Penicillin-Binding Protein 7/8 Contributes to the Survival of *Acinetobacter baumannii* In Vitro and In Vivo

Thomas A. Russo,[1,2,3,4,5] Ulrike MacDonald,[2,4] Janet M. Beanan,[2,4] Ruth Olson,[2,4] Ian J. MacDonald,[3,4] Shauna L. Sauberan,[2,5] Nicole R. Luke,[2,3,5] L. Wayne Schultz,[3,6,7], and Timothy C. Umland[3,6,7]

[1]Veterans Administration Western New York Healthcare System and [2]The Witebsky Center for Microbial Pathogenesis, [3]Center of Excellence in Bioinformatics and Life Sciences, and Departments of [4]Medicine, [5]Microbiology, and [6]Structural Biology, State University of New York–Buffalo, and [7]Hauptman-Woodward Medical Research Institute, Buffalo, New York

Background. *Acinetobacter baumannii* is a bacterial pathogen of increasing medical importance. Little is known about genes important for its survival in vivo.

Methods and results. Screening of random transposon mutants of the model pathogen AB307–0294 identified the mutant AB307.27. AB307.27 contained its transposon insertion in *pbpG*, which encodes the putative low-molecular-mass penicillin-binding protein 7/8 (PBP-7/8). AB307.27 was significantly killed in ascites ($P < .001$), but its growth in Luria-Bertani broth was similar to that of its parent, AB307–0294 ($P = .13$). The survival of AB307.27 was significantly decreased in a rat soft-tissue infection model ($P < .001$) and a rat pneumonia model ($P = .002$), compared with AB307–0294. AB307.27 was significantly killed in 90% human serum in vitro, compared with AB307–0294 ($P < .001$). Electron microscopy demonstrated more coccobacillary forms of AB307.27, compared with AB307–0294, suggesting a possible modulation in the peptidoglycan, which may affect susceptibility to host defense factors.

Conclusions. These findings demonstrate that PBP-7/8 contributes to the pathogenesis of *A. baumannii*. PBP-7/8 either directly or indirectly contributes to the resistance of AB307–0294 to complement-mediated bactericidal activity. An understanding of how PBP-7/8 contributes to serum resistance will lend insight into the role of this low-molecular-mass PBP whose function is poorly understood.

Acinetobacter organisms are emerging pathogens of increasing medical importance [1]. Historically, *Acinetobacter* organisms have been considered primarily as health care–associated pathogens, accounting for 1%–3% of hospital-acquired infections and 2%–10% of infections in intensive care units [2–5]. Importantly, the incidence of *Acinetobacter* infection is increasing worldwide [3, 5, 6]. Favored sites of infection include the respiratory tract, particularly in ventilated patients (*Acinetobacter* infections accounted for 6.9% of hospital-acquired pneumonias in 2003, based on National Nosocomial Infections Surveillance system data), the urinary tract, intravascular access devices, surgical sites, and pressure or diabetic ulcers. Mortality rates associated with *Acinetobacter* infection range from 19% to 54% [6]. Interestingly, *Acinetobacter baumannii* has been described as an uncommon cause of severe community-acquired pneumonia, usually in persons with a comorbid condition (e.g., alcoholics), with the preponderance of cases reported from warm and humid geographic locales [7, 8]. Furthermore, the importance of *Acinetobacter* infections in war-related injuries is now established [9–12]. Finally, *Acinetobacter* organisms emerged as important pathogens in survivors of the Asian tsunami in 2004 [13].

An increasing incidence of infections due to strains with a high level of antibiotic resistance is making treatment challenging [14–16]. Particularly problematic are panresistant strains; rates of infection due to such strains

Received 25 April 2008; accepted 9 September 2008; electronically published 13 January 2009.

Potential conflicts of interest: none reported.

Financial support: US Department of Veterans Affairs (VA Merit Review to T.A.R.); US Army Medical Research Acquisition Activity (contract W81XWH-05–1-0627 to T.A.R.); University at Buffalo Interdisciplinary Research Development Fund (to T.A.R., L.W.S., and T.C.U.).

Reprints or correspondence: Dr. Thomas A. Russo, Dept. of Medicine, Div. of Infectious Diseases, 3435 Main St., Biomedical Research Bldg., Rm. 141, Buffalo, NY 14214 (trusso@acsu.buffalo.edu).

The Journal of Infectious Diseases 2009; 199:513–21

0022-1899/2009/19904-0007$15.00
DOI: 10.1086/596317

are greater outside of the United States. Safe, reliable therapeutic agents with predictable activity against *A. baumannii* are presently nonexistent [17, 18].

The need for an increased understanding of *Acinetobacter* infection pathogenesis, identification of virulence factors, and identification and testing of vaccine candidates and new antimicrobial targets is more pressing than ever [17, 18]. To identify genes important for growth and survival, we performed random mutagenesis on *A. baumannii* strain AB307–0294, which our research group has been studying as a model pathogen [19]. We hypothesized that screening this mutant pool for diminished or absent growth on plates made from human ascites would be an efficient means to identify such factors. As a result of this screen, the AB307–0294 mutant derivative AB307.27 was identified. AB307.27 contains its transposon insertion in *pbpG,* which encodes the putative low-molecular-mass penicillin-binding protein 7/8 (PBP-7/8) in *Acinetobacter* organisms. PBP-7/8 is a hydrolase/endopeptidase that hydrolyzes the D-ananyl-ε-meso-2,6-diaminopimelyl cross-bridge bond in high-molecular-mass sacculi [20]. However, there was no discernible change in phenotype in an *Escherichia coli* PBP-7 mutant, as assessed by growth in laboratory medium [21] and by fluorescence-activated cell sorting [22]. PBP-8 is a OmpT-mediated degradation product of PBP-7, and PBP-7 and PBP-8 have been shown in vitro to stabilize and enhance soluble lytic transglycosylase 70 [20, 23, 24]. PBP-7 is absent from gram-positive bacteria. The precise role of PBP-7/8 in gram-negative bacteria is unclear. It appears to be nonessential for normal cell elongation but has been implicated as an accessory enzyme that modulates cell morphology and in daughter cell separation [22, 25–27]. In this report, we describe a novel phenotype for PBP-7/8 in *A. baumannii*. This protein contributes to growth and survival of *A. baumannii* in human ascites in vitro and in vivo in rat soft-tissue infection and pneumonia models. These data lend new insight into the role of the low-molecular mass penicillin-binding proteins in clinically relevant environments.

