Diagnosis of β-Lactam Resistance in Acinetobacter baumannii Using Shotgun Proteomics and LC-Nano-Electrospray Ionization Ion Trap Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · FEBRUARY 2013

Impact Factor: 5.64 · DOI: 10.1021/ac303326a · Source: PubMed

6 AUTHORS, INCLUDING:

Chih-Jui Chang
Tzu Chi University
13 PUBLICATIONS **248** CITATIONS
SEE PROFILE

Dr. Rohini Rondla
Osmania University
28 PUBLICATIONS **307** CITATIONS
SEE PROFILE

Anren Hu
Tzu Chi University
25 PUBLICATIONS **247** CITATIONS
SEE PROFILE

analytical chemistry

Article

pubs.acs.org/ac

Diagnosis of β-Lactam Resistance in *Acinetobacter baumannii* Using Shotgun Proteomics and LC-Nano-Electrospray Ionization Ion Trap Mass Spectrometry

Chih-Jui Chang,[†] Jyun-Han Lin,[‡] Kai-Chih Chang,[‡,§] Meng-Jiun Lai,[‡] Rondla Rohini,[‡] and Anren Hu*[,‡]

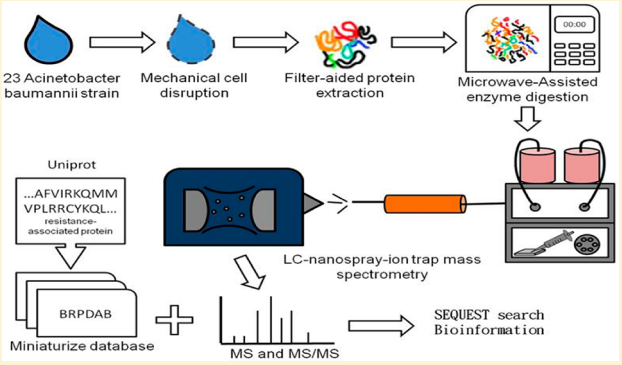
[†]Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien 970, Taiwan

[‡]Department of Laboratory Medicine and Biotechnology, Tzu Chi University, Hualien 970, Taiwan

[§]Buddhist Tzu Chi General Hospital, Hualien 970, Taiwan

Supporting Information

ABSTRACT: *Acinetobacter baumannii* is an important nosocomial pathogen that often affects critically ill patients in intensive care units. β-Lactam antibiotics are the most commonly prescribed drugs for infectious diseases caused by *A. baumannii*. Our aim is to develop an accurate and rapid shotgun proteomics method for the identification of β-lactam-resistant *A. baumannii* pathogens. In the present study, we used automated data-dependent scanning on a nano-LC/ion trap mass spectrometer to characterize proteotypic peptides of *A. baumannii*. Then, we used SEQUEST software to search specific databases, the β-lactam-resistance protein database of *A. baumannii* (BRPDAB). We successfully found a number of associated antibiotic-resistant proteins, including AmpC, β-lactamase, and carO, in clinical resistant strains of *A. baumannii* and differentiated them from wild-type *A. baumannii* strains. We used the results of the search to identify *A. baumannii* pathogens and found a β-lactam-resistant clinical strain of *A. baumannii* using Uniprot annotations, Gene Ontology (GO), and BLAST bioinformatics tools. This proteomic study will provide a platform for the rapid diagnosis of wild-type and resistant strains of *A. baumannii*, which would be useful for the medical treatment of these strains.



In 1968,[1] *Acinetobacter baumannii* was first identified as a gram-negative, aerobic bacteria. It is an important nosocomial pathogen that often affects critically ill patients in intensive care units.[2] β-Lactam-based antibiotics are the most commonly prescribed drugs for *A. baumannii* infectious diseases, and they act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls. However, these drugs are becoming progressively less effective because of the emergence of β-lactam-resistant *A. baumannii*. A major mechanism of β-lactam resistance is the production of a type of hydrolytic enzyme, known as β-lactamase, which disrupts the amide bond of the characteristic four-membered β-lactam ring, thereby decreasing β-lactam efficiency.[3] β-Lactamases can be divided into three main groups according to their functions: (1) cephalosporinases, which are only partially inhibited by clavulanic acid; (2) penicillinases, cephalosporinases, and broad-spectrum β-lactamases, which are inhibited by active-site directed β-lactamase inhibitors; and (3) metallo-β-lactamase, which hydrolyses most β-lactamase antibiotics and is poorly inhibited by almost all β-lactam-containing molecules.[4] Apart from the production of β-lactamases, the resistance to β-lactam antibiotics is often due to the mutation of membrane proteins that reduces the membrane permeability of these antibiotics.[3] Clinical isolates of multidrug-resistant *A. baumannii* have become more significant in hospitals around the world.[5−9] In routine microbiological laboratories, the first step to detect *A. baumannii* infection is to identify the ability of *A. baumannii* to grow on specific types of media; the second step is to test for antibiotic resistance using compounds that inhibit *A. baumannii* growth. However, the traditional phenotypic method for the detection of drug-resistant *A. baumannii* is very time-consuming.

Mass spectrometry provides an alternative approach for identifying microorganisms. Both matrix-assisted laser desorption ionization (MALDI) and electrospray analyzes microorganisms.[10,11] Bisht's group successfully used a proteomic approach to detect streptomycin-resistant *Mycobacterium tuberculosis*.[12] Proteomic analysis also provides a powerful method to study *A. baumannii*.[13] Studies of clinical *A. baumannii* strains have revealed that resistance to antibiotics results from a lack of membrane transport proteins or a change in the amino acid sequences of these proteins.[14−17] Most proteomic analyses are based on two-dimensional gel electrophoresis (2D-PAGE) along with liquid chromatography electrospray ionization mass

Received: November 15, 2012
Accepted: February 2, 2013
Published: February 2, 2013

   dx.doi.org/10.1021/ac303326a | *Anal. Chem.* 2013, 85, 2802−2808

spectrometry (LC-ESI-MS) or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS), which are used to analyze protein expression in biological samples. However, the contamination and reproducibility of 2D-PAGE are important limitations of this technique. Shotgun proteomics has been shown to be more sensitive in whole-cell or membrane protein analyses.[18] Shotgun proteomics primarily involves digesting the whole proteins of cells and separating the resultant peptides by multidimensional protein identification technology (MudPID)[19−21] instead of 2D-PAGE prior to mass spectrometry analysis. Protein digestion is an essential procedure regardless of which proteomic method is used. However, protein digestion is a time-consuming process, usually requiring more than 12 h to complete. A few studies suggest that a microwave-assisted method could significantly reduce the time required for protein digestion.[22−25] Here, we combined microwave-assisted protein digestion and shotgun proteomics to study *A. baumannii*. Our results showed that this combination provided a rapid, sensitive, and specific method to identify *A. baumannii* and detect β-lactam-resistant strains of *A. baumannii*.

