Construction of hypervesiculation Escherichia coli strains and application for secretory protein production

Yoshihiro Ojima, Tomomi Sawabe, Katsuya Konami,
Masayuki Azuma

Citation	Biotechnology and Bioengineering. 117(3); 701-709.
Issue Date	2020-02-11
Type	Journal Article
Textversion	author
Rights	This is the pre-peer reviewed version of the following article: Biotechnology and Bioengineering. Vol.117, Issu.3, p.701-709., which has been published in final form at https://doi.org/10.1002/bit.27239 . This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.
DOI	10.1002/bit.27239

Self-Archiving by Author(s)

Placed on: Osaka City University Repository

Construction of hypervesiculation *Escherichia coli* strains and application for secretory protein production

Yoshihiro Ojima*, Tomomi Sawabe, Katsuya Konami and Masayuki Azuma*

*Department of Applied Chemistry and Bioengineering, Graduate School of Engineering,
Osaka City University, 3-3-138, Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan*

Keywords: *Escherichia coli*, outer membrane vesicle, gene deletion, protein secretion, OmpW, GFP

*Corresponding authors: Yoshihiro Ojima, Masayuki Azuma

Tel.: +81-6-6605-3092

Fax: +81-6-6605-3092

Email: ojima@osaka-cu.ac.jp, azuma@osaka-cu.ac.jp

Abstract

Outer membrane vesicles (OMVs) are extracellular vesicles released from the surface of Gram-negative bacteria, including *Escherichia coli*. Several gene-deficient mutants relating to envelope stress (*nlpI* and *degP*) and phospholipid accumulation in the outer leaflet of the outer membrane (*mlaA* and *mlaE*) increase OMV production. This study examined the combinatorial deletion of these genes in *E. coli* and its effect on OMV production. The *nlpI* and *mlaE* double-gene-knockout mutant ($\Delta mlaE \Delta nlpI$) showed the highest OMV production. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis-based quantitative analysis showed that OMV production by strain $\Delta mlaE \Delta nlpI$ was ~30 times that by the wild-type. In addition, to evaluate the protein secretion capacity of OMVs, green fluorescent protein (GFP) fused with outer membrane protein W (OmpW) was expressed in OMVs. Western blot analysis showed that GFP secretion through OMVs reached 3.3 mg/L in the culture medium of strain $\Delta mlaE \Delta nlpI$ /*gfp*, 500 times that for the wild-type. Our approach using OMVs for extracellular protein secretion in *E. coli* is an entirely new concept compared with existing secretion systems.

1. Introduction

Outer membrane vesicles (OMVs) are nanosized (20–250 nm diameter), spherical bilayered proteolipids that are normally discharged from the surface of Gram-negative bacteria, including *Escherichia coli* (Schwechheimer and Kuehn, 2015; Toyofuku et al., 2019). OMVs contain outer membrane proteins, lipids, periplasmic proteins, lipopolysaccharides, RNA, and DNA. Studies have reported that disturbances in growth, turnover in cell wall components, or exposure to antibiotics initiate vesicle formation (Schwechheimer and Kuehn, 2015; Toyofuku et al., 2019). In particular, envelope stress and phospholipid accumulation in the outer membrane are gaining attention as dominant endogenous factors triggering OMV production by Gram-negative bacteria.

In *E. coli*, deletion of the genes *degP* or *nlpI* induces envelope stress and OMV production (Schwechheimer et al., 2015). The *degP* gene encodes a periplasmic protease/chaperone that manages unfolded and misfolded periplasmic proteins (Lipinska et al., 1990). Enhanced OMV production in the $\Delta degP$ strain is a possible survival strategy that eliminates these undesired proteins from the cell envelope (Schwechheimer and Kuehn, 2013). Recent studies have correlated vesiculation levels with covalent envelope cross-linking. The amount of lipoprotein–peptidoglycan (Lpp–PG) crosslinks is ~40% lower in the hypervesiculating *nlpI* mutant compared with the wild-type (WT) strain (Schwechheimer et al., 2015). It is believed that the breakdown–synthesis balance of PG is modified in the *nlpI* mutant, preventing the formation of proper crosslinks and indirectly leading to OMV production (Schwechheimer and Kuehn, 2015). OMV production is also closely related to the phospholipid transport system. Roier et al. (2016) showed that dysfunction of VacJ (also known as MlaA) and the Yrb ATP-binding cassette (ABC) transport system increases OMV production in the

Gram-negative bacteria *Haemophilus influenzae* and *Vibrio cholerae* because of phospholipid accumulation in the outer leaflet of the outer membrane (Roier et al., 2016). This observation was confirmed by a genome-wide assessment of OMV production in *E. coli*, indicating increased vesiculation of *mlaA* (*vacJ*) and *mlaE* (*yrbE*) mutants (Kulp et al., 2015). Therefore, both envelope stress and phospholipid accumulation are important endogenous factors promoting OMV production. However, it is unclear how combinatorial deletion of such genes affects OMV production in *E. coli*.

