

Joint Transcriptional Control of Virulence and Resistance to Antibiotic and Environmental Stress in *Acinetobacter baumannii*

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ABSTRACT The increasing emergence of antibiotic-resistant bacterial pathogens represents a serious risk to human health and the entire health care system. Many currently circulating strains of *Acinetobacter baumannii* exhibit resistance to multiple antibiotics. A key limitation in combating *A. baumannii* is that our understanding of the molecular mechanisms underlying the pathogenesis of *A. baumannii* is lacking. To identify potential virulence determinants of a contemporary multidrug-resistant isolate of *A. baumannii*, we used transposon insertion sequencing (TnSeq) of strain AB5075. A collection of 250,000 *A. baumannii* transposon mutants was analyzed for growth within *Galleria mellonella* larvae, an insect-based infection model. The screen identified 300 genes that were specifically required for survival and/or growth of *A. baumannii* inside *G. mellonella* larvae. These genes encompass both known, established virulence factors and several novel genes. Among these were more than 30 transcription factors required for growth in *G. mellonella*. A subset of the transcription factors was also found to be required for resistance to antibiotics and environmental stress. This work thus establishes a novel connection between virulence and resistance to both antibiotics and environmental stress in *A. baumannii*.

IMPORTANCE *Acinetobacter baumannii* is rapidly emerging as a significant human pathogen, largely because of disinfectant and antibiotic resistance, causing lethal infection in fragile hosts. Despite the increasing prevalence of infections with multidrug-resistant *A. baumannii* strains, little is known regarding not only the molecular mechanisms that allow *A. baumannii* to resist environmental stresses (i.e., antibiotics and disinfectants) but also how these pathogens survive within an infected host to cause disease. We employed a large-scale genetic screen to identify genes required for *A. baumannii* to survive and grow in an insect disease model. While we identified many known virulence factors harbored by *A. baumannii*, we also discovered many novel genes that likely play key roles in *A. baumannii* survival of exposure to antibiotics and other stress-inducing chemicals. These results suggest that selection for increased resistance to antibiotics and environmental stress may inadvertently select for increased virulence in *A. baumannii*.

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Acinetobacter baumannii is rapidly emerging as a significant human pathogen (1). Current estimates predict that of the approximately 45,000 *A. baumannii* infections per year in the United States (and roughly one million infections annually worldwide), half (~23,000) are due to strains resistant to carbapenem antibiotics and have a mortality rate approaching 20% (2). Indeed, many of the currently circulating strains of this opportunistic pathogen exhibit a multidrug-resistant (MDR) phenotype, and even panresistant strains have been reported (3). In *A. baumannii*, the MDR phenotype can be attributed to a combination of intrinsic and acquired traits (4). While work to identify and characterize the repertoire of *A. baumannii* virulence determinants has begun, additional studies are required to understand the genetic basis of virulence and to translate how these determinants impact human disease (5).

Bacterial genetics represents an extremely powerful tool for the

identification of virulence factors (6, 7), and as genetic technology has advanced, so too has the scale of genetic screens (8–11). We examined genes required for *A. baumannii* virulence by using strain AB5075, a contemporary MDR isolate that represents currently circulating isolates of *A. baumannii* (12–14). Using transposon insertion sequencing (TnSeq), we identified 300 genes required for survival and/or growth within *Galleria mellonella* larvae, an established insect model system for a variety of human microbial diseases (15). In addition to some known virulence factors, the screen reported here identified many novel genes, including several transcriptional regulators that control both virulence-related genes and those required for antibiotic resistance.

As *A. baumannii* clinical isolates are increasingly resistant to antibiotic therapy (16), we hypothesized that environmental virulence factors may be associated with resistance to antimicrobial compounds. Thus, we assessed the antibiotic sensitivity of a subset

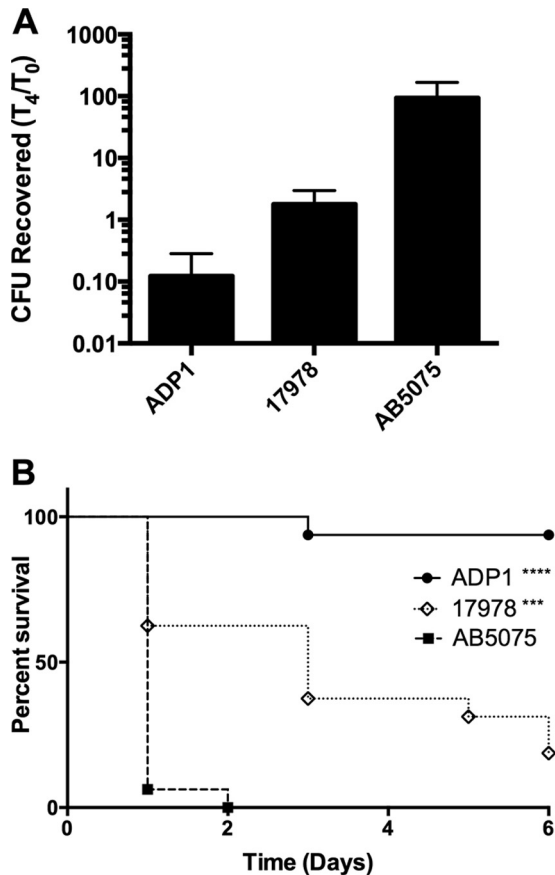


FIG 1 *G. mellonella* differentiates pathogenic and nonpathogenic *Acinetobacter* strains. (A) *G. mellonella* larvae were inoculated with 10^6 CFU of the strains indicated. Larvae were homogenized and bacteria were quantified immediately following infection ($t = 0$) and after 4 h at 37°C ($t = 4$). Data from a representative experiment are presented as the ratio of the number of CFU recovered at $t = 4$ to the number of CFU recovered at $t = 0$ (error bars, 1 standard deviation). (B) *G. mellonella* larvae were inoculated with 10^6 CFU of the strains indicated. Survival was monitored daily for 6 days. ****, $P < 0.0001$; ***, $P < 0.001$. ADP1, *A. baylyi* ADP1; 17978, *A. baumannii* ATCC 17978; AB5075, *A. baumannii* AB5075.

of the mutants. We found that several mutants defective for growth in *G. mellonella* larvae also exhibit increased susceptibility to antibiotics and other environmental stress conditions. These results highlight the existence of links between virulence and resistance to antibiotic and environmental stresses in *A. baumannii* (17–19) and suggest that selection for increased environmental stress resistance may also inadvertently select for strains with enhanced virulence.

RESULTS

TnSeq of *A. baumannii* in *G. mellonella* larvae. In order to screen for virulence determinants in *A. baumannii*, we employed the *G. mellonella* infection model (15, 20, 21). Previous work with several microbial pathogens has demonstrated a positive correlation between the *Galleria* model and mammalian disease models (22–24). Infection of *Galleria* larvae with various *Acinetobacter* strains showed that the model easily distinguishes nonpathogenic from pathogenic *Acinetobacter* spp. For example, Fig. 1 shows that *Acinetobacter baylyi*, a well-characterized, nonpathogenic *Acineto-*

bacter species, does not survive for 4 h within larvae, whereas the human-pathogenic isolates ATCC 17978 and AB5075 survive and grow within the larvae during the same time period. Furthermore, larvae infected with these strains displayed dramatic differences in survival, with strain AB5075 leading to rapid death, *A. baylyi* ADP1 causing minimal larval killing over the course of the experiment, and ATCC 17978 leading to intermediate levels of killing (Fig. 1). The enhanced growth within larvae and the rapid larval killing kinetics are consistent with previous reports that AB5075 is more virulent than the ATCC 17978 strain and supports a correlation between growth in *G. mellonella* larvae with the previously reported virulence of specific *A. baumannii* strains in mammalian models (13) (Fig. 1).

