

Sheltering Effect and Indirect Pathogenesis of Carbapenem-Resistant *Acinetobacter baumannii* in Polymicrobial Infection

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The role of carbapenem-resistant *Acinetobacter baumannii* (CRAB) in polymicrobial infection remains elusive. Having observed the ability of CRAB to shelter other susceptible bacteria from carbapenem killing, we sought to determine the factors contributing to this sheltering effect by transforming different recombinant plasmids into recipient *A. baumannii* cells. The sheltering effects of CRAB were reproduced in recipient *A. baumannii* cells that highly expressed carbapenem-hydrolyzing class D β -lactamases (CHDLs) through their associated strong promoter. With the use of Western blot analysis and a bioassay, the highly expressed CHDLs were found to be extracellularly released and led to hydrolysis of carbapenem. The level of extracellular CHDLs increased after challenge with a higher concentration of CHDL substrates, such as carbapenem and ticarcillin. This increased CHDL may, in part, be attributed to cell lysis, as indicated by the presence of extracellular gyrase. In the planktonic condition, the sheltering effect for the cocultured susceptible bacteria might represent an indirect and passive effect of the CRAB self-defense mechanism, because coculture with the susceptible pathogen did not augment the amount of the extracellular CHDLs. Polymicrobial infection caused by CRAB and a susceptible counterpart exerted higher pathogenicity than monomicrobial infection caused by either pathogen alone in mice receiving carbapenem therapy. This study demonstrated that CHDL-producing CRAB appears to provide a sheltering effect for carbapenem-susceptible pathogens via the extracellular release of CHDLs and, by this mechanism, can enhance the pathogenesis of polymicrobial infection in the presence of carbapenem therapy.

Microorganisms that exist in the same ecological niche can interact in complex ways (1, 2). Different pathogens may act synergistically or in succession to mediate polymicrobial infections (2, 3). Clinical course, disease severity, and antimicrobial therapy outcomes are all affected by the presence of multiple pathogens (2, 4). Under some circumstances, polymicrobial infections have poorer outcomes than monomicrobial infections (5, 6). The contributions of individual pathogens toward the facilitated pathogenesis of polymicrobial infections have been delineated in several cases but remain elusive in many others (2, 3).

Acinetobacter baumannii is a leading pathogen of nosocomial infections worldwide. The rapid evolution of *A. baumannii* strains resistant to multiple antimicrobial agents, including carbapenem, has severely limited therapeutic options (7, 8). Carbapenem resistance in *A. baumannii* has mainly been attributed to the production of carbapenemases, particularly carbapenem-hydrolyzing class D β -lactamases (CHDLs), which include the OXA-23, -40, -51, -58 (9), and -143 classes of β -lactamases (10). Polymicrobial infection has been found in 20 to 50% of *A. baumannii* infections (11–13). Although *A. baumannii* itself is considered to be a low-virulence pathogen, its role in polymicrobial infection has not been delineated.

We observed several patients who had breakthrough polymicrobial bacteremia due to combined infection by carbapenem-susceptible microorganisms and carbapenem-resistant *A. baumannii* (CRAB) during the course of carbapenem therapy (see Table S1 in the supplemental material). The unexpected *in vivo* isolation of carbapenem-susceptible microorganisms in the presence of carbapenem could be due to factors such as inadequate

drug concentration at the site of infection, a compromised host immune system, phenotypic resistance such as biofilm formation of the susceptible pathogen (14), horizontal transfer of the carbapenemase gene from CRAB to the susceptible pathogen (15, 16), or a sheltering effect from the resistant counterpart (17). As described in this report, we observed that CRAB can provide a sheltering effect for susceptible pathogens, thereby protecting them from carbapenem therapy. In this study, we sought to determine the factors that contribute to this sheltering effect, by using recombinant plasmids transformed into *A. baumannii* cells and a mouse pneumonia model.

MATERIALS AND METHODS

Bacterial strains, plasmids, molecular techniques, chemicals, and antimicrobial susceptibility testing. Bacterial strains and plasmids used in this study are listed in Table 1, and primers are shown in Table S2 in the supplemental material. *A. baumannii* was identified using a multiplex

