Infection, Genetics and Evolution 67 (2019) 33–37

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Research paper

Distinct role of outer membrane protein A in the intrinsic resistance of *Acinetobacter baumannii* and *Acinetobacter nosocomialis*

Hyo Il Kwon[a,1], Shukho Kim[a,1], Man Hwan Oh[b,1], Minsang Shin[a], Je Chul Lee[a,*]

[a] Department of Microbiology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea
[b] Department of Nanobiomedical Science, Dankook University, Cheonan, Republic of Korea

ARTICLE INFO

Keywords:
A. baumannii
A. nosocomialis
OmpA
Resistance
Efflux pump

ABSTRACT

Acinetobacter baumannii outer membrane protein A (AbOmpA) contributes to the intrinsic resistance of *A. baumannii* through the OmpA-like domain. The present study investigated the role of *Acinetobacter nosocomialis* OmpA (AnOmpA) in the intrinsic resistance of *A. nosocomialis* and compared it with the role of AbOmpA. The minimal inhibitory concentrations (MICs) of antimicrobial agents against wild-type *A. nosocomialis* ATCC 17903, ΔAn*ompA* mutant, and single-copy An*ompA*-complemented strains were determined by performing E-test or agar dilution. Single-copy *ompA* cross-complemented strains were constructed by cross-inserting An*ompA* and Ab*ompA* open reading frames (ORFs) along with their native promoters into ΔAb*ompA* and ΔAn*ompA* mutant strains, respectively, and the MICs of antimicrobial agents against these strains were determined. The ΔAn*ompA* mutant of *A. nosocomialis* was more susceptible to colistin (20.0-fold) and gentamicin (4.8-fold) than the wild-type strain. The MICs of gentamicin and tetracycline against the ΔAn*ompA* mutant did not decrease in the presence of an efflux pump inhibitor. The MIC of trimethoprim against the ΔAn*ompA* mutant harbouring P_{Abompa} and Ab*ompA* ORF increased by > 4.0-fold compared with that against the wild-type strain. However, the MICs of all the tested antimicrobial agents were similar against the wild-type *A. baumannii* ATCC 17978 and ΔAb*ompA* mutant harbouring P_{Anompa} and An*ompA* ORF. These results indicate that AnOmpA contributes to the intrinsic resistance of *A. nosocomialis* similar to AbOmpA. However, AbOmpA and AnOmpA perform different roles in the intrinsic resistance of trimethoprim.

1. Introduction

Two *Acinetobacter* species, namely, *Acinetobacter baumannii* and *Acinetobacter nosocomialis*, are the most commonly isolated species from clinical specimens worldwide and have many similar phenotypic and genotypic traits (Nemec et al., 2011; Wang et al., 2014). Many clinical isolates of *A. baumannii* and *A. nosocomialis* show multidrug resistance to clinically available antimicrobial agents. However, *A. baumannii* is more resistant to antimicrobial agents and is associated with higher mortality in infected patients than *A. nosocomialis* (Chiang et al., 2011; Park et al., 2013). Acquisition of antimicrobial resistance determinants and development of intrinsic resistance through the expression of antibiotic-permeable porins and efflux pumps contribute to the multidrug resistance of *Acinetobacter* species (Chu et al., 2006; Magnet et al., 2001; Sugawara and Nikaido, 2012; Vila et al., 2007).

Outer membrane protein A of *A. baumannii* (AbOmpA) and *A. nosocomialis* (AnOmpA) plays an important role in bacterial pathogenesis both *in vitro* and *in vivo* (Choi et al., 2005; Kim et al., 2016; Sánchez-Encinales et al., 2017; Sato et al., 2017). We recently showed that AbOmpA contributes to the antimicrobial resistance of *A. baumannii* through a possible interaction between its OmpA-like domain and efflux pump systems (Kwon et al., 2017). AnOmpA of *A. nosocomialis* ATCC 17903 (WP_016804646.1) shows 92% amino acid sequence identity to AbOmpA of *A. baumannii* ATCC 17978 (WP_000777879.1). The C-terminus of AnOmpA (amino acid sequences 226-328) is homologous to that of AbOmpA, suggesting that AbOmpA and AnOmpA play similar roles in the development of intrinsic resistance against antimicrobial agents. The present study determined the role of AnOmpA in the antimicrobial resistance of *A. nosocomialis* and investigated differences in the roles of AbOmpA and AnOmpA in inducing intrinsic resistance.

