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14. ABSTRACT
Bacterial strains from wounded soldiers are iron acquisition and biofilm formation proficient. In the case of the *A. baumannii* AB5075 model strain, the expression of a fully functional acinetobactin-mediated iron acquisition system is critical when tested under laboratory conditions or using *ex vivo* and *in vivo* experimental infection models. Accordingly, the presence of Ga-PPIX significantly reduced the growth of all tested *A. baumannii* strains independently of their site of isolation or resistance phenotypes. However, the activity of Ga-PPIX against bacteria different from *A. baumannii* was variable as it was the activity of Ga nitrate, which also depended on the free-iron content of the media. Thus, these observations support the idea of using iron acquisition activity/functions as targets to treat infections with the biologically inactive Ga-PPIX metalloporphyrin derivative, which negatively impacts bacterial functions involved in critical host-pathogen interactions. These observations also support the potential value of proposed animal experiments that will not only confirm the role of iron acquisition in the virulence of *A. baumannii*, but also test the antimicrobial activity of Ga-PPIX in experimental wound infections. Preliminary studies showed that the value of iron acquisition as a target could be enhanced by the observation that iron-regulated *A. baumannii* hemolysins/cytolysins, which could provide intracellular iron by cell/tissue destruction, could be targeted using a phosphatidylcholine analog as it was done with other relevant human pathogens. In contrast, tested antibiofilm agents not only produced a modest negative effect on their biogenesis, but also increased their formation or enhanced the cell damage caused by the AB5075 wound isolate. The genetic and molecular bases of these unexpected observations could be elucidated by comparative genomics studies once the genomes all isolates are fully sequenced and annotated.

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Table of Contents

Page

1. Introduction……………………………………………………………..…….. 4

2. Keywords…………………………………………………………….……..…….. 4

3. Overall Project Summary………………………………………………………….. 4

4. Key Research Accomplishments……………………………………….……….. 7

5. Conclusions…………………………………………………………………..… 8

6. Publications, Abstract, and Presentations…………………………………..… 8

7. Inventions, Patents and License………………………………………………..… 9

8. Reportable Outcomes……………………………………………………….… 9

9. Other Achievements……………………………………………………………... 9

10. References………………………………………………………….………… 9

11. Appendices…………………………………………………………………… 10

1. Introduction

The overall purpose of this research effort is to determine the role iron acquisition and biofilm functions expressed by Gram-negative pathogens play in the pathogenesis of severe infections in the Wounded Warrior because of polytrauma and blast injuries. The information collected with these studies will be used to explore the efficacy of different chemical and biological agents to block these potential virulence functions using appropriate experimental infection models. These studies have the potential of providing not only new basic information on the pathobiology of bacteria that cause serious infections, but also facilitating the development of new and more effective therapeutics to treat severe Gram-negative infections in wounded military personnel.

2. Keywords

Iron acquisition, biofilms, wound infections, bacterial pathogens, bacterial resistance, bacterial virulence, siderophores, iron acquisition inhibitors, biofilm inhibitors, tissue culture model, animal models, experimental infections, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, phospolipases, genomes, bioinformatics.

3. Overall Project Summary

Targeting iron uptake functions

Work done throughout this project has shown that *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* military wound isolates are able to acquire essential iron under limiting conditions when tested under laboratory conditions. In the case of *A. baumannii*, all 29 strains selected from the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN) strain collection at the Walter Reed Army Institute of Research (WRAIR), showed iron uptake capacity due to the production of mostly catechol-derived siderophores, including *A. baumannii* AB5075. This strain, which was isolated from a wounded soldier, was selected as a model to study the virulence of this pathogen as we reported during this award period (1). The ability of this strain to progress under iron limitation imposed by laboratory conditions as well as to infect and persist in invertebrate and vertebrate hosts, such as *Galleria mellonella* larvae and immunocompromised mice, respectively, clearly depends on the active production and utilization of the catechol siderophore acinetobactin, which we previously described using the *A. baumannii* ATCC 19606^T strain (2).

Interestingly, like ATCC 19606^T, AB5075 produces only acinetobactin but is able to use siderophores produced by other bacteria (xenosiderophores) including baumannoferrin, a novel hydroxamate produced by the *A. baumannii* multi-resistant clinical isolate AYE (Penwell *et al*., submitted for publication). This observation together with our inability as well as that of others to successfully target siderophore-mediated iron uptake systems to develop alternative therapeutics, as it was recently reported for *P. aeruginosa* that could resist the action of a siderophore-conjugated monobactam when tested *in vivo* (3), prompted us to explore other antibacterial alternatives, which could effectively affect the ability of *A. baumannii* to acquire iron during the interaction with the host. As we described in previous reports, we tested the effect of gallium nitrate [$Ga(NO_3)_3$] since this compound is currently used in human medicine to treat serious illnesses (4). Our work with this metal derivative that impairs iron-dependent functions because of its inability to be reduced under physiological conditions, showed that all *A. baumannii*, *P. aeruginosa*, *K. pneumonia* and *E. coli* MRSN and non-MRSN tested isolates are sensitive to this compound. However, bacterial sensitivity to gallium nitrate is significantly variable among all tested strains and dependent on the iron-free content of the culture medium. These observations seriously affect the antimicrobial predictability of this compound when tested under standard laboratory conditions normally used to determine the minimal inhibitory concentrations (MIC) of antimicrobial agents used in human medicine. Furthermore, it was recently reported that the *A. baumannii* LAC-4 hyper-virulent isolate displays high tolerance to gallium nitrate (5), an outcome that could be potentially observed with some of the *A. baumannii* MRSN isolates including AB5075, which is also considered a highly virulent isolate (1). Consequently, we determined the antimicrobial effect of Ga-protoporphyrin IX (Ga-PPIX) on all *A. baumannii* MRSN and non-MRSN strains we have in our collection. When tested under standardized conditions used in clinical microbiology laboratories, Ga-PPIX showed antibacterial activity against all tested *A. baumannii* isolates independently of their site of isolation as well as their resistance phenotype. Furthermore, *ex vivo* and *in vivo* studies using submerged monolayers of A549 human alveolar epithelial cells and *G. mellonella* larvae showed that the addition of 20 and 40 µg/ml of this metalloporphyrin derivative produced significant antibacterial activity against different *A. baumannii* isolates without causing toxic effects to the

human cells or the larvae. All these results and observations will be reported in a manuscript that is almost ready to be submitted for publication to the journal Antimicrobial Agents and Chemotherapy.

We further extended the studies with A549 cells using polarized cells grown on the surface of trans-well membranes, an experimental condition that mimics the interaction of *A. baumannii* with the human host during the pathogenesis of pneumonia. This approach showed not only that AB5075 forms biofilms on the surface of the epithelial cells, but also the capacity of this pathogen to produce a significant reduction in the surfactant layer that covers the polarized cells and extensive cell damage. Interestingly, the addition of Ga-PPIX either to the medium fed to the polarized cells through the trans-well membrane or sprinkled on the surface of the polarized A549 cells significantly reduced their damage as well as that of the surfactant layer when infected with AB5075 without causing detectable cytotoxic effects. Studies completed between the 3rd and 4th quarter of the 3rd year of the award showed that the antimicrobial activity of Ga-PPIX and its effect on bacteria-epithelial cell interactions are different from those detected when adding antibiotics used in human medicine, including kanamycin, chloramphenicol and tetracycline. Overall, these studies and the results they have produced to date have shown the potential value of Ga-PPIX as an effective antibacterial agent and the value of polarized A549 human epithelial cells as an experimental model to study relevant *A. baumannii*-host interactions. Furthermore, the observation that the presence of *A. baumannii* produces significant damage to the surfactant layer that covers the polarized cells suggested to us that this host product could play a critical role in the virulence of this pathogen. We will use these preliminary observations to develop a new research avenue to be supported by extramural funds.

In summary, all studies conducted to date indicate that Ga-PPIX is a convenient antibacterial agent that could be used to treat infections, particularly wound infections, caused by different *A. baumannii* isolates including those that display multi-drug resistance phenotypes. However, the relatively low solubility of Ga-PPIX is a concern. We initiated a collaboration with Dr. Justin Saul, a member of the Miami University Department of Chemical, Paper and Biomedical Engineering who has experience and expertise with methods and techniques used to effectively deliver therapeutic agents including antimicrobial agents. Preliminary studies showed that the combination of Ga-PPIX with the lipids dipalmitoylphosphatidylcholine (DPPC) or 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) resulted in the formation of liposomes that contained this metalloporphyrin derivative as determined by methods normally used to detect the formation of liposomes. Biological tests showed that DPPC and DSPC liposomes are effective tools to deliver Ga-PPIX. We plan to continue this work with Dr. J. Saul and explore the use of hydrogels as a tool to treat wound infections with Ga-PPIX in a research project independent of this award we hope will be supported by extramural funds.

Targeting biofilm biogenesis functions

During the last quarter of this award period we completed the analysis of the antibiofilm activity of the compounds 2AI-1, LED209 and Virstatin, which were described as antibiofilm agents for different bacterial pathogens including *A. baumannii* (6-8). The ability of these compounds to reduce biofilm formation on polystyrene was tested by incubating *A. baumannii* 4795, *A. baumannii* 5711, *P. aeruginosa* 105819 or *E. coli* 105438 in polystyrene culture tubes containing LB broth and 0 μM or 25 μM of the respective compound, which were incubated for 24 h at 37°C without shaking. Each of these four strains represents a member of the four different growth groups we defined by the response of all MRSN tested strains to 2AI-1 described in the last report. Biofilm formation was determined by assessing the retention of crystal violet by bacterial biofilms using the $580_{nm}/600_{nm}$ ratio to normalize the amount of biofilm formed to the cell density of each sample, as previously described (9). Unfortunately, 2AI-1, LED209 nor Virstatin significantly reduced the amount of biofilm formed by the four strains under the tested conditions.

We further investigated the effect of 2AI-1, LED209 and Virstatin on the interaction of *A. baumannii* AB5075 with polarized A549 human alveolar epithelial cells as we described in previous reports. Briefly, A549 cells polarized on the surface of trans-well membrane support plates (Corning) were infected with 10^6 bacterial cells per ml for 72 h. After the first 24 h of infection, some of the polarized cell samples were treated with either 100 μM 2AI-1, 100 μM LED209, or 100 μg/ml Virstatin supplemented in DMEM and fed to the polarized cells from underneath the membrane. As a control, some of the polarized cells were left untreated or were treated with DMEM supplemented with 2AI-1, LED209, or Virstatin. The polarized cells were then fixed after 72 h of infection and prepared for scanning electron microscopy. This approach showed that when left untreated, AB5075 is able to form robust biofilms and cause significant damage to the surfactant layer on the polarized

cells and to the A549 alveolar epithelial cells themselves after 72 h of infection (Fig. 1B) when compared with the uninfected control (Fig. 1A). Treatment of the infected polarized cells with 2AI-1 results in a modest reduction of biofilms formed on the surface of A549 cells and significant cell damage (Fig. 1D) when compared with the untreated infected control sample (Fig. 1B). This response is similar to that detected with AB5075 infected A549 cells (Fig. 1B). Treatment of the infected A549 with LED209 caused an increase in biofilm formation on top of the polarized cell layer. In addition, damage to the surfactant and cell layers could not be determined due to the amount of biofilm on top of the polarized cells (compare panels F and B of Fig. 1). Finally, treatment of the infected polarized cells with Virstatin caused a drastic effect on the samples, there was no detectable mucin layer and most of the A549 cells were absent (Fig. 1H), because they were lysed or detached from the membranes and therefore lost during sample preparation. The few A549 cells that remained attached to the trans-well membranes showed bacteria adhered on their surfaces (Fig. 1H inset). It is important to note that the addition of 2AI-1, LED 209 or Virstatin to uninfected A549 polarized samples did not cause significant changes when compared with uninfected untreated samples (compare panels A, C, E and G of Fig. 1).

In conclusion, 2AI-1, LED209, and Virstatin treatment not only did not prevent biofilm formation on abiotic and biotic surfaces, damage to the mucin layer and epithelial cell damage, but also resulted either in an increase of biofilms on the surface of the epithelial cells or a drastic detrimental effect on the A549 cells when the samples were treated with LED209 and Virstatin, respectively. The observation that Virstatin did not have a significant effect on the ability of *A. baumannii* 4795, 5075 and 5711 to form biofilms may also reflect the observation that only 70% of the tested strains are susceptible to this antibiofilm agent (6). Alternatively, differences in the experimental conditions used in our work when compared with the referenced work could also explain the different outcomes observed with these potential antimicrobial agents. Considering the fact that Virstatin inhibits the activity of the *Vibrio cholerae* ToxT transcriptional factor (8), it is possible to speculate that the enhanced damage we detected with this chemical agent is due to the inactivation of an *A. baumannii* AB5075 ToxT related factor that may negatively control the virulence of this strain. If this is the case, the possibility that the application of this antivirulence agent to bacterial pathogens other than *V. cholerae* may cause undesired results should be carefully considered and investigated.