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description and/or use ^a
Strains	
Ec1003	Clinical carbapenem-susceptible <i>Escherichia coli</i> isolate (IPM MIC, 0.125 mg/liter) recovered concomitantly with <i>A. baumannii</i> Ab1969. This isolate was used in most of the experiments to demonstrate the sheltering effect of carbapenem-resistant <i>Acinetobacter baumannii</i> (CRAb). Clinical characteristics of the patient from whom it was isolated are given in Table S1 in the supplemental material.
EcYT439	Clinical carbapenem-susceptible <i>E. coli</i> isolate (IPM MIC, 0.25 mg/liter), used in the expt in Fig. 1D to demonstrate the sheltering of CRAb.
<i>E. coli</i> strain BL21	Used in antigen production (Invitrogen, Carlsbad, CA).
KpTL425	Clinical carbapenem-susceptible <i>Klebsiella pneumoniae</i> isolate (IPM MIC, 0.25 mg/liter), used in the expt in Fig. 1D to demonstrate the sheltering of CRAb. Clinical characteristics of the patient from whom it was isolated are given in Table S1.
EntCYT240	Clinical carbapenem-susceptible <i>Enterobacter cloacae</i> isolate (IPM MIC, 0.5 mg/liter) recovered from a urine sample. It was used in the expt in Fig. 1D to demonstrate the sheltering of CRAb.
PaTL424	Clinical carbapenem-susceptible <i>Pseudomonas aeruginosa</i> isolate (IPM MIC, 2 mg/liter), used in the expt in Fig. 1D to demonstrate the sheltering of CRAb. Clinical characteristics of the patient from whom it was isolated are given in Table S1.
Ab1969	<i>A. baumannii</i> isolate overproducing OXA-58, isolated from a patient with polymicrobial bacteremia that concomitantly grew Ec1003. Clinical characteristics of the patient from whom it was isolated are given in Table S1.
Ab290	Clinical <i>A. baumannii</i> isolate susceptible to multiple antimicrobials, including TIC, used as a recipient in transformation.
Plasmids	
pET-28a	Novagen commercial vector (Madison, WI) used in gene fragment cloning to generate polyclonal antibodies.
pYMAB-2	<i>A. baumannii</i> - <i>E. coli</i> shuttle vector, in which a replicon of a plasmid from <i>A. baumannii</i> reference strain ATCC 19606 ^T was inserted into pET-28a. No other component of the plasmid, including <i>mob</i> that encodes a protein assisting the mobilization of the plasmid, was included; KAN ^r .
pOXA-58-2	IS1008-ΔISAb3- <i>bla</i> _{OXA-58} amplified by primers IS1008 (XbaI)F and OXA-58 (XhoI)R and cloned into the XbaI and XhoI sites of pYMAB-2.
pOXA-58ΔP2-1	Partial or total P2 promoter on IS1008-ΔISAb3- <i>bla</i> _{OXA-58} deleted by amplification using either primer IS1008-ΔP2-1(XbaI)F, IS1008-ΔP2-2 (XbaI)F, or IS1008-ΔP2-3 (XbaI)F and primer OXA-58 (XhoI)R, and then cloned into the XbaI and XhoI sites of pYMAB-2.
pOXA-23	<i>bla</i> _{OXA-23} and its promoter in ISAb1 amplified by primers ISAb1(XbaI)F and OXA23-like (XhoI)R and cloned into the XbaI and XhoI sites of pYMAB-2. OXA-23 was His tagged.
pOXA-72	<i>bla</i> _{OXA-72} and its promoter amplified by primers OXA-24(XbaI)F and OXA-24-like (XhoI)R and cloned into the XbaI and XhoI sites of pYMAB-2. OXA-72 was His tagged.
pOXA-83	<i>bla</i> _{OXA-83} and its promoter in ISAb1 amplified by primers ISAb1(XbaI)F and OXA-51-like (XhoI)R and cloned into the XbaI and XhoI sites of pYMAB-2. OXA-83 was His tagged.

^a IPM, imipenem; TIC, ticarcillin; KAN^r, kanamycin resistant.

PCR method (18). Bacteria belonging to the family *Enterobacteriaceae* and *Pseudomonas aeruginosa* were identified using a Vitek II system (bioMérieux, Marcy l'Etoile, France). Genes encoding class A, B, and D carbapenemases and the upstream insertion sequence of CHDL genes were detected by a PCR method, as previously described (19, 20). Phenotypic tests were used to identify class B metallo-β-lactamases, as previously described (20). Imipenem, ticarcillin, and kanamycin were purchased from Sigma-Aldrich (St. Louis, MO). Restriction enzymes were purchased from New England BioLabs (Beverly, MA, USA). Antimicrobial susceptibility testing was determined by an agar dilution test (21) or Etest (AB Biodisk, Solna, Sweden). The breakpoint was defined as recommended by the Clinical and Laboratory Standards Institute (22).

Coculture of bacteria and determination of colony count. Overnight cultures of different bacteria and *A. baumannii* strains were diluted 100-fold and cocultured in 5 ml of Luria-Bertani (LB; Difco, Detroit, MI) broth in a Pyrex culture tube (Corning Life Sciences, Manassas, VA) at 37°C for 2 h before antimicrobial agents were added. At given time points, 100-μl portions of 10-fold-diluted coculture were plated on an appropriate agar plate (see Fig. S1 in the supplemental material). *A. baumannii* and other bacterial species were discriminated by the colony characteristics of each species on the plates after overnight culture. Randomly selected colonies were subjected to species validation by the identification methods mentioned above.

Preparation of cellular fractions. Overnight cultures of *A. baumannii* transformants, including strains Ab290(pOXA-58-2), Ab290(pOXA-58ΔP2-1), Ab290(pOXA-58ΔP2-2), and Ab290(pOXA-58ΔP2-3), were

diluted 100-fold and cultured in 100 ml of LB broth. In some experiments, antimicrobials, with or without carbapenem-susceptible Ec1003 (100-fold-diluted overnight culture), were added to the culture of Ab290(pOXA-58-2). Cultures were grown to a logarithmic phase, and the cell densities (CFU/ml) were determined. The cultures were centrifuged at 10,000 × g for 15 min at 4°C. The supernatants, consisting of the extracellular fraction, were collected, filtered with a 0.22-μm Acrodisc filter (Sigma-Aldrich), and concentrated using Amicon Ultra columns (Millipore, Bedford, MA). Periplasmic and cytoplasmic fractions of the cells were fractionated as described previously (23). All cellular fractions were stored at -20°C prior to use.

