RESEARCH PAPER

Outer membrane Protein A plays a role in pathogenesis of Acinetobacter nosocomialis

Sang Woo Kim, Man Hwan Oh, So Hyun Jun, Hyejin Jeon, Seung Il Kim, Kwangho Kim, Yoo Chul Lee, and Je Chul Lee

*Department of Microbiology, Kyungpook National University School of Medicine, Daegu, Korea; †Department of Orthopedic Surgery, Ulsan University Hospital, University of Ulsan College of Medicine, Ulsan, Korea; ‡Department of Nanobiomedical Science, Dankook University, Cheonan, Korea; Division of Life Science, Korea Basic Science Institute, Daejeon, Korea

ABSTRACT

Acinetobacter nosocomialis is an important nosocomial pathogen that causes a variety of human infections. However, the specific virulence factors of this microorganism have not yet been determined. We investigated the role of outer membrane protein A (OmpA) in the pathogenesis of A. nosocomialis. A ∆ompA mutant of the A. nosocomialis ATCC 17903 strain was constructed using markerless gene deletion. The ∆ompA mutant displayed reduced biofilm formation in polystyrene tubes and reduced adherence to A549 cells in comparison to the wild-type strain. These virulence traits of the ∆ompA mutant strain were restored when the ompA gene was complemented. Cytotoxicity was not significantly different between the wild-type strain and the ∆ompA mutant when A549 cells were infected with bacteria or treated with outer membrane vesicles (OMVs). However, OMVs from the wild-type strain induced cytotoxicity in HEp-2 cells, whereas OMVs from the ∆ompA mutant did not induce cytotoxicity. Proteomic analysis of OMVs revealed that OmpA influenced the distribution of envelope and periplasmic proteins. Overall, this study is the first report that links OmpA to A. nosocomialis pathogenesis, and highlights OmpA as a putative target to develop anti-virulence agents or vaccines against A. nosocomialis infection.

Introduction

Acinetobacter species are emerging nosocomial pathogens that cause a variety of human infections, especially in intensive care units. Among the 39 genomic species described to date (http://www.bacterio.net/acinetobacter.html), Acinetobacter baumannii, Acinetobacter nosocomialis, and Acinetobacter pittii are the most clinically relevant species, notorious for the prevalence of nosocomial infections and multi-drug resistant phenotypes. A. baumannii is the most prevalent species among the Acinetobacter species worldwide and the clinical importance of nosocomial infections caused by this microorganism has been well recognized by clinicians. A. nosocomialis is increasingly common as a causative agent of nosocomial infections and accounts for 21% of Acinetobacter species in the United States, 24.4% in Korea, 26.7% and 35.8% in Taiwan, and 46.9% in Norway. A. baumannii and A. nosocomialis are phenotypically similar and genetically closely related. They can be differentiated into different genomic species by molecular methods, including sequence analysis of the 16S rRNA gene, the rpoB gene, and the 16S-23S rRNA gene spacer region, as well as by amplified fragment length polymorphism and amplified 16S rRNA gene restriction analysis. However, previous studies have demonstrated that A. baumannii is associated with greater resistance to antimicrobial agents and higher mortality than A. nosocomialis, which may result from differences in the biological and virulence traits between the two species. A. baumannii possesses several virulence traits, including biofilm formation, cellular adherence and invasion of host cells, serum resistance, iron acquisition, and cytotoxicity in host cells. We recently reported that A. nosocomialis secretes cytotoxic factors via outer membrane vesicles (OMVs), which induce cytotoxicity and an innate immune response in epithelial cells in vitro. However,
the fundamental mechanisms of pathogenicity of *A. nosocomialis* remain to be elucidated.

Outer membrane protein A of *A. baumannii* (AbOmpA) is a major outer membrane porin that contributes significantly to the pathogenesis of *A. baumannii*. AbOmpA inhibits the alternative pathway of complement activation by binding factor H, and is also involved in biofilm formation on abiotic surfaces and adherence to and invasion of host cells. These strategies promote bacterial survival both inside and outside of the host. Furthermore, a large amount of AbOmpA is secreted via OMVs to stimulate innate immune responses and induce host cell death. The secretion of AbOmpA via OMVs may be associated with host pathology upon *A. baumannii* infection *in vivo*. OmpA of *A. nosocomialis* ATCC 17903 shows 92% amino acid sequence homology with that of *A. baumannii* ATCC 17978. Moreover, *A. nosocomialis* OmpA is secreted via OMVs and carries putative nuclear localization signals that may direct OmpA to the nuclei of host cells. Therefore, we postulated that the major outer membrane protein OmpA might directly or indirectly contribute to *A. nosocomialis* pathogenesis, similar to AbOmpA of *A. baumannii*. The aim of this study was to investigate the role of OmpA in the pathogenesis of *A. nosocomialis* by evaluating its effect on biofilm formation, adherence to host cells, and the induction of cytotoxicity in host cells. The virulence traits were compared for wild-type *A. nosocomialis*, the ΔompA mutant of *A. nosocomialis* ATCC 17903, an ompA deletion mutant, and ompA-complemented strains.

### Results

#### Construction of *A. nosocomialis* ΔompA mutant and its ompA-complemented strain

To investigate whether OmpA played a role in the pathogenesis of *A. nosocomialis*, the ΔompA mutant of *A. nosocomialis* ATCC 17903 was constructed using a cloning method based on overlap extension polymerase chain reactions (PCR). Two sets of PCRs were performed using the primer sets of OmpA01F/OmpA01R and OmpA02F/OmpA02R (Table 1), and two PCR products with a size of approximately 1 kb, respectively, were combined by overlap extension PCR using the primers OmpA01F/OmpA02R. The amplicon in which the ompA gene was deleted was cloned into the pOH1 plasmid, resulting in the pOH2 plasmid (Table 2). The ompA gene in the chromosome of *A. nosocomialis* ATCC 17903 was deleted by conjugal gene transfer of pOH2 plasmids and homologous recombination. To determine whether the ompA gene was deleted in the chromosome of *A. nosocomialis* ATCC 17903, a PCR was performed using the primers Omp01F/Omp02R. The PCR products of the wild-type strain were 3.1 kb in size, whereas the ΔompA mutant OH1 strain showed a PCR product of approximately 2.1 kb (Fig. 1A), which was in good agreement with the projected size of the DNA fragment after ompA deletion. For complementation of the ompA gene in the ΔompA mutant OH1 strain, the pOH3 plasmid was constructed by subcloning the ompA coding region under the control of the bla<sub>CTX-M14</sub> gene promoter into the *Acinetobacter*- *E. coli* shuttle vector pWH1266 (Table 2). This recombinant plasmid was transformed into the ΔompA mutant OH1 strain, resulting in the ompA-complemented OH2 strain. PCR analysis using the primers OmpA04F/OmpA04R showed that the wild-type and the ompA-complemented OH2 strains carried the full-length 1,066 bp ompA gene, while it was not amplified in the ΔompA mutant OH1 strain (Fig. 1B). To determine whether the wild-type, ΔompA mutant OH1, and ompA-complemented OH2 strains expressed OmpA in the outer membrane, outer membrane proteins were extracted from each of the three bacterial strains, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent western

