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Bacteriophage membrane protein P9 as a fusion partner for the efficient expression of membrane proteins in *Escherichia coli*

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ABSTRACT

Despite their important roles and economic values, studies of membrane proteins have been hampered by the difficulties associated with obtaining sufficient amounts of protein. Here, we report a novel membrane protein expression system that uses the major envelope protein (P9) of phage φ6 as an N-terminal fusion partner. Phage membrane protein P9 facilitated the synthesis of target proteins and their integration into the *Escherichia coli* cell membrane. This system was used to produce various multi-pass transmembrane proteins, including G-protein-coupled receptors, transporters, and ion channels of human origin. Green fluorescent protein fusion was used to confirm the correct folding of the expressed proteins. Of the 14 membrane proteins tested, eight were highly expressed, three were moderately expressed, and three were barely expressed in *E. coli*. Seven of the eight highly expressed proteins could be purified after extraction with the mild detergent lauryldimethylamine-oxide. Although a few proteins have previously been developed as fusion partners to augment membrane protein production, we believe that the major envelope protein P9 described here is better suited to the efficient expression of eukaryotic transmembrane proteins in *E. coli*.

1. Introduction

Approximately one-third of proteins expressed by an organism are membrane proteins [1]. These membrane proteins, which include receptors, channels, transporters, pumps, and enzymes, perform a variety of functions vital to the survival of organisms. Furthermore, membrane proteins are major targets for small molecule drugs and therapeutic antibodies [2–5]; G-protein-coupled receptors (GPCRs) represent the most important class of drug targets in terms of therapeutic benefit and pharmaceutical sales [6]. However, in spite of their important physiological roles and economic values, current understanding of the structures and functions of membrane proteins lags far behind that of soluble proteins. One of the major reasons for this delay is that it is very difficult to produce membrane proteins [7]; in fact, heterologous expression of membrane proteins remains one of the major challenges in recombinant DNA technology.

A number of valuable techniques for the production of membrane proteins have been developed over the last 2 decades [8–10]. Protein fusion techniques are useful for determining correct folding or to enhance the expression of membrane proteins. For example, Drew et al. employed green fluorescent protein (GFP) as a tool for measuring membrane protein expression [11]. A GFP fusion was also used to monitor the production and purification of membrane proteins in *Escherichia coli* [12] and *Saccharomyces cerevisiae* [13]. Roosild et al. used a small *Bacillus* protein named Mistic to chaperone the production and integration of downstream cargo proteins [14]. Researchers have also attempted to modify host bacterial cells to optimize membrane protein production. By tuning the transcription rate of target genes and reducing the adverse effects of overexpression of membrane proteins, Schlegel et al. optimized an *E. coli* host system for efficient membrane protein expression [15,16]. Massey-Gendel et al. developed a method to select mutant strains of *E. coli* that improve the expression of target membrane proteins and demonstrated high levels of expression of membrane proteins from *Mycobacterium tuberculosis* in these mutant strains [17]. Finally, Plückthun and coworkers developed a method for the directed evolution of integral membrane proteins in the inner membrane of *E. coli* and used this technique to successfully engineer mutant GPCRs with higher levels of expression and stability than their wild-type counterparts [18,19]. Despite these important achievements, producing sufficient amounts of membrane proteins for structural studies or antibody production is still problematic. Therefore, the development of

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an efficient membrane protein expression system remains a major challenge in recombinant DNA technology.

Pseudomonas phage φ6 is unusual in that its nucleocapsid is covered by a membrane envelope originating from the cytoplasmic membrane of its host bacterium, *Pseudomonas syringae* [20]. In addition to phospholipid, the phage envelope contains three proteins: P9, P10, and P13. P9 of φ6, the major envelope protein, comprises 90 amino acids and has a putative single transmembrane segment [21]. When φ6 infects its natural host, *P. syringae*, a large amount of P9 protein is rapidly produced and integrated into the host's cytoplasmic membrane [22,23]. P9 is also integrated into the cytoplasmic membrane when expressed in *E. coli* [24]. Based on these observations, we speculated that the major envelope protein may aid the biosynthesis and integration of foreign membrane proteins. Here, we show that P9 of φ6 assists the production of its chimeric protein in the *E. coli* membrane, suggesting that it may be a valuable fusion partner for efficient membrane protein expression.