MATERIAL AND METHODS

Instrumentation. A Thermo Fisher Scientific LCQ FleetTM (Waltham, MA) Ion Trap Mass Spectrometer with a fused-silica liquid junction tip (pulled in-house) interface was used for nanospray-MS analyses. An Agilent 1200 series capillary LC system and a homemade C18 (150 μm i.d., 15 cm, 5 μm) tip-column were used. All solutions were prepared using analytical grade reagents and Millipore (Bedford, MA, USA) Milli-Q water from a Millipore Milli-Q system.

Bacterial Isolates and Culture. All *Acinetobacter spp.* type strains were obtained from the Bioresource Collection and Research Centre (Hsinchu, Taiwan), and *A. baumannii* ATCC17978 and ATCC19606 strains were used as standard (nonresistant) strains. All clinical isolates were provided by the microbiology departments of hospitals in different areas in Taiwan (Tzu-chi general hospital, Taipei and Hualien, Taichung). Bacteria were grown in Luria−Bertani agar (Becton, Dickinson and company, France) at 37 °C for 18 h.

Minimal Inhibitory Concentration (MIC) Determination. Clinical strains of *A. baumannii* were characterized for their antibiotic sensitivity using a Phoenix 100 ID/AST system (V5.75A/V4.85A) with a NMC/ID-72 panel and EpiCenter microbiology data management system (Becton Dickinson, Sparks, MD, USA). The bacteria were grown in Mueller Hinton agar (Becton, Dickinson and company, France) according to Phoenix manufacturers' protocols. Minimal inhibitory concentration (MIC) determinations were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2009, Wayne, PA, USA).

Protein Extraction. The bacteria were grown in Luria−Bertani agar (Becton, Dickinson and company, France) at 37 °C for 18 h. After the bacterial cells were collected in 1 mL of deionized water, they were harvested by centrifugation at 6000*g* for 15 min at 4 °C. The cell pellets were washed three times, resuspended in 1 mL of deionized water, and thoroughly mixed by vigorous vortexing and disruption using a Bead Beater homogenizer (Biospec Product INC., Oklahoma). The crude cell lysates were centrifuged at 10 000*g* for 15 min at 4 °C. The suspension was then filtered through a 0.45 μm membrane filter (Millipore, Billerica, MA USA) and centrifuged at 15 000*g* for 15 min at 4 °C. The lower solution layer was filtered through a 3000 molecular weight cutoff filter (Millipore, Billerica, MA USA) and centrifuged at 15 000*g* for 80 min at 4 °C. Finally, the upper device was turned upside down and placed in a new tube and then spun for 5 min at 1000*g*. The concentrated proteins were dried via speed-vacuum and stored at −20 °C. The extraction was performed according to methods described in prior studies.[26,27]

In-Solution and Microwave-Assisted Digestion. The proteolytic digestion step was performed according to a previous study.[28] The protein samples were dissolved in 50 μL of 50 mM ammonium bicarbonate. To break the disulfide bonds, the samples were reduced for 5 min at 95 °C in 50 mM ammonium bicarbonate containing 5 mM dithiothreitol and then alkylated for 50 min in the dark by adding 15 mM iodoacetamide. Sequencing-grade modified trypsin (Promega, Madison, WI) was added at an enzyme/substrate ratio of 1:50 (w/w) and allowed to digest the proteins for 15 min in a domestic microwave at 700 W. The digested peptides were dried via speed-vacuum and stored at −20 °C.

LC-nanoESI-MS/MS. The peptide sample was dissolved in 50 μL of deionised water, and 1 μL was injected into the LC-MS system by an autosampler. The homemade C18 tip-column was placed in-line with a Thermo LCQ ion trap mass spectrometer and interfaced with the capillary LC system. The two mobile phases used were as follows: buffer A (100% H_2O, 0.1% formic acid) and buffer B (100% acetonitrile, 0.1% formic acid). Peptides were eluted using a gradient of 0−100% buffer B for 100 min. MS/MS spectra were acquired in data-dependent scanning mode with one full scan followed by four MS/MS scans on the most intense precursor, and the dynamic exclusion of previously selected precursors was enabled for a period of 3 min.

Database Setting. The database in the Uniprot Web site is used to identify β-lactam resistance-associated proteins of *A. baumannii*. On the basis of gene ontology (go)[29] terms and previously identified β-lactam resistance proteins,[3,30] the keywords used to search are: go:0008800 OR go:0046677 OR go:0016999 OR go:0008658 OR name:lactamase OR name:imipenem OR name:carbapenem OR name:penicillin OR name:antibiotic OR name:cephalosporin OR gene:bla OR gene:oxa OR gene:blaADC and organism: "*A. baumannii*". The search results were then saved as a FASTA file, which consists of all the β-lactam resistance related proteins but not the *A. baumannii* specific proteins. The nonredundant NCBI database (2010/0907) search results of the *A.baumanii* ATCC17978, ATCC19606, and MDRAB1 by shotgun proteomics experiment were used to identify the specific peptides of *A. baumannii* (see labeled peptides (× with four ·) in Supplementary Table S1, Supporting Information). There are about 11 *A. baumannii* specific peptides that were identified. BLAST was then performed to search proteins that contain any of the 11 *A.baumannii* specific peptides. The search results were then incorporated into the FASTA files and abbreviated as BRPDAB database (*β-lactam resistance protein database of A. baumannii)*. The BRPDAB database now contains all the β-lactam resistance related proteins and *A. baumannii* specific proteins.