Bacterial OMVs have received considerable attention because of their applicability in biotechnology. The bacterial cellular machinery can be genetically engineered to produce and package heterologous enzymes in OMV nanoparticles. (Alves et al., 2015; Alves et al., 2016). In our previous study, outer membrane protein W (OmpW) efficiently packaged green fluorescent protein (GFP) into OMVs of *E. coli* (WT strain and hypervesiculating mutants $\Delta degP$ and $\Delta nlpI$) by overexpression of an OmpW–GFP fused protein (Ojima et al., 2018). The $\Delta nlpI$ strain showed higher OMV production and GFP secretion into the culture medium than the $\Delta degP$ strain (Ojima et al., 2018). Therefore, using OMVs as a protein secretion tool in microbial engineering is interesting.

In this study, we examined combinatorial deletion of genes involved in envelope stress (*degP* and *nlpI*) and phospholipid accumulation (*mlaA* and *mlaE*) in *E. coli* and its effect on OMV production. To evaluate the protein secretion capacity of OMVs, GFP was packaged into the OMVs. We investigated the application potential of OMVs as a protein secretion tool in *E. coli*.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Table 1 lists the strains and plasmids used in this study. The *E. coli* K-12 strain BW25113 (WT) and its derivatives were obtained from the National BioResource Project (National Institute of Genetics [NIG], Mishima, Japan) (Baba et al., 2006). Double-gene knockout mutants were constructed by P1 transduction using P1kc phage (Koma et al., 2012). Briefly, the kanamycin (Km) cassette was eliminated from the recipient single-gene knockout mutant using flippase/flippase recognition target (Flp/FRT) recombination. Then, P1 transduction was performed on a Km-sensitive recipient strain, with the donor strain harboring an FRT–Km–FRT cassette, which was inserted at the desired locus on the chromosome. The recipient strain was selected by Km resistance. ASKA-*ompW-gfp* was obtained from NIG (Kitagawa et al., 2005), and transformants containing ASKA-*ompW-gfp* were named in the style WT/*gfp*.

E. coli cells were cultured in lysogeny broth (LB; 10 g/L Bacto™ Tryptone, 5 g/L yeast extract, and 10 g/L NaCl). The culture medium of strains harboring ASKA-*ompW-gfp* was supplemented with 50 mg/L chloramphenicol. All test cultures were precultured in LB for 18 h at 37°C and inoculated into 100 mL fresh LB in a 500-mL baffled conical flask such that the optical density at 660 nm (OD_{660}) = 0.01. The cultures were placed on an NR-30 rotary shaker (Taitec, Osaka, Japan), with shaking at 140 strokes/min. Cell growth was recorded by measuring OD_{660} values.

2.2 Gene expression analysis

To examine gene expression, cells of each strain were harvested at 4 and 8 h post-inoculation by centrifugation at 4°C for 10 min at $8,000 \times g$. Total RNA was

1 extracted from the collected cells as described elsewhere (Ojima et al., 2018), and then
2 reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio Inc.,
3 Kusatsu, Japan). Gene expression was analyzed by real-time PCR (Mx3000P, Agilent
4 Technology, Santa Clara, CA), as described in our previous study (Ojima et al., 2018).
5 The gene expression level of *ompW-gfp* was normalized against that of *rrsA* (16S
6 rRNA). The specific primer pairs used are listed in Table 1.

7 8 **2.3 Isolation and transmission electron microscope (TEM) observation of OMVs**

9 OMVs were isolated, as previously described (Gujrati et al., 2014) with some
10 modifications. Briefly, after incubation for 24 h, 100 mL of *E. coli* culture broth were
11 centrifuged at $10,000 \times g$ for 10 min at 4°C to remove the cells. Then, the supernatant
12 was passed through a 0.45- μ m pore filter. Ammonium sulfate was added (final
13 concentration, 400 g/L) for 1 h at room temperature to precipitate the contents. Crude
14 OMVs were obtained by centrifugation at $11,000 \times g$ for 30 min at 4°C. The crude
15 extracts were dissolved in 500 μ L of 15% (v/v) glycerol solution and then concentrated
16 using a CS100FNX ultracentrifuge (Hitachi Koki Co., Tokyo, Japan) at $109,000 \times g$ for
17 1 h. The OMV pellets were resuspended in 100 μ L of 15% (v/v) glycerol solution. The
18 resulting OMV samples were 1000 times more concentrated compared with the original
19 culture broth because of a decrease in the volume from 100 mL to 100 μ L. The OMV
20 samples were dropped onto a mesh copper grid and negatively stained with 4% uranyl
21 acetate for observation under a JEM-2100 TEM (JEOL, Tokyo, Japan).

22 23 **2.4 Evaluation of OMV production and GFP secretion through OMVs**

24 We took 10- μ L samples of the isolated OMVs or *E. coli* cells of each strain for

analysis using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie Blue staining. OMV production was quantified as previously described (Schwechheimer and Kuehn, 2013) with some modifications. To index the OMV concentration, we photographed the SDS-PAGE band at ~37 kDa and analyzed the densitometry using Image J software (National Institutes of Health, Bethesda, MD, USA). The index was normalized against OMV production by the WT strain. OMVs were also quantified on the basis of phospholipid, with minor modifications from a previously described method (Klimentová and Stulík, 2015; Manning and Kuehn, 2011; McBroom et al., 2006). Briefly, isolated OMVs were incubated with FM4-64 (Molecular Probes/Thermo Fisher, IL, USA) at a final concentration of 5 µg/mL in phosphate-buffered saline (pH 7.4) for 20 min. We used samples without OMVs or without FM4-64 as negative controls. After excitation at 558 nm, emission at 734 nm was measured using an INFINITE 200 PRO spectrofluorophotometer (TECAN, Switzerland).