Targeting phospholipases

Since the overall goal of my research program is to understand the cellular and molecular factors involved in the virulence of *A. baumannii*, we have been testing the capacity of this pathogen to lyse vertebrate/human cells as a mechanism that not only cause host cell damage, but also serves as a mean to obtain essential nutrients including iron. Consequently we have investigated the capacity of *A. baumannii* to produce hemolysins since these enzymes have been described as important virulence factors for a wide range of pathogens, including *P. aeruginosa* where the production of phospholipase C has been linked to tissue destruction and pathologies reminiscent of burn infections (10). Since the majority of iron in a vertebrate host is intracellular, the availability of intracellular iron-containing molecules to bacterial pathogens is dependent on the lysis of host cells and subsequent release of these molecules due to cell and tissue damage found in wounds (11, 12). Accordingly, we determined that *A. baumannii* expresses hemolytic activity preferentially against horse and human erythrocytes but not sheep red blood cells, which are normally used to detect hemolytic activity in clinical/diagnostic microbiology. We also determined that the genomes of *A. baumannii* sequenced strains harbor two predicted genes (*plc1* and *plc2*) coding for phospholipase C. Based on these observations, we tested the presence of these genes in 19 *A. baumannii* strains including 10 MRSN strains, including the AB5075 isolate, by PCR using primers that hybridize within the respective coding regions of both phospholipase C genes. Amplicons of the predicted sizes, 993 bp for *plc1* and 1,167 bp for *plc2*, were obtained for all *A. baumannii* isolates confirming the presence of these genes in a variety of *A. baumannii* strains (Fig. 2). This screening study was further complemented by testing the presence of phosphatidylcholine-specific phospholipase activity in the culture supernatants of all 19 *A. baumannii* strains using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit (Fig. 3). These assays showed that although the phospholipase C activity of all culture supernatants was significantly higher than the negative control ($P < 0.001$), there were significant variations among the strains, with the AB3560 and AB3806 wound isolates obtained from injured soldiers being the strains that produced the lowest and highest activities, respectively. However, the phospholipase activity of this particular set of isolates is comparable with the enzymatic activity detected in non-military isolates tested in this study.

Further analysis of the type strain ATCC 19606^T using quantitative RT-PCR and total RNA extracted from cells grown in iron-chelated or iron-repleted medium showed that transcription of *plc1* and *plc2* is indeed significantly higher ($P < 0.01$ and $P < 0.05$, respectively) in bacteria cultured under iron-depleted conditions when compared with medium supplemented with inorganic iron (Fig. 4). These analyses also showed that the iron-regulated expression of *plc1* and *plc2* is similar to that of *bauA*, which codes for the iron-regulated production of the ATCC 19606^T BauA acinetobactin outer membrane receptor protein (2). We are currently investigating the role of the phospholipase C1 and C2 in *A. baumannii* virulence and will study during the next quarter the potential inhibitory effect of the phosphatidylcholine analog miltefosine, which protects lung functions from the activity of phospholipase C produced by *P. aeruginosa* (13).

In summary, our preliminary observations support the value of using miltefosine to treat *A. baumannii* wound infections by blocking a bacterial function that could play a role in host-pathogen interactions that are critical in the pathogenesis of these infections.

Bacterial Genomes

With the assistance of a grant-in-aid from Illumina, because the Miami University Center for Bioinformatics and Functional Genomics (CBFG) purchased a MiSeq next generation nucleotide sequencer, we were able to fully sequence the genomes of one *P. aeruginosa* and three *K. pneumoniae* MRSN isolates provided to us by Dr. Zurawski from WRAIR. We also sequenced the genome to the *K. pneumoniae* ATCC 13883 stain which is being used by other investigators to study different aspects of the pathobiology of this microorganism. This work included not only the proper preparation of the genomic libraries to be sequenced and collection of nucleotide sequence data, but also the utilization of an improved and more convenient gene annotation process that combined the RAST annotation tool with Prokka, a software package designed to annotate bacterial genomes. All this bioinformatics work, which was done in-house in collaboration with Dr. I. Friedberg and his graduate student David Ream, facilitated the overall gene annotation process and produced the properly and conveniently formatted files that were submitted to GenBank to obtain the cognate accession numbers needed to report these sequences in the Genome Announcements manuscript we published early this year (see Reportable Outcomes). Using the same experimental and bioinformatic approach we also sequenced the genome of *A. baumannii* A155, one of the first CC109 isolates from Argentina that includes a AbaR-type island inserted within *comM*, and the *aac(6')-Ib* gene, which confers resistance to numerous aminoglycosides (14-16). This work resulted in a second Genome Announcements we also published early this year (see Reportable Outcomes). We have extended these genome-sequencing efforts to all military isolates obtained from WRAIR and Fort Sam. As seen in Table 1, 42 strains have been sequenced to varying stages. All isolates have had Paired-end libraries sequenced. Twenty-five Mate pair strain libraries have been sequenced and the libraries for the remaining 17 strains have been constructed and will be sequenced in the subsequent quarter. After the DNA sequencing and assembly steps are completed, genomes will be annotated with Prokka version 1.10 on a quadcore i7 workstation with 32 GB DDR3 running Ubuntu 14.04 LTS. These sequences will be submitted to NCBI GenBank and reported in Genome Announcements as we did before for the *K. pneumoniae* strains and the *A. baumannii* A155 isolate. The genomic information to be collected will be very useful for our work, in which we could compare the genomic capacity of the different isolates to express critical iron acquisition and biofilm functions that could be targeted for alternative therapeutics, as well as the projects of other investigators who are interested in the study of these pathogens. As a matter of fact, two investigators interested in the study of *K. pneumoniae* have already contacted us and another colleague of mine, Dr. A. Hauser (Northwestern University, Chicago, IL), has expressed interest in the *A. baumannii* genome sequence data since he is conducting comparative genomic studies with the ultimate goal of understanding the nature of the virulence factors produced by this pathogen.

4. Key Research Accomplishments

- Demonstration of the capacity of all MRSN tested strains to grow under iron limitation and produce biofilms on abiotic surfaces.
- Demonstration of the key role the acinetobactin-mediated iron acquisition system plays in the ability of *A. baumannii* AB5075 strain to persist and cause disease and death when tested using invertebrate and vertebrate hosts.
- Detection of variable and medium-dependent responses to $Ga(NO_3)_3$ by all MRSN tested strain.

- Demonstration of the ability of AB5075 to use xenosiderophores under iron chelated conditions, a response that may reflect a potentially negative outcome of poly-microbial wound infections.
- Although *E. coli*, *P. aeruginosa* and *K. pneumoniae* MDR isolates showed limited sensitivity to Ga-PPIX, all *A. baumannii* tested strains showed sensitivity to this metalloporphyrin derivative that inhibits iron metabolism when tested under standard laboratory conditions.
- Detection of effective antibacterial activity by Ga-PPIX against *A. baumannii* when tested using the *G. mellonella* experimental model without causing detectable host effects.
- Lack of significant effect of antibiofilm agents used with other pathogens when tested with selected MRSN strains using *in vitro* laboratory conditions and abiotic surfaces.
- Detection of undesirable responses to antibiofilm/antivirulence agents successfully used against other pathogens when AB5075 was tested using the *ex vivo* A549 polarized cell infection model. Presence of LED209 and Virstatin increased AB5075 biofilm formation of the surface of the epithelial cells and caused a potential hyper-virulence response against this human epithelial cell line, respectively.
- Availability of MRSN isolates genome sequences that would facilitate comparative genomic studies and the better understanding of genes and gene product potentially involved in their virulence.
- Potential virulence role and target value of phospholipases that could provide iron because of the cell/tissue damage they cause during infection.

5. Conclusions

Experimental data collected during the studies conducted to date clearly indicate that all MRSN isolates obtained from wound infections are iron uptake proficient and capable of forming biofilms on plastic surfaces normally found in medical elements and settings. Regarding the value of iron acquisition as an alternative therapeutic target, which could be used to treat wound infections caused by multi-drug resistant strains, the observation that the virulence of the *A. baumannii* AB5075 MRSN model isolate depends on the expression of the acinetobactin-mediated iron acquisition system supports this possibility. Our data show that Ga-PPIX is the most effective antibacterial agent we tested during our studies because of its negative effect on bacterial iron metabolism. Although this metalloporphyrin derivative displayed variable activity against *P. aeruginosa*, *K. pneumoniae* and *E. coli* strains, it showed antibacterial activity against all *A. baumannii* strains including MRSN and non-MRSN isolates under standard bacteriological conditions. Furthermore, *ex vivo* and *in vivo* studies using the A549 polarized cell system and the *G. mellonella* experimental infection model, respectively, showed that Ga-PPIX has significant antibacterial activity against AB5075. All these observations not only support our hypothesis that targeting iron uptake functions is a logical approach, but also support the implementation of studies using a well-established wound infection model using mice and pigs that will be conducted in the following quarters. Our preliminary studies on the role of phospholipase C activity expressed by all *A. baumannii* MRSN tested isolates, which could provide intracellular iron during infection because of tissue and cell damage, further supports the role of iron in wound infections. Regarding the possibility of using biofilm biogenesis as an antimicrobial target, our studies using compounds already described as antibiofilm/antivirulence agents for other pathogens showed not only moderate inhibitory effects, but also the capacity of the tested compounds to either enhance the AB5075 capacity to form biofilms on the surface of human epithelial cells or even increase the virulence of this MRSN isolate. These responses could be due to the expression of unique *A. baumannii* cellular functions, which could be identified by conducting comparative genomic analysis of the *A. baumannii* MRSN strains we have sequenced during this project.

6. Publications, Abstracts and Presentations

Publications

Jacobs, A. C., M. G. Thompson, C. C. Black, J. L. Kessler, L. P. Clark, C. N. McQueary, H. Y. Gancz, B. W. Corey, J. K. Moon, Y. Si, M. T. Owen, J. D. Hallock, Y. I. Kwak, A. Summers, C. Z. Li, D. A. Rasko, W. F. Penwell, C. L. Honnold, M. C. Wise, P. E. Waterman, E. P. Lesho, R. L. Stewart, **L. A. Actis**, T. J. Palys, D. W. Craft, and D. V. Zurawski. AB5075, a highly virulent isolate of *Acinetobacter baumannii*, as a model strain for the evaluation of pathogenesis and antimicrobial treatments. mBio, 5, e01076-14, 2014.

Arivett, B. A, D. C. Ream, S. E. Fiester, K. Mende, C. K. Murray, M. G. Thompson, S. Kanduru, A. M. Summers, A. L. Roth, D. V. Zurawski, and **L. A. Actis**. Draft genome sequences of *Klebsiella pneumoniae* clinical type strain ATCC 13883 and three multidrug-resistant clinical isolates. Genome Announcements, 3, e01385-14, 2015.

Arivett, B., S. E. Fiester, D. Ream, D. Centrón, M. S. Ramirez, M. Tolmasky, and **L. Actis**. Draft genome of the multidrug-resistant *Acinetobacter baumannii* A155 clinical isolate. Genome Announcements, 3, e00212-15, 2015.

Arivett, B. A., S. E. Fiester, E. Ohneck, C. Kaufman, W. F. Penwell, C. M. Kaufman, R. F. Relich and **L. A. Actis**. Antimicrobial activity of gallium protoporphyrin IX against multidrug-resistant *Acinetobacter baumannii*. Manuscript in preparation.

Fiester, S. E., B. A. Arivett, R. E. Schmidt, A. C. Beckett, T. Ticak, M. V. Carrier, E. J. Ohneck, M. L. Metz, M. K. Sellin Jeffries and **L. A. Actis**. Phospholipase C contributes to the hemolytic activity and virulence of *Acinetobacter baumannii*. Manuscript in preparation.

