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Flagella proteins contribute to the production of outer membrane vesicles from *Escherichia coli* W3110

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ABSTRACT

Gram-negative bacteria, including *Escherichia coli*, release outer membrane vesicles (OMVs) that are derived from the bacterial outer membrane. OMVs contribute to bacterial cell–cell communications and host–microbe interactions by delivering components to locations outside the bacterial cell. In order to explore the molecular machinery involved in OMV biogenesis, the role of a major OMV protein was examined in the production of OMVs from *E. coli* W3110, which is a widely used standard *E. coli* K-12 strain. In addition to OmpC and OmpA, which are used as marker proteins for OMVs, an analysis of *E. coli* W3110 OMVs revealed that they also contain abundant levels of FliC, which is also known as flagellin. A membrane-impermeable biotin-labeling reagent did not label FliC in intact OMVs, but labeled FliC in sonically disrupted OMVs, suggesting that FliC is localized in the lumen of OMV. Compared to the parental strain expressing wild-type *fliC*, an *E. coli* strain with a *fliC*-null mutation produced reduced amounts of OMVs based on both protein and phosphate levels. In addition, an *E. coli* W3110-derived strain with a null-mutation in *flgK*, which encodes flagellar hook-associated protein that is essential along with FliC for flagella synthesis, also produced fewer OMVs than the parental strain. Taken together, these results indicate that the ability to form flagella, including the synthesis of flagella proteins, affects the production of *E. coli* W3110 OMVs.

1. Introduction

Gram-negative bacteria produce outer membrane vesicles (OMVs), the membranes of which are derived from the bacterial outer membrane [1]. OMVs are spherical bilayered proteoliposomes with an average diameter of 20–200 nm. OMVs generally contain the following bacterial components: lipopolysaccharide (LPS), which exclusively occupies the outer leaflet of the outer membrane; phospholipids, which localize to the inner leaflet of the outer membrane; a subset of outer membrane proteins; periplasmic proteins; and nucleic acids [2–6]. Studies on OMVs produced from various bacterial strains demonstrated that they help deliver toxins, such as heat-labile enterotoxin of enterotoxigenic *Escherichia coli*, to host cells [2,6,7], transfer genomic components including drug-resistant gene to other bacteria [5,8], and protect the bacteria from antimicrobial peptides and phage infections [9]. Because virulence factors are present within OMVs, this suggests that OMV production is also involved in bacterial pathogenesis. Consistent with this notion, pathogenic bacteria generally produce more vesicles than their nonpathogenic counterparts [2,10]. Taken together, these observations indicate that the regulation of OMV production is involved in bacterial pathogenesis. In addition, OMVs play a protective role in nonpathogenic bacteria because they enhance bacterial survival by reducing cellular levels of toxic components such as toluene [11].

The release of OMVs from the bacterial surface may be governed by a reduction in the number of cross-links between peptidoglycan and the outer membrane [1,12]. The importance of peptidoglycan in OMV production was also demonstrated by a mutational analysis of *E. coli yfgL*, which encodes a lipoprotein involved in the regulation of peptidoglycan synthesis [10,13]. In addition, OMVs are not thought to be randomly assembled from the outer membrane and periplasm because several outer membrane proteins, such as OmpA and OmpC, and periplasmic proteins are specifically enriched in OMVs [7,10,14]. These observations suggest that outer membrane proteins and periplasmic proteins have some specific affinities for OMVs. However, the molecular basis for these affinities and the roles of OMV proteins in the biogenesis of OMVs are virtually unknown.

E. coli K-12 strain DH5α, which is widely used as a competent cell in molecular biology experiments, is also used to study *E. coli*

Abbreviations: OMVs, outer membrane vesicles; LPS, lipopolysaccharide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight.

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OMVs. Proteomic analyses revealed that OmpA, OmpC, and OmpF are major components of DH5α OMVs [7,14]. Here, we observed that OMVs from *E. coli* K-12 strain W3110, which is widely used as a standard laboratory strain of *E. coli* [15], contains the flagella filament protein FliC as well as OmpA and OmpC as major protein components. FliC has been observed in OMVs produced from various Gram-negative bacteria such as *E. coli* and *Pseudomonas aeruginosa*; however, the role of FliC in OMV biogenesis has not been studied [3,14,16]. We observed an apparent reduction in OMV production in a *fliC*-null mutant, indicating that FliC is involved in OMV production. Taken together, these results, suggest that FliC has a specific affinity for OMVs and is involved in OMV production.

