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Acinetobacter baumannii Outer Membrane Protein A Modulates the Biogenesis of Outer Membrane Vesicles[§]

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Acinetobacter baumannii **secretes outer membrane vesicles (OMVs) during both *in vitro* and *in vivo* growth, but the biogenesis mechanism by which *A. baumannii* produces OMVs remains undefined. Outer membrane protein A of *A. baumannii* (AbOmpA) is a major protein in the outer membrane and the C-terminus of AbOmpA interacts with diaminopimelate of peptidoglycan. This study investigated the role of AbOmpA in the biogenesis of *A. baumannii* OMVs. Quantitative and qualitative approaches were used to analyze OMV biogenesis in *A. baumannii* ATCC 19606[T] and an isogenic ΔAbOmpA mutant. OMV production was significantly increased in the ΔAbOmpA mutant compared to wild-type bacteria as demonstrated by quantitation of proteins and lipopolysaccharides (LPS) packaged in OMVs. LPS profiles prepared from OMVs from wild-type bacteria and the ΔAbOmpA mutant had identical patterns, but proteomic analysis showed different protein constituents in OMVs from wild-type bacteria compared to the ΔAbOmpA mutant. In conclusion, AbOmpA influences OMV biogenesis by controlling OMV production and protein composition.**

Keywords: outer membrane protein A, lipopolysaccharides, peptidoglycan, protein sorting

Introduction

Gram-negative bacteria naturally secrete outer membrane vesicles (OMVs) during growth. OMVs selectively bleb from the outer membrane and form spherical vesicles with diameters ranging from 20–300 nm (Beveridge, 1999; Kuehn and Kesty, 2005; Mashburn-Warren and Whiteley, 2006; Ellis and Kuehn, 2010). OMVs are composed of lipopolysaccharides (LPS), proteins, and phospholipids originating from the outer membrane and periplasmic space. However, recent proteomic studies have shown that OMVs from Gram-negative bacteria contain a considerable portion of proteins originating from the inner membrane and cytosol (Lee *et al.*, 2008a; Kwon *et al.*, 2009; Olofsson *et al.*, 2010; Jin *et al.*, 2011). OMVs have been observed in a variety of environments, including infected tissues, where OMVs play a role in the delivery of toxins and virulence factors to host cells, and the induction of immune responses (Beveridge *et al.*, 1997; Fiocca *et al.*, 1999; Horstman and Kuehn, 2000; Wai *et al.*, 2003; Bergman *et al.*, 2005; Mashburn and Whiteley, 2005; Alaniz *et al.*, 2007).

OMV production is apparently linked to bacterial stress responses and is increased under harsh environmental conditions, such as chemical stress or infection (Kadurugamuwa and Beveridge, 1995; Hellman and Warren, 2001; Namork and Brandtzaeg, 2002; McBroom and Kuehn, 2007). However, the mechanism by which Gram-negative bacteria produce OMVs remains undefined. Deatherage *et al.* (2009) proposed a model of OMV biogenesis in *Salmonella* species. In this model, OMVs are produced at cell envelope regions or the septum where the density of outer membrane-peptidoglycan and outer membrane-peptidoglycan-inner membrane associations are temporarily decreased. They also found that outer membrane proteins identified in OMVs could modulate OMV production via specific domains that interacted with peptidoglycan. *Salmonella* mutants lacking outer membrane proteins, such as OmpA, LppAb, and Pal, produce more OMVs than wild-type bacteria.

Acinetobacter baumannii is an important nosocomial pathogen that causes a variety of human infections in critically ill patients (Dijkshoorn *et al.*, 2007). This microorganism is generally regarded as a low virulent pathogen, but several virulence factors, including phospholipase D (Jacobs *et al.*, 2010), capsular polysaccharides (Russo *et al.*, 2010), serum resistance (Kim *et al.*, 2009; Antunes *et al.*, 2011), biofilm formation (Lee *et al.*, 2008b), iron acquisition (Zimbler *et al.*, 2009), adherence and invasion in host cells (Choi *et al.*, 2008b), and host cell death (Choi *et al.*, 2005, 2008a), have been identified. We have recently demonstrated that outer membrane protein A of *A. baumannii* (AbOmpA) packaged in the OMVs is responsible for host cell death (Jin *et al.*, 2011). Moreover, the structural analysis of AbOmpA showed that the C-terminal periplasmic domain (OmpA-like domain) interacted with diaminopimelate of peptidoglycan (Park *et al.*, 2012). This result suggests