* Corresponding author at: Department of Microbiology, School of Medicine, Kyungpook National University, 680 Gukchaebosang-ro, Jung-gu, Daegu 41944, Republic of Korea.
E-mail address: leejc@knu.ac.kr (J.C. Lee).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.meegid.2018.10.022
Received 28 October 2017; Received in revised form 29 August 2018; Accepted 27 October 2018
Available online 30 October 2018

2. Materials and methods

2.1. Bacterial strains

The present study included *A. nosocomialis* ATCC 17903, its *ompA* deletion (ΔAn*ompA*) OH1 mutant (Kim et al., 2016), and single-copy An*ompA* complemented strain. The cross-complemented ΔAn*ompA* and ΔAb*ompA* mutant strains harbouring Ab*ompA* open reading frame (ORF) and promoter (P_{Abompa}) and An*ompA* ORF and promoter (P_{Anompa}) were constructed by replacing the endogenous ORFs and promoters of these strains, respectively. Bacterial strains and plasmids used in this study are listed in Supplementary Table 1.

2.2. Complementation of the AnompA gene in the ΔAnompA mutant

For single-copy An*ompA* complementation in the ΔAn*ompA* mutant of *A. nosocomialis* ATCC 17903, the An*ompA* ORF with its native promoter was inserted into an *att*Tn7 site located in downstream region of *glmS* on the bacterial genome by a modified markerless gene deletion method (Oh et al., 2015). The An*ompA* ORF with its native promoter, and the upstream and downstream regions of the *att*Tn7 site were amplified from the genomic DNA of *A. nosocomialis* ATCC 17903 using the primer pairs ANompA01F/ANompA01R, ANaTn01F/ANaTn01R, and ANaTn02F/ANaTn02R, respectively (Supplementary Table 2). A kanamycin-resistance cassette was amplified from the pUC4K plasmids using the primer pair U1/U2 (Supplementary Table 2). Four amplicon were subjected to the overlap extension PCR with ANaTn01F and U2 primers. The combined DNA fragment was cloned into pHKD01 to produce pOH5 (Supplementary Table 1). The recombinant plasmid was integrated into the chromosome of the ΔAn*ompA* mutants by using conjugation-based gene transfer and homologous recombination. In the first single cross-over homologous recombination, merodiploids were selected in Luria-Bertani (LB) agar plates containing kanamycin and ampicillin. A second single cross-over homologous recombination of the bacteria was achieved by the *sacB* conferring sucrose sensitivity on LB agar plates with 10% sucrose. The bacteria susceptible to kanamycin were selected, and the insertion of the An*ompA* ORF with its native promoter was confirmed by PCR analysis.

