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Proteomic Analysis of Outer Membrane Proteins from *Acinetobacter baumannii* DU202 in Tetracycline Stress Condition

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Acinetobacter baumannii **readily developed antimicrobial resistance to clinically available antibiotics.** ***A. baumannii*** **DU202 is a multi-drug resistant strain, and is highly resistant to tetracycline (MIC>1,024 μg/ml). The surface proteome of** ***A. baumannii*** **DU202 in response to the sub-minimal inhibitory concentration (subMIC) of tetracycline was analyzed by 2-DE/MS-MS and 1-DE/LC/MS-MS to understand the pathways that form barriers for tetracycline. Membrane expression of major outer membrane proteins (Omps) was significantly decreased in response to the subMIC of tetracycline. These Omps with sizes of 38, 32, 28, and 21 kDa were identified as $OmpA_{38}$, $OmpA_{32}$, CarO, and OmpW, respectively. However, transcription level of these Omps was not significantly changed. 1-DE/LC/MS-MS analysis of secreted proteins showed that $OmpA_{38}$, CarO, OmpW, and other Omps were increasingly secreted at tetracycline condition. This result suggests that** ***A. baumannii*** **actively regulates the membrane expression and the secretion of Omps to overcome antibiotic stress condition.**

Keywords**:** *Acinetobacter baumannii* DU202, outer membrane proteins (Omps), multi-drug resistant (MDR)

Acinetobacter baumannii is a non-motile, obligate aerobic Gram-negative bacterium that has emerged as an important nosocomial pathogen (Smith *et al*., 2007). This species causes a variety of human infections, including pneumonia, urinary tract infections, septicemia, and meningitis (Dupont *et al.*, 2005). *A. baumannii* was known to be susceptible to the majority of antibiotics in the 1970s, but readily developed resistance to commonly used antimicrobial agents over time; 73% of *Acinetobacter* spp. from clinical specimens now show multiple resistance against currently available antibiotics (Kuo *et al*., 2004). Hospital outbreaks of multi-drug resistant (MDR) *A. baumannii* strains have been reported across the world (Bergogone-Berezin and Towner, 1996).

Pathogenic determinants and antibiotic resistance of *A. baumannii* were recently characterized by whole-genomic sequencing and comparative genomic studies. Forty-five clustered, resistant genes were identified in the resistance island of MDR *A. baumannii* AYE (Fournier *et al*., 2006). Six islands containing virulence genes were verified in *A. baumannii* ATCC 17978 through target mutagenesis (Smith *et al*., 2007). Based on the accumulation of genome databases of *Acinetobacter* spp., proteomic approaches recently emerged for the elucidation of antibiotic resistance mechanisms (Dupont *et al.*, 2005; Martí *et al*., 2006). The proteome analysis of bacteria in response to various classes of antibiotics leads us to comprehensive understanding of antimicrobial resistance. More specifically, cell wall and membrane proteomic analyses have become hot issues, because the alteration of membrane permeability is important for antimicrobial resistance (Siroy *et al*., 2006). However, very few outer membrane proteins (Omps) of *A. baumannii* strains have been identified and characterized as porins for the influx of drugs (Magnet *et al*., 2001; Limansky *et al*., 2002; Dupont *et al.*, 2005; Mussi *et al*., 2005; Tomás *et al*., 2005).

Tetracycline is a broad-spectrum antimicrobial agent for Gram-positive and Gram-negative bacterial infections. It inhibits bacterial growth by interfering with protein synthesis at the ribosomal level. Although the clinical use of tetracycline has been increasingly reduced by the emergence and wide-scale spread of resistance, a new glycylcycline antibiotic, tigecycline, has recently been developed and used for challenge studies against MDR *A. baumannii* infections. The association of resistance to tetracyclines with drug permeability in the outer membranes has not yet been characterized in *A. baumannii*.

A. baumannii DU202 showed a high-level resistance to tetracycline (MIC>1,024 μg/ml, MIC; minimal inhibitory concentration assay) (Park *et al*., 2006). In the present study, the membrane proteins of MDR *A. baumannii* DU202 in response to tetracycline were analyzed in order to determine the universal pathways that form barriers for tetracycline. The results show that the membrane expression of Omps was decreased in *A. baumannii* DU202 in response to tetra-

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cycline, whereas the secretion of Omps and periplasmic proteins was increased. These findings suggest that *A. baumannii* regulate its Omp expression to overcome antibiotic stress condition.

