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Quantitative evaluation of recombinant protein packaged into outer membrane vesicles of *Escherichia coli* cells

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Abstract

Outer membrane vesicles (OMVs) are spherical bilayered proteolipids released from the cell surfaces of bacteria, which have gained traction in the biotechnology fields. Bacterial cellular machinery can be genetically engineered to produce and package heterologous enzymes into OMVs, producing nanocarriers and nanoparticle catalysts. However, the productivity or efficiency of packaging the target protein into OMVs has not been quantitatively evaluated. In this study, we packaged green fluorescence protein (GFP) into the OMVs of *Escherichia coli* through N-terminal fused expression to outer membrane protein W (OmpW). The OMV productivity and amount of OmpW-GFP packaged in the OMVs were quantitatively compared between two hypervesiculating mutant strains Δ*nlpI* and Δ*degP*. Both strains increased the OMV production, but the Δ*nlpI* strain additionally enhanced the packaging of OmpW-GFP into OMVs. It was further confirmed that Spr, a peptidoglycan endopeptidase, plays an important role in the enhanced packaging of OmpW-GFP into OMVs through the increased OmpW-GFP expression on the Δ*nlpI* cells. Finally, the amount of OmpW-GFP released in the OMV fraction of both mutants was determined in terms of the OMV productivity and the packaging efficiency of OmpW-GFP into OMVs.

Introduction

Many Gram-negative bacteria form outer membrane vesicles (OMVs) as part of their natural growth cycle.[1-4] OMVs are spherical bilayered proteolipids with diameters of 20–250 nm, and are compositionally similar to the outer membranes of bacteria, which contain outer membrane proteins, lipids, periplasmic proteins, lipopolysaccharides, RNA, and DNA.[5,6] Vesicle formation is reportedly initiated by disturbances in growth, exposure to antibiotics, or turnover in cell wall components.[5,7,8]

Recently, bacterial OMVs have received considerable attention for their applicability to biotechnology. Bacterial cellular machinery can be genetically engineered to produce and package heterologous enzymes into OMV nanoparticles. Specially engineered cells of *Escherichia coli* can simultaneously produce, package, and secrete an active enzyme into their OMVs.[9,10] According to these studies, phosphotriesterase packaged into OMVs is much less susceptible to inactivation by freezing or lyophilization than free enzyme. The enhanced stability was also confirmed through long periods of contaminant digestion. However, although several new techniques have emerged for packaging functional proteins into OMVs, the production and expression of OMV-packaged protein have not yet been quantified.

OMV production is closely related to envelope stress.[11] In a random mutagenesis study of *E. coli*, hypervesiculation was triggered by a transposon insertion immediately following the start codon of *degP*.[12] DegP is a periplasmic protease/chaperon in *E. coli* that manages envelope stress caused by unfolded and misfolded proteins.[13,14] Thus, enhanced OMV production in *E. coli* strains lacking DegP is a possible survival strategy that eliminates these undesired proteins from the cell envelope. Other recent studies have correlated vesiculation levels with more subtle changes in covalent envelope

crosslinking. For instance, the overall content of lipoprotein (Lpp)-peptidoglycan (PG) crosslinks inversely correlates with OMV production. The amount of Lpp crosslinked to PG is approximately 40% lower in the hypervesiculating *nlpI* mutant than in the wild-type *E. coli*.[15] The *nlpI* mutant cannot encode NlpI, an outer membrane lipoprotein that restricts the activity of PG endopeptidase, which cleaves the peptide crosslinks in PG.[15-17] Therefore, it is postulated that the breakdown-synthesis balance of PG is altered in the *nlpI* mutant, preventing the formation of proper crosslinks between PG and Lpp and indirectly leading to increased OMV production.[18]

In this study, we packaged green fluorescence protein (GFP) into the OMVs of *E. coli* through N-terminal fused expression to outer membrane protein W (OmpW), which is constantly and abundantly expressed on the outer membranes of *E. coli* cells and their OMVs.[19,20] The relative OmpW-GFP amounts released in the OMV fraction were quantitatively compared among the wild-type and two hypervesiculating mutants (strains Δ*degP* and Δ*nlpI*). Furthermore, the differences in the released amounts of OmpW-GFP were discussed in terms of the different hypersesiculating mechanisms in the two strains.

Materials and Methods

Bacterial strains and culture conditions

The strains and plasmids used in this study are listed in Table 1. *E. coli* K-12 strain BW25113 and its derivatives were obtained from the National BioResource Project (National Institute of Genetics (NIG), Mishima, Japan).[21] The ASKA-*ompW-gfp* and pNTR-SD-*spr* plasmids were also provided by NIG.[22,23] The transformant containing ASKA-*ompW-gfp* was named WT/*ompW-gfp*.