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that a major outer membrane protein, AbOmpA, can modulate the biogenesis of *A. baumannii* OMVs, because linkages between outer membrane proteins and peptidoglycan are directly responsible for OMV production (Deatherage *et al.*, 2009). However, the role of AbOmpA in OMV production remains unknown. In this study, quantitative and qualitative approaches were used to analyze the production and protein constituents of OMVs from *A. baumannii* ATCC 19606^T and an isogenic ΔAbOmpA mutant. We found that AbOmpA is not only an important virulence factor for inducing host cell death (Choi *et al.*, 2005, 2008a), but also plays a role in biogenesis of *A. baumannii* OMVs.

Materials and Methods

Bacterial strains

A. baumannii ATCC 19606^T and isogenic ΔAbOmpA mutant (Choi *et al.*, 2005) were used in this study. Bacteria were maintained on blood agar plates and cultured in Luria-Bertani (LB) broth for production of OMVs.

Preparation of OMVs

OMVs from *A. baumannii* were prepared as previously described (Wai *et al.*, 2003; Lee *et al.*, 2008a). Briefly, bacteria were grown in 500 ml of LB broth at 37°C with shaking until an optical density of 1.0 at 600 nm was reached. Bacterial cells were removed by centrifugation at 6,000×g at 4°C for 15 min. The supernatants were filtered through a QuixStand Benchtop System (GE Healthcare, USA) using a 0.2 μm hollow fiber membrane (GE Healthcare) to remove bacterial debris. The filtered samples were concentrated via ultrafiltration with a QuixStand Benchtop System using a 500 kDa hollow fiber membrane (GE Healthcare) to exclude molecules with a molecular mass <500 kDa. The samples were ultracentrifuged at 150,000×g at 4°C for 3 h, and OMVs were then resuspended in phosphate-buffered saline (PBS). The concentrations of protein and LPS were determined using a modified BCA assay (Thermo Scientific, USA) and *Limulus* Amebocyte lysate test kits (Sigma, USA), respectively. The purified OMVs were inoculated on blood agar plates to assess sterility and stored at -80°C until used.

Preparation of outer membrane proteins

A. baumannii ATCC19606^T was grown in LB broth with constant shaking at 37°C for 20 h. Bacterial cultures were centrifuged at 4,000 rpm for 30 min. Bacterial pellets resuspended in 10 mM HEPES buffer were sonicated and centrifugated at 1,700×g for 20 min. The supernatant was centrifuged at 100,000×g for 1 h at 4°C. The pellet was resuspended in 10 mM HEPES buffer with 2% sodium lauryl sarcosine and incubated for 30 min at room temperature. The suspension was recentrifuged at 100,000×g for 1 h at 4°C. The pellet containing outer membrane proteins was resuspended in PBS and stored at -80°C until used.

Identification of proteins in OMVs

One-dimensional electrophoresis-liquid chromatography-tandem mass spectrometry (1-DE-LC-MS/MS) was performed as previously described to identify proteins in *A. baumannii* OMVs (Kwon *et al.*, 2009). In brief, proteins in the prepared OMV fractions were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and in-gel digested. The digested proteins were dissolved in 15 μl of 0.02% formic acid in 0.5% acetic acid. The peptide samples were concentrated on an MGU30-C18 trapping column (LC Packings, The Netherlands) and a nano-column (C18 reverse-phase column, Proxeon, USA), and then eluted from the columns by applying a 0–65% acetonitrile gradient for 80 min. All MS and MS/MS spectra were acquired by an LCQ-Deca ESI ion trap mass spectrometer (ThermoFinnigan, USA). The MS/MS spectra were analyzed by the MASCOT program (Matrix Science, USA) using the genome data of *A. baumannii* ATCC 17978 obtained from the NCBInr database (http://www.ncbi.nlm.nih.gov/). Subcellular localization of proteins identified in OMVs was predicted using the PSORTb v.3.0 tool at http://www.psort.org/psortb (Yu *et al.*, 2010).