2.3. Single-copy ompA cross-complementation in the ΔompA mutants of A. baumannii and A. nosocomialis

For single-copy *ompA* cross-complementation, each of the *ompA* ORFs with their native promoters of *A. baumannii* ATCC 17978 and *A. nosocomialis* ATCC 17903 was cross-inserted into the *att*Tn*7* site on each bacterial genome by the modified markerless gene deletion method (Oh et al., 2015). For example, the *A. nosocomialis ompA* ORF with its native promoter, and the upstream and downstream regions of the *A. baumannii att*Tn*7* site were amplified from each bacterial genomic DNA using the primer pairs ANompA01F/ANompA02R, ABaTn01F/ABaTn01R, and ABaTn02F/ABaTn02R (Supplementary Table 2). A mixture of three amplicons with the *npt*I gene was used for the overlap extension PCR with ABaTn01F and U2 primers. The combined DNA fragment was cloned into *Fsp*I-digested pHKD01 to generate pOH814 (Supplementary Table 1). *Escherichia coli* S17-1 λ*pir* strain containing pOH814 was used as a conjugal donor to the ΔAb*ompA* mutant. The conjugation and isolation of the transconjugants were accomplished by the procedure described above. Insertion of the targeted DNA fragment was confirmed by PCR analysis. In a similar way, single-copy Ab*ompA* cross-complementation in the ΔAn*ompA* strain was conducted (Supplementary Table 1). The schematic diagram of the construction of the complemented strains was depicted in Supplementary Fig. S1.

2.4. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of antimicrobial agents were determined by performing E-test or agar dilution methods. Antimicrobial agents included aztreonam, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin, imipenem, nalidixic acid, tetracycline, tigecycline, trimethoprim, and vancomycin (bioMérieux, Marcy L'Etoile, France). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains. Results of the antimicrobial susceptibility test for all the examined antimicrobial agents were obtained under conditions in which MICs of these agents for *E. coli* or *P. aeruginosa* reference strains were within the expected range of quality control according to the Clinical Laboratory Standards Institute (CLSI, 2015). In addition, we determined the susceptibility of antimicrobial agents against *A. nosocomialis* strains in the presence of an efflux pump inhibitor by adding 20 mg/L phenylalanine-arginine β-naphthylamide (PAβN; Sigma-Aldrich, St Louis, MO, USA) to Mueller-Hinton agar plates (Pannek et al., 2006).

2.5. Preparation of outer membrane proteins and western blotting

A. baumannii and *A. nosocomialis* strains cultured in LB broth were harvested and bacterial cells were disrupted by ultrasonic treatment. Cell envelopes were isolated by differential centrifugation. Outer membrane proteins were extracted by adding 2% sodium lauryl sarcosine (Sigma-Aldrich) to the cell envelope proteins and recovered by ultracentrifugation at 100,000 × g for 1 h at 4°C. The outer membrane protein profiles were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% gels. Western blot analysis was performed following SDS-PAGE by incubating the nitrocellulose membrane with a polyclonal anti-mouse AbOmpA immune serum (Choi et al., 2005). The membrane was incubated with a secondary antibody coupled to horseradish peroxidase and developed using an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

3. Results and discussion

3.1. Verification of the constructed mutant strains

Deletion or complementation of the *ompA* genes was verified by analysing the expression of each targeted *ompA* gene or OmpA in the outer membrane. Single-copy An*ompA* complementation in the ΔAn*ompA* mutant of *A. nosocomialis* ATCC 17903 exhibited the expected PCR product of the An*ompA* gene and the exact size of AnOmpA in the outer membrane (Fig. 1). The An*ompA* ORF with P_{Anompa} and the Ab*ompA* ORF with P_{Abompa} were inserted in the ΔAb*ompA* mutant of *A. baumannii* ATCC 17978 and the ΔAn*ompA* mutant of *A. nosocomialis* ATCC 17903, respectively. The An*ompA* and Ab*ompA* cross-complemented strains expressed the outer membrane proteins sized with 37.4 kDa and 38.4 kDa in the outer membrane, respectively (Fig. 2).

3.2. Involvement of AnOmpA in the intrinsic resistance of A. nosocomialis

The MICs of the 11 antimicrobial agents against *A. nosocomialis* ATCC 17903, ΔAn*ompA* mutant (OH1) and An*ompA*-complemented (OH4) strains were determined. The ΔAn*ompA* mutant was more susceptible to colistin (20.0-fold) and gentamicin (4.75-fold) than the wild-type strain. However, the MICs of the remaining antimicrobial agents were the same or decreased by ≤2.0-fold against the ΔAn*ompA* mutant strain (Table 1). The MICs of all the examined antimicrobial agents against the An*ompA*-complemented strain were the same as or similar to those against the wild-type strain (0.67- to 1.57-fold), indicating that the complementation of An*ompA* in the ΔAn*ompA* mutant strain restored its antimicrobial susceptibility to that of the wild-type strain. These results suggest that loss of AnOmpA in the outer membrane altered the antimicrobial susceptibility of *A. nosocomialis*.