Materials and Methods

Bacterial strain and growth

A. baumannii DU202 was pre-cultured in Luria-Bertani (LB) broth until the optical density at 600 nm (OD_{600}) reached 0.7∼0.8. The diluted bacteria at approximately 1.0×10^5 CFU/ml were inoculated into LB broth supplemented with 100, 200, and 500 μg/ml of tetracycline. Bacteria were also inoculated into LB broth of 50 μg/ml of imipenem. The bacteria were grown to an OD_{600} of 0.7∼0.8 (living cells of culture media were estimated as approximately 1.0×10^9∼2.0×10^9 CFU/ml) in LB at 30°C with shaking at 180 rpm and used for proteome analysis.

Fractionation of membrane proteins and 2-D gel electrophoresis

Preparation of membrane proteins was performed according to the modified methods from Molloy *et al.* (2000). The pellet was washed three times with 50 mM Tris- HCl (pH 8.0) and centrifuged at 115,000×g for 20 min. Protein content was quantified by BCA method (Krieg *et al*., 2005). The pellet was used for 2-DE analysis. 2-DE analysis was conducted as described previously (Park *et al.*, 2006).

Preparation of secreted proteins

A. baumannii DU202 was grown in Barth's medium at 30°C with shaking until reaching an OD_{600} of 0.7∼0.8. Whole cells and cell debris were removed from the culture media by centrifugation (6,000×g, 10 min) at 4°C. The supernatant was filtered through a 0.45 mm membrane filter, and was then precipitated in 80% ammonium sulfate solution. The supernatant was centrifuged at 10,000×g for 20 min, and the pellets were dissolved in 20 mM Tris-HCl (pH 8.0). The protein solution was extensively dialyzed against 20 mM Tris-HCl (pH 8.0), or desalting was performed using a PD-10 column (GE Healthcare, Sweden). The protein concentration was calculated by BCA methods. The proteins were fractionated in SDS-polyacrylamide gel before protein identification.

Protein identification with MS/MS analysis using ESI-Q-TOF MS

In order to identify the induced proteins in the presence of tetracycline, the selected protein spots were excised from 2D gels and digested with trypsin (Promega, USA) according to the previously described method (Kim *et al*., 2006). Tryptic peptides that had been dissolved with 0.5% TFA solution were used for MS analysis. All MS/MS experiments for peptide sequencing were performed using a Nano-LC/MS system consisting of an Ultimate HPLC system (LC Packings, Netherlands) and a Q-TOF2 mass spectrometer (Micromass, England) equipped with a nano-ESI source. For protein identification, MS/MS spectra were searched by MASCOT (Matrix science, www.matrixscience.com) or were used for manual sequencing by Masslynx software 3.5 (Micromass, England). Obtained sequences were used for a BLAST search in the NCBI bacterial database (www.ncbi. nlm.gov)

Protein identification with 1-DE and LC-MS/MS using LCQ mass spectrometry

The secreted proteins were fractionated by 12% SDS-PAGE and in-gel digested according to the previously described method (Kim *et al*., 2006). Ten microliter of the peptide sample was concentrated on a MGU30-C18 trapping column (LC Packings). Peptides were eluted from the column and directed onto a 10 cm×5 μm i.d. C18 reverse-phase column (PROXEON, Denmark) at a flow rate of 120 nl/min. Peptides were eluted by a gradient of 0∼65% acetonitrile for 70 min. All MS and MS/MS spectra in the LCQ-Deca ESI ion trap mass spectrometer were acquired in a data-dependent mode. Each full MS (m/z range of 400 to 2,000) scan was followed by three MS/MS scans of the most abundant precursor ions in the MS spectrum with dynamic exclusion enabled. Protein identification was performed using Bioworks version 3.1 (Thermo finnigan, USA) and the SEQUEST algorithm.

Acinetobacter data from NCBInr (www.ncbi.nih.gov) was used for the protein identification The DTA files were generated from the MS/MS threshold of a 10,000 peptide mass tolerance of 1.0 Da. The raw output files were filtered and sorted with DTASelect using the following parameters: ΔCN of at least 0.1 and cross-correlation scores (Xcorrs) values of 2.0 for singly-charged ions (+1), 3.0 for doubly-charged ions (+2), and 4.0 for triple-charged ions (+3).

