Thesis for the Degree Master of Science

Effect of proteorhodopsin production in marine bacterium grown under different culture conditions

by

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ABSTRACT

The proteorhodopsins (PRs) are retinal-containing membrane proteins belonging to the rhodopsin family and catalyze a light-activated proton efflux across cell membrane. PRs are estimated to be present in 13% of all marine bacteria in the photic zone. A Gram-negative and orange pigmented marine bacterium was which produced proteorhodopsin isolated from seawater collected in Gwangalli, Korea, and designated as li, . The a, and designateconditions for growth of Pi, KwereKore25℃, pH 7 and s li, . The a containing 3% NaCi. The proteorhodopsin gene from Pi, Kwas cloned and characterized. The expressional studies of proteorhodopsin gene of PR-J were performed.

Key words : Proteorhodopsin, marine bacterium, rhodopsin, light

INTRODUCTION

Proteorhodopsins (PRs) are retinal-containing proteins that catalyze light-activated proton efflux across the photoactive 7 cell membrane proteins [1,2,17].

Originally microbial rhodopsins were typically associated with Archaea, until PRs were identified in an uncultured ɣ- proteobacterium of the SAR86 [5,19]. PRs are estimated to be present in 13% of all marine bacteria in the photic zone [25]. Planktonic Bacteria, Archaea and Eukarya reside compete in the ocean's photic zone under the pervasive influence of light [18].

The oceanic microbe uses a light energy through proteorhodopsin to acquire ATP [6]. The light energy under using proton: H^+ to cut to send with the cell outside, in compliance with the ion grade it is energy production. Proteorhodopsin is the most important factor from this system. PR is composed with opsin and trans-retinal where they are rhodopsin family [3,8]. They belong to the type I in rhodopsin family [9]. Prokaryotic members of this family include photosensors (sensory rhodopsins), transmembrane proton pump (xanthorhodopsin and PRs) and transmembrane chloride pump (halorhodopsins) [21].

Retinal is converted from β-carotene, a carotenoid [7,26]. Marine bacteria will have the gene to be able to synthesize retinal itself, retinal or precursor [23].

All-trans retinal is produced by the central cleavage of the 15, 15' C=C bond of β-carotene. β-carotene synthesis from isoprenoid precursors is accomplished with the four *crt* genes *crt*E, *crt*B, *crt*I, and *crt*Y (Fig. 1) [13-16]. And one molecule of β-carotene yields two molecules of retinal. Oxidative cleavage of β-carotene yields the rhodopsin chromophore retinal.

This enzyme is termed as 15, 15'-β-carotene-dioxgenase. Some proteobacteria can synthesize β-carotene and convert it to retinal similar to eucaryotes, using the enzyme encoded by the *blh* gene to produce functionally active PRs [12].

Proteorhodopsins are all integral membrane proteins containing seven helical plasma membrane-spanning domains that form channel between the cytoplasm and extracellular environment [10]. It is simple heptahelical proton pumps containing a retinal chromophore covalently bound to helix G via a protonated Schiff's base. Key residues lining the inner surface of the channel bind and coordinate a single molecule of all-trans retinal. The corresponding change in protein conformation enables ion transport accessory effector proteins that interact with the sensory rhodopsin family pf proteins (Fig. 2) [4,22].

In a number of marine bacteria, both retinal biosynthetic genes and PRs are genetically linked, and their lateral transfer and retention appear to be relatively common events, indicatively that the photosystem confers a significant fitness advantage. Abundant evidences exist for PRs function as a transmembrane proton pump, including light-mediated transport of protons in right-side-out PRs vesicles.

The proton motive force (pmf) is the electrochemical potential of protons across the membrane, maintained under aerobic conditions by oxidative phosphorylation. Bacteria use the pmf to synthesize ATP, drive chemiosmotic reactions, and power the rotary flagellar motor [11]. Moreover, the extent to which light-harvesting by PRs can complement or replace other cellular energy sources remains to be quantified. Consequently, this study focuses on the proteorhodopsin genes which use a light energy from the marine resources.

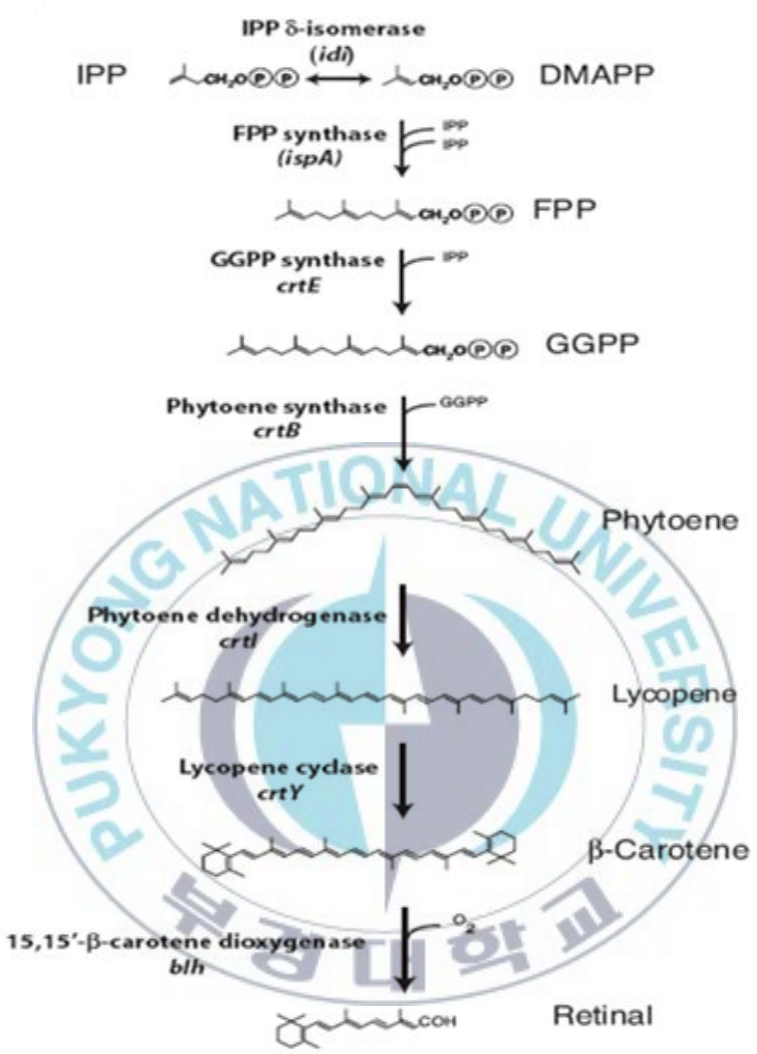

Fig. 1. Scheme of the carotenoid biosynthesis pathways from farnesyl pyrophosphate (FPP) to retinal. (FPP: farnesyl pyrophosphate, IPP: isopentenyl pyrophosphate (diphosphate), *Crt*E: GGPP synthase, GGPP: geranlygeranyl pyrophosphate, *Crt*B: Phytone synthase, *Crt*I: Phytoene desaturase, *Crt*Y: Lycopene cyclase, *Blh*: Bacteriorhodopsin-related-protein-like-homolog protein)

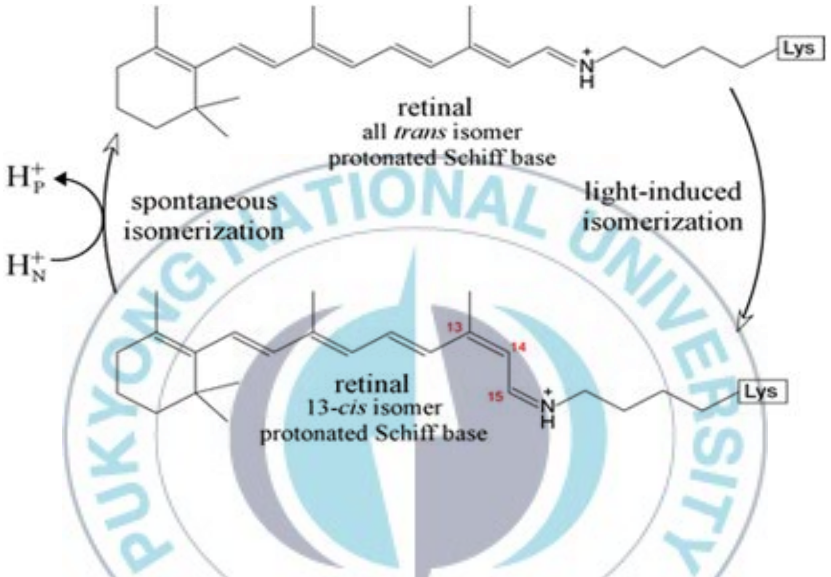

Fig. 2. Scheme of the all trans retinal change to 13-*cis* retinal.