7. Inventions, Patents and License
Nothing to report.

8. Reportable Outcomes
Nothing to report.

9. Other Achievements
Nothing to report.

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11. Appendices

Publications

Appendix 1. Jacobs, A. C., M. G. Thompson, C. C. Black, J. L. Kessler, L. P. Clark, C. N. McQueary, H. Y. Gancz, B. W. Corey, J. K. Moon, Y. Si, M. T. Owen, J. D. Hallock, Y. I. Kwak, A. Summers, C. Z. Li, D. A. Rasko, W. F. Penwell, C. L. Honnold, M. C. Wise, P. E. Waterman, E. P. Lesho, R. L. Stewart, **L. A. Actis**, T. J. Palys, D. W. Craft, and D. V. Zurawski. AB5075, a highly virulent isolate of *Acinetobacter baumannii*, as a model strain for the evaluation of pathogenesis and antimicrobial treatments. mBio, 5, e01076-14, 2014.

Appendix 2. Arivett, B. A, D. C. Ream, S. E. Fiester, K. Mende, C. K. Murray, M. G. Thompson, S. Kanduru, A. M. Summers, A. L. Roth, D. V. Zurawski, and **L. A. Actis**. Draft genome sequences of *Klebsiella pneumoniae* clinical type strain ATCC 13883 and three multidrug-resistant clinical isolates. Genome Announcements, 3, e01385-14, 2015.

Appendix 3. Arivett, B., S. E. Fiester, D. Ream, D. Centrón, M. S. Ramirez, M. Tolmasky, and **L. Actis**. Draft genome of the multidrug-resistant *Acinetobacter baumannii* A155 clinical isolate. Genome Announcements, 3, e00212-15, 2015.

Supporting Data

Table 1. Sequencing of military isolates

Strain	Organism	Source	Collected Date	Isolation source	2 x 250 PE*	2 x 75 Mate-PE$
AB967	*A. baumannii*	WRAIR	9/23/03	Blood	✔	✔
AB2828	*A. baumannii*	WRAIR	3/28/06	Blood	✔	✔
AB3340	*A. baumannii*	WRAIR	10/22/06	Blood	✔	✔
AB3560	*A. baumannii*	WRAIR	12/14/06	Blood	✔	✔
AB3638	*A. baumannii*	WRAIR	1/10/07	Severe Trauma Site	✔	✔
AB3785	*A. baumannii*	WRAIR	3/18/07	Blood	✔	✔
AB3806	*A. baumannii*	WRAIR	3/19/07	Severe Trauma Site	✔	✔
AB3927	*A. baumannii*	WRAIR	5/18/07	Severe Trauma Site	✔	✔
AB4025	*A. baumannii*	WRAIR	6/24/07	Wound	✔	✔
AB4026	*A. baumannii*	WRAIR	6/26/07	Wound	✔	✔
AB4027	*A. baumannii*	WRAIR	6/26/07	Wound	✔	✔
AB4052	*A. baumannii*	WRAIR	7/16/07	War wound (surv)	✔	✔
AB4269	*A. baumannii*	WRAIR	10/16/07	War wound (surv)	✔	✔
AB4448	*A. baumannii*	WRAIR	12/25/07	War wound (surv)	✔	✔
AB4456	*A. baumannii*	WRAIR	12/30/07	Aspirate	✔	✔
AB4490	*A. baumannii*	WRAIR	1/12/08	War wound (surv)	✔	✔
AB4498	*A. baumannii*	WRAIR	1/13/08	Blood	✔	✔
AB4795	*A. baumannii*	WRAIR	5/5/08	Severe Trauma Site bone	✔	✔
AB4878	*A. baumannii*	WRAIR	6/6/08	War wound (surv)	✔	✔
AB4932	*A. baumannii*	WRAIR	7/4/08	Sputum	✔	✔
AB4957	*A. baumannii*	WRAIR	7/17/08	Severe Trauma Site	✔	✔
AB4991	*A. baumannii*	WRAIR	8/3/08	War wound (surv)	✔	✔
AB5001	*A. baumannii*	WRAIR	8/5/08	Blood	✔	✔
AB5197	*A. baumannii*	WRAIR	10/15/08	Severe Trauma Site	✔	✔
AB5674	*A. baumannii*	WRAIR	5/22/09	Blood	✔	✔
105777	*P. aeruginosa*	WRAIR	4/21/2007	Wound	✔	In Progress
105819	*P. aeruginosa*	WRAIR	11/12/2007	Wound	✔	In Progress
105880	*P. aeruginosa*	WRAIR	3/26/2008	Thigh	✔	In Progress
105857	*P. aeruginosa*	WRAIR	1/28/2008	Right knee	✔	In Progress
105738	*P. aeruginosa*	WRAIR	10/7/2006	Chest tube	✔	In Progress
101436	*K. pneumoniae*	WRAIR	5/17/06	Wound	✔	In Progress
101712	*K. pneumoniae*	WRAIR	10/7/07	Wound	✔	In Progress
105371	*K. pneumoniae*	WRAIR	5/22/08	Blood	✔	In Progress
101488	*K. pneumoniae*	WRAIR	11/11/06	Wound	✔	In Progress
101731	*K. pneumoniae*	WRAIR	1/25/08	Wound	✔	In Progress
4640	*K. pneumoniae*	WRAIR	Not Collected	Severe Trauma Site	✔	In Progress
105454	*E. coli*	WRAIR	4/12/08	Urine	✔	In Progress
105547	*E. coli*	WRAIR	2/4/09	Wound	✔	In Progress
109497	*E. coli*	WRAIR	1/20/10	Urine	✔	In Progress
108191	*E. coli*	WRAIR	2/17/09	Urine	✔	In Progress
105433	*E. coli*	WRAIR	11/14/07	Wound	✔	In Progress
105438	*E. coli*	WRAIR	12/17/07	Wound, right thigh	✔	In Progress

* 2 x 250 Paired-End sequencing performed with MiSeq v3 reagent
$ 2 X 75 Mate-Pair Paired-End sequencing performed with MiSeq v3 reagent

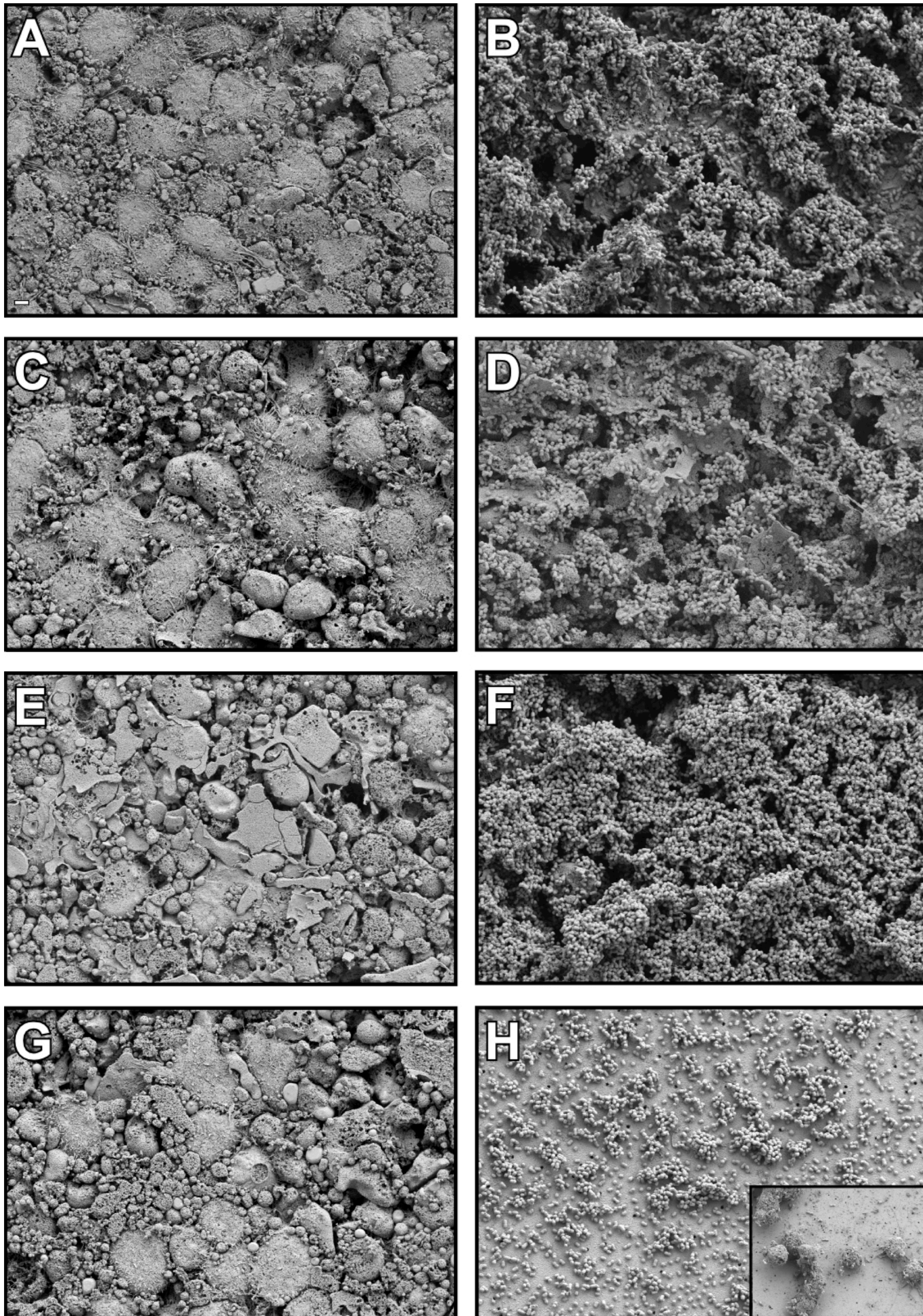

Figure 1. Effect of 2AI-1, LED209 and Virstatin on polarized A549 epithelial cells. Uninfected monolayers were incubated in the absence (A) or presence of 2AI-1 (C), LED209 (E) or Virstatin (G). Infected monolayers were incubated in the absence (B) or presence of 2AI-1 (C), LED209 (E) or Virstatin (H). Inset in H shows the presence of few A549 cells that remained attached to trans-well membranes with bacteria adhered to their surfaces. White bar at the bottom left corner of panel A represents 2 μm.

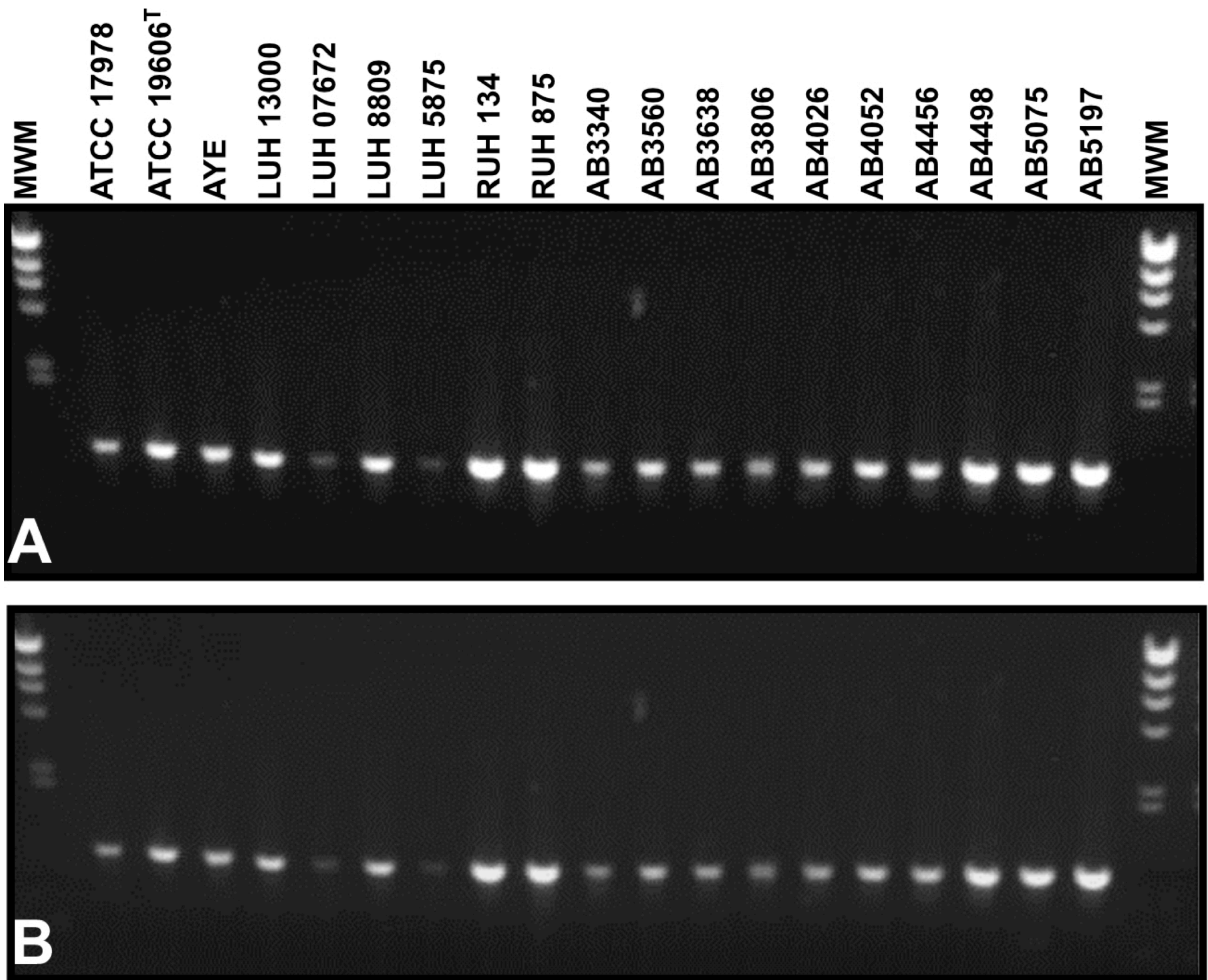

Figure 2. Detection of *plc1* and *plc2* in the genomes of *A. baumannii* strains. Agarose gel electrophoresis of internal amplicons of *plc1* (A) or *plc2* (B) using total genomic DNA isolated from 19 *A. baumannii* strains and primers, which hybridize internally to *plc1* or *plc2*, respectively.

