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 Tn7, 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
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 pGL123, bearing transposon T26, was constructed by the following means: (i) separately amplifying the 3′ 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 (Epigence) after digestion of both the amplicon and the vector with EcoRI and SalI. 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, ATGAGCTCGCATAACTTCGTATAATGTATG; for T26-PgroDN, TAAGAAGGAGATATACATATGAAATCTAACAATGCGCTCATAACTTCGTATAACATTATACGAAGTTATGCGGCCGC. The sequences of the oligonucleotides predicted by Prodigal (41) in regions where no previously known genes were annotated using the PATRIC database (47) and by comparison searching in the Genome 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.

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Ordering 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 possible, represented insertions situated within 5% and 90% of the coding sequence. Insertion sites 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 (34).

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 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.

### 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 GCI 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...
Resistance Island 1

Resistance Island 2

FIG 1 Resistance islands in AB5075-UW. Resistance island 1 (TnAbaR5075) is 13.5 kbp long and, like most other A. baumannii resistance islands, is a TnAbaR-like island inserted in the comM gene. The TnAbaR 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 Tn6018 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 (blaGES-14), aminoglycosides (aacA4, aadA2, aadB, and strAB), chloramphenicol (cmIA5), 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-lactamaseGES-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 nonactivating 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, rearayred, and resequenced. This smaller library is made up of two to three unique, sequence-verified mutants for most genes and is called the
“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 Tn5 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.

![A. baumannii AB5075-UW chromosome](3972672bp)

**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.
flanking the tetracycline resistance marker, enabling excision of the marker by transient expression of Cre site-specific recombination (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 Tn5-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 Tn5 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 isolates 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 I 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 integron prophase.

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 Tn5 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 the 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 another, 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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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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