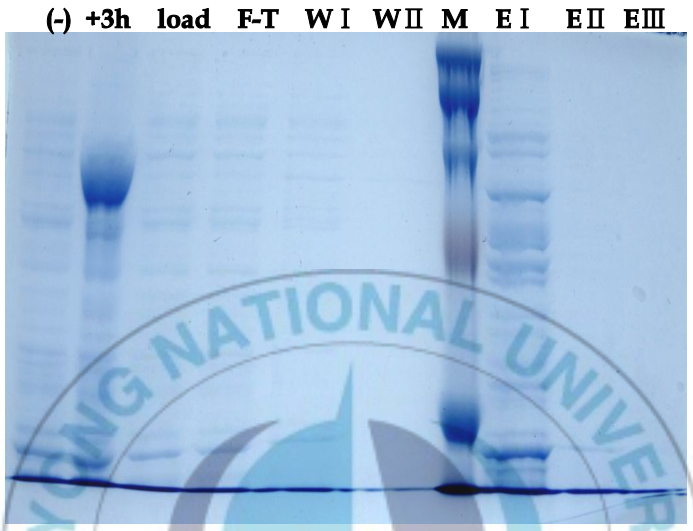


Figure 18. SDS-PAGE analysis of purified recombinant gvpC containing lipase fused with (His)6-tag : Tested samples included total proteins extracted from cells transformed by recombinant gvpC construct without (-) or with IPTG induction for 3 hrs (+3h). Lane M includes protein ladder (240, 140, 100, 70, 50, 35, 25, 20, 15 kDa from top to bottom) and the rightward lanes include the fractions for loading (load) and the fractions obtained from flow through (F-T), washing (W) and elutions (E).

Figure 19. Identification of recombinant gvpC containing lipase and (His)6-tag by western blot : Tested samples included total proteins extracted from cells transformed by recombinant gvpC genes containing lipase fused with (His)6-tag without (-) or with IPTG induction for 3 hrs (+3h). The fractions obtained from the fractions for loading (load), flow through (F-T), washing (W), and elutions (E).

6. Activity assay of recombinant gvpC containing lipase

Activity assay for lipase was carried out on the LB media plate containing rhodamine, IPTG, and antibiotic. For this, transformations were first grown in LB containing amplicillin at 37℃, overnight. Cells were then streaked onto the plate followed by further growth at 37℃, for 3 hrs. Plates were then illuminated under the UV light to compare lipase activity.

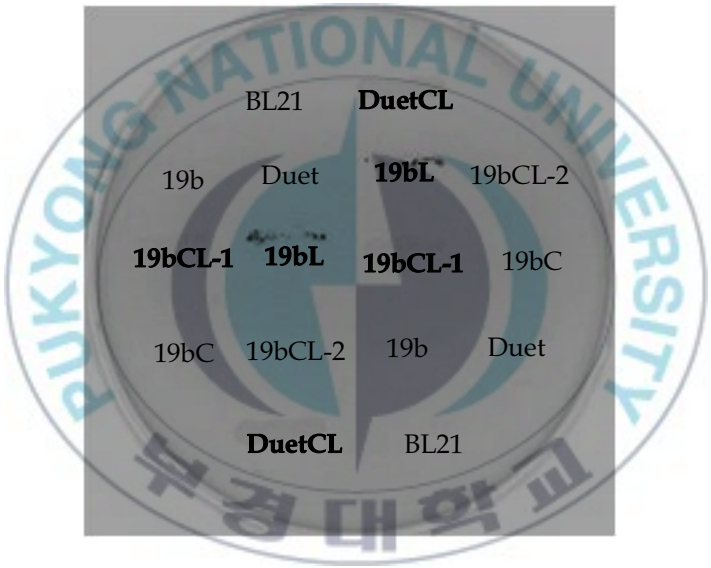
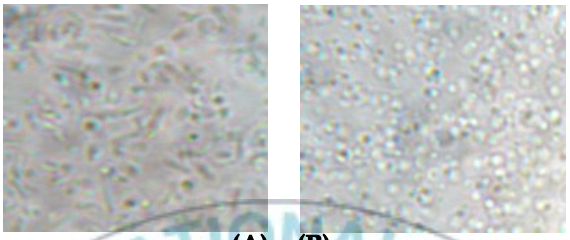


Figure 20. Activity assay of recombinant gvpC containing lipase : Tested samples include *Escherichia* sp. BL21 (BL21), transformed with vector pET19b and pETDuet-1 (19b and Duet), recombinant pET19b plasmid containing lipsae (19bL), recombinant plasmid with gvpC gene in pET19b (19bC), recombinant pET19b plasmids containing gvpC fused with lipase (19bCL-1 and 19bCL-2) and plasmids pETDuet vector containing gvpC gene fused with lipase (DuetCL).