Western blot analysis

Protein samples were separated by 12% SDS-PAGE, followed by electrotransfer onto nylon membranes (Amersham Pharmacia Biotech). The blots were blocked in 5% non-fat skim milk and incubated with a rabbit anti-AbOmpA immune serum produced by our laboratory. The membranes were incubated with a secondary antibody coupled to horseradish peroxidase and developed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech).

Transmission electron microscopy (TEM)

After purifying the OMVs recovered from bacterial supernatants, the vesicles were directly applied to copper grids and stained with 2% uranyl acetate. The samples were washed with PBS and then visualized with a TEM (Hitachi, Japan) operating at 120 kV.

Silver staining of LPS

LPS were extracted from OMVs by an LPS extraction kit (Invitrogen, USA) according to the manufacturer's instructions. To obtain a high purity, the prepared LPS were treated with proteinase K (Roche Applied Science, USA) at 50°C for 30 min. Purified LPS were separated by 10% SDS-PAGE and stained using a PowerStain^{TM} Silver Stain kit (Elpisbio, Korea) according to the manufacturer's instructions.

Statistical analysis

The statistical significance was determined by the Student's *t*-test. A P value of <0.05 was considered to be statistically significant.

Results and Discussion

AbOmpA modulates OMV production

We previously showed that *A. baumannii* ATCC 19606^T produced and secreted OMVs during both *in vitro* culture

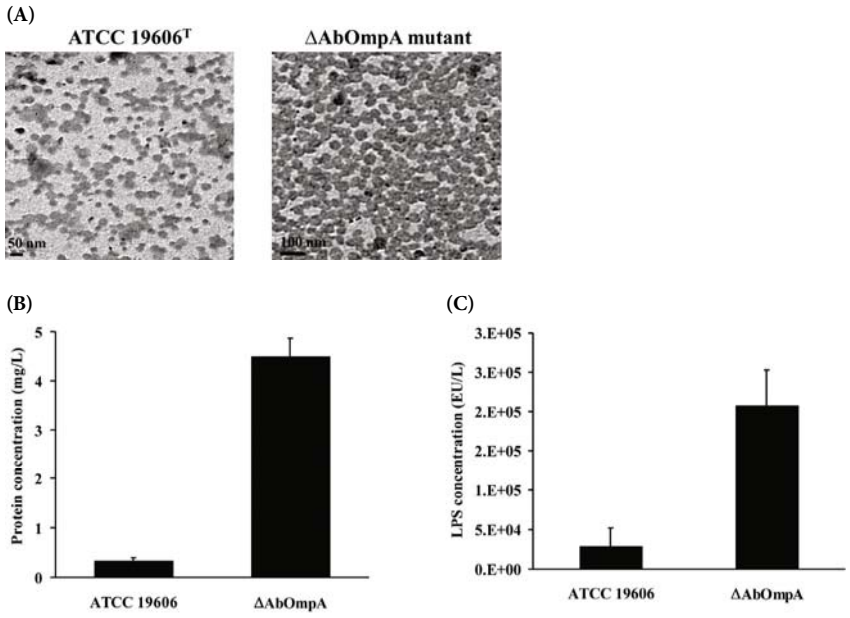

Fig. 1. Quantitative differences in OMV production between *A. baumannii* ATCC 19606^T and the isogenic ΔAbOmpA mutant. (A) Both strains secreted OMVs during *in vitro* culture. The purified OMVs were negatively stained with 2% uranyl acetate and then observed by TEM. (B and C) OMVs were prepared from 1 L of bacterial cultures grown to an optical density of 1.0 at A_{600}. Protein (B) and LPS (C) concentrations were determined. The data are expressed as the mean±standard deviation of two independent experiments

