Peptidoglycan editing provides immunity to *Acinetobacter baumannii* during bacterial warfare

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Peptidoglycan (PG) is essential in most bacteria. Thus, it is often targeted by various assaults, including interbacterial attacks via the type VI secretion system (T6SS). Here, we report that the Gram-negative bacterium *Acinetobacter baumannii* strain ATCC 17978 produces, secretes, and incorporates the noncanonical d-lysine into its PG during stationary phase. We show that PG editing increases the competitiveness of *A. baumannii* during bacterial warfare by providing immunity against peptidoglycan-targeting T6SS effectors from various bacterial competitors. In contrast, we found that d-Lys production is detrimental to pathogenesis due, at least in part, to the activity of the human enzyme d-lysine oxidase (DAO), which degrades d-Lys producing H2O2 toxic to bacteria. Phylogenetic analyses indicate that the last common ancestor of *A. baumannii* had the ability to produce d-Lys. However, this trait was independently lost multiple times, likely reflecting the evolution of *A. baumannii* as a human pathogen.

INTRODUCTION

Peptidoglycan (PG) is a major component of the bacterial cell envelope. Layers of PG surround the cytoplasmic membrane, maintaining cell shape and providing resistance to osmotic stress. PG is composed of glycan chains of alternating N-acetylmuramic acid (GlcNAc) and N-acetylmuramiduramic acid (MurNAc) that are connected through MurNAc-attached peptides (1). Despite its rigidity, PG is necessarily a dynamic structure, requiring constant turnover to enable fundamental processes such as growth and cell division (2). PG synthesis is a multistep process. The PG precursor lipid II, which consists of a GlcNAc-MurNAc pentapeptide (1- Ala-d-dGlu-mDAP-d-Ala-d-Ala in Gram-negative bacteria) linked to a lipid carrier, is synthesized at the cytoplasmic membrane and subsequently translocated into the outer leaflet of the cytoplasmic membrane. Glycan chains are then polymerized by glycosyltransferases, and peptide cross-links are formed through transpeptidation reactions catalyzed by DD-transpeptidases and LD-transpeptidases.

Because of its essentiality, the bacterial cell wall is targeted by various potentially fatal threats, including attacks from bacterial competitors. Competition among Gram-negative bacteria is largely mediated by the type VI secretion system (T6SS) (3). The T6SS is a dynamic nanomachine structurally related to contractile phage tails, and it is used to deliver toxic effector proteins, including PG hydrolases, from an attacking cell (predator) to nearby competitors (prey) in a contact-dependent manner (4–6). Immunity to T6SS-dependent killing is accomplished by the expression of immunity proteins, which specifically bind and inactivate their cognate effector. The limited repertoire of immunity proteins encoded by one bacterium is insufficient to protect against the large diversity of T6SS effectors encoded by bacterial predators. Therefore, the primary role of immunity proteins is to prevent lethal interactions between sister cells (7–9). General resistance mechanisms to prevent T6SS-dependent killing by nonkin competitors remain poorly characterized (10).

Additional threats to the integrity of the bacterial cell wall include the host immune system, antibiotics, and osmotic stress (11–13). Constant exposure to these threats has exerted selective pressure favoring bacterial species capable of modifying their PG to evade or withstand fatal threats. These modifications can occur during PG precursor synthesis or after PG precursors are incorporated into the growing cell wall meshwork (14). PG modifications enabling immune evasion and resistance to antibiotics have been extensively studied and generally include chemical modifications to the glycan backbone or changes in pentapeptide composition or degree of cross-linking (12, 14–18). In addition, PG editing with noncanonical d-amino acids (NCDAAs) has been shown to make bacteria more resistant to osmotic stress (19). NCDAAs have been best characterized in *Vibrio cholerae*, which modifies up to ~5% of its PG subunits with d-Met during stationary phase. In addition to incorporating d-Met into its PG, this organism produces and secretes a wide variety of NCDAAs to millimolar concentrations (19). Some of the secreted NCDAAs inhibit the growth of diverse bacteria at physiological concentrations, making NCDAAs important mediators of interbacterial competition (20). It is currently unknown whether PG editing with NCDAAs plays an important role during bacterial warfare.