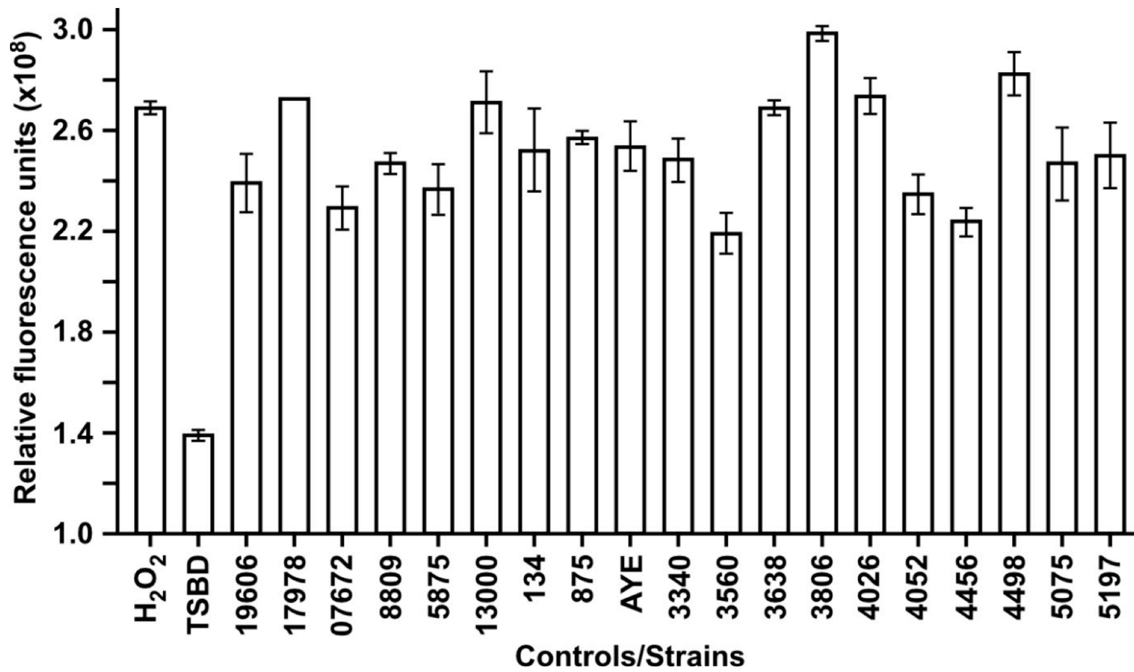

Figure 3. Phosphatidylcholine-specific phospholipase C activity among *A. baumannii* isolates. Culture supernatants from 19 *A. baumannii* strains grown under iron chelation were tested for phosphatidylcholine-specific phospholipase C using the Amplex Red phosphatidylcholine-specific phospholipase C assay kit, using hydrogen peroxide (H_2O_2) or uninoculated medium (TSBD) as positive and negative controls, respectively. Tested strains are identified as follows: 19606 and 17978 represent ATCC strains, 07672-13000 represent strains LUH strains, 134 and 875 represent strains RUH, and 3340-5197 represent MRSN isolates. Error bars represent the standard error (SE) of the mean.

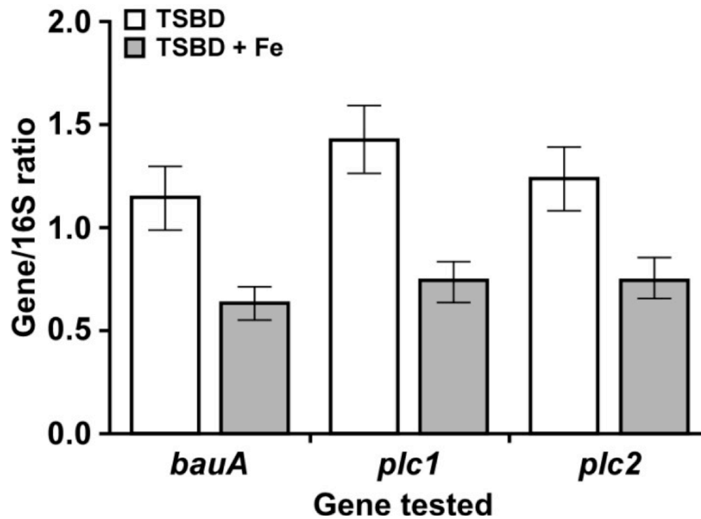

Figure 4. Analyses of *plc1* and *plc2* transcription in ATCC 19606^T and isogenic derivatives. Transcriptional analyses of *plc1* and *plc2* in ATCC 19606^T cells grown under iron chelation (TSBD) or supplemented with 50 µM $FeCl_3$ (TSBD+Fe) and grown for 24 h at 37°C with shaking at 200 rpm. Expression of *bauA* was used as a control of a gene known to be iron regulated. Expression of *plc* genes was normalized to the expression of the 16S gene. Error bars represent the standard error (SE) of the mean.

Appendix 1

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AB5075, a Highly Virulent Isolate of *Acinetobacter baumannii*, as a Model Strain for the Evaluation of Pathogenesis and Antimicrobial Treatments

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ABSTRACT ***Acinetobacter baumannii*** **is recognized as an emerging bacterial pathogen because of traits such as prolonged survival in a desiccated state, effective nosocomial transmission, and an inherent ability to acquire antibiotic resistance genes. A pressing need in the field of *A. baumannii* research is a suitable model strain that is representative of current clinical isolates, is highly virulent in established animal models, and can be genetically manipulated. To identify a suitable strain, a genetically diverse set of recent U.S. military clinical isolates was assessed. Pulsed-field gel electrophoresis and multiplex PCR determined the genetic diversity of 33 *A. baumannii* isolates. Subsequently, five representative isolates were tested in murine pulmonary and *Galleria mellonella* models of infection. Infections with one strain, AB5075, were considerably more severe in both animal models than those with other isolates, as there was a significant decrease in survival rates. AB5075 also caused osteomyelitis in a rat open fracture model, while another isolate did not. Additionally, a Tn*5* transposon library was successfully generated in AB5075, and the insertion of exogenous genes into the AB5075 chromosome via Tn*7* was completed, suggesting that this isolate may be genetically amenable for research purposes. Finally, proof-of-concept experiments with the antibiotic rifampin showed that this strain can be used in animal models to assess therapies under numerous parameters, including survival rates and lung bacterial burden. We propose that AB5075 can serve as a model strain for *A. baumannii* pathogenesis due to its relatively recent isolation, multidrug resistance, reproducible virulence in animal models, and genetic tractability.**

IMPORTANCE **The incidence of *A. baumannii* infections has increased over the last decade, and unfortunately, so has antibiotic resistance in this bacterial species. *A. baumannii* is now responsible for more than 10% of all hospital-acquired infections in the United States and has a >50% mortality rate in patients with sepsis and pneumonia. Most research on the pathogenicity of *A. baumannii* focused on isolates that are not truly representative of current multidrug-resistant strains isolated from patients. After screening of a panel of isolates in different *in vitro* and *in vivo* assays, the strain AB5075 was selected as more suitable for research because of its antibiotic resistance profile and increased virulence in animal models. Moreover, AB5075 is susceptible to tetracycline and hygromycin, which makes it amenable to genetic manipulation. Taken together, these traits make AB5075 a good candidate for use in studying virulence and pathogenicity of this species and testing novel antimicrobials.**

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Acinetobacter baumannii is an opportunistic, Gram-negative pathogen that thrives in clinical settings and is often multidrug resistant (MDR), factors which earn it a place among the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens of clinical importance (1). Some recent isolates are resistant to all typically used antibiotics except colistin and tigecycline and thus are called extensively or extremely drug-resistant (XDR) *A. baumannii* (2). MDR/XDR *A. baumannii* strains are a worldwide problem for clinicians and caregivers in the hospital setting, particularly in the intensive care unit (ICU) (3). *A. baumannii* is also often isolated from infections of severe wounds sustained in military combat. These infections are responsible for increased morbidity, with prolonged wound

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healing and amputations of extremities when limbs cannot be salvaged (4, 5). *A. baumannii* was a predominant isolate from wounded soldiers serving in Iraq (4, 5) and was associated with wartime polytrauma injuries in the past (6). Additionally, there may be a link between *A. baumannii* and crush injuries, as *A. baumannii* infections were also prevalent after the recent large earthquakes in Haiti (7) and China (8).

Another disturbing development that has increased the clinical importance of *A. baumannii* infections is that many strains have become highly antibiotic resistant. For example, in previous decades, *A. baumannii* isolates obtained from both military and civilian settings were often carbapenem sensitive. Now, the majority of U.S. military isolates are carbapenem resistant (9). This trend has also been mirrored in civilian hospitals around the world (10). Recently, even colistin-resistant strains have emerged in the military health care system (11). The latter development is deeply troubling, as colistin is considered the last line of defense against these MDR isolates. Exacerbating this problem is the lack of new treatments in the pharmaceutical pipeline (12); therefore, research on *A. baumannii* virulence factors is urgently needed, as they could constitute potentially novel targets for future antimicrobials.

While previous studies attempted to examine the virulence of different clinical *A. baumannii* strains utilizing *in vivo* model systems (13, 14), the majority of *A. baumannii* researchers still use two American Type Culture Collection (ATCC) strains, ATCC 19606^T and ATCC 17978, which were isolated more than 50 years ago and are not significantly antibiotic resistant. These strains are certainly more amenable to genetic manipulation than most clinical isolates (15, 16) and share considerable genome homology (>90%) to current *A. baumannii* isolates (17), but they are not representative of contemporary isolates of this rapidly evolving pathogen. Some researchers, recognizing that the ATCC isolates are dated, have performed studies with more recent clinical isolates; however, genetic manipulation of such isolates has depended on susceptibility to aminoglycosides (18–20), which is often not found in clinical strains (21). Therefore, our goal was to carry out a systematic study of our own contemporary clinical strains isolated from patients in the U.S. military health care system to identify a strain that is more representative of current clinical isolates, that is highly virulent in established model infections, and that can be genetically manipulated without a potential sacrifice with respect to virulence and antibiotic resistance. Not only does identifying such a strain account for more recent clinical outcomes, but the increased virulence in animal models allows greater statistical power in screening new therapeutics. Moreover, the ability to manipulate the genome allows the study of virulence factors, some of which may be responsible for the emergence of this pathogen in more recent years.

RESULTS

Defining genetic characteristics of *A. baumannii* isolates. In order to identify potential reference strains, a diverse set of 33 *A. baumannii* isolates was chosen based on genetic, isolation site, and antibiotic resistance differences from more than 200 *A. baumannii* strains isolated between 2004 and 2010 from patients in the U.S. military health care system. AB0057, first isolated in 2004 at Walter Reed Army Medical Center, was also included as a comparator because this strain is well characterized, and its genome was previously sequenced (22).

