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Complexity of Complement Resistance Factors Expressed by *Acinetobacter baumannii* Needed for Survival in Human Serum

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***Acinetobacter baumannii* is a bacterial pathogen with increasing impact in healthcare settings, due in part to this organism's resistance to many antimicrobial agents, with pneumonia and bacteremia as the most common manifestations of disease. A significant proportion of clinically relevant *A. baumannii* strains are resistant to killing by normal human serum (NHS), an observation supported in this study by showing that 12 out of 15 genetically diverse strains of *A. baumannii* are resistant to NHS killing. To expand our understanding of the genetic basis of *A. baumannii* serum resistance, a transposon (Tn) sequencing (Tn-seq) approach was used to identify genes contributing to this trait. An ordered Tn library in strain AB5075 with insertions in every nonessential gene was subjected to selection in NHS. We identified 50 genes essential for the survival of *A. baumannii* in NHS, including already known serum resistance factors, and many novel genes not previously associated with serum resistance. This latter group included the maintenance of lipid asymmetry genetic pathway as a key determinant in protecting *A. baumannii* from the bactericidal activity of NHS via the alternative complement pathway. Follow-up studies validated the role of eight additional genes identified by Tn-seq in *A. baumannii* resistance to killing by NHS but not by normal mouse serum, highlighting the human species specificity of *A. baumannii* serum resistance. The identification of a large number of genes essential for serum resistance in *A. baumannii* indicates the degree of complexity needed for this phenotype, which might reflect a general pattern that pathogens rely on to cause serious infections. *The Journal of Immunology*, 2017, 199: 000–000.**

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen responsible for ~2–10% of all hospital-acquired infections, including pneumonia, bacteremia, urinary tract infections, meningitis, and wound infections (1–4). *A. baumannii* infections are notoriously difficult to treat due to intrinsic and acquired antimicrobial resistance, often limiting effective therapeutic options (5). Another essential aspect of these infections is the ability of *A. baumannii* strains to resist the killing action of normal human serum (NHS) (6–8).

The highly complex, multifactorial nature of the complement system provides a myriad of targets for potential interference by bacterial factors. It is therefore not surprising that due to the long coevolution of mammals and microbes, some of the most successful pathogens have developed effective mechanisms for attenuating or escaping complement attack (9). As a countermeasure, targeting microbial factors that promote serum resistance, such as the factor H (FH)–binding protein in *Neisseria meningitidis*, with vaccines can provide protective immunity by a combination of mechanisms including bactericidal activity and increased complement susceptibility (10).

To gain additional insights into the mechanisms of complement resistance in *A. baumannii*, we took advantage of the availability of complete microbial genome sequences and transposon (Tn) mutant libraries to investigate the contribution of the products of all nonessential *A. baumannii* genes to survival in NHS. This screen identified 50 genetic loci needed for complement resistance, and prominent among these were genes encoding the maintenance of lipid asymmetry (Mla) proteins needed for the maintenance of outer membrane (OM) lipid asymmetry, which provided an opportunity for an in-depth analysis of mechanisms of *A. baumannii* serum resistance. These studies showed the Mla system provided resistance to the alternative pathway of complement and deposition of the corresponding complement components, suggesting that this pathway could potentially be targeted for chemotherapeutic or immunotherapeutic interventions.

Materials and Methods

Bacterial strains and growth conditions

All animal experiments performed in this study were carried out in accordance with a protocol approved by the Harvard University or Brigham and Women's Hospital Institutional Biosafety Committees and Institutional Animal Care and Use Committees. These guidelines were established by

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Abbreviations used in this article: COG, cluster of orthologous genes; CVF, cobra venom factor; FB, factor B; FH, factor H; GVB^{++}, gelatin veronal buffer with added Mg^{2+} and Ca^{2+}; HI, heat-inactivated; IM, inner membrane; LB, lysogeny broth; Mla, maintenance of lipid asymmetry; NHS, normal human serum; NMS, normal mouse sera; OD_{650}, OD at 650 nm; OM, outer membrane; Ori, origin of replication; PL, phospholipid; TCC, terminal complement complex; Tn, transposon; Tn-seq, Tn sequencing; WT, wild-type.

the Institute of Laboratory Animal Resources and approved by the Governing Board of the U.S. National Research Council.

All strains and plasmids as well as oligonucleotide primers used in this study are described in Supplemental Tables II and III, respectively. *A. baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa* strains were routinely cultured in lysogeny broth (LB; Lennox; Teknova) and, when required, supplemented with of tetracycline (5 μg/ml), gentamicin (150 μg/ml), or apramycin (50 μg/ml).

Tn mutant library screening in pooled human serum

The *A. baumannii* AB5075 mutant library (11) was used in this study. Strain AB5075 is a clinical isolate from a human case of osteomyelitis that exhibits multiple antibiotic resistances and high virulence in a number of animal models of infection (12). The library includes Tn inserts in 3470 genes inactivated with a Tn conferring resistance to tetracycline (T26) and 447 genes inactivated by a Tn insert encoding hygromycin resistance (T101). Only the 3470 AB5075 genes inactivated with Tn T26 (tetracycline resistant) were investigated in this study.

To prepare the *A. baumannii* AB5075 Tn library for selection in human sera, we first generated a "total Tn mutant library pool" by replica plating the entire Tn mutant library onto rectangular LB agar plates using a 96-pin replicator (Thermo Scientific). After overnight growth, colonies from individual plates were harvested with 10 ml of LB plus 20% glycerol and 1-ml samples from each plate were mixed together, aliquoted, and stored at −80°C for further use.

Four individual vials of the total Tn library were thawed at room temperature and used to inoculate quadruplicate 50-ml LB cultures that were grown with aeration at 37°C to an OD at 650 nm (OD_{650}) of 0.4. Bacterial cultures were then chilled on ice for 15 min and 1-ml aliquots containing ~1.5 $\times 10^8$ CFUs were centrifuged and washed with 1 ml of gelatin veronal buffer with added Ca^{2+} and Mg^{2+} (GVB^{++}). Washed cells were pelleted, resuspended in 100 μl of GVB^{++}, and used to inoculate 4 × 900-μl aliquots of intact NHS or heat-inactivated (HI) NHS (56°C × 20 min) and incubated at 37°C for 2 h with shaking (250 rpm). After this time, 50-μl aliquots from each of these eight library samples (four incubated in intact NHS and four in HI NHS) were removed for viable count determinations by serial dilution and plating, and the remaining 950 μl of the sample was spread onto 245 × 245-mm LB agar plates (Corning) supplemented with 5 μg/ml tetracycline. Plates were incubated at 37°C for 16 h, after which time bacterial colonies were harvested with 20 ml of LB–20% glycerol broth, pelleted, and stored at −80°C until further use.

Preparation of DNA for high-throughput sequencing

Tn–chromosome junctions were prepared and amplified for high-throughput sequencing following protocols described by Fu et al. (13).

Genomic DNA was extracted from 0.5-ml aliquots of each Tn library (eight in total: four intact NHS and four HI NHS samples), sheared into ~500-bp fragments by ultrasonication using a Covaris M220 system, end repaired (Quick Blunting kit; New England BioLabs), and A tailed by Taq polymerase (New England BioLabs). Adapter DNA sequences (Adapter M1.0 and Adapter M2.0; Supplemental Table III) were then ligated to the fragmented DNA and PCR reactions carried out to amplify DNA fragments containing the Tn–chromosomal junction sequences along with the appropriate sequences required for Illumina sequencing (such as P5 and P7 hybridization sequences and barcodes) using primers Tn5-PCR F and multiplex indices 1–12, depending on the number of samples pooled for sequencing (Supplemental Table III). The final PCR products were purified on a 2% agarose gel and DNA fragments of 200–400 bp in length were isolated. Equimolar DNA fragments from each library were combined and sequenced using the MiSeq reagent kit v2 (50 cycle; Illumina) along with the custom sequencing primer Tn5-Seq (Supplemental Table III).

Sequencing analysis

Reads from the Illumina sequencing run were first error-corrected by Musket (14) and then mapped to the *A. baumannii* AB5075-UW genome using the Burrows–Wheeler alignment tool (15), allowing for zero mismatches. Genes that had <10 mapped reads in samples incubated with HI NHS were excluded from further analysis. The combined read counts per gene were further normalized per million mapped reads to account for the differences in sizes of mapped reads among sequenced libraries. Then a genomic location bias correction was performed in each library as described by Chao et al. (16). During bacterial growth and replication, the amount of DNA close to the origin of replication (Ori) increases, which results in more DNA around the Ori becoming available for sequencing. This generally translates into a significant Tn positional bias that becomes apparent as a V-shape in the read counts per Tn relative to their genomic location, with an overall higher average of reads close to the Ori. Therefore, an initial positional bias correction was performed on each sequenced Tn pool to generate normalized sets of data that no longer possessed a skew toward the origin of the chromosome.

To normalize for chromosomal replication biases in read counts in all eight sequenced libraries, the entire AB5075 genome was first divided into 10-kb nonoverlapping windows starting with windows centered around the Ori. Then the number of reads within each of the windows was normalized based on a factor calculated from the average read depth within the window divided by the average depth for the chromosome.