### Table 1. Oligonucleotides used in this study.

| Primers<sup>a</sup> | Sequence (5’→3’)<sup>b</sup> | Use |
|---------------------|-------------------------------|-----|
| OmpA01F             | CTACATAGTATGTCATATTGTAATCCTCCG| Deletion of ompA |
| OmpA01R             | ACTTAAACGAGTCGCTTTTACTGTCAGGAATATCTCCAGAGATA ACAATTG | Deletion of ompA |
| OmpA02F             | CTGAACAGTAATAGGGGACCTCG | Complementation of ompA |
| OmpA02R             | TCTAGAGCTCTAGTCATGCAAGTTGCGCG | Complementation of ompA |
| OmpA03F             | AAGTCAGCAACACCTTCCTTCGACGCCGAGGATCCGACTCACATTACCA AATTGAGGAGGATAGAG | Amplification of ompA |
| OmpA03R             | AAGTCAGCAACACCTTCCTTCGACGCCGAGGATCCGACTCACATTACCA AATTGAGGAGGATAGAG | Amplification of ompA |
| OmpA04F             | AATGGGTCGCGCGATTTGTAATTGCGAGAC | Amplification of bla<sub>CTX-M14</sub> gene promoter |
| OmpA04R             | GGGCCTAGCGTTCTACGTAATAAATTGATGTTGTCCTG | Amplification of npt |
| CtxR                | CTCAAACTACGTTTAGAGTTAGGTTAGCAGGAGGATCCGACTCACATTACCA AATTGAGGAGGATAGAG | Amplification of npt |
| U1                  | GTCTCGTCCTGGAGAAGGAGG | Amplification of npt |
| U2                  | GATCGTCGAGCTGTGAGG | Amplification of npt |

<sup>a</sup>The primers were designed using the genome sequences of *A. nosocomialis* ATCC 17903 (GeneBank accession number AEJ00000000).

<sup>b</sup>Regions of oligonucleotides that are not complementary to the corresponding templates are underlined.
Table 2. Bacterial strains and plasmids used in this study.

| Bacteria or plasmids | Relevant characteristicsa | Reference or source |
|----------------------|---------------------------|---------------------|
| **Bacterial strains** |                           |                     |
| A. nosocomialis       |                           |                     |
| ATCC 17903T          | Type strain               | This study          |
| OH1                  | ATCC 17903T with ΔompA    | This study          |
| OH2                  | OH1 with pOH3             | This study          |
| E. coli              |                           |                     |
| DH56a-Δpir           | supE44 ΔlacU169 (Δp80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA 96 thi-1 relA1 Δpir pahge lysogen; plasmid replication | Laboratory collection |
| S174-Δpir           | Δpir lysogen; thi pro hsdR hsdM1 recA RP4-2 Tc::Mu-Km::Tn7; Tp7 Sm'; host for p-requireing plasmids; conjugal donor | 57 |
| **Plasmids**         |                           |                     |
| pUC4K                | pUC4 with npt; Amp', Km'  | Amersham Pharmacia Biotech 48 |
| pDM4                 | R6K y ori sacB; suicide vector; oriT of RP4; Cm' | This study |
| pOH1                 | pDM4 with npt; Cm', Km'   | This study          |
| pOH2                 | pOH1 with ΔompA; Cm', Km' | This study          |
| pOH3                 | pWH1266 carrying with ompA coding region under control of bla_CTX-M-14 promoter with nptl | This study |
| pWH1266              | Source of replication origin and Tet' for A. baumannii; E. coli-Acinetobacter shuttle vector; Amp', Tet' | 49 |

Note. *Tp7*, trimethoprim-resistant; *Sm'; streptomycin-resistant; *Amp'*; ampicillin-resistant; *Km', kanamycin-resistant; *Cm', chloramphenicol-resistant; *Tet'*; tetracycline-resistant.

blotting with anti-AbOmpA antibodies were performed. The expected 37.4 kDa OmpA protein was expressed in the wild-type and theΔompA-complemented OH2 strains, but was not detected in the outer membrane of theΔompA mutant OH1 strain (Fig. 1C). To determine whether the absence of OmpA in the outer membrane affected bacterial survival and growth, in vitro growth studies were carried out. The three bacterial strains were grown in Luria-Bertani (LB) medium at 37°C for 48 h, and colony forming units (CFUs) were determined at the indicated times. The growth of theompA-complemented OH2 strains was slightly decreased at 24 h when compared to that of wild-type and ΔompA mutant OH1 strains, but no significant difference in growth rates was observed between the three bacterial strains (Fig. 1D).

**OmpA contributes to biofilm formation of A. nosocomialis**

To evaluate the involvement of OmpA in biofilm formation, the ability of the wild-type and ΔompA mutant OH1 strains to produce biofilm on a polystyrene surface was assessed by crystal violet staining at different time points (Fig. 2A). The ability of the wild-type strain to produce the biofilm was gradually increased when the bacteria was cultured for 14, 24, and 48 h. However, theΔompA mutant OH1 strain showed a significant reduction in biofilm formation compared with the wild-type strain when bacteria were cultured during the periods of incubation. Rescue of theompA gene in theΔompA mutant restored the wild-type biofilm phenotype, as demonstrated by both the biofilm assay stained with crystal violet (Fig. 2A) and scanning electron microscopy (SEM) (Fig. 2B). These results suggest that the decreased biofilm formation in theΔompA mutant OH1 strain results from inactivation of theompA gene rather than from any polar effect on genes downstream of theompA gene.

