Outer Membrane Machinery and Alginate Synthesis Regulators Control Membrane Vesicle Production in *Pseudomonas aeruginosa*[▽]

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The opportunistic human bacterial pathogen *Pseudomonas aeruginosa* produces membrane vesicles (MVs) in its surrounding environment. Several features of the *P. aeruginosa* MV production mechanism are still unknown. We previously observed that depletion of Opr86, which has a role in outer membrane protein (OMP) assembly, resulted in hypervesiculation. In this study, we showed that the outer membrane machinery and alginate synthesis regulatory machinery are closely related to MV production in *P. aeruginosa*. Depletion of Opr86 resulted in increased expression of the periplasmic serine protease MucD, suggesting that the accumulation of misfolded OMPs in the periplasm is related to MV production. Indeed, the *mucD* mutant showed a mucoid phenotype and the *mucD* mutation caused increased MV production. Strains with the gene encoding alginate synthetic regulator AlgU, MucA, or MucB deleted also caused altered MV production. Overexpression of either MucD or AlgW serine proteases resulted in decreased MV production, suggesting that proteases localized in the periplasm repress MV production in *P. aeruginosa*. Deletion of *mucD* resulted in increased MV proteins, even in strains with mutations in the *Pseudomonas* quinolone signal (PQS), which serves as a positive regulator of MV production. This study suggests that misfolded OMPs may be important for MV production, in addition to PQS, and that these regulators act in independent pathways.

Pseudomonas aeruginosa is an opportunistic human pathogen, causing infections in cystic fibrosis (CF) and burn patients, as well as other immunocompromised individuals (12, 55). The biofilm-forming ability of *P. aeruginosa* and of other bacteria is thought to contribute to their ability to thrive in hostile host environments and cause chronic infection. The *P. aeruginosa* biofilm matrix is mainly composed of cells, extracellular proteins, DNA, and polysaccharides, including alginate, *pel* product, and *psl* product (13, 16, 50, 70, 75).

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), lipopolysaccharide (LPS), and phospholipids, and they encapsulate periplasmic components (5, 6). *P. aeruginosa* MVs contain some secreted virulence factors for killing host cells or other bacteria, including proelastase, hemolysin, phospholipase C, alkaline phosphatase, and Cif (25, 32), and antibacterial factors, such as murein hydrolases (23, 24, 26, 29, 30). Recent reports indicated that *P. aeruginosa* MVs deliver them directly into the host cell cytoplasm and contribute to the inflammatory response during infection (3, 4, 7). To control *P. aeruginosa* pathogenicity, it is important to know the mechanism of MV production in *P. aeruginosa*. Although MVs have been well studied in terms of

transmission of materials involved in pathogenicity, the production mechanism has not been elucidated completely.

On a relevant note, recent studies have indicated that *P. aeruginosa* MVs contain a quorum-sensing signaling molecule 2-heptyl-3-hydroxyl-4-quinolone (*Pseudomonas* quinolone signal [PQS]) (37), as well as an antimicrobial quinolone (20); these molecules serve as mediators of cell-cell communication (47). This signal serves as a coinducer for the transcriptional activator PqsR (MvfR), which positively regulates PQS production to create an autoregulatory loop that regulates the expression of multiple virulence factors in *P. aeruginosa* (14). PQS is derived from anthranilate through PqsABCD and finally synthesized by PqsH. In *P. aeruginosa*, MV production increases when adjacent lipid A molecules in the outer membrane exhibit charge repulsion (39). PQS interacts strongly with the acyl chains and 4'-phosphate of bacterial LPS and increases MV production (38).

We previously reported that Opr86 depletion causes tremendous blebbing in *P. aeruginosa* (63), but little is known about how Opr86 depletion causes increased MV production. Opr86 belongs to the Omp85/YaeT family, which is conserved in all gram-negative bacteria (51, 66). An OMP belonging to the Omp85/YaeT family was shown to be essential for viability, have a role in assembly or insertion of OMPs, and act as a protective antigen (15, 31, 44, 63, 66, 68, 76, 77). The mechanism of OMP assembly has been studied well in *Escherichia coli*. YaeT is a part of complex that contains four lipoproteins, YfgL, YfiO, NlpB, and SmpA, and plays a central role in assembly of β -barrel OMPs (33, 43, 56, 76).

Stress, such as an elevated temperature, is thought to lead to the accumulation of misfolded OMPs in the periplasm and cause envelope stress (1, 67). Recently, it has been shown that

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misfolded OMPs regulate σ^{22} activity and activate alginate synthesis in *P. aeruginosa* (48). The *P. aeruginosa* sigma factor σ^{22} is encoded by *algU* (*algT*) and is required for the production of alginate (17, 18). The *algU* operon consists of *algU-mucABCD*. MucA is an anti-sigma factor that sequesters σ^{22} (40, 53), and mutations in *mucA* are common in mucoid *P. aeruginosa* isolates from CF patients (35). The products of the other members of this operon, MucB (AlgN), MucC, and MucD, also appear to act in a negative manner along with MucA to inhibit AlgU activity (8, 9, 19, 34). MucD is a periplasmic serine protease and involved in alginate synthesis and virulence (8, 72, 78). AlgW also plays a role in regulating AlgU activity and alginate production (48). Sigma factor σ^{22} of *P. aeruginosa* has homology to the extracytoplasmic-function σ^E of *E. coli*. Recent studies reported that the σ^E pathway is associated with MV production in *E. coli* (11, 41, 42). However, the relationship between envelope stress response and MV production in *P. aeruginosa* is unknown.

Since misfolded OMPs regulate alginate synthesis in *P. aeruginosa* (48), it is believed that misfolded OMPs are not only unnecessary compounds but also intracellular signals to activate cellular phenotypes. In this study, we investigated the effects of OMP machinery-associated factors and alginate synthesis regulators on MV production in *P. aeruginosa*. Moreover, we analyzed a relationship between misfolded OMPs and PQS, which both regulate MV production, and suggest a model of MV production in *P. aeruginosa*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids that were used in this study are listed in Table 1. *E. coli* and *P. aeruginosa* were grown in Luria-Bertani (LB Lennox; Nacalai, Kyoto, Japan) medium. When necessary, the following antibiotics were used: 100 μ g/ml ampicillin and 10 μ g/ml gentamicin for *E. coli* and 200 μ g/ml carbenicillin and 100 μ g/ml gentamicin for *P. aeruginosa*.