E. coli cells were cultured in lysogeny broth (LB) medium (10 g l^{-1} Hipolypeptone, 5 g l^{-1} Bacto-yeast extract and 10 g l^{-1} NaCl). The culture media of the strains harboring the plasmid were supplemented with 33 mg l^{-1} chloramphenicol or 50 mg l^{-1} ampicillin. All test cultures were precultured in LB medium for 18 h at 37°C and inoculated into 80 ml of fresh LB medium in a 200 ml baffled conical flask so as to give an optical density at 660 nm (OD_{660}) = 0.01. The cultures were placed on a rotary shaker (NR-20, Taitec, Osaka, Japan) with shaking at 120 strokes per minute. Cell growth was recorded by measuring the changes in OD_{660}.

Gene expression analysis

To examine gene expression, cells of each strain were harvested at 4, 8, and 12 h post-inoculation by centrifugation at 4°C for 10 min at 8,000 *g*. Total RNA was extracted from the collected cells as described elsewhere,[24] and then reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio Inc., Kusatsu, Japan). Gene expression was analyzed by real-time PCR (Mx3000P, Agilent Technology, Santa Clara, CA), as described in our previous study.[24] The gene expression level of *ompW-gfp* was normalized against that of *rrsA* (16S rRNA). The specific primer pairs are listed in Table 1.

Isolation and microscopic observation of OMVs

OMVs were isolated as previously described[25] with some modifications. Following incubation for 24 h, the *E. coli* culture broth (80 ml) was centrifuged at 3,970 *g* for 10 min at 4°C. The supernatant was then passed through a 0.45 μm pore–size filter. The contents were precipitated by adding ammonium sulfate (final concentration 400 g l^{-1}) at

room temperature for 1 h. The crude OMVs obtained by centrifugation at 12,450 *g* for 30 min at 20°C. The crude extracts were dissolved in 500 µl of 15% (v/v) glycerol, and then concentrated by ultracentrifugation (CS100FNX, Hitachi Koki Co., Tokyo, Japan) at 150,000 *g* for 1 h. The OMV pellets were resuspended in 50 µl of 15% (v/v) glycerol solution. The resulting OMV samples were 1,600 times more concentrated than in the original culture broth by decreasing the volume from 80 ml to 50 µl. The GFP-derived fluorescences of the OMV samples were observed at an excitation wavelength of 488 nm using a BioRevo BZ-9000 microscope (Keyence Corp., Osaka, Japan).

SDS-PAGE and western blotting analyses of OMVs

Five microliter samples of the isolated OMVs or the *E. coli* cells of each strain were analyzed by SDS-PAGE with Coomassie Blue staining. OMV production was quantified as previously described[13] with some modifications. The SDS-PAGE band at ~ 37 kDa was analyzed by densitometry (Image J software, NIH, Bethesda, MD) to index the OMV concentration. The index was normalized against the OMV productivity of the wild type strain.

For western blotting, protein was transferred from the gel to a membrane sheet of Hybond P (GE Healthcare Ltd., Buckinghamshire, England) by the semi-dry transfer method. Hybridization was conducted using an anti-GFP antibody conjugated with horseradish peroxidase (Medical & Biological Laboratories Co., Nagoya, Japan) and an ECL Western Blotting Starter Kit (GE Healthcare Ltd.) following the manufacturer's protocol. The hybridization signals were detected by a ChemiDoc imaging system (Bio-Rad Laboratories Inc., Hercules, CA). The western blotting band was analyzed by densitometry to index the target protein expression. This index was normalized against

the OMV productivity of the wild type strain.

Results and Discussion

Comparison of OMV production among strains

Figure 1A shows representative growth curves of the wild type *E. coli* strain and the deletion mutants Δ*nlpI* and Δ*degP* containing the ASKA-*ompW-gfp* plasmid. These three strains were named WT/*ompW-gfp*, Δ*nlpI*/*ompW-gfp*, and Δ*degP*/*ompW-gfp*, respectively. The WT/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains showed similar growth profiles, reaching OD_{660} = 2.7 and 2.8 at 24 h post-inoculation, respectively. Therefore, deleting the *degP* gene did not repress the cell growth at 37°C. Meanwhile, the Δ*nlpI*/*ompW-gfp* cells exhibited slightly slower growth at 12 h than the WT/*ompW-gfp* cells. The OD_{660} value of Δ*nlpI*/*ompW-gfp* reached 2.5 at 24 h. Overall, these results suggest that deleting the *nlpI* and *degP* genes did not drastically influence the growth of *E. coli*, despite the introduction of ASKA-*ompW-gfp* plasmid. The relative mRNA expression of *ompW-gfp* gene is shown in Figure 1B. The expression level was normalized against that of the WT/*ompW-gfp* strain at each sampling time. At initial growth phase (4 h), the expression levels were not significantly different among strains. At middle growth phase (8 h), mRNA expression in the Δ*degP*/*ompW-gfp* strain was approximately 50% lower than in the WT/*ompW-gfp* strain. At late growth phase (12 h), the expression levels of Δ*nlpI*/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains were 40% lower than that of WT/*ompW-gfp* strain. As a result, it was confirmed that the transcription of recombinant protein in the knockout mutants was at the same level or lower level, as compared to that in the wild type strain.