Given that the *G. mellonella* infection model readily differentiates between pathogenic and nonpathogenic *Acinetobacter* spp., as well as between more and less virulent *A. baumannii* strains, we sought to identify the genetic elements of *A. baumannii* strain AB5075 that are required for growth and/or survival within *G. mellonella* larvae. We performed a TnSeq experiment by the TnSeq Circle method with a previously described library of transposon mutants (8, 12). Aliquots of the transposon library were inoculated into either LB or *G. mellonella* larvae, incubated for 4 h at 37°C (see Fig. S1 in the supplemental material), and processed as described in Materials and Methods. To identify mutants that were underrepresented following growth in *G. mellonella*, a read ratio (RR) was determined for each gene in the *G. mellonella* and LB samples. Using a 10-fold reduction (RR of ≤ 0.10) as a significance cutoff, 300 genes were defined as essential for growth of AB5075 within *G. mellonella* larvae (Fig. 2; see Table S1 in the supplemental material). We anticipated that the *G. mellonella* TnSeq screen would identify three main categories of genes, (i) genes required for nutrient acquisition within *G. mellonella*, (ii) genes required to resist the *G. mellonella* immune responses, and (iii) genes performing an as-yet-unknown role during infection.

Nutrient acquisition/metabolic functions. A recently described concept of innate immune defense to bacterial infection is “nutritional immunity” or the ability of the host to restrict access of essential nutrients (25). The TnSeq screen identified several genes involved in acquisition systems for two essential micronutrients, zinc and iron (Table 1). Several components of the acinetobactin siderophore system were found to be required for growth in *G. mellonella* (Table 1) (26, 27), as well as ABUW_2074, a Fur family transcriptional regulator (RR_{Fur} of 0.050). Additionally, mutants with insertions in each of the three structural genes of the *znuABC* zinc uptake system and the transcriptional regulator controlling the expression of these genes (*zur*) exhibited very low RRs (Table 1). Zinc acquisition has been previously demonstrated as an important virulence determinant for *A. baumannii* disease in a murine lung infection model (28). Consistent with previous reports, a *znuB* mutant showed decreased survival after 4 h of infection, confirming the requirement for zinc acquisition in the *Galleria* model (Fig. 3) (28, 29). Recently, transcriptome data obtained from a *zur* mutant of *A. baumannii* was published and comparison of the *zur*-controlled genes with our TnSeq data set identified eight genes that were both differentially expressed in the *zur* mutant and required for growth in *G. mellonella* (29). These genes include *znuA*, a TetR family regulator (ABUW_1692), *otsB* (ABUW_3122), an isochorismatase hydrolase (ABUW_2374), and four genes of unknown function

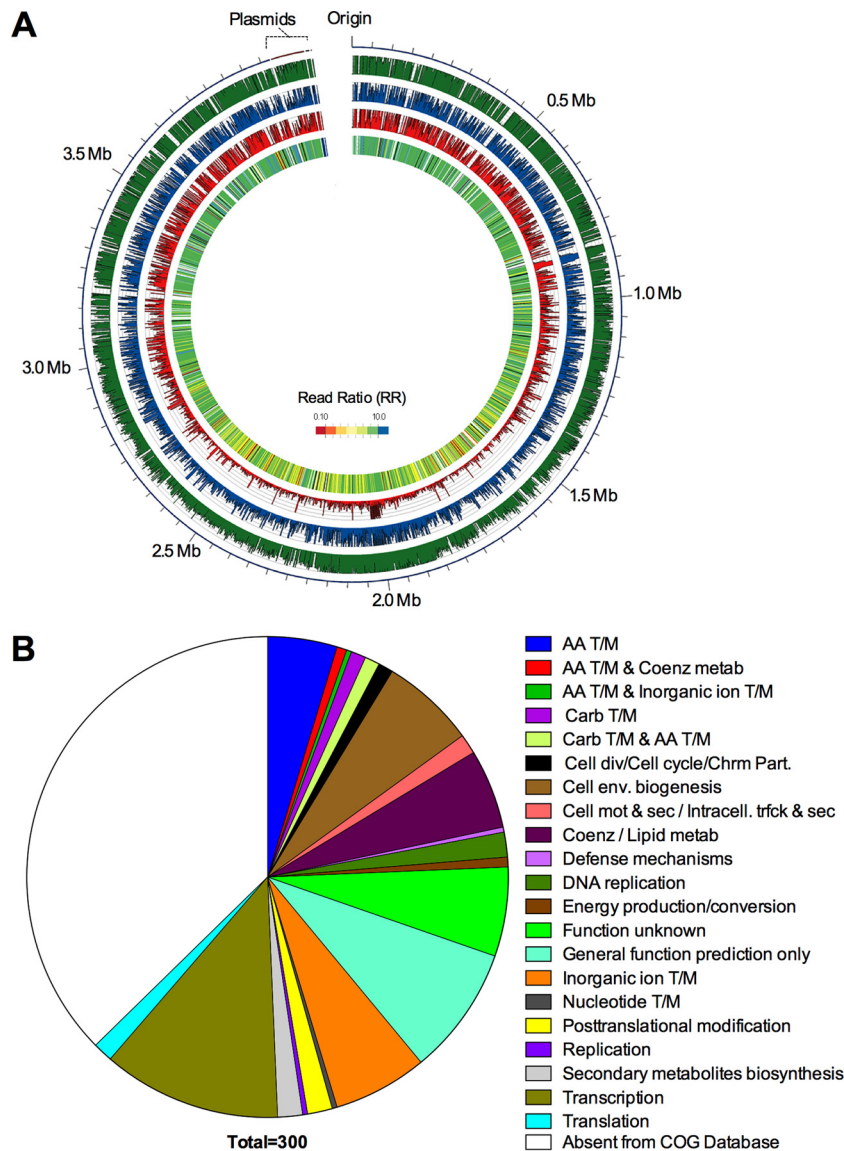


FIG 2 TnSeq experiment. (A) TnSeq experiment data. The outermost ring depicts the AB5075 chromosome and plasmids. The three middle rings depict the numbers of hits per gene in the pregrowth, LB growth, and *Galleria* growth samples (green, blue, and red, respectively). The innermost ring is a heat map of the RR of the Gm to the LB samples (Gm-essential genes are red). Essential genes are white, and genes with a general growth defect are black. The image was created with Circos (61). (B) Pie chart depicting Gm-essential hits grouped by COG categories. AA, amino acid; T/M, transport and metabolism; Coenz, coenzyme; metab, metabolism; Carb, carbohydrate; div, division; Chrm Part., chromosome partitioning; env., envelope; mot & sec, motility and secretion; Intracell. trfck & sec, intracellular trafficking and secretion.

(ABUW_2145, ABUW_2439, ABUW_2442, and ABUW_2679). Identification of these well-known metal ion acquisition systems provides proof of the principle that the *Galleria* model imposes nutrient limitation stresses similar to those in mammalian infection models.

Another pathway required for growth in the larvae is cysteine metabolism/sulfur assimilation (Table 1), which is well conserved and has been characterized in many bacterial species (30, 31). One explanation for the requirement of these genes is that *G. mellonella* limits free cysteine as a pathogen restriction strategy, as shown previously (32). An alternative hypothesis for the cysteine requirement in the *Galleria* model is that cysteine serves as a protectant from oxidative and/or nitrosative stresses, which are widely con-

served antimicrobial defense strategies. Indeed, *cys* genes have been linked to increased susceptibility to oxidative stress in several other bacterial species (33–35).