Construction of mutants. The construction of several mutants of *P. aeruginosa* PAO1 was achieved by conjugation and homologous recombination. Plasmid pG19mucD, which carried deletion cassettes of *mucD*, was constructed as follows. The DNA fragment was amplified from the PAO1 chromosome with primers mucD1/mucD2 and mucD3/mucD4 (listed in Table 1). These flanking DNA fragments were joined using overlap extension PCR. The resulting PCR fragment was digested with BamHI/HindIII and ligated into a multicloning site of pG19II to yield pG19mucD. Plasmids pG19algW, pG19mucA, and pG19mucB were constructed in the same way with restriction enzymes corresponding to their respective primers. Plasmid pG19algU carrying the deletion cassette of *algU* was constructed by the procedure described previously (36). The DNA fragment was amplified from the PAO1 chromosome with primers algUF1/algUR1, digested with HindIII/EcoRI, and ligated into a multicloning site of cloning vector pHSG398. The DNA flanking fragment of *algU* on pHSG398 was amplified using inverse PCR with primers algUF2/algUR2, digested with BglII, and self-ligated. The *algU* flanking fragment was digested with HindIII/EcoRI and ligated into a multicloning site of pG19II to yield pG19algU. The plasmid pG19 series (Table 1) were introduced into the mobilizer *E. coli* strain S17-1 and then were transferred into *P. aeruginosa* PAO1 by conjugation as reported earlier (36, 63). The deletion of the chromosomal gene was confirmed by PCR analysis.

Construction of plasmids for overexpression. The *mucD* and *algW* genes were amplified with primers mucDF/mucDR or algWF/algWR, digested with EcoRI/BamHI, and cloned in pUCP24 under the *lac* promoter to yield plasmids pmucD and palgW. The amplified *mucD* gene with primers mucDF/mucDR2 was digested with EcoRI and cloned into plasmid palgW between the *lac* promoter and the *algW* to yield plasmid pmucDalgW. These plasmids were electroporated in the wild-type (WT) strain PAO1 or in the Δ *mucD*, Δ *algW*, or Δ *algU* mutant.

Isolation and purification of MVs. MVs were isolated using a modified version of the procedure reported by Kadurugamuwa and Beveridge (25). *P. aeruginosa* was grown in LB medium at 37°C for 12 h with shaking at 150 rpm. Cells were removed by centrifugation (6,000 $\times$ g, 15 min), and supernatants were filtered

first through a 0.45- μ m cellulose acetate filter and then a 0.22- μ m cellulose acetate filter. Filtrates were ultracentrifuged (150,000 $\times$ g, 3 h, 4°C) in a Beckman type 45 rotor, and pellets were washed with 10 mM HEPES (pH 6.8) containing 0.85% NaCl (HEPES-NaCl). For MV purification, the method was modified from the procedure reported by Horstman and Kuehn (22). The crude MV sample was adjusted to 45% Optiprep in HEPES-NaCl with EDTA-free protease inhibitor (Roche, Mannheim, Germany) per the manufacturer's instructions. An Optiprep gradient was layered over the 0.5-ml crude MV sample with 0.5 ml Optiprep in HEPES-NaCl (40%, 35%, 30%, 25%, 20%, 15%, and 10%) with protease inhibitor. Gradients were centrifuged at 100,000 $\times$ g for 3 h in a Beckman SW41Ti rotor, and the MV fraction was extracted and washed with HEPES-NaCl.

MV mass measurement. The quantity of MVs was determined by protein assay and phospholipid assay. In the protein assay, extracted MVs were homogenized, and the protein concentration was measured by the Bradford method (10). Bovine serum albumin was used as a reference standard. Since the proteolytic activities of the supernatants were not significantly different among the WT strain and the Δ *mucD*, Δ *algW*, Δ *algU*, Δ *mucA*, and Δ *mucB* mutants (data not shown), we considered that extracellular proteases did not affect the difference of MV production. Proteolytic activity was determined by an azocasein assay as previously reported (27) with some modifications. Briefly, 10 μ 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 CaCl₂ and incubated at 37°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 (A_{400}) in the supernatant was measured. The MV phospholipid concentration was measured using a modified version of the procedure reported by Stewart et al. (60). Briefly, extracted MVs were homogenized, and 10 μ l of the MV sample, 100 μ l of ammonium ferri-thiocyanate solution (27.03 g/liter ferric chloride hexahydrate and 30.4 g/liter ammonium thiocyanate), and 100 μ l of chloroform were vortexed. The absorption of the lower layer was measured at 488 nm. L- α -Phosphatidylethanolamine was used as a reference standard. MV production was measured by the optical density at 600 nm (OD_{600}) and compared with an indicated control strain to determine relative MV production.

Quantification of C23O activities. To examine the transcriptional activities of the *algU* promoter and *mucD* promoter, these DNA fragments were amplified from the PAO1 chromosome with primers PalgUF/PalgUR or mucD1/PmucDR, digested with BamHI/HindIII or EcoRI/HindIII, and ligated into the pMEX9 plasmid upstream of *xylE* to yield plasmids pMEXalgU and pMEXmucD, respectively. These plasmids were introduced into the WT strain PAO1 or the Δ *algW*, Δ *algU*, Δ *mucA*, or Δ *mucB* mutants. To examine the transcriptional activity of *pqsH*, the *xylE* gene cassette was inserted downstream of the chromosomal *pqsH* gene as follows. Two 0.8-kb DNA fragments were amplified from PAO1 chromosomal DNA with primers pqsHX1/pqsHX2 or with primers pqsHX3/pqsHX4, digested with EcoRI/XbaI or XbaI/HindIII, respectively, and ligated into the EcoRI-HindIII site of plasmid pG19II. The *xylE* fragment, digested from pX1918 with XbaI, was then inserted into the XbaI site of the constructed plasmid to yield pG19pqsHX in which *xylE* lies downstream of *pqsH*. This plasmid was introduced into the mobilizer strain *E. coli* S17-1 and transferred into strain PAO1 (WT) and Δ *mucD* mutant using conjugation as reported earlier (36). An assay for activity of catechol 2,3-dioxygenase (C23O) (the *xylE* product) was performed by the method of Maseda et al. (36). Cells cultured for 6 h 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 ($\epsilon^{1\text{ cm}} = 4.4 \times 10^4$).