If any of the 11 *A. baumannii* specific peptides are found after searching from BRPDAB, we can identify that the bacteria is *A. baumannii*. Moreover, if any drug resistance proteins (AmpC, OXA, ...) are found from the same searching results from BRPDAB, it can also provide the information that the *A. baumannii* is β-lactam resistant. It is noteworthy that, if the

dx.doi.org/10.1021/ac303326a | *Anal. Chem.* 2013, 85, 2802−2808

Table 1. β-Lactam Resistance in *A. baumannii* Strains

β-lactam			MIC[a] (μg/mL)									
group	drug		A[b]	SIR[c]	B[d]	SIR	17978	SIR	19606	SIR	MDRAB1	SIR
penicillin	ampicillin	AM	>16	R	ND	ND	8	R	>16	R	>16	R
cephalosporins 3rd	ceftazidime	CAZ	>16	R	≥64	R	4	S	8	S	>16	R
cephalosporins 4th	cefepime	FEP	>16	R	≥64	R	8	S	16	R	ND	ND
monobactam	aztreonam	ATM	>16	R	≥64	R	16	R	16	R	>16	R
carbapenem	imipenem	IMP	>8	R	ND	ND	≤1	S	≤1	S	>8	R
carbapenem	meropenem	MER	>8	R	>8	R	≤1	S	≤1	S	>8	R
penicillin + inhibitor	piperacillin-tazobactam	TZP	>64/4	R	≥128/4	R	≤4/4	S	≤4/4	S	>64/4	R
penicillin + inhibitor	ampicillin-sulbactam	SAM	ND	ND	≥32/8	R	ND	ND	ND	ND	ND	ND

[a]S: susceptible; I: intermediate; R: resistant. [b]10 clinical strains collected from Tzu chi general hospital, Hualien. [c]Drug resistance identified by the Phoenix 100 ID/AST system used in hospitals. [d]10 clinical strains collected from Tzu chi general hospital, Taipei and Taichung.

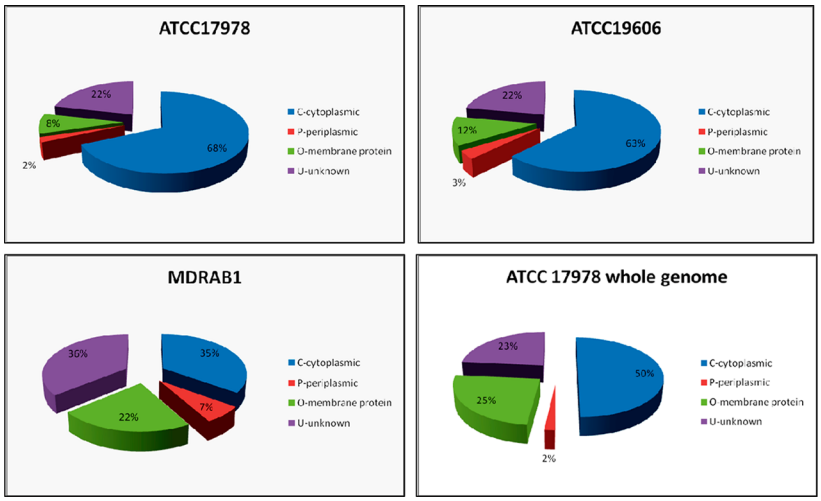


Figure 1. Protein distribution profiles of the analyzed bacteria strains. Shotgun proteomic results were analyzed using a nonredundant NCBI database (2010/0907). The protein distribution profiles of ATCC17978 (A), ATCC19606 (B), and MDRAB1 (C) were analyzed by PSORTb 3.0 software. (D) Predicted proteins from the entire genome of ATCC17978.

peptide sequence is VNNQDFIAGADGVK of CarO rather than NYQQAVPGQEGGVR, it is drug resistant.

Data Analysis and Bioinformatics Tools. MS/MS spectra were analyzed using SEQUEST software to search the BRPDAB database. The oxidation of methionine and the carbamidomethylation of cysteine were considered protein modifications. In the data-dependent experiments, the search tolerances were set as previously described.[28] To analyze the location of all the proteins, we used the publicly available software PSORTb 3.0[31] (www.psort.org). We also used BLAST to align different types of carbapenem-associated resistant outer membrane proteins (CarO), and the PRED-TMBB software predicted the CarO trans-membrane β-barrel secondary structure.[32,33]

RESULTS

β-Lactam Resistance in *A. baumannii* Strains. To test for β-lactam resistance in *A. baumannii* strains, the Phoenix 100 ID/AST system (V5. 75A/V4. 85A) was used to analyze the minimum inhibitory concentrations for different antibiotics (Table 1). Two standard strains, ATCC17978 and ATCC19606, were sensitive to carbapenem and to a combination of penicillin and β-lactamase inhibitors. MDRAB1 and clinical isolates were poorly inhibited by β-lactamase inhibitors.

Identification of *A. baumannii* by Shotgun Proteomics Methods. Two standard strains (ATCC17978 and ATCC19606) and a multidrug resistant strain (MDRAB1) were used for the shotgun proteomics experiments. Their mass spectrometry profiles were used to search the nonredundant database (Supplemental Table S1, Supporting Information). The peptide fragments were analyzed by BLAST to identify their specificity for *A. baumannii*. Eleven specific peptide-associated sequences were selected as signature fragments from the specific protein-associated sequence of *A. baumannii*. The specific proteins were incorporated into the early FASTA file to build a more specific database, BRPDAB (see Supporting Information, Supplemental Table S2).

