Nuclear Translocation of *Acinetobacter baumannii* Transposase Induces DNA Methylation of CpG Regions in the Promoters of *E-cadherin* Gene

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**Abstract**

Nuclear targeting of bacterial proteins has emerged as a pathogenic mechanism whereby bacterial proteins induce host cell pathology. In this study, we examined nuclear targeting of *Acinetobacter baumannii* transposase (Tnp) and subsequent epigenetic changes in host cells. Tnp of *A. baumannii* ATCC 17978 possesses nuclear localization signals (NLSs), 225RKRKRK230. Transient expression of *A. baumannii* Tnp fused with green fluorescent protein (GFP) resulted in the nuclear localization of these proteins in COS-7 cells, whereas the truncated Tnp without NLSs fused with GFP were exclusively localized in the cytoplasm. *A. baumannii* Tnp was found in outer membrane vesicles, which delivered this protein to the nucleus of host cells. Nuclear expression of *A. baumannii* Tnp fused with GFP in A549 cells induced DNA methylation of CpG regions in the promoters of *E-cadherin* (CDH1) gene, whereas the cytoplasmic localization of the truncated Tnp without NLSs fused with GFP did not induce DNA methylation. DNA methylation in the promoters of *E-cadherin* gene induced by nuclear targeting of *A. baumannii* Tnp resulted in down-regulation of gene expression. In conclusion, our data show that nuclear traffic of *A. baumannii* Tnp induces DNA methylation of CpG regions in the promoters of *E-cadherin* gene, which subsequently down-regulates gene expression. This study provides a new insight into the epigenetic control of host genes by bacterial proteins.

**Introduction**

*Acinetobacter baumannii* is an important opportunistic pathogen that causes a variety of human infections in both community and hospitals [1,2]. *A. baumannii* infection causes a high mortality rate in patients with mechanical ventilation and a fatal underlying disease [3]. The fatality of patients infected with *A. baumannii* is primarily due to host factors, but bacterial virulence factors such as biofilm formation [4,5], serum resistance [6,7], bacterial adherence to host cells [8], and host cell death [9,10] are also associated with pathogenic processes and disease development.

Nuclear targeting of bacterial proteins has emerged as a pathogenic mechanism whereby bacterial proteins can directly interact with nuclear molecules or indirectly disturb signal transduction pathways, which result in host cell pathology [11]. To date, very few bacterial proteins, including cytotoxicity distending toxins of Gram-negative bacteria [12–14], IpaH9.8 and OspF of *Shigella* species [13,16], SspH1 of *Salmonella enterica* [17], YopM of *Tersinia* species [18], and a novel nuclear effector (NUE) of *Chlamydia trachomatis* [19], and outer membrane protein A of *A. baumannii* [9], have been found to target the nuclei of host cells and induce cell pathology. However, whole genome analysis revealed that *A. baumannii*, *Escherichia coli*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, and *Shigella sonnei* were found to carry several proteins with nuclear localization signals (NLSs) [20,21]. NLSs are recognized by nuclear transport proteins, importins, and a complex of the NLS-carrying proteins and importins is transported to the nucleus through the nuclear pore complex (NPC) [22,23]. These results suggest that pathogenic bacteria may employ a strategy to target their effector proteins to the nuclei of host cells.

Epigenetic alterations are heritable and reversible changes that alter gene expression without changing the primary DNA sequence and comprise DNA methylation, histone modification, and small, noncoding RNAs [24]. They are involved in transcriptional changes and decisive events determine cell fate and phenotype. DNA methylation occurs on C5 of the cytosine in the dinucleotide CpG sites and closely interacts with histone modifications. In addition, it is required for chromosomal stability, and is a powerful mechanism for maintaining the suppression of...
gene activity. Accumulation evidence indicates that alteration of DNA methylation directly or indirectly contributes to the susceptibility and development of many complex or multifactorial disease [25]. Bacterial infection has recently been shown to induce aberrant DNA methylation of CpG regions in the promoters of host genes, which allow a pathogen to inhibit transcription of host genes. Campylobacter rectus induces hypermethylation in the promoter region of the Igf2 gene [26]. H. pylori infection induces CpG methylation in the promoter regions of mismatch repair and tumor suppressor genes, which are associated with the initiation and progression of gastric cancer [27–29]. In addition to chronic bacterial infection, uropathogenic E. coli also induces DNA methylation in CDKNA2a [p16INK4a] and results in epigenetic down-regulation of this gene in uroepithelial cells [30]. Induction of aberrant DNA methylation and subsequent down-regulation of host genes by bacterial infection are considered to be a new pathogenic mechanism of bacteria.