Quantitative real-time reverse transcriptase PCR (RT-PCR). The cells were grown to an OD_{600} of 0.4 in LB medium. The cells were then harvested by centrifugation, the supernatants were removed, and cell pellets were frozen in an ice-ethanol bath and stored at -80°C until RNA extractions were performed. Total RNA was extracted from approximately 10^8 cells by using the RNeasy minikit (Qiagen, Valencia, CA) as described by the manufacturer. Qiagen on-column DNase was used according to the manufacturer's instructions. Additional DNase treatment was performed using Qiagen DNase I, and RNA was purified using the RNeasy kit again. The cDNA synthesis was performed from 1 μ g RNA using RT SuperScript III (Invitrogen, Carlsbad, CA) with random hexamers. The cDNA was used as a template for PCRs completed with the primers shown in Table 1. Real-time PCR was conducted using LightCycler (Roche) with the LightCycler FastStart DNA Master^{PLUS} SYBR green I kit. The *rplU* gene was used as the internal control, and data were normalized to *rplU* expression.

SDS-PAGE and Western blotting. For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), samples were denatured at 98°C for 10 min using sample buffer containing 50 mM Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 6% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and 0.02% bromophenol

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description, relevant characteristic(s), relevant genotype, or sequence ^a	Reference or source
Bacterial strains		
<i>P. aeruginosa</i> strains		
PAO1	Wild type	21
$\Delta opr86$ mutant	PAO1 $\Delta opr86$ mutant carrying pMMB6786	This study
$\Delta mucD$ mutant	PAO1 $\Delta mucD$ mutant	This study
$\Delta mucD \Delta opr86$ mutant	PAO1 $\Delta mucD \Delta opr86$ double mutant carrying pMMB6786	This study
$\Delta algW$ mutant	PAO1 $\Delta algW$ mutant	This study
$\Delta algU$ mutant	PAO1 $\Delta algU$ mutant	This study
$\Delta mucA$ mutant	PAO1 $\Delta mucA$ mutant	This study
$\Delta mucB$ mutant	PAO1 $\Delta mucB$ mutant	This study
$\Delta pqsH$ mutant	PAO1 $\Delta pqsH$ mutant	65
$\Delta pqsR$ mutant	PAO1 $\Delta pqsR$ mutant	65
$\Delta pqsH \Delta mucD$ mutant	PAO1 $\Delta pqsH \Delta mucD$ double mutant	This study
$\Delta pqsR \Delta mucD$ mutant	PAO1 $\Delta pqsR \Delta mucD$ double mutant	This study
<i>pqsH-xylE</i> strain	PAO1 carrying a chromosomal <i>pqsH-xylE</i> fusion	This study
$\Delta mucD pqsH-xylE$ mutant	$\Delta mucD$ mutant carrying a chromosomal <i>pqsH-xylE</i> fusion	This study
<i>E. coli</i> strains		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi</i> $\Delta(lac-proAB)$ F' (<i>traD36 proAB⁺ lacI^q lacZ</i> Δ M15)	TaKaRa
S17-1	<i>pro thi hsdR recA</i> Tp ^r Sm ^r ; chromosome::RP4-2 Tc::Mu-Km::Tn7	54
Plasmids		
pHSG398	Cloning vector; Cm ^r	TaKaRa
pG19II	Derivative of pK19 <i>mob sacB</i> ; Gm ^r	36
pG19-delopr86	<i>opr86</i> deletion cassette in pG19II	63
pG19mucD	<i>mucD</i> deletion cassette in pG19II	This study
pG19algW	<i>algW</i> deletion cassette in pG19II	This study
pG19algU	<i>algU</i> deletion cassette in pG19II	This study
pG19mucA	<i>mucA</i> deletion cassette in pG19II	This study
pG19mucB	<i>mucB</i> deletion cassette in pG19II	This study
pG19pqsHX	<i>pqsH-xylE</i> cassette in pG19II	This study
pMMB6786	pMMB67EH derivative carrying the <i>opr86</i> gene; Amp ^r	63
pUCP24	<i>P. aeruginosa</i> expression vector	69
popr86	<i>opr86</i> on pUCP24	This study
pmucD	<i>mucD</i> on pUCP24	This study
palgW	<i>algW</i> on pUCP24	This study
pmucDalgW	<i>mucD-algW</i> on pUCP24	This study
pMEX9	pME4510-derived promoter-probe vector; <i>xylE</i> Gm ^r	64
pMEXalgU	<i>algU</i> promoter region in pMEX9	This study
pMEXmucD	<i>mucD</i> promoter region in pMEX9	This study
Primers		
mucD1	5'-CGGGATCCGCGCTCGGAATGGCTGCCG-3'	This study
mucD2	5'-GGCCAGCTCAGCTCCCGTGATGTTTCGAGGG-3'	This study
mucD3	5'-GGGAGCTGAGCTGGCCGAATAAGCCGGCTG-3'	This study
mucD4	5'-CCCAAGCTTCGTACCAGCGAGACCACGCC-3'	This study
algW1	5'-CGGGATCCCCCGTAGCGCTCGGTGGC-3'	This study
algW2	5'-GACGGGGTCCTTGGGGTTGCGAGGCCG-3'	This study
algW3	5'-CCCCAAGGACCCCGTCATTGAAAACGGCGC-3'	This study
algW4	5'-CCCAAGCTTCACACGACCTGCCGCTGC-3'	This study
algUF1	5'-CCGAAGCTTGGAAGGTATCGACTTCGCCGC-3'	This study
algUR1	5'-CGGAATTCAGGGATCGAACTTGCGCACC-3'	This study
algUF2	5'-GCGGATCCACAGCGCAATGCCAAGAGAGG-3'	This study
algUR2	5'-CGGGATCCGAAAGCTCCTCTTCGAACCTGG-3'	This study
mucA1	5'-CGGATCCGATCGGTTCCTCCAAAGCAGG-3'	This study
mucA2	5'-CTGCGGCGCCCTGCTCTTCGCTGTAGC-3'	This study
mucA3	5'-AGAGCAGGGGCGCCGAGGTGATCACC-3'	This study
mucA4	5'-GCTCTAGACCTGCTCCTCGATCATTTCTGG-3'	This study
mucB1	5'-CGGGATCCCTTCATCAAGGCATACCGTGC-3'	This study
mucB2	5'-CGCTCGAGGTCTCTCCTCAGCGTTTCC-3'	This study
mucB3	5'-GGAGAGACCTCGAGCGCTTCCAGTTCACC-3'	This study
mucB4	5'-GCTCTAGAGGTCGGCAGGTCTTCGCCCTCG-3'	This study
PalgUF	5'-GGAATTCGCGCCGATTTCCCGTGGTGG-3'	This study
PalgUR	5'-CCCAAGCTTGTCTCTCTTCGAACCTGGAGG-3'	This study