**OmpA contributes to adherence of A. nosocomialis to epithelial cells**

To investigate whether OmpA was involved in adherence of A. nosocomialis to epithelial cells, A549 cells were infected with the wild-type and theΔompA mutant strains at a multiplicity of infection (MOI) of 100 for 1 h. The wild-type strain showed a dispersed adherence to A549 cells rather than an aggregated adherence at localized areas of the cells (Fig. 3A). There was a significant difference in the percentage of infected cells between the wild-type strain (47.7% ± 9.4%) and theΔompA mutant (31.7% ± 3.9%) (Fig. 3B). Moreover, theΔompA mutant showed a significant reduction in the number of adherent bacteria per 90 cells (80.5 ± 7.8) when compared with the wild-type strain (188.0 ± 8.5) (Fig. 3B). The observed decrease in adherence for theΔompA mutant OH1 strain was restored in theompA-complemented OH2 strain (127.0 ± 5.7). These results suggest that OmpA plays a role in the adherence of A. nosocomialis in A549 epithelial cells.

**OmpA is not associated with epithelial cytotoxicity induced by A. nosocomialis infection**

To determine whether A. nosocomialis induced cytotoxicity of epithelial cells, A549 cells were infected with the wild-type strain at an MOI of 1, 10, and 100 for 16 h. The cells infected with the bacteria were stained with
propidium iodide (PI) and the hypo-diploid cells were counted using flow cytometry. The wild-type strain clearly induced cytotoxicity in A549 cells (Fig. 4, middle panel). To investigate whether OmpA contributed to cytotoxicity of epithelial cells, A549 cells were infected with the ΔompA mutant and the ompA-complemented strains at an MOI of 100 for 16 h. There was no significant difference in the cells with hypo-diploid DNA between the three A. nosocomialis strains (Fig. 4, middle and lower panels). These results suggest that OmpA does not directly contribute to the cytotoxicity of A549 cells induced by A. nosocomialis infection.

**OmpA associated with OMVs is responsible for cytotoxicity of HEp-2 cells**

A. nosocomialis produces and secretes OMVs containing a large amount of OmpA, and A. nosocomialis OMVs induce...
Figure 2. Biofilm formation of *A. nosocomialis* strains. (A) Bacteria were cultured in polystyrene tubes at 30°C for 14 h, 24 h, and 48 h. Biofilm formation was quantified by calculating the ratio of A_{570}/A_{600}. (B) SEM analysis of bacterial biofilms formed on the plastic surfaces at 14 h. Means ± standard deviation were calculated based on the results of three independent experiments. Wild-type, *A. nosocomialis* ATCC 17903; OH1, ΔompA mutant; OH2, ompA complemented strain. *P < 0.05; **P < 0.01.

Figure 3. Adherence of *A. nosocomialis* strains to A549 cells. (A) Cells were infected with *A. nosocomialis* at an MOI of 100 for 1 h and stained with Giemsa solution. Magnification: 100 x. (B and C) A549 cells were infected with *A. nosocomialis* at an MOI of 100 for 1 h, and the percentage of infected cells (B) and cell-associated bacteria per 90 cells (C) were counted. The adherence data are expressed as mean ± standard deviation based on two independent experiments, each performed in triplicate. *P < 0.05.
cytotoxicity of HEp-2 cells. To investigate whether OmpA in the *A. nosocomialis* OMVs was responsible for the cytotoxicity toward the epithelial cells, OMVs were purified from the wild-type, the ΔompA mutant, and the ompA-complemented strains (Fig. 5A). SDS-PAGE and protein gel blotting analysis revealed that OmpA was detected in the OMVs purified from the wild-type and the ompA-complemented strains, but not in the OMVs from the ΔompA mutant (Fig. 5B). We previously showed that cytotoxicity of HEp-2 cells was induced by treatment of 20 μg/ml of OMVs purified from *A. nosocomialis* and, therefore, HEp-2 cells were treated with 5-20 μg/ml OMVs from the wild-type and the ΔompA mutant strains, and cell viability was analyzed using the WST-1 assay. The OMVs from the wild-type strain were cytotoxic to HEp-2 cells at a concentration of 15 μg/ml, whereas the OMVs from the ΔompA mutant did not induce cytotoxicity in HEp-2 cells (Fig. 5C). The OMVs from the wild-type strain, the ΔompA mutant, and the ompA-complemented strain did not induce cytotoxicity in A549 cells treated with ≤20 μg/ml of OMVs (Fig. 5D). These results suggest that OmpA in the *A. nosocomialis* OMVs is associated with epithelial cell death, but that the cytotoxic activity of *A. nosocomialis* OMVs is dependent on the epithelial cell type.

**OmpA determines the composition of proteins in OMVs**

Proteomic analysis of OMVs from the ΔompA mutant was performed to identify proteins associated with
OMVs. We previously identified a total of 147 proteins in the OMVs of *A. nosocomialis* ATCC 17903<sup>T</sup>.<sup>31</sup> In this study, a total of 168 proteins were identified in the ΔompA mutant OMVs (Table S1). Among these, 87 (51.8%) overlapped with the proteins found in the OMVs from the wild-type strain (Fig. 6). Sixty proteins were identified only in the OMVs from the wild-type strain, whereas 81 were unique to the ΔompA mutant OMVs. Hypothetical protein W9I_03611 (gi|407441077), translocation protein TolB (gi|515963786), and hypothetical protein W9I_03176 (gi|407439503) were most abundant in the OMVs from the ΔompA mutant. In contrast, the most abundant proteins specific to the wild-type strain OMVs were hypothetical protein (gi|515187519), hypothetical protein (gi|446575849), and hypothetical protein W9I_00996 (gi|407440186).<sup>31</sup> These results suggest that OmpA may influence the distribution of membrane and periplasmic proteins, which may result in compositional difference in OMVs from the wild-type strain and the ΔompA mutant.

**Discussion**

This study shows that OmpA plays a role in the pathogenesis of *A. nosocomialis*. OmpA expressed in the outer membrane of *A. nosocomialis* does not only contribute to biofilm formation on abiotic surfaces and adherence to human epithelial cells, but OmpA present in OMVs is also responsible for cytotoxicity in epithelial HEp-2 cells. These results indicate that OmpA is a specific virulence factor for *A. nosocomialis*.
factor of *A. nosocomialis* that may contribute to pathogenesis upon infection *in vivo*.