To determine whether using BRPDAB is feasible for the identification of *A. baumannii*, the mass spectra of three *A. baumannii* strains (ATCC17978, ATCC19606, and MDRAB1) and three different species of *Acinetobacter* (*Acinetobacter sp.* ADPI (ATCC33305), *Acinetobacter calcoaceticus* (BCRC11562), and *Acinetobacter* Genomic species 3 (BCRC15420)) were analyzed using BRPDAB. The specific peptides of *A. baumannii* were identified in the *A. baumannii* strains in the BRPDAB search (Supplemental Table S3, Supporting Information). However, no specific peptides of *A. baumannii* were identified in other species of *Acinetobacter* (Supplemental Table S4, Supporting Information). Supplemental Table S5 (Supporting Information) shows a comparison

dx.doi.org/10.1021/ac303326a | *Anal. Chem.* 2013, 85, 2802−2808

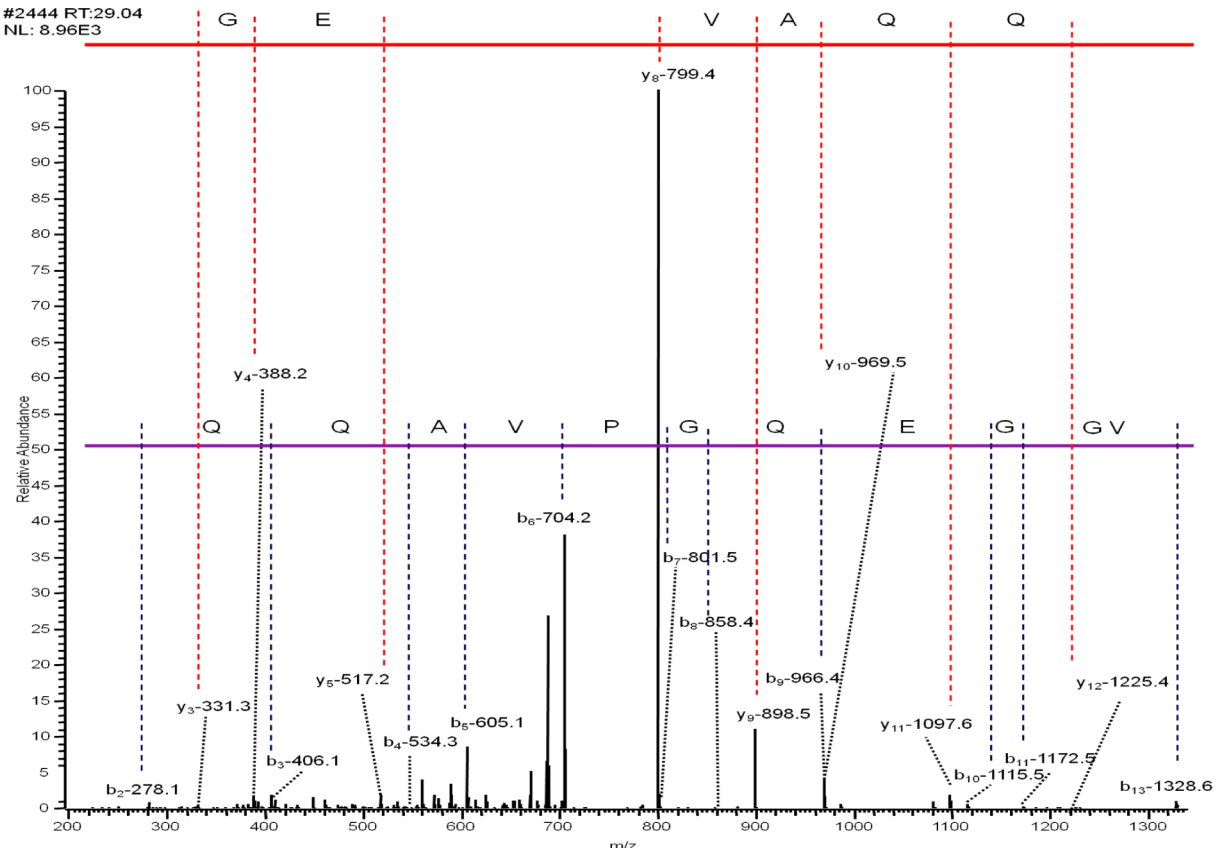


Figure 2. MS-MS spectrum of the NYQQAVPGQEGGVR fragment. Blue lines indicate the b-ions. Red lines indicate the y-ions.

of specific *A. baumannii* peptides and those from other species of *Acinetobacter*. Our results showed that *A. baumannii* can be identified by shotgun proteomics followed by the searching of a specific database (BRPDAB).

Detection of β-Lactam Resistance in *A. baumannii* Strains. Shotgun proteomics has been shown to be both rapid and sensitive in identifying *A. baumannii*. To determine whether this approach could apply to clinical isolates, 20 clinical isolates of *A. baumannii* were analyzed using the same procedure. Extracted proteins from whole cells were digested using a microwave-assisted method followed by shotgun proteomics LC-nanoESI ion trap mass spectrometry. BRPDAB was used for the mass spectrometry data analysis (Supplemental Table S6, Supporting Information). Our results showed that all 20 clinical isolates were identified as *A. baumannii*. Moreover, β-lactam-resistant proteins were also detected in all the 20 *A. baumannii* strains shown in Table S7. Resistance related proteins detected in the strains were labeled with "V". At least two of the resistance related proteins were detected in all the resistant strains of *A. baumannii* in this study when searching from BRPDAB. Overall, shotgun proteomics with a small and specific database (BRPDAB) analysis provides a rapid platform for the identification of β-lactam resistance in *A. baumannii* strains.

DISCUSSION

Mass spectrometry techniques have been shown to be a powerful tool in the proteomic analysis of a variety of clinical isolates. In the present study, we used automated data-dependent scanning using nano-LC/ion trap mass spectrometry to characterize proteotypic peptides of *A. baumannii* pathogens. β-Lactam resistance in *A. baumannii* strains (ATCC17978, ATCC19606, MDRAB1, and twenty clinical isolates) was first tested using a Phoenix 100 ID/AST system. Our results from the shotgun proteomics analysis are consistent with the known resistance of specific *A. baumannii* proteins.