MATERIALS AND METHODS

Sample collection and isolation

Bacteria producing proteorhodopsin substances were collected from Gwangalli, Korea. The samples (seawater) were collected into sterilized glass bottles and transported in the ice box. Bacteria were cultured on PPES-Ⅱ medium with the addition of 1 μM retinal (Table. 1). The plates were incubated at 25℃ for 7 days. Cells were grown under a diurnal light (cool-white light 18 hrs).

Proteorhodopsin Gene cloning

The proteorhodopsin gene was amplified from isolated HTCC1062 (SAR11 strain) and the template samples isolated from seawater by specific polymerase chain reaction using primers 5'- ATGGGTAAAAAACTAAAATT GTTTGCTC -3' (forward) and 5'- CTTAGCTCTACCAGGTTGAG -3' (reverse) [23]. Reaction condition; initial denaturation at 94℃ for 5 min, 30 cycles of denaturation at 94℃ for 30 sec, annealing at 50℃ for 45 sec, extention at 72℃ for 1 min, and then final extention step of 2 min at 72℃. The PCR product was loaded onto 1% agarose gel and the major band of the appropriated size was excised. The DNA from the reaction was purified using the Expin Gel SV kit (GeneAll, Korea).

Table 1. The compositions of PPES-Ⅱ added retinal medium.

Polypeptone	2.0 g
Bacto soytone	1.0 g
Proteose peptone No.3	1.0 g
Bacto yeast extract	1.0 g
NaCl (3%)	30 g
Ferric citrate (0.1%)	10 ㎖
Bacto agar	15 g
Water	1000 ㎖
Retinal concentration	0.1 μM
Final pH	7.0

16S rDNA sequence analysis

16S rDNA sequence analysis was carried out as described by Moyer et al [19]. The 16s rDNA was selectively amplified from purified genomic DNA by using PCR with oligonucleotide primers designed to anneal to conserved positions in the 3' and 5' regions of bacterial 16S rDNA. The forward primer corresponded to positions from 49 to 68 of *E. coli* 16S rDNA and the reverse primer (5'-AGAATTCTAAFACATGCAAGTCAGCG-3') corresponded to the complement of positions 1510 to 1492 (5'-AAGTCGTAACAAGGTAACCGGATCCAC-3').

PCR constituents were PCR premix (GeNet Bio, Korea), 10 pmol forward (1 ㎕), 10 pmol reverse primer (1 ㎕), template DNA (2 ㎕) and sterilized water (16 ㎕). The thermal profile consisted of 25 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 1 min. The PCR product was loaded onto 1% agarose gel and the major band of the appropriated size was excised. The DNA from the reaction is purified using the Expin Gel SV kit (GeneAll, Korea) following the their protocol and ligated into pGEM T-easy vector. The ligation mixture were transformed into XL1-blue competent cells.

Colonies can be picked into 50-80 ㎕ of LB/amp and grown for 2-3 hrs. The proper insert were tested by PCR and analyzed the nucleotide sequences. The resulting sequence data were analysed with programs BLASTN and BLASTX of GenBank.

Bacterial optimum conditions

The temperature range for growth was determined by incubating cells for 3 days in PPES-Ⅱ medium with the addition of 1 μM retinal at the following temperatures; 4, 10, 20, 25, 30, and 37℃, respectively. The pH range for growth was determined by incubating cells for 3 days in PPES-Ⅱ medium with the addition of 1 μM retinal at 25℃ at the following pHs; 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0, respectively. NaCl tolerance was measured in PPES-Ⅱ medium with the addition of 1 μM retinal, at the NaCl concentrations of 0 to 10%. The retinal consistency was measured in PPES-Ⅱ medium at the following concentrations; 0.05, 0.1, 0.2, 0.5, and 0.1 μM.

Growth rate by different component medium

For the best proliferated conditions of isolated bacteria, the growth rates of each constituent element were tested with the following conditions; at 25℃, pH 7, 3% NaCl and 0.1 μM retinal. Each of polypeptone, soytone, proteose peptone, and bacto yeast extracts added into sterilized water and cultivated for 3 days in light or darkness conditions.

Gene knockout

PR gene sequence into algorithm field on the TargeTron Design Site (Sigma-Aldrich, U.S.A) was cut and pasted. PR sequence to generate potential insertion site and the primer design to retarget the intron was submitted [27]. The 3 primer sets (IBS, EBS2, EBS1d) were generated to re-target the intron to PR target site.

PCR reaction was performed in oder to re-target the intron by primer-meditated mutation. The PCR was performed using four primers (Table. 2), included intron PCR template and JumpStarREDTaq ReadyMIX in a single-tube reaction to mutate the intron. PCR reaction consisted of initial denaturation at 94℃ for 30 sec, 30 cycles of denaturation at 94℃ for 15 sec, annealing at 55℃ for 30 sec, extention at 72℃ for 30 sec and then final extention step of 2 min at 72℃. The PCR product was loaded onto 4% agarose gel and the major band of the appropriated size was excised (Fig. 3). The DNA from the reaction was purified using the Expin Gel SV kit (GeneAll, Korea). The purified PCR product was digested with *Hind*Ⅲ and *BsrG* Ⅰ to ligate into the pACD4K-C linear vector (Fig. 4). The reaction was mixed purified PCR product (8 ㎕), 10 × restriction enzyme buffer (2 ㎕), *Hind*Ⅲ (1 ㎕), *BsrG* Ⅰ (1 ㎕), water (8 ㎕), and then incubated at 37℃ for 30 sec, 60℃ for 30 sec, and 80℃ for 10 min. After this reaction, T4 DNA ligase (1 ㎕) was added. This mixture was incubated for 3 hrs for 25℃ at room temperature.

In oder to transform the ligation mixture, PR-J competent cells (100 ㎕) were harvested on ice (20 min) and added ligation mixture (1 ㎕). After heat

shock for 40 sec at 42 °C, cells on ice for 2 min. PPES-Ⅱ medium (900 ㎕, containing of 0.1 mM of retinal) was added. This mixture was incubated for 1 hr at 37℃. After that, Reaction mixture (100 ㎕) was cultured in PPES-Ⅱ added chloramphenicol (25 ㎍/㎖) and 0.1 mM of retinal. The culture fluid w25 ㎍ /㎖) and 0.cultivnd 0.in that medium, 37℃, 0.2 of OD_{600} and cool the incubated to 30℃. At that time, IPTG (100 mM) added,.cultivnd 0.in shaking incubated for 30 min. After this reaction, it was centrifⓄV/㎖d at the maximum speed for 1 min, discarded supernatant,.cultivnd 0.in added PPES-Ⅱ (1 ㎖), 30℃ for 1 hr again. And plnd 0.culture (100 ㎕) on a PPES-Ⅱ agar plate containing Kanamycin (25 ㎍/㎖) and retinal (0.1 mM), incubated at 25℃ for several days. Colonies picked and cultivated in PPES-Ⅱ with retinal. And their growth grade compared with wild type through light existence.

