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Identification and Antibody-Therapeutic Targeting of Chloramphenicol-Resistant Outer Membrane Proteins in *Escherichia coli*

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Bacterial resistance to an antibiotic may result from survival in a suddenly strong antibiotic or in subminimum inhibitory concentration of the drug. Their shared proteins responsible for the resistance should be potential targets for designing new drugs to inhibit the growth of the antibiotic-resistant bacteria. In the current study, comparative proteomic methodologies were used for identification of sharedly altered outer membrane proteins (OM proteins) that are responsible for chloramphenical (CAP)-resistant *Escherichia coli* and for survival in medium with suddenly strong CAP treatment. Six differential OM proteins and another protein with unknown location were determined to be sharedly CAP-resistant-related proteins with the use of 2-DE/MS, Western blotting and gene mutant methods, in which TolC, OmpT, OmpC, and OmpW were critically altered proteins and potential targets for designing of the new drugs. Furthermore, a novel method of specific antibody combating bacterial growth was developed on these OM proteins. Only anti-TolC showed a very significant inhibition on bacterial growth in medium with CAP when antisera to TolC, OmpC, OmpT, and OmpW were separately utilized. The growth of CAP-resistant *E. coli* and its original strain was completely inhibited when they bound with anti-TolC and survived in 1/8 MIC of CAP. This observed result is basically the same to the finding that Δ*tolC* was survived in the same concentration of the antibiotic. Our study demonstrates that the enhancement of expression of antibody target with antibiotic could be very effective approach compared to using a drug alone, which highlights a potential way for treatment of infection by antibiotic-resistant bacteria.

Keywords: antibiotic-resistance • chloramphenical • TolC • *E. coli* • antibody therapy

1. Introduction

The worldwide emergence of antibiotic-resistant bacteria poses a serious threat to human health and animal feed.[1] The development of novel antibacterial classes is a key to keeping pace with the remarkable adaptability of the antibiotic-resistant bacteria, but pharmaceutical companies have made little progress in the development of new bactericidal drugs. Identification of key proteins responsible for the resistance as drug targets and development of a novel therapy to antibiotic-resistant bacteria have been highly concerned.[2]

In an environment rich in an antibiotic, survival of bacteria depends on mounted defense response. Such protective mechanisms include stress and adaptive feedbacks to the exposure to the drugs. When bacteria are suddenly exposed to a strong antibiotic treatment, they alert the stress feedback to take the necessary countermeasure.[3,4] Antibiotic-resistant bacteria may be selected when they survive in treatment with a low concentration of antibiotic. These antibiotic-resistant cells mount an adaptive feedback to the exposure to the antibiotic treatment for their survival.[5,6] Shared proteins between the stress and adaptive responses should be potential targets for the development of new drugs to combat the growth of antibiotic-resistant bacteria because bacteria may be exposed to both of the suddenly increased and persistent antibiotic treatments in the resistance to the antibiotic. Antibiotic resistance can be intrinsic or acquired. Unlike acquired antibiotic resistance, which is based on gene mutant or acquisition of new DNA, intrinsic antibiotic resistance is based on the absence of the drug target or on the lack of permeability of the bacteria to a given drug.[7] Recently, more and more evidence has shown that the intrinsic resistance of many bacteria to antibiotics depends on the constitutive or inducible expression of active efflux system.[7,8] Such mechanism is based on expression regulation of outer membrane (OM) proteome. It is always possible, thus, to find an alternative therapeutic approach for treatment of a disease due to drug-resistant pathogens following the deter-

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mination of the shared outer membrane proteins (OM proteins) as new drug targets.

More recently, a novel therapeutic strategy to combat the evolution of the acquired antibiotic resistance has been developed by the inhibition of gene mutation, in which targets are inside the cells.[2] Unlike the targets inside the cell, which are recognized only by the small molecules that penetrate cell membrane, OM proteins, which locate at the outmost surface of the cell, are readily recognized by an antibody. If the binding of the antibody with the sharedly altered OM proteins responsible for antibiotic resistance negatively regulates the function of these OM proteins, the inhibition may represent a novel approach to combat the growth of drug-resistant bacteria. Since early 20th century, passive administration of a monoclonal, polyclonal, therapeutic antibody has been applied in treatment of many of diseases including infectious diseases, autoimmune diseases, and scorpion stings as well as cancers with the reduce of unwanted side effects of antibody.[9−13] On the other hand, the identification of cell-surface targets suitable for antibody therapy and the preparation of monoclonal antibody aiming at the targets have become a very important research field in tumor immunotherapy.[14,15] Previous studies have demonstrated that the mechanism of antibody against microorganisms include indirect and direct antimicrobial action,[16] whereas the mechanism on eukaryotic cells belong to negative regulation on the function of target proteins which are bound with antibodies.[17] Although many studies have described the antimicrobial action of antibody therapy,[16,18] to our knowledge, no any study has demonstrated the usefulness of antibody against drug-resistant bacteria by regulating function of target proteins as well as the identification of target cell-surface proteins suitable for antibody therapy.

Presently, chloramphenical (CAP) is still a commonly used antibiotic in aquaculture[1] as well as in clinical treatment, especially in patients with bacterial meningitis, which is a recommendation by WHO.[19−21] CAP is also widely used a reagent in the investigation of antibiotic-resistant spectrum in clinical research of drug-resistant bacteria.[22−24] Meanwhile, a line of evidence has indicated that bacterial efflux pump system contributes to their resistance to CAP,[25−27] which makes it possible to determine OM proteins as new drug targets and develop an antibody therapy in infection by the CAP-resistant bacteria, although OM proteome responsible for CAP resistance is ill-defined.

In the current study, we identified CAP-resistant OM proteins in *Escherichia coli* and developed a novel approach to combat the growth of the bacteria with use of the antibody specific to TolC, which highlights a potential way for treatment of infection by antibiotic-resistant bacteria.