and *in vivo* infection (Jin *et al*., 2011). To determine whether the ΔAbOmpA mutant of *A. baumannii* ATCC 19606^T produced OMVs during *in vitro* culture, bacteria were cultured in LB broth and OMVs were purified from culture supernatants. TEM analysis showed that both the *A. baumannii* ATCC 19606^T and ΔAbOmpA mutant produced spherical nanovesicles (Fig. 1A). To quantitatively determine the difference in OMV production between *A. baumannii* ATCC 19606^T and the ΔAbOmpA mutant, OMVs were prepared from 1 L of bacterial cultures and total protein concentrations of OMVs were measured. There was a significant difference in protein concentrations between OMVs from the wild-type bacteria (0.34±0.05 mg/L) and ΔAbOmpA mutant (4.50±0.36 mg/L) (P<0.01) (Fig. 1B). LPS is the single most abundant component of OMVs with LPS content exceeding the total protein content by ratios as high as 10:1 (Ellis and Kuehn, 2010). We determined the LPS concentrations of OMVs prepared from both strains. There was a significant difference in LPS concentration between OMVs from the wild-type bacteria [28,600±23,476 endotoxin units (EU)/L] and the ΔAbOmpA mutant (208,000±45,255 EU/L) (P<0.05) (Fig. 1C). These results suggest that AbOmpA deficiencies in the outer membrane significantly affect OMV production in *A. baumannii*.

AbOmpA is a transmembrane protein that consists of 356 amino acids in *A. baumannii* ATCC 19606^T. A study predicting the tertiary structure of AbOmpA showed that the transmembrane N-terminal residues traversed the outer membrane with eight β-sheet segments and four external loops, while the C-terminal residues were located in the periplasmic space (Jin *et al*., 2011). A BLAST search (http://www.ncbi.nlm.nih.gov/) revealed that the C-terminus of AbOmpA was similar to that of *Escherichia coli* OmpA, and was named the "OmpA-like domain". This domain is known to non-covalently associate with peptidoglycan (De Mot and Vanderleyden, 1994; Koebnik, 1995). In *E. coli* lipoprotein Pal (Cascales *et al*., 2002) and *Neisseria meningitidis* RmpM (Grizot and Buchanan, 2004), the OmpA-like domain interacted with the outer membrane as well as peptidoglycan. The structural analysis of *N. meningitidis* RpmM showed that the C-terminal OmpA-like domain interacted non-covalently with peptidoglycan, and the N-terminal domain stabilized the oligomeric state of PorA-, PorB-, and TonB-dependent transporters in the outer membrane (Jansen *et al*., 2000; Prinz and Tommassen, 2000; Grizot and Buchanan, 2004). *E. coli* MotB and *Vibrio alginolyticus* PomB and MotY were found to interact with the inner membrane (Zhai *et al*., 2003; Okabe *et al*., 2005; Kojima *et al*., 2008). We recently showed that the C-terminus of AbOmpA bound to diaminopimelate, a unique bacterial amino acid from peptidoglycan (Park *et al*., 2012). Two amino acid residues, Asp271 and Arg286, of AbOmpA are key for binding to diaminopimelate of peptidoglycan. These results suggest that the interaction of the C-terminal OmpA-like domain with diaminopimelate of peptidoglycan can control OMV production

AbOmpA controls protein constituents in *A. baumannii* OMVs

To compare the LPS profiles of OMVs from *A. baumannii* ATCC 19606^T and the ΔAbOmpA mutant, LPS was extracted from OMVs obtained from both strains and separated by SDS-PAGE. LPS prepared from OMVs from both the wild-type bacteria and the ΔAbOmpA mutant showed identical patterns (Fig. 2A). To determine differences in protein constituents between the OMVs from wild-type bacteria and the ΔAbOmpA mutant, SDS-PAGE was performed. The protein profiles of the two strains were different (Fig. 2B). Proteins with a molecular mass approximately 38 kDa were the most abundant in OMVs from *A. baumannii* ATCC 19606^T. In contrast, proteins with a molecular mass of about 27 kDa were the most abundant in OMVs from the ΔAbOmpA mutant. Western blot analysis showed that the full-length of AbOmpA with a molecular mass of 38