PCR, RNA preparation, and RT-PCR

Primers for amplification of genes encoding Omps were as follows: 5'-ggt aat gct ggt gtt ggt ge-3' and 5'-acg acg gtt cat agc acg ac-3' for $ompA_{38}$; 5'-tga ccg gtg ctc atg ctt at-3' and 5'-tca aaa ccg att gcc atg tt-3' for $ompA_{32}$; 5'-gct tta ctt gct gct ggt gc-3' and 5'-ccg cat tta ctg cct cct ca-3' for *carO*; 5'-aaa ggc gga cca tga ata g-3' and 5'-agc atc agc agg ttg gaa at-3' for *ompW.* PCR was made up denaturation for 5 min at 94°C, 25 cycles of amplification for 30 sec at 94°C, annealing for 30 sec at 50°C, and extension for 40 sec at 72°C, followed by extension for 7 min at 72°C. Sequencing of the amplicons was performed using a BigDye terminator cycle sequencing kit (Applied Biosystems, England). RT-PCR of $ompA_{38}$, $ompA_{32}$, *carO*, and *ompW* was performed according to the previous method (Yoon *et al*., 2007). Total RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN, Germany). For the reverse transcription reaction, a mixture of total RNA (3 μg), an oligo$(dT)_{20}$ (50 nM), and 10 mM dNTP was incubated at 65°C for 5 min. A mixture of 1× RT buffer, 25 nM $MgCl_2$, 0.1 M DTT, and RNaseOUT™ was incubated at 42°C for 2 min. After incubation at 42°C for 2 min, Invitrogen SuperScriptIII (200 U/μl) was added. This mixture was then incubated at 42°C for 50 min followed by 70°C for 15 min. RNase H (2 U/μl) was then added and incubated at 37°C for 20 min in a 9700 Thermocycler (Perkin-Elmer). PCR products ($ompA_{38}$, $ompA_{32}$, *carO*, and *ompW*) were amplified as described before. Promega Go*Taq* Flexi DNA polymerase was used for amplification.