2. Materials and methods

2.1. Reagents and bacterial strains

Any kD™ Mini-PROTEAN® TGX™ Precast Gels and Ruby protein gel stain were purchased from BIO-RAD. The Molecular Probes® ProQ Emerald 300 Lipopolysaccharide Gel Stain Kit was purchased from Life Technologies. *E. coli* W3110 (NBRC12713) was obtained from the NITE (Kisarazu, Japan). The *E. coli* W3110-derivative strain KP7600, which was used as the parental strain for mini-Tn10 transposon-mutagenesis [17], *fliC*-null (*fliC*::mini-Tn10) mutant strain JD23004, and *flgK*-null (*flgK*::mini-Tn10) mutant strain JD22125 were obtained from the National Bio-resource Project (NIG, Japan). *E. coli* DH5α was obtained from our laboratory stock, which was originally purchased from TAKARA BIO.

2.2. Preparation of OMVs

OMVs were purified as previously described [18] with some modifications. Briefly, 100 ml of LB broth (Miller) was inoculated with 100 μl of pre-culture and then incubated for 16 h at 37 °C with shaking. Then, the culture supernatant was prepared by sequential centrifugations at 2100*g* for 30 min and 13,000*g* for 50 min. The supernatant was filtered with a 0.22-μm pore-sized filter, and OMVs in the filtrate were pelleted by centrifugation at 150,000*g* for 3 h at 4 °C. The pellets were re-suspended in 1 ml of cold 10 mM Tris–HCl (pH 8.0), and then filtered again with a 0.20-μm pore-sized filter.

2.3. Protein and phosphate assays

Protein concentrations were determined using Pierce® BCA protein assay reagent and bovine serum albumin as a standard. Phosphate levels were quantified as previously described [19].

2.4. Quantitative reverse transcription PCR

RNA extracted from *E. coli* W3110 and DH5α using High Pure RNA Isolation kit (Roche Diagnostics) was treated with DNase I and reversetranscribed to cDNA with random hexamer-primer using PrimeScript® RT reagent kit (TAKARA BIO). The cDNA was used as template for PCR reaction [95 °C × 20 s + (95 °C × 5 s + 60 °C × 20 s) × 45 cycles] with Eco™ Real-time PCR System (Illumina) with Lumino Ct®SYBR®Green qPCR Ready Mix™ (SIGMA–Aldrich). Specific primers (*fliC*; 5′-GCTAAAACTCTTGGCCTTGATG-3′ and 5′-GGTTAGTTCCGCCAGTATC-3′, *rrsA*; 5′-CCAGGTGTAGCGGTGAAATG-3′ and 5′-ATCGTTTACGGCGTGGACTAC-3′) were used for the analysis.

2.5. Sucrose-density gradient centrifugation

OMVs (100 μg protein) suspended in 1.25 ml of PBS [137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 2.5 mM Na_2HPO_4 (pH7.4)] were layered over a sucrose density gradient (2.5, 1.6, and 0.6 M sucrose, 1.25 ml each) in a centrifuge tube. Then the sample was centrifuged at 200,000*g* for 16 h at 4 °C, and ten fractions of equal volume were collected from the top. Each fraction was diluted with 6 ml of PBS, and OMVs were collected by centrifuging at 150,000*g* for 1 h at 4 °C. The collected OMVs were suspended in 100 μl of 10 mM Tris–HCl (pH 8.0) and then used for further analysis.