Schwechheimer et al. reported that envelope stress and altered PG balance promotes OMV production by *E. coli* cells.[18] Therefore, the insoluble fractions of the supernatants containing OMVs were obtained from culture broths of the respective strains at 24 h by ultracentrifugation, and were compared with the wild type through an SDS-PAGE analysis. In *E. coli* preparations, the bands observed at ~37 kDa (OmpF, OmpC, and OmpA) provide an index of OMV amount, as these membrane proteins are expressed specifically and abundantly within the outer membrane of cells, and therefore predominate in the OMVs.[13] As shown in Fig. 2A, the protein band at ~37 kDa was much more intense in the fractions from the Δ*nlpI*/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains than in the faction from the WT/*ompW-gfp* strain, suggesting that the *degP* and *nlpI* gene deletions enhanced OMV production even in strains carrying the ASKA-*ompW-gfp* plasmid. Next, the OMV production was quantitatively analyzed based on the densitometry of the ~37 kDa band from each strain. As shown in Fig. 2B, the Δ*nlpI*/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains produced approximately 6 and 8 times more OMVs than the WT/*ompW-gfp* strain, respectively. These tendencies are consistent with those of previous reports.[13,18]

Comparison of OmpW-GFP amount released in OMV fraction

In the preliminary experiment, the packaging efficiency of GFP was compared among major outer membrane proteins (OmpA, OmpC, and OmpW). It was found that among them, OmpW-GFP showed the most significant band by western blotting analysis (data not shown). Therefore, OmpW was selected as the protein that packaged into OMVs. The OMV fractions from the three strains were observed by fluorescence microscopy (Fig. 3A). Green fluorescence was confirmed in the images from all

samples. OMVs are typically 20–250 nm in diameter and the OMV fraction was passed through a 0.45 μm pore–size filter during preparation. Therefore, the micrometer-scale green spots in the fraction were presumed as secondary aggregates of OMVs. Among these samples, the OMV fraction from Δ*nlpI*/*ompW-gfp* culture was most strongly fluorescent. The OmpW-GFP amounts released in the OMV factions (5 μl) were further compared through western blotting analysis using an anti-GFP antibody conjugated with horseradish peroxidase. The bands appeared at the expected molecular weight of OmpW-GFP fused protein (~50 kDa; see Fig. 3B), suggesting that the fused protein was properly expressed on the OMVs. The band was much more intense in the Δ*nlpI* and Δ*degP* strains than in the wild type strain. Similarly to OMV prodcution, the OmpW-GFP amount released in the OMV fraction was quantitatively analyzed based on the densitometry of the band from each strain. As shown in Fig. 3C, the Δ*nlpI* and Δ*degP* cells released approximantely 20 and 12 times more OmpW-GFP than the wild type strain, respectively. Thus, the OmpW-GFP amounts released by the respective strains correspond to the green fluorecsence intensities in the images of the OMV fractions (Fig. 3A). Considering that the relative OMV productivities were 6 and 8 times higher in the Δ*nlpI* and Δ*degP* strains than the wild type strain, respectively (Fig. 2C), we cannnot confirm a linear relashionsip between OMV production and the amount of OmpW-GFP released in the OMV fraction of each strain.

Relationship between OMV production and OmpW-GFP amount

Contrary to expectation, the OmpW-GFP amounts released in the OMV fractions of the Δ*nlpI* and Δ*degP* strains were not liniearly correlated with OMV production. Therefore, some factor that affects the relationship between OMV production and