2. Materials and Methods

2.1. Bacterial Strains. All strains used were from *E. coli* K12 BW25113 and its derivatives, in which *E. coli* K12 BW25113, Δ*ompT*, Δ*ompW*, Δ*lampB*, Δ*fadL*, and Δ*dps* were kindly provided by Nara Institute of Science and Technology, Japan,[29] and Δ*ompC* and Δ*tolC* were collections in our laboratory. CAP-resistant strain (CAP-R) was selected by the use of serial propagations in a Luria−Bertani (LB) medium with 1/2 MIC of CAP. In brief, original *E. coli* K12 BW 25113 was cultured in LB medium. The minimum inhibitory concentration (MIC) of CAP was determined by antimicrobial susceptibility testing when the bacteria grew till 0.5 opitical density (OD600 nm). Then, serial propagations were subcultured in LB mediums with 1/2 MIC of CAP as an experimental group, and without any antibiotics as a control group. The mediums were prepared and used on the same day. After 10 sequential subcultures with 10^5 CFU/mL cells in every passage, the bacteria were transferred onto antibiotic-free LB agar plate at 37 °C overnight and then a colony was randomly selected for the measurement of antimicrobial susceptibility. The CAP-R was achieved when MIC was at least 4-folds higher in the resulting bacteria than in the original strain with the same propagations (CAP-R-O).

2.2. Bacterial Culture and OM Protein Extraction. Four bacterial cultures, CAP-R, CAP-R-O, suddenly increased CAP stress bacteria (CAP-S) and its control (CAP-S-C) were collected for OM protein extraction. For the samples of CAP-R and CAP-R-O, they were separately cultured in LB medium at 37 °C overnight, and then diluted 100-folds in fresh LB medium. The cultures were harvested in 8 h-incubation at 37 °C. For the samples of CAP-S and CAP-S-C, original bacteria were cultured in LB medium at 37 °C overnight, and then diluted 100-folds in fresh LB medium. CAP solution was added into 8 h-culture (1.5×10^9 CFU/mL) at the final concentration of 54.4 μg/mL and the culture continued to keep for 2 h as CAP-S and no CAP was added as CAP-S-C. The resulting CAP-R, CAP-R-O, CAP-S, and CAP-S-C cells were used for OM protein extraction with the use of sarcosine method described previously.[29] Briefly, the bacterial cells were collected by centrifugation at 4000× *g* for 15 min at 4 °C. The cells were then washed in 40 mL sterile saline (0.15 M NaCl) for three times, and then resuspended in appropriate 50 mM Tris-Cl. Cells were disrupted by intermittent sonic oscillation. Supernatant was collected and was further centrifuged at 100 000× *g* for 40 min at 4 °C. The resulting pellet was resuspended in 10 mL of 2% (w/v) sodium lauryl sarcosinate (Sigma) and incubated at room temperature for 1 h, following by centrifugation at 100 000× *g* for 40 min at 4 °C. The collecting pellet was resuspended in appropriate 50 mM Tris-Cl and stored at −80 °C. The concentration of sarcosine-insoluble OM fraction in the final preparation was determined using the Bradford method.

2.3. Two-Dimensional Electrophoresis and Visualization of Protein Spots. 2-DE was performed according to a procedure described previously.[30] About 20 μg of protein sample was separated through the first dimension isoelectric focusing (IEF) electrophoresis using a solution (8 M urea, 2 M thiourea, 4% CHAPS, and 80 mM DTT) and pH 3−10 carrier ampholyte for 8000 V h, and then the gels were transferred to the second dimension electrophoresis using 10% acrylamide gel. Subsequently, 2-DE gel was stained with Coomassie blue and scanned in scanned in an AGFA white-light scanner at a resolution of 600 by 200 mm. The raw images were processed using the 2-D software Melanie 4.0. Altered spots were compared based on their volume percentages in the total spot volume over the whole gel image. Significantly changed spots were selected by rate increased/decreased >2-folds. Each of 2-DE samples was repeated three times.

2.4. MALD-TOF/MS Analysis. The sample solution (30−100 ppm) with equivalent matrix solution was applied onto the MALDI-TOF/Target and prepared for MALDI-TOF/MS analysis and HCCA was used as the matrx. MALDI-TOF spectra were calibrated using trypsin digestion peptide signals and matrix ion signals. MALDI analysis was performed using a fuzzy logic feedback control system (Reflex III MALDI-TOF system Bruker, Karlsruhe, Germany) equipped with delayed ion extraction. Peptide masses were searched against the Swiss-Prot database using the MS-Fit program (http://prospector.ucsf.edu/ucsf-

Table 1. Identification of Altered OM Proteins by PMF Searching in CAP-R with Respect to CAP-R-O

spot no	accession number	variation	character description	subcellular location	MW/p*I*	peptides matched	Moscow score
1	TOLC_ECOLI	↑	multidrug efflux and protein export	OM	53967/5.46	8	86
2	TOLC_ECOLI	↑	multidrug efflux and protein export	OM	53967/5.46	8	86
3	ATPB_ECOLI	↓	ATP synthase subunit beta	IM	50194/4.90	15	200
4	TOLC_ECOLI	↑	multidrug efflux and protein export	OM	53967/5.46	8	86
5	ACEA_ECOLI	↓	socitrate lyase	Cytoplasm	47200/5.44	10	109
6	FADL_ECOLI	↓	long-chain fatty acid transport protein	OM	48742/ 5.09	8	62
7	LamB_ECOLI	↓	Maltose-inducible porin	OM	49941/4.85	7	70
8	OmpT_ECOLI	↑	Protease	OM	35540 /5.76	13	176
9	OmpT_ECOLI	↓	Protease	OM	35540 /5.76	13	176
10	OmpT_ECOLI	↓	Protease	OM	35540 /5.76	13	176
11	OmpT_ECOLI	↓	Protease	OM	35540 /5.76	13	176
12	DpS_ECOLI	↓	DNA protection during starvation protein	unknown	18564/5.72	7	102
13	OmpC_ECOLI	↑	passive diffuse small molecular weight hydrophilic material crossing the outer membrane	OM	40368/4.59	6	76
14	OmpW_ECOLI	↑	a receptor for colicin S4	OM	22928/6.03	6	65
15	OmpW_ECOLI	↑	a receptor for colicin S4	OM	22928/6.03	8	70
16	OmpW_ECOLI	↑	a receptor for colicin S4	OM	22928/6.03	7	65
17	DpS_ECOLI	↓	DNA protection during starvation protein	unknown	18564/5.72	7	95
18	DpS_ECOLI	↓	DNA protection during starvation protein	unknown	18564/5.72	9	92
19	DpS_ECOLI	↓	DNA protection during starvation protein	unknown	18564/5.72	8	80
20	PSPA_ECOLI	↑	Phage shock protein A	IM	25362/5.4	9	80

