

## Variation of Physicochemical Properties and Cell Association Activity of Membrane Vesicles with Growth Phase in *Pseudomonas aeruginosa*<sup>∇</sup>

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***Pseudomonas aeruginosa* and other Gram-negative bacteria release membrane vesicles (MVs) from their surfaces, and MVs have an ability to interact with bacterial cells. Although it has been known that many bacteria have mechanisms that control their phenotypes with the transition from exponential phase to stationary phase, changes of properties in released MVs have been poorly understood. Here, we demonstrate that MVs released by *P. aeruginosa* during the exponential and stationary phases possess different physicochemical properties. MVs purified from the stationary phase had higher buoyant densities than did those purified from the exponential phase. Surface charge, characterized by zeta potential, of MVs tended to be more negative as the growth shifted to the stationary phase, although the charges of PAO1 cells were not altered. *Pseudomonas* quinolone signal (PQS), one of the regulators related to MV production in *P. aeruginosa*, was lower in MVs purified from the exponential phase than in those from the stationary phase. MVs from the stationary phase more strongly associated with *P. aeruginosa* cells than did those from the exponential phase. Our findings suggest that properties of MVs are altered to readily interact with bacterial cells along with the growth transition in *P. aeruginosa*.**

*Pseudomonas aeruginosa* and many other Gram-negative bacteria naturally produce membrane vesicles (MVs), which are released from their outer surface. These bilayered spheres are made up of outer membrane proteins (OMPs) and lipopolysaccharide (LPS) and encapsulate periplasmic components. *P. aeruginosa* MVs also contain some virulence factors for killing host cells or other bacteria, including proteases, phospholipase C, alkaline phosphatase, and antibacterial factors, such as murein hydrolases (13, 15). MVs are also a component of the matrices of biofilm (27, 28). Moreover, *P. aeruginosa* MVs contain a quorum-sensing (QS) signaling molecule, 2-heptyl-3-hydroxyl-4-quinolone (*Pseudomonas* quinolone signal [PQS]) (18), which serves as a mediator of cell-cell communication (24, 34). PQS not only is packaged in MVs but also enhances MV production (18, 19).

*P. aeruginosa* MVs have an ability to interact not only with bacterial cells (12, 14, 16) but also with eukaryotic cells to deliver the pseudomonal toxins (20). *P. aeruginosa* MVs readily fuse with Gram-negative bacteria and attach with Gram-positive bacteria (12). It has been proposed that charge-charge interaction is important in adherence of MVs to bacterial cells (12). The outer surfaces of both Gram-negative and -positive bacteria are usually negatively charged and rich in divalent cations, such as Mg<sup>2+</sup> or Ca<sup>2+</sup>, that stabilize surface charges. In the model of Kadurugamuwa and Beveridge (12), MVs have negatively charged LPSs, and the O side chains of LPSs are loosely packed and separated from one another because of

high curvatures, resulting in little salt bridging by divalent cations between adjacent LPSs. Because of Mg<sup>2+</sup> or Ca<sup>2+</sup> depletion on the MV surface, it is likely that the anionic MV surface would become salt bridged to the bacterial cells. For that reason, MV surface charge would be an important factor that affects the interaction with bacterial cells through physicochemical forces. Surface charge is usually characterized by zeta potential, which is the electrical potential of the interfacial region between the bacterial surface and the aqueous environment (36, 38).

Many bacteria have exquisite regulatory mechanisms that control gene expression during the transition from the exponential phase to the stationary phase (11, 37). An example is QS, the mechanism of which enables it to coordinately tune multiple gene expression in a cell density-dependent manner. Although changes in gene transcription and cell behavior between the exponential phase and the stationary phase have been well studied, changes in the properties of the released MVs have not. To date, *P. aeruginosa* MVs have been well characterized in several studies, which have revealed their potential properties, virulence, and production mechanisms (3, 13, 25). However, each study focused on MVs isolated from a single growth phase. There is a likelihood that MV properties and production mechanisms are, to some degree, altered according to the growth transition.

In this study, we investigated the properties of MVs released during the transition from the exponential phase to the stationary phase. Since *P. aeruginosa* MVs play the role of a PQS carrier, it is thought that MVs take on some functions to easily interact with cells after PQS synthesis is initiated. We hypothesized that MV properties are altered with the growth transition to interact with bacterial cells. We showed that MVs

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developed more negative surface charges, contained more PQS, and interacted with PAO1 cells more readily as the growth shifted to the stationary phase. Our results contribute to understanding the mechanism of how MVs interact with bacterial cells.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The sequenced *P. aeruginosa* PAO1 Holloway strain (10) was used as a standard strain in this study.  $\Delta pqsH$ ,  $pqsH$ - $xylE$ ,  $pqsE$ - $xylE$ , and  $\Delta pqsH$   $pqsE$ - $xylE$  mutants of PAO1 were used (31, 32, 33). Bacterial cells were routinely grown at 37°C in Luria-Bertani (LB) medium at 150 rpm or LB containing 1.5% (wt/vol) agar.

