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Expression of Membrane Proteins in *Escherichia coli* as Fusions to the Phage phi6 Major Envelope Protein

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A large portion of an organism's proteome comprises membrane proteins; these proteins are physiologically important and are often major drug targets. Despite eliciting substantial academic interest and having great economic importance, current understanding of the structures and functions of membrane proteins lags behind that of soluble proteins. One of the major reasons for this delay is the difficulty associated with producing membrane proteins in large quantities. In fact, bacterial expression of membrane proteins remains a major challenge in recombinant DNA technology. Here, we report the use of the major envelope protein (P9) of bacteriophage phi6 as a fusion partner for successful expression of bacterial membrane proteins in *Escherichia coli*. Of the ten membrane proteins included in the study, eight were produced in an intact form in large quantities. One protein degraded and one was not expressed at all. All of the proteins examined in this study contained more than eight trans membrane segments. Future work will focus on the purification of these overproduced proteins and verification of their biological functions.

Key words: Membrane protein; protein expression; phage phi6; protein fusion; fusion partner.

Approximately one third of proteins expressed by an organism are membrane proteins, including receptors, ion channels, transporters, and enzymes[1,2]. A number of membrane proteins are major targets for small molecule drugs and biologicals[3,4]; for example, G-protein-coupled receptors represent the most important class of drug targets in terms of therapeutic benefit and pharmaceutical sales[5]. However, in spite of their economic values and important physiological roles, current understanding of the structures and functions of membrane proteins lags behind that of soluble proteins. One reason for this delay is the difficulty associated with producing membrane proteins in bacteria[6], in fact, bacterial expression of membrane proteins remains one of the major challenges in recombinant DNA technology.

Protein fusion techniques have been used for various purposes in recombinant DNA technology. For example, chaperone fusions have been used to assist folding and improve the solubility of over expressed proteins[7,8]. Green fluorescence protein (GFP) has also been used to monitor protein folding; in the case of membrane proteins, it was initially used as a fusion partner to monitor membrane insertion of an over expressed protein-GFP chimera[9]. Since then, GFP-fusions have been employed widely to monitor expression and folding of a number of chimeric proteins and

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to follow steps in the purification of membrane proteins[10, 11]. Mistic, a small protein with unknown function isolated from *Bacillus subtilus*, has also been used as a chaperone to aid the integration of proteins into the cytoplasmic membrane, although it was not successful in all cases[12-14]. Despite these important developments, overexpression and purification is still a major obstacle to the study of membrane proteins[6].

Bacteriophage phi6 is unique in that it contains a membrane envelope comprising phospholipid and proteins[15]. P9, the major envelope protein of phi6, comprises 90 amino acids and has a single transmembrane segment[16]. When phage phi6 infects its host bacterium *Pseudomonas phaseolicola*, a large amount of P9 is produced rapidly[17]. Therefore, we reasoned that P9 may serve as a chaperone to promote the synthesis of chimeric proteins and their integration into the membrane. This hypothesis was tested by using P9 as an N-terminal fusion partner. The results presented here show that bacterial membrane proteins can be efficiently produced in *Escherichia coli* as P9 chimeras.

MATERIALS AND METHODS

Recombinant DNA technology

The pMP6 plasmid backbone was from pRSETa (Invitrogen). A DNA fragment containing the codon-optimized P9 gene of phi6 was chemically synthesized and inserted into the NdeI and HindIII sites of pRSETa. The DNA sequence of pMP6 is available from the authors upon request. The primers used to amplify selected genes are listed in Table 1.

Immunoblotting

Cells harboring an expression plasmid was induced at the optimal condition described in figure legends and were sonicated. For dot blotting, the protein concentration of each sample was adjusted to 200 ng/ml and then 2-fold serial dilutions were prepared; 1ml of each dilution was loaded onto the membrane. For Western blotting, 250 ng of total cellular proteins were electrophoresed through a 12% SDS-polyacrylamide gel and then transferred to a membrane. P9-cargo fusion proteins were visualized by a mouse anti-P9 antibody[18].