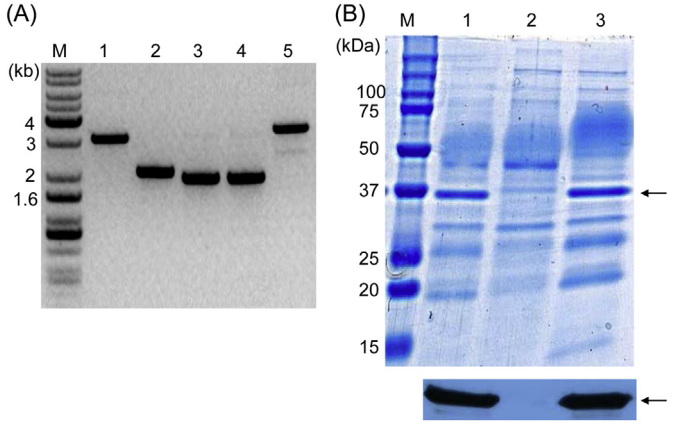

Fig. 1. Construction of *ompA* deletion mutant and *ompA*-complemented strain of *A. nosocomialis*. (A) PCR analysis of the genomic DNA of the wild-type and *ompA* deletion mutant using OmpA01F and OmpA02R primers (lanes 1 and 2). The genomic DNA of *A. nosocomialis* ATCC 17903 and the *ompA* deletion mutant produced the 3.1-kb (lane 1) and 2.1-kb (lane 2) amplicons, respectively. PCR analysis of the genomic DNA of the wild-type, *ompA* deletion mutant and *ompA*-complemented strains using ANompA01F and ANompA01R primers (lanes 3 to 5). Each of the genomic DNAs of the wild-type (lane 3) and *ompA* deletion mutant (lane 4) produced a 1.9-kb amplicon, whereas the PCR using the genomic DNA from the *ompA*-complemented strain (lane 5) resulted in a 3.4-kb amplicon. Lane M, 1-kb DNA molecular marker. (B) SDS-PAGE and western blot analyses of outer membrane proteins prepared from the wild-type, *ompA* deletion mutant, and *ompA*-complemented strains. Protein samples were resolved by SDS-PAGE in 12% gels, transferred to nitrocellulose membranes, and immunoblotted with a polyclonal anti-mouse AbOmpA immune serum. Lane 1, *A. nosocomialis* ATCC 17903; 2, *ompA* deletion mutant of *A. nosocomialis*; 3, *ompA*-complemented strain of *A. nosocomialis*. Arrows indicate the 37.4 kDa AnOmpA.