The ability of nosocomial pathogens, including *A. baumannii*, to form biofilms on abiotic surfaces plays a pivotal role in the persistence and survival of these bacteria in hospital environments. Biofilm formation is considered to be an important virulence factor common to clinical isolates of *A. baumannii*.20,36,37 The expression of pili-like structures on the surface of *A. baumannii* is essential for biofilm formation.19 Although biofilm formation is a complex process involving a wide range of bacterial factors and multiple cellular signals, as well as a sequential expression of genes, several determinants associated with biofilm formation in *A. baumannii* have been characterized, including the CsuAB/ABCDE pilus chaperone-usher system,19,38 the 854 kDa outer membrane protein with a high similarity to the staphylococcal biofilm-associated protein (Bap),39 Abal of the quorum sensing system,40 and the pgaABCD operon responsible for the production of poly-β-1,6-N-acetyl glucosamine.41 Assembly of pili and production of the Bap protein are responsible for the initiation and maturation of biofilms in abiotic surfaces, respectively. Gaddy *et al.*32 reported that OmpA played a role in the biofilm formation of *A. baumannii* on abiotic surfaces. The csuAB/ABCDE genes essential for pili assembly were identified in the *A. nosocomialis* RUH2624 strain,42 suggesting that the pili-like structures might also be associated with biofilm formation in *A. nosocomialis*. The present study clearly showed that OmpA contributed to the biofilm formation of *A. nosocomialis* in polystyrene tubes. The reduction of biofilm mass in the ΔompA mutant was most prominent after 14 h of culture and was significantly different for 48 h of culture (Fig. 2). These results suggest that OmpA may be associated with the initiation of biofilm development, although the present study did not specifically address whether OmpA plays a role in the initiation or maturation step of biofilm formation.

Adherence of pathogenic bacteria to epithelial cells is an essential initial step for colonization and infection. Lee *et al.*21 demonstrated that *A. baumannii* showed two different types of adherence to human bronchial epithelial NCI-H292 cells: an aggregated adherence with connections of thread-like material between bacteria and a dispersed adherence with small pili-like structures connected to the surface of epithelial cells. The latter type of adherence was more frequently observed in clinical isolates of *A. baumannii*. Choi *et al.*22 have demonstrated that OmpA plays a major role in the interactions of *A. baumannii* with epithelial cells in *in vitro* cell culture but also in an *in vivo* mouse pneumonia model. The present study demonstrated that *A. nosocomialis* ATCC 17903T showed a dispersed-like adherence to A549 cells. Although *A. baumannii* adherence to host cells was highly dependent on the epithelial cell type, the adherence efficiency of *A. nosocomialis* in A549 cells was similar to that of *A. baumannii* in NCI-H292 cells.21,22 In the present study, *A. nosocomialis* infected 47% of A549 cells and the number of adhering bacteria per cell was 2.1 (Fig. 3B-C). The ΔompA mutant showed a significant
reduction in both the number of infected cells and adhering bacteria. These results suggest that the OmpA plays a role in the adherence of *A. nosocomialis* to epithelial cells. However, it should be determined whether OmpA is a non-fimbrial adhesin or influences the expression of other adhesins.

*A. baumannii* induces apoptosis of epithelial cells through cell surface death receptors and mitochondrial disintegration. Furthermore, AbOmpA is considered to be an important cytotoxic factor through both mitochondrial and nuclear targeting. The present study showed that *A. nosocomialis ATCC 17903* induced cytotoxicity of A549 cells in an MOI-dependent manner (Fig. 4). In addition, both the ΔompA mutant and the ompA-complemented strains could induce cytotoxicity in A549 cells like the wild-type strain. Epithelial cells infected with *A. nosocomialis* strains showed morphological changes such as cellular shrinkage, round-up and detachment from the culture dish, and nuclear condensation (data not shown). These results suggest that *A. nosocomialis* can induce apoptosis of A549 cells, but that OmpA in the outer membrane is not directly responsible for cytotoxicity. Other bacterial factors are likely associated with cytotoxicity in A549 cells. *A. baumannii* secreted cytotoxic AbOmpA via OMVs. Recently reported that *A. nosocomialis* secreted OMVs and these vesicles were also cytotoxic in HEp-2 cells. These OMVs delivered OmpA to the cytoplasm of host cells, but was unclear whether OmpA was associated with cytotoxicity in the case of *A. nosocomialis* OMVs.

In the present study, OMVs from the wild-type strain could induce cytotoxicity observed upon *A. nosocomial* infection in HEp-2 cells, whereas OMVs from the ΔompA mutant did not induce cytotoxicity in HEp-2 cells (Fig. 5C). HEp-2 cells were treated with 20 μg/ml OMVs from the ompA-complemented OH2 strain, but cytotoxicity was not induced (data not shown). One possible explanation for the defect of ompA complementation in the OMV-induced cytotoxicity is that the genetic elements other than the ompA gene in the pOH3 plasmids or antibiotics to maintain pOH3 plasmids in the ompA-complemented strain may influence on the composition or exact localization of proteins in OMVs. OMVs from the wild-type strain, the ΔompA mutant, and the ompA-complemented strain did not induce cytotoxicity in A549 cells at a concentration of ≤20 μg/ml. Our results suggest that OmpA plays a role in the cytotoxicity mediated by OMVs, and its cytotoxicity is dependent on the type of epithelial cells.

AbOmpA is a transmembrane protein in the outer membrane of *A. baumannii* and its C-terminal OmpA-like domain interacts with dianaminopimelate (DAP) of peptidoglycan. Moon et al. demonstrated that OMV production in the ΔompA mutant constructed by random transposon mutagenesis was significantly higher than that of wild-type *A. baumannii*. Furthermore, proteomic analysis of OMVs showed that there were differences in protein content between the wild-type and the ΔompA mutant. The present study also showed a significant difference between the protein constituents of OMVs from the wild-type strain and from the ΔompA mutant. OmpA of *A. nosocomialis ATCC 17903* and *A. baumannii* 19604 is composed of 347 and 356 amino acids, respectively. Differences in amino acid sequence homology between *A. nosocomialis* OmpA and *A. baumannii* AbOmpA were focused on the transmembrane domain, whereas the C-terminal OmpA-like domain (residues 226 to 328) of *A. nosocomialis* OmpA shows 100% homology with that of *A. baumannii* AbOmpA. Asp271 and Arg286 of AbOmpA are important among the residues in contact with DAP of peptidoglycan.

These two amino acids are also conserved in OmpA of *A. nosocomialis*. These results suggest that the C-terminal OmpA-like domain of *A. nosocomialis* OmpA may also interact with peptidoglycan. This may influence the protein composition of OMVs, especially for proteins located in the membrane and periplasmic space.