After the genomic location bias correction was performed in each library, the average number of normalized, position-corrected reads in all four intact and HI NHS replicate studies was calculated for each gene and these values were used to calculate a HI NHS/intact NHS ratio, which corresponds to the fold change. Genes from intact NHS samples with less than one mapped read per gene were assigned an arbitrary value of 1 to calculate fold change values. Finally, *t* tests were used to assess the statistical significance of changes in the frequency of reads in HI and intact NHS libraries among all four replicate studies. Significance was defined by a p value of ≤ 0.05.

Protein sequences were mapped on the cluster of orthologous genes (COG) database (prokaryotic proteins) via RPSBLAST to identify COG families and classes using WebMGA server (17).

Construction of A. baumannii mlaD*::Tn-p*mla *and* mlaA*:: Tn-p*mlaA *complemented strains*

To construct the *A. baumannii mlaD*::Tn-p*mla* complemented strain, we first amplified the entire *mlaFEDCB* operon including 15 bp upstream of its putative start codon using primer pair XmaI-*mlaFEDCB*-F/PstI-*mlaFEDCB*-R (Supplemental Table III). The 3257-bp-long amplified PCR product was then column purified (Zymo Research) and digested with XmaI/PstI restriction enzymes (New England BioLabs) overnight at 37°C. The digested PCR product was then ligated into XmaI/PstI-restricted pMJG120 plasmid using T4 DNA ligase (New England BioLabs) to yield the complementation plasmid p*mla*. Plasmid p*mla* was transformed into electrocompetent *A. baumannii* AB5075 *mlaD*::Tn, to yield the *A. baumannii mlaD*::Tn-p*mla* complementation strain.

Similar protocols were used for the construction of the *A. baumannii mlaA*::Tn-p*mlaA* complementation strain. In brief, the primer pair XmaI-*mlaA*-F/PstI-*mlaA*-R (Supplemental Table III) was used to amplify a 915-bp amplicon corresponding to the *mlaA* gene plus 15 bp upstream of its putative start codon. The PCR product was then digested with XmaI/PstI restriction enzymes, ligated into pMJG120 (18) as outlined above to yield plasmid p*mlaA* and transformed into *A. baumannii mlaA*::Tn mutant strain to yield the complemented strain *A. baumannii mlaA*::Tn-p*mlaA*.

The correct sequence of the *mlaFEDCB* operon and *mlaA* gene inserts from plasmids p*mla* and p*mlaA*, respectively, was verified by sequencing using primer pair AN180/AN181 (Supplemental Table III).

Serum killing assays with NHS, C1q- and factor B–depleted sera, and normal mouse serum

Overnight cultures of wild-type (WT) *A. baumannii* AB5075 as well as *mlaA*::Tn and *mlaD*::Tn mutant strains were diluted 1:100 into fresh LB and grown to an OD_{650} of 0.4. In the case of *mlaA*::Tn-p*mlaA* and *mlaD*::Tn-p*mla*, these strains were first grown from overnight cultures to an OD_{650} of 0.4 then induced for 2 h with 10 mM isopropyl β-D-1-thiogalactopyranoside, at which point the OD_{650} of the cultures was measured and then diluted back to an OD_{650} of 0.4 with fresh LB to match the cell densities of WT AB5075 and *mlaA*::Tn and *mlaD*::Tn mutant strains. One-milliliter aliquots of these cultures were pelleted by centrifugation (16,000 × *g* 5 min), washed once with 1 ml of GVB^{++}, and cells were suspended in 100 μl of GVB^{++}. The serum-killing assay was performed by combining 45 μl of intact or HI NHS with 5 μl of bacteria prepared as before in a V-bottom, 96-well plate (Corning). Samples were incubated for 2 h at 37°C with shaking, and then the number of CFUs per milliliter was estimated by serial dilution and plating.

The susceptibility of *A. baumannii* AB5075, *mlaD*::Tn, and *mlaD*::Tn-p*mla* complemented strains was also evaluated against C1q- and factor B (FB)–depleted sera (Complement Technology) following exactly the protocol outlined above for NHS. C1q- and FB-depleted sera were also reconstituted by adding back purified C1q or FB at the physiological concentrations of 70 and 200 μg/ml, respectively, and tested against the *mlaD*::Tn as outlined above.

In some studies, serum susceptibility of *A. baumannii* Tn mutant strains was tested in normal mouse serum (NMS) following the protocols previously described. Owing to the unstable nature of the mouse classical pathway components (19), all serum assays involving mouse complement

were carried out with unfrozen NMS prepared from blood freshly collected from male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) following recommendations described by Lachmann (20).

Serum killing with cobra venom factor–depleted human sera

NHS was treated with 25 U/ml cobra venom factor (CVF) (21) (Quidel) following the manufacturer's recommendations or with an equal volume of PBS as control for 1 h at 37°C with shaking (200 rpm). PBS-NHS samples were then split into two aliquots, one HI and the other used directly as active sera, and 45 μl from each of these three serum samples was tested in the standard serum killing assay as outlined above against WT *A. baumannii* AB5075, *mlaD*::Tn, and *mlaD*::Tn-p*mla* complementation strains.

C4 deposition ELISA

The *A. baumannii mlaD*::Tn-p*mla* complemented strain was diluted 1:100 from an overnight culture, grown to an OD_{650} of 0.4, and induced for 2 h with isopropyl β-D-1-thiogalactopyranoside (10 mM), at which point the culture reached OD_{650} of ∼1. Similarly, overnight cultures of WT *A. baumannii* AB5075 and *mlaD*::Tn mutant strains were diluted 1:100 into fresh LB and grown to OD_{650} of 1 to match the final OD_{650} reached by the *mlaD*::Tn-p*mla* complemented strain. Then AB5075, *mlaD*::Tn, and *mlaD*::Tn-p*mla* cultures were diluted back to an OD_{650} of 0.4 with fresh LB. Equal volumes of these bacterial suspensions were centrifuged (16,000 × *g* for 5 min), and pellets resuspended in the same volume of coating buffer (40 mM sodium phosphate buffer [pH 7]) that were then used to coat high-binding ELISA plates (Santa Cruz Biotechnology) with 50 μl of bacterial suspension per well. As a positive control for activation of the lectin pathway, additional wells were coated with 50 μl of a 10 μg/ml solution of mannan (Sigma-Aldrich) in coating buffer. After overnight incubation at 4°C, ELISA plates were blocked with a 0.5% BSA solution in TBS (10 mM Tris-HCl, 140 mM NaCl [pH 7.4]) at 37°C for 1 h and then washed three times with 0.05% Tween 20 in PBS and incubated with 2-fold serial dilutions of C1q-depleted human sera in GVB^{++} for 1 h at 37°C. Plates were washed as above, and wells incubated with polyclonal goat anti-human C4 Abs (Complement Technology) were diluted 1:1000 in dilution buffer (0.05% Tween 20, 5 mM $CaCl_2$, 0.5% BSA in TBS) for 1 h at 37°C. Plates were then washed as above and incubated with secondary rat anti-goat alkaline phosphatase-conjugated Abs (Sigma-Aldrich) diluted 1:1000 in dilution buffer for 1 h at 37°C. Bound Ab was then quantified using the colorimetric substrate *p*-nitrophenyl phosphate (Santa Cruz Biotechnology) measuring OD at 405 nm.

Quantification of C5b-9 binding to A. baumannii *by flow cytometry*

One-milliliter cultures of WT *A. baumannii* AB5075, *mlaD*::Tn mutant, and *mlaD*::Tn-p*mla* complemented strain, prepared as above for serum killing assays, were centrifuged at 16,000 × *g* for 5 min, and pelleted cells were washed once with 1 ml of GVB^{++} buffer and concentrated 10-fold in GVB^{++}. Fifty-microliter aliquots of each of the three washed strains were then incubated with 0.45 ml of a 30% solution of either intact or HI NHS in GVB^{++} at 37°C for 1 h 15 min in a rotating shaker. After this time, the complement reaction was stopped by pelleting cells as above and washing cell suspensions once with 1 ml of ice-cold PBS.

To detect deposition of C5b-9, bacterial suspensions were pelleted by centrifugation and cells stained with a 50-μl solution containing the fluorescent nucleic acid stain SYTO-62 and a mouse mAb to a C9 neoantigen conjugated to FITC (clone aE11) at a final concentration of 30 μM and 10 μg/ml, respectively, in PBS for 1 h at 37°C with shaking (200 rpm). Control samples included unstained cells and cells stained with either SYTO-62 or FITC-conjugated mAb alone. The stained cells were then washed once with 0.5 ml of PBS, fixed with 10% neutral buffered formalin, and analyzed in a MACSQuant (Miltenyi Biotec) flow cytometer. Results were analyzed using FlowJo software (Tree Star).