2.6. Identification of OMV proteins

OMV proteins (9–30 μg) that had been fractionated by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) were visualized using Oriole™ Fluorescent Gel Stain (BIO-RAD), and the protein bands were excised. The gel pieces were treated with 100 μl of acetonitrile for 10 min and then dried. Proteins within the dried gel were reduced with 100 μl of 25 mM ammonium bicarbonate containing 10 mM dithiothreitol at 56 °C for 60 min. The reduced gel was washed with 100 μl of 25 mM ammonium bicarbonate for 10 min, and the proteins in the gel were alkylated with 100 μl of 55 mM iodoacetamide in 25 mM ammonium bicarbonate at room temperature for 45 min. The alkylated gel was washed twice with 100 μl of 25 mM ammonium bicarbonate for 10 min, and the proteins in the gel were treated twice with 200 μl of 50% acetonitrile in 25 mM ammonium bicarbonate at room temperature for 10 min. Then the gel was dried and treated with ~20 μl of 50 mM ammonium bicarbonate containing 10 μg/ml of sequencing-grade modified porcine trypsin (Promega) at 37 °C for 18 h. The resulting peptides were extracted twice with 50 μl of 50% acetonitrile containing 5% trifluoroacetic acid for 30 min at room temperature. The extracted peptides were adsorbed to a C_{18} ZipTip microcolumn (Merck Millipore), and the peptides were eluted with 4–6 μl of 50% acetonitrile containing 0.1% trifluoroacetic acid and 5 mg/ml α-cyano-4-hydroxycinnamic acid. The eluate was subjected to analysis with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) Voyager-DE STR mass spectrometer (Applied Biosystems). Proteins were identified by peptide mass fingerprinting analysis using the MASCOT search engine against the *E. coli* protein database in NCBI.

2.7. Mass spectrometric analysis of lipid A

Lipid A prepared from OMVs or bacterial cells using Tri-reagent (Molecular Research Center) was analyzed with a Voyager-DE STR mass spectrometer as described previously [20].

2.8. Biotin-labeling and detection of labeled proteins

Intact OMVs (40 μg protein) or OMVs (40 μg protein) that had been sonically disrupted three times with a Branson sonifier model S-150D at setting 1 for 10 s were suspended in 200 μl of PBS containing 5 μg/ml of Pierce® Sulfo-NHS-LC-Biotin, and incubated on ice for 20 min. To quench the reaction, 10 μl of PBS containing 1 M glycine was added to the OMVs, and the mixture was incubated on ice for 10 min. Biotin-labeled OMV proteins were fractionated by 12.5% SDS–PAGE under reducing conditions. Proteins separated on the gel were electroblotted onto a nitrocellulose membrane, and biotin-labeled proteins were detected with Pierce® Avidin-conjugated horseradish peroxidase.

3. Results

3.1. Identification of proteins in E. coli W3110 OMVs

We initially examined OMV production during various growth phases of *E. coli* K-12 strain W3110. OMV production was monitored by measuring the protein and phosphate levels in OMVs prepared from the culture supernatant. As shown in Fig. 1A, OMV production from *E. coli* W3110 greatly increased during the late-stationary phase (12 and 16 h cultivation). Increased OMV production in the late-stationary phase was further confirmed by visualizing LPS and proteins within the OMVs (Fig. 1B). Consistent with the well-known fact that *E. coli* K-12 possesses rough-type LPS, LPS in OMVs purified from *E. coli* W3110 exhibited a single band but not the ladder-like pattern that is indicative of smooth-type LPS (Fig. 1B). The major proteins (49, 36, and 34 kDa) that were observed in OMVs prepared from the culture supernatant at the late-stationary phase (12 and 16 h cultivation) were also observed in OMVs from the log and early-stationary phases (3 and 6 h cultivation, respectively), indicating that the protein compositions of OMVs did not significantly change across the bacterial growth phases (Fig. 1B). OMVs prepared from bacterial cultures at the late-stationary phase (16 h cultivation) were used for further analyses.