In conclusion, the present study demonstrates for the first time that OmpA is a specific virulence factor of *A. nosocomialis*. OmpA is considered to be a potential target to develop anti-virulence agents or vaccines against multi-drug resistant *A. nosocomialis*.

**Materials and methods**

**Bacterial strains, plasmids, and culture conditions**

The bacterial strains and plasmids used in this study are listed in Table 2. *A. nosocomialis* and *E. coli* were grown in LB media at 37°C. Chloramphenicol (20 μg/ml) or kanamycin (50 μg/ml) was added to the growth media to maintain the plasmids in *E. coli*. *A. nosocomialis* merodiploids were selected on the medium supplemented with kanamycin (30 μg/ml) and ampicillin (100 μg/ml). Bacterial growth was monitored by determining the optical density at 600 nm (OD at A600).

**Cell culture**

Type II pneumocyte cell line A549 originating from human lung carcinoma and HEp-2 originating from human laryngeal epithelial cells were obtained from the Korean Cell Line Bank (Seoul, Korea). A549 cells and HEp-2 cells were grown in RPMI 1640 medium (HyClone, Logan, UT) and Dulbecco’s modified Eagle medium (HyClone) supplemented with 10% fetal bovine
serum (FBS; HyClone), 2.0 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO₂, respectively. Confluent cells were harvested and seeded into 12-, 24- or 96-well plates for infection with A. nosocomialis strains or incubation with A. nosocomialis OMVs.

**DNA manipulations**

Genomic and plasmid DNA was purified from bacteria using a SolGent™ Genomic DNA prep kit (SolGent, Daejeon, Korea) and AccuPrep® Plasmid Extraction Kit (Bioneer, Daejeon, Korea), respectively. DNA fragments were purified with an AccuPrep Gel Purification Kit (Bioneer). Restriction and DNA modifying enzymes were purchased from New England Biolabs (Ipswich, MA). Routine DNA manipulations were performed as previously described46 or according to the manufacturer’s recommendations. Synthetic DNA fragments containing promoter of the blaCTX-M14 gene were purchased from Integrated DNA Technologies (IDT) (Coralville, IA).

**Construction of the ΔompA mutant**

To construct the ompA deletion mutant, a previously described markerless gene deletion method was modified.35 A new suicide vector was constructed as follows: 1.2 kb nptI DNA conferring resistance to kanamycin was amplified using primers U1 and U2 (Table 1) and the pUC4K vector was used as a template. The PCR product was cloned into pDM4 digested with Smal. The new suicide vector was named pOH1 (Table 2). The ompA gene was inactivated in vitro by deletion of the ompA open reading frame (1,044 bp) using an overlap extension PCR method. To combine the upstream and downstream regions of the ompA gene by overlap extension PCR, a reverse primer, OmpA01R, for the amplification of the upstream region was designed to carry an additional 25 nucleotides at their 5’-end, which are homologous to the downstream region. The upstream and downstream regions of the ompA gene were amplified from the genomic DNA of A. nosocomialis ATCC 17903T using the primer pairs OmpA01F/OmpA01R and OmpA02F/OmpA02R, respectively (Table 1). The two PCR products obtained in the first step were mixed at equimolar concentrations and subjected to overlap extension PCR with the OmpA01F and OmpA02R primers. The resulting 2,075 bp DNA fragment, in which the upstream and downstream regions of the ompA gene were sequentially assembled, was ligated with XbaI-SpeI-digested pOH1 to generate pOH2 (Table 2). The correct assembly of the plasmids was confirmed by DNA sequencing. The plasmid pOH2 was integrated into the chromosome of A. nosocomialis by using conjugation-based gene transfer and homologous recombination. The E. coli S17-1 λ pir tra strain carrying pOH2 was used as a conjugational donor to A. nosocomialis. The conjugation and isolation of the transconjugants were conducted as follows: donor and recipient strains were grown in LB broth until late log phase (OD of 0.8 at A₆₀₀). Bacterial cells were then mixed at an equal ratio and spotted onto an LB plate, and incubated for 12 h at 30°C. The bacteria were resuspended into LB broth and then plated on LB agar plates containing ampicillin and kanamycin to eliminate the donor strain and to select the me- diploid strain produced by first single cross-over homologous recombination. For plasmid excision, second single crossover homologous recombination of the bacteria was achieved by the sacB confering sucrose sensitivity on LB agar plates with 10% sucrose and without NaCl. Bacteria susceptible to kanamycin were selected, and the deletion of the ompA gene was confirmed by PCR analysis.

**Complementation of the ompA gene in the ΔompA mutant**

For complementation of the ompA gene in the mutant strain, the ompA coding region under control of the blaCTX-M14 gene promoter with the kanamycin-resistance cassette was constructed by overlap extension PCR. The primers were designed as follow: A forward primer, OmpA03F, and reverse primer, OmpA03R, for amplification of the ompA coding region were designed to contain additional 25 nucleotides at their 5’-end that are homologous to the blaCTX-M14 gene promoter and kanamycin-resistance cassette, respectively (Table 1). The blaCTX-M14 gene promoter, ompA coding region, and nptI were amplified from the synthetic DNA fragment containing the blaCTX-M14 gene promoter, the genomic DNA of A. nosocomialis ATCC 17903T, and pUC4K using the primer pairs CtxF/CtxR, OmpA03F/OmpA03R, and U1/U2, respectively (Table 1). The three PCR products were mixed at equimolar concentrations and subjected to overlap extension PCR with the OmpA03F and U2 primers. The resulting DNA fragment, in which the blaCTX-M14 gene promoter, ompA coding region, and nptI were sequentially assembled, was ligated with PstI-SphI-digested pWH1266 to generate pOH3 (Table 2). The plasmid pOH3 was transformed into the ompA mutant by electroporation, and kanamycin-resistant colonies were selected.
**Bacterial growth studies**

Overnight cultures of *A. nosocomialis* strains were diluted 1:20 in LB broth and incubated for 48 h at 37°C under static conditions. Bacteria were sampled at the indicated times and CFUs as well as the OD at A_{600} were determined. Bacterial growth studies were performed in duplicate in a total of three independent experiments.