7. Isolation of gas vesicle protein from *Halobacterium* sp.



(A) **(B)**

Figure 21. Analysis of morphology *Halobacterium* sp. SD109 by light microscopy : The figure shows Halobacterium sp. SD109 before (A) and after (B) the treatment with spheroplasting solution.

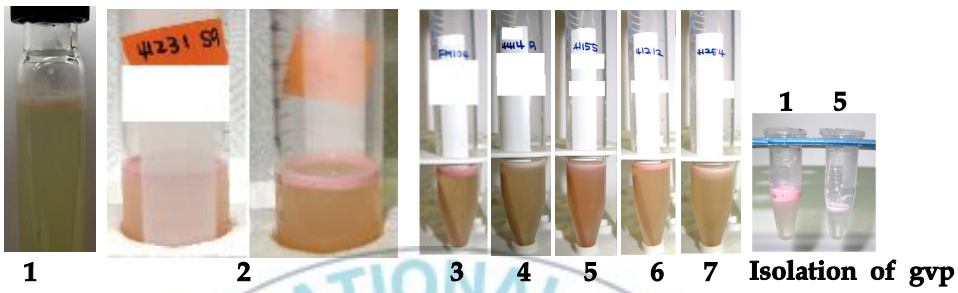


Figure 22. Functional identification of gas vesicle protein in *Halobacterium* sp. : Gas vesicle protein was seen on the top of CM+ media. Samples include *Halobacterium* sp. NRC-1 (1), recombinant plasmid containing gvpA/ gvpC/ and gvpN/ each fused with (His)6-tag (2), shuttle vector pFM104 (3), recombinant plasmid pKJ4414 containing gvp cluster in pKJ408 vector (4), gvpC fused with (His)6-tag cloned into pFM104 (5) and recombinant plasmid pKJ4414 containing gvpA fused with (His)6-tag (6 and 7)

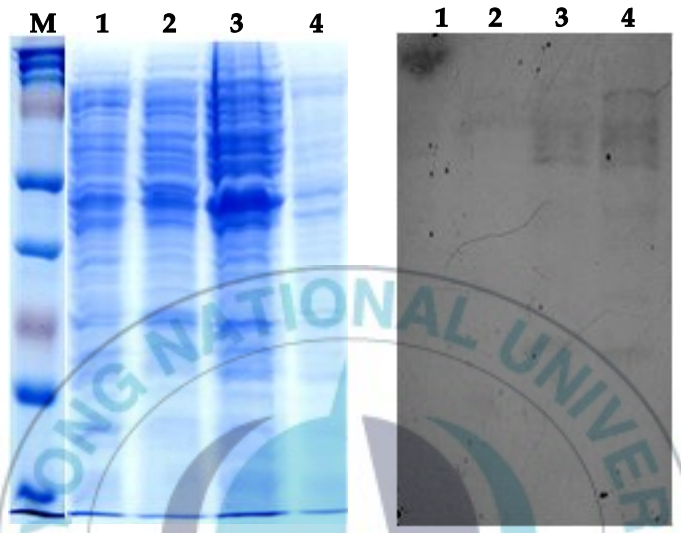


Figure 23. Identification of recombinant gvpC protein in *Halobacterium* by SDS-PAGE followed by western blot : Tested samples include total protein extracted f개m *Halobacterium* sp. NRC-1 (1), purified gas vesicle protein in NRC-1 (2), purified gvpC protein fused with (His)6-tag (3) and total protein gvpC fused with (His)6-tag (4).