Microwave-assisted digestion followed by the shotgun proteomics method was able to detect cytosolic, inter membrane, and outer membrane proteins. The protein ratios of ATCC17978, ATCC19606, and MDRAB1 were analyzed using SEQUEST software (Figure 1). ATCC17978 and ATCC19606 had similar protein distribution profiles. However, the protein distribution profile of MDRAB1 was different from these two standard strains. Moreover, the unknown proteins were much more than standard strains. This multidrug resistant strain was selected in the laboratory by treatment with a high dosage of antibiotics. It is likely that MDRAB1 changes its gene expression to adapt to stress conditions.

Shotgun proteomics techniques have been shown to be a powerful method to detect whole cell proteins. However, mass spectrometry profile analysis using a nonredundant database is a complicated and time-consuming process. To reduce and simplify the database searching process, we followed the concepts of Antibiotic Resistance Genes Online (ARGO)[34] and the Antibiotic Resistance Genes Database (ARDB),[35] developed by groups from Birla Institute of Technology and Science and University of Maryland, respectively, to build a more specific database, BRPDAB, in which the peptide sequences of β-lactam resistance-related and *A. baumannii*-specific proteins were collected. Using the BRPDAB database, we successfully improved upon the disadvantage of shotgun proteomics in whole cell protein analysis. The entire procedure, including

dx.doi.org/10.1021/ac303326a | *Anal. Chem.* 2013, 85, 2802−2808

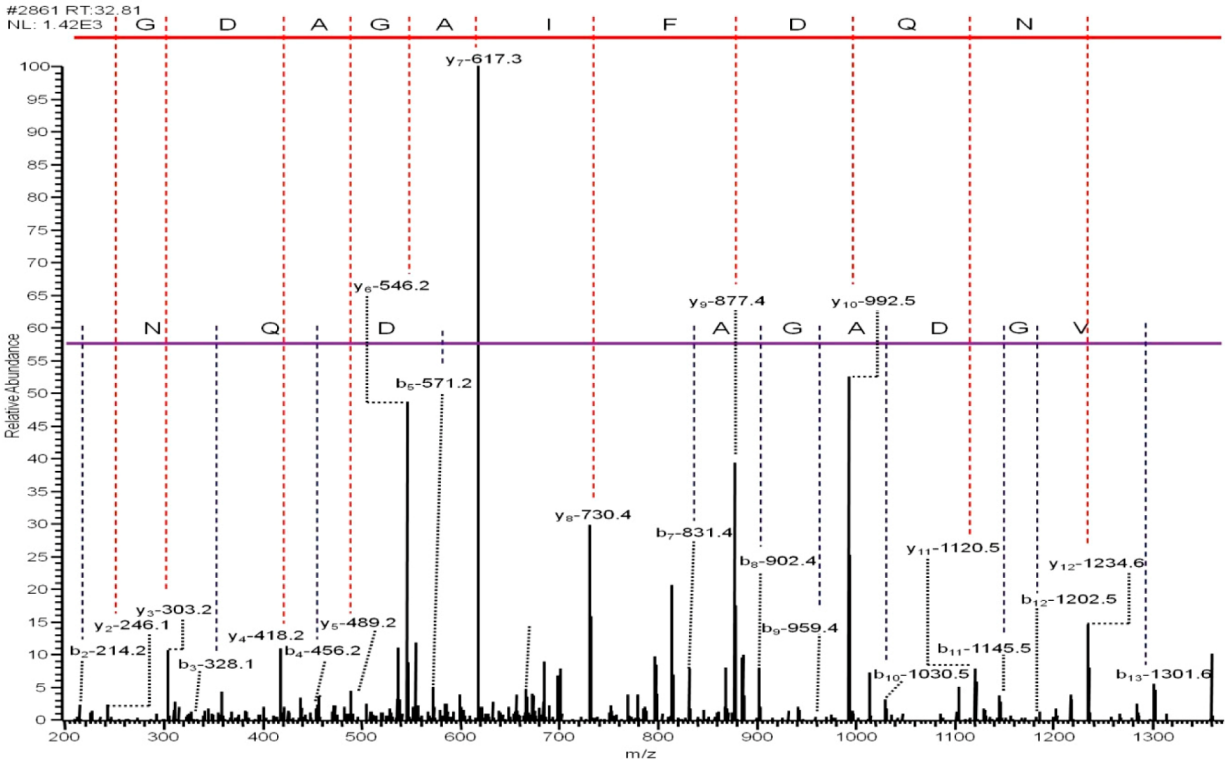


Figure 3. MS-MS spectrum of the VNNQDFIAGADGVK fragment. Blue lines indicate the b-ions. Red lines indicate the y-ions.

microwave-assisted protein digestion, shotgun proteomics, LC-nanoESI ion trap mass spectrometry, and BRPDAB database searching, only requires 5 to 6 h to identify *A. baumannii* strains and resistance mechanisms.

After the shotgun proteomics procedure was standardized in this study, ATCC17978, ATCC19606, MDRAB1, and 20 clinical isolates were analyzed according to these procedures. β-Lactam resistance-related proteins were detected in the 20 clinical isolates (Table S5, Supporting Information). The proteins detected included AmpC cephalosporinase (20/20); *Acinetobacter*-derived cephalosporinases-53 (7/20); carbapenem-associated resistance protein (19/20); beta-lactamase OXA-69-like protein (6/20); beta-lactamase OXA-23-like protein (2/20); and metallo-β-lactamase (1/20). There are two possible mechanisms that result in β-lactam resistance in *A. baumannii*. First, these bacteria may change their gene expression profile to express β-lactamase. Second, they may alter the permeability of their cytoplasmic membrane by changing transport protein expression. β-Lactamases are the major cause of resistance in *A. baumannii*. β-Lactamases are grouped into four classes (A, B, C, and D) according to their similarity in primary structures, known as the Ambler classification. Among those β-lactamases detected in this study, metallo-β-lactamase is class B, AmpC cephalosporinase and *Acinetobacter*-derived cephalosporinases-53 are class C, and OXA β-lactamases are class D. Metal ions are required for the enzyme activity of class B to hydrolyze β-lactams, including penicillin, carbapenem, and cephalosporins. Therefore, class B β-lactamases are inhibited by chelating agents, i.e., EDTA. AmpC cephalosporinase is a strong antibiotic resistance enzyme that hydrolyses most β-lactams, including penicillin, monobactam, and cephalosporins. It is overexpressed in most clinical isolates and is responsible for compromising cephalosporin treatment.[36] OXA β-lactamases are classified according to their amino acid sequence. OXA-23, OXA-24, OXA-69, and OXA-58 could be detected in *A. baumannii*.[37]