For western blot analysis, we transferred protein from a gel to an Immobilon-P membrane sheet (Merck Millipore, Billerica, MA, USA) using the semidry transfer method. Hybridization was performed using an anti-histidine-tag monoclonal antibody as the primary antibody (Medical & Biological Laboratories Co., Nagoya, Japan) and anti-mouse immunoglobulin G (whole molecule)–alkaline phosphatase (Sigma-Aldrich, St. Louis, MO, USA) as the secondary antibody. Hybridization signals were detected using a BCIP-NBT Solution Kit for Alkaline Phosphatase Stain (Nacalai Tesque, Kyoto, Japan). To index target protein expression, we photographed the western blot band and analyzed it using densitometry. GFP secretion was quantified on the basis of a GFP standard sample with a His-tag. GFP secretion was also quantified by fluorescence

1 measurement. The fluorescence intensity of GFP in OMVs was measured by emission at
2 510 nm after excitation at 450 nm using an INFINITE 200 PRO
3 spectrofluorophotometer.

4 5 **3 Results and Discussion**

6 **3.1 OMV production in single- and double-gene knockout mutants**

7 Figure 1A shows the cell growth of each single- and double-knockout mutant of *E.*
8 *coli* at the end of culture for 24 h. OD₆₆₀ of the WT strain was 5.1. Among
9 single-knockout mutants, OD₆₆₀ of the $\Delta nlpI$ and $\Delta degP$ strains decreased slightly
10 compared with that of the WT, as reported previously (Ojima et al., 2018). However,
11 *mlaE* deletion did not affect cell growth. Overall, these results suggest that deleting
12 single genes involved in envelope stress and phospholipid accumulation does not
13 drastically influence *E. coli* cell growth.

14 In contrast, several double-gene and triple-gene knockout mutants showed
15 significantly lower cell growth than the WT. OD₆₆₀ of the $\Delta degP\Delta mlaE$, $\Delta nlpI\Delta mlaA$,
16 $\Delta degP\Delta nlpI$ and $\Delta mlaE\Delta nlpI\Delta degP$ strains was ~3.5; the $\Delta mlaE\Delta nlpI$ strain had the
17 lowest OD₆₆₀ (2.9). Therefore, combinatorial gene knockout represses *E. coli* cell
18 growth.

19 To analyze OMV production, OMVs in supernatants were obtained from the culture
20 broth of respective strains after 24 h by ultracentrifugation. In *E. coli* samples,
21 SDS-PAGE bands observed at ~37 kDa (OmpF, OmpC, and OmpA) provide an index of
22 OMV production, as these membrane proteins are expressed specifically and abundantly
23 within the outer membrane of cells and, therefore, predominate in OMVs
24 (Schwechheimer and Kuehn, 2013). Figure 1B shows the SDS-PAGE band of each

1 OMV sample. Results clearly confirmed the presence of SDS-PAGE bands at ~37 kDa
2 in samples from all strains, indicating that OMVs were successfully isolated from the
3 culture broth. OmpA, OmpC, and OmpF were identified from these bands by MALDI
4 FT-ICR MS in preliminary experiments using the WT strain (data not shown). Next, we
5 quantitatively analyzed OMV production on the basis of densitometry of the ~37 kDa
6 SDS-PAGE bands (Fig. 1C). $\Delta nlpI$ and $\Delta degP$ strains produced, respectively, ~7 and 5
7 times more OMVs than the WT. These results are consistent with those of previous
8 studies (Ojima et al., 2018; Schwechheimer et al., 2015). The OMV production by
9 $\Delta mlaE$ and $\Delta mlaA$ strains was, respectively, ~5 and 3 times higher compared with the
10 WT. The increase in vesiculation upon gene deletion in the Mla pathway is consistent
11 with previous reports (Kulp et al., 2015; Roier et al., 2016).

12 Among double-gene-knockout mutants, only the $\Delta mlaE\Delta mlaA$ strain did not show a
13 further increase in OMV production compared with the single-gene-knockout mutants
14 (Fig. 1C). This is the first study showing that a cumulative gene deletion in the Mla
15 pathway is not effective in increasing OMV production. However, in contrast, other
16 double-gene deletions did significantly increase OMV production compared with
17 single-gene deletions, to >15-fold the level for the WT strain. The $\Delta mlaE\Delta nlpI$ strain
18 showed the highest OMV production, 30 times that by the WT. Studies have reported
19 that increased OMV production following *nlpI* deletion is due to prevention of the
20 formation of proper Lpp-PG crosslinks (Schwechheimer et al., 2015). In contrast,
21 dysfunction of the VacJ/Yrb ABC transport system increases OMV production because
22 of phospholipid accumulation in the outer leaflet of the outer membrane (Roier et al.,
23 2016). Our results demonstrated that combining PG breakdown and phospholipid
24 accumulation in the outer leaflet of the outer membrane is effective in increasing OMV