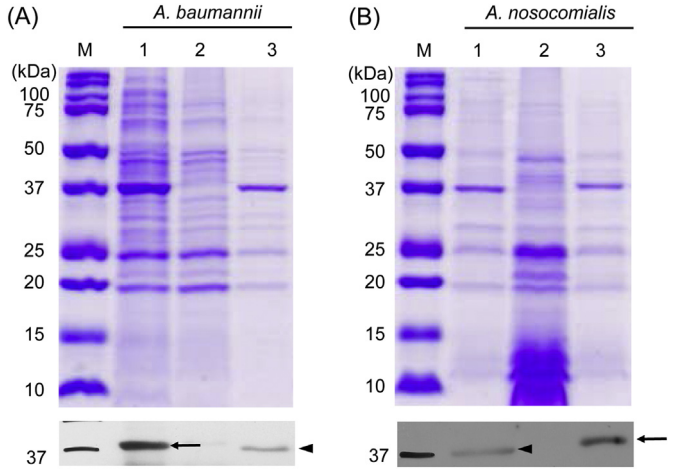

Fig. 2. SDS-PAGE and western blot analyses of outer membrane proteins prepared from *A. baumannii* and *A. nosocomialis* strains. Outer membrane protein samples were resolved by SDS-PAGE in 12% gels, transferred to nitrocellulose membranes, and immunoblotted with a polyclonal anti-mouse AbOmpA immune serum. (A) Lane M, molecular weight marker; 1, *A. baumannii* ATCC 17978; 2, ΔAb*ompA* mutant of *A. baumannii* ATCC 17978; 3, *A. baumannii* ATCC 17978 with P_{AnompA} and An*ompA*. (B) Lane M, molecular weight marker; 1, *A. nosocomialis* ATCC 17903; 2, ΔAn*ompA* mutant of *A. nosocomialis* ATCC 17903; 3, *A. nosocomialis* ATCC 17903 with P_{AbompA} and Ab*ompA*. Arrows and arrow heads indicate the 38.4 kDa AbOmpA and 37.4 kDa AnOmpA, respectively.

3.3. Alteration of cell wall integrity by loss of AnOmpA and its effect on the resistance to colistin

Deletion of the An*ompA* gene may affect the protein composition of the outer membrane, which in turn alters the structure or integrity of the bacterial cell wall. This may affect the non-specific diffusion of various antimicrobial agents or interaction of colistin with the bacterial cell wall. Profiles of outer membrane proteins were different between the wild-type and ΔAn*ompA* mutant strains (Fig. 1B). The most striking finding of the present study was the reduction in the MIC of colistin (20.0-fold decrease) against the ΔAn*ompA* mutant strain. To investigate whether complete loss of AnOmpA in the outer membrane altered the structure or integrity of the bacterial cell wall and subsequently increased the susceptibility of the ΔAn*ompA* mutant strain to colistin, we performed vancomycin-colistin synergy test (Gordon et al., 2010). Vancomycin lacks antimicrobial activity against gram-negative bacteria because of its large size and hydrophilicity. However, the cell-permeabilising property of colistin improves the penetration of vancomycin into gram-negative bacteria (Gordon et al., 2010). The MICs of vancomycin against the wild-type, ΔAn*ompA* mutant, and An*ompA*-complemented strains were 256, 64, and 256 mg/L, respectively. Results of the vancomycin-colistin synergy test showed a significant reduction in the MIC of vancomycin against the wild-type (2.0 mg/L) and An*ompA*-complemented strain (2.0 mg/L). The MIC of vancomycin decreased by > 100-fold (< 0.02 mg/L) against the ΔAn*ompA* mutant. The ΔAn*ompA* mutant strain was more susceptible to colistin (20.0-fold) and vancomycin (4.0-fold) than the wild-type strain in antimicrobial susceptibility test. Moreover, the ΔAn*ompA* mutant strain was more susceptible to vancomycin than the wild-type strain in vancomycin-colistin synergy test. Our results indirectly suggest that loss of AnOmpA in the outer membrane may change the integrity of the bacterial cell wall. However, the integrity of the bacterial cell wall by loss of AnOmpA and its association with the susceptibility to colistin should be determined.

Table 1
The MICs of antimicrobial agents against *A. nosocomialis* strains used in this study.

Antimicrobial agent	MIC (mg/L)[a]			
	P. aeruginosa ATCC 27853	ATCC 17903	OH1 (ATCC 17903 with ΔAn*ompA*)	OH4 (OH1 with An*ompA*)
Aztreonam	2.0	8.0	4.0	7.0
Ceftazidime	1.0	1.5	1.5	1.5
Chloramphenicol[b]	4[c]	64	32	64
Ciprofloxacin	0.75	0.16	0.13	0.16
Colistin	0.5	2.5	0.13[e]	3.5
Tigecycline[b]	0.06[c]	0.5	0.25	0.5
Gentamicin	0.75	0.19	0.04[e]	0.22
Gentamicin + PAβN	ND[d]	0.16	0.04[e]	0.12
Imipenem	1.0	0.09	0.09	0.09
Imipenem + PAβN	ND	0.09	0.09	0.09
Nalidixic acid	2.0[c]	2.0	1.5	3.0
Nalidixic acid + PAβN	ND	1.75	1.0	1.75
Tetracycline	32.0	1.0	0.5	1.5
Tetracycline + PAβN	ND	0.88	0.63	1.75
Trimethoprim	0.50[c]	8.0	12.0	8.0
Trimethoprim + PAβN	ND	10.0	10.0	10.0