Table. 2. Oligonucleotide primers used for the gene knockout

Name	Nucleotide sequence
IBS	5'-AAAAAAGCTTATAATTATCCTTAACTGTCACGATCGTGCGCCCAGATAGGGTG-3'
EBS1d	5'-CAGATTGTACAAATGTGGTGATAACAGATAAGTCACGATCATTAACTTACCTTTCTTTGT-3'
EBS2	5' TGAACGCAAGTTTCTAATTTCGATTACAGTTCGATAGAGGAAAGTGTCT-3'
EBS Universal	5'-CGAAATTAGAAACTTGCGTTCAGTAAAC-3'

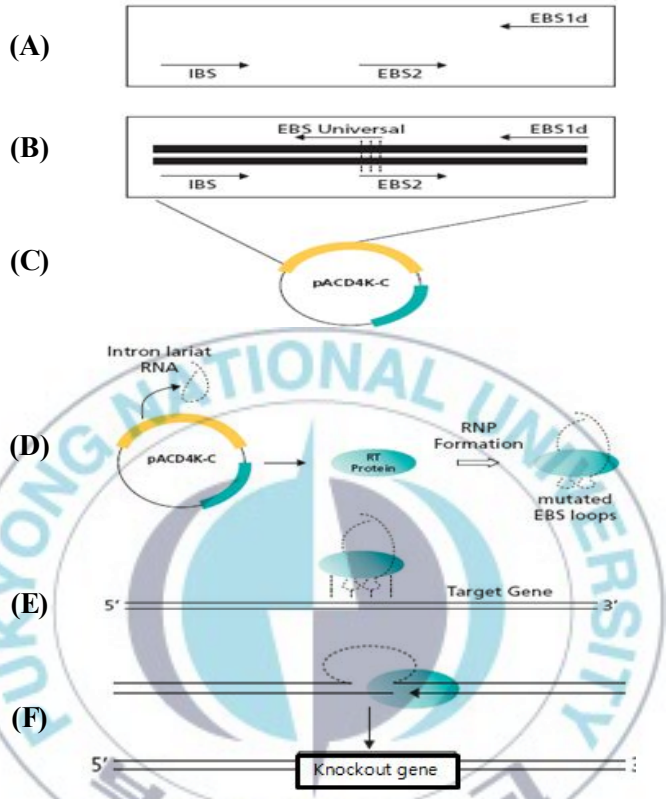

Fig. 3. TargeTron gene knockout system. (A), DNA target site from TargeTron Design Web site and order 3 primers/target site. (B), 1-step assembly PCR to mutate RNA. (C), Clone mutated PCR fragment into expression vector. (D), Transform host and express RNA-protein complex (RNP). (E), Re-targeted RNP locates genomic target. (F), RNP inserts RNA, reverse transcribes cDNA, and host enzymes repair to create a permanent insertion.

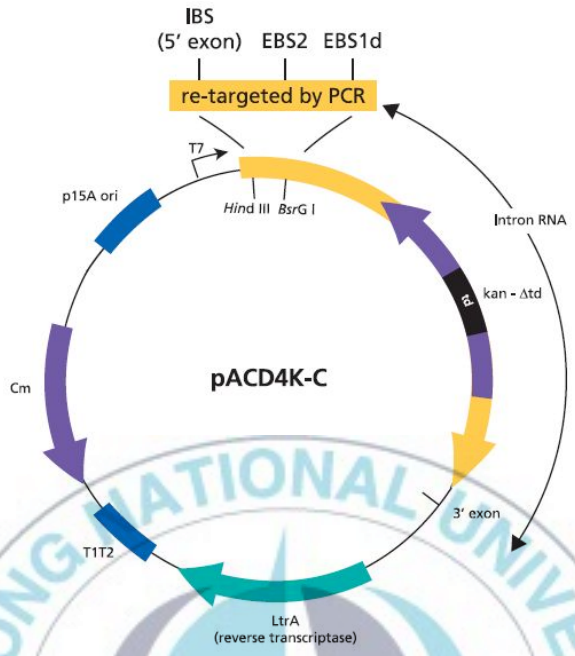

Fig. 4. pACD4K-C plasmid map (7675 bp total). Kan-Δtd; kanamycin RAM marker (for chromosomal insertion selection, not plasmid propagation), LtrA; reverse transcriptase ORF, T1/T2; T1/T2 transcriptional terminator, Cm; chloramphenicol resistance (for plasmid propagation).

RESULTS

Isolation of proteorhodopsin substance-producing microorganisms

Various types of marine bacteria were isolated from the seawater samples collected from Gwangalli, Korea. Among them, some characteristic bacteria were isolated from the plate medium which was cultured on PPES-Ⅱ with supplemented 1 μM retinal. They were cultivated shaking incubator at 25℃ for 5 days (Fig. 5). Each of them performed polymerase chain reaction by specific designed primers for PR gene. The PCR products were identified by electrophoresis on agarose gel. As a result, PCR products were turned out to be 768 bp corresponding to PR gene. This strain was Gram-negative and had a orange pigment (Fig. 6).

Gene cloning of proteorhodopsin

Chromosomal DNA was prepared form isolated strain (PR-J) that was grown to the stationary phase in Marine Broth medium (Difco). The PCR for PR gene was performed using purified chromosomal DNA of PR-J strain as a template. The PCR producthe s8 bp) was used for ligaormed and ducthformation into XL1-bluntoFig. 7) The isolated gene was confirmed after sequn intanalysis oFig. 8) and durned out be 90% sequn intidentity with HTCC1061 by the sequn ce alignment (Fig. 9).

(A) (B)

Fig. 5. Isolation of proteorhodopsin substance-producing microorganisms. (A). Many marine bacteria were isolated on PPES-Ⅱ with additional of 1 μM retinal medium and maintained by serial inoculation. (B). The color and formation on PPES-Ⅱ with additional of 1 μM retinal medium.

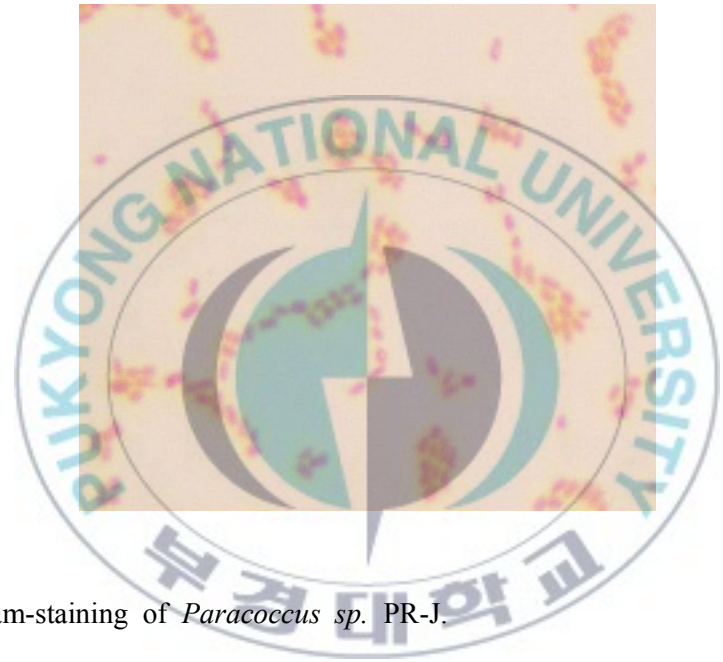

Fig. 6. Gram-staining of *Paracoccus sp.* PR-J.