Of particular interest, the primary structures of carbapenem-associated resistance protein (CarO) are different between standard and β-lactam-resistant *A. baumannii* strains. This membrane protein was first identified in 2002 and is responsible for imipenem resistance.[38] The following fragments were detected in ATCC17978 and ATCC19606: DNKYEWMPVGK, NYQQAVPGQEGGVR (Figure 2), NYQQAVPGQEGGVRGK, IGNGDTLSIDGK, and NDIAPYLGFGFAPK. The BRPDAB analysis indicated that these fragments were derived from a carbapenem-associated resistance protein (A6XB80) (Figure 4A). However, the following fragments were detected in MDRAB1 and 19 clinical isolates: INGQMSYK, VNNQDFIAGADGVK (Figure 3), NDIAPYLGFGFAPK, SFRVNNQDFIAGADGVK, and WLPVGK. The BRPDAB analysis indicated that these fragments were derived from a carbapenem-associated resistance protein (A6YRR1) (Figure 4B). However, these two carbapenem-associated resistance proteins have little similarity in their primary structures (Figure 5); PRED-TMBB predicted these two proteins to have β-barrel structures. The specific fragment VNNQDFIAGADGVK was found in all of the 19 β-lactam-resistant clinical isolates. This fragment is a promising marker for diagnosing β-lactam resistance. AmpC cephalosporinase was found in 20 clinical isolates, and 19 clinical isolates had specific CarO proteins (A6YRR1). This finding suggests that clinical isolates expressing both proteins were likely resistant to penicillin, monobactam, carbapenem, and cephalosporins.

OXA-69 β-lactamase has poor activity for hydrolyzing β-lactam.[39] However, it is overexpressed in *A. baumannii* and is able to compromise carbapenem treatment. It was found that ISAbal was inserted upstream of the OXA-69 gene as a strong promoter.[40,41] Additionally, it has been demonstrated that 88%

dx.doi.org/10.1021/ac303326a | *Anal. Chem.* 2013, 85, 2802−2808

A

>trlA6XB80lA6XB80_ACIBA Carbapenem-associated resistance protein OS=Acinetobacter baumannii GN=carO PE=4 SV=1
MKVLRVLVTTTALLAAGAAMADEAVVHDSYAFDKNQLIPVGARAEVGTTGYGGALLWQAN
PYVGLALGYNGGDISWTDDVSVNGTKYDLDMDNNNVYLNAEIRPWGASTNPWAQGLYIAA
GAAYLDNDYDLAKRIGNGDTLSIDGKNYQQAVPGQEGGVRGKMSYKNDIAPYLGFGFAPK
ISKNWGVFGEVGAYYTGNPKVELTQYNLAPVTGNPTSAQDAVDKEANEIRNDNKYEWMPV
GKVGVNFYW

B

>trlA6YRR1lA6YRR1_ACIBA Carbapenem-associated resistance protein OS=Acinetobacter baumannii GN=carO PE=4 SV=1
MKVLRVLVTTTALLAAGAAMADEAVVHDSYAFDKNQLIPVGARAEVGTTGYGGALLWQAN
PYVGLALGYNGGDISWSDDVKVNGSTYDLDMDNNNVYLNAEIRPWGASTNRWAQGLYVAA
GAAYLDNDYDLTRNVDATRSFRVNNQDFIAGADGVKINGQMSYKNDIAPYLGFGFAPKIN
KNWGVFGEVGAYYTGNPTVKLVSSGSAVTTGDQSLEEAVNAEARKIANDDKYKWLPVGKV
GVNFYW

Figure 4. Two versions of carbapenem-associated resistance protein (CarO) sequences. Letters in blue (A6XB80) represent conserved sequences in wild type *A. baumannii* strains; ATCC17978 and ATCC19606. Letters in red (A6YRR1) represent conserved amino acid sequences in multidrug resistant *A. baumannii* strain MDRAB1 and 19 clinical isolates.

of carbapenem-resistant clinical isolates of *A. baumannii* in Taiwan carry the ISAba1-OXA-69 genotype.[42] ISAba1 was also involved in the overexpression of cephalosporinase.[42,43] In this study, CarO was not detected in clinical isolate 50913. However, it is possible that its carbapenem resistance resulted from ISAba1. Overall, these 20 *A. baumannii* clinical isolates were resistant to carbapenem, monobactam, and cephalosporin; they were also resistant to a combination treatment of penicillin and β-lactamase inhibitors. The shotgun proteomics results were consistent with the MIC results reported in Table 1.

Proteomic analysis provides a powerful method to study *A. baumannii*. The proteomic regulation of *A. baumannii* under antibiotic stress conditions was studied. Using one-dimensional electrophoresis-liquid chromatography-tandem mass spectrometry (1-DE-LC-MS/MS) and isobaric tag for relative and absolute quantitation (iTRAQ), the protein expression profiles of cell wall and plasma membrane in *A. baumannii* were shown to be changed to overcome the stresses induced by different antibiotics.[44] The inner membrane proteins of antibiotic-resistant *A. baumannii* were also identified using a DIGE-based proteomic approach.[45] These studies elegantly revealed the mechanisms of antibiotic resistance in *A. baumannii*. In the present study, we used automated data-dependent scanning on a nano-LC/ion trap mass spectrometer to characterize proteotypic peptides of *A. baumannii*. This proteomic study will provide a platform for the rapid diagnosis of wild-type and resistant strains of *A. baumannii*, which would be useful for the medical treatment of these strains.