Continued on following page

TABLE 1—Continued

Strain, plasmid, or primer	Description, relevant characteristic(s), relevant genotype, or sequence ^a	Reference or source
PmucDR	5'-GGAATTCGCCAGCCGGCTTATTCGGCC-3'	This study
mucDF	5'-GGAATTCGGGAGCTGTAGTCGATGCATACCC-3'	This study
mucDR	5'-CGGGATCCGCCAGCCGGCTTATTCGGCC-3'	This study
mucDR2	5'-GGAATTCGCCAGCCGGCTTATTCGGCC-3'	This study
algWF	5'-GGAATTC AAGGATTTCCCGATGCCCAAGGCC-3'	This study
algWR	5'-CGGGATCCGGATCACTCGCCGCCGTCC-3'	This study
pqsHX1	5'-GGAATTCCTGGATGAGTCGCGCATTGCGG-3'	This study
pqsHX2	5'-GCTCTAGACTACTGTGCGGCCATCTCACCG-3'	This study
pqsHX3	5'-GCTCTAGATGCCAGTGGCGTCTTGGTCGCC-3'	This study
pqsHX4	5'-CCCAAGCTTGATCTCGCCCTCGGACAGCG-3'	This study
mucD-RTF	5'-ACGCTGTATGGCTGCGATGG-3'	This study
mucD-RTR	5'-ATGCTGCGCTCGAGGAAGTC-3'	This study
algW-RTF	5'-CTGATCATCCAGCACCAACCC-3'	This study
algW-RTR	5'-TGTACAGGTTGGCCACTGCC-3'	This study
rplU-RTF	5'-CGCAGTCATTGTTACCGGTG-3'	This study
rplU-RTR	5'-AGGCCTGAATGCCGGTGATC-3'	This study

^a Drug resistance phenotype designations: Tp^r, trimethoprim resistance; Sm^r, streptomycin resistance; Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Amp^r, ampicillin resistance. The added restriction sites in the primers are underlined.

blue. The samples were resolved using 15% SDS-PAGE, fixed in 50% methanol and 10% acetic acid, and visualized by Coomassie brilliant blue staining or silver staining (Daiichikagaku, Tokyo, Japan). Western blot analysis with p-Opr86 antiserum (1/1,000 dilution) was performed as described previously (63). The intensities of protein bands were evaluated using densitometric scanning (Image Master ID Elite; GE Healthcare, Buckinghamshire, United Kingdom).

Bacterial viability assay. The viability of *P. aeruginosa* in planktonic shaking culture was determined by using BacLight live/dead bacterial viability staining kit (Molecular Probes Inc., Eugene, OR) as described previously (63).

TEM. Purified vesicles 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 transmission electron microscope (TEM) (JEOL, Tokyo, Japan) at 80 kV.

PQS detection assay. PQS was extracted from 1 ml of cell-free supernatant, ultracentrifuged supernatant (without MVs) or extracted MVs with acidified ethyl acetate, and separated by thin-layer chromatography (TLC) as described previously (65). The intensities of PQS spots were evaluated using densitometric scanning (Image Master ID Elite; GE Healthcare).

RESULTS

Overexpression of Opr86 decreases MV production. To quantify MVs in the supernatant of *P. aeruginosa* PAO1, cell-free supernatant from overnight culture was ultracentrifuged, and the pellets containing MVs were extracted. To evaluate MV production, the protein concentration in the ultracentrifuged pellets was measured (38, 45). However, it has also been reported that the ultracentrifuged pellets contain other components, such as flagella and pili (4). We fractionated the crude pellets by previously reported methods using an Optiprep density gradient (22). The pellets from *P. aeruginosa* PAO1 supernatant localized to a main band at a medium location (buoyant density, 1.15 g/ml) and a minor band at a heavier layer (buoyant density, 1.20 g/ml). The concentrations of proteins and phospholipids in each fraction were also examined; over 90% of proteins and over 99% of phospholipids were contained in a major band (data not shown). The existence of MVs in the peak fractions could be confirmed by electron microscopy (data not shown). Hence, we concluded that the protein assay of ultracentrifuged pellets roughly represented MV quantities and that the phospholipid assay was more accurate.