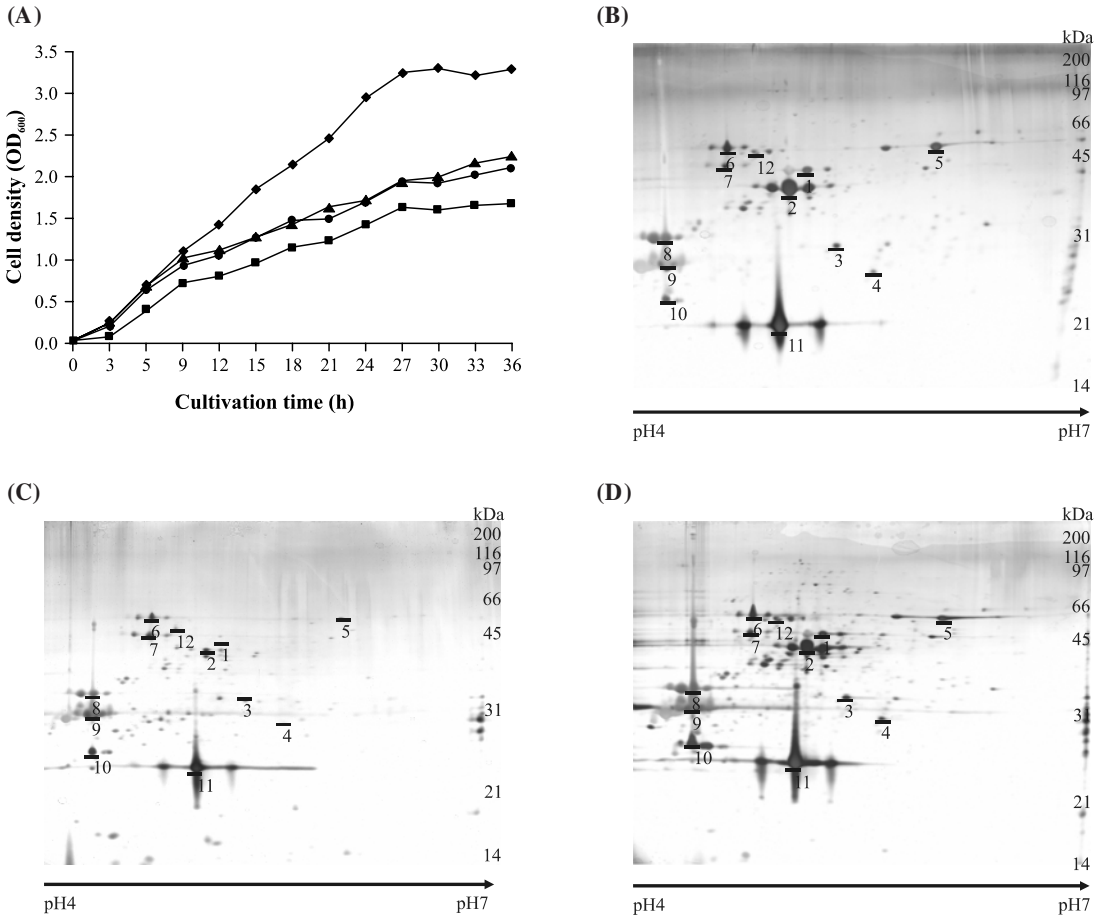

Fig. 1. Growth of *A. baumannii* DU202 in LB medium (◆) supplemented with 100 μg/ml (▲), 200 μg/ml (●), and 500 μg/ml (■) of tetracycline (A). 2-DE of the membrane fraction of *A. baumannii* DU202 cultured in LB medium without antibiotics (B), with 500 μg/ml of tetracycline (C), and with 50 μg/ml of imipenem (D).

Results

Growth inhibition of *A. baumannii* DU202 in the subMICs of tetracycline

A. baumannii DU202 was cultured in LB medium supplemented with tetracycline (100, 200, and 500 μg/ml), and bacterial growth was compared with control bacteria that had been cultured in LB medium. Although the MIC of *A. baumannii* DU202 was >1,024 μg/ml for *in vitro* susceptibility, the growth of *A. baumannii* DU202 in the culture medium supplemented with 100, 200, and 500 μg/ml of tetracycline were depressed as compared with the control bacteria (Fig. 1A). From these results, we selected the subMIC of tetracycline (500 μg/ml) for membrane proteome analysis. We expect that the expression of cell wall components of *A. baumannii* DU202 could be influenced at that concentration.

Proteome analysis of *A. baumannii* DU202 in response to tetracycline

To investigate the protein expression of *A. baumannii* DU202 in response to tetracycline, bacteria that reached the early exponential phase (OD_{A600}, 0.7) were collected, and proteome analysis (soluble and membrane proteins) was then performed. Significant differences in protein levels and expression of each protein in soluble fractions were not found between tetracycline-treated bacteria and -untreated bacteria on the 2-D gels (data not shown). However, protein levels in the membrane fraction from the bacterial cultures with tetracycline were decreased to 81% as compared to those of the bacterial cultures in LB medium. Several abundant protein spots were differentially expressed in response to tetracycline (Fig. 1B and C). Among them, tryptic peptides of 12 protein spots were *de novo* sequenced by MS/MS analysis using the ESI-Q-TOF MS and eleven protein spots were successfully identified by the sequence homology search (Table 1). Eight protein spots were identified for Omps: putative glucose-sensitive porin (spot no. 1), OmpA (spot no. 2, 3, 4, and 8), CarO precursor (spot no. 9 and 10), and putative OmpW (spot no. 11), whereas three protein spots (spot no. 5, 6, and 7) were identified as extracellular proteins. However, significant down-regulation of these Omps was not detected in the imipenem cultured bacteria (Fig. 1D). These results suggest that *A. baumannii* DU202 regulates the expressions of Omps in response to tetracycline.

Table 1. Proteins identified from the membrane fraction of *Acinetobacter baumannii* DU202

Spot no.	pI & Mr (kDa)	Sequence from MS/MS	Identification (Accession no. organism)	Subcellular location[a]	Relative concentration		
					LB	TC	PM
1	4.95 & 40.0	LGVKSFPGEYR VDNMQNLGLVYK LFQPADQTDR	Putative glucose-sensitive porin (OprB-like) (ABO13255, *A. baumannii* ATCC 17978)	Outer membrane	1.00	0.16	1.44
2	4.89 & 38.0	GFAPTDQPLADNK VNEYNVDASR VDGASAGAEYK	Outer membrane protein A (AAR83911, *A. baumannii* ATCC 19606)	Outer membrane	1.00	0.17	1.28
3	5.11 & 20.0	NFYVTVTSDLLTK	Outer membrane protein A (AAR83911, *A. baumannii* ATCC 19606)	Outer membrane	1.00	0.93	0.81
4	5.32 & 28.0	LSEYPNAGDSL	Outer membrane protein A (AAR83911, *A. baumannii* ATCC 19606)	Outer membrane	1.00	0.73	1.01
5	5.62 & 48.0	LGPTTK(Q)LFVSAEK AKQPPVAAPVS AGADVNLYGFVR	Putative (DcaP-like) (ABO 13166, *A. baumannii* ATCC 17978)	Extracellular	1.00	0.32	1.04
6	5.62 & 48.0	FANDYYFPASALK GDLASSSLEK	Putative long-chain fatty acid transport (ABO13185, *A. baumannii* ATCC 17978)	Extracellular	1.00	0.57	1.27
7	5.05 & 42.0	ASGSAFTPALLEVTK NLSFVFG DYYF	Putative long-chain fatty acid transport (ABO13185, *A. baumannii* ATCC 17978)	Extracellular	1.00	1.37	0.97
8	4.18 & 32.0	GATFVGNDSV PALAVGASYMK	Outer membrane protein A (CAJ01528, *Acinetobacter baumannii*)	Outer membrane	1.00	0.82	0.86
9	4.24 & 28.0	APYLGFGFAPK YLDNDYDLTR VVHDSYAFDK	Outer membrane protein CarO precursor (ABG27025, *Acinetobacter baumannii*)	Outer membrane	1.00	0.68	1.08
10	4.24 & 28.0	VHDSYAFDK	Outer membrane protein CarO precursor (ABG27025, *Acinetobacter baumannii*)	Outer membrane	1.00	0.97	1.12
11	4.81 & 21.0	LAPSEDTTTALRVK ADLSPEVHDGCGAK DLNPFVYLNLDNDK	Putative outer membrane protein W (ABO10762, *A. baumannii* ATCC 17978)	Outer membrane	1.00	0.83	1.27
12	4.69 & 45.0	QLAGFDAR PLPYLELQSR	Unidentified Unidentified		1.00	0.25	1.03