The Gram-negative bacterial pathogen *Acinetobacter baumannii* has a remarkable ability to withstand antibiotic treatment and persist in health care settings. As such, it is categorized by the World Health Organization and the U.S. Centers for Disease Control and Prevention as a critical priority for the research and development of
novel antimicrobial therapies. Given the remarkable ability of *A. baumannii* to withstand a wide variety of environmental stressors (21), we investigated whether this organism modifies its PG during stationary phase, as has been reported in other bacterial species (1, 19, 22). To this end, we purified PG from Ab17978 grown to exponential phase (PG_{exp}) or stationary phase (PG_{stat}), digested it with muramidases, and analyzed the resulting muropeptides by high-performance liquid chromatography (HPLC). The muropeptide profile obtained from PG_{exp} is similar to that of *Escherichia coli* (Fig. 1A and figs. S1 and S2). However, the HPLC chromatogram derived from PG_{stat} showed five unique peaks (Fig. 1A). Mass spectrometry (MS) analysis identified these products as monomeric or cross-linked muropeptides containing a lysine residue (Lys) in the fourth (l-Ala-d-iGlu-mDAP-lysine, hereafter referred to as “Tri-Lys”) or fifth (l-Ala-d-iGlu-mDAP-d-Ala-lysine, hereafter referred to as “Tetra-Lys”) position (Fig. 1B, fig. S8, and table S1). Quantification of HPLC profiles showed that the Tri-Lys muropeptide and its cross-linked forms constitute ~24% of the total muropeptides of PG_{stat} (Fig. 1A and table S2). Lys-containing muropeptides were also present in PG_{exp} but in a considerably lower amount (~6% of total muropeptides). In contrast, Tetra-Lys muropeptides were unique to PG_{stat} and accounted for ~10% of the total muropeptides. Together, our results indicate that during stationary phase, Ab17978 is capable of modifying about one-third of its PG with Lys.

MS is unable to differentiate l-Lys from d-Lys. Previous work showed that bacteria that produce and incorporate NCDAAs into their PG meshwork also secrete NCDAAs into the culture supernatant at millimolar concentrations (19, 22). Thus, d-Lys secretion by Ab17978 would suggest editing with the d-enantiomer. We developed a colorimetric assay using the d-Lys–specific oxidase AmaD from *Pseudomonas putida* to detect and quantify the concentration of d-Lys in culture supernatants of Ab17978 (Fig. 2A) (23). Using our AmaD assay, we determined that Ab17978 produces and secretes d-Lys into culture supernatants to a concentration of ~0.3 mM (Fig. 2B), which is comparable to levels of NCDAAs secreted by other bacterial species (19, 24). d-Lys secretion has not been reported in *Acinetobacter*, leading us to investigate how d-Lys is produced.

Amino acid racemases are enzymes that catalyze the conversion between L-amino acids and D-amino acids. Ab17978 encodes five putative amino acid racemases, only one of which is predicted to have a signal peptide (ACX60_11360, hereafter referred to as “RacK”). To identify the racemase involved in d-Lys production, we constructed mutant strains of Ab17978 lacking each racemase and determined the levels of d-Lys in their culture supernatants. Deletion of only one racemase, RacK, completely abrogated d-Lys secretion (fig. S3). Quantification of the racK gene (RacK’) restored d-Lys secretion to levels comparable with wild-type (WT) Ab17978 (Fig. 2B). Having identified RacK as responsible for d-Lys production, we determined whether d-Lys was the Lys enantiomer incorporated into the PG of Ab17978 during stationary phase. We analyzed muropeptides from Ab17978 WT, ΔRacK, and RacK’ by HPLC. PG_{stat} from ΔRacK lacked the peaks corresponding to Tri-Lys and Tetra-Lys subunits, all of which were present in the WT and RacK’ strains (Fig. 2C). Together, our results demonstrate that Ab17978 secretes d-Lys to millimolar concentrations and that PG editing is dependent on the production of d-Lys by RacK. Because RacK is predicted to localize to the periplasm, we propose that PG editing with NCDAAs in Ab17978 occurs in the periplasm, as has been suggested for *V. cholerae* (19, 22). Previous transcriptomics study in Ab17978 showed that racK was also up-regulated in biofilms compared to cells in exponential phase (25).