**MV isolation, purification, and quantification.** MV isolation, purification, and quantification were carried out as described previously (31). Crude MVs were isolated from an LB culture of *P. aeruginosa*. Cells were removed by centrifugation ( $6,000 \times g$ , 15 min), and the supernatant was sequentially filtered through 0.45- and 0.22- $\mu$ m cellulose acetate membranes, followed by ultracentrifugation at  $150,000 \times g$  for 3 h at 4°C. The pellets were washed with 10 mM HEPES (pH 6.8) containing 0.85% (wt/vol) NaCl (HEPES-NaCl). MV protein was quantified by the Bradford method (4), and MV phospholipid was quantified by the Stewart method (29, 31). In MV purification, pellets of ultracentrifuged MVs were resuspended in 500  $\mu$ l of 45% (wt/vol) Optiprep in HEPES-NaCl, transferred to the bottom of an ultracentrifuge tube, and layered with Optiprep-HEPES-NaCl (1 ml of 40%, 35%, 30%, 25%, 20%, and 15% and 0.5 ml of 10%). The gradients were ultracentrifuged ( $100,000 \times g$ , 3 h), and 500- $\mu$ l fractions were collected from each gradient. Protein and phospholipid concentrations were measured in each fraction. Fractions containing MVs were resuspended in HEPES-NaCl buffer and ultracentrifuged again, and purified MVs were obtained. In the protein band pattern analysis, EDTA-free protease inhibitor (Roche, Mannheim, Germany) was added in all processes of MV purification per the manufacturer's instructions. All subsequent steps were carried out at 0 to 4°C to prevent the effect of proteolytic activities in the supernatant. We confirmed that proteolytic activities in the supernatants were substantially inhibited at 4°C (data not shown).

**Proteolytic activities.** Proteolytic activity was determined by an azocasein assay as previously reported, with some modification (31). Briefly, 10  $\mu$ l of cell-free supernatant was added to 0.5 ml of 0.3% azocasein (Sigma) in 50 mM Tris-HCl (pH 7.2) and 0.5 mM CaCl<sub>2</sub> and incubated at 37°C or 4°C for 15 min. The reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid. After centrifugation ( $15,000 \times g$ , 20 min), the absorbance at 400 nm in the supernatant was measured.

**Transmission electron microscopy (TEM) observation.** Purified MVs were placed on Formvar-coated copper grids (200 mesh), which were then stained with 2% aqueous uranyl acetate for 1 min, rinsed, and observed with a JEOL-1010 TEM (JEOL, Tokyo, Japan) at 80 kV.

**SDS-PAGE and protein identification.** OMPs were purified as described previously (30). Protein patterns of MVs and OMPs were examined by SDS-PAGE as previously described (31). MV protein bands were excised from SDS-polyacrylamide gels and placed into microcentrifuge tubes. Gel pieces were washed twice with 40% 1-propanol for 10 min and twice with 100 mM ammonium bicarbonate-50% acetonitrile for 10 min and dried using a centrifugal dryer. In-gel digestion was performed using 5  $\mu$ l of trypsin (20  $\mu$ g/ml) in 50  $\mu$ l of 100 mM ammonium bicarbonate at 37°C for 10 to 16 h. Peptides were extracted twice from the gel with 0.1% trifluoroacetic acid (TFA)-50% acetonitrile for 10 min and dried to 10  $\mu$ l using a centrifugal dryer. Peptide samples were desalted using a C<sub>18</sub> ZipTip microcolumn (Millipore) as described by the manufacturer. For matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) analysis, 2  $\mu$ l of samples was spotted onto the MALDI matrix and analyzed with a Shimadzu Axima-CFR+ (Shimadzu, Kyoto, Japan) mass spectrometer. Proteins were identified by peptide mass fingerprint analysis according to the molecular mass of each tryptic fragment and using the MASCOT search engine of the entire NCBI protein database of *P. aeruginosa*.

**PQS detection.** PQS was quantified using thin-layer chromatography (TLC) as previously described (24). PQS was extracted from sonicated MVs with acidified ethyl acetate, and the solvent was evaporated. Extracts were suspended in 20  $\mu$ l 1:1 ethyl acetate-acetonitrile. A 5- $\mu$ l sample was analyzed using TLC with a 17:2:1 mixture of dichloromethane-acetonitrile-dioxane.

**Measurement of hydrodynamic diameter and surface charge.** Particle size distribution of MVs was analyzed at 25°C with a Zetasizer Nano ZS particle analyzer (Malvern Instruments, Malvern, United Kingdom). Purified MVs were diluted to a concentration of approximately 10  $\mu$ g protein/ml in 10 mM HEPES