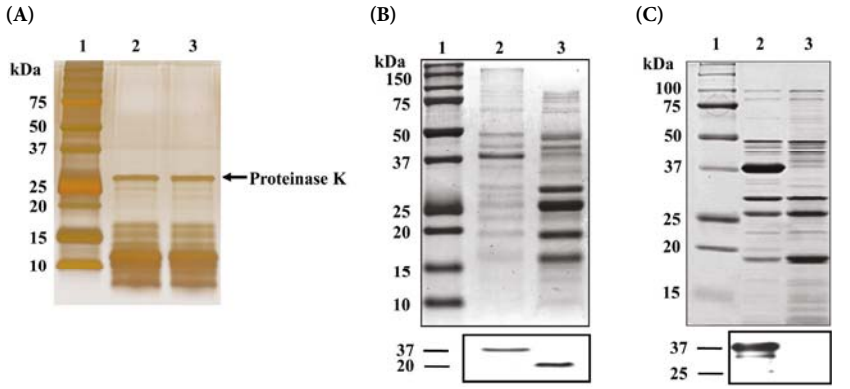

Fig. 2. Qualitative differences in OMVs from *A. baumannii* ATCC 19606^T and the isogenic ΔAbOmpA mutant. (A) Silver staining of LPS extracted from OMVs. (B) SDS-PAGE (upper panel) and Western blot (lower panel) of OMV proteins using an anti-AbOmpA antibody. (C) SDS-PAGE (upper panel) and Western blot (lower panel) of outer membrane proteins prepared from the wild-type and ΔAbOmpA mutant strains using an anti-AbOmpA antibody. Lanes: 1, molecular weight marker; 2, *A. baumannii* ATCC 19606^T; 3, ΔAbOmpA mutant.

kDa was identified in OMVs from *A. baumannii* ATCC 19606^T, whereas the truncated AbOmpA with a molecular mass of approximately 20 kDa was identified in OMVs from the ΔAbOmpA mutant (Fig. 2B). To determine whether the truncated AbOmpA was located in the outer membrane of the ΔAbOmpA mutant, outer membrane proteins prepared from *A. baumannii* ATCC 19606^T and ΔAbOmpA mutant strains were subjected to SDS-PAGE and Western blot using an anti-AbOmpA antibody (Fig. 2C). Full-length AbOmpA was detected in the outer membrane of *A. baumannii* ATCC 19606^T, whereas neither the full-length nor the truncated AbOmpA was detected in the outer membrane of the ΔAbOmpA mutant. These results suggest that the ΔAbOmpA mutant produced the truncated AbOmpA, but that this protein was not localized in the outer membrane.

Proteomic analysis was performed to identify proteins in OMVs. We previously identified a total of 113 proteins in OMVs from *A. baumannii* ATCC 19606^T (Jin *et al.*, 2011), whereas 102 proteins were identified in OMVs from the ΔAbOmpA mutant in this study (Supplementary data Table S1). Among the 102 proteins identified in OMVs from the ΔAbOmpA mutant, 73 (71.6%) were identical to proteins identified in OMVs from *A. baumannii* ATCC 19606^T (Fig. 3). Forty proteins were identified only in OMVs from the *A. baumannii* ATCC 19606^T, while 29 were found only in the ΔAbOmpA mutant OMVs. The most abundant proteins in OMVs from wild-type *A. baumannii* ATCC 19606^T were AbOmpA and putative outer membrane protein (A1S_2840) with a molecular mass of 22.5 kDa (Jin *et al.*, 2011). In contrast, two putative outer membrane proteins with a molecular mass of 27.7 kDa (A1S_3317) and 24.7 kDa (A1S_3297) were the most abundant in OMVs from the ΔAbOmpA mutant (Supplementary data Table S1). We found a discrepancy regarding the abundant proteins between SDS-PAGE gel and proteomic results. This difference is possibly due to the presence of several different proteins with similar molecular masses in one band in SDS-PAGE gels. Three outer membrane proteins, including ferrichrome-iron receptor (gi|126641966), rotamase (gi|126640160), and hypothetical protein A1S_3355 (gi|126643362), were identified in OMVs from the wild-type bacteria, whereas nine outer membrane proteins, including organic solvent tolerance protein precursor (gi|126641591), three putative ferric siderophore receptor proteins (gi|126640547, gi|126641770, and gi|126643331), two putative TonB-dependent receptor proteins (gi|126642916 and gi|126642853), two putative outer membrane proteins (gi|126642177 and gi|126640164), and hypothetical protein A1S_1055 (gi|126641105), were identified in OMVs from the ΔAbOmpA mutant. We also identified different proteins that originated from the periplasmic space and inner membrane when comparing OMVs from wild-type bacteria and the ΔAbOmpA mutant (Supplementary data Table S1). These results suggest that AbOmpA may influence the distribution of envelope proteins, which can regulate protein packing in OMVs.