Table 2. Primers and Restriction Digest Sites Used in This Study

target gene	primers	restriction digest site
tolC	Sense primer: 5′-CGA GAA TTC ATG CAA ATG AAG AAA−3′ antisense primer: 5′-GGT AAG CTT TCA GTT ACG GAA AGG G−3AAG CTT	*Eco*RI− *Hind*III
ompW	Sense primer: 5′-CCC GAA TTC ATG AAA AAG TTA ACA G3′ antisense primer: GGG AAG CTT TTA AAA ACG ATA TCC TGC3′	*Eco*RI− *Hind*III
fadL	Sense primer: 5′-CCC GAA TTC ATG GTC ATG AGC CAG AAA AC−3′ antisense primer: 5′-CCC AAG CTT TCA GAA CGC GTA GTT AAA GTT−3′	*Eco*RI− *Hind*III
ompC	Sense primer: 5′-CGC GGA TCC ATG AAA GTT AAA GTA CTG TC−3′ antisense primer: 5′-GGG AAG CTT TTA TTC GAT GTT AGA C −3′	*Eco*RI− *Hind*III
lamB	Sense primer: 5′-CCC GAA TTC ATG ATG ATT ACT CTG C−3′ antisense primer: 5′-GGG AAG CTT TAT TAC CAC CAG ATT T−3′	*Eco*RI− *Hind*III
ompT	Sense primer: 5′-CCC GAA TTC CTC ATT AAG TTA GAT 3′ antisense primer: 5′-TTT CTC GAG TAG TTG GCG TTC TTA	*Eco*RI-*Xho*I

html4.0/msfit.htm) or the NCBI database using the MASCOT program (http://www.matrixscience.com) against *E. coli* K12 complete genome database in NCBI.

2.5. Gene Cloning, Protein Expression and Purification, and Antiserum Raising as well as Western Blotting. Gene cloning procedures were carried out using standard procedures according to reported complete genome sequence of *E. coli* K12, seven pairs of primers *tolC*, *ompC*, *ompT*, *ompW*, *lamB*, *fadL*, and *dps* were designed as shown as in Table 1. Each PCR fragment was ligated into pET-32a expression vector and transformed into *E. coli* BL21. The resulting *E. coli* separately harboring pET-32a−*tolC*, *ompC*, *ompT*, *ompW*, *lamB*, *fadL*, and *dps* were cultured in 100 mL LB, induced by IPTG and identified by SDS-PAGE. The recombinant proteins (His_6-OM proteins) were purified by affinity chromatography using Ni-NTA resin (Qiagen) and SDS-PAGE. Antisera to the purified recombinant His_6-OM proteins were separately raised by immunizing a rabbit with 500 μg purified protein emulsified with Freund's complete adjuvant. The first injection was followed by three injections of the purified protein with Freund's incomplete adjuvant at two weekly intervals. Antisera were collected and stored −80 °C for use.

These antisera were used as the primary antibody for 2-DE Western blotting. In brief, proteins from 2-D gels were transferred to a NC membrane (Bio-Rad Corp., USA) for 1 h at 70 mA in transfer buffer (48 mM Tris, 39 mM glycine, and 20% methanol) at 4 °C. The NC membrane was blocked for 60 min with 5% skimmed milk in TNT buffer (1.211 g Tris, 8.77 g NaCl, and 500 μL tween-20 in 1L TNT, pH 7.0) at 37 °C. After rinsing three times with TNT buffer for 5 min each, the NC membrane was incubated with the primary antibody at a dilution of TNT buffer containing 5% skimmed milk for 1 h at 37 °C on a gentle shaker. The membrane was rinsed three times, 10 min each, and incubated with goat anti-rabbit-HRP (horseradish peroxidase) at a dilution of 1:2000 in TNT buffer. Subsequently, membrane was scanned in an AGFA white-light scanner at a resolution of 400 by 200 nm. Following background subtraction, the membrane patterns were matched to each other by visual comparison. Altered spots were compared based on their volumes.

2.6. Testing for Antimicrobial Susceptibility by Measurement of MIC. The broth microdilution method was carried out to establish the MIC of CAP according to the NCCLS procedure.[31] The MIC was defined as the lowest concentration that inhibited visible growth. Briefly, the inoculums of CAP-R, CAP-R-O, and these mutants were separately cultured in 5 mL of LB medium at 37 °C overnight, and then the cultures were diluted 1:100 into 5 mL fresh LB medium to obtain the desired cell density (10^5 CFU/mL). Ninety microliters of the CAP solution at the final concentration of 0.94−59.8 μg/mL were used per well, and then 10 μL of the inoculum was added. Controls included sterility control and growth control. Incubation was at 37 °C without shaking for 24 h. The growth was scored visually.