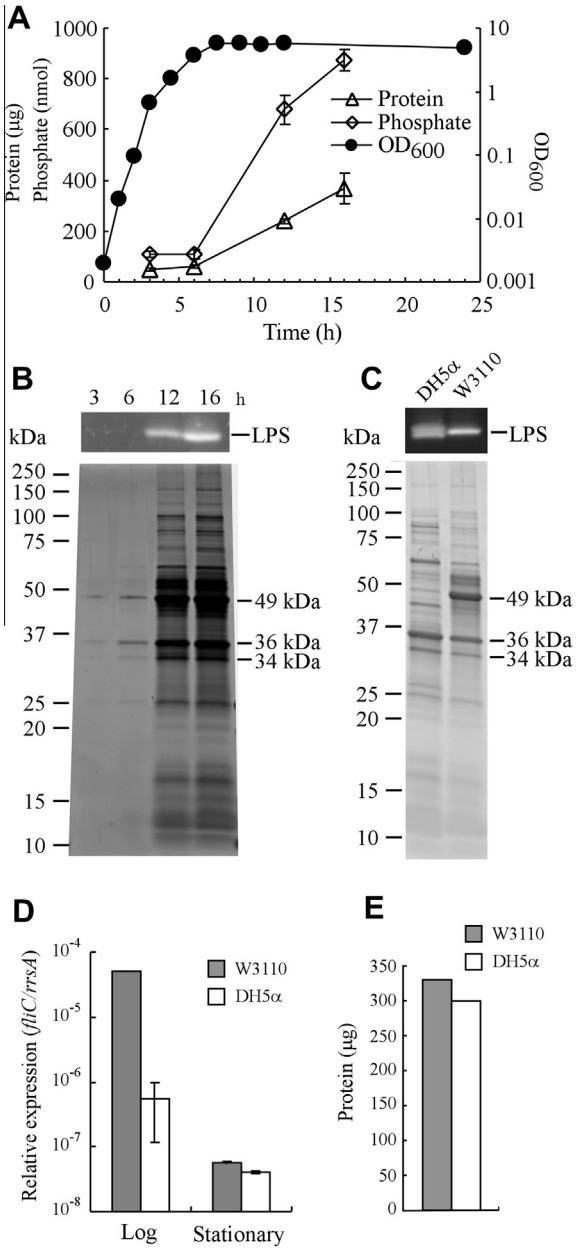

Fig. 1. OMV production by *E. coli* W3110. LB broth (100 ml) was inoculated with 100 μl of a full-growth pre-culture of *E. coli* W3110 and then incubated at 37 °C with shaking. (A) Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}). Protein and phosphate levels in OMVs prepared from the culture supernatant were determined and presented as the mean ± standard deviation of triplicate. (B) *E. coli* W3110 was cultivated for 3, 6, 12 and 16 h, and then OMVs (1 ml) were prepared from the culture supernatant. OMVs (10 μl) subjected to SDS–PAGE was visualized with Lipopolysaccharide staining kit (upper panel) or Ruby gel stain (lower panel). (C) OMVs (3 μg protein) prepared from a 16-h culture of *E. coli* DH5α and W3110 was subjected to SDS–PAGE, and LPS and proteins were visualized as described above. (D) Expression of *fliC* during logarithmic (log) or stationary phase of *E. coli* W3110 and DH5α was quantified by quantitative reverse transcription PCR. Relative expression was defined as the amount of *fliC* transcript normalized with that of *rrsA* 16S ribosomal RNA. Error bars indicate the standard deviation of triplicate. (E) Protein levels in OMVs prepared from the 16 h culture supernatant of *E. coli* DH5α and W3110 were determined.

OMV proteins were subjected to SDS–PAGE, and the protein bands were excised. The excised proteins were digested with trypsin and the resulting peptide fragments were analyzed using a MALDI-TOF mass spectrometer. The analysis revealed that the 49, 36, and 34 kDa proteins in Fig. 1B were FliC, OmpC, and OmpA, respectively. FliC is also known as flagellin, which is a major constituent and exclusive protein of the flagella filament [21]. OmpC and OmpA were previously identified as OMV proteins in proteomic studies, and they are often used as marker proteins for OMVs [7]. A summary of the protein identification analysis is presented in Table 1. In addition to the major proteins, AcnB, AceB, GroEL, RpoC, TnaA, and AldA were identified in OMVs. Among these proteins, AcnB, GroEL, and RpoC were previously identified as *E. coli* OMV proteins [14]. OMVs prepared from *E. coli* K-12 strain DH5α were previously analyzed [7,14], and therefore we could compare OMVs prepared from *E. coli* W3110 with those from *E. coli* DH5α. OmpC and OmpA were identified as major proteins in OMVs from *E. coli* DH5α while FliC was not determined to be a major OMV protein (Fig. 1C). These results were consistent with previous observations that OmpC and OmpA were major proteins in OMVs prepared from *E. coli* DH5α and that FliC was identified as a component of OMVs, although not at high levels [7,14]. Expression levels of FliC mRNA in *E. coli* W3110 and *E. coli* DH5α were examined by quantitative PCR. Expression level of FliC in *E. coli* W3110 was much higher than that in *E. coli* DH5α (Fig. 1D). Furthermore, productions of OMVs from *E. coli* W3110 and *E. coli* DH5α were compared by measuring the protein levels in OMVs. As shown in Fig. 1E, OMVs were produced from the both cells at similar levels. Taken together, these observations indicate that the content of FliC in OMVs differs among bacterial strains, and OMVs from W3110 are unique in that their FliC content is relatively high.