**Preparation of outer membrane proteins and western blotting**

*A. nosocomialis* strains were grown in LB broth to reach late logarithmic phase and were lysed by sonication. Outer membrane proteins were extracted with sodium lauryl sarcosine (Sigma-Aldrich, St. Louis, MO) and recovered by ultracentrifugation at 100,000 × g for 3 h at 4°C. The outer membrane protein profiles were determined by SDS-PAGE using 12% gels. Western blot analysis was performed following SDS-PAGE by incubating the Hybond-ECL membranes (Amersham Pharmacia Healthcare). OMVs were collected by ultracentrifugation.

**Purification of *A. nosocomialis* OMVs and western blotting**

*A. nosocomialis* OMVs were prepared as previously described. Briefly, *A. nosocomialis* strains were grown in 500 ml of LB broth to reach late log phase at 37°C with shaking. Bacteria were removed by centrifugation at 6,000 × g for 20 min at 4°C. The culture supernatants were filtered using a QuiXStand Benchtop System (GE Healthcare, Buckinghamshire, UK) using a 0.2 µm-sized hollow fiber membrane (GE Healthcare) and then concentrated using a 100-kDa hollow fiber membrane (GE Healthcare). The protein concentration was determined using a modified BCA assay (Thermo Scientific, Waltham, MA). The purified OMVs were streaked onto blood agar plates to check sterility and stored at −70°C until use. Western blot analysis was performed to detect OmpA in the OMVs. The purified *A. nosocomialis* OMVs were separated via SDS-PAGE on 12% gels, followed by electrotransfer onto the Hybond-ECL membranes. The membranes were incubated with a polyclonal anti-mouse AbOmpA immune serum and then incubated with a secondary antibody coupled to horseradish peroxidase. The OmpA signals were detected using the ECL plus system.

**Identification of proteins associated with OMVs**

Proteins in the OMVs purified from the ΔompA mutant were identified using 1-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry (1-DE-LC-MS/MS) as previously described. All MS and MS/MS spectra were acquired in data-dependent mode. The MS/MS spectra were analyzed with MASCOT software (Matrix Science, Boston, MA) using all *A. nosocomialis* genome data from NCBI (http://www.ncbi.nlm.nih.gov/) and the decoy sequence database. The locations of proteins were predicted using a subcellular location prediction program, Cello version 2.5 (http://cello.life.nctu.edu.tw/).

**Biofilm assay**

A biofilm formation assay was performed for all *A. nosocomialis* strains as previously described. In brief, overnight cultures were adjusted to OD 2.0 at A_{600} and diluted 200-fold in LB medium containing 0.5% NaCl. Aliquots (1 or 2 ml) of the bacterial suspension were inoculated into 5 ml polystyrene tubes and incubated without shaking at 30°C for 48 h. Planktonic cells were removed and then the tubes were washed twice with 1 ml of PBS. The biofilms on the wall were stained with 0.1% (wt/vol) crystal violet solution for 15 min at room temperature. The biofilms were quantified at A_{570} using biofilm cell-associated dye, which was eluted with 100% ethanol, and normalized to total bacterial growth at A_{600}. The biofilm assay was performed in triplicate in a total of three independent experiments.

**Cell viability**

The cytotoxicity of *A. nosocomialis* infection in A549 cells was measured using flow cytometric analysis as previously described. Briefly, cells were infected with the wild-type, ΔompA mutant, and ompA complemented strains at an MOI of 1, 10, or 100 for 16 h. The adherent and detached cells were collected and resuspended in PBS containing 5% FBS. Cells were stained with PI and the DNA content of 9,000 cells per sample was assessed in a FACSCalibur flow cytometry (BD Biosciences, San Jose, CA) by plotting the PI fluorescent intensity in a histogram plot. The cytotoxicity observed for A549 cells and HEP-2 cells after treatment with *A. nosocomialis* OMVs was measured using the Premix WST-1 cell proliferation assay system (Takara Bio, Otsu, Japan). Cells were seeded at a concentration of 2.0 × 10^5 cells/
ml in 96-well microplates. Cells were treated with different concentrations of OMVs from the wild-type, ΔompA mutant, and ompA complemented strains for 24 h and cellular cytotoxicity was measured at 450 nm, 3 h after treatment with WST1. Each experiment was performed in duplicate in minimum three independent experiments.

**Adherence assay**

The adherence evaluation of *A. nosocomialis* strains to A549 cells was performed as previously described. Briefly, A549 cells were seeded at a density of $6 \times 10^4$ cells in 24-well culture dishes. Cells were infected with the *A. nosocomialis* strains at an MOI of 100 for 1 h. The cells were washed five times with PBS, fixed with methanol for 20 min, and stained with Giemsa solution. Bacterial adherence to the cells was determined by light microscopy. The percentage of infected cells (bacteria-associated cells/bacteria-non-associated cells $\times 100$) and the number of bacteria associated with 90 cells were counted. Adherence assay were performed in triplicate in two independent experiments.

**TEM and SEM analysis**

The purified *A. nosocomialis* OMVs were applied to copper grids and stained with 2% uranyl acetate. The samples were visualized on a transmission electron microscope (TEM) (H-7500; Hitachi Ltd, Tokyo, Japan) operating at 120 kV. Biofilm formation of *A. nosocomialis* was examined by SEM (S-4800; Hitachi Ltd). Bacterial strains were statically cultured in 50-ml conical tubes with plastic coverslips semi-immersed in 5 ml of LB broth for 14 h at 30°C. Samples were pulled out of the LB media and processed as previously described.

**Statistical analysis**

The statistical significance of the data was calculated using Student’s t-test. A $P$ value of $<0.05$ was considered to be statistically significant.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI14C0257).

**Author contributions**

All authors contributed to the design of the experiments, analysis of the data, and writing of the manuscript. SWK and KHK performed the biofilm formation and bacterial adherence assays. MHO constructed the $ompA$ deletion mutant and its complemented strain and performed biofilm assay and SEM analysis. SHJ and HJ performed cytotoxicity assay. SIK and YCL performed proteomic analysis.