In our previous study, it was observed by scanning electron microscopy that the Opr86 deletion mutant releases many

MVs (63). However, it is difficult to quantify accurately how many MVs the Opr86 deletion mutant produces because the growth of Opr86 deletion mutant is remarkably repressed in the absence of isopropyl-β-D-thiogalactopyranoside (IPTG). To confirm the relationship between Opr86 and MV production, we first examined the quantities of MVs in the supernatants of the control strain [PAO1(pUCP24)] and the *opr86* overexpression strain [PAO1(popr86)] after 12 h of incubation in LB medium. Growth curve analysis confirmed that *P. aeruginosa* PAO1 growth was unaffected by the overexpression of

FIG. 1. Overexpression of Opr86 decreases MV production. (A) Western blot analysis using Opr86 antibody. Total proteins were extracted from cells of PAO1 carrying plasmid pUCP24 and PAO1 carrying plasmid popr86 at the exponential phase, and 20 μg of protein was separated by SDS-PAGE. The numbers below the Opr86 bands on the Western blot showed the relative densitometry values compared to the WT strain PAO1(pUCP24). (B) MV production of PAO1 (pUCP24) and PAO1(popr86) after 12 h of growth. The values are compared to the values for PAO1(pUCP24). Data are expressed as means ± standard deviations (error bars) from three independent experiments.

FIG. 2. Relationship between Opr86 and periplasmic protease MucD. (A) Quantitative real-time RT-PCR analysis of *mucD* transcript levels at the exponential phase in *P. aeruginosa* PAO1 and in the $\Delta opr86$ mutant treated with 100 μ M IPTG (+IPTG) or not treated with IPTG (-IPTG). The values are compared to the values for the WT strain (PAO1). Data are expressed as means $\pm$ standard deviations (error bars) from triplicate samples. Similar results were obtained from three independent experiments. (B) Growth curves of LB cultures in the absence and presence of 100 μ M IPTG. After 4 and 8 h of incubation, cultures were diluted to an OD₆₀₀ of 0.01. Data shown are representative of three independent experiments.

Opr86 (data not shown), and immunoblotting analysis confirmed that Opr86 in PAO1(popr86) was overexpressed compared to PAO1(pUCP24) (Fig. 1A). MV production in the Opr86 overexpression strain was less than that of the parent strain: in particular, the protein concentration was decreased in MVs released from the strain expressing Opr86 (Fig. 1B). TLC analysis indicated that PQS quantities in the supernatants are not different for these two strains (data not shown). These data suggest that Opr86 negatively regulates MV production and that this regulation is independent of the PQS pathway.

MucD expression is important under Opr86 depletion conditions. Since Opr86 plays a role in OMP assembly (63) and overexpression of Opr86 resulted in decreased MV production (Fig. 1B), it was hypothesized that accumulation of misfolded OMPs triggers MV production. To elucidate the relationship between Opr86 depletion and the accumulation of misfolded OMPs in the periplasm, we focused on MucD, which is a serine protease that recognizes misfolded OMPs and localizes to the periplasm (8, 72, 73). Quantitative real-time RT-PCR analysis

showed that Opr86 depletion increases *mucD* transcription by about 10-fold (Fig. 2A), suggesting that misfolded OMPs accumulate in the periplasm under Opr86 depletion.

To further examine the effects of Opr86 depletion on accumulation of OMPs in the periplasm, the growth curves were analyzed under Opr86 or MucD depletion. Under Opr86 depletion conditions, the absence of MucD accelerated the cessation of growth compared with when MucD was normally expressed (Fig. 2B). In addition, the cells at the 8-h growth point shown in Fig. 2B were stained with SYTO9 and propidium iodide. The viable cell rates were as follows: 100% ($\pm < 0.1\%$) for the WT strain (PAO1), 97% ($\pm 1\%$) for the $\Delta opr86$ mutant in the presence of IPTG, 68% ($\pm 11\%$) for the $\Delta opr86$ mutant in the absence of IPTG, 98% ($\pm 1\%$) for the $\Delta mucD$ mutant, 88% ($\pm 2\%$) for the $\Delta mucD \Delta opr86$ mutant in the presence of IPTG, and 48% ($\pm 8\%$) for the $\Delta mucD \Delta opr86$ mutant in the absence of IPTG. These results suggest that the activity of MucD against misfolded OMPs is important for viability under Opr86 depletion conditions.

MucD depletion increases MV production. It is known that MucD and AlgW are serine proteases localized in the periplasm in *P. aeruginosa* (8). Here, we examined whether MucD and AlgW are related to MV production. We constructed $\Delta mucD$ and $\Delta algW$ mutants. While the $\Delta mucD$ mutant, which showed a mucoid phenotype (data not shown), displayed increased MV production compared to the WT strain (PAO1), about fourfold-higher protein level and about twofold-higher phospholipid level, MV production in the $\Delta algW$ mutant was barely different from that in the WT (Fig. 3A). Quantitative real-time RT-PCR analysis indicated that *mucD* expression is 7.04-fold (± 0.78 -fold) greater than *algW* expression during the exponential phase in the WT. We concluded that MucD plays the main role of degrading misfolded OMPs, and for that reason, deletion of AlgW did not affect MV production.

It is well-known that deletion of MucD leads to the activation of σ^{22} (8, 48). To examine whether inactivation of σ^{22} leads to decreased MV production, we constructed a $\Delta algU$ mutant and investigated MV production in the $\Delta algU$ mutant. In contrast to our expectation, the $\Delta algU$ mutant showed an approximately twofold increase in MV production (Fig. 3A), suggesting that the effect of MucD on the increase of MV production is not due to σ^{22} activation but that σ^{22} is related to MV production. It is also known that AlgU negatively controls flagellum synthesis in *P. aeruginosa* (18, 62). When crude ultracentrifuged pellets from supernatants of $\Delta mucD$, $\Delta algW$, and $\Delta algU$ mutants were subjected to Optiprep density gradient centrifugation and protein concentrations were examined, over 90% of proteins were found in MVs in all cultures as well as the WT (data not shown). These data indicate that MucD and AlgU negatively control MV production.