2.7. Testing for Antimicrobial Susceptibility by Analysis of Survival Capability in 1/2 MIC of CAP. Bacterial activity assay

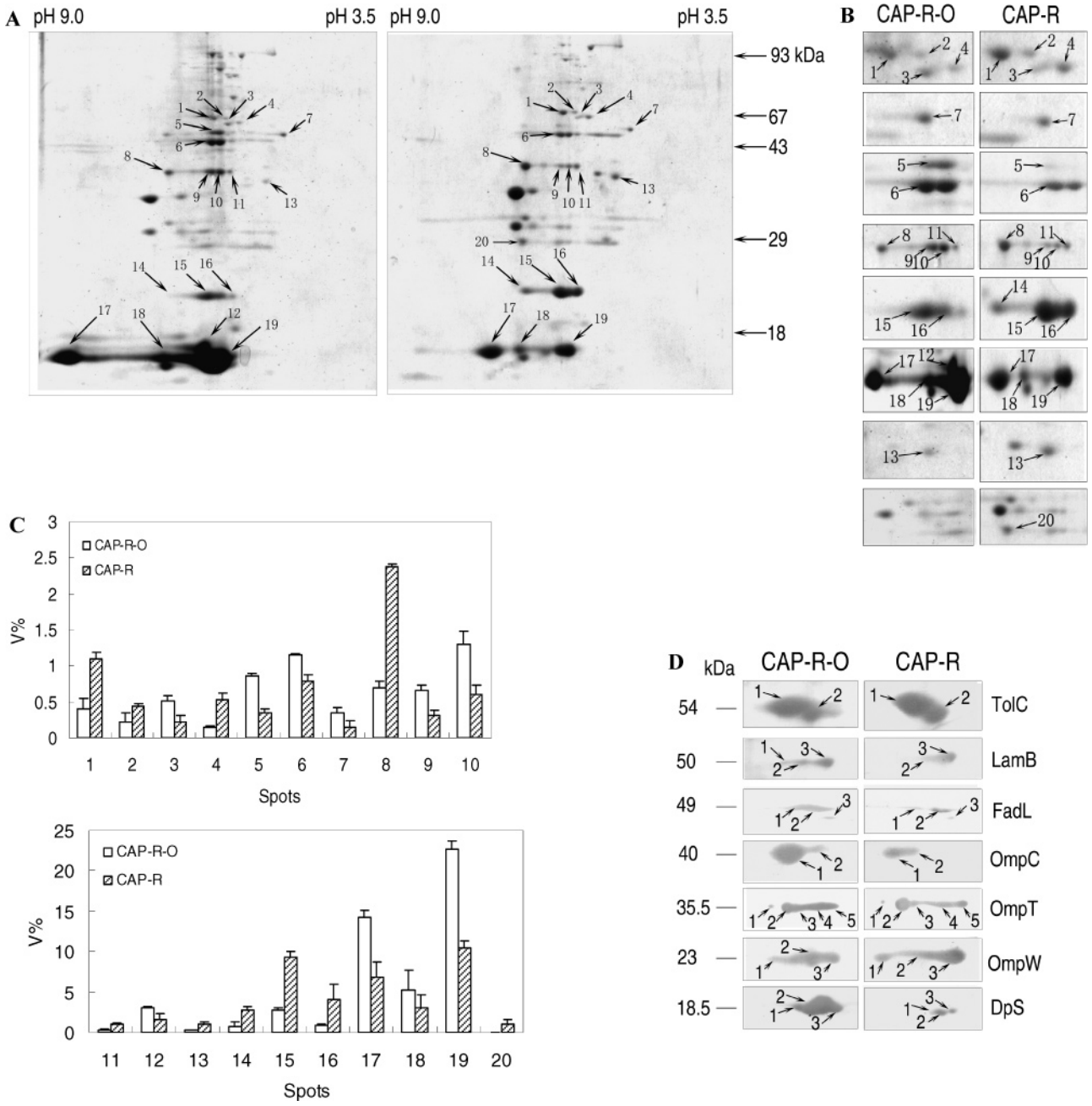

Figure 1. Differential OM protein expression profiles between CAP-R and CAP-R-O detected by 2-DE and confirmed by Western blotting. (A) 2-DE maps of CAP-R-O (left) and CAP-R (right). (B) Enlarged partial 2-DE gels showing altered expression spots. (C) Histogram displays the changes in spot intensity of the altered spots between CAP-R (stripe) and CAP-R-O (white), and bars represent spot intensity with relative volume divided by the total volume over the whole image, according to Melanie 4.0 software description, and their means and standard deviations were from three repeats. (D) Enlarged 2-DE Western blotting results showing target spots detected, which were separately performed on a piece of NC paper for CAP-R or CAP-R-O.

was carried out as described previously with a modification[32] to test the survival capability of bacteria in 1/2 MIC concentration, termed survival capability in 1/2 concentration of an antibiotic. The inoculums of CAP-R, CAP-R-O, and these mutants were separately cultured in 5 mL of LB medium at 37 °C overnight, and then the cultures were diluted 1:1000 into 5 mL fresh LB medium in each of tubes. Six tubes were for each of these bacteria tested. Half of them contained CAP at the last final concentration of 3.4 μg/mL, which was the 1/2 MIC of CAP-R-O, and the other was used for control without any antibiotic. These tubes were incubated at 37 °C for 8 h. Bacterial growth was determined by measurement of the OD600 nm of the cultures. The ability for survival was characterized by comparison between experimental and control groups and was termed an inhibiting rate. The cutoff value of the inhibiting rate was defined as 100 ± 15% of the control and was used to determine significantly changed samples.

2.8. Bacterial Growth-Combating by Binding of Antibody to Corresponding Targeted OM Proteins. A specific antibody combating growth assay was developed to inhibit the activity of CAP-resistant OM proteins. The inoculums of CAP-R, CAP-R-O, Δ*tolC*, Δ*ompC*, Δ*ompT*, and Δ*ompW* were separately cultured in 5 mL fresh LB medium at 37 °C overnight, and then the cultures were diluted 1:100 into 5 mL fresh LB medium to