**References**

[1] Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nat Rev Microbiol 2007; 5:939-51; PMID:18007677; http://dx.doi.org/10.1038/nrmicro1789

[2] Peleg AY, Seifert H, Paterson DL. Acinetobacter baumannii: emergence of a successful pathogen. Clin Microbiol Rev 2008; 21:538-82; PMID:18625687; http://dx.doi.org/10.1128/CMR.00058-07

[3] Nemec A, Krizova L, Maixnerova M, van der Reijden TJ, Deschaght P, Passet V, Vaneechoutte M, Brisse S, Dijkshoorn L. Genotypic and phenotypic characterization of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex with the proposal of Acinetobacter pittii sp. nov. (formerly Acinetobacter genomic species 3) and Acinetobacter nosocomialis sp. nov. (formerly Acinetobacter genomic species 13TU). Res Microbiol 2011; 162:393-404; PMID:21320596; http://dx.doi.org/10.1016/j.resmic.2011.02.006

[4] Wang J, Ruan Z, Feng Y, Fu Y, Jiang Y, Wang H, Yu Y. Species distribution of clinical Acinetobacter isolates revealed by different identification techniques. PLoS One 2014; 9:e104882; PMID:25120020; http://dx.doi.org/10.1371/journal.pone.0104882

[5] Lee YC, Huang YT, Tan CK, Kuo YW, Liao CH, Lee PtdIns, Hsueh PR. Acinetobacter baumannii and Acinetobacter genospecies 13TU and 3 bacteraemia: comparison of clinical features, prognostic factors and outcomes. J Antimicrob Chemother 2011; 66:1839-46; PMID:21653602; http://dx.doi.org/10.1093/jac/dkr200

[6] Schleicher X, Higgins PG, Wisplinghoff H, Körber-Irpgang B, Kresken M, Seifert H. Molecular epidemiology of Acinetobacter baumannii and Acinetobacter nosocomialis in Germany over a 5-year period (2005-2009). Clin Microbiol Infect 2013; 19:737-42; PMID:23034071; http://dx.doi.org/10.1111/1469-0691.12026

[7] Chusri S, Chongsuvivatwong V, Rivera JL, Silpapojakul K, Singhkhamanan K, McNeil E, Doi Y. Clinical outcomes of hospital-acquired infection with Acinetobacter nosocomialis and Acinetobacter pittii. Antimicrobial Agents Chemother 2014; 58:4172-9; PMID:24820079; http://dx.doi.org/10.1128/AAC.02992-14

[8] Lee YT, Huang LY, Chiang DH, Chen CP, Chen TL, Wang FD, Fung CP, Siu LK, Cho WL. Differences in phenotypic and genotypic characteristics among imipenem-non-susceptible Acinetobacter isolates belonging to different genomic species in Taiwan. Int J Antimicrob
Chiang MC, Kuo SC, Chen SJ, Yang SP, Lee YT, Park KH, Shin JH, Lee SY, Kim SH, Jang MO, Kang SJ, Dijkshoorn L, Van Harsselaar B, Tjernberg I, Bouvet PJ, Chang HC, Wei YF, Dijkshoorn L, Vaneechoutte M, Chang TL, Fung CP. Clinical characteristics and outcomes, carbapenem resistance, and outcome of Acinetobacter genomic species. Syst Appl Microbiol 1998; 21:33-41; PMID:938786; http://dx.doi.org/10.1034/j.1230-969X.1998.990201.x

Chung YC, Sheng WH, Li SY, Lin YC, Wang JT, Chen YC, Chang SC. Influence of genospecies of Acinetobacter baumannii complex on clinical outcomes of patients with Acinetobacter bacteremia. Clin Infect Dis 2011; 52:352-60; PMID:21933494; http://dx.doi.org/10.1093/cid/ciq154

Karah N, Haldorsen B, Hegstad K, Simonsen GS, Sundsfjord A, Samuelsen Ø. Species identification and molecular characterization of Acinetobacter spp. blood isolates from Norway. J Antimicrob Chemother 2011; 67:738-44; PMID:21933175; http://dx.doi.org/10.1093/jac/dka552

Gundi VA, Dijkstraorn L, Burignat S, Raoul D, La Scola B. Validation of partial rpoB gene sequence analysis for the identification of clinically important and emerging Acinetobacter species. Microbiol 2009; 155:2333-41; PMID:1938786; http://dx.doi.org/10.1099/mic.0.026054-0

Chang HC, Wei YF, Dijkstraorn L, Vaneechoutte M, Tang CT, Chang TC. Species-level identification of isolates of the Acinetobacter calcoaceticus-Acinetobacter baumannii complex by sequence analysis of the 16S-23S rRNA gene spacer region. J Clin Microbiol 2005; 43:1632-9; PMID:15814977; http://dx.doi.org/10.1128/JCM.43.4.1632-1639.2005

Janssen P, Maquelin K, Coopman R, Tjernberg I, Bouvet P, Kersters K, Dijkstraorn L. Discrimination of Acinetobacter genomic species by AFLP fingerprinting. Int J Syst Bacteriol 1997; 47:1179-87; PMID:936926; http://dx.doi.org/10.1099/00221229-47-4-1179

Dijkstraorn L, Van Hasselaar B, Tjernberg I, Bouvet P, Vaneechoutte M. Evaluation of amplified ribosomal DNA restriction analysis for identification of Acinetobacter genomic species. Syst Appl Microbiol 1998; 21:33-9; PMID:9786720; http://dx.doi.org/10.1016/S0176-1633(98)80006-4

Park KH, Shin JH, Lee SY, Kim SH, Jang MO, Kang SJ, Jung SI, Chung EK, Ko KS, Jang HC. The clinical characteristics, carbapenem resistance, and outcome of Acinetobacter bacteremia according to genospecies. PLoS One 2013; 8:e65026; PMID:23755171; http://dx.doi.org/10.1371/journal.pone.0065026

Chiang MC, Kuo SC, Chen SJ, Yang SP, Lee YT, Chen TL, Fung CP. Clinical characteristics and outcomes of bacteremia due to different genomic species of Acinetobacter baumannii complex in patients with solid tumors. Infection 2011; 40:19-26; PMID:21887526; http://dx.doi.org/10.1007/s15010-011-0187-4

Lee SY, Shin JH, Kim SH, Shin MG, Suh SP, Ryang DW. Analysis of Acinetobacter baumannii complex on clinical outcomes of patients with Acinetobacter baumannii bacteremia. Clin Infect Dis 2011; 52:352-60; PMID:21933494; http://dx.doi.org/10.1093/cid/ciq154

Yoon KJ, Ko EM, Park KH, Shin JH, Bae SH, Kim SH, Lee SY. Analysis of Acinetobacter baumannii complex on clinical outcomes of patients with Acinetobacter baumannii bacteremia. Clin Infect Dis 2011; 52:352-60; PMID:21933494; http://dx.doi.org/10.1093/cid/ciq154