Gram-negative bacteria, including *E. coli*, possess lipid A modification enzymes in their outer membranes and some enzymes, such as *E. coli* PagP and *Salmonella enterica* PagL, can be inactive or latent in the outer membranes [20,22]. We examined the structures of the lipid A portion of LPS in OMVs in order to determine whether these modification enzymes were released from latency. However, an analysis of lipid A with a MALDI-TOF mass spectrometer revealed that the molecular mass of lipid A in OMVs did not differ from that in *E. coli* cells, indicating that lipid A modifications did not occur in OMVs (data not shown).

3.2. FliC is a component of OMVs

Previously, FliC, OmpC, and OmpA were identified as components of OMVs produced by various bacterial strains [3,7,14,16]. However, a previous study suggested that contamination of flagella in OMVs contributes to the detection of FliC in OMVs prepared

Table 1
Summary of proteins identified in OMVs prepared from *E. coli* W3110.

Protein[a]	Description	Localization	MW	AA	Score[b]	Match peptide[c]	Sequence coverage[d] (%)
FliC	Flagellin	Cytosol	51.3	498	170	11	42
OmpA	Outer membrane protein A	Outer membrane	37.2	346	98	7	45
OmpC	Outer membrane protein C	Outer membrane	40.3	367	119	8	40
AcnB	Aconitate hydratase 2	Cytosol	91.1	839	88	14	21
AceB	Malate synthase A	Cytosol	60.4	533	79	8	21
GroEL	60 kDa chaperonin	Cytosol	55.2	548	78	8	21
RpoC	DNA-directed RNA polymerase beta chain	Cytosol	155.9	1407	126	23	22
TnaA	Tryptophanase	Cytosol	52.7	471	98	9	24
AldA	Aldehyde dehydrogenase A	Cytosol	52.2	479	87	8	20

[a] Proteins identified in OMVs prepared from *E. coli* W3110 are listed, along with their molecular weight (MW) and number of amino acid residues (AA).
[b] Score represents the results of the MASCOT analysis against NCBI database.
[c] Match peptide represents the number of peptides that matched the sequence of the identified protein.
[d] Sequence coverage indicates the coverage rate of the protein sequence by the peptides.

from *P. aeruginosa* [3]. In order to examine whether FliC is a component of OMVs prepared from *E. coli* W3110, OMVs were further fractionated by sucrose-density gradient centrifugation. As shown in Fig. 2A, OmpC and OmpA were collected mainly in fractions 6–8, while FliC was collected mainly in fraction 8. On the other hand, LPS was collected mainly in fractions 6 and 8 (Fig. 2A). The densities of fractions 6–8 were 1.16–1.26 g/ml (Fig. 2A), and these observations are consistent with a previous study showing that OMVs prepared from enterotoxigenic *E. coli* migrated around a density of 1.2 g/ml [2]. The co-fractionation of FliC with LPS in fraction 8 suggested that FliC is associated with membranes. These results suggest that FliC is a component of the OMVs collected in fraction 8. The recovery of LPS in two separate fractions (fractions 6 and 8) suggests that at least two different types of OMVs, with differing protein compositions, are produced from *E. coli* W3110.

