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Resources for Genetic and Genomic Analysis of Emerging Pathogen *Acinetobacter baumannii*

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ABSTRACT

***Acinetobacter baumannii* is a Gram-negative bacterial pathogen notorious for causing serious nosocomial infections that resist antibiotic therapy. Research to identify factors responsible for the pathogen's success has been limited by the resources available for genome-scale experimental studies. This report describes the development of several such resources for *A. baumannii* strain AB5075, a recently characterized wound isolate that is multidrug resistant and displays robust virulence in animal models. We report the completion and annotation of the genome sequence, the construction of a comprehensive ordered transposon mutant library, the extension of high-coverage transposon mutant pool sequencing (Tn-seq) to the strain, and the identification of the genes essential for growth on nutrient-rich agar. These resources should facilitate large-scale genetic analysis of virulence, resistance, and other clinically relevant traits that make *A. baumannii* a formidable public health threat.**

IMPORTANCE

***Acinetobacter baumannii* is one of six bacterial pathogens primarily responsible for antibiotic-resistant infections that have become the scourge of health care facilities worldwide. Eliminating such infections requires a deeper understanding of the factors that enable the pathogen to persist in hospital environments, establish infections, and resist antibiotics. We present a set of resources that should accelerate genome-scale genetic characterization of these traits for a reference isolate of *A. baumannii* that is highly virulent and representative of current outbreak strains.**

Acinetobacter baumannii is a Gram-negative opportunistic pathogen that causes infections with serious morbidity and mortality and is one of a group of six pathogens responsible for most multidrug-resistant (MDR) nosocomial infections (the ESKAPE pathogens, i.e., *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (1, 2). The pathogen is infamous for its ability to persist in hospital settings, a feature that reflects its capacity for long-term survival on abiotic surfaces through resistance to desiccation and disinfectants (3).

Genomic and molecular epidemiological studies of *A. baumannii* isolates have helped define the pathogen's global population structure, its antibiotic resistance gene repertoire, the size and content of its pangenome, and phylogenetic relationships among outbreak strains (3–6). Three primary clonal lineages (GC1 to GC3) appear responsible for the majority of hospital outbreaks globally (7). Although these lineages display restricted genetic diversity among core genes (7), the species' genome is actually quite dynamic. Strains display striking variability in accessory gene content (5, 8), including antibiotic resistance genes (9), even among related isolates of a single outbreak (10). This genomic variability presumably reflects the actions of transmissible plasmids, insertion elements, phage, integrons, natural transformation, and recombination (11–18).

The virulence factors responsible for *A. baumannii*'s pathogenicity remain elusive, although a few have been identified. The outer membrane OmpA protein is required for a variety of infection-associated activities (19). The outer membrane porin Omp33-36 induces apoptosis, modulates autophagy, and promotes intracellular persistence in human cells (20). The extracellular polysaccharide capsule is important for survival in ascetic fluid and serum and in an infection model (21). Phospholipase D contributes to serum survival, invasion of epithelial cells, and pathogenicity in a murine pneumonia model (22). Factors protecting bacteria from reactive oxygen, iron limitation, and other stresses are also important for infection (23, 24). The identification of these factors provides a starting point for understanding the basis of *A. baumannii* pathogenicity.

A. baumannii research progress has been limited by the technologies and resources available for studying the pathogen. Although methods for targeted gene disruption, transposon mutagenesis, single-copy complementation using Tn*7*, and plasmid complementation have been developed (25–31), only a few resources have been described for genome-scale experimental studies (31). To facilitate large-scale genetic analysis of *A. baumannii*, we report here a set of genetic and genomic resources based on a

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recently characterized clinical isolate, strain AB5075. AB5075 was developed to provide an experimental reference strain representative of current outbreaks; in the absence of such a strain, different laboratories have worked with different strains, making it difficult to compare results. AB5075 was isolated in 2008 from a combatant wound infection; the strain is highly virulent in a number of animal models, exhibits multiple antibiotic resistances, and can be manipulated genetically (28). We present the completion and annotation of the strain's genome sequence, the creation of an ordered, comprehensive transposon mutant library for the strain, the extension of high-coverage transposon mutant pool sequencing (Tn-seq) technology to it, and an analysis of the strain's essential genes. These resources should help accelerate research to characterize the basis of *A. baumannii*'s success as a tenacious nosocomial pathogen.