production by *E. coli*. In addition, given that the $\Delta mlaE\Delta nlpI$ strain had the lowest OD at the end of culture, a strong increase in OMV production represses cell activity in *E. coli*. A triple-gene-knockout mutant ($\Delta mlaE\Delta nlpI\Delta degP$) was also examined. The OD₆₆₀ of strain $\Delta mlaE\Delta nlpI\Delta degP$ was 3.4 and the OMV production was 13.9-fold that by the WT, suggesting that further cumulative gene deletion did not enhance the OMV production. Figure 2 shows TEM images of isolated OMVs from the WT and the double-gene-deletion mutant with the highest OMV production, i.e. $\Delta mlaE\Delta nlpI$. OMV particles ~20–100 nm in diameter were observed for both strains, suggesting that the $\Delta mlaE\Delta nlpI$ strain produced OMVs in the culture broth.

3.2 OMV production with GFP expression in double-gene-knockout mutants

Previously, we demonstrated that OmpW–GFP fused protein shows excellent efficiency in packaging GFP into OMVs from *E. coli* (Ojima et al., 2018). In addition, the $\Delta nlpI/gfp$ strain showed higher efficiency in GFP secretion through OMVs than the $\Delta degP/gfp$ strain. In the present study, we introduced OmpW–GFP fused protein into double-gene knockout mutants. Figure 3A shows OD₆₆₀ values of WT and double-gene knockout mutants carrying the ASKA-*ompW-gfp* plasmid. OD₆₆₀ of the $\Delta degP\Delta mlaA/gfp$ strain was comparable to that of the WT/*gfp* and $\Delta degP\Delta mlaE/gfp$ strains, whereas the $\Delta mlaE\Delta mlaA/gfp$ and $\Delta nlpI\Delta mlaA/gfp$ strains grew to a slightly lower OD₆₆₀ than the WT/*gfp* strain. Cell growth of the $\Delta degP\Delta nlpI/gfp$ and $\Delta mlaE\Delta nlpI/gfp$ strains was significantly lower than that of the WT/*gfp* strain, and the $\Delta mlaE\Delta nlpI/gfp$ strain showed the lowest OD₆₆₀ (3.6). These results, in comparison with the data in Fig. 1A, suggested that introducing the ASKA-*ompW-gfp* plasmid did

not greatly change the cell growth tendency of the WT or double-gene-knockout mutants, although there was a slight overall increase.

We quantitatively estimated OMV production in recombinant double-gene knockout mutants on the basis of densitometry of the ~37-kDa SDS-PAGE bands (Fig. 3B). Whereas the $\Delta degP\Delta mlaE$ and $\Delta degP\Delta mlaA$ strains showed ~20 times higher OMV production than the WT (Fig. 1C), $\Delta degP\Delta mlaE/gfp$ and $\Delta degP\Delta mlaA/gfp$ strains showed, respectively, ~5 and 3 times more OMV production than the WT/*gfp* strain (Fig. 3B). These results suggested that introducing the ASKA-*ompW-gfp* plasmid represses OMV production in these double-gene-knockout mutants. In contrast, strains $\Delta nlpI\Delta mlaA/gfp$, $\Delta degP\Delta nlpI/gfp$, and $\Delta mlaE\Delta nlpI/gfp$ produced more OMVs than other strains; notably, the *nlpI* gene was deleted from all three of these mutants. In particular, OMV production by strain $\Delta mlaE\Delta nlpI/gfp$ was found to be ~40 times that by the WT. The combinatorial deletion of *mlaE* and *nlpI* is effective in increasing OMV production by *E. coli* regardless of plasmid introduction.

3.3 GFP secretion through OMVs

As shown in Fig. 4A, western blot bands appeared at the expected molecular mass of OmpW–GFP fused protein (~50 kDa) in all lanes, suggesting that it was properly expressed in OMVs. In a preliminary experiment using the WT/*gfp* strain, OmpW–GFP was identified from the corresponding SDS-PAGE band by MALDI FT-ICR MS (data not shown). In single-gene-knockout mutants, the western blot band was much more intense for the $\Delta nlpI/gfp$ strain than for the WT/*gfp* strain, even though the $\Delta nlpI/gfp$ strain sample was diluted 50 times. In double-gene-knockout mutants, with the dilution of each sample taken into consideration, $\Delta nlpI\Delta mlaA/gfp$, $\Delta degP\Delta nlpI/gfp$, and

ΔmlaEΔnlpI/gfp strains secreted much more GFP compared with other strains. The amount of GFP secreted through OMVs in the culture medium was quantified on the basis of densitometry of the western blot band from commercial GFP with known concentration. As shown in Fig. 4B, the WT/*gfp* strain secreted 6.6 μg/L of GFP through OMVs into the culture medium. GFP secretion by strains *ΔnlpI* and *ΔdegP* was 95.1 and 35.6 μg/L, respectively, significantly higher than by the WT. Considering double-gene-knockout mutants, GFP secretion by strains *ΔdegPΔmlaE/gfp*, *ΔdegPΔmlaA/gfp*, and *ΔmlaEΔmlaA/gfp* was less than that by the single-gene knockout mutant *ΔnlpI/gfp* because the OMV production by these strains was strongly repressed by the introduction of the ASKA-*ompW-gfp* plasmid (Fig. 3B). In contrast, three double-gene-knockout mutants from which *nlpI* was deleted secreted a significantly higher amount of GFP; strains *ΔnlpIΔmlaA/gfp*, *ΔdegPΔnlpI/gfp*, and *ΔmlaEΔnlpI/gfp* respectively secreted 1.7, 2.5 and 3.3 mg/L GFP, up to 500 times higher than that by the WT.