We previously predicted the NLS-carrying proteins among the open reading frames (ORFs) of A. baumannii ATCC 17978 based on NLS sequences and found that A. baumannii transposase (Tnp) possessed NLSs, RKKRKK, between amino acid positions 225 and 230 [31]. To obtain a better understanding of A. baumannii pathogenesis regarding nuclear targeting of bacterial proteins, we examined secretion of A. baumannii Tnp from bacteria and its delivery to host cells, nuclear targeting of A. baumannii Tnp, and epigenetic changes and gene expression of host cells. We report here that A. baumannii Tnp induces DNA methylation in CpG regions of E-cadherin (CDH1) gene via nuclear targeting, which subsequently down-regulates expression of this gene.

Results

Nuclear targeting of A. baumannii Tnp via NLS sequences

Tnp of A. baumannii ATCC 17978 (NCBI accession no. gi|126640304) was composed of 362 amino acids and was predicted to carry the putative NLSs, 225RKRKRK230 [31]. To determine whether A. baumannii Tnp targeted the nuclei of host cells, the full-length A. baumannii Tnp gene was cloned into pcDNA3.6.2/N-EmGFP-DEST and incubated for 24 h. The subcellular localization of transposase proteins fused with GFP was observed by confocal laser microscopy. Two A. baumannii transposase proteins with NLSs, Tnp1–362 and Tnp1–230, were located in the nuclei of host cells, whereas transposase proteins without NLSs, Tnp1–37 and Tnp1–224, were located in the cytoplasm.

![Figure 1. A. baumannii transposase targets in the nucleus of host cells via NLSs.](https://example.com/figure1.png)

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DNA Methylation of E-cadherin by A. baumannii Tnp

isolate DU202 secreted OMVs [32,33]. Since OMVs derived from A. baumannii and E. coli contained many bacterial proteins that originated from the outer membrane, periplasmic space, inner membrane, and cytoplasm [32–34], we determined whether A. baumannii Tnp was secreted from bacteria via OMVs. A. baumannii ATCC 17978 was cultured in Luria-Bertani (LB) broth and OMVs were purified from the culture supernatants. Transmission electron microscopic (TEM) analysis showed that A. baumannii ATCC 17978 secreted OMVs during in vitro culture (Fig. 2A). To verify the presence of OMVs, bacterial lysates, culture supernatants, and OMVs were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein profiles were different between three samples (data not shown), suggesting that OMVs purified from A. baumannii ATCC 17978 were not bacterial lysates or artifacts. To determine whether A. baumannii ATCC 17978 could secrete Tnp in vitro culture, bacterial culture supernatants were subjected to Western blot analysis using polyclonal anti-mouse A. baumannii Tnp antibody. A. baumannii Tnp, which has a molecular mass of 39.8 kDa, was detected in the bacterial culture supernatant (Fig. 2B). To verify the presence of Tnp in the A. baumannii OMVs, bacterial cell lysates and OMVs were subjected to Western blot analysis. As a control, recombinant...
A. baumannii Tnp, which has a molecular mass of 44.5 kDa, was used. A. baumannii Tnp with a molecular mass of 39.8 kDa was detected in both the bacterial cell lysates and OMVs (Fig. 2C). Next, to determine whether OMVs could deliver A. baumannii Tnp to host cells, COS-7 cells were treated with A. baumannii OMVs and the subcellular distribution of A. baumannii Tnp was analyzed by confocal laser microscopy. A. baumannii Tnp was mainly distributed in the nuclei of COS-7 cells (Fig. 2D). These results suggest that A. baumannii OMVs deliver their component Tnp to the cytoplasm of host cells.