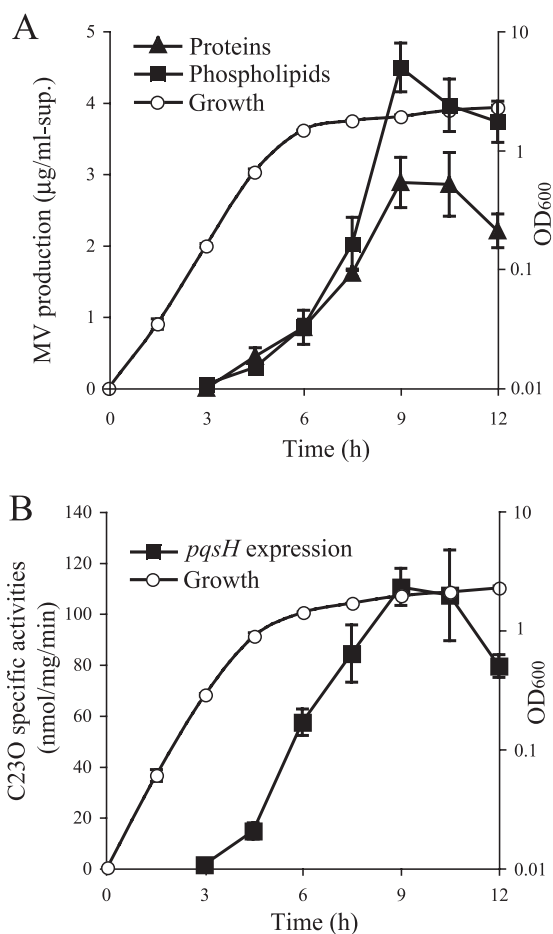


FIG. 1. Time courses of MV production and *pqsH* expression. (A) Growth curve and MV production in *P. aeruginosa* PAO1. The quantity of MVs in LB supernatants was determined by measuring the concentration of proteins and phospholipids in pellets from ultracentrifugation. Data are means  $\pm$  standard deviations from triplicates. The results presented are representative of at least three independent experiments. (B) Growth curve of and *pqsH* transcription in the *P. aeruginosa* *pqsH-xylE* strain. The expression of the *pqsH-xylE* transcriptional fusion was monitored using a catechol-2,3-dioxygenase activity assay. Data are means  $\pm$  standard deviations from triplicates. The results presented are representative of at least three independent experiments.

buffer. MV hydrodynamic zeta average diameter was calculated by the light scattering method.

The relative surface charges were estimated using a Zetasizer Nano ZS particle analyzer. The electrophoresis cell was filled with approximately 10  $\mu$ g MV/ml in 10 mM HEPES buffer or PAO1 cells diluted to an optical density at 600 nm (OD<sub>600</sub>) of 0.1. The Smoluchowski approximation was applied for determining the zeta potential.

**MV association assay.** To monitor the fusion of MVs with bacterial cells, MVs were fluorescently labeled with fluorescein isothiocyanate (FITC) as previously described with some modification (2). Purified MVs (100  $\mu$ g protein) were incubated with FITC (100  $\mu$ g) in 1 ml of 100 mM NaCl-50 mM Na<sub>2</sub>CO<sub>3</sub> (pH 9) for 2 h at 25°C. FITC-labeled MVs were washed three times in HEPES-NaCl buffer ( $150,000 \times g$ , 30 min). PAO1 cells grown to the late exponential phase were washed three times in phosphate-buffered saline (PBS) and diluted to an OD<sub>600</sub> of 1.0. FITC-labeled MVs (10  $\mu$ g/ml) were incubated with cells for 15 min to 120 min at 37°C. Cells were washed twice in PBS. Association of MVs with cells was observed by a scanning confocal laser microscope (LSM 5 Pascal; Carl Zeiss, Oberkochen, Germany) with an FITC filter (485 nm for excitation and 535 nm for emission), and total cells were visualized by differential interference

TABLE 1. MV concentrations in the supernatants and MV-producing activities in the exponential and stationary phases<sup>a</sup>

Cultivation time (h)	MV concn (μg/ml of supernatant)		MV-producing activity (pg/CFU)	
	Protein	Phospholipid	Protein	Phospholipid
4.5	0.60 ± 0.04	0.62 ± 0.15	8.66 ± 0.16	8.91 ± 2.23
12	2.21 ± 0.13	3.28 ± 0.22	7.58 ± 0.63	11.32 ± 1.99

<sup>a</sup> Data are the means ± standard deviations from triplicates.

microscopy. In the fluorometric quantification assay, FITC-labeled cells were lysed with a homogenizer. Fluorescence (485 nm for excitation and 535 nm for emission) was quantitated using a fluorometer (Arvo MX/Light; PerkinElmer, Yokohama, Japan). A standard curve was generated with a dilution series of purified FITC-labeled MVs. The fluorescence intensity of lysed cells, which are

not incubated with MVs, was used for a blank. Cell-associated MV protein level per cell protein was calculated from a standard curve.

**C230 specific activity assay.** To examine the transcription level of *pqsH*, the *pqsH*-*xylE* strain was used (31). To examine the transcription level of the *pqsABCDE* operon under *pqsH* deletion, the  $\Delta pqsH$  *pqsE*-*xylE* mutant was used (32). An assay for activity of catechol-2,3-dioxygenase (C230) (the *xylE* product) was performed as described previously (17). Cells were homogenized, and lysed protein samples were used. The  $A_{375}$  was recorded at 30°C. Specific activity was defined as nanomoles of product formed per minute per milligram of protein ( $e^{1cm} = 4.4 \times 10^4$ ).