Antibodies. The internal fragment of *bla*_{OXA-58} or *gyrA* from *A. baumannii* was amplified with the primer pair OXA-58-21 (BamHI)F (F stands for forward) and OXA-58-21 (XhoI)R (R stands for reverse) or Gyrase-A(KpnI) P1 and Gyrase-A(PstI) P2, respectively (see Table S2 in the supplemental material). These fragments were subcloned into a pET-28a vector (Novagen, Madison, WI) to generate His-tagged proteins. Polyclonal antibodies against the OXA-58 and gyrase proteins of *A. baumannii* were generated from rabbits as previously described (24). The quality of purified polyclonal antibodies was examined using Western blot analysis with the preimmune serum as a negative control. The antibody for the detection of His-tagged protein was purchased from Anogen (Ontario, Canada).

Western blot analysis. Protein samples of cellular fractions from ~2 × 10⁸ CFU bacteria were separated by polyacrylamide gel electrophoresis and immobilized on nitrocellulose membranes. The membranes were incubated with primary antibodies, followed by horseradish perox-

idase-conjugated goat anti-rabbit (Sigma-Aldrich) or goat anti-mouse (Jackson ImmunoResearch Labs, West Grove, PA) secondary antibodies. An enhanced chemiluminescence (ECL) Western blot kit (PerkinElmer, Boston, MA) was used to detect chemiluminescence.

Bioassay for the detection of carbapenem inactivation. Imipenem or kanamycin was diluted in 10 μ l of phosphate-buffered saline (PBS) and preincubated with 10 μ l of a concentrated extracellular fraction of *A. baumannii* transformants for 1 h at 37°C. Each 20- μ l mixture was loaded onto a blank disk (Becton, Dickinson and Company, Franklin Lakes, NJ) that was placed in an agar plate containing a lawn of Ec1003, a carbapenem-susceptible *Escherichia coli*. Inhibitory zones were measured after incubating the plates overnight at 37°C. Antimicrobial inactivation was characterized by a decrease in the area of the inhibitory zone in the presence of the extracellular fraction from carbapenemase-producing transformants compared to that from the vector-alone transformants.

Murine model of polymicrobial infection. Animal experiments were approved by the Ethical Committee for Animal Experiments of National Yang-Ming University. C57BL/6N mice (6 to 7 weeks old, 16 to 18 g) were used (National Laboratory Animal Center, Taiwan). Bacteria were grown to logarithmic phase at 37°C, washed, resuspended in 50 μ l of sterile isotonic saline, and premixed with 50 μ l of 10% porcine mucin (Sigma-Aldrich). Mice were anesthetized, and 100- μ l amounts of bacterial solution were intratracheally introduced into their lungs. The 50% lethal dose (LD₅₀) in mice was calculated using the SigmaPlot (version 7.0) program from SPSS Inc. (Chicago, IL).

In the treatment studies (10 mice/group), mice were intratracheally inoculated with *E. coli* Ec1003 ($\sim 3 \times 10^4$ CFU) and/or *A. baumannii* transformants ($\sim 3 \times 10^6$ CFU). Three hours after inoculation, the mice were intraperitoneally treated with either imipenem (40 mg/kg of body weight, every 8 h) or the equivalent volume of PBS for 2 days. Their survival was recorded. In separate experiments, mice were sacrificed at different time points after inoculation to determine the bacterial counts in the lung. These mice were anesthetized and sacrificed in a CO₂ chamber. The whole lungs were removed, weighed, and homogenized in 1 ml of PBS. Homogenates were serially diluted and plated onto MacConkey agar to determine the *A. baumannii* and *E. coli* colony counts.

Statistical analysis. Continuous variables were analyzed by one-way analysis of variance (ANOVA), followed by the Scheffé posthoc test or Student's *t* tests. Mouse mortality was analyzed with the Kaplan-Meier survival analysis (log rank test). Analyses were performed using SPSS (Statistical Package for the Social Sciences) version 18.0 (Chicago, IL, USA). A *P* value of <0.05 was considered statistically significant.

RESULTS

Sheltering effect of CHDL-producing *A. baumannii*. Isolates from the first patients (see Table S1 in the supplemental material), which included a carbapenem-resistant *A. baumannii* (CRAb) (Ab1969) and a carbapenem-susceptible *E. coli* (Ec1003), were used to demonstrate the carbapenem sheltering effect. Strain Ec1003 could survive only in carbapenem-containing broth (imipenem, 8 mg/liter) in the presence of strain Ab1969 (Fig. 1A). The Ab1969 strain carried a carbapenemase-encoding gene, *bla*_{OXA-58}. Upstream of *bla*_{OXA-58} was *ISAbA3*, which was inserted by *IS1008* (*IS1008-ΔISAbA3-bla*_{OXA-58}). The *ISAbA3* sequence provides a promoter (P1) for *bla*_{OXA-58} expression (25). Together, these elements conferred resistance to ticarcillin (MIC, 256 mg/liter) but not to carbapenem (MIC, 1 mg/liter). Insertion of *IS1008* in *ISAbA3* generates a stronger hybrid promoter (P2) for *bla*_{OXA-58} expression. The construct *IS1008-ΔISAbA3-bla*_{OXA-58} has been shown to confer carbapenem resistance (MIC, 64 mg/liter) and a higher level of ticarcillin resistance (MIC > 1,024 mg/liter) than construct *ΔISAbA3-bla*_{OXA-58} (with deletion of P2) confers (25). Strain Ab1969 also carries an intrinsic *bla*_{OXA-66} that lacks *ISAbA1* as the immediate upstream element (26). Thus, *bla*_{OXA-66} was not