Eight genes predicted to play a role in the catabolism of aromatic compounds were identified as required for growth in *G. mellonella* (Table 1). This could indicate that the *Galleria* hemolymph contains aromatic hydrocarbons as carbon sources for *Acinetobacter*. Mutants predicted to be defective in the metabolism of various aromatic compounds, including benzoic acid and protocatechuate, were reduced following growth in *Galleria* larvae, indicating a link between aromatic compound catabolism and the ability to grow in the larvae. Additionally, two of the identified genes, *paal* and *paay*, predicted to be involved in phenyla-

TABLE 1 Genes required for growth of *A. baumannii* in *G. mellonella*^a

| Function and annotation | Gene | Product | RR |
|--|--------------|---|--------|
| Micronutrient acquisition | | | |
| ABUW_1173 | <i>bauD</i> | Acinetobactin permease | 0.0119 |
| ABUW_1174 | <i>bauC</i> | Acinetobactin permease | 0.0976 |
| ABUW_1176 | <i>bauB</i> | Acinetobactin periplasmic binding protein | 0.0472 |
| ABUW_1177 | <i>bauA</i> | Acinetobactin receptor | 0.0111 |
| ABUW_3740 | <i>znuA</i> | High-affinity Zn transport protein | 0.0312 |
| ABUW_3741 | <i>Zur</i> | Fur family transcriptional regulator | 0.0730 |
| ABUW_3742 | <i>znuC</i> | Zinc import ATP-binding protein | 0.0685 |
| ABUW_3743 | <i>znuB</i> | High-affinity Zn transport protein | 0.0449 |
| Cysteine metabolism/sulfur assimilation | | | |
| ABUW_0643 | <i>cysI</i> | Sulfite reductase | 0.0217 |
| ABUW_0722 | <i>cysH</i> | PAPS ^b reductase | 0 |
| ABUW_0853 | <i>cobA</i> | Uroporphyrin-III C-methyltransferase | 0 |
| ABUW_1760 | | Sulfate permease | 0.0959 |
| ABUW_2218 | <i>cysQ</i> | 3'(2'),5'-Bisphosphate nucleotidase | 0.0503 |
| ABUW_2362 | <i>cysE</i> | Serine acetyltransferase | 0.0361 |
| ABUW_2895 | <i>cysN</i> | Sulfate adenyltransferase subunit 1 | 0 |
| ABUW_2896 | <i>cysD</i> | Sulfate adenyltransferase subunit 2 | 0 |
| Aromatic hydrocarbon metabolism | | | |
| ABUW_1835 | <i>pcaD</i> | 3-Oxo adipate enol-lactonase | 0.0469 |
| ABUW_1837 | <i>pcaC</i> | 4-Carboxymuconolactone decarboxylase | 0.0652 |
| ABUW_1848 | <i>pcaU</i> | <i>pca</i> operon regulatory protein | 0.0852 |
| ABUW_1854 | <i>benP</i> | Benzoate transport porin | 0.0305 |
| ABUW_2090 | | 4-Hydroxybenzoate transporter | 0.0909 |
| ABUW_2349 | | 4-Oxalocrotonate tautomerase | 0.0086 |
| ABUW_2374 | | Isochorismatase hydrolase | 0.0631 |
| ABUW_2523 | <i>paaII</i> | Thioesterase domain protein | 0.0069 |
| ABUW_2524 | <i>paaY</i> | Phenylacetic acid degradation protein | 0.0934 |
| Cell envelope/membrane/Wall | | | |
| ABUW_3360 | <i>lptE</i> | LPS assembly | 0 |
| ABUW_3447 | <i>lpxL</i> | Lipid A biosynthesis acyltransferase | 0 |
| ABUW_3448 | <i>lptB</i> | Glycosyl transferase, group 1 | 0.0248 |
| ABUW_3638 | <i>pbpG</i> | D-Alanyl-D-alanyl carboxypeptidase | 0 |
| ABUW_3831 | <i>Wza</i> | Polysaccharide export protein | 0.0133 |
| ABUW_3832 | <i>Ptp</i> | Protein tyrosine phosphatase | 0 |
| ABUW_3833 | <i>Ptk</i> | Tyrosine protein kinase | 0 |
| Stress response genes | | | |
| ABUW_0655 | <i>typA</i> | GTP-binding protein TypA/BipA | 0.0223 |
| ABUW_1595 | <i>Kef</i> | Ion transport protein | 0.0666 |
| ABUW_1648 | <i>mscS</i> | Mechanosensitive ion channel | 0.0658 |
| ABUW_1740 | <i>rseP</i> | Intramembrane metallopeptidase | 0.0279 |
| ABUW_1763 | <i>uspA</i> | UspA domain protein | 0 |
| ABUW_1804 | <i>trkH</i> | K ⁺ uptake system component | 0.0639 |
| ABUW_2521 | <i>uvrD</i> | UvrD/REP helicase | 0.0460 |
| ABUW_2590 | <i>kefF</i> | NADPH oxidoreductase | 0.0405 |
| ABUW_3122 | <i>otsB</i> | Trehalose phosphatase | 0.0859 |
| Antibiotic resistance | | | |
| ABUW_0842 | <i>adeK</i> | Multidrug efflux protein | 0 |
| ABUW_0843 | <i>adeJ</i> | Multidrug efflux protein | 0.0157 |
| ABUW_0844 | <i>adeI</i> | Multidrug efflux protein | 0.0154 |
| ABUW_1156 | | Drug/metabolite exporter | 0.0442 |
| ABUW_1499 | | EamA-like transporter | 0.0619 |
| ABUW_1520 | | EamA-like transporter | 0.0635 |
| ABUW_1673 | | Bacterial TM ^c pair family protein | 0.0458 |
| ABUW_1851 | | Aminoglycoside phosphotransferase | 0.0912 |
| ABUW_2123 | | Metallo-β-lactamase family protein | 0.0828 |
| ABUW_2550 | | EamA-like transporter | 0.0584 |
| Transcriptional regulation | | | |
| ABUW_1645 | | TetR family transcriptional regulator | 0.0437 |
| ABUW_1672 | | LysR family transcriptional regulator | 0.0676 |
| ABUW_1692 | | TetR family transcriptional regulator | 0.0493 |
| ABUW_1755 | <i>gigD</i> | AsnC family transcriptional regulator | 0 |
| ABUW_1768 | | MarR family transcriptional regulator | 0.0272 |
| ABUW_1849 | | LysR family transcriptional regulator | 0.0547 |
| ABUW_1966 | | LysR family transcriptional regulator | 0.0556 |

(Continued on following page)

TABLE 1 (Continued)

| Function and annotation | Gene | Product | RR |
|-------------------------|-------------|---|--------|
| ABUW_2074 | | Fur family transcriptional regulator | 0.0495 |
| ABUW_2196 | <i>alkR</i> | AraC family transcriptional regulator | 0.0516 |
| ABUW_2236 | | AraC family transcriptional regulator | 0.0550 |
| ABUW_2370 | <i>arsR</i> | ArsR family transcriptional regulator | 0 |
| ABUW_2520 | | TetR family transcriptional regulator | 0.0666 |
| ABUW_2544 | | AraC family transcriptional regulator | 0.0373 |
| ABUW_2555 | <i>soxR</i> | Redox-sensitive transcriptional activator | 0.0934 |
| ABUW_3161 | <i>gigC</i> | LysR family transcriptional regulator | 0.0007 |
| ABUW_3180 | <i>bfmS</i> | TCS sensor kinase protein | 0 |
| ABUW_3260 | <i>gigA</i> | Putative TCS response regulator | 0.0671 |

^a Listed is a subset of genes identified in the TnSeq screen as outlined in the text. For the full list of hits, see the supplemental material.