Virulence of A. baumannii mlaD*::Tn strain in a mouse model of bloodstream infection in immunocompetent and complement-depleted mice*

The animal model of bloodstream infection used was previously described (22). In brief, C57BL/6J mice (n = 13–16 per group, male, 10 wk of age; The Jackson Laboratory) were injected i.p. with 15 μg of CVF (Complement Technology) or 100 μl of PBS as control 36, 24, and 12 h prior and i.v. challenge with 7.6×10^5 CFUs of *A. baumannii mlaD*::Tn strain. Two hours after infection mice were sacrificed, blood was collected via heart stick, and numbers of surviving bacteria were determined by serial diluting and plating of blood samples.

C3 quantification in pooled sera collected from PBS- and CVF-treated mice was performed using a C3 sandwich ELISA (GenWay Biotech) following the manufacturer's instructions.

Phospholipase C treatment

Phospholipase C treatment and TLC were performed using protocols previously described by Nakamura et al. (23). Five hundred milliliters of WT *A. baumannii* AB5075, *mlaD*::Tn mutant, and *mlaD*::Tn-*pmla* complemented strains was grown in LB as described above. Cultures (OD_{650} of 0.4) were then centrifuged at 6000 × *g* for 30 min, pelleted, washed once with 20 ml of a solution of 1× PBS, 0.4 M sucrose, and 15 mM $MgCl_2$ (pH 7.5), and resuspended in 1 ml of the same buffer. Cell suspensions were then treated with 25 U of phospholipase C from *Bacillus cereus* (Sigma-Aldrich) for 1 h at 37°C while mixing on a rotating shaker. After this time, bacterial cells were pelleted at 16,000 × *g* for 5 min and the lipids were extracted by the method of Bligh and Dyer (24). Lipid samples were then separated by TLC on glass-backed silica gel 60 plates (Merck) with chloroform/acetone 94:4 (v/v) solvent system, stained by iodine vapor for 30 min, and the intensity of spots was quantified using ImageJ software. The standard was 100 μg of 1,2-dipalmitoyl-sn-glycerol (Sigma-Aldrich).

Results

A. baumannii *is highly resistant to killing by NHS*

We evaluated the susceptibility to complement-mediated killing in NHS of 15 *A. baumannii* strains, including 5 multidrug-resistant strains from the Singapore General Hospital (strains S1–S5), 5 isolates from American soldiers wounded in the Iraqi war conflict (strains I25–I29), and 5 sequenced strains from multiple geographical locations with various degrees of virulence in animal models of infection (12, 25, 26). Strains were grown to mid-logarithmic phase and then incubated for 2 h in either undiluted, intact NHS, or HI NHS as a control. As depicted in Fig. 1, 12 out of the 15 strains were highly resistant to killing by NHS, indicating that serum resistance is a common feature among virulent *A. baumannii* strains.

Genetic basis of A. baumannii *serum resistance*

We used a Tn library in *A. baumannii* strain AB5075, created and provided to us by Dr. C. Manoil (11), to investigate the genetic basis of *A. baumannii* resistance to killing by human complement. AB5075 is a multidrug-resistant global clone 1 strain isolated in 2008 from a patient with an osteomyelitis infection (12). It displays high virulence in various animal models of infection and is amenable to genetic manipulation (12). These characteristics make AB5075 an attractive model strain for studying *A. baumannii* pathogenicity, including serum resistance. The library encompasses a total of 10,762 Tn mutants and includes 2–3 unique, sequence-verified Tn inserts in virtually all nonessential genes.

A Tn library pool of *A. baumannii* was prepared and then subjected to selection in intact and HI NHS to generate four independent biological replicates for each gene, followed by Illumina sequencing of Tn–chromosome junctions. Reproducibility was determined by comparing the number of mapped reads per Tn mutant among all four intact or HI NHS independent replicates. Data analysis revealed a Pearson correlation coefficient for the sequenced reads that ranged from 0.88 to 0.98 for samples in intact NHS and from 0.97 to 0.98 for samples in HI NHS (Supplemental Table I), indicating that the Tn sequencing (Tn-seq) results were highly comparable among all four independent experiments. In total, 3241 out of the 3470 Tn-disrupted genes (93%) were scored in this analysis. The remaining 229 genes could not be analyzed as they either completely failed to be recovered (109 genes) or had <10 reads per gene in the HI NHS sample libraries (120 genes), which was established as the cut-off read count needed for further analysis. These 229 genes most

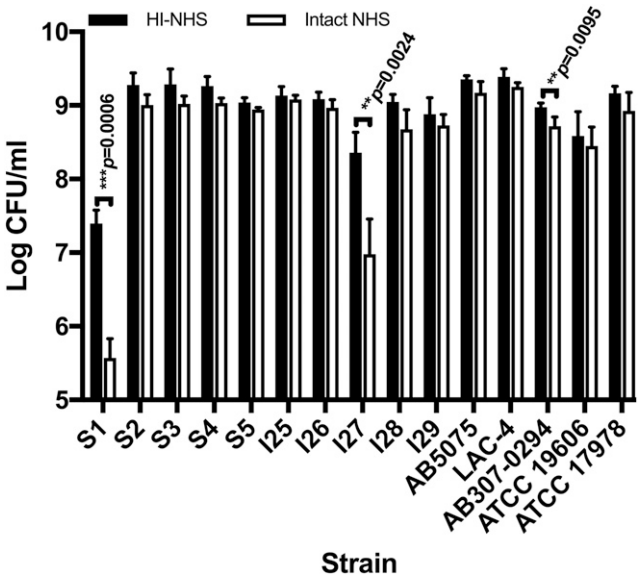

FIGURE 1. Serum sensitivity of *A. baumannii* strains in NHS. Fifteen *A. baumannii* strains were incubated for 2 h at 37°C in the presence of intact NHS (open columns) or HI NHS (solid columns). Data represent mean ± SD of three independent experiments. The *p* values were determined using a two-tailed, unpaired *t* test on $\log_{10}$-transformed data after normality was confirmed using the Shapiro–Wilk test.

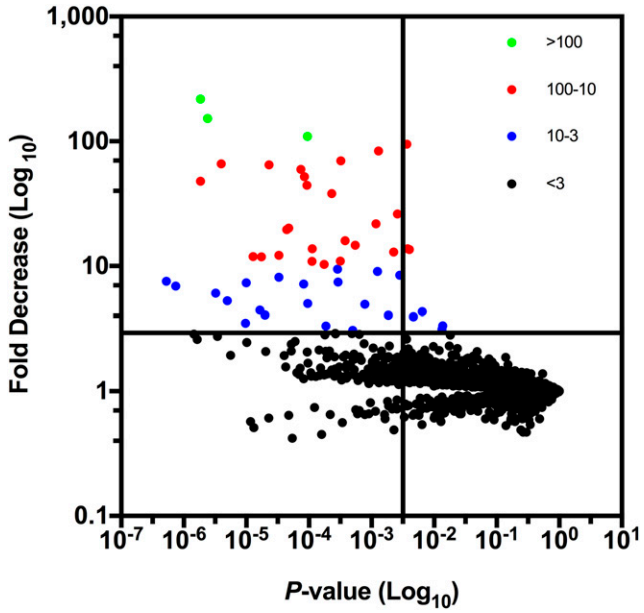

FIGURE 2. Fold decrease and *p* value for survival of *A. baumannii* Tn mutants in human serum based on ratio of normalized mapped reads in HI NHS versus intact NHS from four independent experiments. Each point represents a different Tn mutant and fold change. Mutants with >100, 100- to 10-, 10- to 3-, and <3-fold decreases in survival are shown in green, red, blue and black, respectively. The vertical and horizontal lines in the graph are drawn at $p = 0.05$ and 3-fold change, respectively.

likely represent Tn mutants that displayed a competitive growth disadvantage within the total Tn mutant library pool during the inoculum recovery period and therefore were represented at levels below the lower limit of gene detection by Tn-seq analysis. Alternatively, these genes may encode bacterial factors needed for the survival of *A. baumannii* to killing by some yet uncharacterized, complement-independent, heat-stable serum factors.

The average number of normalized reads in each intact or HI NHS data set was determined for every gene, and these values were used to calculate a ratio of reads between HI and intact NHS. Genes needed for serum resistance were defined as those that had a ≥3-fold decrease in mapped reads after dividing HI NHS by intact NHS values and *p* values of ≤0.05 ($n = 4$; two tails; unpaired *t* test). A total of 50 *A. baumannii* AB5075 genes were identified as needed for serum resistance (Fig. 2, top left quadrant, Table I). Of these, there were three genes with >100-fold decrease (6% of total scored; Fig. 2, green symbols) and 26 with a 10- to 100-fold decrease (52% of total scored; Fig. 2, red symbols) in Tn insert read abundance in HI NHS versus intact NHS. We also found an additional 21 genes with a 3- to 10-fold decrease in Tn insert read abundance (42% of total scored; Fig. 2, blue symbols). The remaining Tn insertions in 3191 genes did not meet the criteria for conferring serum resistance because of either limited decrease in read abundance (<3-fold decrease in HI NHS versus intact NHS) and/or lack of statistical differences shared among all four replicate studies ($p \geq 0.05$).