CONCLUSION

A. baumannii is a strongly antibiotic-resistant pathogen; therefore, rapid diagnosis and detection of resistance in *A. baumannii* is required for appropriate antibiotic treatment in hospitals. It usually takes more than 10 h to perform a biochemical analysis in routine microbiology laboratories using Phoenix, VITEK2. Here, using microwave-assisted digestion and a shotgun proteomics technique with a small and specific database (BRPDAB), we were able to identify *A. baumannii* and its resistance mechanisms simultaneously within 5−6 h. Furthermore, we found that the specific peptide fragment VNNQDFIAGADGVK could be a useful marker of β-lactam resistance in *A. baumannii*. Our results suggest that this method will have great potential for developing new databases and further be useful in the identification of other pathogens in clinical specimens.

ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Address: Department of Laboratory Medicine and Biotechnology, Tzu Chi University, No. 701, Zhongyang Rd., sec. 3, Hualien 97004, Taiwan. Tel.: +886-3-8565301 ext. 2334. Fax: +886-3-8571917. E-mail: anren@mail.tcu.edu.tw.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank the Buddhist Tzu Chi General Hospital, Tzu Chi University, and the National Science Council (NSC), Taiwan for financially supporting this research.

```
1    MKVLRVLVTTTALLAAGAAMADEAVVHDSYAFDKNQLIPVGARAEVGTTGYGGALLWQAN   60  A6XB80
1    MKVLRVLVTTTALLAAGAAMADEAVVHDSYAFDKNQLIPVGARAEVGTTGYGGALLWQAN   60  A6YRR1
     ************************************************************

61   PYVGLALGYNGGDISWTDDVSVNGTKYDLDMDNNNVYLNAEIRPWGASTNPWAQGLYIAA  120  A6XB80
61   PYVGLALGYNGGDISWSDDVKVNGSTYDLDMDNNNVYLNAEIRPWGASTNRWAQGLYVAA  120  A6YRR1
     ****************:***.***:.********************** ******:**

121  GAAYLDNDYDLAKRIGNGDTLSIDGKNYQQAVPGQEGGVRGKMSYKNDIAPYLGFGFAPK  180  A6XB80
121  GAAYLDNDYDLTRNVDATRSFRVNNQDFIAGAD--GVKINGQMSYKNDIAPYLGFGFAPK  178  A6YRR1
     ***********::.:    :: :: :::  ..      :.*:******************

181  ISKNWGVFGEVGAYYTGNPKVELTQYNLAPVTGNPTSAQDAVDKEANEIRNDNKYEWMPV  240  A6XB80
179  INKNWGVFGEVGAYYTGNPTVKLVSSGSAVTTGD-QSLEEAVNAEARKIANDDKYKWLPV  237  A6YRR1
     *.*****************.*:*..   * .**:  * ::**: **.:* **:**:*:**

241  GKVGVNFYW  249  A6XB80   A6XB80_ACIBA
238  GKVGVNFYW  246  A6YRR1   A6YRR1_ACIBA
     *********
```

Figure 5. Comparison of the two carbapenem-associated resistance protein (CarO) sequences. Letters with a blue background were fragments detected in wild type *A. baumannii* strain ATCC19606. Letters with an orange background were fragments detected in multidrug resistant *A. baumannii* strain MDRAB1 and 19 clinical isolates.