MATERIALS AND METHODS

Strains, plasmids, and medium. *A. baumannii* AB5075 was provided by the laboratory of D. Zurawski, as were the hygromycin-resistant transposon mutants described previously (28). The AB5075 isolate studied was designated AB5075-UW to distinguish it as an independently sequenced isolate although it is presumably very similar or identical to the previously described strain (28, 32). Plasmid pLG123, bearing transposon T26, was constructed by the following means: (i) separately amplifying the P_{groE} promoter element and the tetracycline resistance open reading frame (ORF) from plasmid pKK214gfp (33) using, respectively, primer pair T26-PgroUP/T26-PgroDN and primer pair T26-TetUP/T26-TetDN; (ii) joining the two amplicons by overlap extension PCR using primers T26-PgroUP and T26-TetDN; and (iii) ligating the resulting amplicon into pMOD-2 (Epicentre) after digestion of both the amplicon and the vector with EcoRI and SacI. Construction of the plasmid here designated pMOD-hyg, bearing transposon T101, has been described previously (28). Growth medium used for *A. baumannii* was LB (10 g of tryptone, 5 g of yeast extract, and 8 g of NaCl per liter).

Oligonucleotide sequences. The sequences of the oligonucleotides named here were the following: for T26-PgroUP, ATGAATTCT CATAACTTCGTATAGCATACATTATACGAAGTTATGCGGCCGC CCCAAACATCGCAAAAGGTGTA; for T26-PgroDN, CGCATTGTT AGATTTCATATGTATATCTCCTTCTTAAATCTGCAGTG; for T26-TetUP, TAAGAAGGAGATATACATATGAAATCTAACAATGCGCT CATC; for T26-TetDN, ATGAGCTCGCATAACTTCGTATAATGTATG CTATACGAAGTTATGGCGCGCCTCATACTTCCATTCAGGTCGAG GTG; for ME+9-3′(5PH), 5′-phos-CTGTCTCTTATACACATCTCAAC CATCA; for ME+9-5′(5PH), 5′-phos-CTGTCTCTTATACACATCTCA ACCCTGA.

Genome sequencing. Genomic DNA from AB5075-UW was isolated using a PurElute bacterial genomic kit (Edge BioSystems, Gaithersburg, MD) and subjected to long-read sequencing from a single SMRT (single-molecule, real-time DNA sequencing) cell on a Pacific Biosciences RS II sequencer (PacBio). *De novo* assembly of the chromosome was carried out by a hierarchical genome assembly process (HGAP) (34) in SMRT Analysis, version 2.1.1 (http://www.pacb.com/devnet/). The genome size was set to 4 Mb, and target coverage was set to 15 in a Celera assembler. All other parameters were set to defaults. The draft assembly output from HGAP was analyzed with Gepard (35). One contig was found to be spurious and was removed. The overlapping ends of the single contig were trimmed appropriately, and the edited draft assembly was polished and analyzed for circularity and potential misassemblies using RS_Bridgemapper in SMRT Analysis, version 2.1.1. A consensus accuracy of 99.9998% was achieved. Subsequently, AB5075-UW genomic DNA isolated by a DNeasy blood and tissue kit (Qiagen) was subjected to Nextera XT sample preparation (Illumina, Inc.) and sequenced on a MiSeq instrument (Illumina, Inc.) as 250-bp paired-end reads (16.8 million read pairs passed filter). These reads and the raw PacBio reads were aligned using BWA, version 0.7.4 (36), to the single-contig HGAP assembly, achieving greater than 30-times coverage for all bases, and analyzed for variants using SAMTools, version 0.1.18, mpileup (37). No single nucleotide polymorphisms (SNPs) or other variants were found.

To determine the sequence of the large plasmid, the Illumina reads were assembled *de novo* using Abyss, version 1.3.6 (*k*-mer value of 176) (38), resulting in 81 contigs of at least 300 bp. Four of these contigs did not align to the chromosome using Nucmer, version 3.0.6 (39), but showed homology to published *A. baumannii* plasmid sequences and had overlapping sequences at their ends. PacBio reads which aligned to these contigs suggested that they belonged to a single replicon and were used to assign a tentative order. Amplification using unique primers annealing adjacent to the ends of the contigs or adjacent to repeated sequences, followed by sequencing of the PCR products, was used to finalize contig order and close gaps. Realignment of the Illumina reads against the closed sequence confirmed the assembly.

Two smaller plasmids of approximately 8.5 kb and 2 kb were evident from agarose gel analysis of plasmid preparations from AB5075-UW. To determine their sequences, we first prepared plasmid DNA (Qiagen Miniprep) from a pool of over 400,000 random T26 transposon insertion mutants of AB5075-UW and retransformed it by electroporation into wild-type AB5075, selecting for the resistance marker present on the transposon. Plasmid DNA was prepared from 50 transformants and sequenced by capillary sequencing using outward-facing primers at each end of the transposon. Many of the reads aligned to various locations in plasmid pAB0057, achieving greater than 8-times coverage over the entire plasmid sequence with only a single gap, which was closed by targeted PCR and sequencing. The reads which did not align to pAB0057 could be assembled into a single, approximately 2-kb contig, which was confirmed by targeted PCR and sequencing.