OmpW-GFP amount needs to be considered. Here, we hypothesized that besides enhancing the OMV production, deleting the *degP* and *nlpI* genes influenced the level of OmpW-GFP expression on the cells of each *E. coli* strain. As shown in Fig. 1B, the mRNA expression of *ompW-gfp* was at the same level or lower level in the Δ*nlpI* and Δ*degP* strains compared to the wild type, suggesting that the increased OmpW-GFP amounts in the OMV fractions of two knockout mutants were not due to the promoted transcription of *ompW-gfp* gene. Next, the amount of OmpW-GFP in each *E. coli* strain was compared by western blotting analysis. Figure 4A shows the images of western blotting analysis where the same amounts of the whole cell pellets were loaded. Interestingly, the OmpW-GFP band was much more intense in the Δ*nlpI* cells than in the wild type cells, but was similarly intense in the Δ*degP* cells and wild type cells. These results suggest that deleting the *nlpI* gene increased the OmpW-GFP amount on the cells. Quantitatively, OmpW-GFP expression in the Δ*nlpI* cells is approximately 3 times higher than in the wild type cells, and approximately 2 times higher than in the Δ*degP* cells. Thus, the OmpW-GFP release in the OMV faction of the Δ*nlpI* strain was increased not only by the increased OMV amount but also by the increased OmpW-GFP expression per unit of cell. In a previous study, the amount of Lpp crosslinked to PG was approximately 40% lower in the hypervesiculating *nlpI* mutant than in the wild-type *E. coli*.[15] NlpI is an outer-membrane lipoprotein that restricts the activity of Spr (also known as MepS), a PG endopeptidase that cleaves the peptide crosslinks in PG.[15-17] Therefore, it can be hypothesized that Spr plays an important role in the increased OmpW-GFP expression per unit of cell (Fig. 5). Here, we also checked OmpW-GFP expression in the *spr*-overxpressed *E. coli* cells. As shown in Fig. 4, *spr* overexression increased the OmpW-GFP expression to 3 times that of the wild type,

similarly to the Δ*nlpI* strain, supporting our hypothsis mentioned above. Meanwhile, deleting the *degP* gene did not drastically increase the OmpW-GFP expression per unit of cell. In the *degP* mutant, hypervesiculation induced by the accumulation of increased misfolded protein production was not strongly associated with OmpW-GFP expression on OMVs (Fig. 5).

Table 2 summarizes the results obtained in this study. The relative OmpW-GFP amount released in the OMV fraction was 19.8 times higher in the Δ*nlpI* strain than in the wild type strain. This value is close to the product of the relative OMV production (5.9) and OmpW-GFP expression per unit of cell (2.8). In the Δ*nlpI* strain, the relative OmpW-GFP amount released in the OMV fraction was 12.4 times higher than in the wild type, almost equaling the product of 8.0 and 1.6. This clarifies that deleting the *nlpI* and *degP* genes differently affects the OMV production and membrane protein expression on the OMVs, and that both factors determine the amount of OmpW-GFP released in the OMV fraction.

Conclusions

In this study, fusing GFP expression with OmpW in *E. coli* cells enabled the packaging of GFP protein into OMVs. The tendencies of OMV production and packaging of OmpW-GFP into OMVs differed between the two hypervesiculating mutants Δ*nlpI* and Δ*degP*. Whereas the Δ*degP* strain increased only the OMV production, the Δ*nlpI* strain enhanced both OMV production and packaging of OmpW-GFP in OMVs. It was further demonstrated that Spr plays an important role in the enhanced packaging of OmpW-GFP into OMVs through the increased OmpW-GFP expression on the Δ*nlpI* cells. Finally, the amounts of OmpW-GFP released in the OMV

fractions of both mutants were determined in terms of the OMV productivity and the packaging efficiency of OmpW-GFP into OMVs.

Acknowledgement

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FIGURE LEGENDS

Fig. 1 Cell growth and mRNA expression of each *E. coli* strain. (A) Growth profiles of *E. coli* WT/*ompW-gfp*, Δ*nlpI*/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains. (B) Relative mRNA expression of *ompW-gfp* gene in cells of each *E. coli* strain. The expression level was normalized against that of the WT/*ompW-gfp* strain. The vertical bars indicate standard deviations (calculated from more than three independent experiments). Statistically significant differences from the WT/*ompW-gfp* strain (p<0.05) are marked with asterisks.