MATERIALS AND METHODS

Bacterial strains and media. *A. baumannii* strain 307–0294 (blood isolate; sequence type 15 and clonal group 1 [28]) was isolated from a patient hospitalized at Erie County Medical Center (Buffalo, NY) in 1994. AB307–0294 was grown in Luria-Bertani medium, unless stated otherwise. The strain was maintained at −80°C in 50% Luria-Bertani broth and 50% glycerol. Ascites plates consisted of 80% human ascites (pH 7.3; ascites were not sterilized by filtration but were confirmed by culture as sterile) and 20% water. Two-hundred milliliters of water and 15 g of Bacto agar were autoclaved and cooled to 45°C, 800 mL of ascites and kanamycin (final concentration, 40 μg/mL) were added, and plates were poured. For quantitative growth curves, the following were used: 100% human ascites, Luria-Bertani medium, and 100% human urine pooled from 4 healthy donors and filter sterilized before use. Strains were grown overnight in Luria-Bertani medium and diluted in the medium in which the growth curve was being determined, with starting titers ranging from 1×10^4 to 1×10^5 cfu/mL in a final volume of 2 mL. Incubations were at 37°C in a shaking water bath (120 rpm/min). Aliquots were removed at 0, 3, 6, and 24 h, and 10-fold serial dilutions in 1 × PBS were performed to determine the bacterial concentration.

Transposon mutagenesis and screen for lack of growth on ascites plates. Electrocompetent cells were generated by growing AB307–0294 in Mueller-Hinton broth to an $Å_{600}$ of around 0.4. Fifteen milliliters of cells were washed once with 1 mL of ice-cold sterile mqH_2O, followed by 2 washes with 10% ice-cold, sterile glycerol. After the last wash, cells were resuspended in 75 μL and either used immediately or stored at −80°C before use. EZ-Tn5<kan-2>Tnp Transposome (60 ng in 3 μL [Epicentre Biotechnologies]) was electroporated into 75 μL of electrocompetent AB307–0294 (Bio-Rad Gene Pulser; 25 mF/2.5 kV/200 Ohm), using a 0.2-cm gap, in an ice-cold EP chamber (Bio-Rad Laboratories). Immediately after electroporation, cells were resuspended in SOC medium (Invitrogen) and grown at 37°C for 1 h. Aliquots were then plated on Mueller-Hinton plates supplemented with kanamycin (40 μg/mL), and isolated colonies were purified on the same medium. These AB307–0294 mutants (presumably AB307–0294::Tn5<kan-2>) were subsequently gridded onto ascites-kanamycin plates. AB307–0294 mutants that were confirmed to have minimal or no growth on the ascites-kanamycin plates were numbered consecutively and stored at −80°C.

DNA sequencing and analysis. The location of the transposon insertion in mutant derivatives of AB307–0294 was determined by chromosomal sequencing. Chromosomal DNA was prepared from the AB307–0294 mutants of interest by using a Qiagen Genomic-tip 100/G purification column (Qiagen). Cycle sequencing was performed off of the EZ-Tn5 <Kan-2> Transposon (Epicentre Biotechnologies), using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) in accordance with the protocol for sequencing genomic DNA. The KAN-2 FP-1 forward primer included in the transposon kit was used. Cycle sequencing was performed on 10- or 20-μL reactions with the following PCR protocol: step 1, 96°C for 2 min; step 2, 96°C for 30 s, 50°C for 10 s, and 60°C for 4 min for 50 cycles; and step 3, 4°C, ramping 1°C/s. Cycle sequencing products were prepared for sequencing by use of the CleanSEQ (Agencourt) reaction cleanup reagent in accordance with the manufacturer's instructions. Sanger sequencing was performed with a 3130xl Genetic Analyzer DNA sequencer (Applied Biosystems). Sequence comparisons were performed via BLAST analysis of the nonredundant GenBank database.

Cloning of pbpG and construction of a complemented derivative of AB307.27 (PBP-7/8 negative). *pbpG* and 178 bases

upstream and 150 bases downstream were cloned via PCR-mediated amplification (forward primer: 5'-GCTGACGAGCTC-CAATGGAATGACAAAATTAGCAA-3'; reverse primer: 5'-CCT-AGTACCGGTCAATGGACCAAGTAAAAGATTCG-3'). Primers contained capped *Sac*I and *Age*I sites to facilitate ligation into the vector pNLAC1 (tetracycline and ampicillin resistant). The cloned *pbpG* was confirmed to be identical to that in strain AB307–0294 by bidirectional DNA sequencing. The pNLAC1::*pbpG* construct was electroporated into AB307.27, generating the complemented strain AB307.27/pNLAC1::*pbpG*. pNLAC1 without insert was electroporated into AB307–0294 and AB307.27, generating the control strains AB307/pNLAC1 and AB307.27/pNLAC1.

Structural analysis. A 3-dimensional homology model of PBP-7/8 (PBP-78A) was created using the automated first approach mode in SWISS-MODEL [29–31]. The sequence of AB307–0294 PBP-7/8 was entered in FASTA format and submitted to the SWISS-MODEL server (available at: http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1&userid=USERID&token=TOKEN). A BLAST value E-limit for choosing the best template was set to 0.00001. The model PBP-7/8 was returned in Protein Data Bank format. The PBP-7/8 model, the best template, and other templates with identity scores of >25% were superimposed using the secondary structure matching module in Coot [32]. A primary sequence alignment for all templates was performed with ClustalW2 (available at: http://www.ebi.ac.uk/Tools/clustalw2/index.html), using the default settings [33]. The superimposed structures were examined in 3 dimensions, using the graphical display in Coot [34]. The sequence alignment was adjusted by hand to correspond to the overlap of residues in 3-dimensional space.

Rat soft-tissue infection model. The rat pneumonia and soft-tissue infection model animal studies were reviewed and approved by the University at Buffalo and Veterans Administration Institutional Animal Care Committee. An established Long-Evans rat soft-tissue infection model was used as reported elsewhere [35].

Rat pneumonia model. An established Long-Evans rat model for studying pulmonary damage was used as reported elsewhere [36, 37].

Serum bactericidal assay. Complement-mediated bactericidal assays were performed as previously described [38].

Transmission electron microscopy (TEM). TEM was performed as described elsewhere [39].