^b PAPS, phosphoadenosine phosphosulfate.

^c TM, transmembrane.

cetic acid catabolism, have previously been implicated in virulence (36).

Capsule, cell envelope, and membrane biogenesis. Transposon insertions within several genes required for capsule, cell wall, and outer membrane biogenesis reduced the ability of A.

baumannii to survive and grow within *Galleria* larvae (Table 1). This result is not surprising, as many of the immune defense mechanisms employed by the larvae will first encounter the bacterial outer membrane/cell envelope. Indeed, mutants with transposon insertions in several genes required for capsule biosynthesis

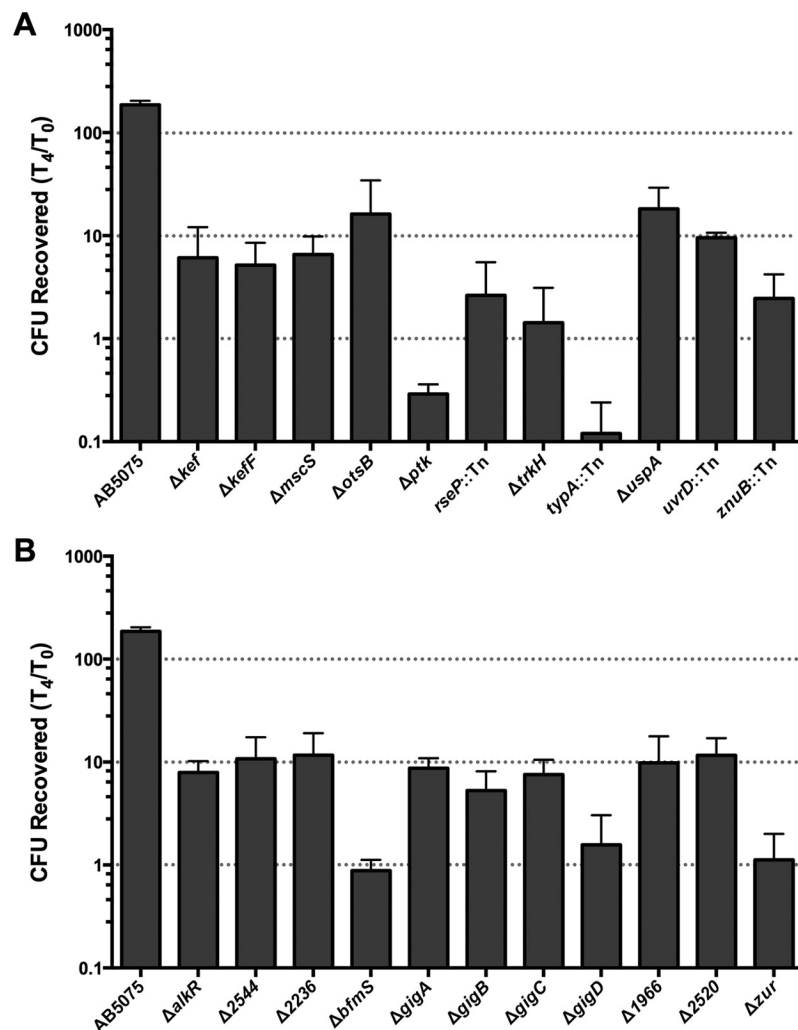


FIG 3 Growth of selected mutants in *G. mellonella* larvae. The mutant strains indicated were inoculated into *G. mellonella* larvae. Data are presented as described in the legend to Fig. 1. Panels: A, stress response genes; B, transcriptional regulators.

were completely absent from the output DNA pool after growth in *Galleria* (see Fig. S2 in the supplemental material). Also, strains harboring insertions in the *pbpG* gene, encoding a D-alanyl-D-alanine endopeptidase required for modification of peptidoglycan, were unable to grow in the larvae (RR_{pbpG} of 0). This gene was previously shown to be required for the growth of *A. baumannii* in human serum, a murine pneumonia model, and a rat pneumonia model (11, 37). In addition to the capsule operon and *pbpG*, transposon insertions in several other genes involved in lipopolysaccharide (LPS) biosynthesis, including *lptE*, *lpxL*, and *lpsB*, were undetected or underrepresented in the larval samples (RRs of 0, 0, and 0.025, respectively). In many Gram-negative bacteria, like *Escherichia coli*, LptE is an essential protein required for the proper insertion of LPS into the outer membrane (reviewed in reference 38). In some Gram-negative organisms, however, such as *Neisseria meningitidis*, LptE is not essential for LPS transport (39). Analysis of a previously published list of candidate essential genes in *A. baumannii* AB5075 indicates that other LPS transport genes (*lptA*, *lptB*, *lptC*, and *lptD*) are essential, whereas *lptE* is not required for growth in rich medium (12). The requirement of *lptE* in the *Galleria* model suggests that there may be environments where LptE is required for proper LPS assembly.

To validate and establish a protective role for capsule production in the *G. mellonella* disease model, as has been shown in other disease models (40), we constructed a strain with an in-frame deletion of the *ptk* gene. Ptk is a structural component of the capsular polysaccharide export machinery, along with Wza and Ptp. Recovery of mutants with changes in all three genes was significantly reduced in the *G. mellonella* larval output pool (RR_{wza} of 0.013, RR_{ptp} of 0, RR_{ptk} of 0). When we infected *G. mellonella* larvae with the *ptk* mutant, larval killing and bacterial growth were dramatically reduced compared to those of wild-type AB5075 (see Fig. S2 in the supplemental material) and were restored by expression of the wild-type *ptk* gene in *trans*, confirming the importance of the capsule as a virulence factor in *A. baumannii*.

Stress response genes. Sixteen genes required for growth within *G. mellonella* are predicted to mediate stress resistance (Table 1). These genes include those involved in the general stress response (*cinA*, *osp*, *typA*, and *uspA*), DNA repair, *uvrD*, membrane/envelope stress (ABUW_1447 [HptX homolog] and *rseP*), oxidation-induced stress (*pqiA* [paraquat] and *arsC* [arsenate]), and osmotic stress resistance (ABUW_2237 [cation transport], *otsB*, *kef*, *kefF*, *mcsS*, *trkA*, and *trkH*) (Table 1). These results suggest that the ability of *A. baumannii* to grow and/or survive in *G. mellonella* may be dependent upon the organism's ability to cope with environmental stresses imposed by the innate immune response to infection. As the *G. mellonella* larva contains hemocytes, phagocytic cells that, much like human neutrophils, generate an oxidative burst and produce antimicrobial peptides, which can mimic osmotic shock (15, 21, 23, 41, 42), we infer that these stress response genes may also play a role in human disease. To confirm the phenotypes of the stress response genes identified in the TnSeq screen, we constructed mutants containing in-frame, unmarked deletions of several of the hits and tested these strains for virulence defects in the *G. mellonella* model (Fig. 3). In the few instances where attempts to generate in-frame deletions were unsuccessful, we used strains harboring a transposon insertion in the gene of interest. Deletion strains showing defective survival and/or growth within *G. mellonella* include both genes with known/predicted functions, including *ptk*; *bfmS* (the gene for a