Genome annotation and genome comparisons. Genome annotation was derived from bioinformatics predictions and manual curation using the Prokaryotic Genome Analysis Tool (PGAT) (40). Briefly, protein coding genes were predicted by homology to annotated *Acinetobacter* and other bacterial genes. Open reading frames were predicted by Prodigal (41) in regions where no previously known genes were detected. Genes for which no functional annotation was available were annotated using InterProScan classification (42). Phage regions were identified using the Phage Search tool (Phast) (43), insertion sequences (ISs) were identified using the ISfinder database (44), tRNA sequences were identified using tRNAscan (45), and rRNA sequences were identified using RNAmmer (46). Potential antibiotic resistance genes were identified using the PATRIC database (47) and by comparison searching in PGAT using published lists of *A. baumannii* resistance functions (14, 48, 49). Gene presence or absence queries in comparison to other *A. baumannii* genomes were carried out using PGAT. The genome sequence and annotation details can be viewed at the PGAT site (http://tools.nwrce.org/cgi-bin/pgat_acinetobacter/elementlist.cgi?id=AB5075UW).

Phylogenetic analysis. Phylogeny of 13 *A. baumannii* strains with complete genomes was generated using the kSNP software package, version 2.1.1, in which SNP discovery was based on *k*-mer analysis, i.e., single variant positions within sequences of nucleotide length *k* (50). The maximum-likelihood tree was based on SNPs in 21-mers that were identified in at least 50% of the strains. Tree branches are expressed in terms of changes per total number of SNPs, not changes per site, as SNP-based trees do not include invariant sites. The tree was drawn using Dendroscope (51). The distantly related *Acinetobacter baylyi* strain ADP1 was included as the outgroup strain for the phylogenetic reconstruction but was subsequently removed to better visualize branching patterns.

Transposon mutagenesis. T26 mutagenesis was carried out by transformation of transposon-transposase complexes (transposomes) (Epicentre). The transposon was amplified from plasmid pLG123 using primers ME+9-3′(5PH) and ME+9-5′(5PH) and purified by a Qiagen MinElute PCR purification kit with elution into Tris-EDTA (TE) buffer. Transposomes were assembled by mixing the amplified transposon (in TE

buffer at a concentration greater than 700 ng/μl) with 1 volume of 100% glycerol and 2 volumes of EZ-Tn5 Transposase (Epicentre), incubating the mixture for 30 min at 20°C and then for 45 min at 30°C, and then storing the mixture at 4°C. Electrocompetent AB5075-UW was prepared by diluting overnight LB cultures approximately (5×10^9)-fold in 50 ml of LB without salt, incubating cultures for 17 to 18.5 h as multiple 5-ml cultures with rolling at 37°C to reach an optical density at 600 nm (OD_{600}) of 5.5 to 7.5, pelleting and washing samples three times with fresh 10% glycerol at room temperature, and finally pelleting and resuspending samples in a minimal volume of 10% glycerol. Electrocompetent cells were electroporated with a Bio-Rad Gene Pulser using, per electroporation in a 1-mm cuvette, 50 μl of electrocompetent cells and 0.25 μl of the transposome preparation with pulsing at 100 Ω, 25 μF, and 1.6 KV. The cells were then incubated in 1 ml of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, and 20 mM glucose) with agitation at 37°C for 1 h before they were plated on selective medium or rapidly frozen in the presence of 5% dimethyl sulfoxide (DMSO) for later use. Transformants were selected on LB agar, usually with 5 μg/ml tetracycline, though occasionally with 7.5 or 10 μg/ml tetracycline. One transformation typically yielded 15,000 to 35,000 independent insertion mutants.

Ordered mutant library construction. Generation of the T101 mutants has been described previously (28). T26 insertion mutant colonies were picked following mutagenesis and arrayed into 384-well plates containing LB with 5% DMSO using a QPix2 colony-picking robot (Genetix). Plates were incubated for 24 h at 37°C and then stored at −80°C. Insertion sites were identified by semidegenerate PCR and sequencing of the transposon-genome junctions (52, 53). Specific protocols and oligonucleotide sequences used are available upon request. To assemble the three-allele library, custom scripts and manual curation were used to choose, when available, three mutants for each gene or pseudogene that, whenever possible, represented insertions situated between 5% and 90% of the coding sequence, were spaced at distance from one another within the gene, and had yielded high-quality sequence mapping data during the sequencing of the primary library. T26 insertions were favored over T101 insertions. Strains were cherry picked, re-colony purified, incubated, and stored in a manner similar to one previously described (54).