Statistical analyses. Data are presented as mean values (±SEM). *P* values of .05/*n* (where *n* is the number of comparisons) are considered statistically significant, based on use of Bonferroni correction for multiple comparisons, and *P* values of >.05/*n* but <.05 are considered as representing a trend. To normalize in vitro and in vivo data, $\log_{10}$-transformed values were used. The area under each curve was calculated, and the areas were compared using 2-tailed unpaired *t* tests (Prism 4 for MacIntosh [GraphPad Software]).

RESULTS

Identification of AB307–0294 PBP-7/8. To identify factors in *Acinetobacter* organisms that are necessary for growth and survival in human infection, we used an experimental approach that was a modification of the method we previously used to identify virulence factors in extraintestinal pathogenic *E. coli* [40, 41]. First, random transposon mutagenesis was performed, and transposon mutants were selected on plates with nutrient-rich laboratory medium (i.e., Mueller-Hinton agar). Next, mutants were gridded onto ascites plates, which consisted of 80% human ascites (fluid that accumulates in the peritoneal cavity in pathologic states) and agar. Ascites plates are an ex vivo modified minimal medium and in essence are roughly reflective of inflammatory extracellular fluid, a common environment for extracellular bacterial pathogens such as *Acinetobacter* organisms. This screen resulted in the identification of AB307.27. Quantitative growth curves confirmed that AB307.27 was significantly killed in ascites ($P < .001$), but its growth in laboratory medium (i.e., Luria-Bertani broth) was similar to growth of its parent AB307–0294 ($P = .13$) (figure 1*A*). Chromosomal sequencing, priming off of the EZ-Tn5 <Kan-2> transposon, was performed on DNA purified from AB307.27. The transposon insertion within AB307.27 was in *pbpG* (between nucleotides 462 and 463), which encoded a putative D-alanyl-D-alanine endopeptidase or PBP-7/8. The complete sequence of AB307–0294 PBP-7/8 and its surrounding genes was determined (Genbank accession number EU676123). The open reading frame of *pbpG* contained 1023 nucleotides, which encoded a protein of 340 amino acids with a predicted molecular weight of 35,949 Da. Because the direction of transcription for a putative threonine synthase, encoded by the open reading frame 3' to *pbpG,* was in the opposite direction, the transposon insertion in *pbpG* should not have a polar effect. To confirm this, quantitative growth curves were performed in ascites with the constructs AB307–0294/pNLAC1 (a wild-type parent containing the cloning vector without an insert), AB307.27/pNLAC1 (a PBP-7/8 mutant derivative of AB307–0294 containing the cloning vector without an insert), and AB307.27/pNLAC1::*pbpG* (a PBP-7/8 mutant derivative containing cloned *pbpG*). Growth of the wild-type parent AB307–0294/pNLAC1 was similar to growth of the complemented mutant AB307.27/pNLAC1::*pbpG* ($P = .17$), confirming that inactivation of PBP-7/8 was responsible for decreased growth in ascites. As expected, the noncomplemented strain AB307.27/pNLAC1 demonstrated a significant decrease in survival in ascites, compared with AB307–0294/pNLAC1 ($P < .001$) and AB307.27/pNLAC1::*pbpG* ($P < .001$) (figure 1*B*).

In silico analysis of AB307–0294 PBP-7/8. The SWISS-MODEL server created a model covering residues 92–332 of

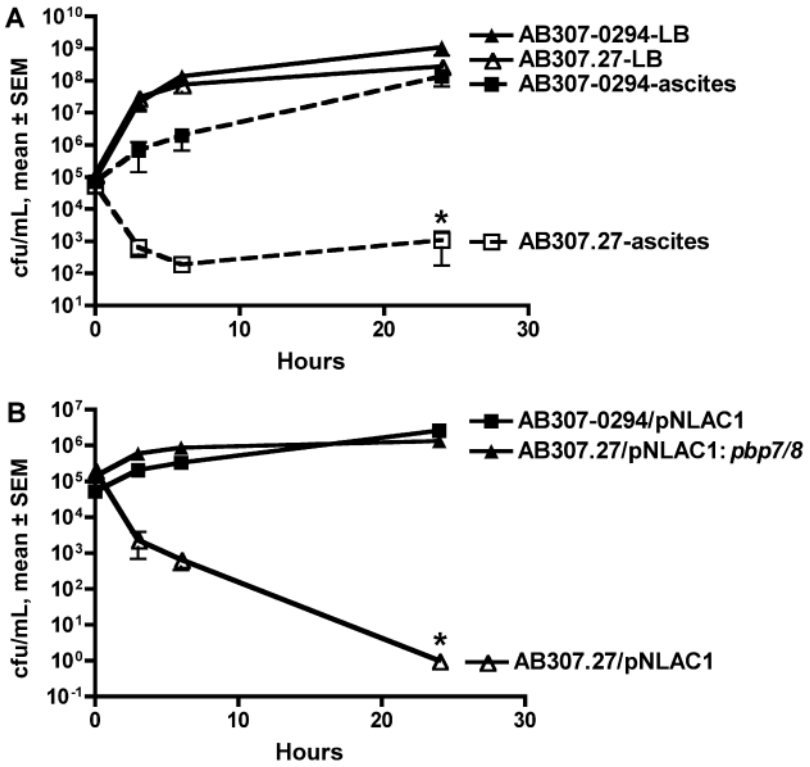

Figure 1. Growth/kill curve for *Acinetobacter baumannii* strains AB307–0294 (wild-type) and derivatives in Luria-Bertani medium and 100% human ascites. *A,* Growth of AB307–0294 and AB307.27 was assessed at 0, 3, 6, and 24 h in each medium. Growth of AB307–0294 ($n = 4$) and AB307.27 ($n = 4$) in Luria-Bertani medium was similar ($P = .13$, by a 2-tailed unpaired t test). In contrast, survival of AB307.27 ($n = 6$) was significantly decreased in ascites, compared with AB307–0294 ($n = 6$) (*$P < .001$). *B,* Growth of AB307–0294/pNLAC1 (a wild-type parent containing a cloning vector without an insert), AB307.27/pNLAC1 (a penicillin-binding protein [PBP]–7/8 mutant derivative of AB307–0294 containing a cloning vector without an insert), and AB307.27/pNLAC1::*pbp7/8* (a PBP-7/8 mutant derivative containing cloned *pbp7/8*) was assessed at 0, 3, 6, and 24 h in ascites. Growth of AB307–0294/pNLAC1 ($n = 4$) and AB307.27/pNLAC1::*pbp7/8* ($n = 4$) was similar ($P = .17$). In contrast, survival of AB307.27/pNLAC1 ($n = 3$) was significantly decreased in ascites, compared with AB307–0294/pNLAC1 (*$P < .001$) and AB307.27/pNLAC1::*pbp7/8* (*$P < .001$).