DNA methylation of CpG regions in the promoters of E-cadherin gene and down-regulation of gene expression by nuclear targeting of A. baumannii Tnp

To determine whether nuclear targeting of A. baumannii Tnp induced cellular damage, cells were transfected with plasmid constructs containing the full-length A. baumannii Tnp gene cloned in the pCDNA™6.2/N-EmGFP-DEST and incubated for 48 h. The viability of COS-7 cells transfected with the full-length A. baumannii Tnp gene was slightly increased (126±2.8%) as compared to that of COS-7 cells transfected with the empty destination vector. Expression of A. baumannii Tnp fused with GFP in the nuclei of A549 cells did not induce any morphological change relative to control cells transfected with the destination vector (Fig. 1). To determine whether A. baumannii Tnp induced epigenetic changes in host cells, A549 cells were transfected with plasmid constructs of the full-length A. baumannii Tnp gene cloned in pCDNA™6.2/N-EmGFP-DEST and incubated for 48 h. A549 cells that originated from human lung carcinoma were used because the respiratory tract is the most common infection site of A. baumannii [2]. Genomic DNA was extracted from A549 cells and methylation-specific polymerase chain reaction (MSP) was performed using primers specific for the CpG regions of p16INK4A, hMLH1, and E-cadherin genes, which are involved in inhibiting cell cycle progression, DNA mismatch repair, and adhesion of epithelial cells to one another, respectively [33-39]. A. baumannii Tnp specifically induced DNA methylation of CpG regions in the promoters of E-cadherin gene (Fig. 3A), but not in CpG regions of p16INK4A and hMLH1 (data not shown). To determine whether DNA methylation of CpG regions in the promoter of E-cadherin gene was dependent on nuclear targeting of A. baumannii Tnp, A549 cells were transfected with three mutant clones, Tnp1–37, Tnp1–224, and Tnp1–230, fused with GFP and then MSP specific for the CpG regions of E-cadherin gene was performed. The truncated Tnp1–230 with NLSs induced DNA methylation, whereas the two mutant clones without NLSs, Tnp1–37 and Tnp1–224, did not induce DNA methylation (Fig. 3A). An aberrant DNA methylation in the promoters of genes can down-regulate transcription level. We determined mRNA expression of E-cadherin gene in A549 cells transfected with plasmid constructs of the full-length of A. baumannii Tnp fused with GFP. When transfection efficiency reached to 60–70%, total RNA of cells was harvested and quantitative reverse transcriptase-PCR (qRT-PCR) was performed. As a control, A549 cells were transfected with pCDNA™6.2/N-EmGFP-DEST vector. A. baumannii Tnp down-regulated mRNA expression of E-cadherin gene (0.82±0.16) as compared to the empty destination vector (1.0±0.06) (p<0.05) (Fig. 3B). These results suggest that nuclear targeting of A. baumannii Tnp specifically induces DNA methylation of CpG regions in the promoters of E-cadherin gene and then down-regulates gene expression.

Figure 2. A. baumannii OMVs deliver transposase to the nucleus of host cells. (A) TEM observation of OMVs from A. baumannii ATCC 17978. (B) Detection of A. baumannii transposase in the bacterial culture supernatant. Bacteria were cultured in LB broth and proteins in the culture supernatants were subjected to 12% SDS-PAGE and Western blot analysis using the polyclonal anti-mouse transposase antibody. (C) Secretion of A. baumannii transposase from bacteria via OMVs. Bacterial cell lysates (lane 1), OMVs (lane 2), and recombinant A. baumannii transposase (lane 3) were subjected to 12% SDS-PAGE and Western blot analysis using the polyclonal anti-mouse transposase antibody. (D) COS-7 cells were treated with A. baumannii OMVs (20 µg/ml of protein concentrations) for 24 h. Cells were fixed, permeabilized with Triton X-100, and stained with a mouse anti-A. baumannii transposase polyclonal immune sera, followed by Alexa Fluor 488-conjugated mouse immunoglobulin G (green), DAPI was used to stain the nuclei (blue). Subcellular distribution of A. baumannii transposase was analyzed by confocal microscopy. Analytical sectioning was performed from the top to the bottom of the cells. The figure represents all projections of the sections in one picture.