Tn-seq methodology. To generate a mutant pool for Tn-seq analysis, mutagenesis reaction products from several T26 transposome electroporations (frozen as described above) were combined and plated on eight Q-Trays (Genetix), each containing 250 ml of LB agar with 5 μg/ml tetracycline. Plating density corresponded to approximately 57,000 transposon mutant colonies per Q-Tray. After incubation for 14 h at 37°C, cells were collected by multiple washings into a total of 30 ml of 10% glycerol per Q-Tray. Equal volumes of these eight plate harvests were combined, mixed by vortexing, flash frozen in aliquots, and stored at −80°C. Genomic DNA from an aliquot of this pool was prepared by a DNeasy blood and tissue kit (Qiagen). Tn-seq analysis of this DNA was carried out multiple times using either the circle method (55) or the terminal deoxynucleotidyl transferase (TdT) method (56) and sequenced on an Illumina MiSeq or Genome Analyzer II instrument. Minor modifications of each Tn-seq method were made for use with transposon T26. Detailed Tn-seq protocols, including oligonucleotides used, are available upon request. Total mapped reads ranged from 1.6 million to 8.2 million per technical replicate. Read counts were normalized to 10 million total mapped reads per technical replicate. For chromosomal insertions, counts were also normalized for local read density, as described previously (55). For essential gene analysis, normalized read counts for all technical replicates were averaged at each insertion location. Reads which could be mapped with equal confidence to multiple genomic locations were assigned randomly to one of the possible locations. Mapping of reads and tallying of hit and read counts by gene were carried out using custom Python scripts.

Nucleotide sequence accession numbers. The complete AB5075-UW genome sequence (BioProject PRJNA243297) has been deposited in the GenBank under accession numbers CP008706.1 (chromosome), CP008707.1 (plasmid p1AB5075), CP008708.1 (plasmid p2AB5075), and CP008709.1 (plasmid p3AB5075). The Pacific Biosciences reads used for genome assembly have been deposited in the Sequence Read Archive (SRA) under accession number SRX803116. The Illumina reads used for genome assembly and assembly validation have been deposited in the SRA under accession number SRX751631. The Illumina reads used for Tn-seq analysis have been deposited in the SRA under accession numbers SRX802078, SRX802079, SRX802080, SRX802081, SRX802082, and SRX802083.

TABLE 1 The AB5075-UW genome

Feature	Value for the replicon			
	Chromosome	Plasmid p1	Plasmid p2	Plasmid p3
Length (bp)	3,972,672	83,610	8,731	1,967
Plasmid replication group[a]		GR6	GR2	Novel
%GC	39.1	37.0	34.4	39.2
Genetic elements (no.)				
Protein coding genes	3,771	108	13	3
Pseudogenes	34	10	1	0
IS elements	8	2	0	0
rRNA genes	18	0	0	0
tRNA genes	74	0	0	0
Resistance island	RI-1 (Tn*AbaR5075*)	RI-2	None	None

[a] According to reference 57.

RESULTS

Complete genome sequence of *A. baumannii* AB5075. To provide a framework for large-scale analysis of AB5075, we completed its genome sequence and annotation (Table 1). Although partial sequence information was available for other isolates of the strain, we carried out a fully independent analysis of the isolate (AB5075-UW) that served as the immediate parent of most of the ordered mutant library we constructed (described below).

The AB5075 genome consists of a 4-Mbp chromosome and three plasmids. One of the plasmids is unusually small (less than 2 kbp) and has only three genes, a replication initiation gene and two genes of unknown function. The replication initiation gene corresponds to a previously undefined plasmid replication group (57). The other two plasmids are closely related to previously described plasmids (8, 58), the larger of which was shown to be transmissible by conjugation (58). Strain AB5075 is closely related to three other strains of the GC1 group (AB0057, AYE, and 307-0294), which are diverse in origin and which represent both MDR and drug-susceptible phenotypes (see Fig. S1 in the supplemental material).

The genome contains two islands of resistance genes: one in the chromosome interrupting the *comM* gene (resistance island 1 [RI-1]) and a second in the largest plasmid (RI-2) (Fig. 1). Resistance island insertions in *comM* are found in most MDR *A. baumannii* strains but are usually larger than those found in AB5075 (10, 59, 60) (Fig. 1; also see Text S1 in the supplemental material). The plasmid resistance island represents a novel insertion in a known plasmid (58) and exhibits the remarkable feature that it is flanked by direct repeats of a 439-bp miniature inverted-repeat transposable element (MITE)-like sequence (61–64) (Fig. 1; see Text S1 in