Predicted functions of gene products required for A. baumannii *serum resistance*

All genes identified as important for *A. baumannii* survival in human serum were subjected to bioinformatic analysis and classified into functional categories. The complete list of the functional categories of *A. baumannii* AB5075 serum resistance genes is presented in Table I. Genes belonging to the "cell wall/membrane/envelope biogenesis" category had the most impact on serum resistance, accounting for 28% of all genes identified (Fig. 3A, Table I). Among these we found four genes, ABUW_0383 (*mlaF*), ABUW_0384 (*mlaE*), ABUW_0385 (*mlaD*), and ABUW_3259 (*mlaA*), annotated as encoding proteins for the "maintenance of lipid asymmetry" (*mla*) pathway (27). We also identified the gene ABUW_0386 from the lipid transport and metabolism category, which encodes a putative phospholipid (PL)-binding protein MlaC that is part of the Mla pathway, as needed for serum resistance. In *E. coli,* the products of the *mlaFEDCB* operon and *mlaA* gene function together to remove PLs from the outer leaflet of the OM, which can accumulate under certain stress conditions (27, 28). The Mla pathway therefore restores the asymmetric lipid distribution of the OM with LPS and PLs in the outer and inner leaflets, respectively (27).

As expected, genes in the cell wall/membrane/envelope biogenesis category–encoding proteins needed for capsular polysaccharide biosynthesis, as well as their surface transport and retention, were identified as factors strongly contributing to serum resistance, as it is known that the capsule plays an important role in this phenotype (29). Validation studies carried out with two mutants in genes ABUW_3831 (*wza*) and ABUW_2898 (*wzi* homolog), which encode predicted OM proteins required for the surface transport and retention of capsule, respectively, demonstrated the critical role of capsule for *A. baumannii* survival in intact NHS. Tn inserts in these genes led to 7.6- and 3.8-$\log_{10}$ killing of ABUW_3831 and ABUW_2898 mutants, respectively, when comparing intact NHS versus HI NHS (both $p < 0.0001$; Fig. 4A).

Genes annotated in the categories of general function prediction, unknown function, and signal transduction mechanisms that are needed for capsule production were also identified following serum selection (ABUW_3822, ABUW_3825, homologs of *qhbA* and *wzy*, respectively, and ABUW_3832 [*ptp*]). Additionally, we identified a significant number of genes (20%) needed for serum resistance with unknown functional predictions (Fig. 3A, Table I).

Analysis of the subcellular localization of *A. baumannii* serum resistance gene products revealed that 34% of all products were

Table I. *A. baumannii* AB5075 Tn mutants with decreased survival in intact NHS versus HI NHS

AB5075 Locus Tag	Reads in HI NHS	Reads in Intact NHS	Fold Change: Reads in HI NHS/Intact NHS	*p* Value[a]	Gene Name	Annotation
Amino acid transport and metabolism						
ABUW_2418	246.53	46.70	5.28	4.97×10^{-6}		Lysine exporter protein
ABUW_2456	41.56	10.58	3.93	4.67×10^{-3}		Hydroxymethylglutaryl-CoA lyase
Carbohydrate transport and metabolism						
ABUW_1134	115.01	16.66	6.90	7.45×10^{-7}		Major facilitator superfamily MFS_1
ABUW_3041	194.12	9.64	20.15	4.77×10^{-5}	*algC*	Phosphomannomutase
Cell wall/membrane/envelope biogenesis						
ABUW_0090	14.50	2.02	7.17	8.20×10^{-5}	*glmU*	Bifunctional protein GlmU
ABUW_0383	170.98	4.48	38.13	2.31×10^{-4}	*mlaF*	Uncharacterized ABC transporter, ATP-binding protein YrbF
ABUW_0384	103.29	13.90	7.43	2.92×10^{-4}	*mlaE*	Uncharacterized ABC transporter, permease component YrbE
ABUW_0385	189.42	3.18	59.52	7.44×10^{-5}	*mlaD*	Putative phospholipid ABC transporter-binding protein MlaD
ABUW_0386[b]	304.05	5.83	52.14	8.47×10^{-5}	*mlaC*	Putative phospholipid-binding protein MlaC
ABUW_1868	89.41	9.87	9.06	1.25×10^{-3}		Hypothetical protein
ABUW_3259	156.77	1.43	109.68	9.43×10^{-5}	*mlaA*	VacJ family lipoprotein
ABUW_3448	31.65	3.07	10.31	1.75×10^{-4}		Glycosyl transferase, group 1
ABUW_3638	26.16	1	26.16	2.60×10^{-3}	*pbpG*	D-alanyl-D-alanine carboxypeptidase family protein
ABUW_3280	21.82	1	21.82	1.18×10^{-3}		Nucleotide sugar epimerase/dehydratase
ABUW_3824	64.65	1	64.65	2.31×10^{-5}		Family 1 glycosyl transferase
ABUW_3828	15.97	1	15.97	3.79×10^{-4}		Hypothetical protein
ABUW_3829	47.91	1	47.91	1.84×10^{-6}		Hypothetical protein
ABUW_3830	27.28	1.39	19.56	4.43×10^{-5}		UDP-glucose/GDP-mannose dehydrogenase
ABUW_3831	10.88	1	10.88	1.12×10^{-4}	*wza*	Polysaccharide export protein
Coenzyme transport and metabolism						
ABUW_2736	12.96	1	12.96	2.26×10^{-3}	*cinA*	Competence/damage-inducible protein CinA
Energy production and conversion						
ABUW_2489	13.74	1	13.74	1.13×10^{-4}	azo*R*	Acyl carrier protein phosphodiesterase
Function unknown						
ABUW_0135	188.72	15.50	12.17	3.32×10^{-5}		Signal peptide protein
ABUW_0986	13.49	1.60	8.42	2.83×10^{-3}		Restriction endonuclease
ABUW_1191	80.33	6.73	11.93	1.28×10^{-5}		Hypothetical protein
ABUW_1192	128.51	15.82	8.12	3.32×10^{-5}		Hypothetical protein
ABUW_2168	18.86	4.64	4.06	1.98×10^{-5}		Hypothetical protein
ABUW_2637	42.68	7.05	6.06	3.22×10^{-6}		Peptidase M15
ABUW_2735	83.53	1	83.53	1.29×10^{-3}		Hypothetical protein
ABUW_3758	69.64	1	69.64	3.23×10^{-4}	*dedA*	Protein DedA
ABUW_3825	14.71	1	14.71	5.50×10^{-4}		Hypothetical protein
ABUW_3881	11.85	1	11.85	1.74×10^{-5}		Matrixin superfamily
General function prediction only						
ABUW_1060	102.07	22.96	4.45	1.65×10^{-5}		Hypothetical protein
ABUW_2363	66.02	1	66.02	3.95×10^{-6}		Tetratricopeptide repeat family protein
ABUW_3822	10.96	1	10.96	3.16×10^{-4}		Bacterial transferase hexapeptide (three repeats) family
Inorganic ion transport and metabolism						
ABUW_2898	94.75	1	94.75	3.65×10^{-3}		Hypothetical protein
ABUW_3369	152.10	1	152.10	2.40×10^{-6}		Rhodanese domain protein
Intracellular trafficking, secretion, and vesicular transport						
ABUW_0532	44.31	1	44.31	9.32×10^{-5}	*yajC*	Preprotein translocase, YajC subunit
Lipid transport and metabolism						
ABUW_0312	49.27	14.06	3.51	9.64×10^{-6}	*ispH*	Hydroxymethylbutenyl pyrophosphate reductase
ABUW_0729	217.50	1	217.50	1.85×10^{-6}	*uppP*	Undecaprenyl-diphosphatase UppP

(*Table continues*)

Table I. (*Continued*)

AB5075 Locus Tag	Reads in HI NHS	Reads in Intact NHS	Fold Change: Reads in HI NHS/Intact NHS	*p* Value[a]	Gene Name	Annotation
ABUW_1737	26.29	5.25	5.01	9.58×10^{-5}	*uppS*	Undecaprenyl diphosphate synthase
ABUW_2957	181.73	59.25	3.07	4.98×10^{-4}		Enoyl-CoA hydratase/ isomerase
Mobilome: prophages, transposons						
ABUW_2662	57.04	7.55	7.56	5.25×10^{-7}		Hypothetical protein
Posttranslational modification, protein turnover, chaperones						
ABUW_3385	54.74	13.50	4.05	1.86×10^{-3}	*prc*	Carboxy-protease
ABUW_3642	36.05	3.83	9.42	2.85×10^{-4}		Putative periplasmic C-terminal protease
Signal transduction mechanisms						
ABUW_3302	35.38	7.14	4.95	7.79×10^{-4}	*relA*	GTP pyrophosphokinase (ppGpp synthetase I)
ABUW_3408	67.30	20.19	3.33	1.86×10^{-4}		Sel1 domain protein
ABUW_3639	11.93	2.76	4.31	6.46×10^{-3}	*gacA*	Response regulator
ABUW_3832	13.56	1	13.56	4.00×10^{-3}	*ptp*	Protein–tyrosine–phosphatase ptp
Translation, ribosomal structure and biogenesis						
ABUW_1608	13.74	1	13.74	3.75×10^{-3}	*miaA*	tRNA δ(2)-isopentenylpyrophosphate transferase
ABUW_1736	43.68	5.96	7.33	9.91×10^{-6}	*frr*	Ribosome recycling factor

[a]*t* test was used to compare normalized mapped reads between HI and intact NHS samples among four independent experiments and derive corresponding *p* values.
[b]Gene ABUW_0386 (*mlaC*) belongs to the lipid transport and metabolism category but as part of the *mlaFEDCB* operon was placed here for clarity.

predicted to be localized to the bacterial cell envelope (either cytoplasmic membrane, periplasm, or OM) and an additional 4% predicted to reside in an extracellular compartment. Thirty-four percent of the gene products had a predicted cytoplasmic localization and 28% had unknown bacterial cell localizations (Fig. 3B). That 38% of the genes encoding proteins were predicted to be either a cell wall or secreted factor indicates that the selection and sequencing strategies employed correctly identified many of the essential *A. baumannii* serum resistance factors, inasmuch as cell envelope components maintaining bacterial OM and cell surface integrity are known to promote resistance to killing by complement system components.