PBP-7/8, resulting in a final E-score of 3.56e-16. PBP-5 from *E. coli* (Protein Data Bank code 1nj4) was chosen as the best template (27% identity to PBP-7/8) [42]. Other homologous proteins with crystal structures available and >25% identity with PBP-7/8 were PBP-4 from *Staphylococcus aureus* (Protein Data Bank code 1tvf) (Rajashankar et al., unpublished data), PBP-3 from *Streptococcus pneumoniae* (Protein Data Bank code 1xp4) [43], and DD-transpeptidase from *Streptomyces* strain K15 (Protein Data Bank code 1skf) [44]. Surprisingly, even with the low levels of sequence identity, the active site residue motifs SXXK, SXN, and KTG were completely conserved (figure 2). The superposition of all structures placed the active site residues within a root mean square deviation of 1.5 Å from their positions in each structure. Although the sequences surrounding the active site residues were varied, there was strict conservation of the secondary structural elements creating the active site scaffold (figure 2).

```
PBP78A      121---IASITKLMTAVVTADA---(34)---RAEVLLFALMKSENPAAAALARTY---(87)---NINLSKTGYI--340
PBP5E        95---PASLTKMMTSYVIGQA---(41)---VSQLIRGINLQSGNDACVAMADFA---(85)---NVDGIKTGHT--427
PBP4SA       59---PASMTKLMTMYLTLEA---(39)---IADLLQITVSNSSNAAALILAKKV--(102)---GTDGLKTGSS--369
PBP3SP       39---IASITKLITVYLVYEA---(38)---VEELLEATLVSSANSAAIALAEKI--(102)---GFDGLKTGTT--379
DDPEPS       33---TGSTTKIMTAKVVLAQ---(36)---VRQLLYGLMLPSGCDAAYALADKY---(99)---GAIGVKTGSG--262
SS                HHHHHHHHHHHHHHH           HHHHHHHHHTTTTHHHHHHHHH           SSSSSSSSS
```

Figure 2. Structural alignment of the AB307–0294 penicillin-binding protein 7/8 (PBP-7/8) model (PBP78A) with homologous PBP crystal structures. The conserved active site residue motifs SXXK, SXN, and KTG are boxed. The secondary structural (SS) elements α-helix (H), β-strand (S), and turn (T) that arrange the active site residues are conserved in each structure. Although PBP78A has low identity with PBP5E (27%), the secondary structural elements serve to present the conserved active site residues in 3-dimensional space. Structures of other homologous proteins are PBP-5 from *Escherichia coli* (PBP5E; Protein Data Bank [PDB] code 1nj4), PBP-4 from *Staphylococcus aureus* (PBP4SA; PDB code 1tvf), PBP3 from *Streptococcus pneumoniae* (PBP3SP; PDB code 1xp4), and DD-transpeptidase from *Streptomyces* strain K15 (DDPEPS; PDB code 1skf). Structures were superimposed using the SSM superposition module of Coot [33].

Table 1. Homology of AB307–0294 PBP-7/8 with other homologues/orthologues.

This table is available in its entirety in the electronic edition of the *Journal of Infectious Diseases*.

Next, the DNA/predicted protein homology of AB307–0294 PBP-7/8 was compared with various homologues/orthologues (table 1, which appears only in the electronic edition of the *Journal*). Finally, a promoter prediction analysis was performed on the 5' DNA sequence to the predicted transcriptional start site of *pbpG* (BPROM [SoftBerry]; available at: http://www.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). A putative promoter, whose transcription is predicted to be directed by the σ^{70} factor, was identified.

AB307.27 (PBP-7/8 negative) has an abnormal morphology. The cell morphology of AB307.27 (PBP-7/8) was assessed via TEM. When grown in logarithmic phase in Luria-Bertani medium, more coccobacillary forms of AB307.27 (PBP-7/8 negative) were observed, compared with its wild-type parent AB307-0294; however, both coccobacillary and bacillary forms were seen with each strain (figure 3). This finding suggests that AB307.27 may possess an abnormal peptidoglycan and suggests that, in *Acinetobacter* organisms, PBP-7/8 may play a more critical role in modulating cell morphology than has been described for other species [22].

AB307.27 (PBP-7/8 negative) is killed in the rat soft-tissue infection model. An in vivo validation of our in vitro findings was needed to confirm that PBP-7/8 was a factor that contributed to *Acinetobacter* infection. Initially, we compared the growth/survival of AB307–0294 (wild-type) and its isogenic derivative AB307.27 (PBP-7/8 negative) in a rat model of soft-tissue infection. A major advantage of this infection model is that multiple samples can be obtained over time from each animal, making it time- and cost-efficient for initial assessment of strains in vivo. Furthermore, it is clinically relevant given that *A. baumannii* has been increasingly recognized as a cause a variety of soft-tissue infections [10, 12]. Compared with AB307–0294, AB307.27 demonstrated a significant decrease in survival in this