## RESULTS

**MV production in the exponential and stationary phases.** MVs from a *P. aeruginosa* LB culture were isolated by ultracentrifugation of the cell-free supernatant, and the MV quantities were assayed by measuring the protein and phospholipid

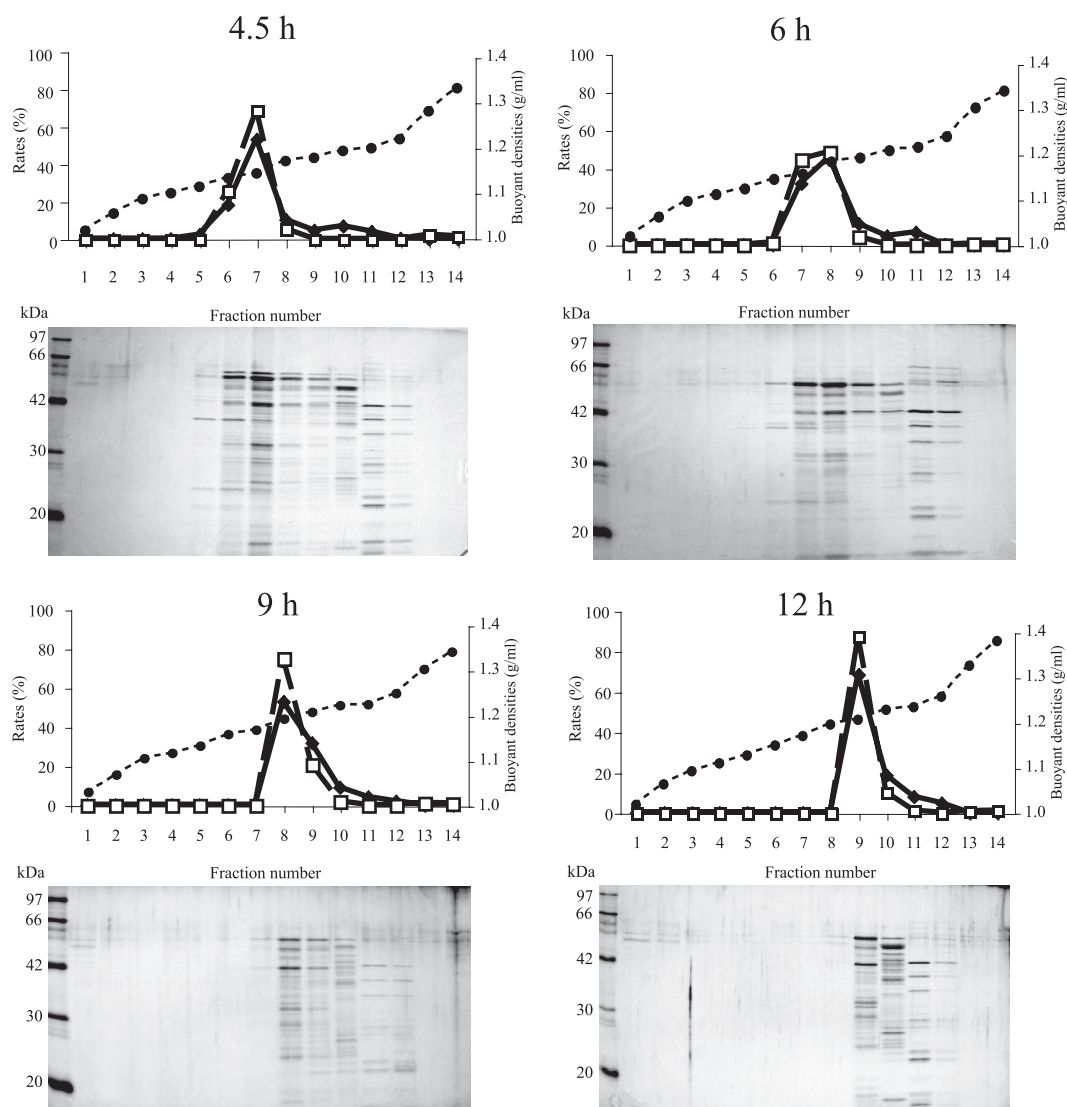


FIG. 2. Protein concentrations, phospholipid concentrations, buoyant densities, and SDS-PAGE profiles of density gradient fractions. MVs were extracted from LB medium at 4.5, 6, 9, and 12 h of growth. Crude MV protein (100 μg) was separated on a 10% to 45% Optiprep gradient, and 14 fractions were collected (fraction 1 is the lightest and fraction 14 is the heaviest). The upper panels show the protein concentration (diamonds; left longitudinal axis), phospholipid concentration (squares; left longitudinal axis), and buoyant density (circles; right longitudinal axis) of each fraction. In the bottom panels, equal volumes of each fraction were subjected to SDS-PAGE (15% acrylamide) and silver stained.

TABLE 2. Temporal changes in properties of MVs and cells

Cultivation time (h)	MVs			Cell zeta potential <sup>c</sup> (mV)
	Buoyant density <sup>a</sup> (g/ml)	Hydrodynamic diam <sup>b</sup> (Z avg [nm])	Zeta potential <sup>c</sup> (mV)	
4.5	1.08, 1.11	105.2 ± 7.2	-8.4 ± 1.3	-35.0 ± 1.2
6	1.10, 1.14	107.3 ± 12.1	-14.4 ± 1.8	-34.3 ± 0.8
9	1.13, 1.14	111.8 ± 16.2	-18.2 ± 1.9	-34.2 ± 0.6
12	1.15, 1.17	118.8 ± 8.3	-24.8 ± 1.4	-34.7 ± 0.3

<sup>a</sup> Data are minimum and maximum densities of fractions including MVs.

<sup>b</sup> Data are the means ± standard deviations of the average intensity from three independent assays.

<sup>c</sup> Data are the means ± standard deviations from three independent experiments carried out in triplicate.

concentrations of the pellets. We previously showed that over 90% of proteins and 99% of phospholipids of the ultracentrifuged pellets consist of MVs (31). Figure 1A shows that MV production was initiated at 4.5 h of growth and increased as the growth switched to the stationary phase.