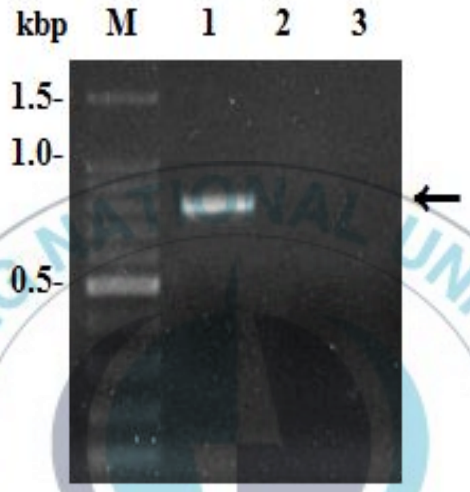

Fig. 7. PCR product amplified with PR-F and PR-R, The lane M 100 bp DNA ladder (Takara, Japan); lane 1: orange pigment bacteria (amplified proteorhodopsin gene), lane 2: orange-red pigment bacteria, lane 3: pinkish pigment bacteria, lane

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     M  G  K  K  L  K  L  F  A  L  T  A  V  A  L  M  G  A  S  G  V  A  N  A   24
1    ATGGGTAAA AAACTAAAA TTGTTTGCT CTTACAGCT GTTGCCCTA ATGGGTGCT TCAGGTGTA GCAAATGCC
     E  T  V  L  L  A  S  D  D  F  V  G  I  S  F  W  L  V  S  M  A  C  L  A   48
73   GAAACTGTG TTGTTAGCA TCTGATGAC TTTGTTGGA ATTTCATTC TGGCTTGTG TCAATGGCT TGTTTAGCA
     A  T  V  F  F  F  L  E  R  S  S  V  P  A  G  W  R  V  S  I  T  V  A  G   72
145  GCAACTGTG TTCTTCTTT TTAGAAAGA AGTTCAGTT CCAGCTGGC TGGAGAGTT TCAATCACT GTTGCAGGA
     L  V  T  G  I  A  F  I  H  Y  M  Y  M  R  E  V  W  I  M  T  G  E  S  P   96
217  CTAGTTACT GGTATTGCA TTCATACAT TACATGTAC ATGAGAGAA GTATGGATC ATGACTGGT GAGTCACCA
     T  V  Y  R  Y  I  D  W  L  I  T  V  P  L  L  M  L  E  F  Y  F  V  L  A  120
289  ACAGTTTAT AGATATATT GACTGGTTA ATTACAGTT CCACTATTG ATGCTAGAA TTCTATTTT GTTCTTGCG
     A  V  G  K  A  N  S  G  V  F  W  R  L  M  L  G  T  L  V  M  L  V  G  G  144
361  GCAGTAGGT AAAGCAAAC TCTGGAGTA TTCTGGAGA TTAATGCTT GGTACTTTA GTAATGCTA GTTGGAGGA
     Y  L  G  E  A  G  Y  I  N  S  M  L  G  F  I  I  G  M  A  G  W  I  Y  I  168
433  TACTTAGGA GAAGCAGGT TACATCAAT TCTATGCTT GGTTTCATT ATCGGTATG GCTGGATGG ATCTACATC
     L  Y  E  V  F  S  G  E  A  G  K  M  A  A  K  S  G  N  K  A  L  V  T  A  192
505  TTATATGAA GTATTTTCA GGTGAAGCA GGTAAAATG GCAGCGAAA AGTGGAAAC AAAGCTCTT GTTACTGCG
     F  G  A  M  R  M  I  V  T  V  G  W  A  I  Y  P  L  G  Y  V  F  G  Y  L  216
577  TTTGGTGCT ATGAGAATG ATCGTTACA GTTGGTTGG GCTATTTAC CCATTAGGT TATGTATTT GGTTACTTA
     T  G  G  V  D  A  N  S  L  N  V  I  Y  N  A  A  D  F  L  N  K  I  A  F  240
649  ACAGGTGGT GTAGATGCT AACTCACTA AACGTGATT TACAACGCA GCTGACTTC TTGAATAAG ATCGCTTTT
     G  L  I  I  W  A  A  A  M  T  Q  P  G  R  A  K                        256
721  GGTCTAATC ATTTGGGCA GCAGCAATG ACTCAACCT GGTAGAGCT AAG
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Fig. 8. Nucleotide and deduced amino acid sequences of proteorhodopsin biosynthesis gene of *Paracoccus sp.* strain PR-J.

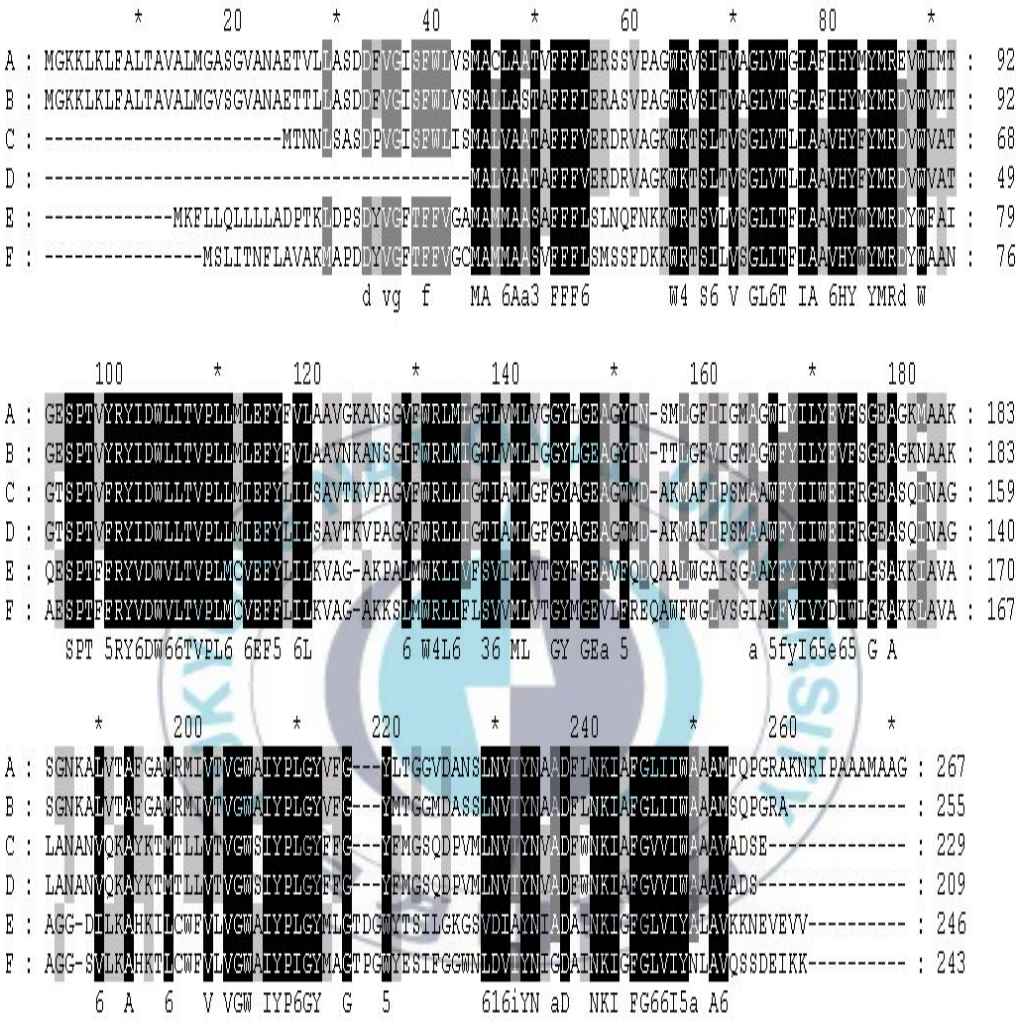

Fig. 9. Multiple alignment of the deduced amino acid sequence of proteorhodopsin biosynthesis gene sequences taken from Genbank. A, PR-J, B, *Candidatus Pelagibacter ubique* strain HTCC1062, C, Gamma proteobacterium HTCC2207, D, Gamma proteobacterium HTCC6216, E, *Dokdonia donghaensis.*

16S rDNA sequence analysis

Extraction of genomic DNA and amplification of 16S rDNA were carried out. The PCR product of 16S rDNA from PR-J strain was ligated into pGEM-T vector (Promega) and sequenced using a Termination Sequencing Ready Reaction Kit (Perkin Elmer, U.S.A) and then electrophoresed using an ABI 377 Genetic Analyzer (Perkin Elmer, U.S.A) (Fig. 10). The resulting sequence data were analyzed with the programs BLASTN and BLASTX at the GenBank database. The sequence similarity of 16S rDNA from PR-J strain with *Paracoccus sp.* was very high, therefore it was designated to *Paracoccus sp.* strain PR-J.