The diversity set of *A. baumannii* isolates was determined via pulsed-field gel electrophoresis (PFGE) analysis and a multiplex PCR assay previously developed to identify the international clonal complexes (ICC). Separately, antibiograms were determined using two different automated bacterial identification systems. The majority of strains were found to be multidrug resistant, typical of current clinical strains (see Table S1 in the supplemental material). As shown in Fig. 1, the genetic similarity of the strains, as determined by PFGE, ranged from 45 to 100%. PFGE types were considered to represent the same clones when their genetic similarity was >80% (23); based on this cutoff, the 33 strains represent 19 unique clones. When the genetic relatedness of these 19 clones was compared, it was found that the majority of them clustered into three groups, which generally aligned with the ICC designations determined by multiplex PCR (Fig. 1 and Table 1) (24). Exceptions were the isolates AB3560, AB4456, and AB4857, which were determined to be ICC III by the multiplex assay but appeared to be ICC I via PFGE. In this case, we relied on the multiplex data (Table 1) to be definitive. These data were used to select four representative strains for genome sequencing and evaluation in animal models.

Three of the strains chosen each represented one of the three ICC groups, AB5075 (ICC I), AB5711 (ICC II), and AB4857 (ICC III). The fourth strain, AB5256 was an outlier, as the OXA-51 allele from this strain was amplified with group 1 primers (24), while the *csuE* allele was not. The isolates were sequenced (25) and compared to previously sequenced *A. baumannii* genomes using the BLAST score ratio (BSR) approach (26). This method compares putative peptides encoded in each genome based on the ratio of BLAST scores to determine if they are conserved (BSR value $\geq$ 0.8), divergent ($0.8 > BSR > 0.4$), or unique ($BSR < 0.4$). The majority of the proteomes were similar among strains, meaning they had a BSR of >0.4; however, each isolate also had a set of unique proteins (see Table S2 in the supplemental material). These results are similar to what has been found previously with MDR *A. baumannii* clinical isolates (17), suggesting that the strains used in this study are not genetic outliers.

Virulence assessed in the *Galleria mellonella* model. Strains were first tested in a *Galleria mellonella* infection model, as this model is well established to assess virulence and novel therapeutics for bacterial pathogens, including *A. baumannii* (27, 28). *G. mellonella* larvae were infected with an approximate dose of 1.0×10^5 CFU with each of the four sequenced *A. baumannii* isolates, AB4857, AB5075, AB5256, and AB5711, as well as control strain AB0057. Worms were observed for 6 days, and death was recorded. Within 24 h postinfection, approximately 25% of AB5075-infected worms remained, while the other four strains had survival rates of 70% or higher (Fig. 2). By the end of the 6-day study, AB5075-infected worms had a survival rate of 16%; strains AB4857 and AB5256 were considered moderately pathogenic in this model, with survival rates of 50% and 35%, respectively. The least lethal strains were AB5711 and AB0057, with survival rates of 85% and 83%, respectively. Phosphate-buffered saline (PBS)-injected control worms displayed 100% survival through the course of the study. Based on these data, it was hypothesized that AB5075 was more virulent than the other four strains tested. Using the Mantel-Cox test with Bonferroni correction for multiple comparisons, Kaplan-Meier curves were compared, and AB5075 was found statistically to be more lethal than AB4857, AB5711, and AB0057 (all P values < 0.0125). While AB5256 had a higher

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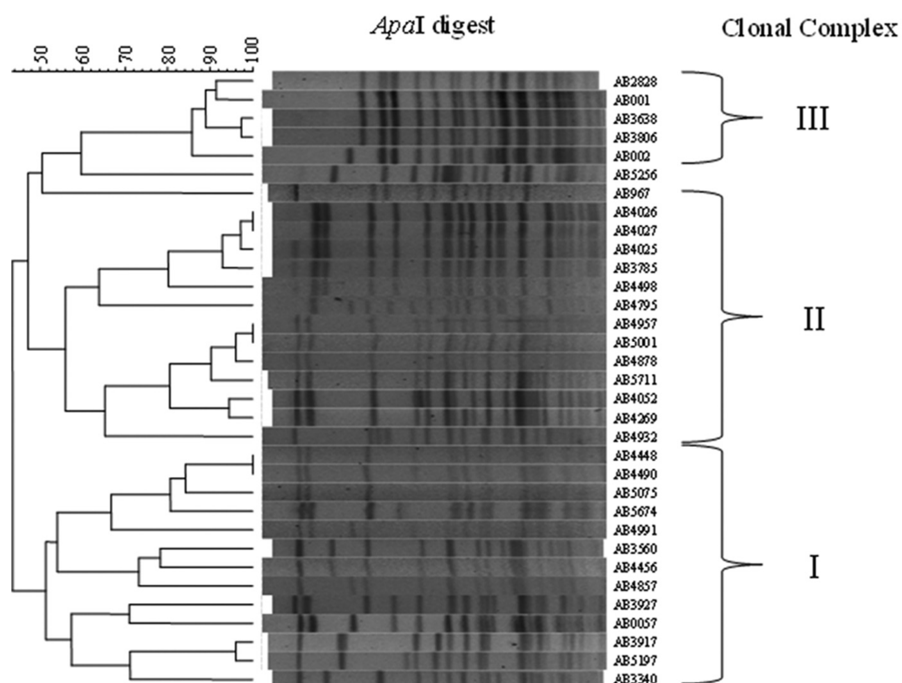

FIG 1 Pulsed-field gel electrophoresis of *A. baumannii* strains. Genomic DNA was isolated from 33 *A. baumannii* clinical isolates, digested with ApaI, and separated by pulsed-field gel electrophoresis (PFGE). Patterns of electrophoresis were compared using BioNumerics 6.0 software. The ICC was determined by multiplex PCR analysis, and brackets delineate the approximate grouping of each strain.

survival rate in this study than AB5075, the difference was not significant after the Bonferroni correction.

Separately, to compare the lethality of AB5075 to that of more commonly utilized *A. baumannii* model strains, the 50% lethal doses (LD_{50}) of AB5075, ATCC 17978, and ATCC 19606^T were determined in *G. mellonella*. The LD_{50} of AB5075 was 1.0×10^4 CFU. In contrast, the LD_{50} of ATCC 17978 and ATCC 19606^T were 5.0×10^5 and 1.0×10^6, respectively.

Virulence assessed in the mouse pulmonary model. *A. baumannii* strains were examined in a murine pulmonary model of infection, because this model is commonly used to assess bacterial virulence and drug efficacy, and survival can be assessed rapidly after an inoculum is delivered (14, 29). The animals were immunocompromised with two doses of cyclophosphamide before inoculation, a treatment that allows *A. baumannii* to establish an infection (14). Mice were inoculated on day 0 with one of the five representative *A. baumannii* strains at a dose of 5.0×10^6 CFU and monitored for 6 days. Consistently, mice infected with AB5075 had a mortality rate of 70% within 48 to 72 h and a 6-day survival rate of 25% (Fig. 3). The other four strains tested, AB0057, AB5711, AB5256, and AB4857, were less lethal than AB5075 in this model, with 6-day survival rates of 65, 80, 80, and 85%, respectively. The log-rank (Mantel-Cox) test with Bonferroni correction determined that AB5075 time-to-death results were statistically significant compared to the other clinical isolates ($P < 0.0125$). The clinical scores of each infected animal also correlated with the survival plots; AB5075-infected mice displayed more severe illness than mice infected with the other strains. In a separate experiment, mice inoculated with 5.0×10^6 cells of ATCC 17978 or ATCC 19606^T resulted in minimal clinical scores and no animal death (data not shown).

Lung bacterial burden in these infections was assessed on days 2 and 3, at the height of illness. The lung CFU/g values for the five infecting strains were compared using a Kruskal-Wallis test followed by a Dunn's multiple comparison test. On day 2, the lungs of AB5256-infected mice had significantly less *A. baumannii* than AB4857-, AB0057-, and AB5075-infected lungs ($P \leq 0.05$) but not AB5711-infected lungs. On day 3, AB5256 displayed a significant decrease in CFU/g compared only to AB5075 (Fig. 4). The four other strains assessed in the model had no significant difference in lung bacterial burden, with the median log CFU/g ranging from 8.5 to 9.3 on day 2 and 8.0 to 8.9 on day 3.

Development of genetic tools in an *A. baumannii* model strain. Our success in establishing AB5075 infections in multiple animal models suggests that the isolate would be an attractive model strain for studying *A. baumannii* virulence. However, in addition to an ideal model strain being highly virulent in animal models, the ability to genetically manipulate the isolate is vital for the study of *A. baumannii* pathogenicity. Because antibiotic resistance determinants are central to many types of genetic manipulations, such as transposon mutagenesis, the antibiotic sensitivity profile of AB5075 was examined in detail. It was observed that AB5075 is susceptible to tetracycline, doxycycline, and related antibiotics (see Table S1 in the supplemental material) and to high levels of erythromycin and hygromycin.

With these known susceptibilities in mind, a method previously developed in *A. baumannii* (30) was adapted for creating AB5075 isogenic mutants by utilizing the *hph* gene, encoding hygromycin resistance, from pMQ300 (31) and the EZ Tn*5* (Epicentre Biotechnologies, Madison, WI) to develop a Tn*5*-based mutagenesis system. This system was used to generate a library of ~6,700 transposon mutants. DNA sequencing of the library was

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TABLE 1 *A. baumannii* strains used in this study[a]

Strain	MRSN	Isolation site	Yr isolated	Clonal complex[b]	Source
AB001	1332	ND	ND	ND	C. Murray
AB002	1333	ND	ND	ND	C. Murray
AB0057	1311	Blood/sepsis	2004	I	This study
AB967	1308	Blood/sepsis	2003	III	This study
AB2828	846	Blood/sepsis	2006	III	This study
AB3340	847	Blood/sepsis	2006	I	This study
AB3560	848	Blood/sepsis	2006	III	This study
AB3638	849	Posterior wound	2007	III	This study
AB3785	853	Blood/sepsis	2007	II	This study
AB3806	854	Leg wound	2007	III	This study
AB3917	1309	Blood/sepsis	2007	ND	This study
AB3927	856	Tibia/osteomyelitis	2007	I	This study
AB4025	858	Femur/osteomyelitis	2007	II	This study
AB4026	859	Fibula/osteomyelitis	2007	II	This study
AB4027	860	Femur/osteomyelitis	2007	II	This study
AB4052	863	War wound	2007	II	This study
AB4269	877	War wound	2007	II	This study
AB4448	899	War wound	2007	I	This study
AB4456	903	Tracheal aspirate	2007	III	This study
AB4490	906	War wound	2008	I	This study
AB4498	907	Blood	2008	II	This study
AB4795	930	Bone/osteomyelitis	2008	II	This study
AB4857	939	Ischial/osteomyelitis	2008	III	This study
AB4878	941	War wound	2008	II	This study
AB4932	949	Sputum	2008	II	This study
AB4957	951	Sacral/osteomyelitis	2008	II	This study
AB4991	953	War wound	2008	I	This study
AB5001	954	Blood/sepsis	2008	II	This study
AB5075	959	Tibia/osteomyelitis	2008	I	This study
AB5197	960	STS/tissue	2008	I	This study
AB5256	961	Blood/sepsis	2009	NA	This study
AB5674	963	Blood/sepsis	2009	I	This study
AB5711	1310	Blood/sepsis	2009	II	This study
ATCC 19606[T]	NA	Urine	1948	ND	ATCC
ATCC 17978	NA	Spinal meningitis	1951	ND	ATCC
RUH134	NA	Urine	1982	II	L. Dijkshoorn
RUH875	NA	Urine	1984	I	L. Dijkshoorn
RUH5875	NA	Unknown, Netherlands	1997	III	L. Dijkshoorn
ACICU	NA	Outbreak isolate, Rome, Italy	2005	II	M. Tolmasky

[a] MRSN, The Multidrug-resistant Organism Repository and Surveillance Network; ND, no data; NA, not applicable; STS, sterile swab site (most likely from an infected wound).
[b] As determined by multiplex assay performed in this study. AB5256 was considered NA because only the OXA-51 amplicon was amplified from group 1 primer set (31).

performed as previously described (32), yielding 2,548 unique transposon insertions and 68.5% coverage of the genome.