Considering that the relative OMV production by strain *ΔmlaEΔnlpI/gfp* was 40 times that by the WT/*gfp* strain as determined by SDS-PAGE, a 500-fold increase in GFP secretion cannot be completely explained by increased OMV production (Table 2). In addition to evaluation of GFP secretion using western blot analysis, fluorescence-based measurement again showed 3.3 mg/L of GFP secretion in the culture of strain *ΔmlaEΔnlpI/gfp*. Therefore, we hypothesized that besides enhancing the OMV production, deleting both *mlaE* and *nlpI* influenced the level of GFP production in the *E. coli* cell. Supplementary Figure S1 shows western blot analysis, where the same amounts of whole-cell pellets were loaded to compare the GFP production in the cells. As reported previously (Ojima et al., 2018), the band was more intense in *ΔnlpI/gfp*

cells than in WT cells. Also, the band in $\Delta mlaE\Delta nlpI/gfp$ cells was about twofold the intensity of that in WT cells, suggesting that GFP production in $\Delta mlaE\Delta nlpI/gfp$ cells is approximately double that in the WT. Thus, the GFP secretion through OMV by strain $\Delta mlaE\Delta nlpI/gfp$ was increased not only by increased OMV production but also by increased GFP expression per unit cell. However, a 500-fold increase in GFP secretion was not completely explained. We also used real-time PCR analysis to determine the mRNA expression of the *ompW-gfp* gene in WT and $\Delta mlaE\Delta nlpI$. Relative mRNA expression of *ompW-gfp* in $\Delta mlaE\Delta nlpI$ compared with WT was 1.4 ± 0.6 at 4 h and 1.3 ± 0.8 at 8 h. Therefore, the transcription level was not significantly different between the WT and $\Delta mlaE\Delta nlpI$ mutant, suggesting that the increased amount of OmpW-GFP in the OMV fraction of the $\Delta mlaE\Delta nlpI$ mutant was not due to a dramatically increased transcription level of the *ompW-gfp* gene.

Next, we hypothesized that double-gene deletion of $\Delta mlaE$ and $\Delta nlpI$ influences the protein and phospholipid balance of the OMVs and that SDS-PAGE analysis thus underestimates OMV production. So, OMV production was also compared among strains WT/*gfp*, $\Delta mlaE/gfp$, $\Delta nlpI/gfp$, and $\Delta mlaE\Delta nlpI/gfp$ on the basis of the amount of phospholipid. As shown in Table 2, relative OMV production by strain $\Delta nlpI/gfp$ determined using FM4-64 was 10.9 times that by the WT/*gfp* strain, corresponding to the result obtained by SDS-PAGE. However, strain $\Delta mlaE/gfp$ showed 9.6 times higher OMV production, whereas the value determined by SDS-PAGE was 1.7. With regard to this difference, it was reported that Mla pathway mutants increased OMV production because of phospholipid accumulation in the outer leaflet of the outer membrane (Roier et al., 2016). Therefore, we suggest that protein-based evaluation underestimated OMV production by strain $\Delta mlaE/gfp$. Meanwhile, phospholipid-based evaluation determined

~15 times higher OMV production by strain $\Delta mlaE\Delta nlpI/gfp$ compared with WT/*gfp*, whereas the value determined by SDS-PAGE was ~40 times. Considering these values, it can be suggested that OMV production by strain $\Delta mlaE\Delta nlpI/gfp$ is at least ~15 times higher than that by the WT. Therefore, evaluation by SDS-PAGE did not underestimate OMV production by strain $\Delta mlaE\Delta nlpI/gfp$, and underestimation of OMV production by SDS-PAGE was not the reason for the nonlinearity of GFP secretion against OMV production.

To check for the presence of free GFP in OMV fraction from strain $\Delta mlaE\Delta nlpI/gfp$, OMV samples were treated by spin-filtration using a desalting column, 100K MWCO (Amicon Ultra, Merck Millipore). The resultant samples were analyzed by western blot using an anti-GFP antibody. GFP was not detected in the flow through of the spin-filtered sample, suggesting that free GFP is not present in the OMV sample. Further studies are required to explain the additional increase in GFP secretion through OMVs in strain $\Delta mlaE\Delta nlpI/gfp$.

Quantitative estimation of GFP production in the cells is summarized in Table 2. GFP production in $\Delta mlaE\Delta nlpI/gfp$ cells was 261 mg/L, which was almost equal to that in WT cells (269 mg/L) because OD₆₆₀ of the $\Delta mlaE\Delta nlpI/gfp$ strain was around half that of the WT. The percentage of secreted GFP was calculated as 0.003% in WT/*gfp* strain culture and approximately 1.3% in $\Delta mlaE\Delta nlpI/gfp$ culture, suggesting a significant increase of GFP secretion in the double-gene-knockout mutant.

