Studies on Selective Protein Loading onto Extracellular Membrane Vesicles of a Novel Cold-Adapted Bacterium, *Shewanella vesiculosa* HM13

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A recombinant protein expression system working at low temperatures is expected to be useful for the production of thermolabile proteins and enzymes with toxic activity to the host cell, such as proteases that attack proteins of the host cell. Extracellular membrane vesicles (EMVs) produced by bacteria generally have a spherical structure surrounded by lipid membranes with a size ranging from 20 nm to 250 nm in diameter and play an important role in various bacterial activities. In this study, a novel cold-adapted bacterium secreting a single major protein as a cargo of EMVs was isolated and characterized. Furthermore, the selective cargo-loading mechanism of this bacterium was analyzed to construct a system to produce heterologous proteins as cargoes of EMVs.

In Chapter I, I described identification of a novel cold-adapted bacterium, *Shewanella vesiculosa* HM13, which secretes a large amount of a protein, named P49, to the culture supernatant as a cargo of EMVs, and characterization of a unique protein secretion system of this strain. In Chapter II, to construct a protein expression system by using *S. vesiculosa* HM13 as the host, I identified the promoter region of the P49 gene that is useful for heterologous protein production at low temperatures. In Chapter III, I constructed a system for secretory production of foreign proteins as cargoes of EMVs and found that P49 functions as a carrier that transports its fusion partner to EMVs.

CHAPTER I

Isolation of a novel bacterial strain capable of producing abundant extracellular membrane vesicles carrying a single major cargo protein and analysis of its transport mechanism

I isolated a novel cold-adapted strain, *Shewanella vesiculosa* HM13, as a prospective host for heterologous protein secretion at low temperatures. This strain produced a single major secretory protein, named P49, in the culture supernatant both at 4 °C and 18 °C, and the yield of this protein was 3.6 mg/L-culture and 5.3 mg/L-culture, respectively. No proteins of known function showed significant sequence identity to P49. Interestingly, P49 was co-purified with the extracellular membrane vesicles (EMVs) released from the cell surface of this strain, and it was also found that this protein is embedded in the EMVs. This indicated that *S. vesiculosa* HM13 has a P49-selective cargo-loading system and is expected to be useful as the host for secretory production of foreign proteins. Whole-genome sequence of *S. vesiculosa* HM13 revealed the presence of a gene cluster coding for components of a non-canonical type II protein secretion system (T2SS) in addition to a gene cluster coding for a canonical T2SS of the general secretory pathway. The P49-coding gene was located downstream of the former gene cluster. Deletion of the gene coding for a putative outer membrane channel of the T2SS-like translocon, named GspD2, suggested that the T2SS-like machinery functions as a novel type of protein translocon responsible for the P49-selective cargo loading to the EMVs.

CHAPTER II

Construction of a low-temperature protein expression system using *Shewanella vesiculosa* HM13 as the host

A low-temperature protein expression system by using *S. vesiculosa* HM13 as the host was constructed. To apply a promoter of the P49 gene in this protein production system, a DNA fragment with high expression activity was searched for from the 5'-untranslated region of the P49-coding gene. For this purpose, DNA fragments containing the putative promoter were introduced into a promoter-assay plasmid harboring the gene coding for enhanced green fluorescence protein, eGFP, as a reporter. The maximum yield of eGFP was obtained when the 1,000-bp upstream region of the P49-coding gene was used, and the amount of eGFP was estimated to be 19 mg/L-culture at 18 °C. I also used this system for the production of ß-lactamase (BLA) and analyzed the expression levels of BLA at different growth phases. As the result, maximum specific activity of BLA was observed when the recombinant cells were harvested at the late-log phase. SDS-PAGE analysis of the cytosolic fraction demonstrated that the protein band of BLA was detected as one of the major protein bands, indicating the usefulness of the heterologous protein production system using *S. vesiculosa* HM13 as the host.

CHAPTER III

Secretory protein production as a cargo of extracellular membrane vesicles and analysis of the cargo-loading mechanism

I constructed a system for secretory production of heterologous proteins as cargoes of extracellular membrane vesicles (EMVs) of *S. vesiculosa* HM13. Enhanced green fluorescent protein (eGFP) fused to the C-terminus of P49 produced by recombinant *S. vesiculosa* HM13 cells was detected from the cell and EMV fractions, indicating that modification of the C-terminus of P49 does not abolish the cargo loading to the EMVs and that P49 functions as a carrier to deliver the fusion partner to EMVs. According to the domain structure prediction, P49 has two hydrophobic segments (H1 and H2), which are predicted to form transmembrane helices. To test the involvement of these hydrophobic segments in the loading of P49 onto EMVs, a series of C-terminal truncated P49 was fused to eGFP. eGFP fused to the full length of P49 and eGFP fused to truncated P49 that lacks the C-terminal hydrophilic region were detected from the EMVs. In contrast, deletion of the H2-containing region led to disappearance of the fusion proteins from the EMVs and their accumulation in the insoluble cellular fraction, suggesting that this hydrophobic region is not involved in its transport to the outer membrane, but in its loading to the EMVs. These findings contribute to understanding of the cargo-selective protein loading mechanism of *S. vesiculosa* HM13 and to construction of a system for the production of heterologous proteins as cargoes of bacterial EMVs.