overexpressed in this strain and, therefore, was not responsible for the carbapenem resistance in this isolate (26). No other known class A or B carbapenemase gene was detected.

Next, we sought to determine whether OXA-58, but not other factors from strain Ab1969, is responsible for sheltering strain Ec1003 from carbapenem killing. Ab290(pOXA-58-2) is a transformant carrying an *A. baumannii-E. coli* shuttle vector (pYMAB-2) harboring *IS1008-ΔISAbA3-bla*_{OXA-58}. When Ab290(pOXA-58-2) was cocultured with Ec1003 in the presence of imipenem, Ec1003 was sheltered from carbapenem killing in the presence of a high inoculum of Ab290(pOXA-58-2) (2×10^6 CFU/ml; Fig. 1B and Fig. S1 in the supplemental material). In contrast, the Ab290 transformant bearing the shuttle vector pYMAB-2 failed to exert a sheltering effect (Fig. 1D). Ec1003 cells recovered from the plate cocultured with either strain Ab1969 or Ab290(pOXA-58-2) retained their susceptibility to imipenem after subculture in the absence of carbapenem-resistant *Acinetobacter* strains. In addition, PCR also did not detect *bla*_{OXA-58} in these Ec1003 cells (data not shown), indicating that the growth of these Ec1003 cells in the presence of carbapenem was not due to spontaneous genetic changes or to the acquisition of resistant determinants, including *bla*_{OXA-58}.

Ab290(pOXA-58-2) also resisted ticarcillin (conferred by *bla*_{OXA-58}) and kanamycin (conferred by *aph* on the shuttle vector). However, while this variant exhibited a sheltering effect against ticarcillin, it did not protect Ec1003 against kanamycin-induced death (Fig. 1C). These results demonstrate the presence of a specific sheltering effect conferred by OXA-58 expression in the Ab290 variant. The carbapenem sheltering effect of Ab290(pOXA-58-2) could also be demonstrated using another carbapenem-susceptible *E. coli* strain (EcYT439), as well as *Klebsiella pneumoniae* (strain KpTL425), *Enterobacter cloacae* (strain EntC YT240), and *P. aeruginosa* (strain PaTL424) (Fig. 1D; see Fig. S1 in the supplemental material). Ab290 transformants producing other proteins in the CHDL family, including OXA-23, -72, and -83, were also shown to exert a similar carbapenem sheltering effect (Fig. 1E).

Detection of active OXA-58 in the extracellular fraction of *A. baumannii* overproducing OXA-58. We have shown that the CRAb sheltering effect could be demonstrated in planktonic cultures. Therefore, we hypothesized that OXA-58 is released extracellularly by the CRAb strain to confer carbapenem shelter to susceptible bacteria. Western blot analysis confirmed the presence of extracellular OXA-58 in *A. baumannii* Ab290(pOXA-58-2) cultures (Fig. 2A), but not Ab290(pYMAB-2) cultures (see Fig. S2 in the supplemental material). Other CHDL proteins, including OXA-83, OXA-72, and OXA-23 could also be detected in the extracellular fraction of transformants producing these enzymes (Fig. S2). This extracellular OXA-58 likely resulted from cellular secretion or leakage, as opposed to cell lysis, as indicated by the absence of gyrase immunoreactivity that is typically observed in the bacterial cytoplasmic fraction (Fig. 2A).

OXA-58 production by the hybrid promoter (P2) driving *IS1008-ΔISAbA3* was required for the presence of extracellular OXA-58 (Fig. 2A). In *A. baumannii* Ab290(pOXA-58ΔP2-3) cells, in which the P1 *bla*_{OXA-58} promoter was retained by deletion of the P2 promoter, the concentration of OXA-58 in the cytoplasmic fraction was decreased, and OXA-58 immunoreactivity was not detected in the periplasmic and extracellular fractions. The extracellular fraction of Ab290(pOXA-58-2) could hydrolyze imipenem (Fig. 2B), but not kanamycin (Fig. 2B), indicating the specificity of the hydrolytic activity of extracellular OXA-58. This