3.4 Possibility of OMVs as a tool for protein secretion

A recent study summarized the development of Gram-negative bacteria, including *E. coli*, for the secretion of heterologous proteins (Burdette et al., 2018). Although *E. coli*

1 is a robust, inexpensive expression host for recombinant protein production, the typical
2 production process involves intracellular protein expression, which requires cell lysis
3 and multiple purification steps. Secretion of heterologous proteins into the extracellular
4 medium increases product purity from cell culture, decreases downstream processing
5 requirements, and reduces overall cost; therefore, the utility of *E. coli* would expand if it
6 secreted the protein product. Protein secretion from *E. coli* operates through highly
7 specialized systems that can translocate proteins from the cytosol to the extracellular
8 medium in either one or two steps (Burdette et al., 2018). The secretion systems for
9 native proteins in *E. coli* are categorized into types I, II, V, and VIII, and these systems
10 also successfully secrete recombinant proteins (Burdette et al., 2018).

11 Table 3 shows a list of heterologous proteins secreted by *E. coli*. For proteins with a
12 relatively large molecular mass (>30 kDa), 2–30 mg/L are secreted, mainly by the type I
13 secretion system (Fernandez et al., 2000; Khosa et al., 2018). The amount of GFP
14 secreted by OMVs in this study (3.3 mg/L) is comparable with these values. The type I
15 secretion system involves one-step secretion achieved by a HlyA-tagged guide. In this
16 pathway, the protein cargo must be either completely or partially unfolded to be secreted.
17 In the case of secretion of peptides or smaller proteins, studies have reported extremely
18 high production (Browning et al., 2017; Chen et al., 2014), for example 6 g/L of
19 13.1-kDa Fab (Voulgaris et al., 2015). Small proteins or peptides are secreted into the
20 periplasm by the type II secretion system with extracellular secretion using a leaky outer
21 membrane. The type V and VIII systems were applied for secretion of pertactin (1
22 mg/L) and cecropin A (294 mg/L after purification) (Sevastyanovich et al., 2012; Wang
23 et al., 2017), respectively. Compared with the existing secretion systems, our approach
24 for extracellular protein secretion from *E. coli* using OMVs is an entirely new concept.

4 Conclusions

E. coli strain $\Delta mlaE\Delta nlpI$ showed the highest OMV production, ~30 times higher than that by the WT strain. GFP secretion through OMVs reached ~3 mg/L in the culture medium of strain $\Delta mlaE\Delta nlpI$. Extracellular protein secretion using OMVs is an entirely new concept compared with the existing secretion systems in *E. coli*. Considering the downstream purification of recombinant protein from OMVs, it is already confirmed that ultrasonication is effective for disruption of OMVs. Therefore, insertion of protease site-specific cleavage sequences between OmpW and the target protein will enable a smart purification process for the target protein.

Acknowledgment

The authors acknowledge financial support by a Grant-in-Aid for Young Scientists (B) (No. 17K14869) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Chemical Innovation Encouragement Prize from the Japan Association for Chemical Innovation. The authors also thank Dr. Koma and Dr. Ohmoto, Osaka Institute of Industrial Science and Technology, for technical support in P1 transduction. We thank James Allen, DPhil, from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Author contributions

YO and MA proposed the research concept and provided necessary tools for experiments and experimental instructions. YO designed experiments, analyzed data and wrote the manuscript. TS and KK conducted experiments and analyzed data. All authors read and approved the manuscript.

Literature Cited

- Alves, N. J., Turner, K. B., Daniele, M. A., Oh, E., Medintz, I. L., Walper, S. A. (2015). Bacterial nanobioreactors—directing enzyme packaging into bacterial outer membrane vesicles. *ACS Applied Materials & Interfaces*, 7(44), 24963-24972.
- Alves, N. J., Turner, K. B., Medintz, I. L., Walper, S. A. (2016). Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. *Scientific Reports*, 6, 24866.
- Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K. A., Tomita, M., Wanner, B. L., Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. *Molecular Systems Biology*, 2, 2006.0008.
- Browning, D. F., Richards, K. L., Peswani, A. R., Roobol, J., Busby, S. J. W., Robinson, C. (2017). *Escherichia coli* "TatExpress" strains super-secrete human growth hormone into the bacterial periplasm by the Tat pathway. *Biotechnology and Bioengineering*, 114(12), 2828-2836.
- Burdette, L. A., Leach, S. A., Wong, H. T., Tullman-Ercek, D. (2018). Developing Gram-negative bacteria for the secretion of heterologous proteins. *Microbial Cell Factories*, 17(1), 196.
- Chen, Z. Y., Cao, J., Xie, L., Li, X. F., Yu, Z. H., Tong, W. Y. (2014). Construction of

leaky strains and extracellular production of exogenous proteins in recombinant
Escherichia coli. *Microbial Biotechnology*, 7(4), 360-370.

Fernandez, L. A., Sola, I., Enjuanes, L., de Lorenzo, V. (2000). Specific secretion of
active single-chain Fv antibodies into the supernatants of *Escherichia coli* cultures
by use of the hemolysin system. *Applied and Environmental Microbiology*, 66(11),
5024-5029.