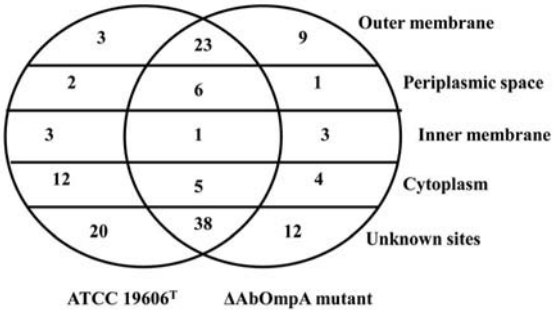

Fig. 3. Venn diagram of proteins identified in OMVs from *A. baumannii* ATCC 19606^T and the isogenic ΔAbOmpA mutant. The number of proteins identified in OMVs is presented. A total of 113 proteins were identified in OMVs from *A. baumannii* ATCC 19606^T and 102 were found in ΔAbOmpA mutant OMVs. Seventy-three proteins identified in OMVs from both the wild-type and ΔAbOmpA mutant bacteria were identical.

Certain proteins are enriched in OMVs, whereas others are excluded, suggesting that OMVs are created by a specific mechanism rather than by random blebbing of the outer membrane (Wensink and Witholt, 1981; Kato *et al.*, 2002; Kulp and Kuehn, 2010). Lpp is the most abundant protein in the cell envelope of *E. coli*, but not found in *E. coli* OMVs (Wensink and Witholt, 1981). Moreover, differences in protein composition have been observed between septum- and cell body-derived OMVs in *Salmonella* species (Deatherage *et al.*, 2009). Septum-derived OMVs are highly enriched with TolB, whereas cell body-derived OMVs are enriched with flagellar proteins, FliD and FlgK. These results suggest that a distribution of specific envelope linkages control OMV protein composition. Accordingly, a structural analysis of the N-terminus of AbOmpA and its association with other outer membrane proteins should be performed to under-

stand the regulation of protein packing in *A. baumannii* OMVs.

Our previous study showed that AbOmpA was not present in the outer membrane of the ΔAbOmpA mutant, as demonstrated by SDS-PAGE of outer membrane fractions (Choi *et al.*, 2005). We generated the ΔAbOmpA mutant using transposon mutagenesis. The transposon was inserted within the *AbOmpA* gene at nucleotides 557. However, the truncated AbOmpA was found in OMVs from the ΔAbOmpA mutant in this study (Fig. 2B). Although the ΔAbOmpA mutant produced the truncated N-terminus of AbOmpA, this protein was not correctly positioned in the outer membrane. This result suggests that the outer membrane of the ΔAbOmpA mutant cannot interact with peptidoglycan via AbOmpA, which reduces the association between the outer membrane and peptidoglycan in the ΔAbOmpA mutant.

In conclusion, AbOmpA provides cell integrity, and as a structural protein, enhances the permeability of small solutes. This major outer membrane protein also acts as a virulence factor and is responsible for host cell death, serum resistance, and adherence to and invasion of bacteria in host cells (Choi *et al.*, 2005, 2008a, 2008b; Kim *et al.*, 2009). In addition to the pathogenic potential of AbOmpA, the present study demonstrates that AbOmpA can modulate biogenesis of *A. baumannii* OMVs.

Acknowledgements

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