[a] PSORTb program

Gene identification of the differentially expressed Omps

Because the Omps were identified by the sequences of two or three tryptic peptides, the sequencing coverage of the identified proteins was low (<13%). In order to verify Omps (protein spots 2, 8, 9, and 11) on the gene level, PCR was performed using degenerate primers, which were designed on the sequences of tryptic peptides and the amplicons were then sequenced. Sequencing result showed that these genes have identical sequences OmpA of *A. baumannii* ATCC 19606^T, 33 to 36 kDa Omp from *A. baumannii*, CarO from *A. baumannii*, and OmpW from *A. baumannii*, respectively.

Transcriptional analysis of the differentially expressed Omps by RT-PCR

To determine whether the reduced expression of Omps from *A. baumannii* DU202 in response to tetracycline was due to the reduction of mRNAs, RT-PCR was performed. Total RNA was prepared from three stages (OD; 0.4, 0.7, and 0.9) of bacterial growth (Fig. 2). However, transcriptional levels of four genes (*ompA$_{38}$*, *ompA$_{32}$*, *carO*, and *ompW*) were not noticeably changed in response to tetracycline.

Secretion of Omps from *A. baumannii* DU202 in response to tetracycline

Several Omps of *Acinetobacter* spp. are known to be secreted in response to chemical stresses, such as alcohols and aromatic compounds (Pessione *et al*., 2003; Walzer *et al*., 2006). To determine whether *A. baumannii* regulated their Omp secretion in response to tetracycline, *A. baumannii* DU202 was cultured in the medium with tetracycline. To minimize