Lee SY, Shin JH, Kim SH, Shin MG, Suh SP, Ryang DW. Evaluation of matrix-assisted laser desorption ionization-time of flight mass spectrometry-based VITEK MS system for the identification of Acinetobacter species from blood cultures: comparison with VITEK 2 and MicroScan systems. Ann Lab Med 2015; 35:62-8; PMID:25553282; http://dx.doi.org/10.3343/alm.2015.35.1.62

Chang HC, Wei YF, Dijkstraorn L, Vaneechoutte M, Chang SC, Jang HC. Species identification and molecular characterization of Acinetobacter spp. blood isolates from Norway. J Antimicrob Chemother 2011; 67:738-44; PMID:21933175; http://dx.doi.org/10.1093/jac/dka552

Chung YC, Sheng WH, Li SY, Lin YC, Wang JT, Chen YC, Chang SC. Influence of genospecies of Acinetobacter baumannii complex on clinical outcomes of patients with Acinetobacter bacteremia. Clin Infect Dis 2011; 52:352-60; PMID:21933494; http://dx.doi.org/10.1093/cid/ciq154

Lee SY, Shin JH, Kim SH, Shin MG, Suh SP, Ryang DW. Infection 2011; 40:19-26; PMID:21887526; http://dx.doi.org/10.1007/s15010-011-0187-4

http://dx.doi.org/10.1007/s15010-011-0187-4
[30] Rumbo C, Tomás M, Fernández Moreira E, Soares NC, Carvajal M, Santillana E, Beceiro A, Romero A, Bou G. The Acinetobacter baumannii Omp33-36 porin is a virulence factor that induces apoptosis and modulates autophagy in human cells. Infect Immun 2014; 82:4666-80; PMID:25156738; http://dx.doi.org/10.1128/IAI.02034-14

[31] Nho JS, Jun SH, Oh MH, Park TI, Choi CW, Kim SI, Choi CH, Lee JC. Acinetobacter nosocomialis secretes outer membrane vesicles that induce epithelial cell death and host inflammatory responses. Microb Pathog 2015; 81:39-45; PMID:25778390; http://dx.doi.org/10.1016/j.micpath.2015.03.012

[32] Gaddy JA, Tomaras AP, Actis LA. The Acinetobacter baumannii 19606 OmpA protein plays a role in biofilm formation on abiotic surfaces and the interaction of this pathogen with eukaryotic cells. Infect. Immun 2009; 77:3150-60; PMID:19470746; http://dx.doi.org/10.1128/IAI.00096-09

[33] Jin JS, Kwon SO, Moon DC, Gurung M, Lee JH, Kim SI, Lee JC. Acinetobacter baumannii secretes cytotoxic outer membrane protein A via outer membrane vesicles. PLoS One 2011; 6:e17027; PMID:21386968; http://dx.doi.org/10.1371/journal.pone.0107027

[34] Jun SH, Lee JH, Kim BR, Kim SI, Park TI, Lee JC, Lee YC. Acinetobacter baumannii outer membrane vesicles elicit a potent innate immune response via membrane proteins. PLoS One 2013; 8:e71751; PMID:23977136; http://dx.doi.org/10.1371/journal.pone.0071751

[35] Oh MH, Lee JC, Kim J, Choi CH, Han K. Simple method for markerless gene deletion in multidrug-resistant Acinetobacter baumannii. Appl Environ Microbiol 2015; 81:3357-68; PMID:25746991; http://dx.doi.org/10.1128/AEM.03975-14

[36] Rodríguez-Baño J, Martí S, Soto S, Fernández-Cuenca F, Cisneros JM, Pachón J, Pascual A, Martínez-Martínez L, McQueary C, Actis LA, et al. Biofilm formation in Acinetobacter baumannii: associated features and clinical implications. Clin Microbiol Infect 2008; 14:276-8; PMID:18187832; http://dx.doi.org/10.1111/j.1469-0691.2007.01916.x

[37] King LB, Szwatol E, Swiatlo A, McDaniel LS. Serum resistance and biofilm formation in clinical isolates of Acinetobacter baumannii. FEMS Immunol Med Microbiol 2009; 55:414-21; PMID:19220466; http://dx.doi.org/10.1111/j.1574-695X.2009.00538.x

[38] de Breij A, Gaddy J, van der Meer J, Koning R, Koster A, van den Broek P, Actis L, Nibbering PH, Earl AM, Ward DV, Paterson DL, et al. The success of acinetobacter species; genetic, metabolic and virulence attributes. PLoS One 2012; 7:e46984; http://dx.doi.org/10.1371/journal.pone.0046984

[39] Choi CH, Hyun SH, Kim J, Lee YG, Seol SY, Cho DT, Lee JC. Nuclear translocation and DNase I-like enzymatic activity of Acinetobacter baumannii outer membrane protein A. FEMS Microbiol Lett 2008; 288:62-7; PMID:18783439; http://dx.doi.org/10.1111/j.1574-6968.2008.01323.x

[40] Park JS, Lee WC, Yeo KJ, Ryu KS, Kumaraasiri M, Heseck D, Lee M, Mobashery S, Song JH, Kim SI, et al. Mechanism of anchoring of OmpA protein to the cell wall peptidoglycan of the gram-negative bacterial outer membrane. FASEB J 2012; 26:219-28; PMID:21965596; http://dx.doi.org/10.1096/fj.11-188425

[41] Moon DC, Choi CH, Lee JH, Choi CW, Kim HY, Park JS, Kim SI, Lee JC. Acinetobacter baumannii outer membrane protein A modulates the biogenesis of outer membrane vesicles. J Microbiol 2012; 50:155-60; PMID:22367951; http://dx.doi.org/10.1007/s12275-012-1589-4

[42] Sambrook J, Russell D. Molecular cloning: a laboratory manual, 3rd ed. 2001. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

[43] Simon R, Priefe U, Pühler A. A broad host range mobilization system for in vivo genetic engineering transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1983; 1:784-91; http://dx.doi.org/10.1038/nbt1183-784

[44] Milton DL, O’Toole R, Horstedt P, Wolf-Watz H. Flagellin A is essential for the virulence of Vibrio anguillarum. J Bacteriol 1996; 178:1310-9; PMID:8631707

[45] Hunger M, Schmucker R, Kishan V, Hillen W. Analysis and nucleotide sequence of an origin of DNA replication in Acinetobacter calcoaceticus and its use for Escherichia coli shuttle plasmids. Gene 1990; 87:45-51; PMID:2185139; http://dx.doi.org/10.1016/0378-1119(90)90494-C