As a further means of modifying the genome, the same hygromycin cassette was inserted into the pUC18T-mini-Tn*7*T-Zeo vector, and this vector was introduced via conjugation into AB5075. Tn*7* insertion into the chromosome was selected for by growth on 250 μg/ml hygromycin and confirmed by PCR across the *att*Tn*7* site on the 3′ end of the *glmS* gene in the AB5075 chromosome. As proof of concept for the use of Tn*7* for gene insertion in the chromosome, the *lux* operon was inserted into the *att*Tn*7* site. This resulted in bioluminescence of this strain, and subsequent subculturing of AB5075::Tn*7*-*lux* over 7 days without antibiotic selection did not affect the bioluminescent signal (see Fig. S1A in the supplemental material), suggesting that the Tn*7* insertion in the chromosome is stable. Additionally, when this strain was cultured in LB broth, there was no growth defect compared to the wild-type isolate AB5075 (see Fig. S1B in the supplemental material). These methods provide us with a means of interrupting and inserting genes on the chromosome, both of which are essential in studying bacterial pathogenesis.

Evaluation of rifampin as proof of concept. As a proof of concept for the use of AB5075 as a model strain for assessing novel antimicrobials, rifampin treatment against AB5075 was tested in the *G. mellonella* and murine pulmonary models. In *G. mellonella*, 30 min after worms were inoculated with 6.0×10^5 CFU of AB5075, they received a treatment injection of 5 or 10 mg/kg rifampin. By 41 h postinfection, all worms infected with AB5075 and receiving PBS treatment had died (Fig. 5). Conversely, worms treated with 5 or 10 mg/kg rifampin had survival rates of 94 and 100%, respectively, at this time point. At the end of the 4-day study, survival rates were 50 and 78%, respectively. The Mantel-Cox test determined that the time to death for AB5075-infected worms was statistically reduced compared to that for rifampin-treated worms ($P < 0.001$).

To further assess AB5075 as a model strain, the mouse lung model was used to examine the efficacy of rifampin. Mice infected intranasally with 5.0×10^6 CFU of AB5075 were treated once daily intraperitoneally (IP) with 2.5, 5, or 10 mg/kg rifampin, and survival was monitored for 48 h. At time of death, or euthanasia at 48 h postinfection, lungs were collected to determine bacterial

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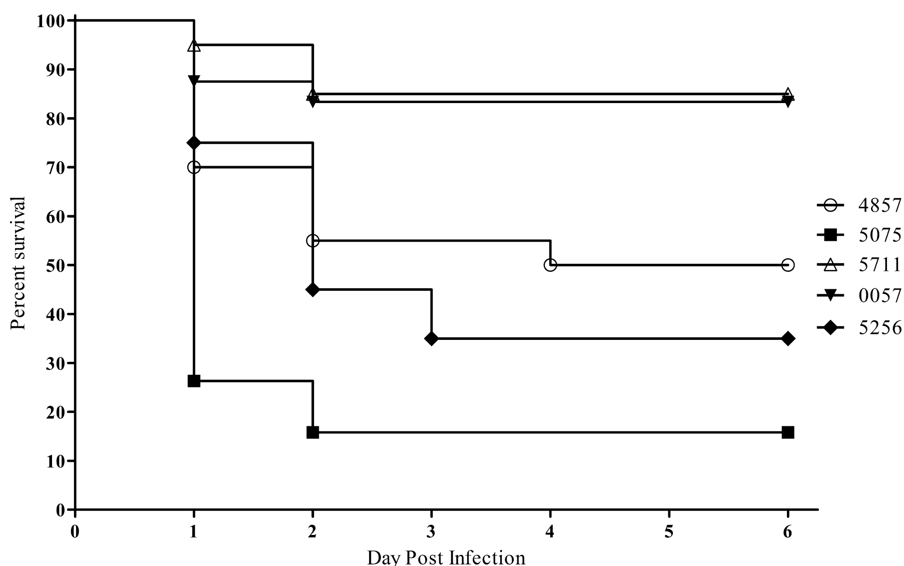

FIG 2 Survival of *Galleria mellonella* larvae infected with *A. baumannii*. Kaplan-Meier survival curves of *G. mellonella* infected with 1.0×10^5 CFU of selected strains of *A. baumannii* are shown. Curves were compared via the Mantel-Cox test with the Bonferroni correction for multiple comparisons. AB5075 showed significantly increased mortality compared to AB4857, AB5711, and AB0057 ($P < 0.0125$).

load. As shown in Fig. 6A, at 48 h after inoculation, only 12.5% of mice infected but not treated with rifampin survived, whereas infected mice receiving 5 and 10 mg/kg rifampin had survival rates of 87.5 and 100%, respectively. These differences in survival were found by the Mantel-Cox test to be statistically significant ($P =$ 0.0035 and 0.0008, respectively) compared with mice infected but not treated with rifampin. Mice treated with 2.5 mg/kg rifampin had a 48-h survival rate of 20%, which was not statistically different from that of the control group.

The levels (CFU/g) of bacteria in lung tissue correlated with the survival curves. The median levels in the control and 2.5 mg/kg-treated groups were almost identical, at 9.04 and 9.07 log CFU/g, respectively. Figure 6B shows a decrease in the median log CFU/g for mice treated with 5 and 10 mg/kg rifampin, with values of 8.10 and 4.86, respectively. The Kruskal-Wallis test, followed by Dunn's multiple comparison test, found a statistically significant difference in bacterial burden when the control group was compared to animals treated with 5 or 10 mg/kg rifampin ($P < 0.05$).

DISCUSSION

The goal of the work presented in this report was to identify a model strain of *A. baumannii* that represented current infection

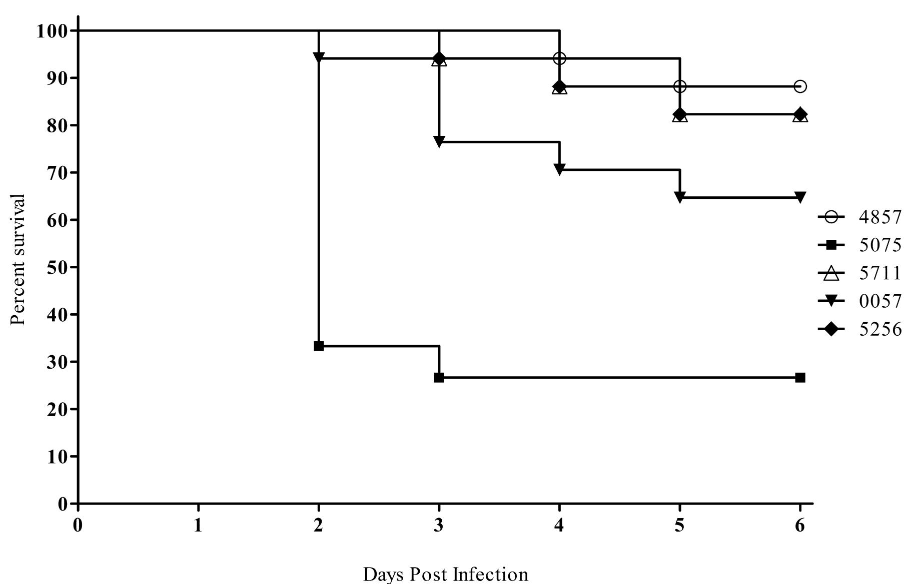

FIG 3 Assessment of *A. baumannii* virulence with the mouse pulmonary model. Kaplan-Meier survival curves of mice infected with 5.0×10^6 CFU of selected *A. baumannii* strains. Curves were compared via the Mantel-Cox test with the Bonferroni correction for multiple comparisons. AB5075 showed significantly increased mortality compared to AB4857, AB5711, AB0057, and AB5256 ($P < 0.0125$).

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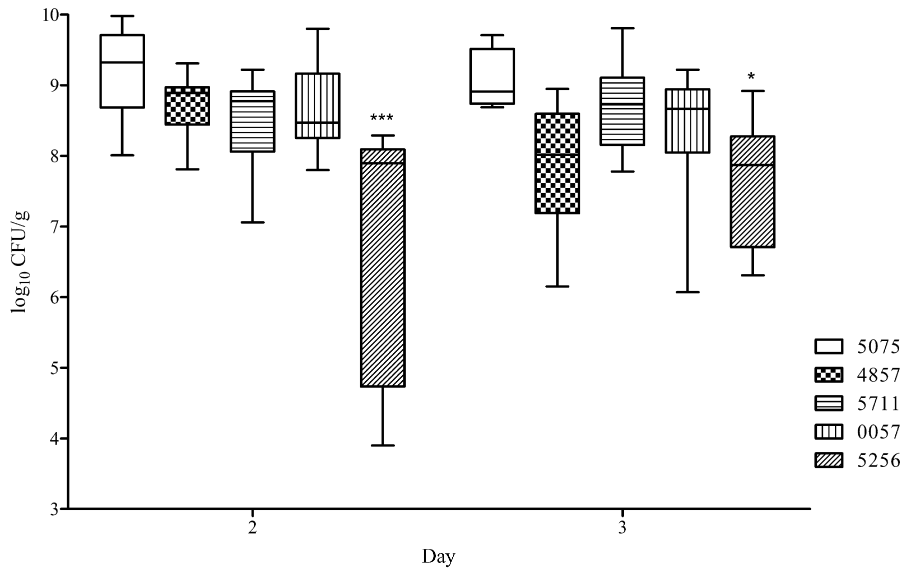

FIG 4 Bacterial levels in lung tissue in the mouse pulmonary model. Box-and-whisker plots of CFU burdens in lungs are shown for days 2 and 3 postinfection. Boxes show median and interquartile ranges, while whiskers represent the 95% confidence interval CI. Strains were compared via the Kruskal-Wallis test followed by Dunn's multiple comparisons posttests. * and ***, $P < 0.05$ and 0.001, respectively.

isolates, was highly virulent in multiple animal models, and was amenable to genetic manipulation. A strain fitting these characteristics would be clinically relevant and could be utilized in various models to study novel therapeutics, and the ability to create isogenic mutants would be critical in investigating genes required for virulence and the pathogenesis of severe infections in the human host. In our comprehensive studies of isolates representative of each major clonal complex group, we identified one such strain, AB5075.

Previous studies compared the virulence of *A. baumannii* strains in single infection models, including a murine pulmonary model (13, 14) and a *G. mellonella* model (33). Other work also compared the virulence of a single strain, ATCC 19606^{T}, in multiple models *in vivo* (34); however, to our knowledge, this is the first study that used several infection models in parallel to compare multiple strains using representative isolates from each clonal group. Importantly, when we compared infections caused by other *A. baumannii* strains, including the widely used ATCC

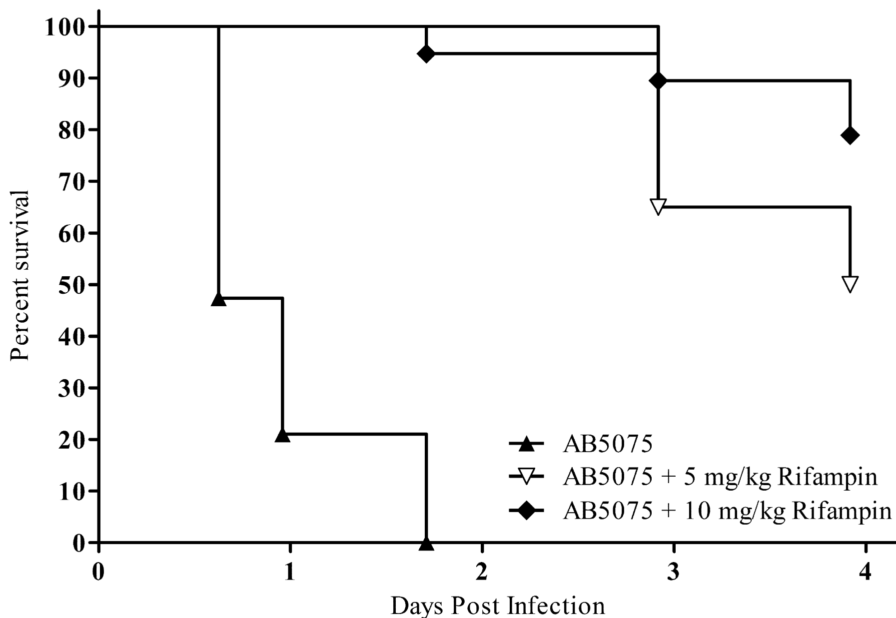

FIG 5 Rifampin as proof of concept in the *G. mellonella* model. Kaplan-Meier survival curves of *G. mellonella* infected with 6.0×10^5 CFU of AB5075 are shown. Worms received a single treatment, 30 min postinfection, of DMSO, 5 mg/kg rifampin, or 10 mg/kg rifampin. Curves were compared via the Mantel-Cox test. The control-treated worms showed significantly increased mortality compared to rifampin-treated worms ($P < 0.001$).