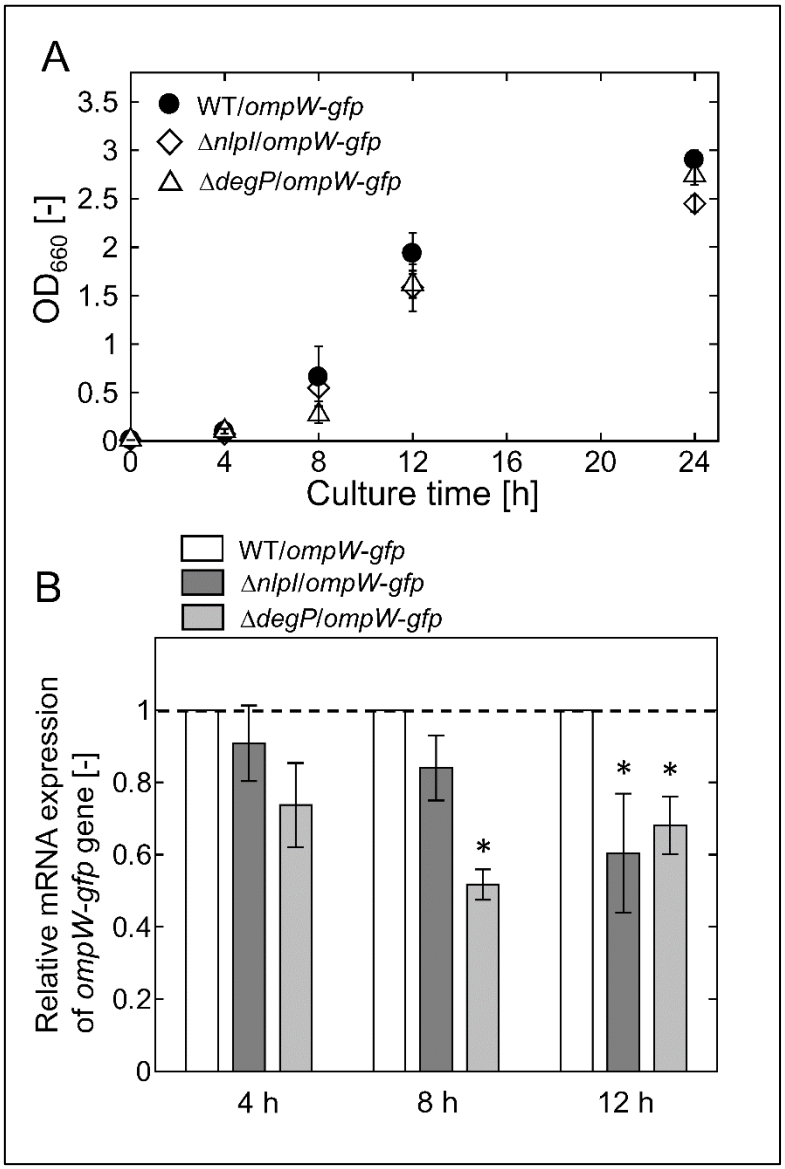

Fig. 2 OMV production of each *E. coli* strain. (A) SDS-PAGE analysis of OMVs isolated from *E. coli* WT/*ompW-gfp*, Δ*nlpI*/*ompW-gfp* and Δ*degP*/*ompW-gfp* strains. (B) Comparison of OMV production among the *E. coli* strains. The amounts of OMVs produced were normalized against that of the WT/*ompW-gfp* strain. The vertical bars indicate standard deviations (calculated from more than three independent experiments). Statistically significant differences from the WT/*ompW-gfp* strain (p<0.05) are marked with asterisks.