RESULTS AND DISCUSSION

Construction of the expression vector and selection of membrane proteins

To test the hypothesis that P9 assists the synthesis and integration of proteins into the membrane, we constructed vector pMP6, which contained the P9 open reading frame under the control of the T7 promoter[18] (Fig.1). Plasmid pMP6 also containeda multiple cloning site with a His tag downstream of the P9 open reading frame, which enabled the insertion of a target gene and the

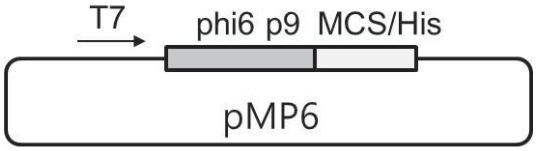

Fig. 1. Schematic illustration of the pMP6 expression plasmid. The plasmid contains the P9 gene under the control of the T7 promoter. Target genes were inserted at the multiple cloning site (MCS) to produce His-tagged P9-target protein chimeras.

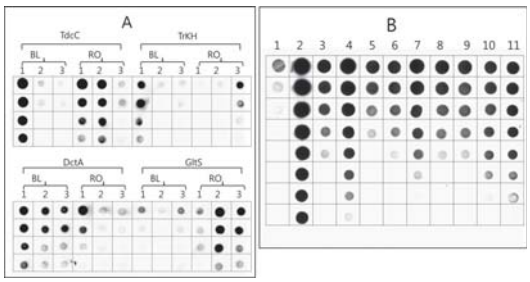

Fig. 2. Dotblot analyses of the P9 fusion proteins expressed in *E. coli*. The protein concentration of each sample was adjusted to 200 ng/ml and then 2-fold serial dilutions were prepared; 1ml of each dilution was loaded onto the membrane. P9-cargo fusion proteins were visualized by immunoblotting with an anti-P9 antibody. (A) The results of screening for optimal conditions for induction of expression in the BL21(DE3) (indicated by BL) and Rosetta(DE3) (indicated by RO) strains. Representative data for four proteins are shown. The induction conditions were as follows: lane 1, 37°C for 3 h; lane 2, 25°C for 4 h; lane 3, 25°C for 8 h. (B) Dotblot analysis of optimized P9 fusion protein expression. Cells induced under the optimal conditions for each protein were harvested and sonicated. Lane 1, BL21(DE3) with pMP6 vector alone; lane 2, ExuT; lane 3, GltS; lane 4, NhaA; lane 5, NupC; lane 6, TrKH; lane 7, TdcC; lane 8, TyrP; lane 9, XylE; lane 10, DctA; and lane 11, P9 positive control