Validation of serum resistance genes not previously associated with this phenotype

Two additional Tn mutants encoding the cytoplasmic membrane proteins, DedA and an L-lysine permease (ABUW_2418), not previously associated with serum resistance, were selected for further validation in the serum sensitivity assay. DedA belongs to a partially characterized, highly conserved family of proteins that appears to play a role in multiple cellular processes, including cell division, temperature sensitivity, membrane lipid composition, envelope-related stress responses, loss of proton motive force, and virulence (30). The L-lysine exporter protein is involved in the efflux of excess L-lysine to control for intracellular levels of L-lysine (31, 32). Tn mutants in the DedA protein and L-lysine permease were decreased 69.6- and 5.2-fold, respectively, in HI NHS versus intact NHS (Table I). As seen in Fig. 4A, incubation of DedA protein and L-lysine permease Tn mutants in intact NHS resulted in ∼3.3- and 2.8-$\log_{10}$ of killing when compared with the HI NHS controls.

Tn mutants in four proteins annotated in the categories of unknown or general function predictions (ABUW_1192, ABUW_2363, ABUW_2735, and ABUW_3881) were additionally selected for validation in the complement killing assay. Tn insertions in these genes showed a strong decrease in viability in intact NHS versus HI NHS by Tn-seq (8.1-, 66-, 83.5-, or 11.8-fold reductions for ABUW_1192::Tn, ABUW_2363::Tn, ABUW_2735::Tn, and ABUW_3881 Tn mutants, respectively) and upon validation resulted in 0.6-, 3.2-, 1-, and 7.7-$\log_{10}$ reductions in CFU in intact NHS versus HI NHS (Fig. 4A, Table I). As a negative control, we tested a Tn mutant strain, ABUW_2540::Tn, with an unchanged number of mapped reads between HI NHS and intact NHS by Tn-seq (fold change of 1) and, as expected, it remained completely serum resistant. Taken together, these results highlight the accuracy of Tn-seq to predict the requirement of specific *A. baumannii* genes needed for human serum resistance.

In addition to human sera, we also investigated susceptibility of these eight selected *A. baumannii* Tn mutants to killing by freshly prepared, unfrozen NMS. As shown in Fig. 4B, all eight *A. baumannii* Tn mutant strains previously shown to be susceptible to NHS (Fig. 4A) were fully resistant to killing by intact NMS. We used a *P. aeruginosa* LPS-rough mutant strain, PAO1 *galU*, as a control to demonstrate functional activity of the NMS, as this strain is significantly killed by mouse complement (Fig. 4B).

Detailed analysis of the mla *pathway and* A. baumannii *serum resistance*

We next focused our attention on a set of five genes belonging to the Mla pathway. The genetic organization of the *mla* genes in *A. baumannii* AB5075 is depicted in Supplemental Fig. 1A. In *E. coli,* this pathway is comprised of five proteins (the periplasmic chaperone MlaC and the inner membrane (IM) MlaFEDB ATP-binding cassette family transporter that functions together with OM lipoprotein MlaA, encoded elsewhere on the chromosome, to prevent the accumulation of PLs in the outer leaflet of the OM (27). Mutations in the *mla* genes are not lethal but result in increased OM permeability (33–35). Specific Tn abundance reductions in HI NHS versus intact NHS were as follows (fold reductions are indicated in parentheses): *mlaF* (38.1), *mlaE* (7.4), *mlaD* (59.5), *mlaC* (52.1), *mlaB* (0.8), and *mlaA* (109.6).

To investigate the role of *mla* homolog genes in *A. baumannii* OM lipid asymmetry, we directly compared the content of surface exposed PLs of WT AB5075, *mlaD*::Tn mutant, and the complemented strain *mlaD*::Tn-p*mla* following whole-cell treatment with phospholipase C and detection of diacylglycerol released

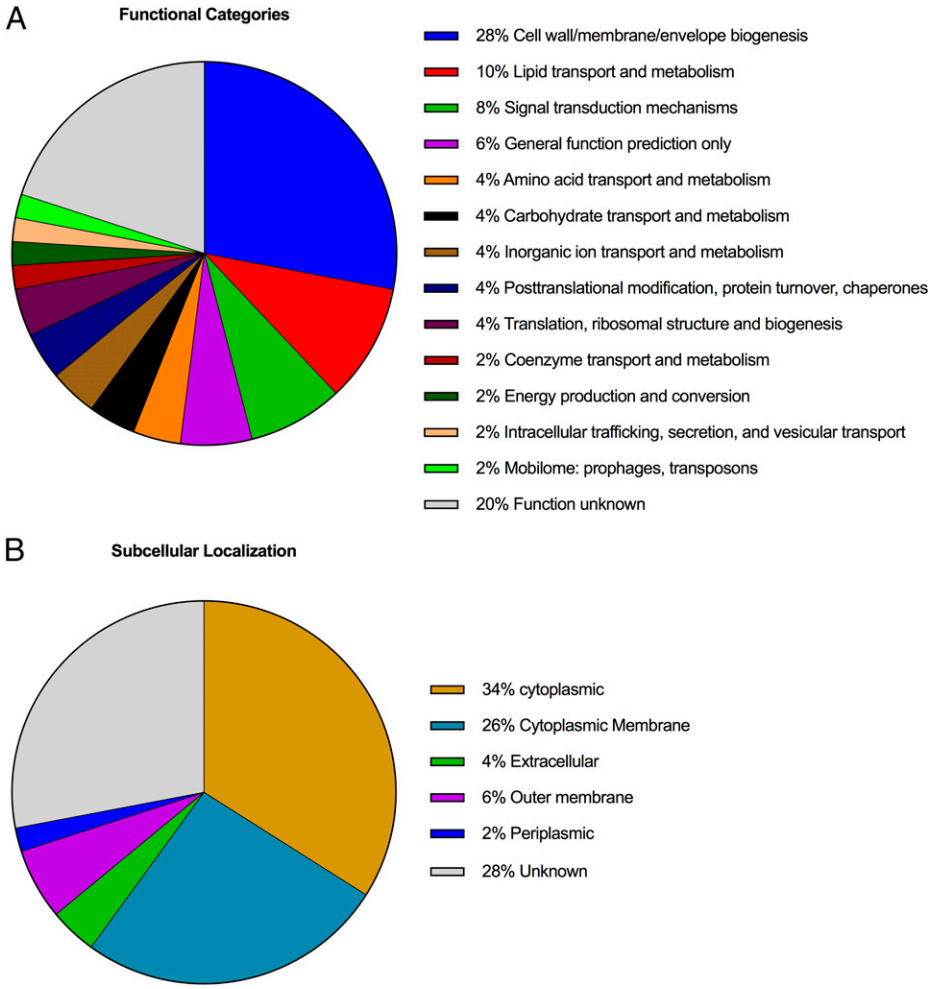

FIGURE 3. (**A**) Distribution into functional categories of 50 serum resistance genes in *A. baumannii* AB5075 identified by Tn-seq. Functional annotation was performed using the WebMGA tool that uses the RPSBLAST algorithm with the COG database for prokaryotic proteins. (**B**) Predicted subcellular localization of protein products encoded by Tn mutants identified by Tn-seq. Subcellular localization prediction was performed with the PSORTb v3.0 server.

from cells using TLC as described by Nakamura et al. (23). As seen in Supplemental Fig.1B, the overall amount of diacylglycerol released from the *mlaD*::Tn mutant strain was higher (spot density increase of 107%) than that extracted from WT AB5075 and *mlaD*::Tn-p*mla* complemented strain (spot density increase of 120%). This increase in surface-exposed PLs exhibited by the *mlaD*::Tn mutant strain was accompanied by a significant increased susceptibility of this strain to the detergent SDS when compared with WT AB5075 and *mlaD*::Tn-p*mla* strains (Supplemental Fig. 1C) but not against a sublethal concentration of SDS (0.0075%) plus increasing concentrations of EDTA, ranging from 1.5 to 0.00073 mM (data not shown). This indicates that similar to *E. coli* mutants in the *mla* pathway, the *A. baumannii mla* mutants have an increase in the OM permeability, rendering the cells susceptible to detergent by an increase in surface-exposed PLs (27).