Bacterial optimum conditions

In order to investigate the optimum conditions for the cultivation of isolated PR-J, the effect against temperature, pH, NaCl, and retinal were examined. For optimum temperature, the bacteria were cultured at different temperatures; 4℃, 10℃, 20℃, 30℃, and 37℃, respectively. The optimum temperature was 25℃ (Fig. 11). For optimum pH, the bacteria were cultured at different pH; 3 to 11. The growth appeared to be most prominently from pH 7 (Fig. 12). The optimum NaCl concentration for growth was 3%. When the strain was cultivated at 0% NaCl in the medium, the growth was slow. No growth occurred in the prese No gromore than 5% (w/v) NaCl (Fig. 13). In oder to investigate optimum retinal concentration, the bacteria were cultured

```
   1  AGAATTCTG ATACATGCA AGTCGAGCG AGACCTTCG GGTCTAGCG GCGGACGGG TGAGTAACG CGTGGGAAC
  73 GTGCCCTTC TCTACGGAA TAGCCCCGG GAAACTGGG AGTAATACC GTATACGCC CTTTGGGGG AAAGATTTA
 145 TCGGAGAAG GATCGGCCC GCGTTGGAT TAGGTAGTT GGTGGGGTA ATGGCCCAC CAAGCCGAC GATCCATAG
 217 CTGGTTTGA GAGGATGAT CAGCCACAC TGGGACTGA GACACGGCC CAGACTCCT ACGGGAGGC AGCAGTGGG
 289 GAATCTTAG ACAATGGGG GCAACCCTG ATCTAGCCA TGCCGCGTG AGTGATGAA GGCCTTAGG GTTGTAAAG
 361 CTCTTTCAG CTGGGAAGA TAATGACGG TACCAGCAG AAGAAGCCC CGGCTAACT CCGTGCCAG CAGCCGCGG
 433 TAATACGGA GGGGGCTAG CGTTGTTCG GAATTACTG GGCGTAAAG CGCACGTAG GCGGACTGG AAAGTCAGA
 505 GGTGAAATC CCAGGGCTC AACCTTGGA ACTGCCTTT GAAACTATC AGTCTGGAG TTCGAGAGA GGTGAGTGG
 577 AATTCCGAG TGTAGAGGT GAAATTCGT AGATATTCG GAGGAACAC CAGTGGCGA AGGCGGCTC ACTGGCTCG
 649 ATACTGACG CTGAGGTGC GAAAGCGTG GGGAGCAAA CAGGATTAG ATACCCTGG TAGTCCACG CCGTAAACG
 721 ATGAATGCC AGACGTCGG CAAGCATGC TTGTCGGTG TCACACCTA ACGGATTAA GCATTCCGC CTGGGGAGT
 793 ACGGTCGCA GATTAAAAC TCAAAGGAA TTGACGGGG CCCGCACAA GCGGTGGAG CATGTGGTT TATTCGAGC
 865 ACGCGCAGA ACCTTACCA ACCCTTGAC ATGGCAGGA CCGCTGGAG AGATTCAGC TTTCTCGTA AGAGACCTG
 937 CACACAGGT GCTGCATGG CTGTCGTCA GCTCGTGTC GTGAGATGT TCGGTTAAG TCCGGCAAC GAGCGCAAC
1009 CCACGTCCC TAGTTGCCA GCATTCAGT TGGGCACTC TATGGAAAC TGCCGATGA TAAGTCGGA GGAAGGTGT
1081 GGATGACGT CAAGTCCTC ATGGCCCTT ACGGGTTGG GCTACACAC GTGCTACAA TGGTGGTGA CAGTGGGTT
1153 AATCCCCAA AAGCCATCT CAGTTCGGA TTGTCCTCT GCAACTCGA GGGCATGAA GTTGGAATC GCTAGTAAT
1225 CGCGGAACA GCATGCCGC GGTGAATAC GTTCCCGGG CCTTGTACA CACCGCCCG TCACACCAT GGGAGTTGG
1297 TTCTACCCG ACGACGCTG CGCTAACCT TCGGGGGGC AGGCGGCCA CGGTAGGAT CAGCGACTG GGGTGAAGT
1369 CGTAACAAG GTAACCGGA TCCAC
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Fig. 10. 16S rDNA nucleotide sequence of *Paracoccus sp.* strain PR-J

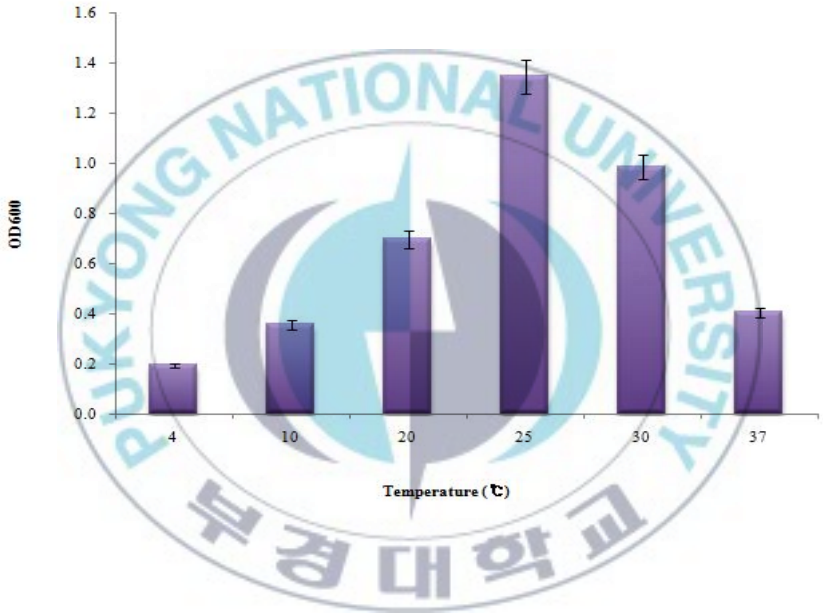

Fig. 11. Effect of temperature on the growth *Paracoccus sp.* PR-J in PPES-Ⅱ with addition of 0.1 μM retinal medium (pH 7.0 and 3% NaCl).

Fig. 12. Effect of pH on the growth *Paracoccus sp.* PR-J in PPES-Ⅱ with addition of 0.1 μM retinal medium (25℃ and 3% NaCl).

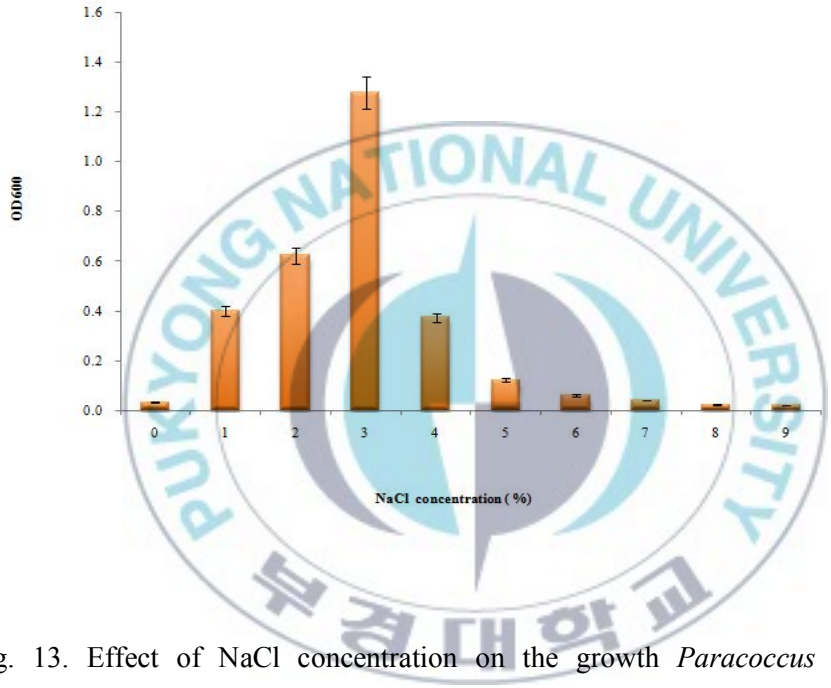

Fig. 13. Effect of NaCl concentration on the growth *Paracoccus sp.* PR-J in PPES Ⅱ (25℃ and pH 7).

at 25℃, pH 7 and NaCl 3% the following different retinal concentrations; 0.05 μM, 0.1 μM, 0.2 μM, 0.5 μM and 1 μM, respectively.

The optimum retinal concentration is 0.1 μM when comparing their growth rate (Fig. 14). Growth was monitored by measuring the turbidity after a 10-day incubation at 25℃, pH 7, 0.1 μM retinal, and 3% NaCl with shaking. At 16 hours after cultivation, *Paracoccus* sp. PR-J entered into logarithmic phase (Fig. 15).

Growth rate by different component medium test of light existence

In order to investigate the optimum proliferated condition of the bacterium, the growth was monitored in the medium containing ingredients which are necessary to proliferation. The result was 25℃, pH 7, 3% NaCl, and 0.1 μM retinal.