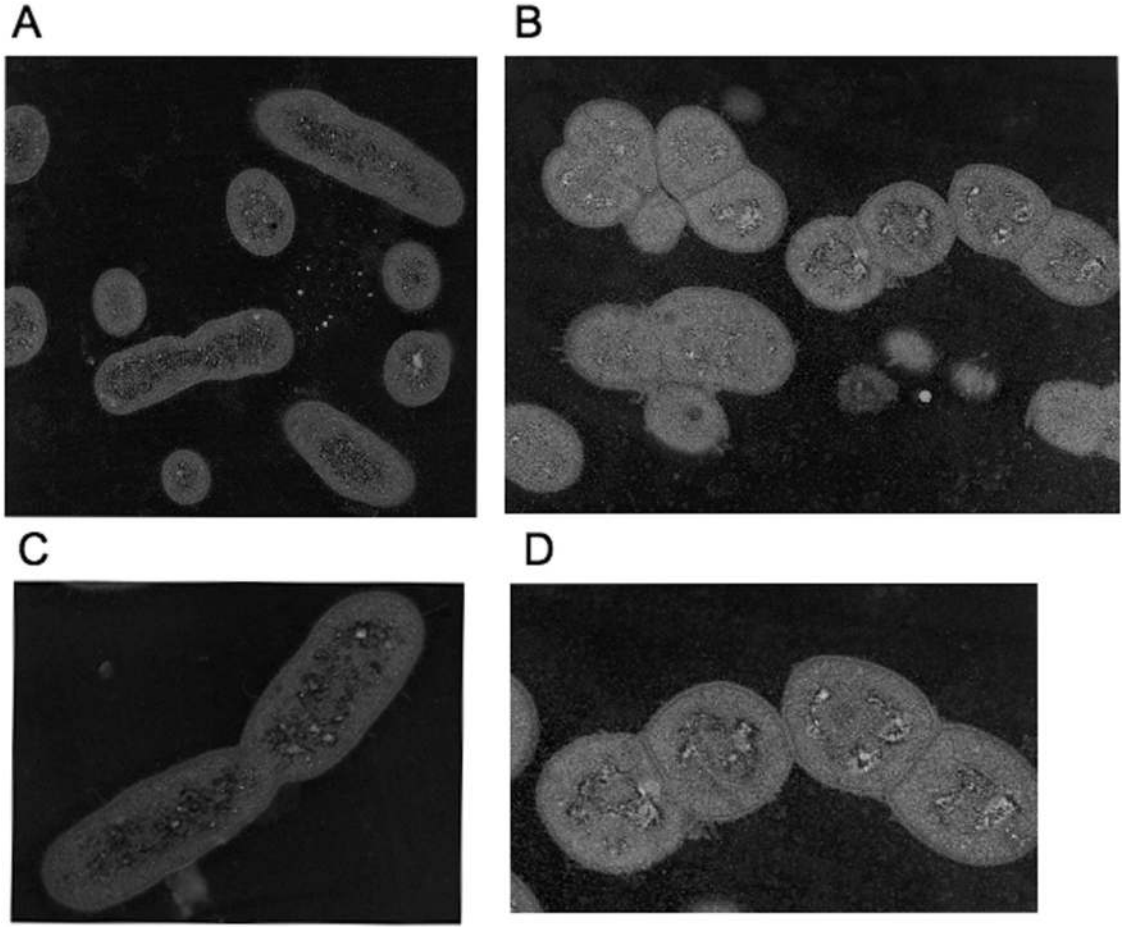

Figure 3. Transmission electron microscopy was performed on AB307–0294 (wild-type) and AB307.27 (penicillin-binding protein 7/8 negative) as described elsewhere [39]. Cells were grown in Luria-Bertani medium in logarithmic phase. *A* and *C,* AB307–0294 (10,000× and 20,000× original magnification, respectively); *B* and *D,* AB307.27 (10,000× and 20,000× original magnification, respectively).

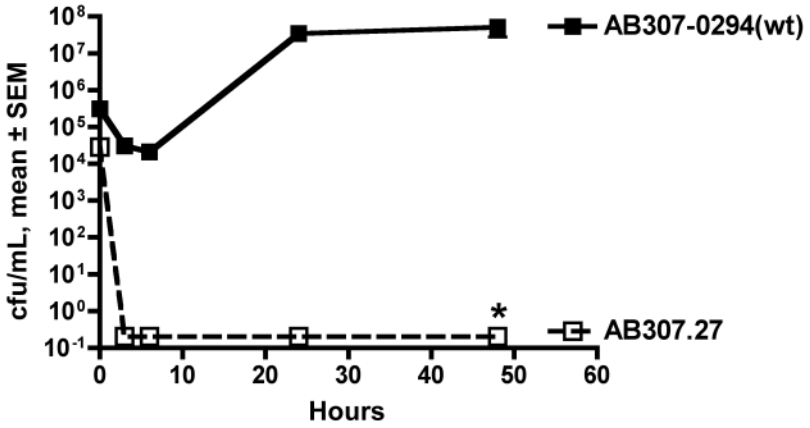

Figure 4. Survival of *Acinetobacter baumannii* strain 307–0294 (wild-type [wt]) and its isogenic derivative AB307.27 (penicillin-binding protein 7/8 negative) in the rat soft-tissue infection model. Rats were prepared and challenged with AB307–0294 and AB307.27 as described elsewhere [35]. Bacterial titers were determined at 0, 3, 6, 24, and 48 h. Survival of AB307.27 ($n = 4$–6 for each time point) was significantly decreased in this model, compared with AB307–0294 ($n = 5$ for each time point) (*$P < .001$, by a 2-tailed unpaired t test).

model ($P < .001$) (figure 4). These data demonstrate that PBP-7/8 is important for the survival of AB307–0294 in soft-tissue infection.

AB307.27 (PBP-7/8 negative) is killed in the rat pneumonia model. Next, we compared the growth and survival of AB307–0294 (wild-type) and its isogenic derivative AB307.27 (PBP-7/8 negative) in a rat pneumonia model, another common type of *Acinetobacter* infection. Compared with AB307–0294, AB307.27 demonstrated a significant decrease in survival in this model ($P = .002$) (figure 5). These data demonstrate that PBP-7/8 is important for the survival of AB307–0294 in pulmonary infection.

AB307.27 demonstrates an increase in susceptibility to complement-mediated bactericidal activity. The innate response is instrumental in determining whether extracellular bacterial pathogens such as *Acinetobacter* organisms are successfully cleared or establish an infection. The complement system is a critical component of the host's innate immune system. Therefore, we assessed whether AB307.27 had increased susceptibility to complement-mediated bactericidal activity, compared with its parent, AB307–0294. Compared with AB307–0294, AB307.27 demonstrated a significant decrease in survival in 90% human serum ($P < .001$) (figure 6). These data support the concept that complement-mediated bactericidal activity in vivo is at least one mechanism responsible for the clearance of AB307.27 in the rat soft-tissue infection and pneumonia models.

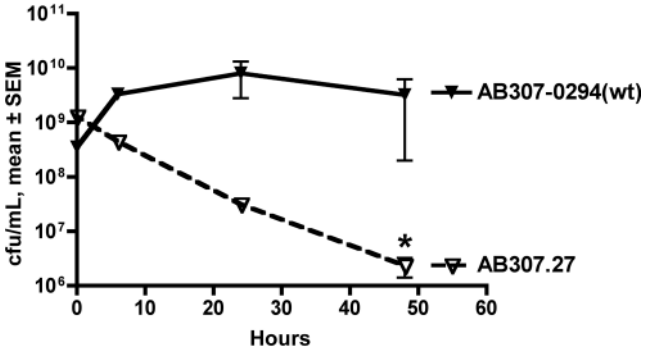

Figure 5. Survival of *Acinetobacter baumannii* strain 307–0294 (wild-type [wt]) and its isogenic derivative AB307.27 (penicillin-binding protein 7/8 negative) in the rat pneumonia model. Rats were challenged with 3.9×10^8 cfu of 307–0294 and 1.3×10^9 cfu of AB307.27 by intratracheal instillation, and total lung bacterial titers were determined at 0, 6, 24, and 48 h. Survival of AB307.27 ($n = 3$) was significantly decreased in this model, compared with AB307–0294 ($n = 3$) (*$P < .002$, by a 2-tailed unpaired t test).

