Studies on Production Mechanisms of Extracellular Membrane Vesicles of Cold-Adapted Bacteria

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Extracellular membrane vesicles (EMVs) are secreted by Gram-negative bacteria. These nanoparticles are produced by budding and the following pinching-off of the outer membrane and contain outer membrane proteins and various other molecules. Interestingly, the composition of these molecules in EMVs is remarkably different from that of the cells, with particular molecules being enriched in EMVs. This implies operation of a cargo selection mechanism for EMVs. Due to this property, EMVs also have attracted a great deal of attention for biotechnological applications as a platform to produce membrane proteins in extracellular space. Extracellular production of foreign proteins using recombinant bacterial EMVs makes it feasible to obtain desired proteins and separate them from the host cells by simple centrifugation or filtration. By combination of EMV-based protein production system and cold-adapted bacteria, it will become more feasible to obtain heat-labile proteins from the culture supernatant.

In Chapter I, I analyzed effects of membrane phospholipid alteration on vesicle production in a cold-adapted bacterium, *Shewanella livingstonensis* Ac10. In Chapter II, I analyzed regulation mechanisms of vesicle production and biofilm dispersion of another cold-adapted bacterium, *Shewanella vesiculosa* HM13, in response to extracellular environment. In Chapter III, I identified a gene related to vesicle production by screening mutants obtained by random transposon mutagenesis. Together, this study contributes to understanding of molecular production mechanisms and roles of EMVs in cold-adapted bacteria as well as future application of EMVs to EMV-based foreign protein production at low temperatures.

CHAPTER I

Characterization of extracellular membrane vesicles of *Shewanella livingstonensis* Ac10 and enhancement of its vesicle productivity by membrane phospholipid alteration

A cold-adapted bacterium, *Shewanella livingstonensis* Ac10, which produces eicosapentaenoic acid (EPA) as a component of its membrane phospholipids, is useful as a host for heterologous production of thermolabile proteins at low temperatures. Although the protein production system with this bacterium was shown to be more beneficial than that with other bacteria for soluble cytosolic enzymes from another cold-adapted bacterium, production system for membrane proteins and extracellular proteins have not been established. In this chapter, I investigated the involvement of EPA in the vesicle production and suggested the future application of EMVs for extracellular protein production. Quantitative analysis demonstrated that the vesicle production was significantly increased (3−5 folds) by alteration of membrane compositions. The lack of EPA facilitated incorporation of an outer membrane protein, OmpC176, into EMVs. Induction of vesicle production and specific protein incorporation into EMVs provide a basis for an EMV-based protein production system at low temperatures and show the involvement of EPA in the regulation of vesicle production, where the lack of EPA probably accumulates abundant misfolded OmpC176 in the cells, possibly followed by induction of EMV production to reduce envelope stress.

CHAPTER II

Regulation of vesicle production and biofilm dispersion of *Shewanella vesiculosa* HM13 in response to extracellular environment

Many kinds of bacteria secrete and utilize EMVs for survival in their growing environments. Therefore, the amount and the components of EMVs should be tuned in response to the environments. Although multiple vesiculation mechanisms are suggested, little is known about how bacteria regulate vesiculation in response to extracellular environment. In this chapter, I identified a sensor protein to regulate vesicle production, HM1275, in EMVs of a Gram-negative bacterium, *Shewanella vesiculosa* HM13. This sensor protein sensed lysine concentration in a poor nutrient medium and induced vesicle production. Furthermore, the protein has sequence homology with a biofilm dispersion protein, BdlA, and induced biofilm dispersion in response to lysine in the medium. There may be a linkage between these lysine-induced phenomena, where the lysine-sensing cells secrete HM1275-containing EMVs that are delivered to other cells to induce collective biofilm dispersion. This study suggests that EMVs work as a carrier of a switch to make a decision in biofilm life cycle, providing possible application of EMVs as a biofilm disruption tool.

CHAPTER III

Identification of a gene related to vesicle production of *Shewanella vesiculosa* HM13

Vesiculation is a universal phenomenon conserved in the three domains of life. Despite its ubiquitousness, the vesiculation mechanism has not yet been fully elucidated. To understand bacterial vesiculation mechanism, I constructed multi-dot blotting method for *S. vesiculosa* HM13 to comprehensively explore genes involved in vesicle production. Using this method, vesicle production of random transposon mutants in a 96-well plate was simultaneously quantified and the mutants with decreased vesicle productivity were characterized in detail. These experiments identified a gene coding for cyclopropane-fatty-acyl-phospholipid synthase, of which disruption by transposon insertion caused hypo-vesiculation phenotype. This enzyme catalyzes post-synthetic modification of unsaturated fatty-acyl chains of phospholipids into cyclopropane-containing ones to moderate membrane fluidity in prokaryotic and eukaryotic cells. These results suggest that vesicles are formed around the rigid membrane regions made by this enzyme and propose a novel mechanism of vesicle formation, possibly conserved among different domains of life.