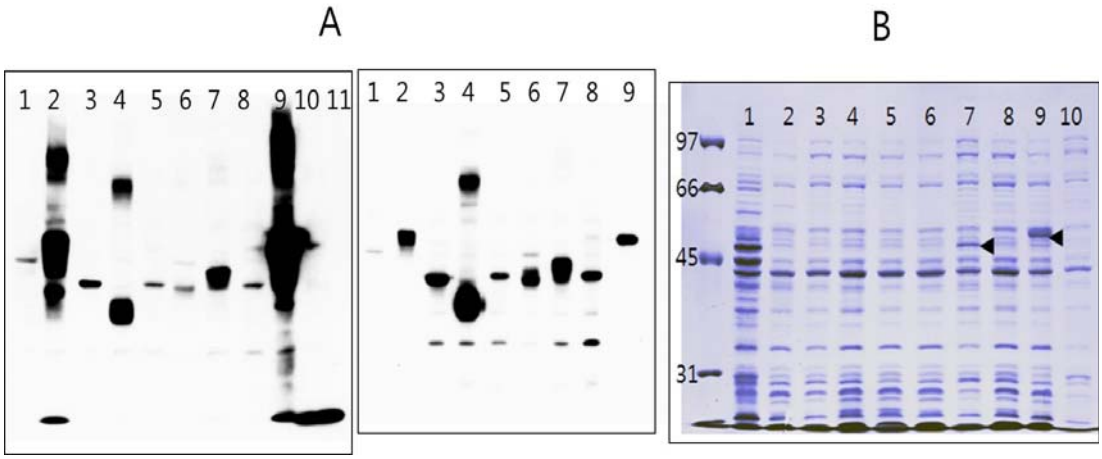

Fig. 3. Immunoblot and SDS-PAGE analyses of the P9 fusion proteins expressed in *E. coli*. Total cellular proteins were electrophoresed through a 12% SDS-polyacrylamide gel and then transferred to a membrane and immunoblotted with an anti-P9 antibody (A), or stained with Coomassie Blue (B). In the left panel of (A), each lane contains 250ng of total protein. In order to see clear bands in the samples of ExuT (lane 2) and XylE (lane 10), their loaded amounts in the right panel were reduced to 50ng [ExuT (lane 2)] and 20 ng [XylE (lane 10)]. Lane 1, BL21(DE3) with pMP6 vector alone; lane 2, ExuT; lane 3, GltS; lane 4, NhaA; lane 5, NupC; lane 6, TrKH; lane 7, TdcC; lane 8, TyrP; lane 9, XylE; lane 10, DctA; lane 11, P9 positive control

subsequent production of a P9-fusion protein. This plasmid enabled easy purification of P9 protein and the subsequent production of an anti-P9 antibody inmouse.

Ten membrane transporters with diverse properties were selected from *E.coli* (Table 2)[19]. The sizes of these proteins varied from 389 to 492 amino acids, and the number of transmembrane segments ranged from 8 to 12. Because the N-termini of the membrane proteins were fused to the C-terminus of P9, we reasoned that the topology of the membrane proteins might affect membrane integration; therefore, five proteins with extracellular N-termini (N_{out} topology) and five proteins with intracellular N-termini (N_{in} topology) were selected. We also reasoned that the length of the region between the transmembrane segment of P9 and the first transmembrane segment of the fused protein might also affect integration; therefore, proteins containing various lengths of this region were selected (Table 2).

Expression levels of membrane proteins determined by dotblotting

DNA fragments encoding the membrane proteins listed in Table 2 were amplified and cloned into pMP6 to produce P9-cargo protein chimeras. Their expression levels in two *E. coli* hosts, namely BL21(DE3) and Rosetta(DE3), were examined by dotblotting using an anti-P9 antibody. The cells were induced at 37°C for 3 h, or 25°C for 4 or 8 h. Some of the proteins, such as TdcC, were produced readily in both strains, but others, such as GltS, were produced only in the Rosetta strain. After determination of the optimal induction conditions for each protein (see examples in Figure 2A), the cells were collected, sonicated, and dotblotted onto a nylon membrane at2-fold serial dilutions (Fig. 2B). Among the ten proteins examined, nine were produced at detectable levels (Fig. 2B). DcuC, which contains 12 transmembrane segments and has N_{out} topology, was not detected in the dotblot screening (data not shown). ExuT, which contains 11 transmembrane segments, was produced at the highest level. We did not observe any relationship between expression levels and topological parameters such as the location of the N-terminus or the number of transmembrane segments.

Western blotting and SDS-PAGE

Although dotblotting can be used to determine the level of protein production, it does not reveal whether a protein is intact or degraded; therefore, SDS-PAGE and western blots were performed to identify the sizes of the expressed proteins (Fig. 3).TdcC and XylE produced strong bands in both immunoblots and Coomassie-stained gels (Fig. 3; lanes 7 and 9, respectively).