[a] MICs were obtained as mean values from duplicate assays in which MICs of the antimicrobial agents against *P. aeruginosa* ATCC 27853 or *E. coli* ATCC 25922 were within the quality control range.
[b] Antimicrobial susceptibility of chloramphenicol and tigecycline was determined by agar dilution methods.
[c] *E. coli* ATCC 25922 was used as a quality control strain.
[d] ND, not determined.
[e] Values indicate ≥4-fold difference in susceptibility between ATCC 17903 and OH1 strains.

Table 2
The MICs of antimicrobial agents against *A. nosocomialis* and *A. baumannii* strains used in this study.

Antimicrobial agent	MIC (mg/L)[a]			
	ATCC 17903	OH1/Ab*ompA* with P_{Abompa}	ATCC 17978	HKD14/An*ompA* with P_{Anompa}
Aztreonam	7.0	8.0	20.0	16.0
Ceftazidime	1.25	1.25	2.5	2.0
Chloramphenicol[b]	64	64	128	128
Ciprofloxacin	0.09	0.09	0.09	0.09
Colistin	2.25	2.25	0.25	0.25
Tigecycline[b]	0.5	0.25	0.5	0.5
Gentamicin	0.25	0.22	0.22	0.22
Imipenem	0.16	0.16	0.18	0.18
Nalidixic acid	3.0	1.75	3.5	3.5
Tetracycline	0.75	0.5	0.75	0.75
Trimethoprim	8.0	> 32.0[c]	> 32.0	> 32.0

[a] MICs were obtained as mean values from duplicate assays in which MICs of the antimicrobial agents against *P. aeruginosa* ATCC 27853 or *E. coli* ATCC 25922 were within the quality control range.

[b] Antimicrobial susceptibility of chloramphenicol and tigecycline was determined by agar dilution methods.

[c] Values indicate ≥4-fold difference in susceptibility between ATCC 17903 and OH1 strain harboring Ab*ompA* with P_{Abompa}.

3.4. Possible association of AnOmpA with efflux pumps

Ab*ompA*-disrupted mutant is more susceptible to efflux pump substrates aztreonam, chloramphenicol, and nalidixic acid than wild-type *A. baumannii* ATCC 17978 (Smani et al., 2014). Moreover, AbOmpA mediates the extrusion of these antimicrobial agents through the outer membrane (Smani et al., 2014). We recently found that AbOmpA contributes to the intrinsic resistance of *A. baumannii* through its OmpA-like domain (Kwon et al., 2017). The OmpA-like domain of AbOmpA may interact with resistance-nodulation-division efflux pumps. To investigate whether AnOmpA was associated with efflux pumps, we determined the MICs of five antimicrobial agents that serve as efflux pump substrates in the presence of PAβN (Table 1). The MICs of gentamicin, nalidixic acid, and tetracycline against the wild-type strain decreased slightly in the presence of PAβN, but the MICs of gentamicin and tetracycline against the ΔAn*ompA* mutant did not decrease in the presence of PAβN. A previous study reported a similar reduction in the MICs of gentamicin, imipenem, and nalidixic acid against *A. baumannii* ATCC 17978 (Kwon et al., 2017). The MIC of nalidixic acid and trimethoprim against the ΔAn*ompA* mutant strain also decreased slightly in the presence of PAβN. Thus, our results suggest that AnOmpA is possibly associated with efflux pumps. However, we did not characterize mechanisms underlying the interaction between AnOmpA and efflux pumps for inducing intrinsic resistance of *A. nosocomialis*.