Abstract (Korean)

*Escherichia coli*과 *Halobacteria halobium*에서 재조합 gas vesicle 단백질 발현 분석

강 경 묘

부경대학교 대학원 수산생물학과

고세균에 속하며 호염성 미생물인 *Halobacterium*은 민물의 환경에서는 삼투압에 의해 세포가 생존할 수 없는 특성을 가진 생물종이다. *Halobacterium*은 생태계에 적용될 때 유전자 변형생물에 대한 오염을 막으면서도 재조합 단백질 제작 시 유용하게 쓰일 수 있는 다양한 단백질들을 생산하는 종이다. 이 논문에서는 해양환경오염의 주요인이라 생각되는 유류와 중금속 제거를 응용방안 중 하나로 Halobacterium의 주요 단백질 중 하나인 gas vesicle 단백질을 이용하여 재조합 단백질 제작 가능성에 대해 연구하였다. 실험을 위해 먼저 gas vesicle 단백질을 만드는 유전자를 PCR을 통해 증폭하였고 이것을 *Escherichia coli* 에 도입하여 재조합 단백질 유전자의 삽입 여부와 발현유무를 확인하였다. 재조합 유전자를 설계할 때에 Histidine-tag을 함께 재조합하여 재조합 단백질 정제를 용이하게 하였고 단백질 분석 시에 사용되는 SDS-PAGE와 western blot으로 단백질을 확인할 수 있었다.

Ⅳ. Acknowledgement

가장 먼저 2년 동안 함께 석사 생활한 것과 마찬가지이신 부모님 사랑하고 감사드립니다. 몸은 멀리 있지만 심적으로 든든한 힘이 된 오빠 강경훈에게도 고맙다는 말 전하겠습니다. 항상 제 몸 걱정뿐이신 조부모님께도 진심으로 감사의 인사 전하고 싶습니다.

4년의 학부, 2년의 대학원 생활 중 사랑과 많은 가르침을 주신 김창훈 교수님, 조재윤 교수님, 장영진 교수님, 김동수 교수님, 배승철 교수님, 남윤권 교수님, 공승표 교수님 그리고 잊지 못할 조언을 아낌없이 주셨던 허성범 교수님께 감사의 인사 전합니다. 분자 생화학 실험실의 노경언 선배님, 김윤숙 선생님, 친절한 황인철선배, 정이 많이 들어버린 Vo Thi Thu Em 언니, 항상 믿고 의지되는 우리 김초원, 최미진, 장준철 다들 너무 고맙습니다. 그 외에 많은 충고주신 이상윤선배, 힘들 때면 이야기 들어주셨던 송지효선배, 윤현호선배, 김기태선배 그리고 저 많이 챙겨주신 양성진선배, 김대근선배 감사드립니다. 매번 도움 주셨던 최수희언니, 강경림언니, 대학원 생활동안 몸도 마음도 달래 주셨던 이혜정언니, 김성완 선배 진심으로 고맙습니다. 항상 못난 친구 이해해줬던 류유성, 배려 깊은 귀염둥이 김민정, 나의 쌍둥이 정혜정, 내 이야기 묵묵하게 들어주던 조다래, 건강하게 출산하기를 바라는 오유라, 타지에서 고생하는 박주영, 건강 챙기길 바라는 최란 다들 고맙습니다. 힘나게 만드는 신영섭 이제 시작하는 대학원 생활 잘해나가길 바라고, 좋은 결과 있길 바라는 이현주, 후회 없는 한해가 되었으면 하는 항상 미안하고 고마운 김보경 진심으로 고맙습니다. 그 외 정혜영, 이향미언니, 박현식선배, 이종훈선배, 장재영선배, 이보람, 정주연 감사드립니다.

마지막으로 저를 믿어주시고 아껴주셨던 지도 교수님 김종명 교수님께 기대에 미치지 못한 것 같아 죄송스럽기도 하지만 항상 존경하고 감사드린다는 말 전하고 싶습니다. 교수님 진심으로 감사드립니다.

Ⅴ. References

Blaseio, U. and F. Pfeifer. 1990. Transformation of *Halobacterium halobium*: development of vectors and investigation of gas vesicle synthesis. Proc Natl Acad Sci. **87**:6772-6776

Cline, S. W. and W. F. Doolittle. 1987. Efficient transfection of the archaebacterium *Halobacterium halobium*. J Bacteriol. **169**:1341-1344

DasSarma, P., J. A. Coker, V. Huse, and S. DasSarma. 2009. Microorganisms, Halophiles, Industrial Applications. Wiley Encyclopedia of Industrial Biotechnology.