ExuT, which showed the highest level of expression in the dotblot analysis (Fig. 2B), also produced a strong band in the immunoblot (Fig. 3A; lane2); however, a distinct band corresponding to this protein could not be identified in the Coomassie-stained gel. This result could be explained by the fact that strongly hydrophobic proteins may not bind Coomassie Brilliant Blue R250 well (20). Similarly, although the dotblots indicated that the expression levels of GltS and NhaA were greater than or equal to those of TdcC and XylE, bands corresponding to these proteins were not detected

Table 1. Primers used in this study

gene	Forward primer	Reverse primer
dcaA	5'-CCCGGGAAAAAACCTCTCTGTTTAAAAGC-3'	5'-AAGCTTCAGAGGATAATTCGT GCGTTT-3'
dcuC	5'-CCCGGGCACTGACATTCATTGAGCTCCTT-3'	5'-CCATGGACTTGCCTGTGACCG CTGCTGC-3'
exuT	5'-GATATCCAGCAACGTTCGGGGCGTGCCGG-3'	5'-AAGCTTCATGTTGCGGTGCGG GATCGTT-3'
gltS	5'-CCCGGGCATTTCATCTCGATACTTTAGCA-3'	5'-CCATGGAACCGGCAAAAATCG GCAACAT-3'
nhaA	5'-CCCGGGCAAAACATCTGCATCGATTCTTT-3'	5'-AAGCTTCAACTGATGGACGCAA ACGAAC-3'
nupC	5'-CCCGGGCAGACCGCGTCCTTCATTTTGTA-3'	5'-AAGCTTCCAGCACCAGTGCTGC GATTGA-3'
tdcC	5'-CCCGGGCAAGTACTTCAGATAGCATTGTA-3'	5'-AAGCTTCAAACAGTTTGTATAC GATGTT-3'
trkH	5'-CCCGGGCACATTTTCGCGCCATTACCCGA-3'	5'-AAGCTTCTTCACGCCAGAAAGT CGGGGT-3'
tyrP	5'-CCCGGGCAAAAAACAGAACCCTGGGAAGT-3'	5'-AAGCTTCCCCCACTTCTGGTAAC AACCC-3'
xylE	5'-CCCGGGCAAATACCCAGTATAATTCCAGT-3'	5'-AAGCTTCCAGGGTAGCAGTTTG TTGTGT-3

Table 2. The ten selected proteins used in this study

Protein	Amino acids	Topology[a]	Transmembrane domains[a]	Amino acids from the N-terminus to the first transmembrane domain[a]	Function
DctA	429	N_{in}	8	4	Proton motive force-dependent C_4-dicarboxylate transporter
DcuC	462	N_{out}	12	3	Dicarboxylate uptake transporter
ExuT	473	N_{out}	11	46	Aldohexuronate transporter
GltS	402	N_{out}	8	69	Sodium-dependent glutamate transporter
NhaA	389	N_{out}	11	6	Sodium ion/proton antiporter
NupC	401	N_{out}	8	3	High-affinity nucleoside transporter
TdcC	444	N_{in}	11	22	Threonine/proton symporter
TrkH	484	N_{in}	10	8	Potassium ion transporter
TyrP	404	N_{in}	11	6	Tyrosine-specific permease
XylE	492	N_{in}	12	12	D-xylose/proton symporter

[a]As taken from Daley et al. (19).

in the Coomassie-stained gel (Fig. 3B; lanes 3 and 5, respectively). Again, this result may suggest that some membrane proteins are not efficiently stained with Coomassie Brilliant Blue R250. DctA, which was strongly detected in the dotblot analysis, produced only a small band similar to the size of P9 in the immunoblot analysis. This result suggests that, although it was highly expressed, the DctA portion of the P9-protein chimera was completely degraded.

CONCLUSION

This study demonstrates the use of the major envelope protein of bacteriophage phi6 as a fusion partner for successful expression of membrane proteins in bacteria. Of the ten membrane proteins tested, eight were produced as intact proteins in large amounts. One protein degraded and one was not expressed at all. Considering that all of the proteins included in the study were membrane proteins with more than eight transmembrane segments, this level of performance is high. Future work will focus on purifying and examining the biological functions of these over expressed fusion proteins.

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