To validate the predicted role of the Mla pathway in *A. baumannii* resistance to human complement, we compared the susceptibility in the serum killing assay of WT AB5075 strain and two Tn mutants in the *mlaD* and *mlaA* genes. We also tested two strains complemented with either the entire *mla* operon, *mlaD*::Tn-p*mla*, or the *mlaA* gene, *mlaA*::Tn-p*mlaA*. As shown in Fig. 5A, incubation of *A. baumannii mlaA*::Tn in intact NHS resulted in ~1.5-$\log_{10}$ of killing when compared with WT AB5075 ($p < 0.0001$) and the complemented strain, *mlaA*::Tn-p*mlaA* ($p < 0.0001$) tested under the same conditions or the same strain incubated in HI NHS ($p < 0.0001$). Similar results were seen when *mlaD*::Tn mutant and *mlaD*::Tn-p*mla* were tested in the assay. These results demonstrate the critical importance of the *mla* pathway system in maintaining full resistance of *A. baumannii* to killing by human sera.

Consistent with our previous observations of the lack of NMS bactericidal activity against *A. baumannii* Tn mutant strains, the *A. baumannii mlaD*::Tn strain was also resistant to killing by NMS (Fig. 5B).

Analysis of the virulence of the *A. baumannii mlaD*::Tn strain in a mouse model of bloodstream infection, comparing mice with an intact complement system to those depleted of complement component C3 following treatment with CVF did not result in a significant increase in *A. baumannii* blood burdens (Fig. 5C) The effectiveness of mouse C3 depletion by CVF treatment was confirmed by demonstrating a 93.5% reduction in C3 in serum of CFV-treated versus PBS control mice.

The discrepancy in *A. baumannii* susceptibility to NHS and NMS highlights a limitation of mouse models to evaluate microbial virulence when differences in mouse and human C systems lead to discrepant interactions with microbial pathogens. Moreover, similarly to NMS, serum samples obtained from various other animals species, including Wister and Lewis rats, rabbit, and guinea pigs, also failed to kill the *mlaD*::Tn mutant strain (data not shown), corroborating the human species specificity of our findings.

Role of specific complement components in killing of the A. baumannii mlaD*::Tn mutant strain*

We analyzed the contribution of the different complement system pathways to the susceptibility of WT *A. baumannii* AB5075,

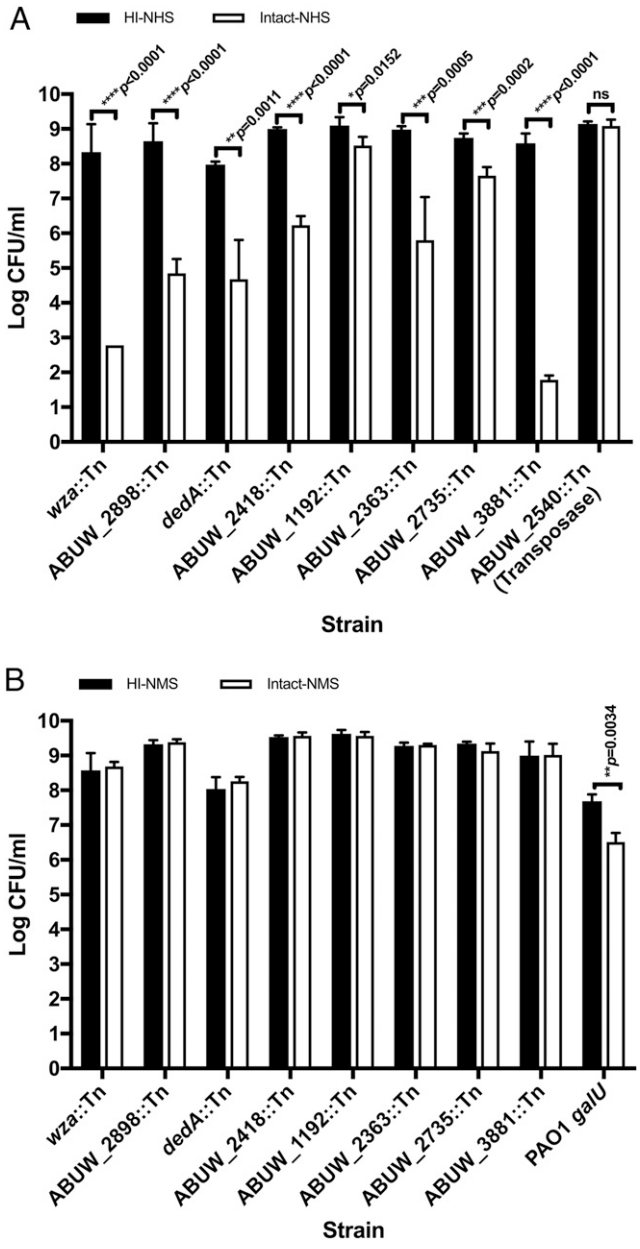

FIGURE 4. Validation of eight *A. baumannii* AB5075 genes identified by Tn-seq as required for human serum resistance. Susceptibility of Tn mutants tested in (**A**) NHS and (**B**) NMS using intact (open columns) or HI-sera (filled columns). Two strains, *A. baumannii* ABUW_2540::Tn and *P. aeruginosa* PA01 *galU* mutant, were included as negative and positive controls in the assays performed with NHS and NMS, respectively. Data represent the mean ± SD of three to five independent experiments. The *p* values were calculated using a two-tailed, unpaired *t* test on $\log_{10}$-transformed data after normality was confirmed using the Shapiro–Wilk test.

mlaD::Tn, and *mlaD*::Tn-p*mla* complemented strains to killing by NHS, using human sera selectively depleted of C1q or FB, needed for activation of the classical or alternative complement pathways, respectively. We further evaluated the deposition of the C4 component in C1q-depleted serum by an ELISA, indicative of the lectin pathway–mediated killing. As seen in Fig. 6A (left panel), the *A. baumannii mlaD*::Tn mutant remained susceptible to killing by human serum that was selectively depleted of C1q when compared with AB5075 and *mlaD*::Tn-p*mla* complemented strains. Furthermore, no significant increase in the killing of *A. baumannii mlaD*::Tn was observed when the C1q-depleted serum was reconstituted with C1q (70 μg/ml). In FB-depleted human serum the survival of the *A. baumannii mlaD*::Tn strain was similar to that of the WT AB5075 and *mlaD*::Tn-p*mla* complemented strains (Fig. 6A, right panel). Serum killing of the *mlaD*::Tn strain was, however, fully

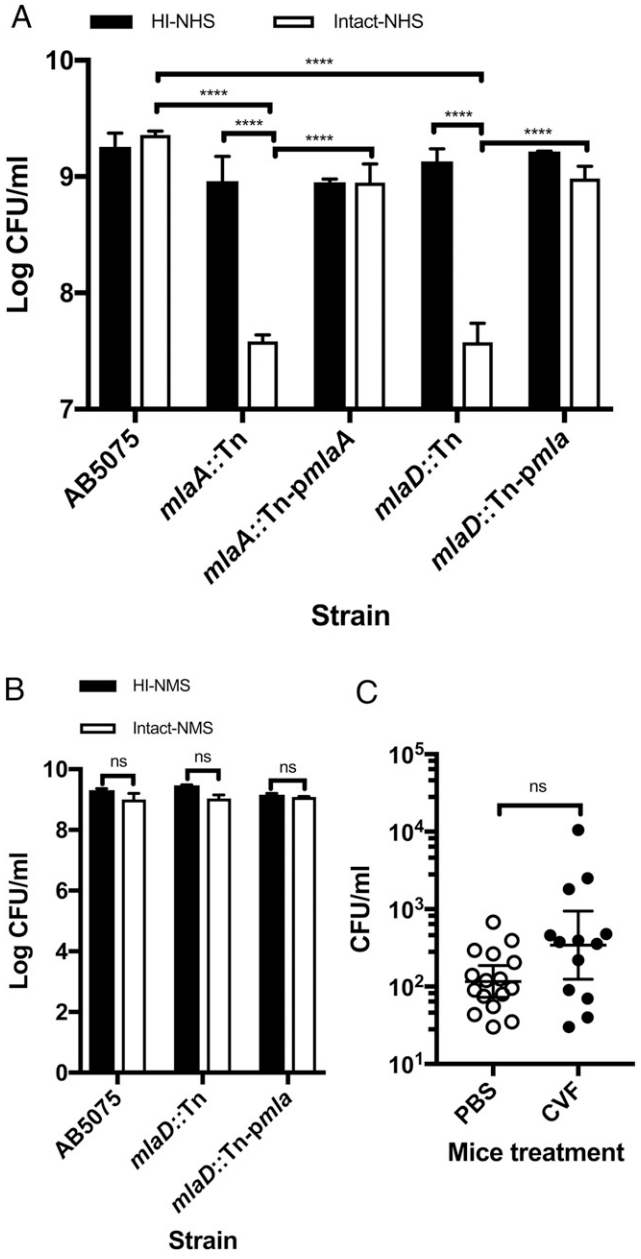

FIGURE 5. (**A**) Susceptibility of WT AB5075, *mlaA*::Tn, and *mlaD*::Tn mutants as well as *mlaA*::Tn-p*mlaA* and *mlaD*::Tn-p*mla* complemented strains in intact NHS (open columns) or HI NHS (filled columns). Columns represent the mean ± SD of three independent experiments. (**B**) Susceptibility of AB5075, *mlaD*::Tn mutant, and *mlaD*::Tn-p*mla* complemented strains in intact NMS (open columns) and HI-NMS (filled columns). Columns represent the mean ± SD of three independent experiments. (**C**) Survival of *A. baumannii mlaD*::Tn mutant strain in murine model of bloodstream infection in complement-depleted mice via treatment with CVF (solid symbols) or control animals given PBS (open symbols) (13–16 mice per group). Normality was confirmed using the Shapiro–Wilk test on $\log_{10}$-transformed data, and statistical significance was calculated using one-way ANOVA ($p < 0.0001$) followed by a Tukey post hoc test. Statistical comparison between PBS- and CFV-treated mice was performed using the two-tailed Mann–Whitney *U* test (C). ****$p < 0.0001$ (A) or two-tailed, unpaired *t* test (B).