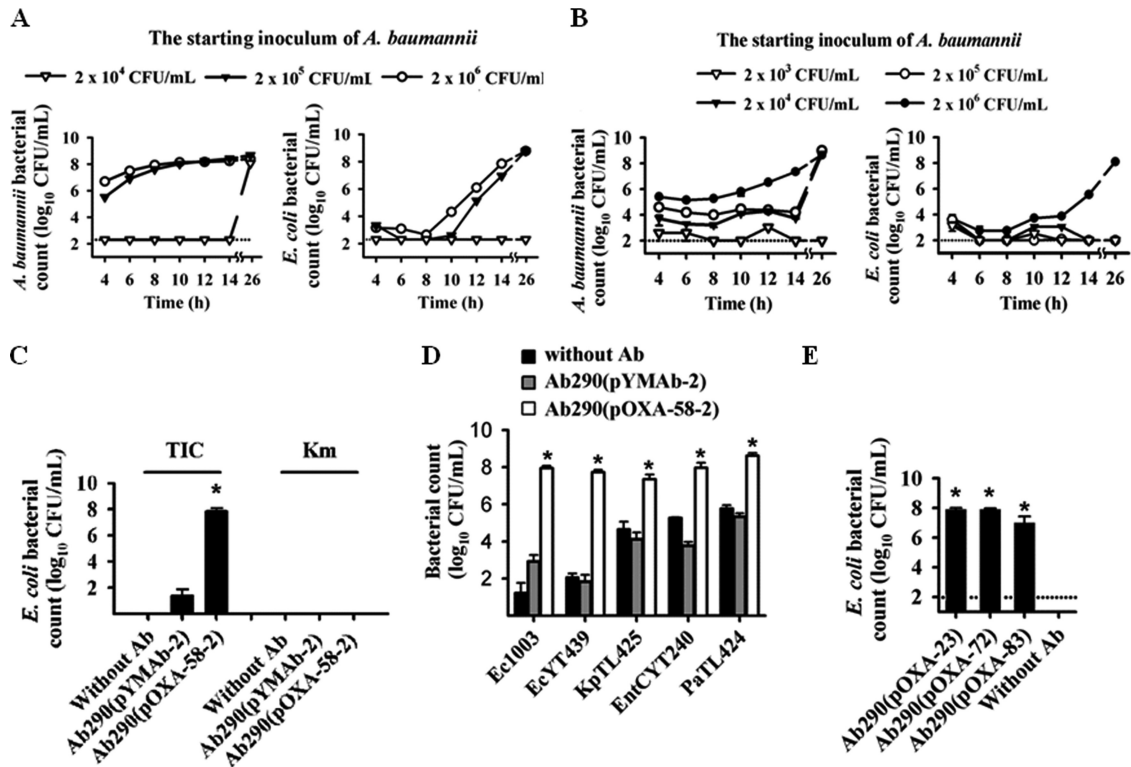


FIG 1 Carbenem-resistant *Acinetobacter baumannii* producing carbenem-hydrolyzing class D β -lactamases (CHDLs) confer shelter to carbenem-susceptible bacteria. (A and B) A carbenem-susceptible *Escherichia coli* strain (Ec1003) (10^4 CFU/ml) was cocultured with different amounts of a clinical *A. baumannii* isolate producing OXA-58 (Ab1969) (A) or an *A. baumannii* transformant producing OXA-58 [termed Ab290(pOXA-58-2)] (B) in the presence of imipenem. In panel B, although *A. baumannii* Ab290(pOXA-58-2) could survive under the culture condition at an inoculum of 10^4 CFU/ml or higher (left panel), *E. coli* Ec1003 was protected from carbenem killing only at 10^6 CFU/ml of Ab290(pOXA-58-2) (right panel). (C) At 10^6 CFU/ml, Ab290(pOXA-58-2) sheltered Ec1003 against ticarcillin (TIC) but not kanamycin (Km) killing. The control Ab290(pYMAb-2) transformant, which carries an empty shuttle vector, failed to exert the sheltering effect. The experiment was also performed without *A. baumannii* (Without Ab). (D) Sheltering effect of *A. baumannii* Ab290(pOXA-58-2) for other Gram-negative bacteria against carbenem-induced death. The first two or four letters of the strain designations indicate the species as follows: Ec, *E. coli*; Kp, *Klebsiella pneumoniae*; EntC, *Enterobacter cloacae*; Pa, *Pseudomonas aeruginosa*. (E) Ab290 transformants producing other CHDL proteins, including OXA-23, -72, and -83, sheltered Ec1003 against carbenem-induced death. Each symbol in panels A and B is the mean of triplicate results. The dotted lines in panels A, B, and E indicate the detection limit. The bars and error bars in panels C to E indicate the means and standard deviations, respectively, of triplicate tests. Values that are significantly different ($P < 0.05$) are indicated by an asterisk.

hydrolytic activity was positively correlated with the amount of OXA-58 that was overexpressed (Fig. 2B). No imipenem hydrolysis was observed in the extracellular fraction of Ab290(pOXA-58 Δ P2-3), in which the imipenem inhibitory zone was comparable to that of the Ab290(pYMAb-2) control. The coculture data also indicated a crucial role for OXA-58 production by hybrid promoter P2 in the CRAb sheltering effect (Fig. 2C).

Treatment with antimicrobial agents increases extracellular CHDL. The amount of extracellular OXA-58 of *A. baumannii* Ab290(pOXA-58-2) was increased in the presence of higher concentrations of OXA-58 substrate, including imipenem (8 mg/liter) and ticarcillin (1,000 mg/liter), but not in the presence of higher concentrations of kanamycin (Fig. 3A). This increased OXA-58 may, in part, be attributed to cell lysis, as indicated by the presence of extracellular gyrase. No augmentation of the extracellular OXA-58 from Ab290(pOXA-58-2) was detected when this strain was cocultured in planktonic culture with *E. coli*, as indicated by the results of Western blot analysis (Fig. 3B) and the imipenem hydrolysis assay (Fig. 3C).