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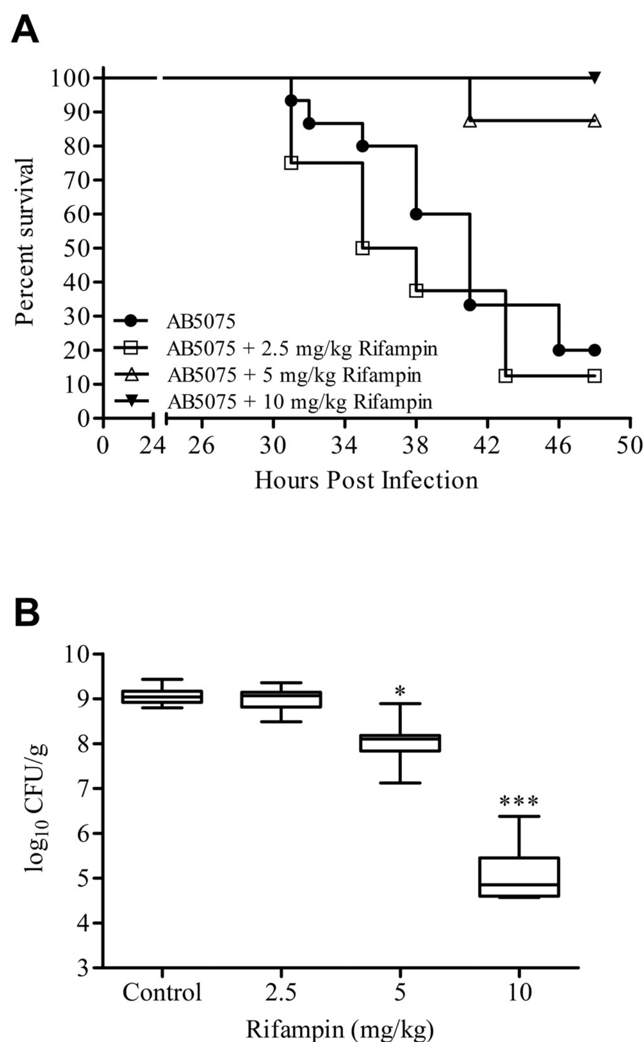

FIG 6 Rifampin as proof of concept in the mouse lung model. (A) Kaplan-Meier survival curves of mice infected with 5.0×10^6 CFU of selected strains of *A. baumannii* are shown. Mice were treated once daily IP with 0, 2.5, 5, or 10 mg/kg rifampin. Curves were compared via the Mantel-Cox test. Rifampin treatments of 5 and 10 mg/kg resulted in significantly increased survival compared to the untreated control ($P = 0.0035$ and 0.0008, respectively). (B) Box-and-whisker plots of CFU burdens within lungs are shown for day 2 postinfection. Boxes show median and interquartile ranges, while whiskers represent 95% CI. Treatments were compared via the Kruskal-Wallis test followed by Dunn's multiple comparisons posttests. * and ***, $P < 0.05$ and 0.001, respectively.

19606^T and ATCC 17978, we did not observe the same severe infection as was consistently observed with AB5075.

In the *G. mellonella* and mouse pneumonia models, AB5075 infection resulted in survival rates consistently below 25%, providing a wide spectrum between infected and uninfected animals to assess novel therapies or genetically mutated strains. Additionally, in the mouse pulmonary model, blood samples and lung histopathology from AB5075-infected mice were consistently positive for the presence of *A. baumannii* (data not shown) and, combined with the high lung bacterial burden observed, offer further means of evaluation in this model. Furthermore, while a recent publication claimed that *A. baumannii* could not cause osteomyelitis in a rat fracture model (35), our data showed that AB5075 could establish an infection in this model and that *A. baumannii* could still be cultured from the bone after 28 days postinfection (see the supplemental material). Recently, we also successfully developed a murine wound model of infection with AB5075, using a small inoculating dose (36). Therefore, the use of AB5075 in additional animal models further demonstrates the great utility of AB5075, as it can serve as a model strain for a variety of studies. The increased virulence of AB5075 in these models also results in larger differences between infected and uninfected animals compared to other tested strains, which allows for less ambiguous results, high statistical power, and thus a requirement for fewer animals to obtain publishable data.

In addition to the successful use of AB5075 in animal models, we were able to exploit the susceptibility of AB5075 to hygromycin to generate a Tn*5* transposon insertion library and use a Tn*7* transposon derivative to insert genes into the genome of this strain. In collaboration with the Manoil laboratory at the University of Washington, we were able to sequence the Tn*5* transposon library using a convenient high-throughput sequencing method. Further, Manoil and his team illustrated the genetic utility of AB5075 by generating a Tn*5* insertion library utilizing a tetracycline-based transposon system (C. Manoil, personal communication). It is expected that these sequenced transposon insertion libraries will be powerful tools for future investigations, as similar libraries have been utilized with success with other bacterial pathogens (37, 38). In the future, the Manoil laboratory will distribute wild-type and mutant derivatives of AB5075 (http://www.gs.washington.edu/labs/manoil/baumannii.htm).

The ability to interrupt or mutate specific genes via transposon insertion and complement mutations via Tn*7*-mediated chromosomal insertion provides a powerful toolkit to answer important questions, such as the nature of specific genes and gene products that are critical for virulence in the host. We believe that AB5075, and the genetic tools and animal models we have designed around this strain, will serve as a platform to readily and reproducibly test mutants in putative virulence factor genes and assess novel antimicrobials. Moreover, we encourage other research groups to use AB5075 as a model strain and to take advantage of the tools we are developing to test their own hypotheses about virulence determinants of *A. baumannii*.

MATERIALS AND METHODS

Bacterial strains, growth media, and clinical microbiology. All work was carried out under biosafety level II or II+ conditions. All the *A. baumannii* strains used in this study can be found in Table 1. Routine growth and strain maintenance was carried out in Luria-Bertani Lennox (LB) broth and agar. Bacterial identification, antibiograms, and MIC were determined using the Vitek 2 (bioMérieux, France) and Phoenix (Becton, Dickinson and Co., Franklin Lakes, NJ) automated systems according to the manufacturer's instructions. Rifampin was obtained from Sigma-Aldrich (St. Louis, MO), prepared in dimethyl sulfoxide (DMSO), and then further diluted in sterile saline.

Pulsed-field gel electrophoresis. The pellet from an overnight broth culture was resuspended in 2 ml 10 mM Tris-HCl, 10 mM EDTA (pH 8.0) to a density equivalent to 0.5 McFarland. Suspensions were mixed with equal volumes of melted 1.6% SeaKem Gold agarose (Lonza, Walkersville, MD), dispensed into wells of a plug mold, and allowed to solidify. The plugs were incubated for 2 h in 20 mg/ml proteinase K and cell lysis buffer (50 mM Tris-HCl, 50 mM EDTA [pH 8.0], 1% *N*-lauroylsarcosine, sodium salt) at 54°C. Subsequently, the plugs were washed four times in TE buffer (10 mM Tris-HCl, 10 mM EDTA [pH 8.0]) and then incubated in a digestion buffer consisting of 50 U of ApaI restriction enzyme, NEBuffer

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4, and bovine serum albumin (New England BioLabs, Ipswich, MA) at 25°C for at least 2 h. Electrophoresis was performed at a constant voltage of 200 V by the CHEF-DR II system (Bio-Rad Laboratories, Hercules, CA) with pulse times ramping from 7 to 20 s for 18.5 h. Gels were stained with ethidium bromide and photographed under UV light. PFGE clustering was determined by using the unweighted-pair group method with arithmetic averages (UPGMA) and Dice's coefficient (BioNumerics version 6.0, created by Applied Maths NV).

Multiplex PCR assay. Multiplex PCR methods were followed directly from the work of Turton et al. (24), with modified reaction volume and reagents. Briefly, *A. baumannii* templates were prepared from a single colony grown on Luria-Bertani (LB) agar and resuspended in Lyse-N-Go PCR reagent according to the manufacturer's instructions (Thermo Scientific, Rockford, IL). PCRs were prepared in 20-μl volume using DreamTaq master mix (Thermo Scientific).

Genome sequencing and bioinformatic analysis. Genome analysis was performed as previously described (17), with AB5075, AB5711, AB4857, and AB5256 included in the bioinformatic comparisons. Unique genes were determined using the BLAST score ratio analysis (26). BLAST score ratio (BSR) is an *in silico* approach to conduct comparative proteomic analyses based on proteins predicted to be encoded in a genome (26). BSR was used to compare the proteins encoded in the newly sequenced strains with three isolates from the University of Maryland (17) and eight previously sequenced reference isolates (SDF, AYE, ATCC 17978, ADP1, AB0057, ATCC 19606^T, ACICU, and AB307). The BSRs were calculated as the ratio of raw BLASTP score for the query to the raw BLASTP score of the reference strain. BSR cutoffs of $\geq$0.8, $<$0.8 to $>$0.4, and $<$0.4 were used to determine whether a gene is conserved, divergent, or unique, respectively. A BSR value of 0.8 corresponds to ~85 to 90% identity over 90% of the length of a protein sequence, indicating a highly conserved sequence, while a BSR value of 0.4 corresponds to 30% identity over 30% of the length of a protein sequence, indicating a unique sequence (26).

***Galleria mellonella* infection model.** *A. baumannii* strains were grown overnight in an orbital shaker (37°C, 200 rpm), and overnight cultures were then diluted 100-fold into fresh medium and grown for 3 h. Cells were collected by centrifugation (5 min, 5,000 $\times$ *g*), washed once in phosphate-buffered saline solution (PBS), and resuspended in PBS to a final OD_{600} of 1.0. Further dilutions were done in PBS. The number of bacterial cells in the injected sample was enumerated by plating 10-fold serial dilutions on LB agar plates and counting CFU after overnight incubation.

G. mellonella larvae (Vanderhorst Wholesale, Saint Marys, OH) were used within 10 days of shipment from the vendor. Larvae were kept in the dark at 21°C before infection. Larvae weighing 200 to 300 mg were used in the LD_{50} and survival assays as described previously (28), with slight modifications. Briefly, 5 μl of the sample was injected into the last left proleg of the larvae using a 10-μl glass syringe (Hamilton, Reno, NV) fitted with a 30G needle (Novo Nordisk, Princeton, NJ). Each experiment included control groups of noninjected larvae or larvae injected with 5 μl sterile PBS. For rifampin experiments, approximately 30 min postinfection, worms were injected with 2 μl of rifampin in the second-to-last left proleg using a 10-μl glass syringe. Injected larvae were incubated at 37°C, assessing death at 24 h intervals over 6 days. Larvae were considered dead if they did not respond to physical stimuli. Experiments in which 10% or more of the larvae in either of the control groups died were omitted from the statistical analysis. Experiments were repeated three times using 10 or 20 larvae per experimental group.

The LD_{50} for *A. baumannii* strains AB5075, ATCC 19606^T, and ATCC 17978 were determined by preparing a series of 2-fold dilutions of a PBS suspension of the bacterial strain, starting with a bacterial concentration that caused death of all the larvae within 24 h and going down to a concentration at which no deaths were recorded within this time frame. Twenty larvae were injected with 5 μl of the appropriate dilution, and larvae were determined to be alive or dead after 24 h. Two independent biological repeats of LD_{50} determination were performed. LD_{50} were determined using the Spearman-Karber method.

Murine pulmonary model. The animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the Walter Reed Army Institute of Research (IB02-10). All research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in reference 39. Six-week-old female BALB/c mice (National Cancer Institute, Frederick, MD) were housed at 3 to 4 animals per cage and allowed access to food and water *ad libitum* throughout the experiment. The pulmonary infection model was adapted from reference 14. Briefly, to promote infection, mice were rendered neutropenic via intraperitoneal administration of 150 mg/kg and 100 mg/kg cyclophosphamide in sterile saline on day -4 and day -1 prior to infection (day 0), respectively.