It has been reported elsewhere that a QS signal, PQS, which is released during the stationary phase, enhances MV production and that PQS is enclosed within MVs (18). To investigate the relationship between the initiation of MV production and that of PQS synthesis in *P. aeruginosa*, the reporter gene *xylE* was inserted downstream of the chromosomal *pqsH* gene, the product of which catalyzes the final reaction in PQS synthesis, and the expression was examined by assaying catechol-2,3-dioxygenase activity. The result showed that the transcription of *pqsH* was initiated from 4.5 h of growth and peaked in the early stationary phase (Fig. 1B).

We further examined how many MVs bacteria produce in the exponential (4.5-h growth) and stationary (12-h growth) phases. MVs were produced in both phases (Table 1), although the PQS synthesis might be very low in the exponential phase compared to the stationary phase. Interestingly, while MV protein concentration and MV phospholipid concentration were similar in the exponential-phase MVs (ex-MVs), phospholipid concentration was higher than protein concentration in the stationary-phase MVs (st-MVs). These results suggest that MVs are produced in both the exponential and stationary phases but that their properties are not identical in each growth phase.

**Buoyant densities of MVs.** To investigate the properties of MVs with the growth transition, the differences in the buoyant density of MVs at each growth phase were examined. We fractionated crude MVs from 4.5, 6, 9, and 12 h of growth by an Optiprep density gradient method (31). For each sample, gradient fractions were collected and the concentrations of proteins and phospholipids were measured (Fig. 2). When MVs from the exponential phase (4.5 h of growth) were fractionated, both the protein and phospholipid concentrations were comparatively high in the light fractions (fractions 4 to 7), indicating that exponential-phase MVs fractionated to the light fractions. Indeed, the existence of MVs in the peak fractions could be confirmed by electron microscopy (data not shown). On the other hand, MVs from the stationary phase (12 h of growth) were found in the heavier fractions (fractions 8 and 9), suggesting that the buoyant density of st-MVs is higher than that of ex-MVs. As growth continued, the buoyant densities had a tendency to increase (Table 2). To further analyze the density of MVs, each fraction was subjected to SDS-PAGE and

bands were detected by silver staining (Fig. 2). Probable MV bands were detected in the fractions with high protein and phospholipid concentrations. Some bands were detected in the heavier fractions (fractions 10 to 12) of each sample, and these fractions seemed to include flagellin (53 kDa), pilin (19 kDa), and other components of cells, as seen by Bauman and Kuehn (3). Thus, the buoyant density of MVs increases as the cells grow, suggesting that the MV component alters depending on the growth phase.

**Comparison of the exponential- and stationary-phase MV proteomes.** To investigate whether the protein profiles of MVs are altered according to the growth transition, MVs purified from 4.5-, 6-, 9-, and 12-h growth were compared using SDS-PAGE. Each band pattern was very similar but not identical to the others (Fig. 3). Some major bands of the exponential-phase MVs were excised and identified using MALDI-TOF MS after in-gel tryptic digestions. Major outer membrane proteins (OMPs), including OprD, OprF, and OprH, and extracellular proteins, including putative aminopeptidase PaAP and phospholipase C PlcB, and the bacteriophage protein PA0622 were identified (Table 3). The most abundant MV protein is ami-

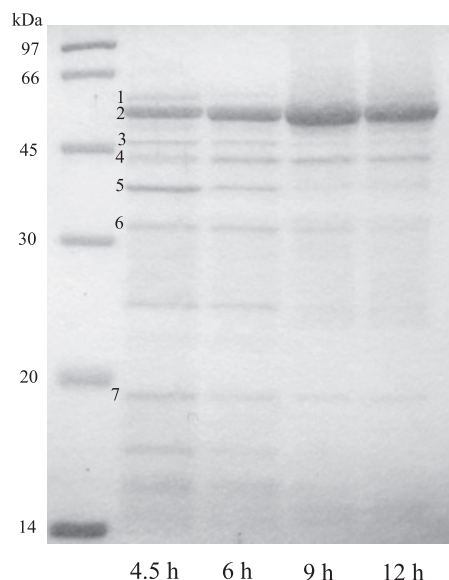


FIG. 3. Protein profiles of MVs purified from 4.5, 6, 9, and 12 h of growth. Purified MV proteins (10 µg) were separated by SDS-PAGE (15% acrylamide) and stained with Coomassie brilliant blue. The number of each band corresponds to the numbers in Table 3.

TABLE 3. Identified proteins in PAO1 MVs

No.	Annotation name	PA no.	Score	tMW <sup>a,b</sup>	MP <sup>b</sup>	Cov. <sup>c</sup>
1	Putative aminopeptidase PaAP	PA2939	143	57.5	13/15	29
2	Putative aminopeptidase PaAP	PA2939	143	57.5	13/15	29
3	OprD	PA0958	67	48.4	7/11	26
4	Bacteriophage protein	PA0622	65	41.2	8/15	30
5	OprF	PA1777	76	37.6	5/11	24
6	Phospholipase C, PlcB	PA0026	84	36.7	9/22	42
7	OprH	PA1178	105	21.6	9/17	44

<sup>a</sup> Theoretical mass.