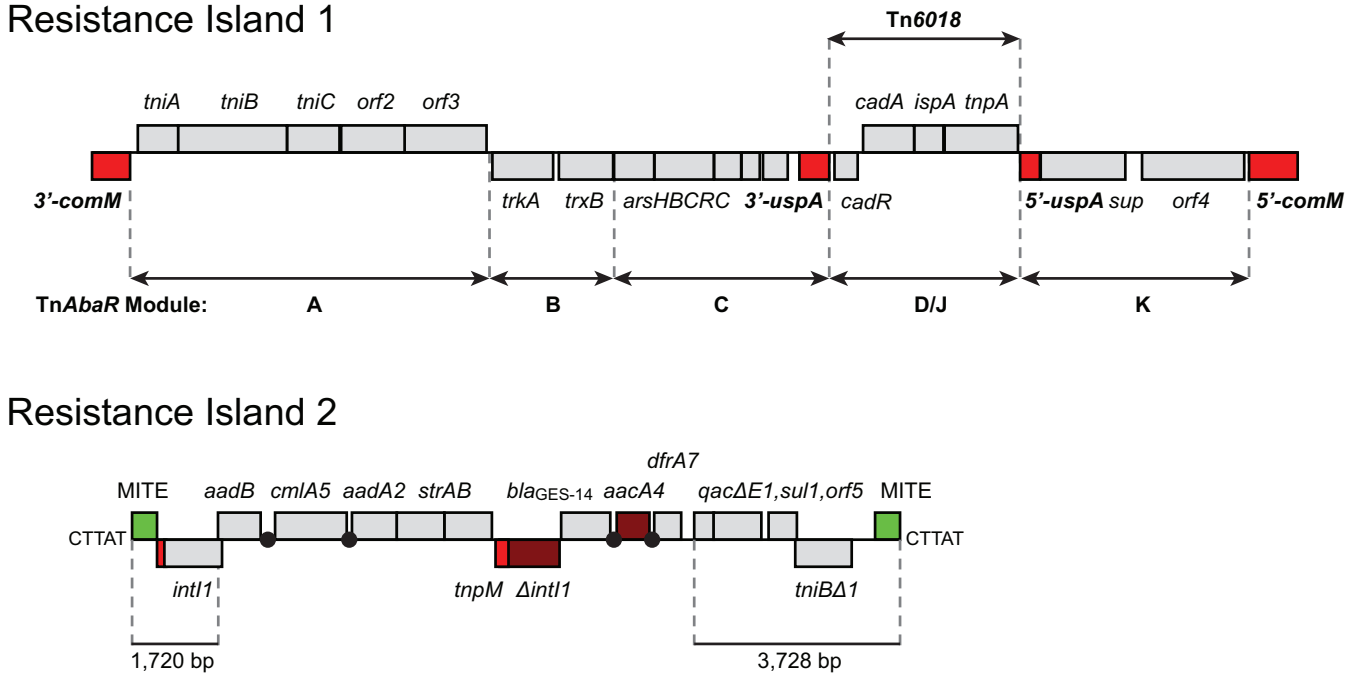

FIG 1 Resistance islands in AB5075-UW. Resistance island 1 (Tn*AbaR5075*) is 13.5 kbp long and, like most other *A. baumannii* resistance islands, is a Tn*AbaR1*-like island inserted in the *comM* gene. The Tn*AbaR* modules are labeled according to a previous convention (59). Compared to most known *comM* islands, RI-1 is small and contains one rather than two copies of the Tn*6018* module. RI-1 carries predicted arsenic and antimony resistance genes (*arsCRCBH*), a cadmium resistance locus (*cadAR*), and a sulfate transporter gene (*sup*). Resistance island 2 is a 13.5-kbp island in plasmid p1 consisting of a complex class 1 integron truncated at both ends by direct repeats of a 439-bp miniature inverted-repeat transposable element (MITE)-like sequence (green). The indicated 1,720- and 3,728-bp segments (including the MITE-like sequences) are 100% identical to sequences defining the ends of different class 1 integrons in other *Acinetobacter* strains (see Text S1 in the supplemental material). The 5-bp target site duplication of the plasmid at the RI-2 insertion site is shown. RI-2 includes genes for resistance to β-lactams (bla_{GES-14}), aminoglycosides (*aacA4*, *aadA2*, *aadB*, and *strAB*), chloramphenicol (*cmlA5*), and trimethoprim (*dfrA7*). Circles, intact *attC* sites ("59-bp elements"); red, interrupted gene fragments; dark red, putative pseudogenes. Additional gene abbreviations follow a previous convention (59).

the supplemental material). The insertion site shows a 5-bp duplication, suggesting that the entire island was inserted into the plasmid by transposition (58, 62, 64). The island has a complex class 1 integron structure seen in one other *A. baumannii* strain (65) and carries genes for resistance to β-lactams (the extended-spectrum β-lactamase GES-14), aminoglycosides, chloramphenicol, and trimethoprim (see Text S1 in the supplemental material). The presence of these resistance genes in a putatively mobile genetic element on a transmissible plasmid could greatly enhance their spread to other bacteria.

Phage and insertion sequences (IS) are additional genetic elements that can facilitate horizontal transfer and alter resistance phenotypes in *Acinetobacter* (9–11, 14, 66, 67). Approximately 7% of the AB5075 chromosome comprises phage-like regions (see Table S1 in the supplemental material). One of these regions appears to be an intact prophage with several genes similar to genes of *Pseudomonas* phage phiCTX. The prophage, which we name phiOXA, carries the OXA-23 extended-spectrum β-lactamase gene (68, 69) and a 5′-adjacent IS element (see Text S1 in the supplemental material). Compared to closely related strains, the AB5075 genome contains an intermediate number of IS elements. Several of these elements may contribute to the strain's resistance profile (see Table S2 and Text S1 in the supplemental material).