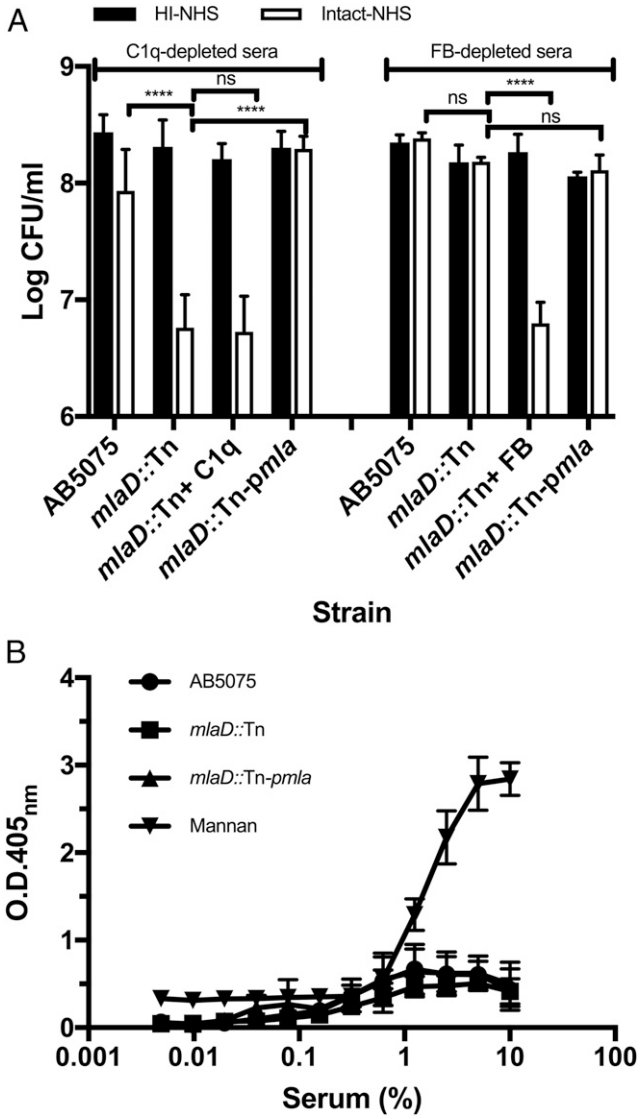

FIGURE 6. (**A**) Susceptibility of *A. baumannii* AB5075, *mlaD*::Tn mutant, and *mlaD*::Tn-p*mla* complemented strains to killing by C1q-depleted (left panel) or FB-depleted (right panel) human sera. *A. baumannii* strains were tested against intact (open columns) or HI (filled columns) C1q- or FB-depleted human sera. The susceptibility *A. baumannii mlaD*::Tn was also tested against C1q- or FB-depleted sera reconstituted with 70 or 200 μg/ml purified C1q or FB, respectively, either directly or after heat inactivation. Columns represent the mean ± SD of four to six independent experiments. After normality was confirmed using the Shapiro–Wilk test on $\log_{10}$-transformed data, statistical significance was calculated using one-way ANOVA ($p < 0.0001$) followed by a Tukey post hoc test. (**B**) C4 deposition onto *A. baumannii*. ELISA plates were coated with either *A. baumannii* AB5075, the *mlaD*::Tn mutant or the *mlaD*::Tn-p*mla* complemented strain or with mannan as a positive control, and incubated with serial dilutions of C1q-depleted sera at 37°C for 1 h. Bound C4b was detected with anti-C4 Abs. Results represent the means ± SD of four independent experiments. ****$p <$ 0.0001.

restored when FB-depleted sera were reconstituted with FB at 200 μg/ml (*mlaD*::Tn versus *mlaD*::Tn+FB; $p < 0.0001$). Measuring the relative deposition of the C4 serum component on *A. baumannii* strains in C1q-depleted human sera to determine whether the lectin pathway–mediated killing of the *mlaD*::Tn strain (Fig. 6B) showed that deposition of C4 was overall low and almost identical among all three *A. baumannii* strains, suggesting minimal activation of the lectin pathway by the *mlaD*::Tn mutant. C4 deposition via the lectin pathway was readily detected on the control Ag, mannan. These findings indicate that an aberrant Mla pathway results in susceptibility of *A. baumannii* to killing via the alternative pathway of complement.

Owing to the relevance of complement FH, as a key negative regulator of the alternative complement pathway in both the fluid phase and on cellular surfaces (36), we investigated the ability of WT AB5075 strain to bind FH and its potential contribution to the serum resistance of this strain. Results from FACS studies as well as complement killing assays performed with NHS and FH-depleted human sera revealed little to no binding of purified FH, tested at the physiologic concentration of 500 μg/ml, to WT AB5075. There was a similar killing activity of both intact and FH-depleted human sera against this strain (data not shown). Taken together, these results demonstrated that FH does not play a role in the resistance of AB5075 to killing by human complement.

To positively confirm that killing was mediated by the C3-dependent formation of the terminal C complex (TCC) C5b-9, human serum was depleted of complement component C3 using CVF, resulting in the complete abrogation of the killing of the *mlaD*::Tn strain (Fig. 7) and a susceptibility comparable to that of the same strain incubated in HI-NHS (Fig. 7). Conversely, CVF treatment did not disrupt the resistance of the WT AB5075 or *mlaD*::Tn-p*mla* complemented strains to NHS.

An evaluation by FACS of the deposition of the TCC components by intact and HI NHS on WT AB5075 (Fig. 8, top row), the *mlaD*::Tn mutant (Fig. 8, middle row) and the *mlaD*::Tn-p*mla* complemented strain (Fig. 8, bottom row) revealed little deposition of C5b-9 by intact NHS on WT AB5075 cells (17.3% positive cells) or the *mlaD*::Tn-p*mla* complemented strain (8% positive cells), whereas the serum-sensitive *A. baumannii mlaD*::Tn strain exhibited high levels of surface C5b-9 binding (61.4% of positive cells). There was only a very low level of C5b-9 deposition on the cells in HI NHS samples (Fig. 8, right column). Collectively, these results clearly demonstrate that *A. baumannii* Mla pathway mutants are killed via the alternative pathway–mediated deposition of the TCC deposition onto bacterial cells.

Discussion

The ability to resist complement-mediated killing is considered an important determinant of pathogenicity for many Gram-negative bacteria, and the results from this study demonstrate that serum resistance is widespread among *A. baumannii* strains. To expand our current understanding into the molecular mechanisms underlying serum resistance in *A. baumannii*, we conducted a Tn-seq analysis using a mutant library created in the serum-resistant strain AB5075. Findings from this screen identified 50 novel complement resistance genes of *A. baumannii*, and follow-up studies confirmed the role of eight of these genes in resistance to killing by NHS, implicating the remaining genes as also likely to encode proteins providing serum resistance to this organism. Interestingly, parallel studies conducted in NMS revealed that none of these eight *A. baumannii* Tn mutant strains was susceptible to killing by mouse complement, indicating species specificity of this organism's interactions with the complement components. Additionally, depleting functional complement activity in vivo in mouse sera did not enhance the virulence of *A. baumannii*. This discrepancy in human/mouse serum killing could be explained in terms of concentrations of complement proteins, which are significantly lower in mouse than in human sera (37) or differences in complement regulatory systems between these species (38, 39). The ability to recruit serum complement regulatory proteins to microbial surfaces is a prominent mechanism of complement evasion used by many pathogens (40), and this binding occurs, for most complement regulatory proteins, in a