To further examine the effects of alginate synthetic regulators, we tested MV production in $\Delta mucA$ and $\Delta mucB$ mutants. The $\Delta mucA$ mutant showed a hypermucoid phenotype, and the $\Delta mucB$ mutant showed a mucoid phenotype compared to the WT (data not shown). In the results from the quantitative MV assay, $\Delta mucA$ and $\Delta mucB$ mutants showed decreased MV production (Fig. 3A). When extracted MVs from the WT were added to the MV-free supernatant of the WT or $\Delta mucA$ mutant and the MV-resuspended solutions were ultracentrifuged to examine the effect of alginate on precipitation, the protein

FIG. 3. Effects of chromosomal *mucD* deletion on MV production. (A) MV production of the WT strain (PAO1) and $\Delta mucD$, $\Delta algW$, $\Delta algU$, $\Delta mucA$, and $\Delta mucB$ mutant strains after 12 h of culture. The values are compared to the values for the WT strain. Data are expressed as means $\pm$ standard deviations (error bars) from three independent experiments. (B) Electron micrographs of negatively stained MVs purified from supernatants from the WT and $\Delta mucD$ strains. Bar, 100 nm. (C) SDS-PAGE protein profiles of MVs purified from supernatants from the WT and the $\Delta mucD$ mutant. One microgram of protein was separated by SDS-PAGE and stained with silver. The positions of molecular mass markers (in kilodaltons) are shown to the left of the gel.

and phospholipid contents of the pellets were not different in these samples (data not shown), suggesting that alginate in the supernatant of the $\Delta mucA$ mutant does not affect the precipitation of MVs. These results support the idea that alginate synthesis regulators are linked to MV production.

We presumed that alginate synthesis regulators affect *mucD* expression and hence lead to altered MV production. To verify this presumption, we examined *mucD* expression in the WT strain (PAO1) and in $\Delta algW$, $\Delta algU$, $\Delta mucA$, and $\Delta mucB$ mutant strains by quantitative real-time RT-PCR. Expression of

FIG. 4. *MucD* expression in the WT strain and in $\Delta algW$, $\Delta algU$, $\Delta mucA$, and $\Delta mucB$ mutant strains. (A) Quantitative real-time RT-PCR analysis of *mucD* transcript levels in cells at the exponential phase. The values are compared to those for the WT. Data are expressed as means $\pm$ standard deviations (error bars) from triplicate samples. Similar results were obtained from three independent experiments. (B) Organization of the *algU-mucABCD* gene cluster. The fragments inserted upstream of the *xyIE* gene in plasmids pMEXalgU and pMEXmucD are shown. (C and D) Catechol 2,3-dioxygenase (C23O) activity assays of *PalgU* (C) and *PmucD* (D) transcript levels. C23O activity in the cells in which plasmid pMEXalgU or pMEXmucD was introduced were measured and normalized against the C23O activity of the cells in which pMEX9 was introduced. Data are expressed as means $\pm$ standard deviations (error bars) from three independent experiments.

FIG. 5. Effects of *mucD* and/or *algW* multicopies on MV production. Values are compared to the values for PAO1 carrying plasmid pUCP24. Data are expressed as means $\pm$ standard deviations (error bars) from three independent experiments. Δ *mucD* pmucD, Δ *mucD* mutant carrying plasmid pmucD.

mucD was almost inversely related to MV production; in particular, the deletion of *algU* decreased *mucD* expression, and deletion of *mucA* resulted in increased *mucD* expression (Fig. 4A). It is known that *mucD* transcription is activated by the *algU* promoter (*PalgU*) and another independent *mucD* promoter (*PmucD*) (73). Each promoter was inserted into the front of *xylE* in reporter plasmid pMEX9 (Fig. 4B), and the effects of alginate synthesis regulators on those promoter activities were analyzed. The activity of *PalgU* was much higher than that of *PmucD* in the WT (Fig. 4C and D), suggesting that *mucD* expression for the most part is affected by *PalgU* activity. The results of *PalgU* transcription were similar to the result from quantitative real-time RT-PCR (Fig. 4A and C). Deletion of *algU* resulted in decreased *PalgU* expression (Fig. 4C), leading to positive autoregulation by AlgU. In contrast, deletion of *mucA* resulted in increased *PalgU* expression (Fig. 4C). This effect was suggested by the previous report that MucA binds to AlgU and inhibits it from acting as a positive transcriptional regulator (40, 53). In the same way, depletion of MucB, which is also a negative regulator of AlgU, led to increased *PalgU* expression (Fig. 4C). These results suggest that some alginate synthesis regulators control MV production by altering *mucD* expression.

To examine the effects of MucD on MV production in detail, MVs were purified from supernatants of WT or Δ *mucD* cultures. The TEM observation analysis showed that MVs isolated from the Δ *mucD* culture were not altered in terms of size and configuration compared to those from the WT culture (Fig. 3B). However, analysis of SDS-PAGE protein profiles indicated that MVs released from the Δ *mucD* mutant contained many kinds of bands that are not detected in the MVs released from the WT (Fig. 3C), and it is believed that these bands are misfolded OMPs. As presumed before, this result supports the idea that misfolded OMPs regulate MV production.

FIG. 6. Effect of MucD on MV production in PQS-defective strains. Values are compared to the values for the WT. Data are expressed as means $\pm$ standard deviations (error bars) from three independent experiments.

Overexpression of MucD or AlgW represses MV production.

To further analyze the effects of serine proteases localized in the periplasm, we tested MV production during overexpression of MucD or AlgW. PAO1 carrying plasmid pmucD and PAO1 carrying plasmid palgW showed decreased MV production compared to PAO1(pUCP24) (Fig. 5). PAO1(pmucDalgW), in which both MucD and AlgW are overexpressed, also displayed decreased MV production (Fig. 5). We also tested the effects of overexpression of MucD and AlgW on MV production in Δ *mucD* and Δ *algW* mutants. While the Δ *mucD* mutant carrying plasmid pmucD and the Δ *algW* mutant carrying plasmid palgW showed decreased MV production at the same level of PAO1(pmucD) or PAO1(palgW), the Δ *mucD* mutant carrying palgW and Δ *algW* mutant carrying pmucD did not repress MV production to the same degree (Fig. 5). These results suggested that overexpression of periplasmic proteases represses MV production, but the selectivity of OMP degradation in MucD and AlgW may not be completely identical.