A total of 133 candidate resistance genes in diverse functional categories (48) reside outside the two AB5075 resistance islands and potentially contribute to resistance (see Table S3 in the supplemental material). All of the strain's resistance genes have orthologues in one or more of the closely related GC1 strains (AB0057, AYE, and 307-0294), implying highly overlapping resistance armamentaria (see Table S3 in the supplemental material).

More detailed information about the features of the AB5075 genome and its relationship to other strains is provided in Text S1 in the supplemental material. Overall, the strain displays a number of features that distinguish it from its close GC1 relatives.

Comprehensive ordered transposon mutant library. As a resource for genetic analysis of AB5075, we created an arrayed library of mutants with defined transposon insertions in most nonessential genes of the organism. Our goal was to create a colony-purified library with relatively complete genome coverage that was small enough to facilitate efficient phenotype screening. We also wanted it to include several different mutations for each gene to minimize missed genotype-phenotype associations arising from noninactivating mutations or library cross-contamination and to provide immediate confirmation of associations observed. To meet these objectives, we created a library made up of two to three different insertion mutants per nonessential gene. The library was created in two stages (Table 2). First, a large primary collection of individual mutants generated by random insertion mutagenesis and selection on LB agar was arrayed and sequence defined. This collection contained an average of over 10 unique insertion mutants per coding gene. Second, individual mutants from this primary collection were colony purified, rearrayed, and resequenced. This smaller library is made up of two to three unique, sequence-verified mutants for most genes and is called the

TABLE 2 Ordered transposon mutant library

Library and parameter	No. for transposon(s)		
	T26	T101	T26 + T101
Primary collection			
Insertion mutants arrayed	51,744	10,368	62,112
Insertions successfully mapped	38,340	6,964	45,304
Duplicate, discrepant, or ambiguous insertions	2,341	308	2,649
Unique insertions	35,999	6,656	42,655
Within genes (coding + pseudogenes)	33,137	6,156	39,293
Intergenic	2,862	500	3,362
Genes hit internally (of 3,940)	3,471	2,266	3,527
Avg unique hits per gene in genome	9.1	1.7	10.8
Genes not hit internally	469	1,674	413
Three-allele library			
Total mutants	10,187	575	10,762
Within genes	10,025	562	10,587
Intergenic	162	13	175
Mutants confirmed by resequencing	8,243	319	8,562
Genes with insertions			
Total	3,470	447	3,523
1 unique insertion	364	350	305
2 unique insertions	444	80	305
3 unique insertions	2,098	16	2,243
4 unique insertions	420	1	505
>4 unique insertions	144	0	165
Genes without insertions	470	3,493	417

"three-allele library." The three mutants chosen for each gene corresponded, where possible, to insertions distributed between 5% and 90% of the coding sequence and were chosen irrespective of their colony phase type (P. Rather, personal communication). The mutants in the three-allele ordered library are listed in Data Set S1 in the supplemental material, and a browser (University of Washington Transposon Mutant Library Browser [http://tools.uwgenomics.org/tn_mutants]) provides a means to scan the genome for mutants and to request individual strains (Fig. 2). Copies of the entire arrayed three-allele library will also be available (www.gs.washington.edu/labs/manoil/baumannii.htm).

Two Tn*5* transposons with different resistance markers were used to generate the mutants (see Fig. S2A in the supplemental material), with a transposon conferring tetracycline resistance (T26) accounting for the majority (95%) of the mutants in the three-allele set (Table 2). Transposon T26 includes *loxP* sites