The addition of polypeptone, soytone, proteose peptone and yeast extract were used for PPES-Ⅱ medium's created ingredients, under adding with the condition which has the light or dark during 56 hrs cultivated. The proliferation of the bacterium is good from the medium where it added polypeptone, soytone, proteose, yeast extract from the condition which the light (Fig. 16.).

When these four conditions compared with bacterium growth rate in PPES-Ⅱ, there is not so much difference. After that, under the identical optimum conditions it tried to compare bacterial growth rate from mixed medium with PPES-Ⅱ. The result, the possibility of knowing the fact that there

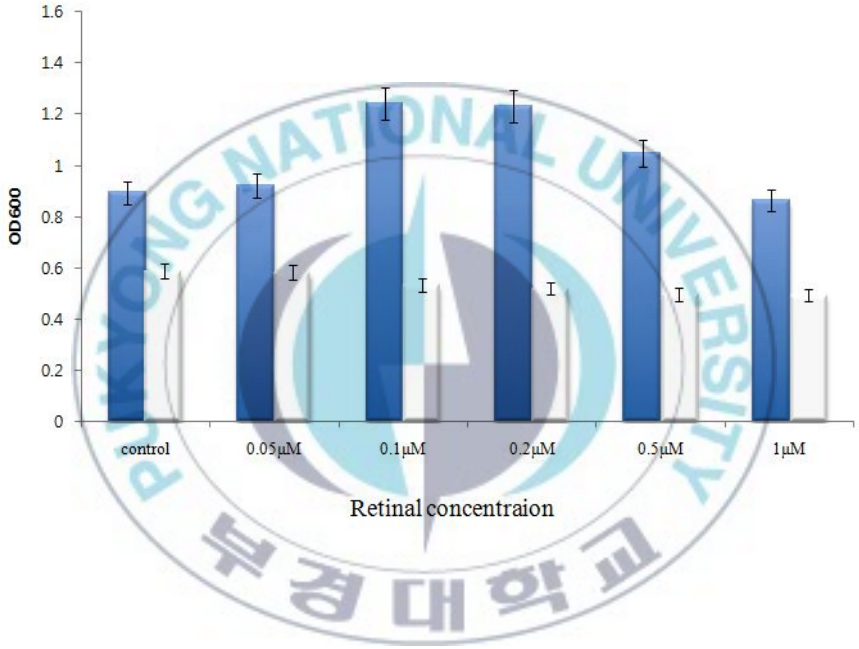

Fig. 14. Effect of retinal concentration on the growth *Paracoccus sp.* PR-J in PPES-Ⅱ (25℃, pH 7 and 3% NaCl). (Light condition, Darkness condition)

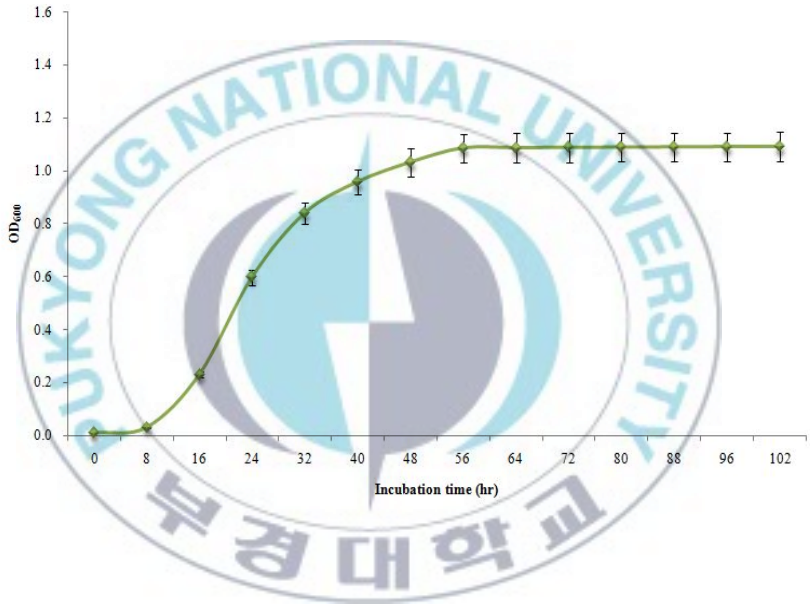

Fig. 15. Time course on the growth *Paracoccus sp.* PR-J at optimum culture (25℃, pH 7, 3% NaCl and 0.1 μM retinal) in PPES-Ⅱ medium.

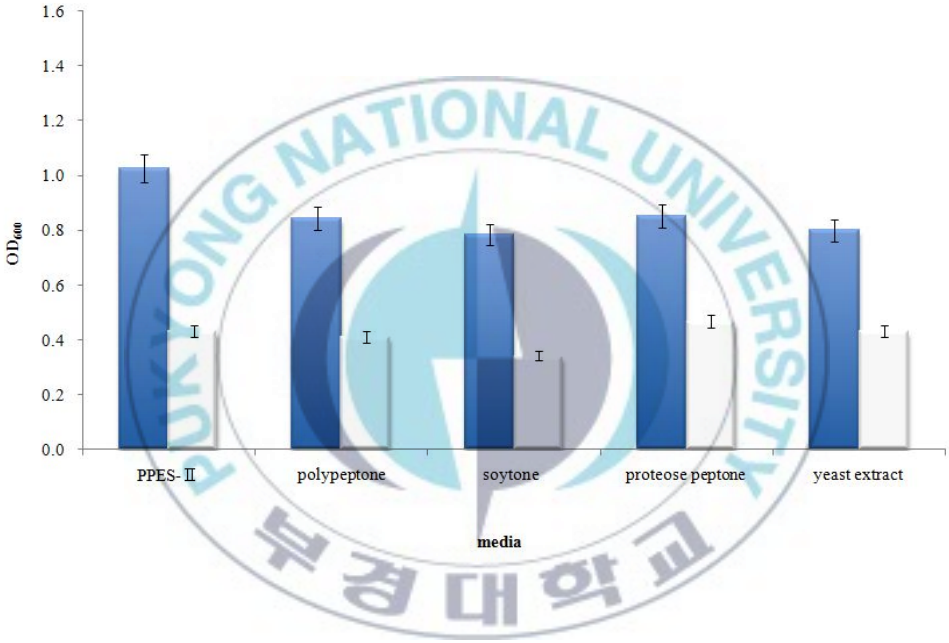

Fig. 16. The growth rate which it follows in each ingredient (25℃, pH 7 and 3% NaCl). (Light condition, Darkness condition)

was not the difference whose growth rate of the bacteria which it raised from different mixed medium and PPES-Ⅱ in the condition which the light. Growth of bacteria in condition without light is less than that of the condition on light (Fig. 17).

Proteorhodopsin gene knockout

TargeTron gene knockout system (Sigma-Aldrhch, U.S.A) made the bacterium where proteorhodopsin becomes mutation. Wild type strain from the conditions which are the light was compared to mutant type bacterium on the growth rate. The culture conditions were 25℃, pH 7, 3% NaCl, for 56 hrs, 0.1 μM retinal concentration, and 150 rpm shaking in PPES-Ⅱ medium. When the light exists, bacteria growth rate wild type was much higher than that of mutant type bacterium. From the dark conditions, both of two cases was not grown well and the growth rate was slow (Fig. 18).

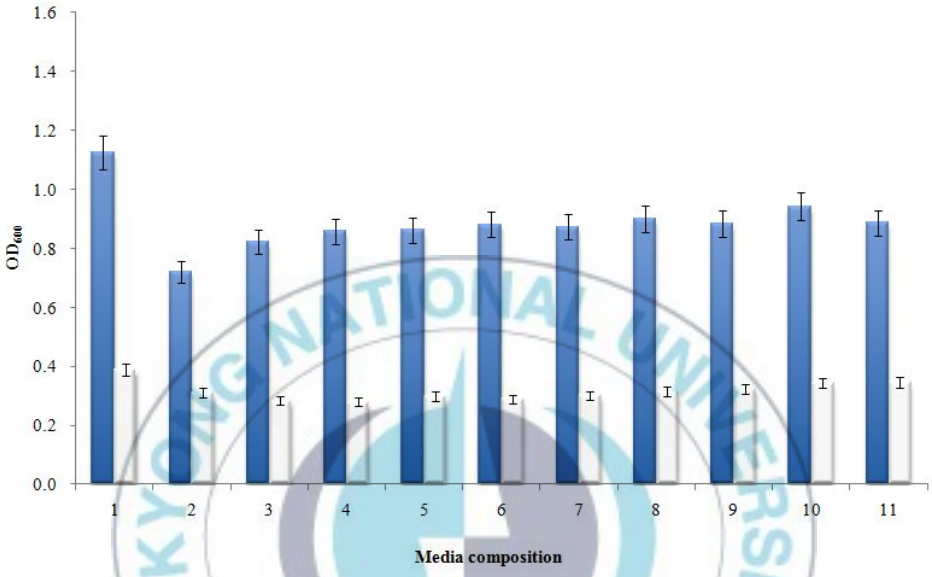

Fig. 17. The growth rate which it follows in mixed media. 1; PPES-Ⅱ, 2; polypeptone, 3; polypeptone and soytone, 4; polypeptone and yeast extract, 5; polypeptone and proteosoe peptone, 6; soytone and yeast extract, 7; soytone and proteose peptone, 8; yeast extract and proteose peptone, 9; polypeptone, soytone and yeast extract, 10; polypeptone, soytone and proteose peptone, 11; soytone, yeast extract and proteose peptone (Light condition, Darkness condition).

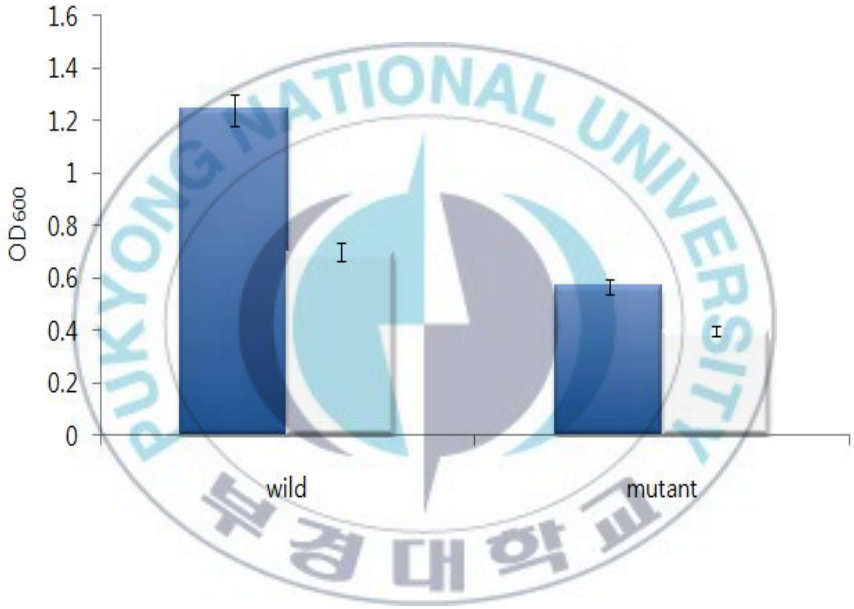

Fig. 18. Growth difference on between wild type and mutant type by existence of light. (Light condition, Darkness condition)

DISCUSSION

Proteorhodopsins are bacterial light-dependent proton pumps. Rhodopins are found in the domains Archaea, Bacteria and Eukarya. Rhodopsin in Archaea functions as energy-transducing light-driven proton or chloride pumps, or as photoactive sensory proteins. In Eukarya, rhodopsins function primarily as sensory proteins. Among them, rhodopsins in Bacteria (proteorhodopsin; PR) are retinal binding integral membrane pigment that are predicted to have a phototrophic potential.

This research was focused on the separation of the microbe which has proteorhodopsin gene from Gwangalli, Korea. This bacterium was Gram-negative and had a orange pigment. The PR gene was turned out to be 768 bp. Isolated strain was examined for the optimum growth conditions corresponding to PR gene. This strain has proteorhodopsin gene, designated as *Paracoccus sp.* strain PR-J. This strain showd the optimum growth from 25℃ temperature, hydrogen ion concentration optimum showed a growth from pH 7. Also, both NaCl and retinal consistency were 3% and 0.1 μM, respectively. It had the bacterium growth rate of optimum from 56 hours.