Growth of AB307.27 (PBP-7/8 negative) in human urine is similar to that of AB307–0294 (wild-type). The growth of AB307–0294 and AB307.27 in human urine was assessed because the urinary tract is another site that *Acinetobacter* organisms commonly infect. However, in urine, complement levels are low and anticomplement activity may be present [45, 46]. The growth of AB307–0294 and AB307.27 was similar in human urine ($P = .07$) (figure 7). These data demonstrate that PBP-7/8 is not important for the growth/survival of AB307–0294 in human urine.

DISCUSSION

To our knowledge, this is the first report to describe a role for PBP-7/8 in the pathogenesis of infection. We demonstrated that the PBP-7/8–deficient mutant AB307.27, an isogenic derivative of the wild-type *A. baumannii* strain AB307–0294, was killed in human ascites (figure 1). Next, we demonstrated that AB307.27 was also killed in vivo in rat soft-tissue infection and pneumonia models (figures 4 and 5), both important types of *Acinetobacter* infection. We then demonstrated that AB307.27 was killed in 90% human serum in vitro (figure 6). TEM demonstrated more

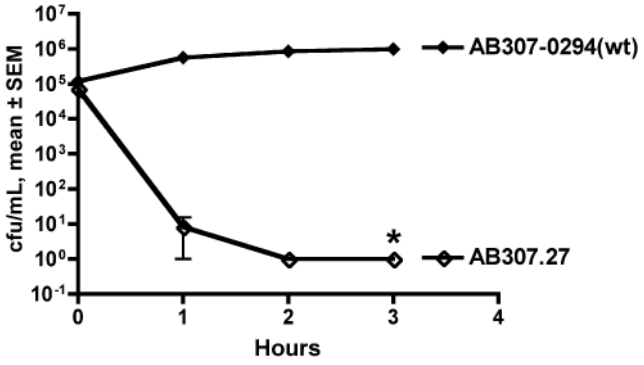

Figure 6. Effect of 90% normal human serum on the viability of the *Acinetobacter baumannii* strain AB307–0294 (wild-type [wt]) and its isogenic derivative AB307.27 (penicillin-binding protein 7/8 negative). Assays were performed as described elsewhere [38]. All strains were also assessed in the presence of 90% heat-inactivated (at 56°C for 30 min) normal human serum, and their growth was similar; therefore these data are not shown. Bacterial titers were determined at 0, 1, 2, and 3 h. Survival of AB307.27 ($n = 4$) was significantly decreased, compared with AB307–0294 ($n = 4$) (*$P < .001$, by a 2-tailed unpaired t test).

coccobacillary forms of AB307.27, compared with AB307–0294 (figure 3), suggesting a possible modulation in the peptidoglycan, which may affect susceptibility to host defense factors. Finally, the growth of AB307.27 was similar to AB307–0294 in human urine (figure 7), an environment in which complement levels are low and anticomplement activity may be present [45, 46]. Taken together, these results demonstrate that PBP-7/8 contributes to the pathogenesis of *A. baumannii* in the rat soft-tissue infection and pneumonia models. Furthermore, PBP-7/8 either directly or indirectly contributes to the resistance of AB307–0294 to complement-mediated bactericidal activity.

PBPs and their role in peptidoglycan synthesis have been extensively reviewed [27, 47, 48]. In brief, bacterial peptidoglycan consists of cross-linked N-acetylglucosamine and N–acetylmuramic acid glycan chains. It is a critical cell structure that provides the bacterium shape and is instrumental in resisting various physical forces [27, 47, 49]. PBPs have been classified as having a high or low molecular mass. High-molecular-mass PBPs enable peptidoglycan polymerization and insertion into the preexisiting cell wall [27, 47]. low-molecular-mass PBPs contribute to cell separation and peptidoglycan remodeling [22, 27, 49]. The low-molecular-mass PBPs have been less well studied than the high-molecular-mass class A and class B enzymes [47, 48]. However, in limited studies involving *E. coli,* low-molecular-mass PBPs, including PBP-7/8, have in general been shown not to be essential when grown in Luria-Bertani medium [21, 22, 25, 26]. PBP-7/8 has been postulated to play a role in cell wall remodeling [20, 50]. In contrast to AB307–0294, in *Salmonella* organisms the expression of PBP-7 is under the direction of the σ^s factor and is induced in carbon-starved medium (starvation-stress response), but a PBP-7–negative mutant was equally virulent as its wild-type parent after oral gavage in a BALB/c mouse sepsis model [50]. Therefore, the dramatic phenotype observed with AB307.27 both in human serum and ascites in vitro and in rat pneumonia and soft-tissue infection models was novel and surprising. We have established that at least 1 mechanism for this phenotype is an increase in susceptibility to complement-mediated bactericidal activity (figure 6). It remains possible that PBP-7/8 directly or indirectly contributes to the resistance of AB307–0294 to the bactericidal activity mediated by professional phagocytes or antimicrobial peptides, but these possibilities were not directly assessed in this report. The increase in coccobacillary forms of AB307.27, compared with AB307–0294, suggests that its peptidoglycan may be altered. However, further quantitative definition of the probable abnormalities in the peptidoglycan in AB307.27 via flow cytometry and/or biochemical methods are required to confirm that the peptidoglycan is truly abnormal in AB307.27 (PBP-7/8 negative)