<sup>b</sup> Number of peptide masses matching the top hit from MASCOT peptide mass fingerprinting/total number of peptide masses.

<sup>c</sup> Sequence coverage (%) in peptide mass fingerprinting.

nopeptidase PaAP, and its identification is similar to the results from the work of Bauman and Kuehn (3). Interestingly, OMPs, including OprD, OprF, and OprH, were enriched in the exponential MVs compared to those in the stationary MVs (Fig. 3). On the other hand, the expression level of OprD, OprF, or OprH localized in PAO1 outer membrane remained constant irrespective of the growth phase based on SDS-PAGE analysis (data not shown). The proteolytic activity of the supernatant in the stationary phase was higher than that in the exponential phase (data not shown), suggesting that OMPs localized in MV may be degraded by extracellular proteases in the supernatant.

**Size and appearance of MVs.** To investigate whether the sizes of MVs vary according to the growth transition, the hydrodynamic diameter of MVs was examined in 10 mM HEPES (pH 6.8) by the light scattering method (Table 2). The hydrodynamic diameter of MVs did not vary among MVs at 4.5, 6, 9, and 12 h of growth (Table 2). Moreover, the distributions of MV diameters were also similar among these samples (data not shown). These data indicate that the MV's average size is invariable, regardless of the growth phase.

Moreover, the appearance of MVs was observed by TEM (Fig. 4). While changes in both the size and shape of MVs could not be observed, the translucence was different between two growth phases: ex-MVs were more translucent than st-MVs, suggesting that stationary-phase MVs contain a higher concentration of some substances, such as PQS or metals. This result reflects the earlier finding that MV density is enhanced as growth develops (Table 2).

**PQS content in MVs.** It has been known that MV production is regulated by PQS in *P. aeruginosa* (18). However, while PQS synthesis occurred at the onset of the stationary phase (9), MV production occurred detectably from the exponential phase. Hence, it is presumed that MV production is independent of

PQS during the exponential phase. To corroborate the presumption, the existence of PQS in MVs was examined using TLC. The result showed that the amount of PQS in ex-MVs was smaller than that in st-MVs (Fig. 5). To further investigate the effect of PQS on MV production during the exponential phase, MV production of the  $\Delta pqsH$  mutant, which does not synthesize PQS, was examined. While MV production of the  $\Delta pqsH$  mutant was 1.94 ( $\pm 0.19$ )-fold decreased in the exponential phase (4.5-h culture) over that of PAO1 wild type (WT), it was 4.15 ( $\pm 1.35$ )-fold decreased in the stationary phase (12-h culture) over that of PAO1 WT, suggesting that the effect of PQS on MV production in the exponential phase is smaller than that in the stationary phase. Thus, it can be considered that dependence of MV production on PQS is low in the exponential phase.

**Zeta potential of MVs.** To consider the behavior of MVs, it is important to understand the zeta potential of MVs because it has been suggested that salt bridging by divalent cations between MVs and bacterial cells is important (12). Zeta potentials varied widely among MVs from different growth phases: MVs tended to be more negative as the growth switched to the stationary phase (Table 2). It was reported previously that the zeta potential of *P. aeruginosa* cells is as negative as  $-25$  mV at pH 7 (6). In our experiment, the zeta potential of *P. aeruginosa* cells was around  $-35$  mV and constant regardless of the growth phase (Table 2). These data indicate that MVs, which have different zeta potentials, were

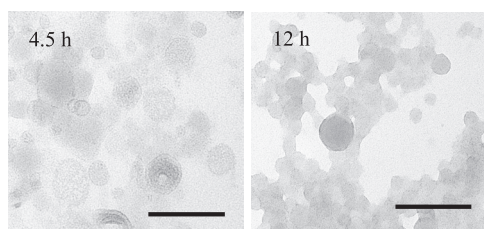


FIG. 4. Electron micrographs of exponential (4.5-h)- and stationary (12-h)-phase MVs. MVs were visualized by negative staining. Results are representative images. Bars, 100 nm.

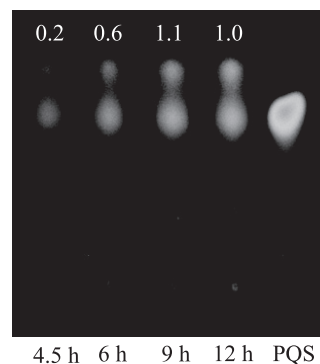


FIG. 5. Contents of PQS in MVs at 4.5, 6, 9, and 12 h of growth. PQS extracted from purified MVs (2.5  $\mu$ g protein) was subjected to TLC. Chemically synthesized PQS (25 nmol) was used as a reference (right). Inset numbers are spot intensities relative to that of 12-h growth.