A. baumannii isolates AB4857, AB5075, AB5711, AB0057, and AB5256 were grown overnight in LB broth with aeration at 37°C, subcultured to mid-exponential phase, washed, and resuspended in PBS with optical density at 600 nm (OD_{600}) values corresponding to 2×10^8 CFU/ml. For infection, mice were anesthetized with oxygenated isoflurane immediately prior to intranasal inoculation with 25 μl of bacterial cultures, corresponding to 5×10^6 CFU. For rifampin experiments, mice were injected IP daily, starting at 4 h postinfection. Animal morbidity was scored twice daily for 6 days using a system evaluating mobility, coat condition, and conjunctivitis as previously described (14). As mice became exceedingly moribund based on clinical score, they were humanely euthanized according to protocol.

To assess CFU burden in the lungs, mice were humanely euthanized according to protocol on days 2 and 3 postinfection via an injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). To quantify the pulmonary CFU burden, lungs were homogenized in 1 ml PBS, and serial dilutions were plated using the Autoplate spiral plating system (Advanced Instruments, Norwood, MA) onto LB agar supplemented with 50 μg/ml carbenicillin. Bacterial load was reported as CFU per gram of lung tissue.

Transposon library generation. Transposon mutants were constructed using the EZ-Tn*5* transposon construction vector pMOD-5<R6Kγ*ori*/MCS> (Epicentre, Madison, WI). The transposable element was created by PCR amplifying *hph*, encoding hygromycin resistance, from vector pMQ300 (31) using the primers pMODHygForKPN (AAAAAAGGTACCggaaatgtgcgcggaacccc) and pMODHygRevPST (AAAAAACTGCAGttggtctgacaatcgatgcgaattgg). The amplicon was then cloned into the multicloning site (MCS) of pMOD-5<R6Kγ*ori*/MCS>. The transposome was constructed according to manufacturer's instructions and introduced into cells via electroporation. The transformed cells were selected for on LB agar supplemented with 250 μg/ml hygromycin. Colonies were picked from plates and grown overnight in 96-well plates containing 100 μl of low-salt LB supplemented with 250 μg/ml hygromycin. After overnight incubation, 100 μl of 50% glycerol was added to each well, and plates were immediately moved to -80°C for storage. The Tn*5* mutant library was subjected to high-throughput sequencing as previously described (32).

Construction of hygromycin mini-Tn*7* vector and insertion onto the chromosome. To make Tn*7*-based genetic tools usable in AB5075, the *hph* gene, coding for hygromycin resistance, was cloned into pUC18T-mini-Tn*7*T-Zeo (40). Briefly, *hph* was amplified from pMQ300 (31) using primers pMOD Hyg For (AAAGCATGCggaaatgtgcgcggaacccc) and pMOD Hyg Rev (AAAGCATGCttggtctgacaatcgatgcgaattgg) (lowercase letters represent the actual primer; capital letters are the restriction site for each primer and a poly-A overhang) and ligated into pUC18T-mini-Tn*7*T-Zeo, which was digested with NcoI and then blunted with the Klenow fragment of the DNA polymerase I (Fermentas). A derivative of the pUC18T-mini-Tn*7*T-*hph* vector containing the *lux* operon was constructed by amplifying the *luxABCDE* operon out of pUC18T-mini-Tn*7*T-Gm-*lux* (40) with the primers Lux For (TCAAGGTTCTGGACCAGTTG) and Lux Rev (AAAAAAAAGCTTGGTGTAGCGTCGTAAGCTA

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ATA). The PCR product was digested with BamHI and HindIII and then cloned into the MCS of pUC18T-mini-Tn*7*T-*hph*.

The mini-Tn*7* elements were transposed into the *att*Tn*7* site of AB5075 via the method of Kumar et al. (41). Conjugation mixtures were scraped from LB plates, resuspended in 1 ml of PBS, and plated on LB agar supplemented with 250 μg/ml of hygromycin and 25 μg/ml of chloramphenicol. Insertion into the *att*Tn*7* site was confirmed with the primers AB5075 attTn*7* FWD (AACACAAGTGGAAGTGATTTCT) and AB5075 attTn*7* REV (TGGCTTGCACCAATCATTTATAG), which flanked the *att*Tn*7* site.

Statistical analyses. All statistical analyses were carried out using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA). Survival curves were compared via Kaplan-Meier curve analysis with the Bonferroni correction for multiple comparisons. Recovered bacterial burdens were compared via either the Mann-Whitney U test or the Kruskal-Wallis test followed by Dunn's multiple-comparison test. All results were considered significant at a P value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01076-14/-/DCSupplemental.

Figure S1A, TIF file, 0.6 MB.
Figure S1B, TIF file, 2.2 MB.
Figure S2, TIF file, 0.5 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Text S1, DOCX file, 0.1 MB.

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The findings and opinions expressed herein belong to the authors and do not necessarily reflect the official views of the WRAIR, the U.S. Army, or the Department of Defense.

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Appendix 2

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Draft Genome Sequences of *Klebsiella pneumoniae* Clinical Type Strain ATCC 13883 and Three Multidrug-Resistant Clinical Isolates

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***Klebsiella pneumoniae* is a Gram-negative human pathogen capable of causing hospital-acquired infections with an increasing risk to human health. The total DNA from four clinically relevant strains was sequenced to >100× coverage, providing high-quality genome assemblies for *K. pneumoniae* strains ATCC 13883, KP4640, 101488, and 101712.**

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Klebsiella pneumoniae is the fourth most common cause of Gram-negative-associated hospital-acquired infections, including urinary tract infections, pneumonia, septicemia, and wound infections (1, 2). The nosocomial prevalence of *K. pneumoniae* is exacerbated by the emergence of multidrug-resistant strains, especially those producing carbapenemase (KPC-1). This has made *K. pneumoniae* a threat to human health worldwide. Many reports have explored the multidrug resistance and capsular properties of *K. pneumoniae*; however, there remains a paucity of literature regarding the elucidation of virulence factors and the general physiology of this pathogen. The dearth of information is highlighted by the absence of the genome sequence of the *K. pneumoniae* clinical type strain ATCC 13883 from publicly available databases. To this end, the genome sequence of strain ATCC 13883, as well as those of *K. pneumoniae* strains KP4640, 101712, and 101488, three strains isolated from wounded warriors at the Walter Reed Army Medical Center (WRAMC) and San Antonio Military Medical Center (SAMMC), Fort Sam Houston, TX, were determined using next-generation sequencing methods.

The strains were routinely stored at -80°C in 10% glycerol. DNA was isolated from overnight LB cultures grown with agitation at 37°C using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). The absorption at 260 nm and 280 nm was measured for each sample to determine quantity and quality using the NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The DNA concentrations for library preparation were determined by the SYBR green (Life Technologies, Grand Island, NY) standard curve method in a black 96-well plate (Corning, Tewksbury, MA, USA) using a FilterMax F5 spectrophotometer with Multimode Analysis software version 3.4.0.25 (Molecular Devices, Sunnyvale, CA, USA). The Nextera XT kit (Illumina, San Diego, CA, USA) was used to simultaneously fragment and adapter tag the libraries, as per the manufacturer's instructions. Library production was visualized with a Bioanalyzer 2100 high-sensitivity DNA analysis kit (Agilent Technologies, Santa Clara, CA) using the version B.02.08.SI648 software to analyze the fragmentation of the resultant libraries. Individual libraries were normalized by bead-based affinity, pooled, and then sequenced using the MiSeq v3 600-cycle kit (Illumina, San Diego, CA, USA) to perform 300-bp paired-end sequencing on a MiSeq instrument (Illumina), per the manufacturer's instructions. *De novo* assembly was performed using Genomics Workbench 7.5 with the Bacterial Genome Finishing Module (CLC bio, Boston, MA), run on a workstation with an AMD Opteron 2.10 GHz 16-core processor with 128 GB DDR3 ECC random access memory (RAM). The genomes were annotated with Prokka version 1.10 on a quadcore i7 workstation with 32 GB DDR3 running Ubuntu 14.04 long-term support (LTS) (3).

The *de novo* assembly resulted in a 5,725,870-bp genome containing 68 tRNAs and 5,525 genes with 5,456 proposed coding sequences (CDS) for the clinical type strain ATCC 13883. The remaining three genomes were 5,590,832, 5,570,720, and 5,575,268 bp for strains KP4640, 101488, and 101712, respectively. The strain KP4640 genome contains 71 tRNAs and 5,270 genes with 5,198 CDS. Strains 101488 and 101712 have 75 and 73 tRNAs, 5,375 and 5,208 genes, and 5,299 and 5,134 CDS, respectively.

Nucleotide sequence accession numbers. The whole-genome shotgun projects were deposited into GenBank under Bioproject ID PRJNA261239 with accession numbers JSZI00000000 (ATCC 13883), JSZJ00000000 (101712), JSZK00000000 (101488), and JSZL00000000 (KP4640). The versions described in this paper are versions JSZI01000000 (ATCC 13883), JSZJ01000000 (101712), JSZK01000000 (101488), and JSZL01000000 (KP4640).

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The findings and opinions expressed herein belong to the authors and do not necessarily reflect the official views of the WRAIR, the U.S. Army, or the Department of Defense.

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Appendix 3

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Draft Genome of the Multidrug-Resistant *Acinetobacter baumannii* Strain A155 Clinical Isolate

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***Acinetobacter baumannii* is a bacterial pathogen with serious implications on human health, due to increasing reports of multidrug-resistant strains isolated from patients. Total DNA from the multidrug-resistant *A. baumannii* strain A155 clinical isolate was sequenced to greater than 65× coverage, providing high-quality contig assemblies.**

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Acinetobacter baumannii, a Gram-negative pathogen, causes a variety of nosocomial infections such as bacteremia, meningitis, skin and soft tissue infections such as necrotizing fasciitis, ventilator-associated pneumonia, and urinary tract infections (1, 2). These infections are becoming harder to treat due to the rise in the number of multidrug-resistant *A. baumannii* strains present in clinical settings (1, 3). *A. baumannii* strain A155 is a multidrug-resistant strain originally isolated from a urinary sample at a hospital in Buenos Aires, Argentina, in 1994 (4). At that time, unlike in most of the world where clonal complex 109 (CC109) and CC92 (also known as international clonal lineage 1 and 2, respectively) were predominant, most strains isolated in Argentina belonged to CC113 (5). *A. baumannii* A155 was among the first CC109 isolates in Argentina (4). This strain includes the AbaR-type island inserted within *comM*, and the *aac(6′)-Ib* gene, which confers resistance to numerous aminoglycosides (4, 6, 7).

A. baumannii A155 whole-genome sequencing and annotation were performed as described previously (8). Briefly, the A155 isolate was routinely stored at −80°C in 10% glycerol, passaged to overnight LB cultures grown with agitation at 37°C, and total DNA was isolated using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA). DNA quantity and quality were assessed using Nanodrop 2000 (Thermo Scientific, Wilmington, DE, USA). The SYBR Green (Life Technologies, Grand Island, NY, USA) standard curve method was used to estimate DNA concentration for library preparation in a black 96-well plate (Corning, Tewksbury, MA, USA), and fluorescence values were obtained using a FilterMax F5 spectrophotometer with Multi-Mode Analysis software version 3.4.0.25 (Molecular Devices, Sunnyvale, CA, USA). The Nextera XT kit (Illumina, Inc., San Diego, CA, USA) was used to simultaneously fragment and construct adapter-tagged libraries per the manufacturer's instructions. The Bioanalyzer 2100 High Sensitivity DNA analysis kit (Agilent Technologies, Santa Clara, CA, USA) with version B.02.08.SI648 software was used to determine the fragmentation of the resultant libraries. Individual libraries were normalized by bead-based affinity, pooled, and then sequenced using the MiSeq version 3 600-cycle kit (Illumina) to perform 300-bp paired-end sequencing on a MiSeq instrument (Illumina) per the manufacturer's instructions. *De novo* assembly was performed using Genomics Workbench version 7.5 with the Bacterial Genome Finishing module (CLC bio, Boston, MA, USA) on a workstation with an AMD Opteron 2.10-GHz 16-core processor and 128-GB DDR3 ECC RAM. Genomes were annotated with Prokka version 1.10 on a quadcore i7 workstation with 32-GB DDR3 running Ubuntu 14.04 LTS (9).

The *de novo* assembly resulted in a 3,933,455-bp genome encoding 55 tRNAs and 3,760 genes with 3,704 proposed CDSs for the *A. baumannii* A155 clinical strain.

Nucleotide sequence accession numbers. The first version of the *de novo* whole-genome assembly of *A. baumannii* A155 was deposited into GenBank under Bioproject ID PRJNA261239 with the accession number JXSV00000000, version JXSV01000000.

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