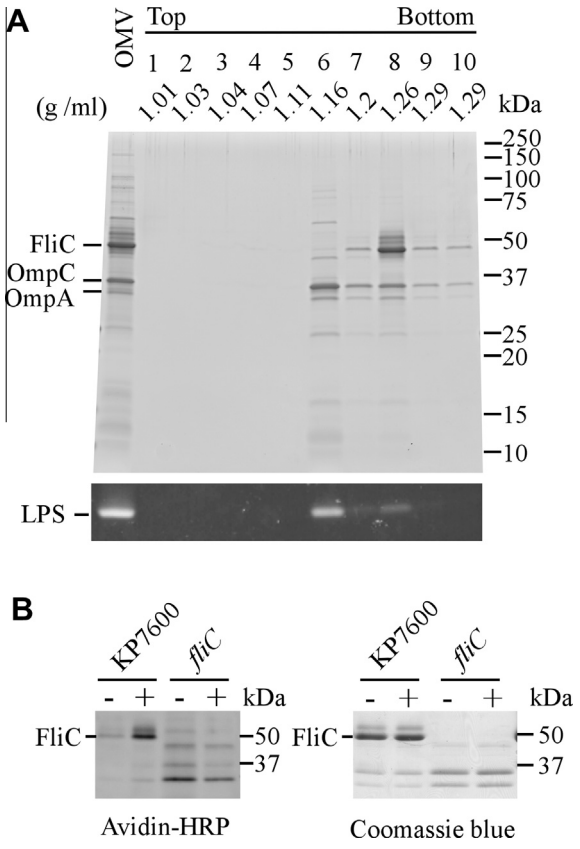

Fig. 2. FliC is a component of *E. coli* W3110 OMVs. (A) OMVs prepared from *E. coli* W3110 were separated by sucrose-density gradient centrifugation, and the total OMV input (2 μg protein) as well as 10 μl of the each fraction was subjected to SDS–PAGE, and visualized as described in Fig. 1. The fraction numbers (1–10) and the density (g/ml) are indicated. (B) Sonically disrupted OMVs (+) and intact OMVs (−) prepared from the *fliC*-null strain or parental *E. coli* W3110–derivative strain KP7600 were labeled with Sulfo-NHS-LC-Biotin. Biotin-labeled proteins fractionated by SDS–PAGE were visualized by avidin-conjugated horseradish peroxidase (avidin-HRP, left panel). Alternatively, the proteins were visualized with Coomassie blue (right panel).

To further analyze the association of FliC with membrane vesicles, proteins on the surface of intact OMVs were labeled with biotin using a water-soluble and membrane-impermeable reagent. Alternatively, OMVs were sonically disrupted, and proteins in the sonicated preparation were labeled with the same reagent. FliC was labeled with biotin in the sonically disrupted but not in the intact OMVs (Fig. 2B left), suggesting that FliC is localized in the lumen of OMV.

3.3. Flagella proteins are involved in OMV production in E. coli W3110

The presence of FliC in OMVs prompted us to examine whether FliC is involved in OMV production in *E. coli* W3110. OMV production in the *fliC*-null mutant strain and parental wild-type strain were examined by measuring the protein and phosphate levels in the OMVs. As shown in Fig. 3C, OMV production was reduced in the *fliC*-null mutant compared to the parental wild-type strain based on both the protein and phosphate levels. Bacterial growth was similar between the *fliC*-null mutant strain and parental wild-type strain, excluding the possibility that bacterial growth affected OMV production (Fig. 3B). Furthermore, OMV production was examined with a *flgK*-null mutant strain, in which flagella formation was defective due to the absence of the flagellar hook–associated protein FlgK (Fig. 3A). OMV production in the *flgK*-null strain was also significantly reduced compared to the parental wild-type strain (Fig. 3C). In contrast, cell growth was similar between the *flgK*-null strain and parental wild-type strain, excluding the possibility that bacterial growth affects OMV production (Fig. 3B). OMV production and protein composition were similar between the *fliC*-null mutant and *flgK*-null mutant (Fig. 3C and D), suggesting that *fliC* and *flgK* have similar effects on OMV production. These results suggested that the ability to form flagella, including synthesis of the flagella protein FliC, is involved in OMV production by *E. coli* W3110.

4. Discussion

In this paper we demonstrated that a major component of *E. coli* W3110 OMVs is FliC, which is an exclusive protein unit of the flagella filament and determined that FliC is localized in the lumen of OMV. Furthermore, *fliC*-null or *flgK*-null mutations greatly reduced OMV production, suggesting that flagella synthesis affects the production of OMVs from *E. coli* W3110.