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Bacterial proteins that target the nucleus of host cells play a crucial role in bacterial pathogenesis. In this study, we demonstrated that *A. baumannii* Tnp is a new bacterial effector that induces DNA methylation of CpG regions in the promoters of *E-cadherin* gene via nuclear targeting. *A. baumannii* Tnp does not only catalyze ‘cut-and-paste’ reactions, which promotes the movement of DNA segments to new sites, but also induces epigenetic modification of host genes. This is the first study to report that the *A. baumannii* protein directly induces epigenetic alteration via nuclear targeting.

Whole genome analysis of bacteria is a highly useful tool to predict nuclear targeting proteins based on NLSs. We identified 34 proteins with the putative NLSs among the 3,367 ORFs of *A. baumannii* ATCC 17978 [31]. Of the *A. baumannii* proteins predicted to carry the putative NLSs, 14 were found to target in the nuclei of host cells. Among the nuclear targeting proteins identified, we selected *A. baumannii* Tnp to determine the DNA methylation of CpG regions in the promoters of genes because several bacterial transposons encoded their own DNA modifying enzymes to regulate gene expression [40]. Aberrant DNA methylation of CpG regions in the promoters of genes allows a pathogen to inhibit or down-regulate transcription of specific genes, which may alter host cell biology. DNA methylation in CpG regions of host genes by bacterial infection has been demonstrated in several previous studies [26,29,30]. Bacterial infection and inflammatory mediators released from host cells have been found to trigger CpG methylation in the promoters of eukaryotic genes [41]. However, specific bacterial molecules that induce DNA methylation in promoters of host genes have not yet been identified.

Nuclear targeting of *A. baumannii* Tnp did not induce cytotoxicity of host cells, although several nuclear targeting proteins of *A. baumannii*, such as transcriptional regulator, 50S ribosomal protein L20, putative transcriptional regulator, and DNA cytosine methyltransferase, induce host cell death [31]. Instead, *A. baumannii* Tnp specifically induced DNA methylation in...
the CpG regions of E-cadherin gene, but not in the CpG regions of p16\(^{N^R\delta}\) and hMLH1. Aberrant DNA methylation in CpG regions of E-cadherin gene was specifically induced by nuclear targeting of A. baumannii Tnp with NLSs, but not induced by cytoplasmic localization of mutant A. baumannii Tnp without NLSs. Our results suggest that A. baumannii Tnp may exert epigenetic alterations of host cells after nuclear targeting. Moreover, we demonstrated that DNA methylation in the CpG regions of E-cadherin gene down-regulates expression of this gene. Expression of E-cadherin gene was significantly different between A549 cells transfected with the empty destination vector and plasmid clones of A. baumannii Tnp fused with GFP. We did not determine the molecular mechanisms of DNA methylation in the CpG regions of E-cadherin gene such as activation of DNA methyltransferases, but this study identified a novel pathogenic mechanism by which bacterial proteins regulate expression of host genes via epigenetic alterations.

There are some variations in methylation frequency of tumor-associated genes in tumors. We used A549 cells originated from human lung carcinoma for DNA methylation of CpG regions in the promoters of tumor-associated genes because the respiratory tract is the most common site for colonization and infection of A. baumannii. E-cadherin and p16\(^{N^R\delta}\) genes are frequently methyated in lung cancer, whereas hMLH1 gene is rarely methylated and its methylation correlates with late stage of lung cancer [42]. It is thus plausible to guess that acute and transient transfection of A. baumannii Tnp cannot induce DNA methylation of hMLH1 gene in A549 cells. E-cadherin is the key components for adherence junctions between epithelial cells, which allow the body to maintain internal homeostasis as a physical barrier [43]. Many pathogenic bacteria can destroy junctional complexes that comprise the protective functions of epithelial cells. In particular, the virulence factors CagA and VacA secreted by H. pylori disrupt the tight and adherent junctions and cytoskeleton architecture, and increase cell proliferation through gene modification, finally contributing to gastric carcinogenesis [44]. Interestingly, E-cadherin also has a growth suppressor function by inducing cell cycle arrest via up-regulation of the cyclin-dependent kinases, p27 [45]. It is thus tempting to speculate that E-cadherin gene may be a good target of A. baumannii Tnp.