Virtually all bacteria have the ability to incorporate NCDAAs, either produced by their own racemases or present in the environment, into their PG meshwork. NCDAAs can inhibit the growth of diverse pathogens, with the strongest effect being observed when they interfere with the PG cross-linking activity of transpeptidases. Since d-Lys interferes with the PG cross-linking activity of transpeptidases (table S4), previous studies have shown that PG editing with NCDAAs can inhibit the growth of diverse bacteria (20). However, we found that the concentration of d-Lys present in the spent media of Ab17978 was insufficient to inhibit the growth of *Pseudomonas aeruginosa* PAO1, *Acinetobacter nosocomialis* strain M2 (M2), or *A. baumannii* strain 19606 (Ab19606) (fig. S5).

We next hypothesized that PG editing with d-Lys could constitute a protective mechanism against T6SS-mediated attacks from nonkin bacteria. In this model, PG editing masks the target of T6SS PG hydrolase effectors, similar to how bacteria become resistant...
to antibiotics by modifying the structure of the antibiotic target. The H1-T6SS of *P. aeruginosa* PAO1 delivers at least two PG hydrolase effectors into target cells: the amidase (or di-endopeptidase) Tse1 and the muramidase Tse3 (4). Thus, to test our hypothesis, we performed bacterial competition assays with *P. aeruginosa* PAO1 as the predator and *A. baumannii* strains expressing (Ab17978 WT or UPAB1 RacK+) or not expressing (Ab17978ΔRacK or UPAB1 WT) RacK as prey. We found that ΔRacK was ~15-fold more susceptible to T6SS killing compared with WT Ab17978 (Fig. 3A). Consistently, UPAB1 RacK+ showed ~25-fold higher survival than WT UPAB1 when co-incubated with PAO1 (Fig. 3A and fig. S6). Similarly, we observed that PG editing by d-Lys increased the survival of *A. baumannii* against *Serratia marcescens* and *A. nosocomialis* M2 (Fig. 3A). Collectively, these results indicate that PG editing with d-Lys provides *A. baumannii* immunity against T6SS-dependent attacks from diverse bacteria.

To gain better insight into the mechanism of PG editing–based immunity to T6SS-mediated attacks, we compared the activity of *P. aeruginosa* PAO1 Tse1 toward nonedited (TetraTetra) and d-Lys–edited (TetraTri-d-Lys) dimeric muropeptides. A mix of both muropeptides was incubated with purified Tse1, and samples were taken at different time points and analyzed by HPLC (Fig. 3B). At 0.5 min, we found that most of the TetraTetra muropeptides were hydrolyzed by Tse1, whereas nearly 40% of TetraTri-d-Lys remained intact. The hydrolysis of TetraTri-d-Lys was significantly lower than that of TetraTetra, indicating that d-Lys editing reduces the susceptibility of PG to Tse1-mediated hydrolysis.

Fig. 2. Racemase RacK is responsible for d-Lys secretion and PG editing. (A) Overview of the colorimetric assay used to quantify d-Lys secretion. Purified d-Lys oxidase AmaD from *P. putida* catalyzes the oxidative deamination of d-Lys to release 6-amino-2-oxohexanoate, hydrogen peroxide (H2O2), and ammonium (NH3). H2O2 produced was then quantified by treatment with horseradish peroxidase (HRP) and a fluorophore dye. The amount of fluorescence is converted to d-Lys concentration through a standard curve. (B) Quantification of d-Lys present in supernatant fractions of Ab17978 WT, 17978ΔRacK, and the complemented strain (17978 RacK+). Data represent means ± SD of three biological replicates. (C) Chromatograms of purified, stationary phase muropeptides from the indicated strains. Muropeptide structures are shown in fig. S2.

