Studies on the Transport Mechanism and Physiological Roles of a Cargo Protein of Extracellular Membrane Vesicles from *Shewanella vesiculosa* HM13

(*Shewanella vesiculosa* HM13 の細胞外膜小胞積荷タンパク質の輸送機構と生理的役割に関する研究)

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Gram-positive and Gram-negative bacteria secrete spherical nanostructures, called extracellular membrane vesicles (EMVs). EMVs are attracting great attention in various biotechnological applications, including the development of vaccines and drug delivery systems. However, information on the molecular basis of vesiculation and selective cargo-loading is very limited. *Shewanella vesiculosa* HM13 secretes a protein of unknown function, P49, as a single major cargo of EMVs and is expected to be useful as a host for secretory production of heterologous proteins via EMVs. To promote understanding of the bacterial vesiculation mechanism and the application of bacterial EMVs, I analyzed the molecular mechanism of P49 loading to EMVs and physiological function of P49.

CHAPTER I

Genetic characterization and functional implications of the gene cluster for protein transport to extracellular membrane vesicles of *Shewanella vesiculosa* HM13

Here, I analyzed the transport mechanism of P49 to EMVs. The P49 gene is found in a gene cluster containing the genes encoding homologs of surface polysaccharide biosynthesis proteins (Wza, WecA, and Wzx), a lipopolysaccharide (LPS) modification protein (LptA), a protein involved in phospholipid metabolism (GdpD), a protein for redox reaction (NfnB), and components of type II secretion system (T2SS). I disrupted the genes coding for Wzx, LptA, GdpD, NfnB, and the homologs of the T2SS components and analyzed the productivity and morphology of EMVs and the localization of P49. EMV production and morphology were only moderately affected by the gene disruption, demonstrating that these gene products are not essential for EMV synthesis. In contrast, the localization of P49 was significantly affected by the gene disruption. The lack of homologs of the T2SS components resulted in deficiency in secretion of P49. When *gdpD*, *wzx, lptA,* and *nfnB* were disrupted, P49 was released to the extracellular space without being loaded to the EMVs. These results suggest that P49 is translocated across the outer membrane through the T2SS-like machinery and subsequently loaded onto EMVs through interaction with surface components of EMVs synthesized by the proteins encoded by the genes in the vicinity of the P49 gene.

CHAPTER II

Protein loading onto extracellular membrane vesicles of a hypervesiculating bacterium, *Shewanella vesiculosa* HM13, mediated by their surface polysaccharides

The results shown in Chapter I suggested that P49 is loaded onto EMVs via the interaction with the surface component synthesized by the function of Wzx. It is known that Wzx family proteins are responsible for the biosynthesis of bacterial surface polysaccharides, such as extracellular polysaccharide (EPS) and O-antigen of LPS. Because it was reported that the LPS of *S. vesiculosa* HM13 lacks O-antigen, I analyzed the EPS of EMVs and found that Wzx is responsible for the EPS synthesis in *S. vesiculosa* HM13. To examine whether P49 is loaded onto the EMVs via the interaction with the EPS, I conducted the *in vitro* P49 binding assay to the EMVs. This assay revealed that P49-free EMVs harboring EPS associate with purified P49 *in vitro*. In contrast, P49 was not associated with the EMVs from the mutants that do not produce EPS due to the loss of Wzx, LptA, and GdpD, indicating that P49 is loaded onto EMVs via the interaction with the EPS synthesized by these proteins. It was also found that P49 is not loaded onto EMVs from the mutant that lacks NfnB although this mutant produces EMV-associated EPS. This result suggests that NfnB is not essential for the production of EPS but is responsible for EPS modification required for its interaction with P49.

CHAPTER III

Functional analysis of the major cargo protein of extracellular membrane vesicles of *Shewanella vesiculosa* HM13

Although P49 is expected to be useful as a carrier to produce heterologous proteins as cargo proteins of EMVs, little is known about its physiological functions because of its low sequence similarity with reported proteins of known function. In this chapter, I examined the involvement of P49 in the dispersion of EMVs and cell adhesion. Nanoparticle tracking analysis of the EMVs demonstrated that P49 enhances the dispersion of the EMVs. This result suggests that P49 increases the surface area of EMVs and facilitates long-distance delivery of EMVs. On the other hand, the cell adhesion test using fibronectin and mucin demonstrated that P49 inhibited the cell adhesion to these adhesion factors. This function might contribute to preventing undesirable cell adhesion. These findings provide new insights into the physiological function of P49.