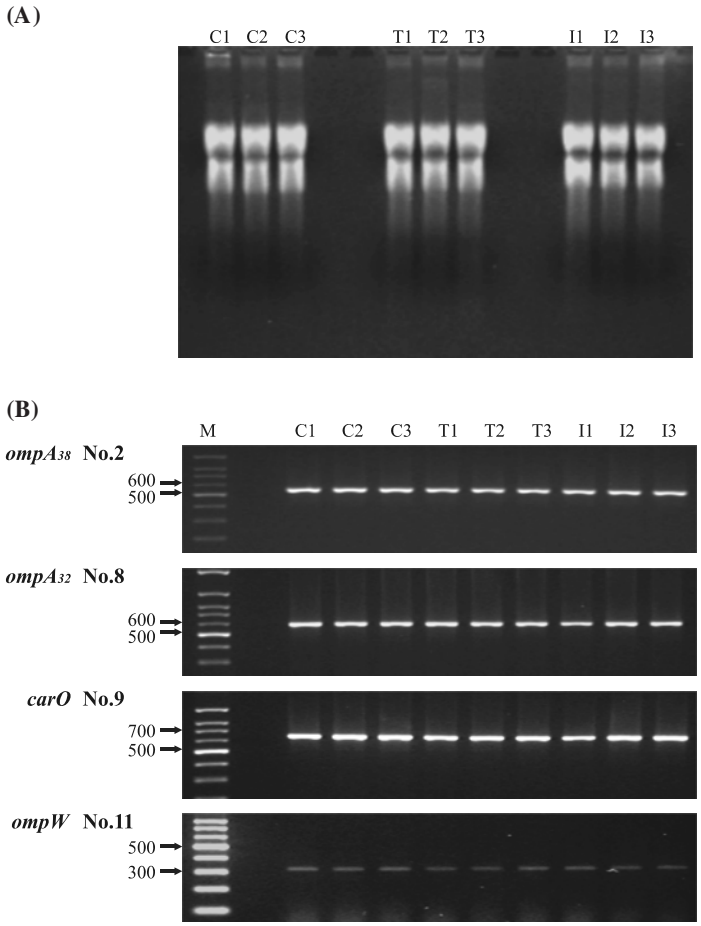

Fig. 2. Extraction of total RNA from *A. baumannii* DU202 cultured in antibiotic-free (C), tetracycline (T), and imipenem (I) using QIAGEN RNeasy Mini Kit (A). The bacteria was harvested at OD 0.4 (C1, T1, I1), 0.7 (C2, T2, I2), and 0.9 (C3, T3, I3). RT-PCR of four *omp* genes (*ompA*$_{38}$, *ompA*$_{32}$, *carO*, and *ompW*) (B).

bacterial disruption, the culture supernatant was collected at the early exponential phase. The secreted proteins from *A. baumannii* DU202 were prepared using two different procedures. To maximize and double-check the identified proteins, we used two different preparation methods. As compared with control cultures, the secreted proteins from *A. baumannii* DU202 cultured in the medium with 500 μg/ml of tetracycline increased by 413% (68.8±7.4 mg/500 ml culture media) in the ammonium sulfate precipitation and dialysis preparation, and by 172% (68.8±7.4 mg/500 ml culture media) in the ammonium sulfate precipitation and desalting column preparation (Fig. 3). However, the secreted proteins from *A. baumannii* DU202 cultured in the medium with 100 μg/ml of tetracycline were not significantly increased as compared with the control cultures. To identify the secreted proteins from *A. baumannii* DU202 in response to tetracycline, LC-MS/MS analysis was applied. Each gel lanes (control and 500 μg/ml of tetracycline) of SDS-PAGE was divided into five fractions according to molecular sizes, and the secreted proteins were subjected to in-gel digestion (Fig. 3). *Acinetobacter* data from NCBInr was used as database. 56, 46, 30, and 30 proteins were identified from each four lanes in Fig. 3 (data not shown). Among them, the Omps and periplasmic proteins are listed in Table 2. In order to identify the major secretory proteins from *A. baumannii* DU202 in response to tetracycline, gel bands T1 through T4 were sliced and analyzed by LCQ MS spectrometry. Analytic result revealed that OmpA$_{38}$ (T1), β-Lactamase (T2), glutamate/aspartate transporter (T3), and hypothetical proteins (T4) were the major secretory proteins produced by *A. baumannii* DU202 in response to tetracycline. The secretion of cytoplasmic proteins, glutamine synthetase, and cofactor-independent phosphoglycerate mutase III (S1) was decreased in *A. baumannii* DU202 in response to tetracycline.

Discussion

We investigated the cellular responses of *A. baumannii* DU202 to tetracycline by performing a proteome analysis. *A. baumannii* DU202 down-regulated the expression of Omps in the outer membranes and up-regulated the secretion of Omps and periplasmic proteins in response to tetracycline. To our knowledge, this is the first study to demonstrate the regulation of Omps in *A. baumannii* in response to tetracycline.