Fig. 3. PG editing protects *A. baumannii* from T6SS amidase effector. (A) PG editing with d-Lys provides protection to T6SS-dependent attack from *P. aeruginosa* PAO1. Competition assay between PAO1, *S. marcescens* (Serratia), or *A. nosocomialis* M2 as predator and *A. baumannii* strains expressing (Ab17978 WT or UPAB1 RacK+) or not expressing (Ab17978ΔRacK or UPAB1 WT) RacK as prey. Bar graphs represent the means ± SD from four biological replicates. (B) Nonedited (TetraTetra) and edited muropeptides (TetraTri-d-Lys) were treated together with purified PAO1 amidase effector Tse1. Reaction products were analyzed by HPLC at time t = 0 (Ctrl), t = 0.5 min, and t = 5 min. Undigested muropeptides were quantified, and specific activity of Tse1 against TetraTetra and TetraTri-d-Lys was calculated. Specific activity values shown on graph are expressed in milligrams of muropeptide degraded/min/Tse1 concentration. Data present means ± SD of two biological replicates. Statistical analyses were performed using the Student’s unpaired t test, *P < 0.05, **P < 0.01, and ***P < 0.001.
Fig. 4. d-Lys secretion impairs A. baumannii virulence. (A) RacK is detrimental in a murine model of acute pneumonia. Mice were intranasally inoculated with either the Ab17978 WT or ΔRacK (left) or the UPAB1 WT or RacK+ (right). Following 36 hours of infection, the bacterial burdens of the kidneys, heart, liver, spleen, lungs, and blood were determined. Each symbol represents an individual mouse. Statistical analyses were performed using the Mann-Whitney U test, and LOD represents the limit of detection. (B) DAO has increased antibacterial activity on A. baumannii strains having RacK. WT and ΔRacK Ab17978 (left) or WT and RacK+ UPAB1 (right) were incubated with DAO or DAO + catalase for 4.5 hours, and CFU were enumerated. Bar graphs represent the means ± SD of four biological replicates. Statistical analyses were performed using the unpaired Student’s t test, *P < 0.05, **P < 0.01, and ****P < 0.0001.

uncleaved. We observed a ~4-fold decrease in the specific activity of Tse1 toward the d-Lys–modified muropeptide compared with the nonedited muropeptide, consistent with the results of our bacterial competition assays (Fig. 3A). Together, these results support the hypothesis that PG editing in Ab17978 provides an immunity protein–dependent mechanism to prevent T6SS-dependent killing by masking the target PG from the injected effector. Some bacterial species modify their PG to counteract the host innate immune response (12, 33). We examined whether the presence of RacK would affect A. baumannii pathogenesis in the murine acute pneumonia model of infection (34). Ab17978 or UPAB1 strains either encoding or lacking RacK were introduced by intranasal inoculation, and bacterial burdens were enumerated 36 hours after infection. Unexpectedly, strains unable to produce d-Lys showed substantially higher bacterial burdens [1 to 2 log colony-forming units (CFU)] in the kidney, heart, and liver, suggesting that d-Lys production is disadvantageous for bacterial dissemination (Fig. 4A).

Recent findings have suggested that the d-amino acid oxidase (DAO), expressed by human neutrophils, macrophages, and epithelial cells, contributes to host defense against various pathogens through the production of H$_2$O$_2$, a by-product of the DAO-dependent oxidative deamination of d-amino acids (24, 35, 36). Thus, we hypothesized that d-Lys–producing bacteria would be more susceptible to DAO-dependent killing. To test this, we incubated stationary phase Ab17978 or UPAB1 strains either encoding or lacking RacK with purified DAO and enumerated surviving CFU after 4.5 hours. DAO showed significant antibacterial activity against A. baumannii strains harboring RacK, resulting in a 10$^3$-fold reduction in CFU compared with strains not producing d-Lys (Fig. 4B). The addition of purified catalase, an enzyme that eliminates H$_2$O$_2$, provided full protection against DAO, indicating that A. baumannii killing by DAO is H$_2$O$_2$-dependent. Together, our results demonstrate that d-Lys production is detrimental to pathogenesis, at least in part, due to DAO activity.