Gujrati, V., Kim, S., Kim, S. H., Min, J. J., Choy, H. E., Kim, S. C., Jon, S. (2014).
Bioengineered bacterial outer membrane vesicles as cell-specific drug-delivery
vehicles for cancer therapy. *ACS Nano*, 8(2), 1525-1537.

Khosa, S., Scholz, R., Schwarz, C., Trilling, M., Hengel, H., Jaeger, K. E., Smits, S. H.
J., Schmitt, L. (2018). An A/U-Rich Enhancer Region Is Required for High-Level
Protein Secretion through the HlyA Type I Secretion System. *Applied and
Environmental Microbiology*, 84(1), e01163.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H.,
Mori, H. (2005). Complete set of ORF clones of *Escherichia coli* ASKA library (a
complete set of *E. coli* K-12 ORF archive): Unique resources for biological
research. *DNA Research*, 12(5), 291-299.

Klimentová, J., Stulík, J. (2015). Methods of isolation and purification of outer
membrane vesicles from gram-negative bacteria. *Microbiological Research*, 170,
1-9.

Koma, D., Yamanaka, H., Moriyoshi, K., Ohmoto, T., Sakai, K. (2012). A convenient
method for multiple insertions of desired genes into target loci on the *Escherichia
coli* chromosome. *Applied Microbiology and Biotechnology*, 93(2), 815-829.

Kulp, A. J., Sun, B., Ai, T., Manning, A. J., Orench-Rivera, N., Schmid, A. K., Kuehn,

- 1 M. J. (2015). Genome-wide assessment of outer membrane vesicle production in
2 *Escherichia coli*. *PLoS One*, 10(9), e0139200.
- 3 Lipinska, B., Zylicz, M., Georgopoulos, C. (1990). The HtrA (DegP) protein, essential
4 for *Escherichia coli* survival at high temperatures, is an endopeptidase. *Journal of*
5 *Bacteriology*, 172(4), 1791-1797.
- 6 Manning, A. J., Kuehn, M. J. (2011). Contribution of bacterial outer membrane vesicles
7 to innate bacterial defense. *BMC Microbiology*, 11, 258.
- 8 McBroom, A. J., Johnson, A. P., Vemulapalli, S., Kuehn, M. J. (2006). Outer membrane
9 vesicle production by *Escherichia coli* is independent of membrane instability.
10 *Journal of Bacteriology*, 188(15), 5385-5392.
- 11 Ojima, Y., Yamaguchi, K., Taya, M. (2018). Quantitative evaluation of recombinant
12 protein packaged into outer membrane vesicles of *Escherichia coli* cells.
13 *Biotechnology Progress*, 34(1), 51-57.
- 14 Roier, S., Zingl, F. G., Cakar, F., Durakovic, S., Kohl, P., Eichmann, T. O., Klug, L.,
15 Gadermaier, B., Weinzerl, K., Prassl, R., Lass, A., Daum, G., Reidl, J., Feldman,
16 M. F., Schild, S. (2016). A novel mechanism for the biogenesis of outer membrane
17 vesicles in Gram-negative bacteria. *Nature Communications*, 7, 10515.
- 18 Schwechheimer, C., Kuehn, M. J. (2013). Synthetic effect between envelope stress and
19 lack of outer membrane vesicle production in *Escherichia coli*. *Journal of*
20 *Bacteriology*, 195(18), 4161-4173.
- 21 Schwechheimer, C., Kuehn, M. J. (2015). Outer-membrane vesicles from
22 Gram-negative bacteria: Biogenesis and functions. *Nature Reviews Microbiology*,
23 13(10), 605-619.
- 24 Schwechheimer, C., Rodriguez, D. L., Kuehn, M. J. (2015). NlpI-mediated modulation

1 of outer membrane vesicle production through peptidoglycan dynamics in

2 *Escherichia coli*. *Microbiologyopen*, 4(3), 375-389.

3 Sevastsyanovich, Y. R., Leyton, D. L., Wells, T. J., Wardius, C. A., Tveen-Jensen, K.,

4 Morris, F. C., Knowles, T. J., Cunningham, A. F., Cole, J. A., Henderson, I. R.

5 (2012). A generalised module for the selective extracellular accumulation of

6 recombinant proteins. *Microbial Cell Factories*, 11, 69.

7 Toyofuku, M., Nomura, N., Eberl, L. (2019). Types and origins of bacterial membrane

8 vesicles. *Nature Reviews Microbiology*, 17(1), 13-24.

9 Voulgaris, I., Finka, G., Uden, M., Hoare, M. (2015). Enhancing the selective

10 extracellular location of a recombinant *E. coli* domain antibody by management of

11 fermentation conditions. *Applied Microbiology and Biotechnology*, 99(20),

12 8441-8453.

13 Wang, M., Huang, M., Zhang, J., Ma, Y., Li, S., Wang, J. (2017). A novel secretion and

14 online-cleavage strategy for production of cecropin A in *Escherichia coli*.

15 *Scientific Reports*, 7(1), 7368.

16

FIGURE LEGENDS

Fig. 1 Cell growth and relative outer membrane vesicle (OMV) production in each *Escherichia coli* strain. (A) OD₆₆₀ of each *E. coli* strain after 24 h of culture. (B) SDS-PAGE analysis of OMVs isolated from each *E. coli* strain. (C) Relative OMV production by each *E. coli* strain. OMV production was normalized to that by the wild-type (WT). Vertical bars indicate standard deviations (calculated from more than three independent experiments).