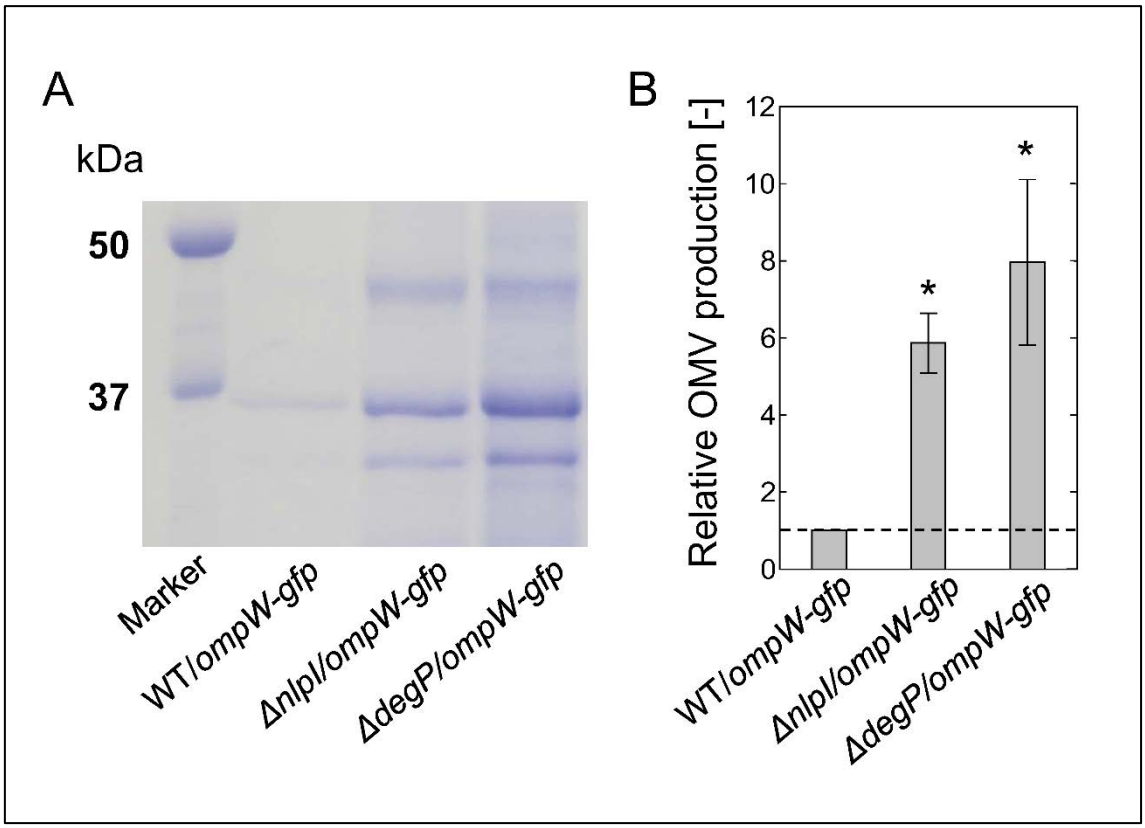

Fig. 3 OmpW-GFP expression in OMV fraction isolated from each *E. coli* strain. (A) Fluorescence microscopic images of OMV fractions isolated from the respective *E. coli* strains. (B) Western blotting analysis of OmpW-GFP. The hybridization was carried out using an anti-GFP antibody conjugated with horseradish peroxidase. (C) Comparison of OmpW-GFP amounts released in the OMV fractions of the respective *E. coli* strains. The values were normalized against that of the WT/*ompW-gfp* strain. The vertical bars indicate standard deviations (calculated from more than three independent experiments). Statistically significant differences from the WT/*ompW-gfp* strain ($p<0.05$) are marked with asterisks.