Based on the optimum growth rate conditions which were completed, PPES-Ⅱ medium it follows respectively ingredients growth rate test. And they divided under the conditions which have the light and the darkness experimented. Proteorhodopsin synthesis gene becomes activation from light condition, this had all about twice higher cell growth rate that of darkness condition.

Whether proteorhodopsin synthesis gene was the active or not in compliance with lights, gene-knockout experiments were performed. Both wild type and mutant growth rate were compared. It appeared that growth rate wild type *Paracoccus sp.* strain PR-J cell is higher that of mutant strain, suggesting that light give an effect to proteorhodopsin activation.

국문초록

Proteorhodopsins (PRs)는 rhodopsin family에 속하는 막단백질로 opsin (seven transmembrane α-helices)과 trans-retinal의 공유결합으로 이루어져 있다. PRs은 빛에너지를 이용해 proton (H^+)이온을 세포 외부로 내보내는 작용으로 생체에너지원을 생성한다. PR 유전자는 planktonic Bacteria, Archaea 그리고 Eukarya에 다양하게 나타난다. 또한, PR 유전자는 유광층에 존재하는 모든 해양미생물 중 13%에 존재하며 빛의 영향을 받는 것으로 알려져 있다.

오렌지색을 띠며, 그람 음성균인 이 해양세균은 광안리 연안으로부터 분리되어졌다. 이 균은 *Paracoccus sp.*와 가장 유사성이 높았으며, proteorhodopsin을 활성화하는 유전자를 가지고 있었다. PR 유전자의 클로닝을 통해 사이즈를 확인한 결과 768 bp였다.

이 세균의 세포 성장률이 가장 최적인 조건은 25℃, pH 7, 3% NaCl, 그리고 0.1 μM retinal 일 때, 성장 시간이 56시간일 때 가장 높았다. 이 균의 성장률은 빛이 있는 조건에서 PPES-Ⅱ 배지에서 가장 높았다. 또한 최소배지에서 빛이 있는 조건에서의 세포 증식률과의 비교 시, PR 유전자 knockout을 통해서 proteorhodopsin은 빛이 존재할 때 증식률이 높아진다는 것을 알 수 있었다.

본 논문에서는 균주에서 proteorhodopsin생성 조건 연구를 통해서 유용 에너지 자원 확보의 기초단계 확립하고자 이루어졌다.

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REFERENCES

1. Béjà, O., E. N. Spudich, J. L. Spudich, M. Leclerc, and E. F. DeLong. 2001. Proteorhodopsin phototrophy in the ocean. *Nature* **411**, 786-789.

2. Béjà, O., L. Aravind, E. V. Koonin, M. T. Suzuki, A. Hadd, L. P. Nguyen, S. B. Jovanovich, C. M. Gates, R. A. Feldman, J. L. Spudich, E. N. Spudich, and E. F. DeLong. 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* **289**, 1902-1906.

3. Bielawski, J., K. Dunn, G. Sabehi, and O. Béjà. 2004. Darwinian adaptation of proteorhodopsin to different light intensities in the marine environment. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 14824−14829.

4. Birge, R. R. 1990. Nature of the primary photochemical events in rhodopsin and bacteriorhodopsin. *Biochim. Biophys. Acta* **1016**, 293−327.

5. Boichenkoa, V. A., J. M. Wangb, J. Antónc, J. K. Lanyib, and S. P. Balashovb. 2005. Functions of carotenoids in xanthorhodopsin and archaerhodopsin, from action spectra of photoinhibition of cell respiration. *Science* **309**, 2061-2064.

6. Frigaard, N., A. Martinez, T. J. Mincer1, and E. F. DeLong. 2006.

Proteorhodopsin lateral gene transfer between marine planktonic Bacteria and Archaea. *Nature* **439,** 847-850.

7. Gazalah, S., L. Alexander, K. H. Jung, R. Partha, J. L. Spudich, T. Isaacson, J. Hirschberg, M. Wagner, and O. Béjà. 2005. New insights into metabolic properties of marine bacteria encdedoding proteorhodopsins. *PLoS Biol.* **3**, e273.

8. Giovannoni, S. J., L. Bibbs, J. C. Cho, M. D. Stapels, R. Desiderio, K. L. Vergin, M. S. Rappe, S. Laney, L. J. Wilhelm, H. J. Tripp, E. J. Mathur, and D. F. Barofsky. 2005. Proteorhodopsin in the ubiquitous marine bacterium SAR11. *Nature* **438**, 82-85.

9. Gonzalez, M. R., and I. Marin. 2004. New insights into the evolutionary history of type 1 rhodopsins. *J. Mol. Evol.* **58,** 348-358.