In vivo sheltering effect and indirect pathogenic role of CHDL-producing *A. baumannii*. In the murine model, *E. coli*

Ec1003 alone was more virulent than either *A. baumannii* Ab290(pYMAb-2) or Ab290(pOXA-58-2) alone, with LD₅₀ values of 1.795×10^4 , 1.563×10^6 , and 7.096×10^7 CFU, respectively. However, only Ab290(pOXA-58-2) (with inoculums of $\sim 3 \times 10^6$ CFU), but not Ec1003 ($\sim 3 \times 10^4$ CFU) or Ab290(pYMAb-2) ($\sim 3 \times 10^6$ CFU), was able to survive in imipenem-treated mice for 48 h after inoculation (data not shown).

In the polymicrobial model mice, the amount of *E. coli* Ec1003 in mice coinfecting with *A. baumannii* Ab290(pOXA-58-2) was significantly higher than in those coinfecting with Ab290(pYMAb-2) at 24 and 36 h after inoculation in the imipenem-treated group (Fig. 4A). The amount of Ec1003 in mice coinfecting with Ab290(pOXA-58-2) did not differ between those treated with imipenem or PBS (Fig. 4A). These results indicate that Ab290 expressing OXA-58 demonstrated an *in vivo* sheltering effect for Ec1003.

In the imipenem-treated groups (Fig. 4B), the 72-h mortality was significantly higher in mice coinfecting with *E. coli* Ec1003 and *A. baumannii* Ab290(pOXA-58-2) (80%) or Ab290(pOXA-72) (50% [see Fig. S3 in the supplemental material]) than in those coinfecting with Ec1003 and Ab290(pYMAb-2) (0%) or in those