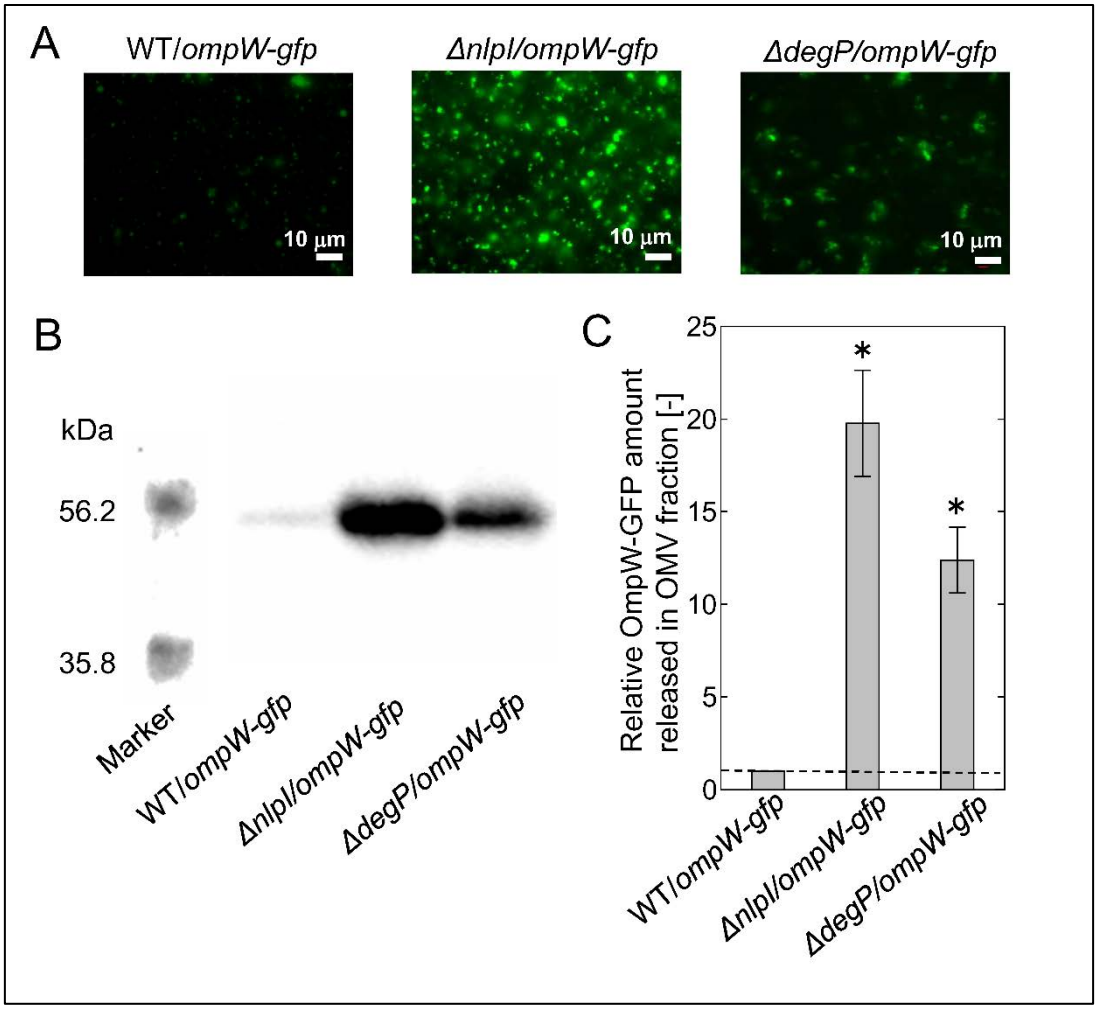

Fig. 4 OmpW-GFP expression in whole cell pellets of each *E. coli* strain. (A) Western blotting analysis of OmpW-GFP. The loading samples were prepared so as to be from the same amount of cells on an OD_{660} basis. (B) Comparison of OmpW-GFP expression among the *E. coli* strains. The values were normalized against that of the WT/*ompW-gfp* strain. The vertical bars indicate standard deviations (calculated from more than three independent experiments). Statistically significant differences from the WT/*ompW-gfp* (p<0.05) strain are marked with asterisks.

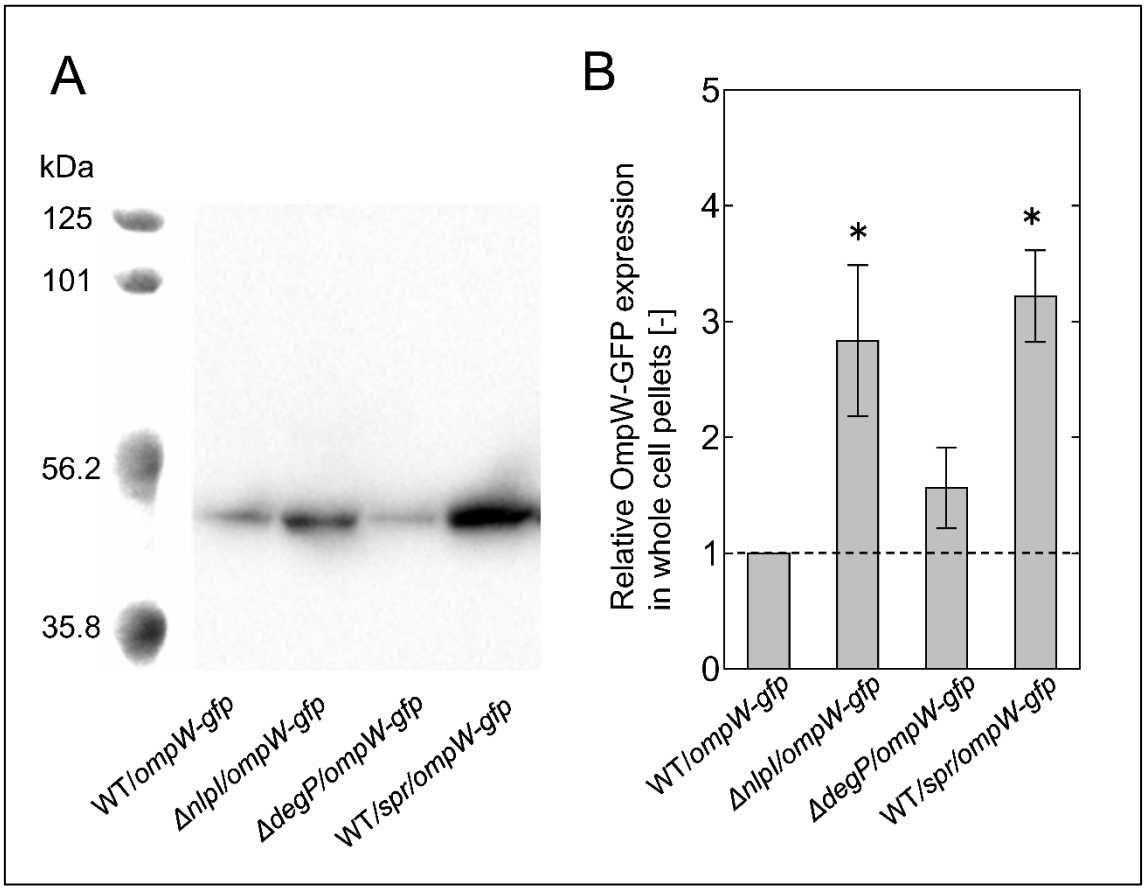

Fig. 5 Possible mechanisms of enhanced OMV production and/or OmpW-GFP expression induced by deleting the *nlpI* and *degP* genes.