10. Jose´, R. T., L. M. Christianson, O. Be´ja`, M. T. Suzuki, D. M. Karl, J. Heidelberg, and E. F. DeLong. 2003. Proteorhodopsin genes are distributed among divergent marine bacterial taxa. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 12830–2835.

11. Kashket, E. R. 1985. The Proton Motive Force in Bacteria : A Critical Assessment of Methods. *Ann. Rev. Micro.* **39,** 219-242.

12. Kim, S. Y., A. Stephen, W. L. S. Brown, and K. H. Jung. 2008. Screening and characterization of proteorhodopsin color-tuning mutations in *Escherichia coli* with endogenous retinal synthesis. *Biochim. Biophys. Acta* **1777**, 504–513.

13. Lee, J. H., Y. B. Seo, S. Y. Jeong, S. W. Nam, and Y. T. Kim. 2007. Functional Analysis of Combinations in Astaxnathin Biosynthesis Genes from *Paracoccus haeundaensis*. *Biotechnol. Bioprocess Eng.* **12**, 312-317.

14. Lee, J. H., and Y. T. Kim. 2006. Cloning and characterization of the astaxanthin biosynthesis gene cluster from the marine bacterium *Paracoccus haeundaensis*. *Gene* **370**, 86-95.

15. Lee, J. H., and Y. T. Kim. 2006. Functional expression of the astaxanthin biosynthesis genes from a marine bacterium, *Paracoccus haeundaensis*. *Biotechnol. Lett.* **28**, 1167-1173.

16. Lee, J. H., Y. S. Kim, T. J. Choi, W. J. Lee, and Y. T. Kim. 2004. *Paracoccus haeundaensis* sp. nov., a Gram-negative, halophilic, astaxanthin-producing bacterium *Int. J. Syst. Evol. Microbiol.* **54**, 1699-1702.

17. Martinez, A., A. S. Bradley, J. R. Waldbauer, R. E. Summons, and E. F. DeLong. 2007. Proteorhodopsin photosystem gene expression enables

photophosphorylation in a heterologous host. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 5590-5595.

18. McCarren, J., and E. F. DeLong. 2007. Proteorhodopsin photosystem gene clusters exhibit co-evolutionary trends and shared ancestry among diverse marine microbial phyla. *Environ. Microbiol.* **9**, 846-858.

19. Morris, R. M., M. S. Rappe´, S. A. Connon, K. L. Vergin, W. A. Siebold, C. A. Carlson, and S. J. Giovannoni. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. *Nature* **420**, 806-810.

20. Moyer, C. L., F. C. Dobbs, and D. M. Karl. 1994. Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ Microbiol.* **60**, 871-879.

21. Oesterhelt, D., and W. Stoechenius. 1973. Funtions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2853-2857.

22. Ottolenghi, M., and M. Sheves. 1989. Synthetic retinals as probes for the binding site and photoreactions in rhodopsins. *J. Membr. Biol.* **112**. 193−7212.

23. Peck, R. F., C. E. Erasun, E. A. Johnson, V. N. Wailap, S. P. Kennedy, L. Hood, S. DasSarma, and M. P. Krebs. 2001. *Brp* and *blh* are required for synthesis of the retinal cofactor of bacteriorhodopsin in *Halobacterium salinarum*. *J. Biol. Chem.* **276**, 5739-5744.

24. Rappe´, M. S., S. A. Connon, K. L. Vergin, and S. J. Giovannoni. 2002. Cultivation of the ubiquitous SAR11 marine bacterioplankton clade. *Nature.* **418,** 630-633.

25. Stingl, U., R. A. Desiderio, J. C. Cho, K. L. Vergin, and S. J. Giovannoni. 2007. The SAR92 Clade: an Abundant Coastal Clade of Culturable Marine Bacteria Possessing Proteorhodopsin. *Appl. Environ. Microbiol.* **73**, 2290-2296.

26. Walter, J. M., D. Greenfield, C. Bustamante, and J. Liphardt. 2007. Light-powering *Escherichia coli* with proteorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 2408-2412.

27. Zhong, J., M. Karberg, and A. M. Lambowitz. 2003. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retro transposition-activated selectable marker. *Nucleic Acids Res.* **31**, 1656-1664.