

# Screening and functional analysis of genes involved in extracellular membrane vesicle production in *Shewanella vesiculosa* HM13

Hiromu Inoue

Bacteria secrete nanometer-sized lipid particles, called extracellular membrane vesicles (EMVs). The physiology and biotechnological applications of EMVs have been attracting significant attention. However, the molecular mechanisms underlying EMV production in bacteria have not been fully elucidated. *Shewanella vesiculosa* HM13, a Gram-negative bacterium, produces unusually large amounts of EMVs with lower particle-size variation than other well-characterized *Shewanella* species and *Escherichia coli*. Elucidation of the molecular basis of EMV production in this strain is expected to provide valuable insights into the mechanisms of bacterial EMV production and morphogenesis. The aim of this thesis was to identify the genes involved in EMV production in *S. vesiculosa* HM13 and to analyze their physiological functions.

## CHAPTER I

### Rapid screening and identification of genes involved in bacterial extracellular membrane vesicle production using a curvature-sensing peptide

In this study, I established a rapid screening method for identifying genes involved in EMV production in *S. vesiculosa* HM13 using random transposon mutagenesis and a curvature-sensing peptide, N-terminus-substituted FAAV peptide labeled with nitrobenzoxadiazole (nFAAV5-NBD). This peptide selectively binds to lipid packing defects on EMVs, where lipids are loosely packed due to high membrane curvature, even in the presence of cells. By applying this peptide to a genome-wide screening of hyper- and hypo-vesiculating transposon mutants of *S. vesiculosa* HM13, 18 hyper- and eight hypo-vesiculating strains were obtained. I identified the transposon insertion sites in these strains and conducted targeted gene-disruption experiments to investigate the involvement of the transposon-disrupted genes in EMV production. Through characterization of EMV production, cell proliferation, and EMV-mediated protein secretion of the targeted gene-disrupted strains, I identified eight and four genes whose disruption increased and decreased EMV productivity, respectively. These results suggest that EMV productivity in *S. vesiculosa* HM13 is regulated by protein quality control, extracellular environmental signal sensing, and glutamate metabolism. This study provides a novel screening method using a curvature-sensing peptide and new insights into genes related to EMV production.

## CHAPTER II

### Physiological function of LetB, a phospholipid-trafficking protein, in extracellular membrane vesicle production in *Shewanella vesiculosa* HM13

The physiological role of LetB, a phospholipid-trafficking protein, in EMV biogenesis was previously unclear. In this study, I found that the expression of LetB<sup>HM13</sup>, a LetB homolog, was significantly decreased in one of the hyper-vesiculating mutants of *S. vesiculosa* HM13. The predicted hexameric model of LetB<sup>HM13</sup> showed structural features consistent with a phospholipid transporter spanning the periplasm. Furthermore, I identified homologous genes encoding a proposed proton-motive force-driven pump, *letA1*<sup>HM13</sup> and *letA2*<sup>HM13</sup>, upstream of *letB*<sup>HM13</sup>, suggesting conservation of a lipid-trafficking system involving LetAB in strain HM13. The *letB*<sup>HM13</sup>-disrupted strain produced EMVs with varied morphology, including large and wrinkled EMVs, which were not observed in the parental strain. Disruption of *letB*<sup>HM13</sup> also altered the lipid profiles of EMVs, likely due to impaired outer membrane lipid homeostasis, and resulted in hyper-vesiculation. These results suggest that LetB<sup>HM13</sup> regulates EMV production and morphology by maintaining outer membrane lipid homeostasis.

## CHAPTER III

### Novel bacterial dynamin-like proteins VdyA and VdyB regulating membrane fusion and curvature in extracellular membrane vesicle biogenesis

I investigated the physiological functions of the newly identified bacterial dynamin-like proteins VdyA and VdyB in EMV production by *S. vesiculosa* HM13 through *in vivo* and *in vitro* analyses. Deletion of either *vdyA* or *vdyB* decreased EMV production. Overexpression of either *vdyA* or *vdyB* alone did not affect EMV production, whereas co-overexpression of *vdyA* and *vdyB* increased EMV production. These results suggest that VdyA and VdyB function cooperatively in EMV production. Furthermore, heterologous co-expression of *vdyA* and *vdyB* in *E. coli* increased EMV production, suggesting that these genes could be applied to increase EMV production by diverse bacteria. *In vitro* liposome assays using purified proteins showed that VdyA promoted membrane fusion in a GTP hydrolysis-dependent manner, and VdyB regulated membrane curvature with Bin/Amphiphysin/RVS (BAR) domain-like activity. These results suggest that VdyB forms membrane protrusions and that VdyA closes the membrane at the protrusion neck. In strain HM13, VdyA and VdyB were suggested to localize to the inner leaflet of the inner membrane, which suggests that these proteins contribute to outer-inner membrane vesicle (O-IMV) production or to the supply of phospholipids to the outer membrane for outer membrane vesicle (OMV) production.