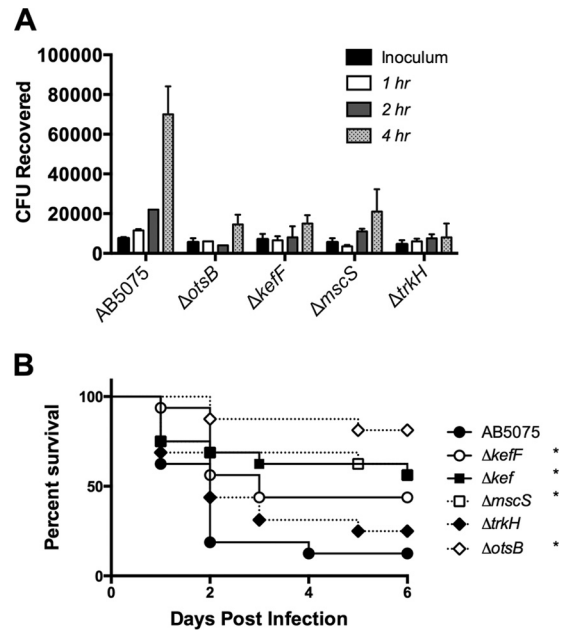


FIG 4 Osmotic stress genes are required for virulence in *G. mellonella*. (A) Growth of osmotic stress mutants in freshly collected hemolymph monitored over time. (B) *G. mellonella* larvae ($n = 16$) were infected with 10^5 CFU of wild-type AB5075 or isogenic mutants. Larval survival was monitored daily for 6 days. *, $P < 0.05$.

two-component system [TCS] sensor kinase described in further detail below); *znuB*; stress response genes *rseP*, *typA*, and *uspA*; and genes with uncharacterized functions (Fig. 3).

As several of the stress response genes identified in the TnSeq screen are predicted to mediate osmotic stress resistance, we conducted additional experiments to demonstrate a role for osmotic stress resistance in the *G. mellonella* model. The osmotic stress gene mutants used in the experiment shown in Fig. 3 were defective for growth in freshly collected larval hemolymph, consistent with the results obtained in the TnSeq experiment (Fig. 4A). Decreased killing of larvae by several strains harboring deletions of osmotic stress genes was also observed (Fig. 4B).

Furthermore, we performed additional complementation experiments with a subset of genes (*rseP*, *typA*, and *uspA*) predicted to be involved in both stress sensing and survival. In other proteobacteria, the *rseP* gene encodes an intramembrane protease that transmits outer membrane stress via RpoE/ σ^E (43), while *typA* and *uspA* are predicted to be involved in the general stress response and have previously been shown to play a role in virulence (44–46). We confirmed the initial TnSeq results by assessing the ability of these mutants to grow within and kill *G. mellonella*. Strains defective for these genes displayed a *Galleria* growth defect, which was restored to nearly wild-type levels by complementation with wild-type copies of the genes expressed in *trans* (Fig. 5).

Antibiotic resistance genes. All strains of *A. baumannii* harbor efflux pumps associated with the removal of toxic compounds from bacterial cells (47). The TnSeq screen identified genes belonging to three different types of efflux systems, *adeI*, *adeJ*, and *adeK* (RRs of 0.0247, 0.0233, and 0, respectively), which encode the resistance-nodulation-cell division pump AdeIJK (48); four different genes containing EamA-like transporter domains of the metabolite/drug transporter family (ABUW_1156 [RR of 0.044],

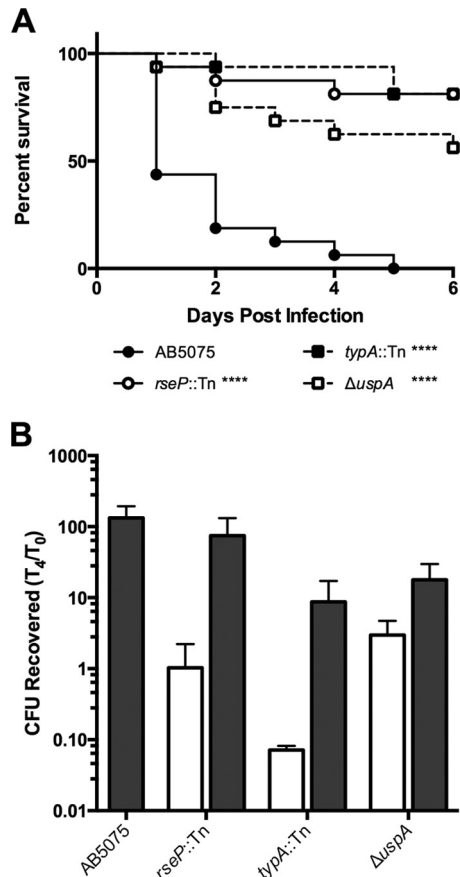


FIG 5 Stress response genes are required for *G. mellonella* killing and growth in *Galleria*. (A) *G. mellonella* larvae were infected with the strains indicated as described in the legend to Fig. 1. (B) Growth of wild-type AB5075 (gray bar) or the strains indicated harboring a hygromycin resistance gene (white bars) or a wild-type copy of the deleted gene at the Tn7 locus (gray bars) following inoculation of *G. mellonella* larvae as described in the legend to Fig. 1. ****, $P < 0.0001$.

ABUW_1499 [RR of 0.062], ABUW_1520 [RR of 0.064], ABUW_2550 [RR of 0.058]) and a member of the proteobacterial antimicrobial compound efflux family (ABUW_1673 [RR of 0]) (49) (Table 1). Decreased recovery of strains harboring interruptions of these genes following growth in *Galleria* larvae relative to rich medium suggests that *Galleria* produces toxic compounds during infection that *A. baumannii* must export for survival. In addition to these drug efflux systems, the recovery of strains harboring insertions within two genes (ABUW_1851 and ABUW_2123) encoding a putative aminoglycoside phosphotransferase and a beta-lactamase family protein was also reduced following *G. mellonella* growth. Although annotated as encoding potential antibiotic resistance mechanisms, we hypothesize that the proteins produced by these genes may perform other functions, as *G. mellonella* larvae likely do not produce either aminoglycosides or β -lactams.

Transcriptional regulators. Other than genes of unknown function, the largest single category of hits from the TnSeq screen includes genes annotated as having signal transduction or transcriptional regulation functions (Table 1; Fig 2). Among the 32 genes in this group, only a few have previously recognized functions, i.e., *arsR* (arsenic resistance), *alkR* (alkane metabolism),

bfmS (biofilm formation), and *soxR* (redox homeostasis) (50–53). An insufficient number of transposon insertions mapped to *bfmS* (the cognate response regulator for *bfmS*) in the LB sample to be included in the TnSeq analysis. Several other putative transcriptional regulatory genes were found (see Table S1 in the supplemental material), i.e., two TCS response regulators, seven LysR family regulators, five TetR family regulators, four AraC family regulators, two AsnC/Lrp family regulators, a MarR family regulator, and several others. One of the TetR family regulators (ABUW_1692) and one of the AsnC/Lrp regulators (ABUW_1755, described below) were reported to be differentially expressed in the *zur* mutant (29), as well as during biofilm growth (54). Coupled with the *bfmS* requirement for growth in *G. mellonella*, this finding suggests that similar genetic pathways are required for both growth within and killing of *G. mellonella* larvae. We term these *gig* genes for growth in *Galleria*. While many additional genes listed in Table 1 (see also Table S1 in the supplemental material) could be considered to encode the *gig* phenotype, we formally classify only a small subset as *gig* genes because loss of these particular genes leads to a robust defect in both growth within and killing of *Galleria* larvae. The *gig* genes include ABUW_3260, a predicted TCS response regulator with a PP2C protein phosphatase domain (*gigA*); ABUW_3261, a putative anti-anti-sigma factor (*gigB*); ABUW_3161, a LysR family regulator (*gigC*); and ABUW_1755, an AsnC/Lrp family regulator (*gigD*) (Fig. 6). Expression of the *gigB*, *gigC*, and *gigD* genes in *trans* restored the *Galleria* growth defect of these strains (Fig. 6). Attempts to complement the *gigA* mutant yielded ambiguous results.