We observed that the deletion of AlgU resulted in increased MV production (Fig. 3A) and considered the idea that the effect of AlgU on MV production was due to upregulation of MucD expression (Fig. 4). To further verify this notion, we tested the effect of overexpression of MucD on MV production in a Δ *algU* mutant. The result showed that the Δ *algU* mutant carrying plasmid pmucD partially complemented production compared to PAO1(pUCP24) (Fig. 5), suggesting that AlgU downregulation of MV production is related to MucD proteolytic activity but does not completely depend on it. In contrast, the Δ *algU* mutant carrying plasmid palgW showed decreased MV production as well as PAO1 (palgW) (Fig. 5). These results suggest that overexpression of periplasmic proteases represses MV production, even in the σ^{22} -defective strain.

Effect of MucD on MV production in PQS-defective strains.

It has been reported that PQS is a positive regulator of MV production in *P. aeruginosa* (37). In addition, the results of this study suggested that periplasmic proteases repress MV production in *P. aeruginosa* (Fig. 3A and 5). To examine whether the effect of periplasmic proteases on MV production depends on the PQS pathway, we constructed Δ *pqsH* Δ *mucD* and Δ *pqsR* Δ *mucD* double mutants, which both accumulate misfolded OMPs in the periplasm and do not produce PQS, and

FIG. 7. Effects of MucD on PQS synthesis and localization. (A) C23O activity assays of *pqsH* transcript levels. Data are expressed as means $\pm$ standard deviations (error bars) from three independent samples. (B to D) Detection of PQS by TLC and UV light. Spot intensity is shown in arbitrary units. PQS/OD₆₀₀ values are shown compared to the values for the WT strain carrying plasmid pUCP24. PAO1 carrying plasmid pUCP24 or plasmid pmucD and the Δ *mucD* mutant carrying plasmid pUCP24 (Δ *mucD* pUCP24) or plasmid pmucD (Δ *mucD* pmucD) are shown. (B) PQS in supernatants including MVs [Sup. (+MV)]. The rightmost lane shows synthesized PQS (25 nmol). (C) PQS in MVs. (D) PQS in supernatants without MVs [Sup. (-MV)].

tested MV production. The levels of MVs in Δ *pqsH* and Δ *pqsR* mutants were decreased at both the protein level and phospholipid level (Fig. 6), and these results are consistent with a previous report by Mashburn and Whiteley (37). Deletion of *mucD* in these mutants showed the same level of MV phospholipid quantities as in Δ *pqsH* and Δ *pqsR* mutants but showed high MV protein quantities, suggesting that MucD is related to MV proteins. Moreover, since MucD also affects MV production in a PQS-defective strain, it is thought that these two processes are in independent pathways.

Alteration of MucD expression changes PQS localization. It has been reported that MVs act as PQS carriers in the *P. aeruginosa* supernatants (37). We asked whether change in MV production by MucD regulation affect PQS synthesis. To test this, the *pqsH*-xylE allele was introduced into the WT strain (PAO1) or the Δ *mucD* mutant strain, and the effects of MucD on *pqsH* transcription were examined. The level of *pqsH* transcription was not different even when MucD was depleted or overexpressed (Fig. 7A). TLC analysis also indicated that the PQS quantities in the supernatants are not different in these strains (Fig. 7B). However, the localization of PQS was different when the expression level of MucD was altered. The amount of PQS contained in MVs was elevated in the Δ *mucD* mutant carrying plasmid pUCP24 than in PAO1(pUCP24), while the amount of PQS in MVs was decreased in the WT strain PAO1(pmucD) and the Δ *mucD* mutant carrying plasmid pmucD (Fig. 7C). In contrast, the PQS amount in the supernatant was high in the strains expressing a high level of MucD (Fig. 7D). These results indicated that MucD regulation of MV production affects PQS localization without changing the quantity of PQS in the supernatant.

DISCUSSION

Analysis of Opr86 and MucD provides a useful tool for understanding the effects of misfolded OMPs on phenotypic changes in *P. aeruginosa*. Since the Omp85/YaeT family, which includes Opr86, is essential for viability and has been difficult

to analyze, there has been little study of the relationship between the factor and a physiological phenotype. In *E. coli*, lipoproteins such as YfgL, YfiO, NlpB, and SmpA exist in a heterooligomeric complex with YaeT and coordinate the overall OM assembly (57, 75). While cells lacking YfgL, NlpB, or SmpA are viable and exhibit only minor OMP assembly defects, YfiO and YaeT are essential, and depletion of these proteins in cells results in severe OMP targeting defects. Previously, we found that depletion of Opr86, which result in OMP assembly defects, causes hypervacuolation (63). Moreover, we showed that overexpression of *opr86* results in decreased MV production in this study (Fig. 1B). These data suggest that misfolded OMPs upregulate MV production in *P. aeruginosa*, and the data correspond to the fact that stress, such as accumulation of toxic misfolded OMPs, causes increased MV synthesis in *E. coli* and *Salmonella enterica* (42). We also observed that overexpression of SurA resulted in decreased MV production in *P. aeruginosa* (unpublished data). It has been reported that SurA is a chaperone responsible for the periplasmic transit of the bulk mass of OMPs to the outer membrane in *E. coli* (28, 49), but *P. aeruginosa* SurA has been not characterized. It has also been shown that Skp and DegP play the role of chaperone with OMPs, but the loss of either DegP or Skp has no effect on the outer membrane composition in *E. coli* (57). We examined the effect of PA3647, which seems to be an *E. coli* *skp* homolog, on MV production. Overexpression of this gene did not affect the quantity of MVs in supernatants compared to that in the WT (unpublished data), suggesting that either PA3647 does not operate as a chaperone or it also plays a supplementary role in the mechanism in *E. coli*. In contrast, overexpression of MucD, which is the DegP homolog in *P. aeruginosa*, resulted in decreased MV production (Fig. 5). DegP has been shown to have not only chaperone activity but also a periplasmic serine protease that degrades improperly folded or mislocalized OMPs (59, 61), and it is believed that repression of MV production by MucD is attributed to the latter function.