We found that A. baumannii Tnp was secreted from bacteria and delivered to host cells via OMVs. To determine the mechanisms underlying secretion and delivery of A. baumannii Tnp to host cells, recognition sites necessary for type I or II secretion systems were searched. However, A. baumannii Tnp did not harbor signal peptides or recognition sites necessary for type I or II secretion systems. A. baumannii does not have type III or IV secretion systems, although several genes encoding type IV secretion systems have been found [46]. Instead, A. baumannii OMVs contained more than 100 proteins derived from the outer membrane, periplasmic space, inner membrane, and even cytoplasm [32,33]. A. baumannii Tnp was not found in the proteome of OMVs from A. baumannii ATCC 19606\(^t\) and DU202 [32,33], but this nuclear targeting protein was identified in OMVs from A. baumannii ATCC 17978 using Western blot analysis in this study. Discrepancy of A. baumannii Tnp in the OMVs is possibly due to limitations in the proteomic analysis or differences between A. baumannii strains.

In conclusion, the present study demonstrated that nuclear targeting of A. baumannii Tnp induces DNA methylation of CpG regions in the promoters of E-cadherin gene and down-regulates expression of this gene. Our study may contribute to a novel pathogenic mechanism by which bacterial proteins directly regulate gene expression of host cells via epigenetic alterations.

### Table 1. Oligonucleotide primers used for methylation-specific PCR.

| Primers and sequences (5' to 3') | Amplicon (bp) | Annealing temperature (°C) | References |
|---------------------------------|---------------|---------------------------|------------|
| Methylated                     |               |                           |            |
| P16 (F): TTATTAGGGGTTGGCCGATCGC | 150           | 58                        | 49         |
| P16 (R): GACCCCGAAGCGCGAACGTTA |               |                           |            |
| hMLH1 (F): AGGTAGAGGTGTTGTTATG | 115           | 58                        | 49         |
| hMLH1 (R): CCTATCGTAACTACCGGG  |               |                           |            |
| E-cadherin (F): TGTAGTACGTTATG  | 112           | 57.5                      | 50         |
| E-cadherin (R): CGA ATA CGA TCG AAT CGA ACC G | | | |
| Unmethylated                    |               |                           |            |
| P16 (F): TTATTAGGGGTTGGCCGATCGC | 151           | 58                        | 49         |
| P16 (R): CAACCCGAACCCCAACCATAA |               |                           |            |
| hMLH1 (F): TTTGTATGATGTTTATAGGT | 124           | 56                        | 49         |
| hMLH1 (R): ACCACCTCATATAAATTACCCACA | | | |
| E-cadherin (F): TGTAGTACGTTATG  | 120           | 57.5                      | 50         |
| E-cadherin (R): ACACCAATACCAATCAAACAAAA | | | |

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### Materials and Methods

#### Bacterial strains and DNA manipulations

A. baumannii ATCC 17978 was grown on blood agar plates at 37°C. Genomic DNA was purified from bacteria cultured in LB broth using a genomic DNA preparation kit (SolGent, Korea) and then used as a template for PCR. E. coli DH5\(^\alpha\) and BL21 (DE3) were used for DNA cloning and production of recombinant proteins, respectively. E. coli strains were grown on blood agar plates or in LB broth at 37°C. Routine DNA manipulations were performed as previously described [47] or according to the manufacturer’s instructions of the reagents used.