Mutations in *gig* genes produce decreased antibiotic resistance. As discussed above, several genes required for growth in *G. mellonella* appeared to be involved in resistance to environmental stress and/or antibiotics (Table 1). This finding led to the hypothesis that genes required for virulence may also have an important role in resistance to environmental stress(es), such as exposure to antibiotics. In order to test this hypothesis, we performed antibiotic susceptibility testing according to the CLSI guidelines (55) and discovered that several genes that exhibited an RR of < 0.10 in the *G. mellonella* model also mediate resistance to antibiotics. For example, strains with either *gigA* or *gigB* deleted showed increased sensitivity to several antibiotics, including meropenem, aminoglycosides, and tigecycline (Table 2; Fig. 7). The Δ *ptk* mutant strain showed enhanced susceptibility to several antibiotic categories, suggesting that capsular polysaccharides also serve to protect *A. baumannii* from antibiotic killing (Table 2). In addition, deletion of a LysR family regulator (ABUW_1966) or *zur* led to increased susceptibility to peptidoglycan-targeting antibiotics, including penicillins and meropenem (Table 2). The strain harboring a transposon insertion in *typA* also exhibited sensitivity to several antibiotics (Table 2). The finding that these genes are also required for growth within and/or killing of *G. mellonella* larvae supports the hypothesis that there is a link between virulence and the ability to resist environmental and/or antibiotic stresses.

DISCUSSION

A. baumannii is a serious health risk because of its rapid acquisition of antimicrobial resistance and the inherent ability of the species to survive in the clinical environment by resisting decon-

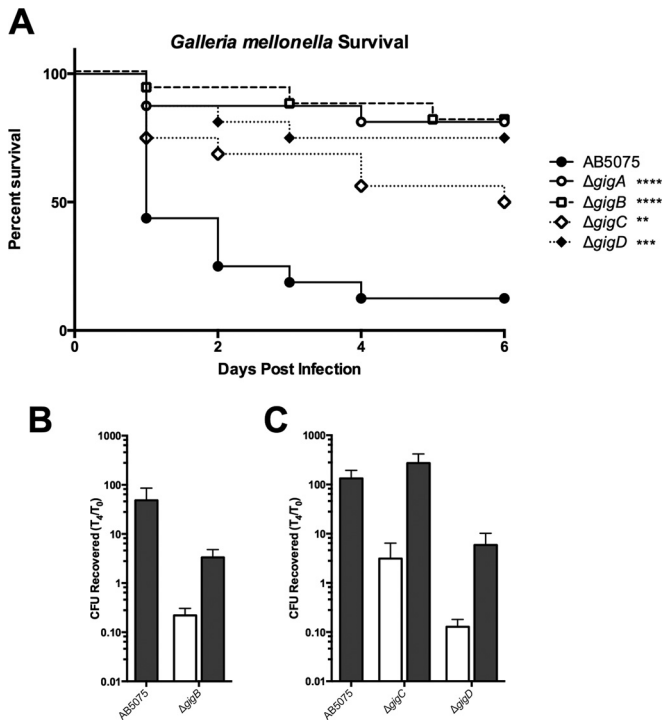


FIG 6 *Gig* genes are required for virulence in *G. mellonella*. (A) *G. mellonella* larvae were infected with the strains indicated and monitored for survival as described in Fig. 1. (B) Growth of AB5075 with the empty vector (left gray bar) or the *gigB* mutant harboring the empty vector (white bar) or a complementing clone of *gigB* (right gray bar). (C) Growth of wild-type AB5075 (left gray bar) or the strains indicated harboring a hygromycin resistance gene (white bars) or a wild-type copy of the deleted gene at the Tn7 locus (center and right gray bars). In panels B and C, growth is depicted as described in the legend to Fig. 1. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$.

tamination practices employed in health care facilities (56), thus providing the potential for spread to susceptible patients. Despite the increasing prevalence of MDR isolates, however, the molecular mechanisms underlying the pathogenesis of *A. baumannii* remain poorly defined. In this work, we confirmed many known virulence genes and also identified novel genes that contribute to *A. baumannii* virulence as assessed in a well-established invertebrate model.

Recently, Geisinger and Isberg published the results of experiments investigating the capsule synthetic genes of *A. baumannii* (18). Other work indicates that the capsule locus is quite variable across the sequenced genomes of *A. baumannii* isolates, suggesting that this important surface structure is under positive selection by environmental stresses and perhaps immune recognition (57), and as our data suggest, the presence of capsule is protective during antibiotic stress (Table 2). Indeed, there is a consensus that production of exopolysaccharide capsule is important for *A. baumannii* virulence in all models of infection, including the *Galleria* model used for the studies described here (18, 40). In accordance with this, we confirmed the requirement for capsule synthesis in AB5075 by showing that deletion of *ptk*, a gene required for capsule export, impaired growth within the larval cavity and attenuated larval killing (see Fig. S2 in the supplemental material). Geisinger and colleagues reported difficulty in generating *ptk* mutants of strain ATCC 17978 and found that strains with a *ptk* mutation

had acquired suppressor mutations in the *bfmRS* locus (18). We readily obtained *ptk* null mutants of the AB5075 strain, and sequencing of the *bfmRS* locus in four independent *ptk* deletion strains did not reveal any differences from the wild-type *bfmRS* sequence (data not shown), although we cannot exclude the possibility that these strains have acquired other suppressor mutations. Additional observations from our lab and others demonstrate that *A. baumannii* strains harboring deletions of the BfmS sensor kinase generate excess polysaccharides (data not shown; 18), suggesting that the BfmRS TCS may modulate capsule production. On the basis of this observation, we speculate that BfmS may act as a phosphatase for BfmR, and loss of BfmS leads to increased BfmR-P, which in turn activates capsule production.

As capsular polysaccharides are commonly associated with protection from host defenses and antibiotics and because *G. mellonella* produces antimicrobial peptides in response to bacterial infection, we originally hypothesized that the increased capsule production of the *bfmS* mutant may confer protection from the insect defenses. On the contrary, we observed that *bfmS* null strains were defective in growth/persistence in *G. mellonella* (Fig. 3). For the cognate response regulator *bfmR*, we were unsuccessful in obtaining a deletion strain in the AB5075 background, and strains harboring a transposon insertion in this gene appear to have a growth defect. In light of these observations; we propose that the *bfmRS* TCS has a broader role beyond the mediation of biofilm formation.

Wang et al. recently published the results of an investigation of the genetic requirements for *A. baumannii* persistence in a murine pneumonia model (11). Despite the use of different strains (Wang and colleagues used strain ATCC 17978) and model systems, the two studies did identify many of the same genes. For instance, both experiments identified known virulence factors for *A. baumannii*, including zinc and iron acquisition systems, capsule and LPS biosynthesis genes, amino acid metabolism and acquisition genes, and the *bfmRS* TCS (11). The correlation between the present study and that of Wang et al., which used a mammalian infection model, demonstrates the utility of the *G. mellonella* system for studying *A. baumannii* infection. Beyond these similarities, however, the work described here, as discussed below, identified additional virulence genes that were not previously identified.