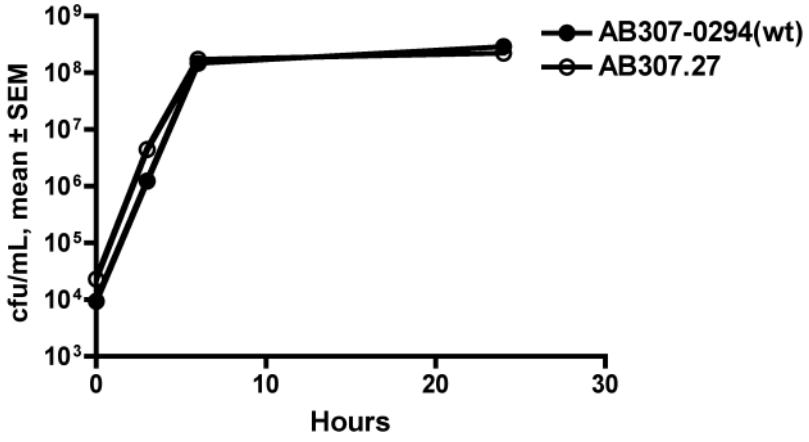

Figure 7. Growth/kill curve for *Acinetobacter baumannii* strain AB307–0294 (wild-type [wt]) and its isogenic derivative AB307.27 (penicillin-binding protein 7/8 negative) in 100% human urine. Growth of AB307–0294 and AB307.27 was assessed at 0, 3, 6, and 24 h in 100% human urine. Growth of AB307–0294 ($n = 4$) and AB307.27 ($n = 3$) in urine was similar ($P = .07$, by a 2-tailed unpaired t test).

and, if so, to define the nature of the change. Furthermore, whether this is a direct or an indirect result of the loss of PBP-7/8 is unresolved. A direct effect, resulting in an alteration in the structure of peptidoglycan due to the loss of PBP-7/8, would seem most likely. However, because PBP-7/8 is a hydrolase, one might predict that overexpression, not a lack of expression, would increase the susceptibility of the bacterium to complement-mediated bactericidal activity. An indirect effect on other PBP or glycolases (e.g., soluble lytic transglycosylase 70), which in turn affects the peptidoglycan structure, or an indirect effect independent of peptidoglycan remain possibilities. Future studies that assess binding of various proteins in the complement system will generate new insights on the mechanism by which PBP-7/8 contributes to complement resistance.

Selection for transposon mutant derivatives of AB307–0294 on laboratory medium and then screening for essentiality on ascites plates were critical for the efficiency of our approach. This simple innovation enabled us able to identify, in one step, genes that are both essential and expressed in vivo. The strength of this approach is that it is unbiased and highly efficient. We did not select the targets but allowed the genetic screen, designed to identify the phenotype of in vivo essentially, to dictate the choices. Furthermore, although we did not identify genes that are essential for growth in laboratory medium, we were able to identify those that are essential for growth/survival in ascites but not Mueller-Hinton medium, a more interesting mutant set. Subsequent identification of the gene into which the transposon inserted in mutants of interest by chromosomal sequencing and an in vivo assessment in the soft-tissue infection model resulted in a relatively efficient approach for identifying previously unrecognized and unknown virulence factors, as well as potential drug targets.

In summary, we have established that PBP-7/8 is critical for the survival of *A. baumannii* strain AB307–0294 in the rat soft-tissue infection and pneumonia models. Furthermore, PBP-7/8 either directly or indirectly contributes to the resistance of AB307–0294 to complement-mediated bactericidal activity. An understanding of how PBP-7/8 contributes to serum resistance will lend insight into the role of this low-molecular-mass PBP whose function is poorly understood.

Acknowledgments

We thank Steven R. Gill for assistance with the promoter prediction analysis and Thomas W. Loehfelm for helpful discussions regarding the construction of the cloning vector pNLAC1.

References

1. Munoz-Price LS, Weinstein RA. *Acinetobacter* infection. N Engl J Med **2008**; 358:1271–81.
2. Fournier PE, Richet H. The epidemiology and control of *Acinetobacter baumannii* in health care facilities. Clin Infect Dis **2006**; 42:692–9.
3. Richet H, Fournier PE. Nosocomial infections caused by *Acinetobacter baumannii*: a major threat worldwide. Infect Control Hosp Epidemiol **2006**; 27:645–6.
4. Joly-Guillou ML. Clinical impact and pathogenicity of *Acinetobacter*. Clin Microbiol Infect **2005**; 11:868–73.
5. Falagas ME, Karveli EA. The changing global epidemiology of *Acinetobacter baumannii* infections: a development with major public health implications. Clin Microbiol Infect **2007**; 13:117–9.
6. Gaynes R, Edwards JR. Overview of nosocomial infections caused by gram-negative bacilli. Clin Infect Dis **2005**; 41:848–54.
7. Anstey NM, Currie BJ, Hassell M, Palmer D, Dwyer B, Seifert H. Community-acquired bacteremic *Acinetobacter* pneumonia in tropical Australia is caused by diverse strains of *Acinetobacter baumannii,* with carriage in the throat in at-risk groups. J Clin Microbiol **2002**; 40:685–6.
8. Chen MZ, Hsueh PR, Lee LN, Yu CJ, Yang PC, Luh KT. Severe community-acquired pneumonia due to *Acinetobacter baumannii*. Chest **2001**; 120:1072–7.
9. Tong MJ. Septic complications of war wounds. JAMA **1972**; 219:1044–7.
10. Centers for Disease Control and Prevention. *Acinetobacter baumannii* infections among patients at military medical facilities treating injured US service members, 2002–2004. MMWR **2004**; 53:1063–6.
11. Scott P, Deye G, Srinivasan A, et al. An outbreak of multidrug-resistant *Acinetobacter baumannii-calcoaceticus* complex infection in the US military health care system associated with military operations in Iraq. Clin Infect Dis **2007**; 44:1577–84.
12. Davis KA, Moran KA, McAllister CK, Gray PJ. Multidrug-resistant *Acinetobacter* extremity infections in soldiers. Emerg Infect Dis **2005**; 11: 1218–24.
13. Maegele M, Gregor S, Steinhausen E, et al. The long-distance tertiary air transfer and care of tsunami victims: injury pattern and microbiological and psychological aspects. Crit Care Med **2005**; 33:1136–40.
14. Rice LB. Challenges in identifying new antimicrobial agents effective for treating infections with *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. Clin Infect Dis **2006**; 43(Suppl):S100–5.
15. Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob Agents Chemother **2007**; 51:3471–84.
16. Sunenshine RH, Wright MO, Maragakis LL, et al. Multidrug-resistant *Acinetobacter* infection mortality rate and length of hospitalization. Emerg Infect Dis **2007**; 13:97–103.
17. Paterson DL, Doi Y. A step closer to extreme drug resistance (XDR) in gram-negative bacilli. Clin Infect Dis **2007**; 45:1179–81.
18. Talbot GH, Bradley J, Edwards JE Jr, Gilbert D, Scheld M, Bartlett JG. Bad bugs need drugs: an update on the development pipeline from the Antimicrobial Availability Task Force of the Infectious Diseases Society of America. Clin Infect Dis **2006**; 42:657–68.
19. Loehfelm TW, Luke NR, Campagnari AA. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. J Bacteriol **2008**; 190:1036–44.
20. Romeis T, Holtje JV. Penicillin-binding protein 7/8 of *Escherichia coli* is a DD-endopeptidase. Eur J Biochem **1994**; 224:597–604.
21. Denome SA, Elf PK, Henderson TA, Nelson DE, Young KD. *Escherichia coli* mutants lacking all possible combinations of eight penicillin binding proteins: viability, characteristics, and implications for peptidoglycan synthesis. J Bacteriol **1999**; 181:3981–93.
22. Meberg BM, Paulson AL, Priyadarshini R, Young KD. Endopeptidase penicillin-binding proteins 4 and 7 play auxiliary roles in determining uniform morphology of *Escherichia coli*. J Bacteriol **2004**; 186:8326–36.
23. Henderson TA, Dombrosky PM, Young KD. Artifactual processing of penicillin-binding proteins 7 and 1b by the OmpT protease of *Escherichia coli*. J Bacteriol **1994**; 176:256–9.
24. Romeis T, Holtje JV. Specific interaction of penicillin-binding proteins 3 and 7/8 with soluble lytic transglycosylase in *Escherichia coli*. J Biol Chem **1994**; 269:21603–7.
25. Priyadarshini R, de Pedro MA, Young KD. Role of peptidoglycan amidases in the development and morphology of the division septum in *Escherichia coli*. J Bacteriol **2007**; 189:5334–47.