The benefit of PG editing in bacterial competition and detriment to pathogenesis present an interesting evolutionary paradox, making it difficult to predict whether natural selection would favor Acinetobacter strains capable or incapable of modifying their PG. To this end, we performed a phylogenetic profiling analysis of RacK across 3052 Acinetobacter strains representing 53 species. We found that RacK is largely confined to the Acinetobacter calcoaceticus/A. baumannii (Acb)–complex, occurring only sporadically in other species (fig. S7). Within the Acb–complex, RacK is prevalent in the two earliest branching lineages, suggesting its presence in the last common ancestor of the Acb–complex (Fig. 5). Furthermore, RacK is always flanked by the same four genes, and this gene order is maintained irrespective of the presence of RacK (Fig. 5). A phylogenetic analysis provided no evidence that the evolutionary history differs between the four flanking genes and RacK, indicating that the five genes were likely present in the last common ancestor of A. baumannii. We found that only 7% of A. baumannii strains encode
RacK, and these strains are distributed across different clades in the A. baumannii phylogeny (Fig. 5 and fig. S6). Thus, our results indicate that RacK was encoded by the last common ancestor of the Acb-complex and was lost multiple times independently during A. baumannii evolution.

Our results are summarized in Fig. 6. During stationary phase, the periplasmic racemase RacK catalyzes the conversion of L-Lys to D-Lys in the periplasm. The presence of D-Lys in the periplasmic space leads to PG editing by Ldts and PBPs in competition with their canonical cross-link activity. This PG editing mechanism provides a form of innate immunity against foreign PG targeting T6SS. PGase, Peptidoglycanase (B) As a trade-off, secretion of D-Lys during infection increases host DAO activity and thus decreases A. baumannii virulence. PGase, peptidoglycanase; OM, outer-membrane; IM, inner-membrane.

Fig. 6. Proposed model of D-Lys production role in Acinetobacter spp. (A) The lysine racemase RacK converts L-Lys to D-Lys in the periplasm. The presence of D-Lys in the periplasmic space leads to PG editing by Ldts and PBPs in competition with their canonical cross-link activity. This PG editing mechanism provides a form of innate immunity against foreign PG targeting T6SS. PGase, Peptidoglycanase (B) As a trade-off, secretion of D-Lys during infection increases host DAO activity and thus decreases A. baumannii virulence. PGase, peptidoglycanase; OM, outer-membrane; IM, inner-membrane.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions**

Bacterial strains used in this study are listed in table S5. Unless otherwise noted, strains were grown in lysogeny broth (LB) liquid medium at 37°C with shaking (200 rpm). The antibiotics rifampicin (150 µg/ml), igrasan (25 µg/ml), kanamycin (7.5 or 50 µg/ml), gentamicin (20 µg/ml), chloramphenicol (15 µg/ml), carbenicillin (100 or 200 µg/ml), and zeocin (50 µg/ml) were added when necessary. Spontaneous rifampicin-resistant mutant strains were obtained by plating an overnight culture on LB agar with rifampicin.

**PG isolation and analysis**

PG from exponential and stationary cells from A. baumannii strains was isolated and analyzed by HPLC as previously described (41, 42). Briefly, A. baumannii strains were grown at 37°C in 500 ml of LB media to a final OD$_{600}$ (optical density at 600 nm) of 0.6 for exponential and grown for 24 hours for stationary phase samples. Cells were collected by centrifugation for 15 min at 4°C and 7000 g. Cell pellets were resuspended in 50 ml of cold water and lysed by dropwise addition to 50 ml of boiling 8% SDS under vigorous stirring. Samples were boiled for further 30 min to ensure complete solubilization of the membranes and degradation of the high–molecular weight DNA. Crude PG samples were collected by ultracentrifugation for 60 min at 110,000 g at 25°C. Pellets were washed several times with phosphate buffer (pH 6.0) to remove SDS. Crude PG samples were treated with α-amylase (1 mg/ml) for 1 hour at 37°C and then with Pronase (2 mg/ml) for overnight at 60°C to remove trapped high–molecular weight glycogen and PG-associated proteins, respectively. Further PG preparation steps were performed as previously described (41). Briefly, muropeptides were released from PG by the muramidase cellosyl (Hoechst, Frankfurt am Main, Germany), reduced by sodium borohydride, and separated on a 250 nm by 4.6 mm 3-µm ProntoSIL 120-3-6C18 AQ reversed-phase column (Bischoff, Leonberg, Germany). The eluted muropeptides were detected by their absorbance at 205 nm. The PG composition from exponentially and stationary growing cells was analyzed in two biological replicates. Muropeptides were assigned according to their retention times of the known muropeptides from E. coli and A. baumannii (42) and quantified using the Laura software (LabLogic Systems).