In *E. coli*, the relationship between MV production and the

signaling system, including Bae, Cpx, Psp, and the σ^E pathway, which monitor bacterial cell envelope systems, has been studied (42). The σ^E pathway is activated by the binding of DegS to the C-terminal YYF or YQF sequence present on OMPs when accumulation of misfolded OMPs is caused by a stress, such as an elevated temperature (1, 2, 67). Since the periplasmic protein cytochrome b_{562} fused with C-terminal YYF increased MV production and since it is enriched in MVs (42), it is hypothesized that this C-terminal sequence acts as an inducible signal for MV production. In *E. coli*, σ^E pathway regulators, including DegS, RseA, and DegP, act as negative regulators of MV production (42). These three factors, DegS, RseA, and DegP, are homologous to AlgW, MucA, and MucD of *P. aeruginosa*, respectively. In *P. aeruginosa*, however, strains with mutations in the sigma factor σ^{22} pathway, including $\Delta algW$, $\Delta algU$, $\Delta mucA$, $\Delta mucB$, and $\Delta mucD$ mutants showed different quantities of MV production (Fig. 3A). While DegS and RseA repress MV production in *E. coli* (42), deletion of *algW* did not alter MV levels significantly and deletion of *mucA* resulted in decreased MV levels in *P. aeruginosa*, suggesting that the mechanism of this pathway in *P. aeruginosa* is a little different from the σ^E pathway in *E. coli*. It is believed that the different mechanism is derived from the gene location: while *degP* is located in a different position from that of *rpoE* or *rseA* in *E. coli*, *mucD* is located in *algU-mucABCD* in *P. aeruginosa*. We considered the possibility that alteration of MV levels in some alginate synthetic regulators is a consequence of alteration of *mucD* expression in *P. aeruginosa* (Fig. 4). In our experiment, *mucD* expression mostly depended on the *algU* promoter, although Wood and Ohman previously reported that the *mucD*-specific promoter leads to about half of the amount of MucD (73). Since we analyzed *mucD* expression at the transcriptional level, while Wood et al. analyzed it at the translational level, there may be some posttranscriptional regulation. In addition, it is known that MucD (DegP, HtrA) sensitively responds to several stresses, such as osmotic pressure and temperature (2, 71, 73). Therefore, the differences in the growth conditions, such as the medium and growth stage, may have resulted in the different levels of *mucD* expression.

The relationship between the sigma factor σ^E pathway and the release of MVs has also been reported in *Vibrio cholerae* (58). A small regulatory noncoding RNA VrrA, whose expression requires σ^E activity, positively regulates MV production through downregulation of an outer membrane protein OmpA. Meanwhile, another study reported that activation of the σ^E pathway causes a rapid downregulation of major *omp* mRNAs and downregulates the accumulation of misfolded OMPs in *Salmonella enterica* serovar Typhimurium (46). Since the *algU* deletion caused an increase in MV production in our results, it is possible that the latter regulation exists in *P. aeruginosa*. Indeed, Wood and Ohman recently reported that small RNAs were upregulated by σ^{22} activation in *P. aeruginosa* (74). Further investigation is needed to elucidate the detailed mechanism of MV regulation by σ^{22} .

In *P. aeruginosa*, it has been shown that PQS is one of the most important factors for releasing MVs by interacting strongly with LPS (38). A proposed model for MV production is that charge-to-charge repulsion between neighboring negatively charged LPS is enhanced by PQS, resulting in curvature of the outer membrane (39). Divalent cations stabilize the

FIG. 8. Model of brief MV production and stress response in the periplasm of *P. aeruginosa*. MV production is enhanced by PQS and misfolded OMPs, and these processes are in independent pathways. Misfolded OMPs accumulate in the periplasm through the decline in function of OMP assembly machinery. Accumulated misfolded OMPs proceed in three processes. To alleviate periplasmic stress, serine protease MucD degrades misfolded OMPs selectively (process I). In another process, misfolded OMPs are released as MVs (process II). According to the level of expression of MucD, MV production changes. When there is an extreme accumulation of misfolded OMPs in the periplasm, alginate synthesis seems to be activated (process III). OM, outer membrane; IM, inner membrane.

gram-negative outer membrane by forming salt bridges between negatively charged phosphates of neighboring LPS molecules. Since several quinolones have been shown to interact with cations (38), PQS probably functions by sequestering positive charge from LPS.

Taking all the results together, MV production seems to be induced by at least two independent processes as shown in Fig. 8. While PQS operates on LPS from outside, misfolded OMPs increase MV proteins from the periplasm. We also propose a model for the presumed process of misfolded OMPs as shown in Fig. 8. It is thought that elimination of misfolded OMPs can be divided into three processes. One is degradation by the serine protease MucD. The second process is the induction of MV production. Accumulation of misfolded OMPs in the periplasm resulting from decreased MucD would cause excretion of misfolded OMPs to the extracellular milieu through MVs and increased MV production. This process in *E. coli* has been previously reported in a study by McBroom and Kuehn (42). The third process is alginate synthesis. However, it seems that this process is low priority in terms of misfolded OMP elimination. Alginate synthesis usually occurred in small amounts in *P. aeruginosa* PAO1. Qiu et al. showed that overexpression of MucE, which has a WVF motif in its C-terminal region and is a positive regulator of alginate synthesis to activate AlgU activity, did not change appearance after induction from a *lac* promoter or a *tac* promoter (48). However, a higher

level of induction by an *aaC1* or a P_{BAD} promoter caused mucoid conversion. Therefore, it is believed that a slight fluctuation in the quantity of misfolded OMPs in the periplasm does not affect alginate synthesis.

In the airways of CF patients, mucoid conversion frequently occurs mostly through mutations in *mucA* (35). As chronic CF lung disease progresses, mucoid, alginate-overexpressing strains emerge and become the predominant form. Alginate is one of the components in exopolysaccharides in *P. aeruginosa* biofilms. On the other hand, it has been reported that MVs are also an important particulate component of the matrix of not only *P. aeruginosa* biofilms but also of mixed bacterial biofilms (52). However, its function in biofilms has not been elucidated. Investigation of the role of MVs in biofilm production should be particularly interesting.

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