1 Fig. 2 Transmission electron microscopy images of OMVs isolated from *E. coli* WT and
2 $\Delta mlaE\Delta nlpI$ strains. The OMVs were stained with uranyl acetate.
3

4
5

Fig. 3 Cell growth and relative OMV production by each *E. coli* strain. (A) OD₆₆₀ of each *E. coli* strain after 24 h in culture. (B) Relative OMV production by each *E. coli* strain. OMV production was normalized to that by the WT. Vertical bars indicate standard deviations (calculated from more than three independent experiments).

1 Fig. 4 Green fluorescent protein (GFP) secretion in the OMV fraction isolated from
2 each *E. coli* strain. (A) Western blot analysis of secreted GFP. Hybridization was
3 performed using an anti-His-tag primary antibody. (B) Quantitative evaluation of
4 GFP secretion in OMV fractions of respective *E. coli* strains. Values were
5 quantified on the basis of a GFP standard sample. Vertical bars indicate standard
6 deviations (calculated from more than three independent experiments).
7

1 Supplementary Fig. S1 Western blot analysis of GFP produced in each *E. coli* strain.
2 Loading samples were prepared on an OD₆₆₀ basis so as to include the same
3 quantity of cells.
4

5

Table 1 *E. coli* strains and plasmid used in this study

Strains	Note	Reference
<i>E. coli</i>		
BW25113	<i>rrnBT14 ΔlacZ</i> WJ16 <i>hsdR514 ΔaraBAD</i> AH33	Baba et al., 2006
<i>ΔnlpI</i> (JW3132)	BW25113, <i>ΔnlpI::FRT-Km-FRT</i>	Baba et al., 2006
<i>ΔdegP</i> (JW0157)	BW25113, <i>ΔdegP::FRT-Km-FRT</i>	Baba et al., 2006
<i>ΔmlaE</i> (JW3161)	BW25113, <i>ΔmlaE::FRT-Km-FRT</i>	Baba et al., 2006
<i>ΔmlaA</i> (JW2343)	BW25113, <i>ΔmlaA::FRT-Km-FRT</i>	Baba et al., 2006
<i>ΔdegPΔmlaE</i>	<i>ΔdegPΔmlaE::FRT-Km-FRT</i>	This study
<i>ΔdegPΔmlaA</i>	<i>ΔdegPΔmlaA::FRT-Km-FRT</i>	This study
<i>ΔmlaEΔmlaA</i>	<i>ΔmlaEΔmlaA::FRT-Km-FRT</i>	This study
<i>ΔnlpIΔmlaA</i>	<i>ΔnlpIΔmlaA::FRT-Km-FRT</i>	This study
<i>ΔdegPΔnlpI</i>	<i>ΔdegPΔnlpI::FRT-Km-FRT</i>	This study
<i>ΔmlaEΔnlpI</i>	<i>ΔmlaEΔnlpI::FRT-Km-FRT</i>	This study
Plasmid		
ASKA- <i>ompW-gfp</i>	pCA24N carrying <i>ompW</i> and <i>gfp</i> under P _{T5-lac} control, Cm ^r	Kitagawa et al., 2005

Table 2 Summary of GFP secretion and production, cell growth, relative OMV production of each *E. coli* strain

	WT/ <i>gfp</i>	$\Delta nlpI$ / <i>gfp</i>	$\Delta mlaE$ / <i>gfp</i>	$\Delta mlaE\Delta nlpI$ / <i>gfp</i>
GFP secretion in the medium [$\mu\text{g/L}$]	6.6 ± 0.2	95.1 ± 16	35.6 ± 7.7	3309 ± 736
GFP production in the cells [mg/L]	269 ± 37	420 ± 179	400 ± 110	261 ± 86
Percentage of secreted protein [%]	0.003	0.023	0.009	1.268
Relative OMV production (SDS-PAGE) [-]	1	10.6 ± 3.4	1.7 ± 0.3	38.3 ± 1.2
OD ₆₆₀ [-]	6.4 ± 0.6	5.0 ± 0.3	5.8 ± 0.8	3.4 ± 0.4

Table 3 Selection of heterologous proteins secreted by *E. coli* cells

Secretion system	Mechanism	Secretion product	Extracellular titer	References
Type I	HlyA	scFv	2 mg/L	Fernández et al. 2000
Type I	HlyA	Interferon alpha 2	6 mg/L	Khosa et al. 2018
Type I	TliDEF, HlyA	Lipase	3 mg/L	Khosa et al. 2018
Type II and generalized two-step	TatExpress	Human growth hormone	30 mg/L	Browning et al. 2017
Type II and generalized two-step	Leaky OM	Human parathyroid hormone (9.4 kDa with thioredoxin)	680 mg/L	Chen et al. 2014
Type II and generalized two-step	Leaky OM	Fab	6 g/L	Voulgaris et al. 2015
Type V	Type V	Pertactin	1 mg/L	Sevastyanovich et al. 2012
Type VIII	Type VIII	Cecropin A	294 mg/L (after purification)	Wang et al. 2017
OMVs	Leaky OM	GFP	3.3 mg/L	This study