Table 2
Primers used for mutagenesis and construction of GFP fusions.

Primer name	DNA sequence
P9 FOR	5′-GGAGATATACATATGCCGTTTC-3′
P9CDEL71REV	5′-CCTCGAGCCGATCAGCTCGCAGATCA-3′
P9CDEL51REV	5′-CCTCGAGTCCTGACGGGTCACGAACTCGTG-3′
P69 S12C FOR	5′-GCAAAGCGTTCACCGAAGCC-3′
P69 S12C REV	5′-AGGTCGGGTCCTGTTTCACC-3′
P69 S22C FOR	5′-GCACCGGCACCCAGATCCTGG-3′
P69 S22C REV	5′-AGCGTTCGCTGGCTTCGGTGA-3′
P69 S77C FOR	5′-TGGTCGCAAAGCGATCCTGGC-3′
P69 S77C REV	5′-GCACCGGCACCCAGATCCTGG-3′
P69 F3C FOR	5′-CATATGCCGTGTCCGCTGGTGAAACAGGAC-3′
P69 A90C REV	5′-GCTCGAGATGCACAGAAACGGAATGTTGGC-3′
StoCuniV FOR	5′-GTAGCTCTGGATCCGGCAAACAAACCACCG-3′
StoCuniV REV	5′-GTTTGCCGGATCCAGAGCTACCAACTCTTTT-3′
69TM FOR	5′-GTCGACCCGTCAGGAACAAGCGGTC-3′
P9EcoRV	5′-TACATATGGGATATCCGCTGGTGAAACAGGACC-3′
GFPfor	5′-AGCTAGCTCAAGCTTGGAATTCTGGATCCGCTGGCTCCGCTG-3′
GFPrev	5′-GAGGCCTTAAGTTATTTGTAGAGCTCATCCAT-3′
P9RevAfl	5-TCCGGACTTAAGTTAATGGTGATGGTGATGGT-3

2. Materials and methods

2.1. Plasmid construction and protein analysis

The pMP6 plasmid backbone was generated from an NdeI-HindIII fragment of pRSETa (Invitrogen). A DNA fragment containing the codon-optimized P9 gene of φ6 and linker DNA (with a thrombin cleavage site), a multiple cloning site, and a 6X His-tag was chemically synthesized and inserted into the NdeI and HindIII sites of pRSETa. An unanticipated mutant clone (pMP6), in which Gly64 was mutated to Cys (G64C), performed slightly better than wild-type pMP6; therefore, the former plasmid was used in all experiments. The DNA sequence of pMP6 is available from the authors upon request.

Target proteins were randomly chosen among membrane proteins whose full length cDNA clones were available from Korea Human Genbank (http://genbank.kribb.re.kr/Search/HumanCDna/SearchForm.aspx). The primers used to amplify the cDNAs encoding the proteins examined in this study are listed in Table 1. These cDNAs were inserted into the multiple cloning site of pMP6 to produce the P9 chimeric proteins. Template cDNA clones were obtained from The Center for Functional Analysis of Human Genome (Taejon, Korea). All clones were confirmed by DNA sequencing.

To express target proteins without P9 tagging, the P9 ORF in pMP6 was amplified with primers P9EcoRV and P9RevAfl (Table 2) and the Nde-HindIII fragment of pMP6 was then replaced with an NdeI-HindIII fragment of the above amplified DNA. This process generated a plasmid (pXP6) with an EcoRV restriction site positioned immediately after the initiation codon of P9. PCR-amplified cDNA fragments were then inserted into the EcoRV-HindIII sites of pXP6 to produce plasmids expressing target genes without P9 tagging. To generate the target-GFP fusions, the GFP ORF was amplified by PCR using the GFPfor and GFPrev primers (Table 2), and then cloned into a *T*-vector. The DNA fragment containing the GFP ORF was excised from the *T*-vector and cloned into the expression plasmids directly after the membrane protein.