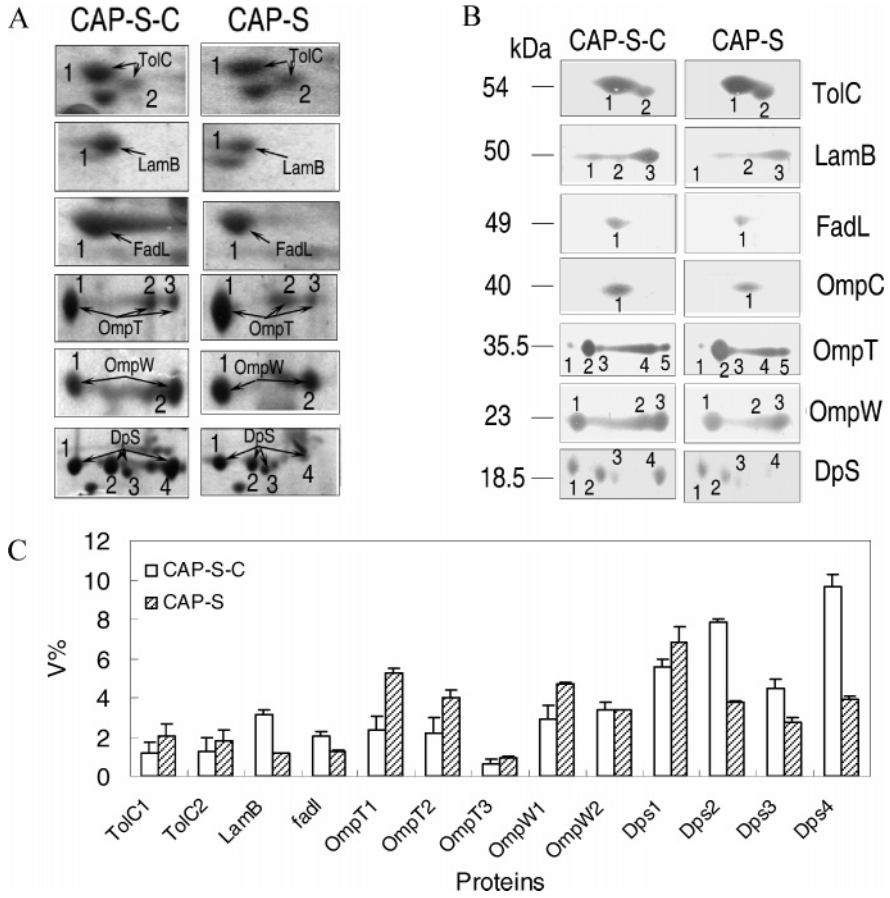

Figure 2. Differential OM protein expression profiles of *E. coli* in 2 h after a suddenly strong CAP treatment detected by 2-DE and confirmed by Western blotting. (A) Enlarged partial 2-DE gels showing altered expression spots. (B) Histogram displays the changes in spot intensity of the altered spots between CAP-S (stripe) and CAP-S-C (white), and bars represent spot intensity with relative volume divided by the total volume over the whole image, according to Melanie 4.0 software description, and their means and standard deviations were from three repeats. (C) Enlarged 2-DE Western blotting results showing target spots detected, which were separately performed on a piece of NC paper for CAP-S or CAP-S-C.

obtain the desired cell density (OD600 nm = 0.5, 10^8 CFU/mL). A pellet from 200 μL of each of six cultures was obtained by centrifugation at 9000× *g* for 4 min and was separately incubated with 50 μL of rabbit preimmune serum and immune serum against TolC, OmpC, OmpT, or OmpW (the antibody titer 1:4000 for Western blotting) at 37 °C for 1 h. After centrifugation at 9000× *g* for 4 min, the bacteria were suspended in 200 μL of fresh LB medium and then were diluted 1:1000 into 5 mL of LB medium without or with 1/8 MIC CAP. These cultures were incubated at 37 °C for 9 h and were measured at OD600 nm for survival capability.

3. Results

3.1. Altered OM Proteome of CAP-Resistant *E. coli* Was Achieved by 2-DE and its Western Blotting. To investigate how bacterial OM protein expression changed in response to CAP resistance, we obtained CAP-R by 10 sequential of subcultures in 1/2 MIC of CAP. The MICs of CAP-R and CAP-R-O, respectively, were 27.2 and 6.8 μg/mL, which showed four folds of increased after the 10 sequential subcultures. CAP-R and CAP-R-O were separately harvested, and their OM proteins were extracted. The OM proteins were separately by 2-DE, and about 50 spots were detected in each of the gels (Figure 1A). Out of the 50 spots, 20 showed differential expression change. The 20 differential spots were identified by MALDI-TOF/MS and belonged to 10 proteins: TolC (spots 1, 2, 4). AtpB (spot 3), AceA (spot 5), FadL (spot 6), LamB (spot 7), OmpT (spot 8−11), OmpC (spot 13), OmpW (spots 14−16), Dps (spots 12, 17−19) and PspA (spot 20), in which TolC, OmpC, OmpW, OmpT, LamB, and FadL belong to OM proteins, and AceA, Dps and AtpB, PspA are cytoplasimic, unkown location and inner membrane proteins, respectively, according to the genome annotation from the Swiss-Prot database (Table 1). Image-analysis revealed that TolC, OmpC, OmpW, and PspA were up-regulated and AtpB, AceA, FadL, LamB, and Dps were down-regulated. Of the four spots of OmpT, interestingly, spot 8 up-regulated, whereas spots 9−11 down-regulated. Enlarged differential spots and their changes are shown in Figure 1B, C.

Furthermore, Western blotting was applied for conformation of these results. First, seven genes of *tolC*, *ompC*, *ompT*, *ompW*, *fadL*, *lamb*, and *dps* were cloned and expressed, and their recombinant proteins were purified and used for antibody preparing. Then the prepared antibodies were utilized as the primary antibody in Western blotting. The Western blotting was performed in 2-DE rather than 1-DE because there was more than one spot for TolC, OmpT, OmpW, and Dps. Equally importantly, comparison between altered proteins, some of which showed up-regulation and the other showed down-regulation in one NC paper, could achieve more reliable results.

The Western blotting results indicated that there was better correction in TolC, OmpW, FadL, LamB, Dps between 2-DE and its Western blotting but in OmpC, which was adverse between the two methods, showing up-regulated OmpC in 2-DE against down-regulated OmpC in 2-DE Western blotting (Figure 1D).