**MS/MS analysis**

New muropeptide fractions with retention times other than standard muropeptides were collected and analyzed by tandem MS (MS/MS) as infections (36–39). Despite the competitive advantages conferred by the expression of RacK during interbacterial competition, modern clinical Ab (Acinetobacter baumannii) strains and other Acinetobacter species of the Acb-complex have relinquished their copy of the racK gene. Strains from the most common lineages of clinical A. baumannii strains, such as international clone 1 (IC1), IC2, and IC3, do not carry the racK gene. This is reminiscent of the loss of the T6SS via genetic disruptions to the T6SS gene locus or the accumulation of inactivating point mutations in ~40% of A. baumannii clinical isolates (40, 41). Both PG editing and a functional T6SS are crucial in the environment but appear to impose a fitness cost during infection. These examples illustrate the multiple adaptations that A. baumannii has undertaken to become a pathogen capable of successfully infecting the human host.
Carbenicillin. The bacteria were grown in LB to an OD600 of 0.6, and Rosetta II (Invitrogen) and selected on electroporated into E. coli assembly mix (New England Biolabs). The pET22b+_amaD vector was sequence using Hi-Fi DNA AspelB in pET22b+ vector void of the sequence.

The muropeptide fractions a and b were also acetylated using an established protocol (17) prior MS/MS analysis to confirm the number of amino groups.

Construction of A. baumannii mutant and complement strains

The primers used in this study are listed in table S5. Mutants were constructed as described previously (44). Briefly, an antibiotic resistance cassette was amplified with primers pair P1_kan Fwd-Rev (Integrated DNA Technologies) with homology to the flanking regions of the racK gene with additional 3’ 18 to 25 nucleotides of homology to the FRT (FLP recognition target) site–flanked kanamycin resistance cassette from plasmid pKD4. This polymerase chain reaction (PCR) product was electroproporated into competent Ab17978 carrying pAT04, which expresses the RecAB recombination (44). Mutants were selected on kanamycin (10 μg/ml), and integration of the resistance marker was confirmed by PCR. To remove the kanamycin resistance cassette, electrocompetent mutants were transformed with pAT03 plasmid, which expresses the FLP recombinase. To create Ab17978 RacK+ strain, the racK gene was cloned into pSH vector and under an arabinose inducible promoter, and then the pSH_RacK plasmid was electroproporated into Ab17978 ΔracK strain. The racK gene was introduced into UPAB1 via a four-parent conjugal strategy as described by Kumar et al. (45). Briefly, 100 μl of stationary cultures were normalized to an OD600 of 2.0 of each recipient strain, and HB101(pRK2013), EC100D(pTNS2), and EC100D containing the pUC-miniTn7-racK plasmid were added to 600 μl of warm LB. Each suspension was washed twice by centrifugation at 7000g, followed by resuspension of the bacterial pellet in 1 ml of warm LB. On the final wash, the bacterial pellet was resuspended in 25 μl of LB, and the suspension was spotted on a prewarmed low-salt LB agar plate and incubated overnight at 37°C. After 4 hours at 37°C, the spots were harvested, and supernatants (0.5 ml) were collected. Supernatants were deproteinized using Amicon Ultra 0.5-ml Centrifugal Filters with 3000 MWCO (molecular weight cut-off). Supernatants were incubated with AmaD (0.4 mg/ml) for 1 hour at 37°C. Hydrogen peroxide coproduct was then measured using Hydrogen Peroxide Assay Kit (Abcam) as instructed by the manufacturer.