3.5. Different roles of AbOmpA and AnOmpA in trimethoprim resistance

In the present study, the MIC of trimethoprim against the ΔAn*ompA* mutant strain increased slightly (1.5-fold) in the absence of PAβN; however, no difference in the MIC of trimethoprim was observed against the wild-type ATCC 17903 and ΔAn*ompA* mutant strains in the presence of PAβN (Table 1). The ΔAb*ompA* mutant strain was more susceptible to trimethoprim (MIC, 6.0 mg/L) than the wild-type *A. baumannii* ATCC 17978 (MIC, > 32.0 mg/L) (Kwon et al., 2017). Moreover, *A. baumannii* mutant strain harbouring AbOmpA that lacked the OmpA-like domain was more susceptible to trimethoprim (MIC, 14.0 mg/L) than the wild-type *A. baumannii* ATCC 17978 strain. The MIC of trimethoprim against both the wild-type *A. baumannii* ATCC 17978 strain (MIC, 32.0 mg/L) and ΔAb*ompA* mutant strain (MIC, 12.0 mg/L) decreased in the presence of PAβN (Kwon et al., 2017). These results suggest that AbOmpA and AnOmpA play different roles in trimethoprim resistance. To investigate the different roles of AbOmpA and AnOmpA in intrinsic resistance to antimicrobial agents, we constructed a ΔAn*ompA* mutant strain harbouring P_{Abompa} and Ab*ompA* ORF and a ΔAb*ompA* mutant strain harbouring P_{Anompa} and An*ompA* ORF and lacking their endogenous promoters and ORFs, and determined the MICs of the antimicrobial agents against these strains. Surprisingly, the MIC of trimethoprim against the ΔAn*ompA* mutant strain harbouring P_{Abompa} and Ab*ompA* ORF increased by > 4.0-fold compared with that against the wild-type *A. nosocomialis* ATCC 17903 (Table 2). However, the MIC of trimethoprim against the wild-type *A. baumannii* ATCC 17978 and the ΔAb*ompA* mutant stain harbouring P_{Anompa} and An*ompA* ORF remained unchanged. The MICs of other antimicrobial agents against the wild-type and mutant strains did not changed by ≥2-fold. These results suggest that AbOmpA and AnOmpA play a different role in the intrinsic resistance to trimethoprim.

This is the first study to describe that Δ*ompA* mutant strain of *A. nosocomialis* shows increased susceptibility to aztreonam (2.0-fold), chloramphenicol (2.0-fold), colistin (20.0-fold), gentamicin (4.75-fold), and tetracycline (2.0-fold). The present study highlighted the different roles of AbOmpA and AnOmpA in intrinsic resistance to trimethoprim. AbOmpA plays a role in intrinsic resistance of trimethoprim through the OmpA-like domain (Kwon et al., 2017). However, the present study shows that AnOmpA may not associated with the intrinsic resistance to trimethoprim, although the OmpA-like domain of AnOmpA of *A. nosocomialis* ATCC 17903 is homologous to that in AbOmpA of *A. baumannii* ATCC 179783. The mismatched regions in the amino acid sequences of AbOmpA and AnOmpA are mainly located in extracellular loops. Differences in amino acid sequences, especially in the extracellular loops, between AbOmpA and AnOmpA may account for the different biological functions of these proteins in the pathogenesis of the two *Acinetobacter* species, including toxicity in host cells induced by outer membrane vesicles derived from the bacteria (Kim et al., 2016; Nho et al., 2015). Further studies are needed to characterize functional differences in AbOmpA and AnOmpA associated with antimicrobial susceptibility and bacterial pathogenesis based on the structural analysis of AbOmpA and AnOmpA.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2018.10.022.

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).

Competing interests

None declared.

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