3.2. Altered OM Proteome in Response to Suddenly Strong CAP Treatment by 2-DE and its Western Blotting. To investigate whether the altered OM proteins in CAP-R were found in CAP-S, OM proteins were extracted from both of CAP-S and CAP-S-C and then were analyzed by 2-DE. Our results showed that two TolC (TolC 1, 2), a LamB, a FadL, three OmpT (OmpT 1−3), two OmpW (OmpW 1, 2), four Dps (Dps1−4), and no OmpC were detected in these gels, in which up-regulated TolC 1 and TolC 2, OmpT 1, OmpW, and down-regulated LamB, FadL, Dps 4 were determined in CAP-S compared with CAP-S-C (Figure 2A, B). These results indicated that CAP-S showed the same changes for FadL, LamB, and OmpT as CAP-R did, but not for TolC, OmpC, OmpW, and Dps. For TolC, one more up-regulated spot was determined in CAP-R than in CAP-S, sharing two. For OmpC, one spot was up-regulated in CAP-R and no any spot was found in CAP-S. For OmpW, three spots increased in CAP-R, but one in CAP-S, sharing one. For Dps, four were up in CAP-R and one up in CAP-S, sharing one. Furthermore, Western blotting was applied for confirmation of the 2-DE results as shown in Figure 2C. These altered OM proteins identified by 2-DE were confirmed by Western blotting in CAP-S. Meanwhile, down-regulated OmpC was determined when anti-OmpC was used as the primary antibody in Western blotting as a function of its sensitivity over 2-DE, suggesting the agreement in OmpC between CAP-R and CAP-S based on analysis of Western blotting. These results suggest the diversity of OM proteins in CAP-S with respect to CAP-R, but at least the change trend for the six OM proteins and Dps is similar between CAP-R and CAP-S. In summary, TolC, OmpC, OmpT, OmpC, FadL, LamB, and Dps are shared proteins from the two treatments.

3.3. TolC, OmpC, OmpT, and OmpW were Critical OM Proteins Responsible for CAP Resistance by Investigation of Their Mutants. To test the role of *tolC*, *ompC*, *ompT*, *ompW*, *fadL*, *lamb*, and *dps* in response to CAP resistance, MICs of these gene mutants, CAP-R and CAP-R-O, were determined using the broth microdilution method, as described by the NCCLS assay. Each experiment was repeated three times, and the typical MIC values obtained are reported in Figure 3A. When these mutants were cultured in MH medium with different concentrations of CAP, significant differences in MIC were observed in Δ*tolC*, Δ*ompC*, Δ*ompT*, and Δ*ompW* with respect to CAP-R, CAP-R-O, showing a 4-fold decrease in the Δ*tolC* and 2-fold decrease in the Δ*ompC*, Δ*ompT*, and Δ*ompW*. On the other hand, the function in CAP resistance of the seven genes was further investigated using the survival capability assay. CAP-R nad CAP-R-O and Δ*purA*, Δ*udp*, Δ*frdB*, and Δ*tsx* were used as original and irrelative-protein mutant controls, respectively. We set the cutoff value of inhibiting rates from 65.2 to 88.2% (original strain ± 15%) based on the 76.7% inhibiting rate of CAP-R-O. The cutoff value was used for the determination of relative and irrelative proteins to CAP resistance. Our results showed that the growth of Δ*tolC*, Δ*ompC*, Δ*ompT*, Δ*ompW*, Δ*fadL*, Δ*lamB*, and Δ*dps* was significantly inhibited in the range from 89.5 to 98.8% when they were cultured in 1/8 MIC of CAP-R-O in which few growth of Δ*tolC*, with OD 0.02 at 8 h incubation, was measured. The inhibiting

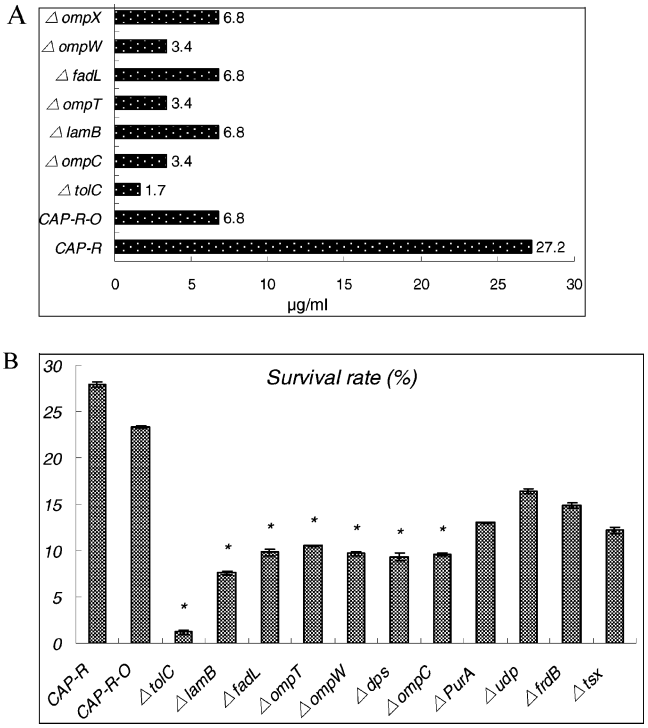

Figure 3. Testing for antimicrobial susceptibility. (A) MICs of CAP-R, CAP-R-O, and its mutants by broth microdilution method. *ompX* as irrelative control. (B) Survival capability of CAP-R, CAP-R-O, and its mutants in medium with 1/2 MIC CAP of CAP-R-O.

rates of control Δ*purA*, Δ*udp*, Δ*frdB*, and Δ*tsx* were from 83.6 to 87.8%, locating in the range of the cutoff value and showing no difference with CAP-R-O. The survival rates are summarized in Figure 3 B. Thus, the altered OM proteins determined by 2-DE were all related to CAP resistance with the use of the survival capability method. The sensitivity of the survival capability over the standard MIC measurement by broth microdilution method may be related to: (1) different concentrations of CAP used in the two methods, (2) function in antibiotic-related proteins with CAP concentration, and (3) the difference of sensitivity between the two methods. Of the seven proteins, nevertheless, TolC, OmpC, OmpT, and OmpW were closely related to CAP resistance. Down-regulated FadL, LamB, Dps, and OmpC encountered up-regulated TolC and OmpW in analysis of 2-DE and its Western blotting (decreased OmpC only identified by Western blotting), but Δ*fadL*, Δ*lamB*, Δ*dps*, and Δ*ompC* showed the increased inhibiting rates as up-regulated Δ*tolC* and Δ*ompW* did. As to OmpT, it had both up-regulated and down-regulated spots. In our other study on chlortetracycline-resistant *E. coli*, down-regulated LamB and up-regulated TolC were companied with significantly faster and lower growth than the control *E. coli* K12 BW 25113, respectively (data not shown). The reason why Δ*fadL*, Δ*lamB*, Δ*dps*, and Δ*ompC* showed the inhibited growth waits investigation.