#### Cell culture

Two eukaryotic cell lines, COS-7 originating from African green monkey kidney and A549 cells originating from human lung...
carcinoma, were purchased from Korean Cell Line Bank (Seoul, Korea) and used in this study. COS-7 cells were grown in Dulbecco’s modified Eagle’s medium (HyClone) supplemented with 10% fetal bovine serum (FBS; HyClone), 2.0 mM l-glutamine, 100 U/ml penicillin, and 20 μg/ml streptomycin at 37°C in 5% CO2. A. baumannii ATCC 17978 was used to transform E. coli DH5α, and template DNA (100 ng). The PCR products were added and each reaction was further incubated at 37°C for 45 min at room temperature. The mixture was added to 1×107 cells and the cells were incubated in a CO2 incubator for 24 h. Subcellular localization of GFP-tagged proteins was observed using a confocal laser microscope (Carl Zeiss).

Construction and expression of A. baumannii Tnp-GFP fusion proteins

Genomic DNA was purified from A. baumannii ATCC 17978 and used as a template to perform PCR. The Gateway recombinational cloning system was used for these experiments. The specific primer set used for the full-length A. baumannii Tnp was as follows: forward primer-5′-AAA AAG CAG GCT CCA CCA TGA TGG CTG AAT AGC TGG GTT-3′, reverse primer-5′-AGA ACG CTT GCT TCC TTA AAT CCT GAA ATG CAG TTA A-3′. To generate A. baumannii Tnp mutant clones, a forward primer (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAC CAT CGT CTT GAT GTA CAA AGA TGG TTG ATG AAT AGT ATG CAG TTA A-3′) and three reverse primers (5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT ATG AAT TGG TTT CAT GTA CAA AGC AAG TGG GTT AAT TGG TTG TTT TGC GGT TTC GAT TAT T-3′ for Tnp1–224, and 5′-AGA AGA AAG TTT GTA CAA AAA AGC AGG CTC CAC C-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT AAT TGG TTG TTT TGC GGT TTC GAT TAT T-3′ for Tnp1–290) were used. PCR was performed in a total volume of 20 μl containing the following: 1.5 U Platinum Pfx DNA polymerase (Invitrogen, USA), 2 μl of 10× Pfx amplification buffer, 0.3 mM dNTP mixture, 1 mM MgSO4, 0.3 μM of each primer, and template DNA (10 ng). The PCR products were amplified again with the attB adapter primers (5′-GGG GAC AAG TTT GTA CAA AAA AGC AGG CTC CAC C-3′ and 5′-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTT AAT TGG TTG TTT TGC GGT TTC GAT TAT T-3′), which generated the full-length attBl and attB2 sites flanking A. baumannii Tnp ORFs. The Gateway-compatible amplified gene was recombined into the pDONR221 vector (Invitrogen) using the BP reactions. The plasmid pDONR207 was mixed with 2 μl of the attB-linked PCR product in 15 μl of BP reaction mixture containing 3 μl BP Clonase I enzyme mix (Invitrogen). After incubation at 25°C for 60 min, proteinase K (4 μg in 1.5 μl was added and then each reaction was incubated at 37°C for 10 min. BP reaction mixtures were used directly for bacterial transformation. Aliquots (5 μl) of the entry clone were used to transform E. coli DH5α cells (Library Efficiency) and bacteria were plated on LB medium containing 50 μg/ml of gentamicin. A single colony from the transformed plates was tested by colony-PCR with specific primers for the A. baumannii Tnp gene and sequenced using an ABI Prism 3730XL Analyzer (Applied Biosystems). The entry clone was used for the generation of GFP-tagged clones or production of recombinant proteins in a reaction mixture containing 2 μl LR Clonase II enzyme mix (Invitrogen), 150 ng pcDNA3.1/N-EmGFP-DEST vector (Invitrogen) for the GFP-tagged clones or pET160-DEST (Invitrogen) for the production of recombinant proteins, and TE buffer (pH 8.0). After incubation at 25°C for 3 h, proteinase K (1 μg/ml) was added and each reaction was further incubated at 37°C for 10 min. The LR reactions were used to transform E. coli DH5α or BL21 (DE3).