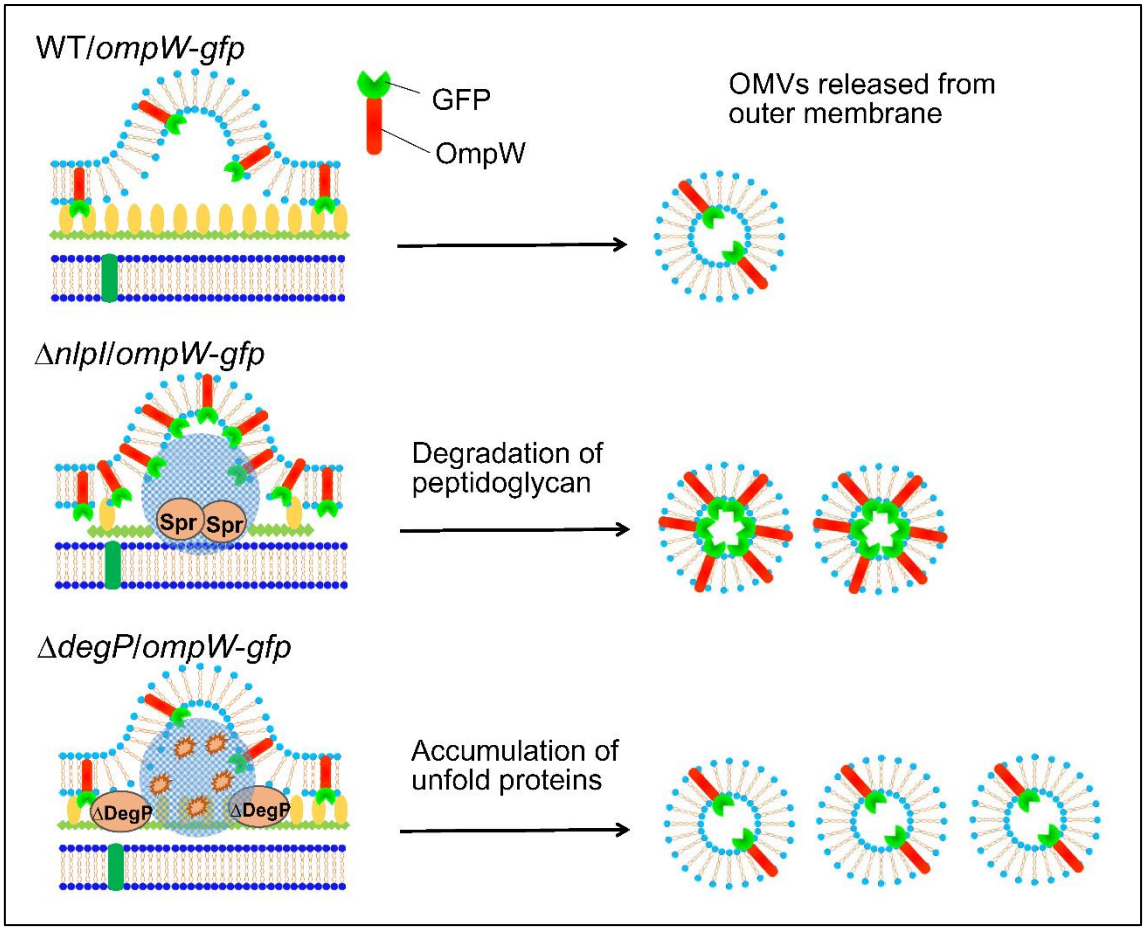

Table 1 *E. coli* strains and plasmids used in this study

Strains or plamisds	Note	Reference
Strains		
BW25113	Host strain of Keio collection	21
JW3132 (Δ*nlpI*)	BW25113, Δ*nlpI* ::*FRT-Km-FRT*	21
JW0157 (Δ*degP*)	BW25113, Δ*degP*::*FRT-Km-FRT*	21
Plasmids		
ASKA-*ompW-gfp*	pCA24N carrying *ompW* and *gfp* under $P_{T5\text{-}lac}$ control, Cm^r	22
pNTR-SD-*spr*	pNTR-SD carrying *spr* under P_{lac} control, Amp^r	23

Table 2 Summary of relative OMV production and OmpW-GFP expression of *E. coli* strains

	WT/*ompW-gfp*	Δ*nlpI* /*ompW-gfp*	Δ*degP* /*ompW-gfp*
Relative OMV production	1	5.9 ± 0.8	8.0 ± 2.1
Relative OmpW-GFP expression	1	2.8 ± 0.7	1.6 ± 0.3
Relative OmpW-GFP amount released in OMV fraction	1	19.8 ± 2.9	12.4 ± 1.8