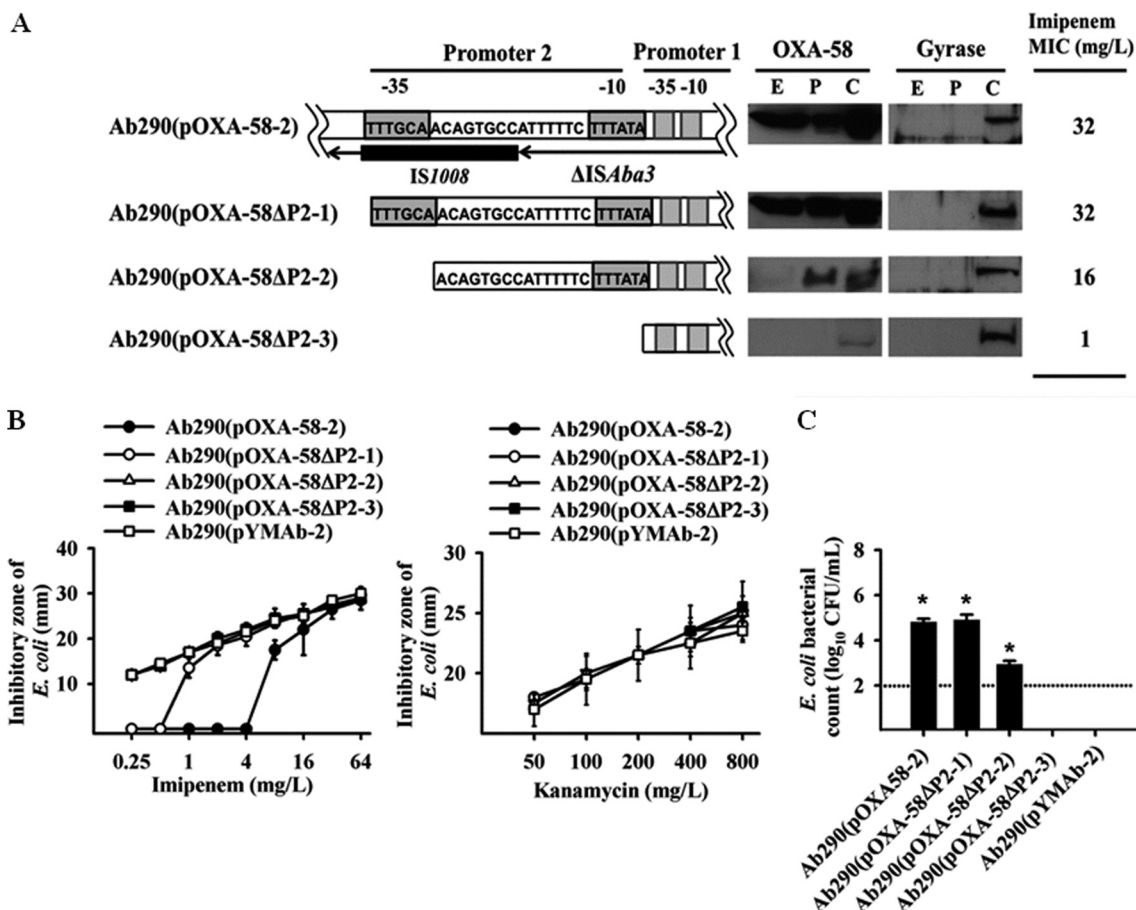


FIG 2 Detection of active CHDL in extracellular fractions from *A. baumannii* cells highly expressing OXA-58. (A) OXA-58 was detected in the extracellular fraction of *A. baumannii* Ab290(pOXA-58-2) by Western blotting. Constructs carrying a deletion at promoter 2 (P2) are demonstrated in the schematic map. Deletion of the -35 component of P2, or the entire P2, drastically diminished extracellular levels of OXA-58. The extracellular fraction (E), periplasmic fraction (P), and cytoplasmic fraction (C) of the transformants are indicated in the gels. (B) The imipenem (IPM) hydrolytic activity of several transformants was detected using a bioassay, in which failure of the transformants to hydrolyze kanamycin indicated specific IPM hydrolysis by OXA-58. (C) The sheltering effect of carbapenem-resistant *A. baumannii* (CRAB) was associated with a high level of OXA-58 expression. Colony counts were determined after 6-h cultures to determine the difference in the sheltering effect by transformants harboring different deleted promoters. Values that are significantly different ($P < 0.05$) are indicated by an asterisk. The dotted line indicates the detection limit. In panels B and C, data are presented as the means and standard deviations (error bars) of triplicate tests.

that received monomicrobial Ab290(pOXA-58-2) (0% [data not shown]). Taken together, these results indicate that the *in vivo* imipenem sheltering effect of Ab290(pOXA-58-2) for Ec1003 was associated with enhanced pathogenicity in polymicrobial, compared to monomicrobial, CRAB infection during imipenem therapy.

DISCUSSION

In this study, we demonstrated a novel role for CRAB in the clinical setting in addition to its impact in severely ill patients (11). In polymicrobial infection, CRAB may shelter carbapenem-susceptible bacteria from carbapenem killing. In a murine pneumonia model, polymicrobial infection resulting from inoculation with CRAB and a carbapenem-susceptible, but more virulent, pathogen displayed higher pathogenicity than did a monomicrobial infection caused by either pathogen alone during carbapenem therapy. This result has emphasized the importance of early identification of CRAB in polymicrobial infection and highlighted the

necessity of antibiotic regimens that can eradicate CRAB in polymicrobial infections.

In this study, the sheltering effect of CRAB could be attributed, at least in part, to extracellularly released carbapenem-hydrolyzing CHDLs. Bacteria can also provide antimicrobial resistance to susceptible recipients through horizontal gene transfer (15), formation of biofilm (27), the conveyance of antimicrobial inactivation enzymes through type VI secretion systems (28), membrane vesicles (29), or nanotube connections (30), and the production of volatile compounds (e.g., ammonia) to reduce the membrane permeability of susceptible recipients (31). The advantage of the mechanism we describe (i.e., sheltering conferred by extracellular CHDL) is that even relatively remote bacteria can be protected.

Extracellular β -lactamases, including those found in the biofilm matrix, were previously considered to be the result of cell lysis after exposure to antimicrobials (27). Our results indicate that the CHDLs were extracellularly released in the absence of antimicrobials. The extracellular CHDLs were not the results of cell lysis, as

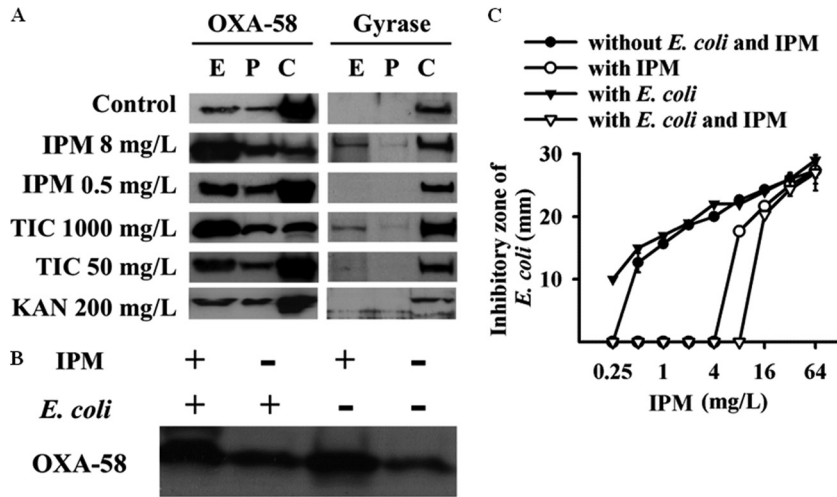


FIG 3 Extracellular OXA-58 is increased in the presence of antimicrobials in CRAB cultures. (A) Extracellular OXA-58 in *A. baumannii* Ab290(pOXA-58-2) cultures was increased in the presence of higher concentrations of imipenem (IPM) and ticarcillin (TIC), but not kanamycin (KAN). The extracellular fraction (E), periplasmic fraction (P), and cytoplasmic fraction (C) of the transformants are indicated. (B and C) The presence of *E. coli* (Ec1003) did not augment the extracellular release of OXA-58, as demonstrated by Western blotting (B) and IPM hydrolysis assay (C). In panel C, values are means and standard deviations (error bars) of triplicate tests.

indicated by the absence of gyrase. This extracellular release of CHDLs was intimately linked to their high expression with a strong promoter. Although the exact mechanism is currently undetermined, the extracellular release of highly expressed periplasmic proteins has been demonstrated previously (32, 33). The maintenance of some extracellular CHDLs might act as a front-line protective strategy for *A. baumannii* to hydrolyze relevant

antimicrobials, such as carbapenem or ticarcillin, before they reach the cells. We observed that an increase in the concentrations of these antimicrobials can trigger cell lysis and increase the extracellular CHDL concentration, which represents a more rapid and effective method to cope with large amounts of antimicrobial challenges.