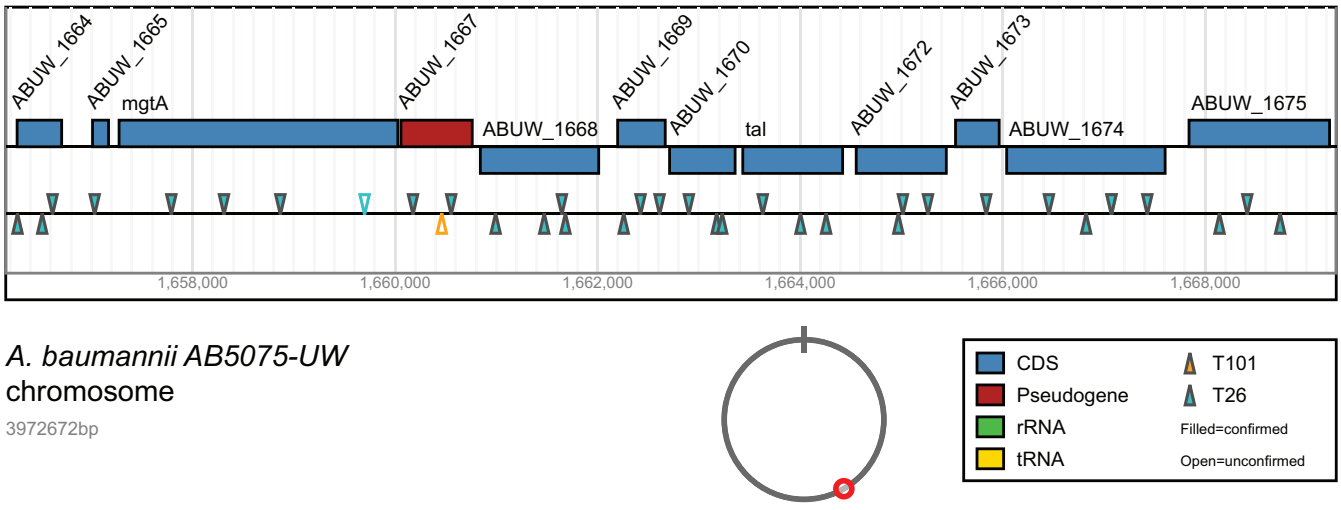

FIG 2 Web browser map of three-allele library mutants. A partial screen shot of the transposon locations in a representative region of the AB5075 genome from the Transposon Mutant Library Browser (http://tools.uwgenomics.org/tn_mutants) is shown. Transposons are represented as triangles, with positions above or below the line corresponding to their orientations in the genome. Filled triangles represent insertions whose locations were confirmed by multiple sequencings, and open triangles represent locations determined by single sequencings. Placing the mouse cursor over individual triangles in the browser window reveals information about the insertions and facilitates ordering the corresponding mutants. CDS, coding sequence.

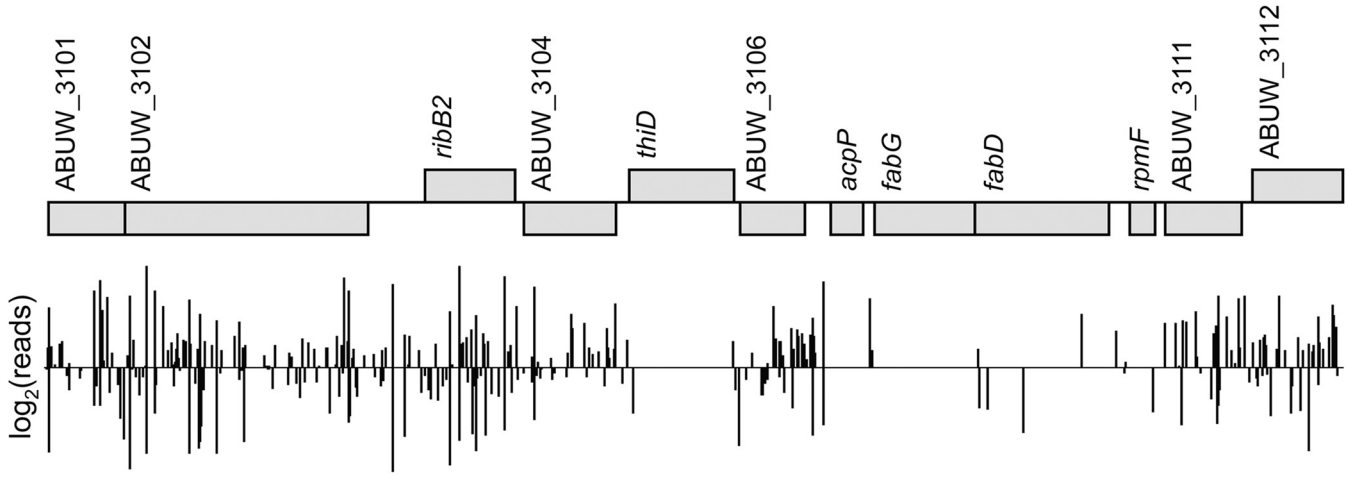

FIG 3 Profile of Tn-seq reads from the high-saturation mutant pool. The genes in a representative segment of the genome are shown. Vertical bars in the lower part of the diagram represent $\log_2$-transformed normalized read counts per insertion site (counts averaged from the six technical replicates), with bars above or below the line reflecting insertion in forward or reverse orientation relative to the genome, respectively. Scale maximum corresponds to a $\log_2$ value of 12. The genes *thiD*, *acpP*, *fabG*, and *rpmF*, which encode a thiamine metabolism protein, acyl-carrier protein, a lipid metabolism protein, and a ribosomal protein, respectively, lack insertions and are candidate essential genes. The *fabD* gene (lipid metabolism) is also classified as a candidate essential gene based on its low density of insertions (see Materials and Methods; also see Fig. S3 in the supplemental material). The significance of the few insertions detected in *fabD* is unknown, but one possibility is that they are not fully inactivating, e.g., due to insertion in transiently duplicated regions.

flanking the tetracycline resistance marker, enabling excision of the marker by transient expression of Cre site-specific recombinase (see Fig. S2B in the supplemental material). For insertions in one of the six possible translational reading frames, the recombination results in an in-frame insertion of 73 codons without a stop codon, generating presumptive nonpolar mutations. In addition, double mutants may be constructed by first excising the resistance marker from one mutant and then introducing a second insertion by transformation of genomic DNA from another mutant (see Fig. S2B in the supplemental material). Through iteration, strains bearing three or more mutations may also be constructed.