Donovan, R. S., C. W. Robinson and B. R. Glick. 1996. Review: optimizing inducer and culture conditions for expression of foreign proteins under the control of the *lac* promoter. J Ind Microbiol. **16:**145-154

Halladay, J. T, J. G. Jones, F. Lin, A. B. MacDonald and S. DasSarma. 1992. The Rightward gas vesicle operon in Halobacterium plasmid pNRC100: Identification of the gvpA and gvpC gene products by use of antibody probes and genetic analysis of the region downstream of gvpC. J Bacteriol. **175(3):** 684-692

Halladay, J. T., W. L. Ng and S. DasSarma. 1992. Genetic transformation of a halophilic archaebacterium with a gas vesicle gene cluster restores its ability to float. Gene. **119:**131-136

Holmes, M. L. and M. L. Dyall-Smith. 1990. A plasmid vector with a selectable marker for halophilic archaebacteria. J Bacteriol. **172**:756-761

Inoue, H., H. Nojima and H. Okayama. 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene. **96:**23-28

Kushner, D.J. 1966. Mass culture of red halophilic bacteria. Biotechnol Bioeng. **8**:237-245

Jeon, J. H., J. T. Kim, Y. J. Kim, H. K. Kim, H. S. Lee, S. G. Kang, S. J. Kim and J. H. Lee. 2009. Cloning and characterization of a new cold-active lipase from a deep-sea sediment metagenome. Appl Microbiol Biotechnol. **81:**865-874

Kirschke, A. 2006. Engineered Proteins for Binding and Organization of

Inorganic Particles. Biological Appl. p14-15.

Offner, S., U. Ziese, G. Wanner, D. Typke and F. pfeifer. 1998. Structural characteristics of halobacterial gas vesicles. Microbiol. **144:**1331-1342

Oren, A., N. Pri-El, O. Shapiro and N. Siboni. 2005. Gas vesicles isolated from Halobacterium cells by lysis in hypotonic solution are structurally weakened. FEMS Micobiol. **253:**337-341

Pazirandeh, M., B. M. Wells and R. L .Ryan. 1998. Development of bacterium-based heavy metal biosorbents: enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl Envion Microbiol. **64(10):**4068-4072

Pfeifer, F., D. Gregor, A. Hofacker, P. Plößer and P. Zimmermann. 2002. Regulation of Gas Vesicle Formation in Halophilic Archaea. J Mol Microbiol Biotechnol. **4(3):**175-181

Robb, F. T., A. R. Place, K. R. Sowers, H. J. Schreier, S. DasSarma and E. M. Fleischmann. 1995. Archaea : a Laboratory Manual - Halophiled. Cold Spring Harbor Laboratory Press, Plainview, NY.

Roesijadi, G. 1986. Mercury-binding proteins from the marine mussel, Mytilu edulis. Environ Health perspectives. **65:**45-48

Sambrook, J. and D. W. Russell. 2001. Molecular Cloning : A Laboratory Manual. Third edition. Cold Spring Harbor Laboratory Press, Plainview, NY.

Shukla, H.D. and S. DasSrma. 2004. Complexity of gas vesicle biogenesis in *Halobacterium* sp. strain NRC-1 : identification of five new proteins. J Bacteriol. **186(10):**3182-3186

Soppa, J. 2006. From genomes to function : haloarchaea as model organisms. Microbiol. **152:**585-590

Sremac, M., Stuart, E. S., 2008. Recombinant gas vesicles from Halobacterium sp. displaying SIV peptides demonstrate biotechnology potential as a pathogen peptide delivery vehicle. BMC Biotechnol. **8**:9

Thompson, J. D., D.G. Higgins and T. J. Gibson. 1994. CLUSTAL W : improving the sensitivity of progressive multiple sequence alignment through sequence weight matrix choice. Nucl Acids Res. **22:**4673-4680

Walsby, A. E. 1994. Gas vesicles. Microbiol. Rev. **58:**94-144