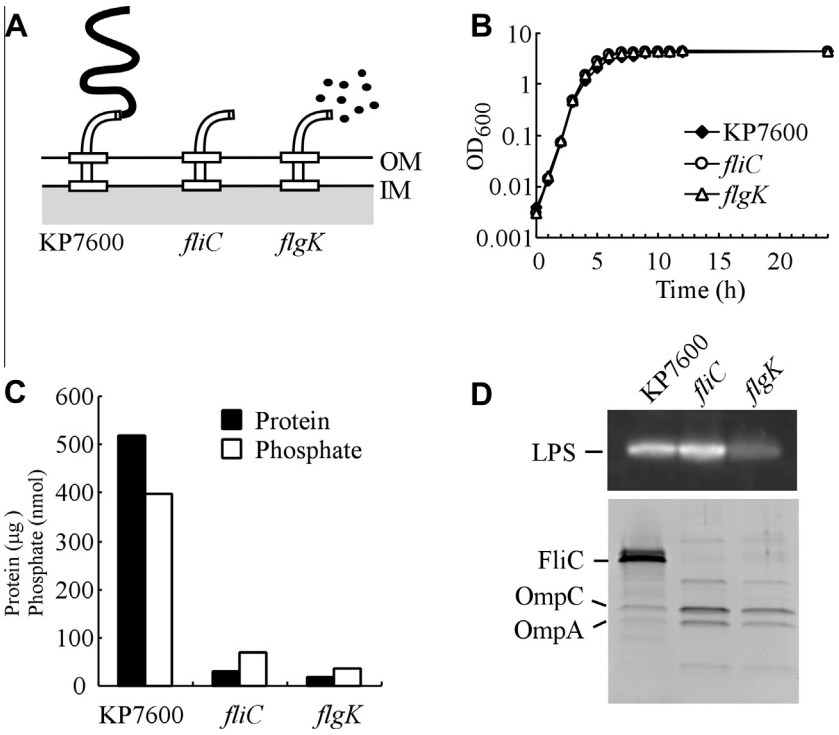

Fig. 3. Bacterial ability to produce flagella affects OMV production. (A) Schematic representations of flagella synthesis in the *fliC*-null and *flgK*-null strains and the parental wild-type strain. IM and OM represent the inner membrane and outer membrane, respectively. (B) Growth of the *fliC*-null and *flgK*-null strains and the parental KP7600 strain was monitored as described in Fig. 1A. (C) OMVs were prepared from 100 ml of culture supernatant (16 h cultivation) from the *fliC*-null and *flgK*-null strains and the parental KP7600 strain, and the protein and phosphate levels were determined. (D) OMV (5.3 nmol phosphate, upper panel) and OMV (1 μg protein, lower panel) prepared from *fliC*-null and *flgK*-null strains and the parental KP7600 strain were subjected to SDS–PAGE and visualized as described in Fig. 1.

FliC, which is better known as flagellin, induces inflammatory responses by activating cell surface Toll-like receptor 5 and/or intracellular NLRC4 [23,24]. These responses are important for innate immunity against bacterial invasion; however, excessive responses can cause sepsis. In general, pathogenic bacteria produce more vesicles than their nonpathogenic counterparts [2,10], suggesting that OMVs contain virulence factors derived from these pathogens. Consistent with this notion, our results demonstrated that the *fliC*-null mutant produced much less OMVs than the parental strain. OMVs might be used as a means to deliver flagellin to other bacteria or host cells.

Our results demonstrated that flagella synthesis, including synthesis of FliC, affects OMV production by *E. coli* W3110. Our results also demonstrated that the bacterial growth phase affects OMV production. However, the precise molecular cross-talk between flagella synthesis and OMV production remains unknown. In addition, *E. coli* DH5α, which express much less amount of fliC than *E. coli* W3110, produces OMVs similar levels with that of *E. coli* W3110. These results, taken together, suggest that quantity of flagella synthesis is not important for the production of OMV, but flagella synthesis exert key regulatory role on OMV production. Furthermore, several factors, such as stationary phase stress that leads to activation of σ^E, a regulator of peptidoglycan synthesis, and the number of cross-links between peptidoglycan and the outer membrane, have been shown to be involved in OMV production [10,12,25]. In addition to flagella synthesis and the bacterial growth phase, these factors might also cooperatively regulate OMV production, and this potential cooperativity should be the focus of future studies.

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