Tn-seq analysis of AB5075. To provide an efficient procedure for screening pools of mutants for genotype-phenotype associations, we extended transposon insertion pool screening (Tn-seq) to AB5075. We used transposon Tn*5*-based methodology to provide high genome coverage (70). We compared two different Tn-seq procedures, the circle method we developed (55) and an adaptation to Tn*5* of a method that utilizes terminal deoxynucleotidyl transferase (TdT) (56). Approximately 450,000 T26 insertion mutants selected on LB agar were pooled, and DNA from the pool was analyzed by the two methods. Both methods successfully identified insertions comparable in number to the predicted pool complexity (see Text S2 in the supplemental material). The profile of reads mapping to a representative segment of the genome illustrates the high density of insertions found in most (nonessential) regions and provides examples of several genes with very few reads that are putative essential genes (Fig. 3). The two Tn-seq methods both worked well for *A. baumannii* AB5075 and provided remarkably similar lists of essential genes (see below and Text S2 in the supplemental material).

Candidate essential genes. The transposon insertion profiles from Tn-seq and the primary ordered mutant library represent independent data sets that can be used to identify AB5075 genes essential for growth on nutrient-rich agar. The data sets reflect complementary advantages and disadvantages of the two procedures for identifying essential genes. The Tn-seq analysis provides high genome coverage but does not distinguish well between slow-growing and nongrowing mutants because the strains in the pool are grown in competition. The ordered library was generated from isolated colonies and should include slow-growing mutants but provides lower genome coverage and is therefore expected to lack insertions by chance in more nonessential genes than Tn-seq analysis. We therefore defined candidate essential genes as those with low representation in both data sets (see Fig. S3A to C in the supplemental material). The 438 candidate essential genes are listed in Data Set S2 in the supplemental material. Nearly all of these genes have orthologues in a different strain of *A. baumannii* (ATCC 17978), and about three-quarters of the orthologues were found to be essential using a Tn-seq procedure different from the procedures used here (31) (see Fig. S3D in the supplemental material).

DISCUSSION

Comparative genomic studies have provided detailed descriptions of the global population structure, antibiotic resistance gene repertoire, virulence capabilities, and genome plasticity of *A. baumannii*. In contrast, genome-scale experimental analysis of antibiotic resistance and virulence has lagged, in part because resources for functional genomic studies have been unavailable. The work presented here provides several such resources for strain AB5075.

Strain AB5075 is a multidrug-resistant global clone 1 strain isolated in 2008 from a soldier with osteomyelitis under treatment at Walter Reed Army Medical Center. Its MDR phenotype and recent isolation make it more representative of current outbreak strains than older type strains often used in experimental studies (28). In addition, AB5075 displays robust virulence in multiple animal models and is amenable to genetic manipulation, making it a promising model strain for analysis of a variety of traits (28).

The AB5075 genome sequence revealed several unique features. The strain carries an unusually small plasmid with only three genes, as well as a large plasmid with a novel resistance island made up of a class 1 integron flanked by miniature inverted-repeat transposable element (MITE)-like units. AB5075 also carries the clinically important extended-spectrum β-lactamase OXA-23 gene within a novel intact prophage.

The principal contribution of this study was the generation of a comprehensive ordered library of colony-purified, sequence-defined transposon mutants. Such defined mutant libraries have proved useful for genome-scale studies of other pathogens (71–73). The *A. baumannii* library provides redundant, multiple-allele coverage of most nonessential genes, with most mutant identities verified by two independent sequencings. Library mutants can be browsed using a web-based tool (Transposon Mutant Library Browser [http://tools.uwgenomics.org/tn_mutants]) and are available to the research community.

To provide methodology for rapid genome-scale genetic analysis of growth-related traits, we adapted transposon mutant pool sequencing (Tn-seq) technology to AB5075. Two Tn-seq methods employing Tn*5* transposition were examined, and both provided high-density insertion site coverage of the genome. We combined the coverage data from the Tn-seq experiments with that from generation of the ordered mutant library to define the AB5075 genes essential for growth on nutrient-rich medium. The two experimental data sets used for identifying essential genes complement one other, and the final list of genes based on both data sets should thus be more accurate than those provided by either method alone.

In summary, we created a set of genetic and genomic resources for analyzing a reference isolate of *A. baumannii*. These resources should facilitate in-depth genetic dissection of virulence, antibiotic resistance, and other clinically relevant traits of this important nosocomial pathogen.

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