

Identification and characterization of proteins involved in extracellular membrane vesicle production of *Shewanella vesiculosa* HM13

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Gram-negative bacteria secrete nano-sized spherical extracellular membrane vesicles (EMVs) via blebbing of the outer membrane or explosive cell lysis. EMVs not only have essential biological functions but also have great potential in the biotechnological applications. Decades of exploration proposed several EMV biogenesis mechanisms, such as the reduced outer membrane-peptidoglycan crosslinking and the periplasmic accumulation of proteins. However, our understanding of the EMV biogenesis mechanism is still very limited. A cold-adapted Gram-negative bacterium *Shewanella vesiculosa* HM13 was isolated from the intestinal contents of horse mackerel. The bacterium produces a large quantity of EMVs carrying a single major cargo protein of unknown function, named P49. Because of these properties, *S. vesiculosa* HM13 is expected to be useful for extracellular production of heterologous proteins as cargoes of EMVs at low temperatures. To facilitate the applications of *S. vesiculosa* HM13, it is important to elucidate its vesiculation mechanisms at the molecular level.

CHAPTER I: Enhancing extracellular membrane vesicle productivity of *Shewanella vesiculosa* HM13 by weakening outer membrane-peptidoglycan linkage

In Gram-negative bacteria, the outer membrane is connected to peptidoglycans predominantly through Braun's lipoprotein (Lpp), and the formation of this linkage is catalyzed by an L,D-transpeptidase (Ldt). To increase the utility of *S. vesiculosa* HM13 as a host for EMV-mediated protein production, I constructed gene-disrupted mutants of Lpp and Ldt and assessed the EMV productivity and the effects on the morphology of the mutants. As a result, an approximately 2.5-fold increase in EMV production was achieved, whereas the morphology of the EMVs of these mutants remained almost identical to that of the parent strain. In accordance with the increase in EMV production, the mutants secreted approximately 2-fold higher amounts of P49 than the parent strain into the culture broth associated with EMVs. These

findings will contribute to the development of an EMV-based secretory production system for heterologous proteins using *S. vesiculosa* HM13 as a host.

CHAPTER II: Comprehensive identification and functional characterization of cargo proteins of extracellular membrane vesicles of *Shewanella vesiculosa* HM13

To better understand the function and biogenesis pathway of EMVs produced by *S. vesiculosa* HM13, I performed a proteomic analysis of the EMVs produced by the P49-deficient mutant Δ P49 and its parent strain Δ pyrF^{HM13}. The involvement of several EMV-cargo lipoproteins in the EMV biogenesis was shown by their gene disruption experiments. Most notably, disruption of the genes coding for LpoA and LpoB, which are supposed to activate peptidoglycan biosynthesis, resulted in about 2.7- and 14.1-fold increase, respectively, in EMV production compared to the parent strain. The proteomic analysis of EMVs produced by *S. vesiculosa* HM13 contributed to the understanding of the mechanism of EMV production.

CHAPTER III: Involvement of multiple predicted glycosyl transferases in the biogenesis of extracellular membrane vesicles of *Shewanella vesiculosa* HM13

To elucidate the mechanism of EMV production and promote application of *S. vesiculosa* HM13, transposon (Tn)-mediated random mutagenesis was performed, and the mutants with altered EMV productivity were selected. As a result, two mutants with significant reduced EMV production were obtained. Surprisingly, the hypo-vesiculation phenotype was found to be caused not by Tn-insertion but by spontaneous mutations that occurred in a common gene, *hm3339*. This gene and its nearby genes were predicted to encode glycosyl transferases (GTs). Their disruption caused reduced EMV production and also resulted in dissociation of P49 from EMVs. Meanwhile, I found that disruption of *hm3339* caused a reduced surface capsular polysaccharide (CPS) content. Notably, abnormal bubble-like membrane structures were found on the cell surface of Δ *hm3339*. These results suggest that CPS produced by these GTs plays an important role in the biogenesis of EMVs.