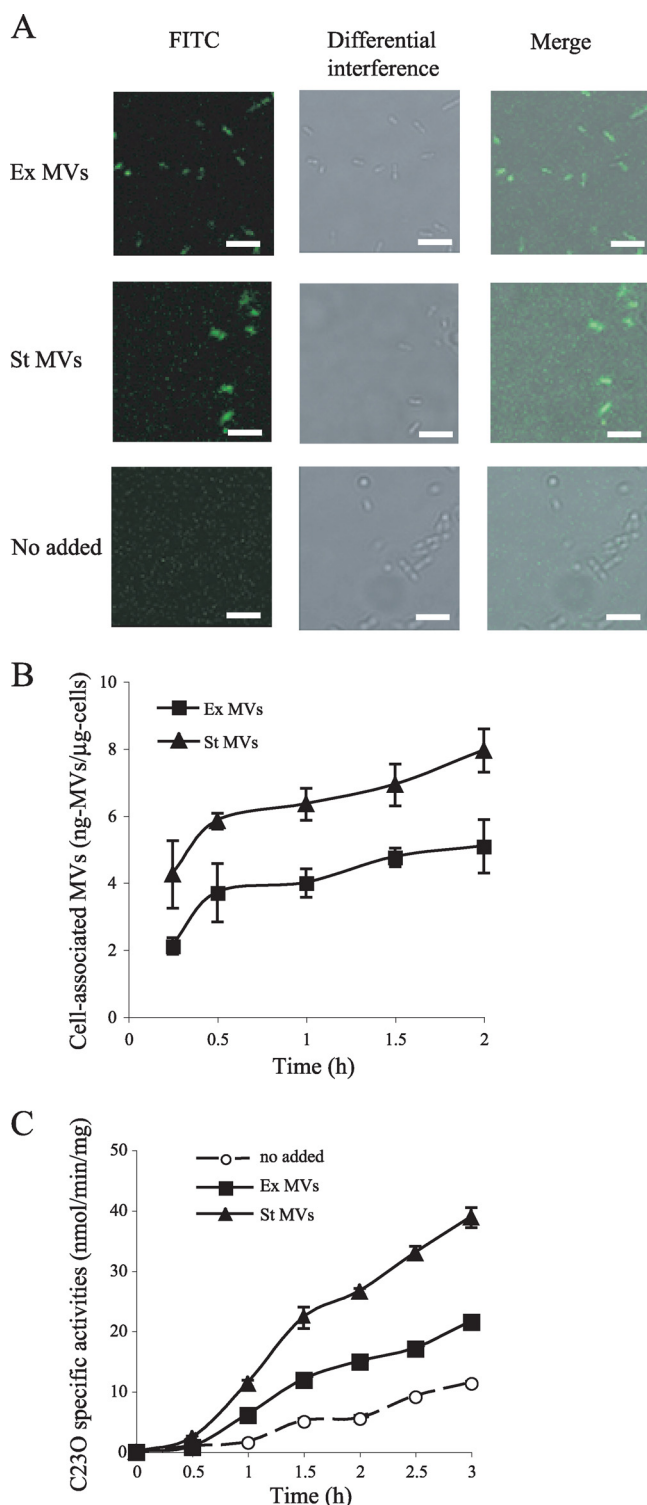


FIG. 6. Association of MVs with PAO1 cells. (A) Visualization of MV association with PAO1 cells. FITC-labeled ex-MVs and st-MVs were incubated with PAO1 cells for 1 h at 37°C. Cells were visualized by confocal microscopy. Experiments were repeated three times, and representative images are shown. Scale bars, 5  $\mu$ m. (B) Cell association activity of MVs. FITC-labeled ex-MVs and st-MVs were incubated with PAO1 cells for 15 min to 2 h at 37°C. Data are means  $\pm$  standard deviations from triplicates. Representative data from three independent experiments are shown. (C) Catechol-2,3-dioxygenase activity assays of *PpqsA* transcriptional level in a  $\Delta pqsH pqsE-xylE$  mutant in

released according to growth phase without altering cellular zeta potentials.

**Fusion of MVs to bacterial cells.** It has been reported elsewhere that *P. aeruginosa* MVs are fused to the Gram-negative bacterium *Escherichia coli* and attached to the Gram-positive bacterium *Staphylococcus aureus* (12). Since *P. aeruginosa* MVs are used as a carrier of interspecies communication (18), it can be considered that *P. aeruginosa* MVs fuse to their own cells. Purified ex-MVs and st-MVs were fluorescently labeled with FITC and incubated with *P. aeruginosa* PAO1 cells grown to the late exponential phase. When we incubated PAO1 cells with ex- or st-MVs at 37°C for 1 h, clear fluorescence was observed in both samples by microscopic observation (Fig. 6A). In contrast, no fluorescence was detected in cells when cells were not incubated with MVs. These results indicate that the FITC-labeled MVs were associated with PAO1 cells.

To test whether different potentials between ex- and st-MVs contribute to fusion activity with PAO1 cells, we quantified MVs associated with PAO1 cells. Cell-associated MV proteins were calculated from a standard curve relating fluorescence to ng of FITC MVs for each of the MV preparations. The results showed that st-MVs were 1.5- to 2-fold more highly associated with cells than were ex-MVs (Fig. 6B). When we tested temperature dependence of the MV-cell association, the amounts of st-MVs, which interact with cells, were not different between 37°C and 4°C (data not shown), suggesting that an enzymatic mechanism was not related to MV-bacterial cell association.

To further confirm the difference of MV-cell association activity between the ex- and st-MVs, the response of cells to PQS in MVs was examined. PQS acts as a ligand for PqsR, the transcriptional activator that controls the *pqsABCDE* operon (39). The gene products of *pqsABCD* catalyze the synthesis of 2-heptyl-4-quinolone (HHQ) from anthranilic acid, and the gene product of *pqsH* converts HHQ to PQS. The expression of *pqsABCDE* is autoregulated positively (7). To monitor the response of PQS, we used a  $\Delta pqsH pqsE-xylE$  strain, and C23O (*xylE* product) specific activities were measured. Figure 6C shows that st-MVs promoted the expression of the *pqsABCDE* operon more than did ex-MVs. This result reflects the data that st-MVs possess higher levels of PQS and interact more easily with *P. aeruginosa* cells than do ex-MVs.