Muropeptide isolation

TetraTetra and TetraTri-d-Lys were purified by HPLC on an Aeris peptide column (250 mm by 4.6 mm; 3.6-μm particle size; Phenomenex, USA), concentrated, and desalted using a water-methanol gradient on the same column before MS analysis. TetraTetra was obtained from muramidase treatment of E. coli sacculi (46). Chromatographic analyses of muropeptides were performed on ACQUITY Ultra Performance Liquid Chromatography (UPLC) BEH C18 column (130 Å, 1.7 μm, 2.1 mm by 150 mm; Waters), and peptides were treated muropeptides were analyzed by UPLC.

In vitro reaction

TetraTri-d-Lys was obtained by mixing 10 μg of TetraTetra with 20 mM d-Lys and 1 μM LdtA for 4 hours in 20 mM tris (pH 8.0). PG digestion was analyzed over time (0.5, 1, and 5 min) using a final concentration of purified Tse1 enzyme (either 0.01 or 0.02 mg/ml) from P. aeruginosa at 37°C in 20 mM tris-HCl (pH 8.0). The specific activity was calculated from two independent experiments in duplicates (n = 4). The dataset resulting from the lower enzyme concentration (0.01 mg/ml; n = 2) was plotted to illustrate the differential substrate consumption by Tse1 on canonical versus noncanonical dimers. Individual muropeptides were quantified from their integrated areas using samples of known concentration as standards. Reactions were terminated by inactivation at 100°C for 10 min and centrifuged at 14,000 g for 30 min to discard the coagulated Tse1. Next, Tse1-treated muropeptides were analyzed by UPLC. Determination of the extent of Tse1-dependent degradation was performed by comparing the integration areas with respect to a nontreated sample.

T6SS competition assay

Competition assays were performed as previously described (47). Briefly, predator and prey overnight cultures were pelleted, washed three times in fresh LB, and resuspended at an OD600 of 1.0. The cultures were mixed at a predator:prey ratio of 5:1 (PAO1:Ab or M2:Ab) or 10:1 (Serratia:Ab), and 10 μl of drops was spotted on a LB 3% agar plate. After 4 hours at 37°C, the spots were harvested, resuspended in 0.7 ml of LB, and serially diluted and plated on rifampicin or gentamicin LB agar plates to determine the number of colonies.
surviving prey cells. In parallel, the number of surviving PAO1 predator was also enumerated on igsan LB agar plates.

**Murine model of *A. baumannii* acute pneumonia**
All infection experiments were approved by the Vanderbilt University Institutional Animal Care and Use Committee and are in compliance with the guidelines set by the Animal Welfare Act, the National Institutes of Health, and the American Veterinary Medical Association. Lung infection experiments were performed essentially as previously described (32). Briefly, before infection, overnight cultures were subcultured 1:100 in 30 ml of liquid LB media and incubated at 37°C with shaking. Bacteria in mid-exponential phase growth were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS), and resuspended in PBS to the same concentration. Anesthetized 7- to 9-week-old C57BL/6 mice (the Jackson laboratory) were inoculated intranasally with 4 × 10⁸ to 6 × 10⁸ CFU of Ab17978, UPAB1, or their respective isogenic mutants in 35 µl of PBS. Lungs, livers, spleens, kidneys, hearts, and blood were aseptically harvested from mice euthanized at 36 hours after infection. Organs were homogenized and then serially diluted and plated to LB agar to determine bacterial burdens.

**DAO antimicrobial in vitro assay**
Bacteria cultures (3 ml) grown for 20 hours were centrifuged at 10,000g, and supernatants (900 µl) were collected. One hundred microliters of fresh LB was added as well as purified porcine kidney 10,000 g, and supernatants (900 µl) of Ab17978, UPAB1, or their respective isogenic mutants in 35 µl of PBS. Lungs, livers, spleens, kidneys, hearts, and blood were aseptically harvested from mice euthanized at 36 hours after infection. Organs were homogenized and then serially diluted and plated to LB agar to determine bacterial burdens.

**Supplementary Materials**
Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/30/eaab5614/DC1

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Peptidoglycan editing provides immunity to Acinetobacter baumannii during bacterial warfare

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