When we looked more closely at the genes involved in amino acid biosynthesis, we observed that many genes involved in the cysteine metabolism/sulfur assimilation pathway were required for *A. baumannii* to grow in *Galleria*. This pathway is well conserved across bacterial species (58), and aside from generating cysteine for protein synthesis, the sulfur assimilation pathway appears to prevent damage due to oxidative stress, possibly by maintaining a reducing environment inside the cell (34). As oxidative stress is a widely conserved pathogen restriction factor, we hypothesize that this pathway may be involved in the oxidative stress resistance of *A. baumannii*. Interestingly, when we interrogated the data of Wang and colleagues, we observed that several genes in the cysteine/sulfur assimilation were also required for persistence in the murine pneumonia model, even though the authors did not comment on these genes (11). The correlation between the two studies further emphasizes the importance of the cysteine/sulfur pathway for *A. baumannii* virulence. As the sulfur assimilation pathway has recently attracted renewed interest as a target for novel antimicrobial therapeutics (reviewed in reference 30), it will be important to investigate whether pharmacological perturba-

TABLE 2 Antibiotic susceptibilities of selected TnSeq hits

| Strain ^a | Sensitivity to: ^{b,c} | | | | | | | | | |
|---------------------|--------------------------------|------------|-------------|-------------|-------------|-----------|-------------|-------------|-----------|----------------------|
| | Amp-sulb | Pip-tazo | Merop | Amik | Tobra | Levofl | Col | Minocyc | Gent | Tigecyc ^d |
| ATCC 17978 | 29 | 23 | 27 | 25 | 24 | 26 | 14 | 28 | 25 | 0.13 |
| AB5075 | 6 | 6 | 8.2 | 8.4 | 10 | 11.2 | 12.7 | 24 | 6 | 1.5 |
| $\Delta alkR$ | 6 | 6 | 7 | 9 | 10.5 | 11 | 12 | 23 | 6 | 1 |
| $\Delta 2544$ | 6 | 6 | 8 | 8 | 10 | 11 | 13 | 25 | 6 | 1 |
| $\Delta 2236$ | 6 | 6 | 7 | 9 | 10 | 11 | 13 | 24 | 6 | 1 |
| $\Delta arsR$ | 6 | 6 | 8 | 8 | 10 | 12 | 12 | 21 | 6 | 2 |
| <i>bfmR</i> ::Tn | 6 | 6 | 8 | 9 | 10.5 | 12 | 14 | 25 | 6 | 1 |
| $\Delta bfmS$ | 8 | 6 | 9 | 6 | 8 | 8 | 12 | 21 | 6 | 2 |
| $\Delta gigA$ | 6 | 6 | 9.2 | 14.6 | 17.8 | 12.1 | 13.5 | 24 | 13 | 0.75 |
| $\Delta gigB$ | 8.4 | 7.5 | 10.8 | 18.2 | 20.7 | 13 | 14.7 | 27.5 | 16 | 0.125 |
| $\Delta gigC$ | 6 | 6 | 8 | 6 | 9 | 12 | 14 | 27 | 6 | 0.5 |
| $\Delta gigD$ | 9 | 6 | 9 | 6 | 10 | 11 | 14 | 27 | 6 | 0.75 |
| Δfur | 6 | 6 | 8 | 9 | 10 | 10 | 14 | 25 | 6 | 1.5 |
| $\Delta 1849$ | 6 | 6 | 8 | 7 | 10 | 11 | 12 | 22 | 6 | 1 |
| $\Delta 1966$ | 9 | 6.5 | 9.8 | 7.1 | 8.7 | 10.7 | 13.1 | 25 | 6 | 1.5 |
| $\Delta 1672$ | 6 | 6 | 8 | 7.5 | 10 | 11 | 14 | 27 | 6 | 0.75 |
| $\Delta 1768$ | 6 | 6 | 8 | 9 | 9 | 11.5 | 13 | 25 | 6 | 1 |
| Δptk | 10 | 7.8 | 10.5 | 7.4 | 10 | 9.3 | 14.5 | 27.5 | 6 | 0.75 |
| $\Delta 1645$ | 6 | 6 | 8 | 9 | 11 | 11 | 13 | 24 | 6 | 0.75 |
| $\Delta 1692$ | 6 | 6 | 8 | 6 | 10 | 11 | 12 | 20 | 6 | 2 |
| $\Delta 2520$ | 6 | 6 | 8 | 9 | 9 | 11 | 14 | 25 | 6 | 1 |
| <i>typA</i> ::Tn | 6 | 6 | 9 | 15 | 11 | 10 | 15 | 28 | 6 | 1.0 |
| $\Delta uspA$ | 8 | 6 | 9 | 6 | 9 | 9 | 12 | 22 | 6 | 2 |
| Δzur | 12.5 | 10 | 11 | 9 | 11.5 | 11.4 | 12 | 26 | 6 | 2.0 |

^a Numbers indicate strains harboring a deletion/interruption of the annotation number (ABUW_0000). Amp-sulb, ampicillin-sulbactam; Pip-tazo, piperacillin-tazobactam; Merop, meropenem; Amik, amikacin; Tobra, tobramycin; Levofl, levofloxacin; Col, colistin; Minocyc, minocycline; Gent, gentamicin; Tigecyc, tigecycline.

^b Unless indicated otherwise, sensitivity is represented as the zone of growth inhibition (in millimeters) surrounding a filter disc impregnated with the antibiotic indicated.

^c Bold values are statistically significant different from those of wild-type AB5075.

^d Tigecycline sensitivity is reported as the MIC (in micrograms per milliliter) determined by *E* test.

tion of this pathway represents a viable strategy to treat *A. baumannii* infection.

Our experiment also identified many genes involved in transcriptional regulation ($n = 32$), which is significant because one of the key differences distinguishing *A. baumannii* from the closely related but nonpathogenic species *A. baylyi* is the presence of transcription regulatory genes. Indeed, Adams and colleagues recently found that one major difference between *A. baylyi* and *A. baumannii* is the presence of substantially more transcriptional regulators in *A. baumannii* (~10% of the pan-*A. baumannii* genes) (59). We conclude that these transcriptional regulators may control genes that allow *A. baumannii* to be a human pathogen. Of the 32 regulator genes identified in this study, half do not have an ortholog in *A. baylyi*, supporting the conclusions of the aforementioned study. Future studies to define genes controlled by the transcriptional regulators identified in our screen will likely highlight the key regulatory networks involved in *A. baumannii* sensing and responding to the host environment.

Interestingly, several of the genes required for growth in *Galleria* identified in our screen also show a growth defect in subinhibitory concentrations of various antibiotics (Fig. 7; Table 2). The functions of genes required for surviving both within *Galleria* and during environmental and/or antibiotic stress encompass signal transduction, transcriptional regulation, and genes predicted to play a role in stress survival. We thus conclude that the stresses imposed upon *A. baumannii* during infection overlap those faced by bacteria during exposure to antibiotics and other environmental stressors. Antibiotics mediate cell killing through a variety of mechanisms, and we have shown that strains harboring deletions

of specific transcriptional regulators display increased antibiotic sensitivity; therefore, we infer that these regulators may also control genes required for detoxification of stress or for alteration of cell/envelope permeability—a property that could impact sensitivity to both antimicrobial peptides and antibiotics. We further propose that selection for increased survival of environmental stresses, such as clinical disinfectant strategies, antimicrobial compounds, etc., inadvertently selects for strains displaying a hypervirulence phenotype and may, in fact, select for MDR in clinical settings. This hypothesis is supported by work by Geisinger and Isberg in which they observe increased virulence after exposure to sublethal levels of antibiotics (18) and Roux et al., who recently reported loss of virulence in antibiotic-sensitive bacterial mutants (19). Future experiments will be aimed at identifying genes controlled by the transcriptional regulators described here.