3.4. Bacterial Growth in Medium with CAP was Sharply Inhibited by Anti-TolC. To find an approach to inhibit the function of OM proteins responsible for antibiotic resistance, we developed the method of bacterial growth combating by specific antibody in the current study. Effect of CAP on growth of CAP-R and CAP-R-O was investigated as shown in Figure 4 A. The growth was a bit faster in CAP-R than in CAP-R-O, especially in the medium with 1/2 MIC of CAP from CAP-R-O, suggesting the phenotypic characteristic of CAP-R in response

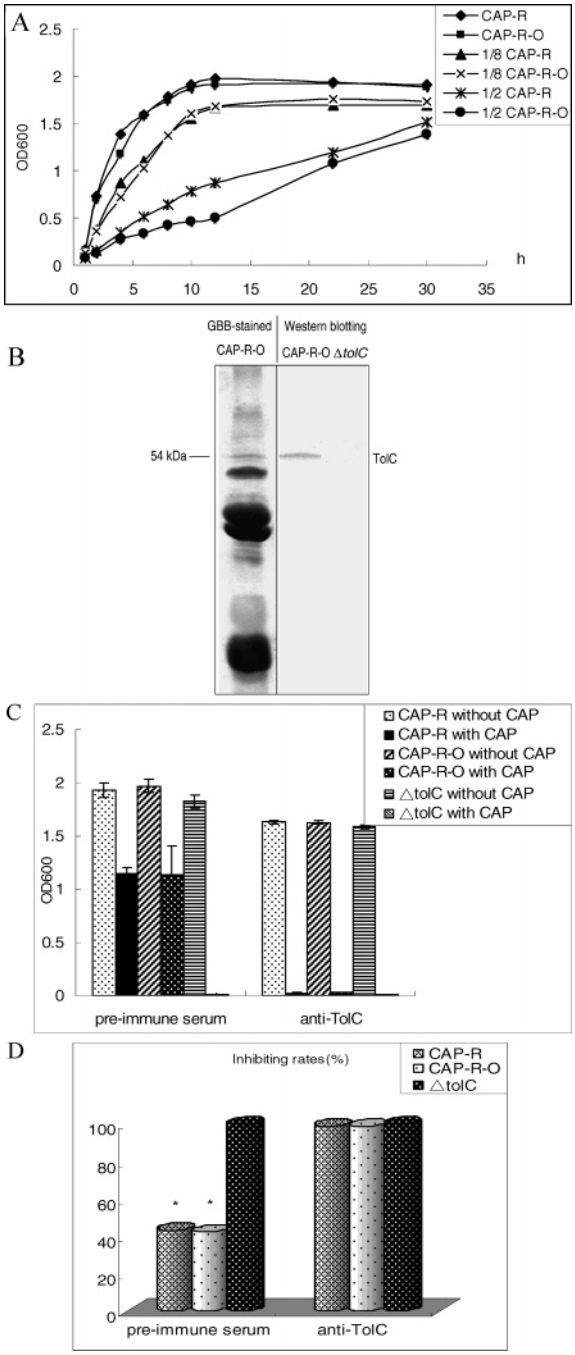

Figure 4. Growth of *E. coli* was combated by specific anti-TolC. (A) Growth curve of CAP-R, CAP-R-O in medium with and without CAP. CAP-R, CAP-R was cultured in LB medium; CAP-R-O, CAP-R-O was cultured in LB medium; 1/8 CAP-R, CAP-R was survived in LB medium with 1/8 MIC CAP of CAP-R-O; 1/8 CAP-R-O, CAP-R-O was survived in LB medium with 1/8 MIC CAP of CAP-R-O; 1/2 CAP-R, CAP-R was survived in LB medium with 1/2 MIC CAP of CAP-R-O; 1/2 CAP-R-O, CAP-R-O was survived in LB medium with 1/2 MIC CAP of CAP-R-O. (B) Identification of anti-TolC specificity, showing only a specific band at the TolC location was observed using sarcosine-insoluble fractions of CAP-R-O and Δ*tolC* as antigens in Western blotting of anti-TolC as the primary antibody. (C) CAP-R, CAP-R-O, and Δ*tolC* were separately treated with preimmune serum and anti-TolC serum and then cultured in LB medium without or with 1/8 MIC CAP of CAP-R-O. (D) Comparison of inhibiting rates between CAP-R, CAP-R-O, and Δ*tolC* separately treated by preimmune serum and anti-TolC serum. * showing significant difference ($P < 0.01$).