For SDS–PAGE analysis, cells were harvested and resuspended in 20 mM Tris–HCl (pH 7.5), and then sonicated. After the addition of 5X SDS–PAGE loading buffer, the samples were incubated at 25 °C for 20 min and then separated by SDS–PAGE. Proteins were visualized by Coomassie brilliant blue R250 staining. For the GFP fusions, fluorescent bands were observed before staining using a UV transilluminator. 5 μg and 0.1 μg of protein per well was loaded for Coomassie stained gels and immunoblots, respectively. Boiling caused aggregation of membrane proteins and delayed migration through the gel; therefore, it was important not to boil the sample before gel loading.

2.2. Localization and purification of P9 and antibody generation

E. coli BL21(DE3) cells harboring pMP6 were cultivated at 25 °C; once they reached an OD_{600} of 0.5, the cells were induced by the addition of isopropylthio-β-galactoside (0.5 mM final concentration). After 4 h of induction, the cells were harvested and resuspended in P9 buffer comprising 20 mM Tris–HCl (pH 8.0), 0.3 M

Table 1
Primers used for amplification of cDNAs.

Gene	Forward primer	Reverse primer
ADORA3	5′-AAGCTGCAGATATCCCCAACAACAGCACTGCT-3′	5′-GGGGTACCAATTGCTACTCAGAATTCTTCTC-3′
EDNRA	5′-TGACCAGCTGAAACCCTTTGCCTCAGGG-3′	5′-AGCTAAGCTTGGTTCATGCTGTCCTTATGG-3′
LPAR1	5′-TGACCAGCTGCTGCCATCTCTACTTCCATCCC-3′	5′-AGCTAAGCTTGAACCACAGAGTGGTCATTG-3′
NPYR1	5′-ATGACAATATTCAACATTATTTTCCCAGG-3′	5′-AGCTAAGCTTGATTTTTTCATTATCATCATTG-3′
SLC19A2	5′-GACAGCTGATGTGCCCGGCCCGGTGTC-3′	5′-GGAAGCTTCTGAAGTGGTTACTTGAGAACT-3′
HTR3A	5′-GACAGCTGTGCTGCTGTGGGTCCAGCAG-3′	5′-GGAAGCTTCAGCGTACTGCCAGATGGACCA-3′
P2RX4	5′-GAGATATCTGGCGGGCTGCTGCGCCGCG-3′	5′-GGAAGCTTCCTGGTCCAGCTCACTAGCAAG-3′
SLC2A4	5′-GAGATATCTGCCGTCGGGTTTCCAGCAG-3′	5′-GGAAGCTTCGTCATTCTCATCTGGCCCTAA-3′
PTGES	5′-GACAGCTGTGCCTGCCCACAGCCTGGTGA-3′	5′-GGAAGCTTCCAGGTGGCGGGCCGCTTCCCA-3′
DRD2	5′-CCCGGGTGGATCCACTGAATCTGTCCTGG-3′	5′-AAGCTTCGCAGTGGAGGATCTTCAGGAA-3′
CHRM1	5′-GATATCCCAACACTTCAGCCCCACCTGCTG-3′	5′-AAGCTTCGCATTGGCGGGAGGGAGTGCGG-3′
CYSLTR1	5′-CCCGGGACGATGAAACAGGAAATCTGACA-3′	5′-AAGCTTCTACTTTACATATTTCTTCTCC-3′
MC1R	5′-GATATCCTGCTGTGCAGGGATCCCAGAGA-3′	5′-AAGCTTCCCAGGAGCACGTCAGCACCTC-3′
PTGER3	5′-GATATCACAAGGAGACCCGGGGCTACGGA-3′	5′-AAGCTTCATTTCCCCAAAATTCCTCTTG-3′