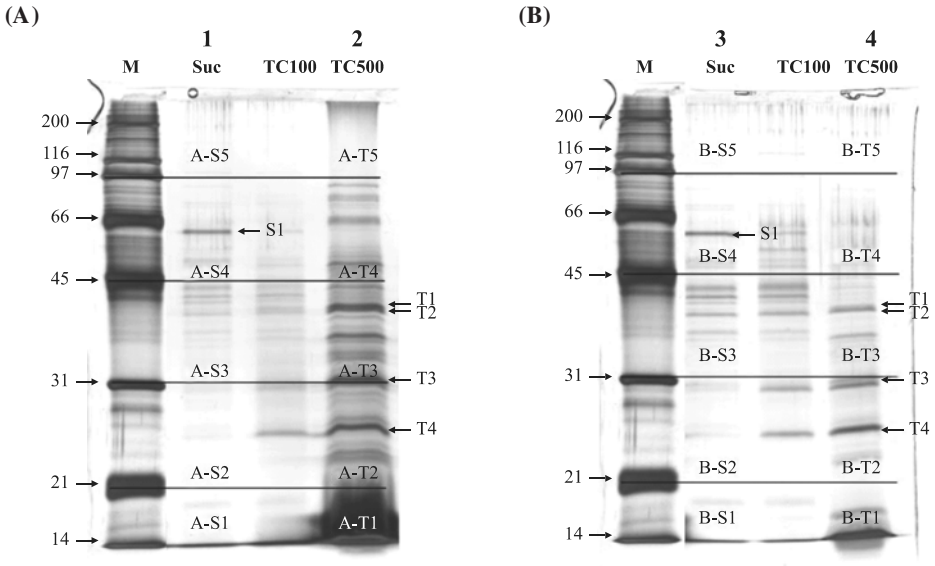

Fig. 3. The secreted proteins from *A. baumannii* DU202 in response to the subMIC of tetracycline. Secreted proteins were prepared by ammonium sulfate precipitation and dialysis (A) and by the ammonium sulfate precipitation and desalting column preparation (B). Suc, succinate culture; TC100, succinate culture including 100 μg/ml of tetracycline; TC500, succinate culture including 500 μg/ml of tetracycline. Numbered four lanes were treated with trypsin and used for LC-MS/MS analysis.

The modification of outer membrane permeability is associated with antibiotic resistance. The role of Omps in antibiotic influx was partially characterized in carbapenem-resistant *A. baumannii* strains (Tomás *et al*., 2005). Expression of Omps, including CarO and 33 to 36 kDa Omp, was significantly decreased with increased resistance to carbapenems in *A. baumannii* strains. Since these Omps form channels and participate in the influx of carbapenems, the loss or reduced expression of these porins influences drug permeability. Resistance to tetracycline antibiotics is usually mediated by substrate-specific *tet* genes in *Acinetobacter* species.

Energy-dependent efflux systems, which are encoded by genes *tet*(A), *tet*(B), and *tet*(H), confer resistance to tetracycline and/or minocycline, whereas *tet*(M) protects ribosomes and confers resistance to both tetracycline and minocycline. Apart from the substrate-specific resistance to tetracyclines, a non-specific resistance mechanism, the AdeABC efflux pump system, was identified in an MDR *A. baumannii* strain (Magnet *et al*., 2001). However, the relationship between outer membrane permeability and resistance to tetracyclines has not yet been characterized. In the present study, we demonstrated that membrane expression of four Omps, especially $OmpA_{38}$, was clearly down-regulated in response to tetracycline (Fig. 1).

$OmpA_{38}$ is the most abundant protein in the outer membranes of *A. baumannii*. Although $OmpA_{38}$ primarily functions as a porin in the outer membranes, it seems to be an important virulence factor in the induction of apoptosis of epithelial cells (Choi *et al*., 2005). In addition, $OmpA_{38}$ is highly homologous to AlnA, which is known as a bioemulsifier protein. Some *Acinetobacter* species secrete AlnA and produce oil-in-water emulsions for efficient biodegradation of polyaromatic hydrocarbons (Walzer *et al*., 2006). In the present study, we demonstrated that $OmpA_{38}$ of *A. baumannii* is related to the resistance of tetracycline, and is down-regulated under tetracycline stress conditions. Interestingly, the truncated $OmpA_{38}$ (protein spots 3 and 4 on the 2-D gel in Fig. 1) was observed in the membrane fractions, and its expression was also decreased in response to tetracycline. Further study is needed to characterize the biological meaning of the truncated $OmpA_{38}$ in the membranes. The loss of the 33 to 36 kDa Omp is known to be associated with resistance to carbapenems. The amino acid sequences of our $OmpA_{32}$ are identical to the 33 to 36 kDa Omp (Tomás *et al*., 2005). The expression of $OmpA_{32}$ was decreased in *A. baumannii* in response to tetracycline. However, RT-PCR and northern blotting (data not shown) result revealed that down-regulation of these Omps in the bacterial membranes was not due to the transcriptional regulation. Since tetracycline can bind to 30S ribosomes, the reduced expression of Omps in the bacterial membranes may be possibly due to the translational regulation. We only identified some major Omps in *A. baumannii* DU202 in response to tetracycline by 2-DE/MS-MS, although many unidentified Omps that are expressed at low levels in the outer membranes may be associated with the permeability of tetracycline. For further searching of the other Omps, more sensitive techniques, such as LC/MS-MS analysis by nano LC-MS spectrometry, should be applied.