26. Priyadarshini R, Popham DL, Young KD. Daughter cell separation by penicillin-binding proteins and peptidoglycan amidases in *Escherichia coli*. J Bacteriol **2006**; 188:5345–55.
27. Sauvage E, Kerff F, Terrak M, Ayala JA, Charlier P. The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. FEMS Microbiol Rev **2008**; 32:234–58.
28. Ecker JA, Massire C, Hall TA, et al. Identification of *Acinetobacter* species and genotyping of *Acinetobacter baumannii* by multilocus PCR and mass spectrometry. J Clin Microbiol **2006**; 44:2921–32.
29. Arnold K, Bordoli L, Kopp J, Schwede T. The SWISS-MODEL workspace: a web-based environment for protein structure homology modelling. Bioinformatics **2006**; 22:195–201.
30. Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis **1997**; 18: 2714–23.
31. Schwede T, Kopp J, Guex N, Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucleic Acids Res **2003**; 31: 3381–5.
32. Emsley P, Cowtan K. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr **2004**; 60:2126–32.
33. Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. Bioinformatics **2007**; 23:2947–8.
34. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Crystallogr D Biol Crystallogr **2004**; 60:2256–68.
35. Russo TA, Beanan JM, Olson R, et al. Rat pneumonia and soft-tissue infection models for the study of *Acinetobacter baumannii* biology. Infect Immun **2008**; 76:3577–86.
36. Russo TA, Bartholomew LA, Davidson BA, et al. Total extracellular surfactant is increased but abnormal in a rat model of gram-negative bacterial pneumonia. Am J Physiol Lung Cell Molec Physiol **2002**; 283: L655–63.
37. Russo TA, Davidson BA, Carlino-MacDonald UB, Helinski JD, Priore RL, Knight PR 3rd. The effects of *Escherichia coli* capsule, O-antigen, host neutrophils, and complement in a rat model of gram-negative pneumonia. FEMS Microbiol Lett **2003**; 226:355–61.
38. Russo T, Sharma G, Brown C, Campagnari A. The loss of the O4 antigen moiety from the lipopolysaccharide of an extraintestinal isolate of *Escherichia coli* has only minor effects on serum sensitivity and virulence in vivo. Infect Immun **1995**; 63:1263–9.
39. Nazareth H, Genagon SA, Russo TA. Extraintestinal pathogenic *Escherichia coli* survives within neutrophils. Infect Immun **2007**; 75:2776–85.
40. Russo TA, Jodush ST, Brown JJ, Johnson JR. Identification of two previously unrecognized genes *(guaA, argC)* important for uropathogenesis. Mol Microbiol **1996**; 22:217–29.
41. Russo T, Carlino U, Mong A, Jodush S. Identification of genes in an extraintestinal isolate of *Escherichia coli* with increased expression after exposure to human urine. Infect Immun **1999**; 67:5306–14.
42. Nicholas RA, Krings S, Tomberg J, Nicola G, Davies C. Crystal structure of wild-type penicillin-binding protein 5 from *Escherichia coli:* implications for deacylation of the acyl-enzyme complex. J Biol Chem **2003**;278: 52826–33.
43. Morlot C, Pernot L, Le Gouellec A, et al. Crystal structure of a peptidoglycan synthesis regulatory factor (PBP3) from *Streptococcus pneumoniae*. J Biol Chem **2005**; 280:15984–91.
44. Fonze E, Vermeire M, Nguyen-Disteche M, Brasseur R, Charlier P. The crystal structure of a penicilloyl-serine transferase of intermediate penicillin sensitivity: the DD-transpeptidase of streptomyces K15. J Biol Chem **1999**; 274:21853–60.
45. Acquatella H, Little PJ, de Wardener HE, Coleman JC. The effect of urine osmolality and pH on the bactericidal activity of plasma. Clin Sci **1967**; 33:471–80.
46. Beeson PB, Rowley D. The anticomplementary effect of kidney tissue; its association with ammonia production. J Exp Med **1959**; 110:685–97.
47. Macheboeuf P, Contreras-Martel C, Job V, Dideberg O, Dessen A. Penicillin binding proteins: key players in bacterial cell cycle and drug resistance processes. FEMS Microbiol Rev **2006**; 30:673–91.
48. Stewart GC. Taking shape: control of bacterial cell wall biosynthesis. Mol Microbiol **2005**; 57:1177–81.
49. Vollmer W, Joris B, Charlier P, Foster S. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev **2008**; 32:259–86.
50. Kenyon WJ, Nicholson KL, Rezuchova B, et al. σ^s-dependent carbon-starvation induction of *pbpG* (PBP 7) is required for the starvation-stress response in *Salmonella enterica* serovar Typhimurium. Microbiology **2007**; 153:2148–58.