to CAP resistance. This investigation contributes to the evaluation of growth combating of CAP-R and CAP-R-O by antibodies. The bacterial growth-combating was performed using bacteria strains CAP-R, CAP-R-O, Δ*tolC*, Δ*ompC*, Δ*ompT*, and Δ*ompW*. They were separately mixed with a preimmune serum and a corresponding immune serum and then were cultured in LB medium with or without CAP. The survival ability was measured by monitoring the fraction of viable cells at 9 h as a function of the exposure of the 1/8 MIC antibiotic treatment. The significant effect of anti-OmpC, -OmpT, and -OmpW on bacterial growth was not observed (data not shown), but bacterial growth combating by anti-TolC was determined. The high specificity of anti-TolC and the resulting OD value at 600 nm for CAP-R, CAP-R-O, and Δ*tolC* is plotted in Figure 4B and C, respectively. The OD values decreased from 1.97 to 1.13 (inhibiting rate 42.64%) and from 1.63 to 0.03 (inhibiting rate 98.16%) in CAP-R-O, from 1.93 to 1.14 (inhibiting rate 40.09%) and from 1.62 to 0.02 (inhibiting rate 98.77%) in CAP-R, and from 1.83 to 0.01 (inhibiting rate 99.45%) and from 1.59 to 0.01 (inhibiting rate 99.37%) in Δ*tolC*, when they were cultured in medium with and without 1/8 MIC of CAP following an incubation with preimmune serum and anti-TolC serum, respectively. The inhibiting rates were shown in Figure 4C, which is significantly higher in Δ*tolC* than in CAP-R-O and in CAP-R after preimmune serum treatment, indicating more sensitive to CAP in Δ*tolC* than CAP-R-O and CAP-R. Interestingly, there were no differences between CAP-R-O, CAP-R, and Δ*tolC* with anti-TolC treatment, suggesting similar sensitiveness to CAP were shared between the three strains when CAP-R-O and CAP-R were bound with anti-TolC. These results suggest the anti-TolC targeting functions in bacterial growth-combating in medium with CAP, and the combating capability is equal to *tolC* deletion in 9 h observation. Therefore, bacterial growth in medium with antibiotics can be combated by an antibody specific to a drug-sensitive OM protein.

4. Discussion

In the present study, we try to set up a novel strategy for growth-combating by identification and antibody immunotherapeutic targeting of OM proteins in response to antibiotic resistance. Our strategy is based on the high-accuracy, high-efficiency, and high-throughput protein analyses by proteomic methodologies and the action on negative regulation of cell-surface protein function by antibody. We target the shared proteins responsible for the antibiotic-resistant bacteria both from the exposure to a suddenly strong antibiotic treatment and from persistent exposure to 1/2 MIC of the drug because the two exposures may be encountered by pathogens in resistance to the drug. Six OM proteins and an unknown location Dps were determined to be the shared proteins responds to CAP resistance and used for further analysis because our aim was to investigate the functional OM proteome in response to the resistance of this drug for drug targets. The six OM proteins belong to different functional classes, i.e., porins (LamB, OmpC), usher protein (FadL), otrher (TolC, OmpW, and OmpT) according to the Swiss-Prot databases.[29] A line of evidence has indicated TolC- and OmpC-mediated antibiotic resistance. TolC functions as a multidrug efflux system and OmpC regulates OM permeability for natural antibiotic resistance.[33,34] Recently, reports have shown the antibiotic-resistant proof in involvement of LamB, FadL, OmpW, and OmpT in some of bacterial species. Two clinical strains of *Enterobacter aerogenes* with overproduction of LamB and

OmpX exhibited phenotypes of multiresistance to beta-lactam antibiotics, fluoroquinolones, chloramphenicol, tetracycline, and kanamycine.[35] The resistant mutations were found in *fadL*, encoding a long-chain fatty acid transporter, which could decrease the intracellular concentration of novel antibacterial compound A-344583 in *Haemophilus influenzae* using restriction enzyme modulation of transformation efficiency.[36] OmpW was involved in the antibiotic resistance in *E. coli*, *Samonella enterica*, and *serovar typhimurium*.[30,37] The OmpT-mediated antibiotic flux was suggested, and its expression and activity increased in response to recombinant CAP acetyltransferase overexpression.[38,39] Thus, of the six OM proteins and Dps, behavior in resistance to CAP is ill-defined in OmpC, LamB, FadL, and OmpW. Our results provide the evidence of up-regulated TolC, OmpC, OmpW and down-regulated FadL, LamB, Dps as well as up- and down-regulated spots of OmpT responsible for CAP resistance. Of the seven proteins, TolC, OmpC, OmpW, and OmpT, especially TolC, were confirmed by their mutants to be strong carries in response to this drug resistance with the use of MIC measurement, and survival capability methods, and may become shared proteome for the targets of the new drug inhibiting CAP-resistant bacteria.

The elucidation of the molecular details of drug resistance is a very active area of research that crosses many disciplinary boundaries since an understanding of the mechanism(s) by which drug resistance develops leads to improvements in extending the efficacy of current antimicrobials.[9,40,41] Today, two major strategies are used in antibiotic drug discovery by the evaluation of the structural variation among existing antibiotic drug discovery, by the evaluation of the structural variations among existing antibiotic classes, and by the evaluation of novel antibiotic substance.[4] However, the development of new antibiotics does not keep pace with antibiotic resistance, and new therapy is urgently needed.[2,4,42,43] In the current study, we developed a novel approach of antibody immunotherapeutic targeting at a critical OM proteins responded to antibiotic resistance for bacterial growth-combating in environment with an antibiotic. Antisera to TolC, OmpC, OmpT, and OmpW were applied for this study, of which anti-TolC showed a very significant inhibition on bacterial growth in medium with CAP. This inhibition is so strong that the growth behaved as Δ*tolC* did. Thus, we demonstrate that TolC is an idea antibody immunotherapeutic target in combating of CAP-resistant bacteria. Thus, the novel approach developed significantly increases the sensitivity of bacteria to an antibiotic. Compared with the development of a new antibiotic to control the drug-resistant bacteria, there were several advantages for using this approach. First, the high specificity and affinity of antibody guaranty the specific binding with bacterial target proteins so that possible side-effects that commonly appear in patients with antibiotic treatment may be avoided in clinic. Second, specific antibody therapy may exclude drug resistance that rises from the use of antibiotics. Third, specific antibody may be an ideal factor that must complement an antibiotic for the treatment of a multifactorial disease, for example the bacterial and fungal sepsis of newborn babies.[39] Fourth, antibody therapy could avoid the long period of discovering new antibiotics. And last, antibody is inexpensive so that it may be widely used in clinic. Importantly, the specific antibody approach can greatly reduce dose of antibiotic used and inhibit the growth of antibiotic-resistant bacteria.

In summary, a novel approach was developed to combat the growth of bacteria in medium with CAP based on screening drug-resistant-related OM proteins and negatively regulating the critical OM proteins by proteomics and specific antibody, respectively. Our study suggests that combination therapy involving antibiotics that enhance the expression of an antibody target could be far more effective than either drug alone.

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