MATERIALS AND METHODS

Strains and growth conditions. For the strains, plasmids, and oligonucleotide primers used in this study, see Tables S2 and S3 in the supplemental material. *E. coli* and *A. baumannii* strains were cultured in LB medium at 37°C. When required, the antibiotics added for selection were tetracycline (10 and 5 $\mu\text{g/ml}$), hygromycin (500 and 250 $\mu\text{g/ml}$), and apramycin (50 and 25 $\mu\text{g/ml}$) for growth on solid agar and in liquid medium, respectively.

***G. mellonella* infection.** *G. mellonella* larvae were purchased from Knutson's Live Bait (Brooklyn, MI). Infection of *G. mellonella* was performed with overnight bacterial cultures as previously described (13). For larval survival experiments, infected larvae were stored in the dark at 37°C and monitored daily for survival for 6 days. Significance of survival differences was assessed with the log rank test. For bacterial recovery exper-

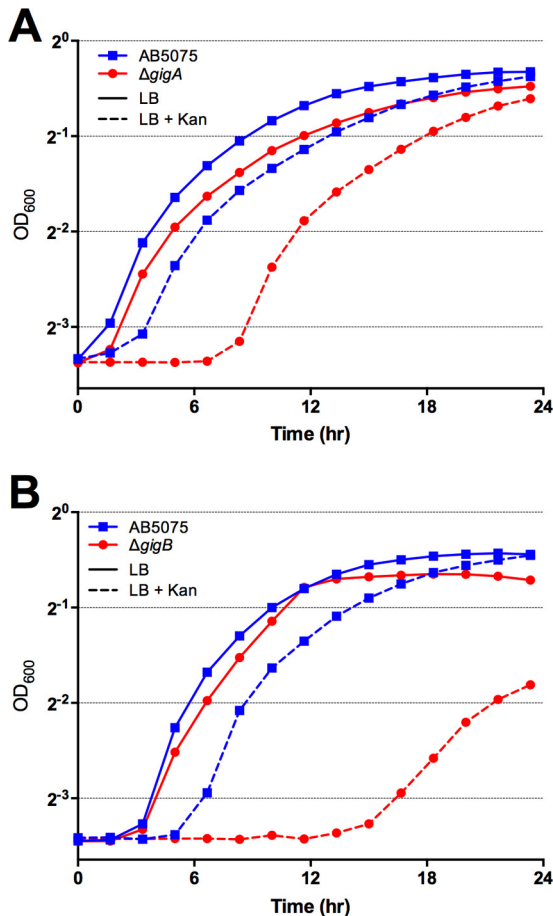


FIG 7 Genes required for growth in *Galleria* are also required for growth in subinhibitory concentrations of antibacterials. The strains indicated were grown for 24 h at 37°C in LB with or without 625 μ g/ml kanamycin (Kan). Growth was measured by determining the OD₆₀₀ every 10 min in a Tecan 96-well plate reader.

iments, larvae were infected with $\sim 10^6$ CFU of the strains indicated. The CFU recovery experiments were repeated at least three times, and data from a representative experiment are shown. Larvae ($n = 2$ per time point) were homogenized with a D1000 Benchtop homogenizer in 2 ml of phosphate-buffered saline (PBS) immediately following infection ($t = 0$) and after 4 h of incubation at 37°C ($t = 4$). Homogenates were diluted and plated onto LB agar with 5 μ g/ml chloramphenicol to eliminate the growth of the normal flora of *G. mellonella* without impacting the growth of AB5075 and its derivatives. Bacterial growth is represented as the ratio of the number of CFU per larva at $t = 4$ to the number of CFU at $t = 0$. To assess bacterial growth in hemolymph, the bacterial strains indicated ($\sim 10^4$ CFU) were mixed with freshly collected hemolymph. Larvae were lanced, and hemolymph was collected from several larvae and pooled prior to bacterial inoculation. Bacterium-hemolymph mixtures were incubated at 37°C, and bacteria were enumerated at the times indicated. Hemolymph experiments were repeated three times, and the results of a single experiment are shown.

Transposon mutant library and TnSeq experiment. The *A. baumannii* AB5075 transposon mutant library was prepared as previously described (8, 12). For the TnSeq experiment, aliquots of the transposon library (stored at -80°C) were thawed on ice for 30 min and diluted and $\sim 10^6$ CFU were inoculated either into 5 ml of LB or into *G. mellonella* larvae. The LB and *G. mellonella* pools were grown at 37°C for 4 h, at which time larvae were homogenized as described above and aliquots of the LB

cultures or larval homogenates were plated onto LB agar with tetracycline (10 μ g/ml) to eliminate the normal bacterial flora of *G. mellonella*. The plates were incubated at 37°C for 4 h. Bacteria were then washed from the plates with 1.5 ml of sterile PBS. Genomic DNA was prepared with the Qiagen DNeasy kit according to the manufacturer's recommendations and processed and sequenced as previously described (8). Following the mapping of reads to the AB5075 genome, genes with fewer than five unique insertions mapped in the LB sample were omitted from further analysis. To identify underrepresented genes following growth in *G. mellonella*, the number of reads per gene in the *G. mellonella* sample was divided by the number of reads per gene in the LB sample to generate an RR. An RR of 0 is reported for cases in which no reads mapped to a given gene in the *G. mellonella* sample. A hit was defined as a gene with an RR of < 0.1 , i.e., a 1-log reduction in the number of reads following passage through *G. mellonella*. For the full TnSeq data set, see Data Set S1 in the supplemental material.

Generation of mutant strains and mutant strain complementation.

Gene deletions were performed with an allelic exchange plasmid (pMJG42) harboring the *sacB* gene for counterselection via growth on medium containing sucrose as previously described (60). Sucrose-resistant colonies were subjected to colony PCR to identify clones that had lost the wild-type gene, and deletions were confirmed by sequencing. For complementation at the Tn7 site, DNA containing the entire open reading frame of the deleted gene, including the native promoter region of the targeted gene, was amplified by PCR, cloned into pMJG111, and introduced into AB5075 as previously described (13, 60). For plasmid-based complementation of the *gigB* mutant strain, the *gigB* region was amplified and cloned into pMJG120. For the oligonucleotides used for Tn7- and pMJG120-based complementation, see Table S3 in the supplemental material.

Antibiotic susceptibility testing. Antibiotic sensitivity testing was performed according to CLSI guidelines (55). Disc diffusion assay (DDA) and *E* test experiments were performed five times. Data are reported as the mean zone of inhibition (DDA)/MIC (*E* test). Growth curves were performed with a Tecan M200 microtiter plate reader. Overnight cultures of the strains indicated were diluted 1:100 into fresh LB medium with or without kanamycin, and growth was monitored for 24 h. The growth curves were repeated three times, and results are shown as the mean optical density at 600 nm (OD₆₀₀) obtained from triplicate wells of a representative experiment.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01660-15/-/DCSupplemental>.

Data Set S1, XLSX file, 0.4 MB.

Figure S1, TIF file, 2.8 MB.

Figure S2, TIF file, 0.4 MB.

Table S1, PDF file, 0.1 MB.

Table S2, PDF file, 0.1 MB.

Table S3, PDF file, 0.1 MB.

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