REFERENCES

(1) Baumann, P. *J. Bacteriol.* **1968**, *96*, 39−42.
(2) Dent, L.; Marshall, D.; Pratap, S.; Hulette, R. *BMC Infect. Dis.* **2010**, *10*, 196.
(3) Poole, K. *Cell. Mol. Life Sci.* **2004**, *61*, 2200−2223.
(4) Bush, K.; Jacoby, G. A.; Medeiros, A. A. *Antimicrob. Agents Chemother.* **1995**, *39*, 1211−1233.
(5) Shibata, N.; Doi, Y.; Yamane, K.; Yagi, T.; Kurokawa, H.; Shibayama, K.; Kato, H.; Kai, K.; Arakawa, Y. *J. Clin. Microbiol.* **2003**, *41*, 5407−5413.
(6) Turton, J. F.; Kaufmann, M. E.; Warner, M.; Coelho, J.; Dijkshoorn, L.; van der Reijden, T.; Pitt, T. L. *J. Hosp. Infect.* **2004**, *58*, 170−179.
(7) Da Silva, G.; Dijkshoorn, L.; Van Der Reijden, T.; Van Strijen, B.; Duarte, A. *Clin. Microbiol. Infect.* **2007**, *13*, 190−195.
(8) Zong, Z.; Lü, X.; Valenzuela, J. K.; Partridge, S. R.; Iredell, J. *Int. J. Antimicrob. Agents* **2008**, *31*, 50−54.
(9) Choi, W. S.; Kim, S. H.; Jeon, E. G.; Son, M. H.; Yoon, Y. K.; Kim, J.-Y.; Kim, M. J.; Sohn, J. W.; Kim, M. J.; Park, D. W. *J. Korean Med. Sci.* **2010**, *25*, 999−1004.
(10) Ho, Y.-P.; Reddy, P. M. *Mass Spectrom. Rev.* **2011**, *30*, 1203−1224.
(11) Ho, Y.-P.; Reddy, P. M. *Clin. Chem.* **2010**, *56*, 525−536.
(12) Sharma, P.; Kumar, B.; Gupta, Y.; Singhal, N.; Katoch, V. M.; Venkatesan, K.; Bisht, D. *Proteome Sci.* **2010**, *8*, 59.
(13) Soares, N.; Cabral, M.; Parreira, J.; Gayoso, C.; Barba, M.; Bou, G. *Proteome Sci.* **2009**, *7*, 37.
(14) Dupont, M.; Pagès, J.-M.; Lafitte, D.; Siroy, A.; Bollet, C. *J. Proteome Res.* **2005**, *4*, 2386−2390.
(15) Martí, S.; Sánchez-Céspedes, J.; Oliveira, E.; Bellido, D.; Giralt, E.; Vila, J. *Proteomics* **2006**, *6*, S82−S87.
(16) Kwon, S.-O.; Gho, Y. S.; Lee, J. C.; Kim, S. I. *FEMS Microbiol. Lett.* **2009**, *297*, 150−156.
(17) Vashist, J.; Tiwari, V.; Kapil, A.; Rajeswari, M. R. *J. Proteome Res.* **2009**, *9*, 1121−1128.
(18) Yates, J. R. *J. Mass Spectrom.* **1998**, *33*, 1−19.
(19) Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. *Nat. Biotechnol.* **1999**, *17*, 676−682.
(20) Washburn, M. P.; Wolters, D.; Yates, J. R. *Nat. Biotechnol.* **2001**, *19*, 242−247.
(21) Speers, A. E.; Wu, C. C. *Chem. Rev.* **2007**, *107*, 3687−3714.
(22) Lin, S.-S.; Wu, C.-H.; Sun, M.-C.; Sun, C.-M.; Ho, Y.-P. *J. Am. Soc. Mass Spectrom.* **2005**, *16*, 581−588.
(23) Sun, W.; Gao, S.; Wang, L.; Chen, Y.; Wu, S.; Wang, X.; Zheng, D.; Gao, Y. *Mol. Cell. Proteomics* **2006**, *5*, 769−776.
(24) Reddy, P. M.; Hsu, W.-Y.; Hu, J.-F.; Ho, Y.-P. *J. Am. Soc. Mass Spectrom.* **2010**, *21*, 421−424.
(25) Vaezzadeh, A. R.; Deshusses, J. M. P.; Waridel, P.; François, P.; Zimmermann-Ivol, C. G.; Lescuyer, P.; Schrenzel, J.; Hochstrasser, D. F. *J. Microbiol. Methods* **2010**, *80*, 56−62.
(26) Manza, L. L.; Stamer, S. L.; Ham, A.-J. L.; Codreanu, S. G.; Liebler, D. C. *Proteomics* **2005**, *5*, 1742−1745.
(27) Wisniewski, J. R.; Zougman, A.; Nagaraj, N.; Mann, M. *Nat. Methods* **2009**, *6*, 359−362.
(28) Hu, A.; Chen, C.-T.; Tsai, P.-J.; Ho, Y.-P. *Anal. Chem.* **2006**, *78*, 5124−5133.
(29) Ashburner, M.; Ball, C. A.; Blake, J. A.; Botstein, D.; Butler, H.; Cherry, J. M.; Davis, A. P.; Dolinski, K.; Dwight, S. S.; Eppig, J. T.; Harris, M. A.; Hill, D. P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J. C.; Richardson, J. E.; Ringwald, M.; Rubin, G. M.; Sherlock, G. *Nat. Genet.* **2000**, *25*, 25−29.
(30) Fournier, P.-E.; Vallenet, D.; Barbe, V.; Audic, S.; Ogata, H.; Poirel, L.; Richet, H.; Robert, C.; Mangenot, S.; Abergel, C.; Nordmann, P.; Weissenbach, J.; Raoult, D.; Claverie, J.-M. *PLoS Genet.* **2006**, *2*, No. e7.
(31) Yu, N. Y.; Wagner, J. R.; Laird, M. R.; Melli, G.; Rey, S.; Lo, R.; Dao, P.; Sahinalp, S. C.; Ester, M.; Foster, L. J.; Brinkman, F. S. L. *Bioinformatics* **2010**, *26*, 1608−1615.
(32) Bagos, P.; Liakopoulos, T.; Spyropoulos, I.; Hamodrakas, S. *BMC Bioinf.* **2004**, *5*, 29.
(33) Bagos, P. G.; Liakopoulos, T. D.; Spyropoulos, I. C.; Hamodrakas, S. J. *Nucleic Acids Res.* **2004**, *32*, W400−W404.
(34) Scaria, J.; Chandramouli, U.; Verma, S. K. *Bioinformation* **2005**, *1*, 5−7.
(35) Liu, B.; Pop, M. *Nucleic Acids Res.* **2009**, *37*, D443−D447.
(36) Corvec, S.; Caroff, N.; Espaze, E.; Giraudeau, C.; Drugeon, H.; Reynaud, A. *J. Antimicrob. Chemother.* **2003**, *52*, 629−635.
(37) Walther-Rasmussen, J.; Høiby, N. *J. Antimicrob. Chemother.* **2006**, *57*, 373−383.
(38) Limansky, A. S.; Mussi, M. A.; Viale, A. M. *J. Clin. Microbiol.* **2002**, *40*, 4776−4778.
(39) Heritier, C.; Poirel, L.; Fournier, P.-E.; Claverie, J.-M.; Raoult, D.; Nordmann, P. *Antimicrob. Agents Chemother.* **2005**, *49*, 4174−4179.
(40) Hu, W. S.; Yao, S.-M.; Fung, C.-P.; Hsieh, Y.-P.; Liu, C.-P.; Lin, J.-F. *Antimicrob. Agents Chemother.* **2007**, *51*, 3844−3852.
(41) Turton, J. F.; Ward, M. E.; Woodford, N.; Kaufmann, M. E.; Pike, R.; Livermore, D. M.; Pitt, T. L. *FEMS Microbiol. Lett.* **2006**, *258*, 72−77.
(42) Lin, Y.-C.; Hsia, K.-C.; Chen, Y.-C.; Sheng, W.-H.; Chang, S.-C.; Liao, M.-H.; Li, S.-Y. *Antimicrob. Agents Chemother.* **2010**, *54*, 2078−2084.
(43) Rodriguez-Martinez, J.-M.; Poirel, L.; Nordmann, P. *Antimicrob. Agents Chemother.* **2010**, *54*, 4930−4933.
(44) Yun, S. H.; Choi, C. W.; Kwon, S. O.; Park, G. W.; Cho, K.; Kwon, K. H.; Kim, J. Y.; Yoo, J. S.; Lee, J. C.; Choi, J. S.; Kim, S.; Kim, S. I. *J. Proteome Res.* **2011**, *10*, 459−469.
(45) Tiwari, V.; Vashistt, J.; Kapil, A.; Moganty, R. R. *PLoS One* **2012**, *7*, No. e39451.