Another response of *A. baumannii* to the subMIC of tetracycline is the regulation of protein secretion. Two preparation methods were applied to identify secretory proteins of *A. baumannii* DU202 in response to tetracycline. Some cytoplasmic proteins such as metabolic enzymes or ribosomal proteins were detected in the culture supernatants, but the majority of the secretory proteins were membrane proteins and periplasmic proteins in the preparation of tetracycline-treated bacteria (Table 2). Of the secretory proteins

Table 2. Membrane proteins secreted from *Acinetobacter baumannii* DU202

Preparation	Accession no. and Identified proteins	Subcellular location[a]
Ammonium precipitation and dialysis		
Lane 1		
Succinate media without tetracycline		
	YP_001084684.1, Putative ferric siderophore receptor protein,	Outer membrane
	A3M8K2, OMP38_ACIBT Outer membrane protein omp38 precursor	Outer membrane
	YP_001086288.1, Putative outer membrane protein	Outer membrane
	YP_001083260.1, Cysteine synthase A/O-acetylserine sulfhydrolase A subunit PLP-dependent enzyme	Cytoplasmic Membrane
	YP_001083364.1, Putative outer membrane protein W	Outer membrane
Lane 2		
Succinate media with 500 μg/ml of tetracycline		
	A3M8K2, OMP38_ACIBT Outer membrane protein omp38 precursor	Outer membrane
	YP_001083364.1, Putative outer membrane protein W	Outer membrane
	YP_001084520.1, Glutamate/aspartate transport protein	Periplasmic
	YP_001085388.1, β-lactamase	Periplasmic
	YP_001085787.1, Putative long-chain fatty acid transport protein	Outer membrane
	YP_001085900.1, Putative TonB-dependent receptor protein	Outer membrane
	YP_001086288.1, Putative outer membrane protein	Outer membrane
	YP_001083918.1, Putative outer membrane protein	Outer membrane
	YP_001085557.1, Outer membrane protein CarO precursor	Outer membrane
	YP_001083999.1, Mutarotase precursor	Periplasmic
	YP_001083104.1, Putative Cytochrome b precursor	Periplasmic
Ammonium precipitation and desalting column		
Lane 3		
Succinate media without tetracycline		
	A3M8K2, OMP38_ACIBT Outer membrane protein omp38 precursor	Outer membrane
	YP_001083260.1, Cysteine synthase A/O-acetylserine sulfhydrolase A subunit PLP-dependent enzyme	Cytoplasmic Membrane
Lane 4		
Succinate media with 500 μg/ml of tetracycline		Outer membrane
	A3M8K2, OMP38_ACIBT Outer membrane protein omp38 precursor	Outer membrane
	YP_001083364.1, Putative outer membrane protein W	Periplasmic
	YP_001084520.1, Glutamate/aspartate transport protein	Periplasmic
	YP_001085388.1, β-lactamase	Outer membrane
	YP_001086288.1, Putative outer membrane protein	

[a] PSORTb program

from *A. baumannii* in response to tetracycline, $OmpA_{38}$, CarO, and OmpW which exhibited the reduced expression in the membranes were detected. This result suggests that the subMIC of tetracycline may regulate the membrane expression of these Omps by secretory systems. Moreover, our results showed that ammonium precipitation and dialysis procedures were more powerful than ammonium precipitation and desalting column procedures for determining the secretory proteins. Since small numbers of secretory proteins were identified by ammonium precipitation and desalting column procedures, several proteins were thought to be trapped on the desalting column. The 1-DE/LC/MS-MS analysis was a useful method for secretome analysis of *A. baumannii* strains.

We also investigated the proteome analysis of *A. baumannii* DU 202 in response to the subMIC of imipenem. The growth rate of *A. baumannii* DU202 cultured in LB medium supplemented with 50 μg/ml of imipenem was identical to that of bacteria cultured in LB medium without imipenem. 2-DE results showed that membrane proteomes from bacteria cultured supplemented with imipenem were nearly the same as those of the bacteria cultured in LB without imipenem (Fig. 1D). Since *A. baumannii* DU202 carried two Ambler class D β-lactamases, $bla_{OXA\text{-}23}$ and $bla_{OXA\text{-}51}$ (data not shown), the enzyme degradation of carbapenems is more likely responsible for resistance to carbapenems, instead of drug influx.

In conclusion, our results suggest that resistance to tetracycline is strongly associated with the regulation of Omps in *A. baumannii*. The expression of Omps in cell membranes was significantly decreased in *A. baumannii* in response to tetracycline, whereas the secretion of major Omps was increased. The membrane expression of some Omps that are possibly involved with the permeability of tetracycline may be regulated at translational level or by secretory systems. Our study indicates that proteome analysis of bacterial membrane proteins is powerful technique to extend our understanding of resistance mechanisms in antibiotic-resistant bacteria.

Acknowledgements

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