## DISCUSSION

Although MVs are produced in both the exponential and stationary phases (Table 1), little attention has been given to changes in the properties of MVs within the growth phase. Under standard laboratory growth conditions, our data demonstrate that MVs released from the exponential phase and the stationary phase have different properties in terms of buoyant densities, surface charges, electron microscopic appearances, and PQS contents, whereas MVs retain the same hydrody-

the presence and absence of MVs. The cells were grown to an OD<sub>600</sub> of 1.0 in LB medium, MVs were added to cultures (final concentration, 5  $\mu$ g protein/ml), and C23O was examined for 30 min to 3 h. Data are means  $\pm$  standard deviations from triplicates. Representative data from three independent experiments are shown.

namic diameter. As far as we know, this is the first report to demonstrate a potential difference in MVs released during different growth phases.

Electron charge is an important factor in the interaction between MVs and bacterial cells. *P. aeruginosa* MVs have been reported to interact with both Gram-negative and Gram-positive bacteria (13). MVs are negatively charged because of LPS and small amounts of salt bridging by  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  between adjacent O side chains. For that reason, it has been proposed that MVs can be readily salt bridged to the  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -rich surface of bacterial cells. We showed that st-MVs were highly negatively charged (Table 2) and that they interact with bacterial cells more easily than do ex-MVs. Moreover, the st-MVs possessed high levels of PQS (Fig. 5) and induced the transcription of the *pqsABCDE* operon (Fig. 6C). Hence, such highly negatively charged MVs liberated during the stationary phase could be useful in cell-cell communication in *P. aeruginosa*.

The difference in the electron charges of MV surfaces and the PQS contents also provides a possible explanation for the difference in MV production mechanisms between the exponential phase and the stationary phase. Several studies have proposed that the electronegative charge of LPS causes charge-to-charge repulsion between adjacent molecules of LPS, which results in outer membrane blebbing in *P. aeruginosa* (13, 22, 26). Moreover, recent studies show that PQS interacts strongly with LPS, and ionic interactions between PQS and  $\text{Mg}^{2+}$  in the outer membrane enhance the anionic repulsion between neighboring LPS molecules, which enhances membrane blebbing (19, 20). In our study, st-MVs certainly contained PQS (Fig. 5), and their surfaces were highly negatively charged (Table 2), while PQS levels were low and the surface was more neutral in ex-MVs. It is possible that MVs are liberated by anionic repulsions that are dependent on PQS during the stationary phase but that another mechanism independent of PQS affects MV production during the exponential phase in *P. aeruginosa*. Our results also showed that st-MVs had higher densities and were less translucent than ex-MVs (Table 2 and Fig. 4). One possible explanation is that these differences would be derived from the increase of PQS concentration in MVs. We recently reported that misfolded OMPs would be an important factor to accelerate MV production and increase protein concentration in MVs (31). Since MV protein concentration was the same as that of phospholipid in the supernatant at the exponential phase, unlike in the case of the stationary phase (Table 1), it is possible that these proteins may be related to MV production during the exponential phase.

*P. aeruginosa* is a notorious pathogen that secretes many virulence exoproducts, most of which are produced when growth switches to the stationary phase. Cell density-dependent gene expression occurs in response to environmental signals, a process known as QS. Quinolones and phenazines are excreted from cells at specific times during growth, and their production is controlled by QS in *P. aeruginosa* (8, 9). The production of several secreted proteins such as azurin and proteases (e.g., elastase, PrlP, PasP, and PaAP) is regulated by QS (5, 23, 35). It has been suggested that secreted elastase is capable of degrading MV proteins (3). In this study, OMPs, including OprD, OprF, and OprH, were contained at lower

levels in st-MVs than in ex-MVs (Fig. 3). Since many proteases are secreted during the stationary phase, these OMPs may be more labile than other MV proteins to proteases. PaAP has been characterized as a zinc-dependent leucine aminopeptidase and is secreted via Xcp-mediated type II secretion and regulated by the Las QS system (21, 23). A recent study demonstrated that PaAP is abundant in MVs from clinical strains compared to those from PAO1, and it promotes the association of MVs with lung cells (2). In our experiment, PaAP was consistently a most abundant protein in PAO1 MVs (Fig. 5). Since PaAP expression depends upon QS expression and oxygen concentration (1, 23), this discrepancy may be related to different culture conditions. In any event, the MVs that include large amounts of PaAP seem to be capable of attacking host cells. However, it has been unknown if PaAP is related to association with bacterial cells. Since cell association activity was not altered between 37°C and 4°C in our results, the effect of physicochemical factors, but not enzymatic factors, may be mostly related to MV-cell association.

In conclusion, we demonstrated that properties of MVs were altered as growth switches to the stationary phase in *P. aeruginosa*. We have shown that MVs released at the stationary phase possess negatively charged surfaces and high activity of association with cells. These findings would be useful for understanding the mechanism of interaction between bacterial cells and MVs.

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