Transfection of the constructed plasmids in host cells

The plasmid constructs obtained from the LR reactions were used to transform E. coli BL21 (DE3) and recombinant proteins were overexpressed after induction with 1 mM of isopropyl β-D-1-thiogalactopyranoside at 37°C for 4 h. Recombinant proteins were purified using a nickel-column (Amersham Biosciences) and endotoxins were removed by polymyxin B-coated beads (Sigma). The protein concentration was determined using a modified BCA assay (Thermo Scientific). Concentrations of endotoxins were determined using a Limulus Amebocyte lysate test kit (Sigma) and the quantity of endotoxin in the recombinant proteins was ≤0.01 ng/mg.

Purification of OMVs

OMVs were prepared from A. baumannii ATCC 17978 as previously described [34,48]. Bacteria were grown in LB broth at 37°C with shaking until the optical density at 600 nm reached 1.0. After removing bacterial cells, culture supernatants were filtered through a 0.2 μm hollow fiber membrane equipped with a QuixStand Benchtop System (GE Healthcare) to remove residual bacterial debris. The samples were then concentrated via ultracentrifugation with a QuixStand Benchtop System using a 500 kDa hollow fiber membrane (GE Healthcare). The OMV fractions were ultracentrifuged at 150,000 × g at 4°C for 3 h. The purified OMVs were resuspended in phosphate-buffered saline (PBS) and checked for sterility. The OMVs were applied to copper grids and stained with 2% uranyl acetate. The OMVs were visualized using a transmission electron microscope (Hitachi, Japan) that was operated at 120 kV.

Western blot analysis

Bacteria were cultured in LB broth for the indicated time periods and then culture supernatants were collected. Proteins in the culture supernatants were precipitated with trichloroacetic acid. Protein concentrations of each bacterial culture supernatant and bacterial cell lysates were quantified using a modified BCA assay (Thermo Scientific). The samples were separated by 12% SDS-PAGE, followed by electrotransfer onto nitrocellulose membranes (Hybond-ECL; Amersham Pharmacia Biotech). Membrane blots were blocked in 5% non-fat skim milk and incubated with a mouse anti-A. baumannii Tnp immune sera, which were produced in our laboratory. The membranes were incubated with a secondary antibody coupled to horseradish peroxidase and developed using an enhanced chemiluminescence system (Amer- sham Pharmacia Biotech).

Cytotoxicity assay

The cellular cytotoxicity was measured using the Premix WST1 cell proliferation assay system (TaKaRa) [10]. Cells were transfected with the destination vector pcDNA3.1/N-EmGFP-DEST and A. baumannii Tnp constructs cloned in the destination vector, and then incubated for 48 h. When transfection efficiency of the cloned plasmids reached to 65–70%, WST1 was added and incubated.
ethidium bromide. Each MSP was repeated at least once to verify the results. PCR products were analyzed on 2% agarose gel and stained with ethidium bromide for the methylated and unmethylated genes, respectively. PCR amplification steps were carried out using the StepOnePlus Real-Time PCR System (Applied Biosystems) according to the manufacturer’s protocol. The specific primer set for E. coli hemin gene (5’TAC TAT GAT GAA GAA GGA GG-3’ and 5’-CGG AAC CGC TTC CTT CAT AG-3’) and glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH: 5’-GAG GAG TGG GTG CTC TG-3’ and 5’-GGG CCT GAC CTG CCG TCT AG-3’) were used. qRT-PCR was performed in a total volume of 20 μl with the following components: 10 μl of SYBR Green master-mix (Applied Biosystems), 2 μl of each forward and reverse primers (0.5 μM final concentration), 2 μl of cDNA (100 ng), and 6 μl of dH2O. The amplification conditions were: initial denaturation (95°C, 10 min), followed by 40 cycles of denaturation (95°C, 15 s), annealing, and extension (60°C, 1 min). Melting curve analysis was used to confirm amplicon specificity. The normalization and quantification of mRNA expression were performed using the StepOne™ Software version 2.2 supplied by the manufacturer.

**Author Contributions**

Conceptualized and designed the experiments: DCM DSK JCL. Performed the experiments: DCM SML JHL. Analyzed the data: DCM CHC DSK SIK. Contributed reagents/materials/analysis tools: SIK. Wrote the paper: DCM DSK JCL.

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