We did not determine whether the carbapenem sheltering ef-

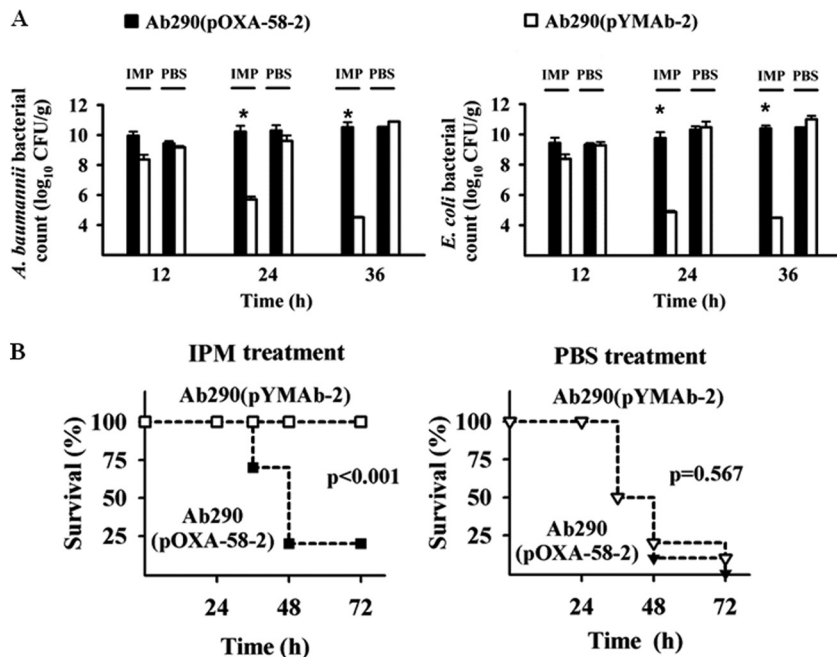


FIG 4 *A. baumannii* producing OXA-58 exhibits an *in vivo* sheltering effect. Mice were coinfecting with *E. coli* Ec1003 and *A. baumannii* Ab290(pOXA-58-2) or Ec1003 and Ab290(pYMAb-2), and treated with IPM or PBS 3 h later. The numbers of bacteria in the mouse lung and the mortality rates of mice were determined at different time points after inoculation. (A) *A. baumannii* Ab290(pOXA-58-2) shelters *E. coli* Ec1003 from IPM killing *in vivo*. (B) Together, these bacteria exhibited enhanced pathogenicity and resulted in higher mortality. The bars and error bars in panel A indicate the means and standard deviations of triplicate tests. *, $P < 0.05$.

fect depended on additional mechanisms in the cocultured susceptible bacteria, such as changes in membrane permeability or increased expression of efflux pumps. In planktonic cultures, the sheltering effect for the cocultured susceptible bacteria might represent an indirect and passive effect of the CRAb self-defense mechanism, because the presence of *E. coli* did not significantly augment the amount of the extracellular CHDLs. Another explanation for this result is that the limited sensitivity of our detection methods may have failed to discriminate subtle differences in CHDL levels.

Interestingly, the kanamycin-resistant determinant did not display similar sheltering effects. One reason for this finding may be that the kanamycin-resistant determinants did not secrete into the extracellular space. Another explanation may be the high energy cost for aminoglycoside modification, which would inhibit the extensive inactivation of aminoglycoside (34).

Previous studies have not consistently demonstrated that β -lactamase-producing bacteria (BLPB) can shelter susceptible partners from β -lactam killing (17, 35–37). The BLPB sheltering effect may vary with the type of β -lactamase produced, as well as with the microorganisms that produce them (37). In the current study, we demonstrated that the inoculum of CRAb and the level of CHDL expression play deterministic roles in the sheltering effect.

In our model, the susceptible bacteria, including *A. baumannii*, are not eradicated in the mice after treatment with imipenem for 36 h. However, all the mice that survived 48 h of imipenem treatment had their carbapenem-susceptible pathogen eradicated (data not shown). The survival of carbapenem-susceptible *A. baumannii* in the lungs of mice treated with imipenem (120 mg/kg/day, divided into 3 doses) had also been demonstrated in a previous study which used the same pneumonia model (38). The mucin included in the inoculation might hinder the clearance of these susceptible pathogens.

In conclusion, we have demonstrated that CRAb plays a novel role in polymicrobial infection by sheltering carbapenem-susceptible pathogens, thereby exacerbating the pathogenesis of polymicrobial infection during carbapenem therapy. These results indicate a greater importance for the presence of CRAb in clinical settings and suggest a need for more-aggressive controls of CRAb in polymicrobial infection.

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