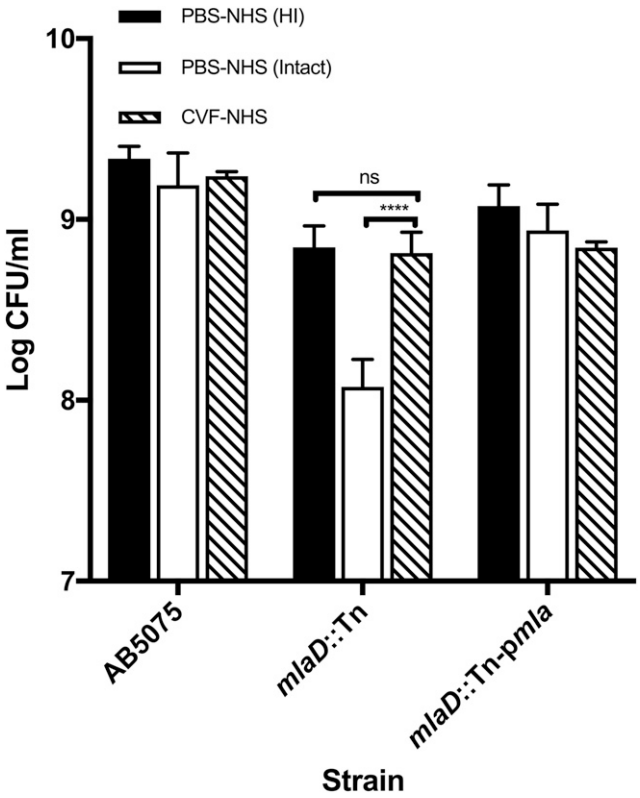

FIGURE 7. Effect of CVF depletion of C3 on NHS killing activity of WT *A. baumannii* AB5075, *mlaD*::Tn, and complemented *mlaD*::Tn-p*mla* strains. Following CVF (striped columns) or PBS treatment (open columns), sera were tested against *Acinetobacter* strains in complement killing assays. Also tested in the assay was a PBS-treated NHS sample following HI (solid columns). Data represent mean ± SD of three independent experiments. Statistical significance was calculated using one-way ANOVA ($p < 0.0001$) followed by a Tukey post hoc test after normality was confirmed using the Shapiro–Wilk test on $\log_{10}$-transformed data. ****$p < 0.0001$.

species-specific manner (41). Overall, the results in the present study highlight the limitations of murine systems for studying serum resistance and virulence of human pathogens, and they also show the utility of working within a human system with the genetic and analytic tools used in our study.

In contrast to genes encoding proteins that synthesize the capsular polysaccharide, Tn mutants in LPS biosynthesis and export genes were not identified as essential for serum resistance in our Tn-seq screen. This absence is consistent with the fact that at least 19 genes belonging to LPS biosynthesis and export pathways in AB5075 were classified as putative essential genes by Gallagher et al. (11) in AB5075 and are either absent from the AB5075 Tn library or the Tn inserts are located at the extreme 3′ or 5′ end of coding sequences, suggesting that these inserts do not disrupt the function of the protein encoded by such a gene.

One of the most interesting factors identified in this study contributing to *A. baumannii* survival in human sera was the *mla* system. The Mla pathway is an intermembrane PL transport system whose main function is to maintain the asymmetry of the OM of Gram-negative bacteria by preventing the accumulation of PLs in the outer leaflet of the OM by transporting them to the IM (27). In the Mla system the lipoprotein MlaA participates in the extraction of PLs from the outer leaflet of the OM, transferring them to the periplasmic transporter MlaC, which then delivers the substrate to the MlaFEDB transporter complex that finally reinserts them into the IM (27).

Similarly, to other bacterial species (23, 27), this study demonstrated the role that the *mla* pathway of *A. bamannii* plays in preventing the accumulation of surface-exposed PLs and maintaining the barrier function of the OM of this bacterium (Supplemental Fig. 1B, 1C).

In the present study, we validated the contribution of the Mla pathway to *A. baumannii* resistance to human serum using Tn mutants in their coding sequences, as well as their corresponding complemented strains, targeting two of the system's components: the initial OM lipoprotein MlaA and the cytoplasmic membrane protein MlaD, which is part of the terminal MlaFEDB transporter complex. Additional complement killing assays carried out in NHS pretreated with CVF, used to selectively deplete C3 protein, validated the complement system as the killing effector of *A. baumannii mla* mutants. This finding was further supported by FACS studies showing significantly higher levels of the C5b-9 terminal complex deposited by intact NHS on the *mlaD*::Tn mutant strain than on WT AB5075 or the *mlaD*::Tn-p*mla* complemented strain or the three strains incubated with HI NHS. Additional complement killing studies conducted with C1q- and FB-depleted human sera, which are defective in the classical and alternative complement pathways, respectively, as well as ELISA C4 deposition assays on *Acinetobacter* strains by C1q-depleted sera, used to measure lectin pathway activation, conclusively demonstrated the exclusive role of the alternative pathway in the killing activity of NHS against the *A. baumannii mlaD*::Tn mutant strain.

Taken together, these results highlight the importance of the Mla system of *A. baumannii* in maintaining full resistance against complement killing. Our findings are consistent with a previous study reporting the role of *mla* homolog genes in maintaining resistance of nontypable *Haemophilus influenza* to killing by human sera (23). However, whereas in this study the expression of the *mla* genes protects *A. baumannii* against killing by the alternative complement pathway, in nontypable *H. influenza* the loss of serum resistance in *mla* mutants was mediated by the activation of classical pathway via binding of natural IgMs in serum to surface oligosaccharide epitopes (23). The nature of the surface structures of *A. baumannii mla* mutant strains triggering the activation of the alternative pathway remains to be elucidated. However, because the hallmark of *mla* mutations in Gram-negative bacteria is the accumulation of surface-exposed PLs, it would be possible to speculate that these macromolecules could indeed be playing a key role activating the alternative pathway of the complement system in the *A. baumannii mlaD*::Tn mutant strain. Future studies, however, will be needed to further investigate this hypothesis.

The identification of bacterial factors conferring resistance to killing by NHS represents a potentially new approach to vaccine and drug development. The recent licensing of two new vaccines in Europe and the United States against serogroup B *N. meningitidis* that contain FH-binding protein from *N. meningitidis* as one of its main active components validates the vaccine development pathway by targeting bacterial factors involved in complement resistance. Alternately, our approach could aid in finding drugs that might have minimal bactericidal activity on their own but would nonetheless be effective in treating infections by enhancing serum sensitivity by binding to and disrupting the function of bacterial factors needed for serum resistance.

In conclusion, the availability of a high-throughput screening technology and data analysis pipeline such as the one used in this study has proven highly successful in identifying novel essential genes and functional factors needed for *A. baumannii* serum resistance. Future studies will be needed to unravel the exact

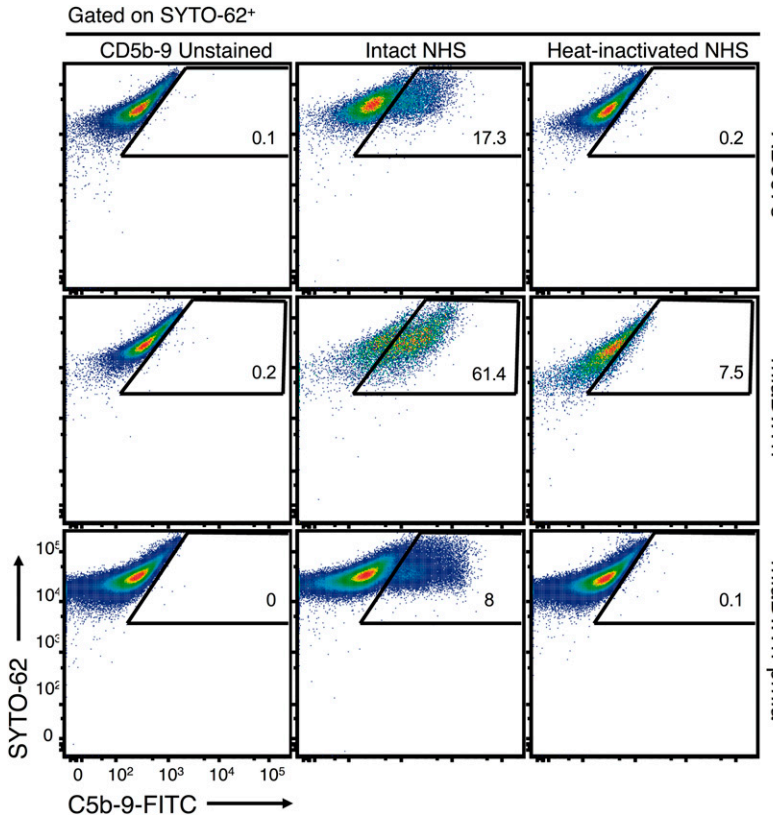

FIGURE 8. C5b-9 deposition on *Acinetobacter* strains measured by flow cytometry. Representative flow cytometry dot plots of C5b-9 deposition on WT *A. baumannii* AB5075 (top row), the *mlaD*::Tn mutant (middle row), or the *mlaD*::Tn-p*mla* complemented strain (bottom row) are shown. C5b-9 deposition was measured after the incubation of bacterial strains with either 30% intact NHS (middle column) or HI NHS (right column) for 1 h 15 min followed by labeling with a FITC-conjugated mouse mAb to a C5b-9 neoantigen (*x*-axis) and the nucleic acid stain SYTO 62 (*y*-axis). The percentage of FITC-positive cells in all strain/treatment combinations, which is indicated in every panel, was calculated after gating on a C5b-9 unstained, SYTO 62-positive cell population (left column).

roles of these uncharacterized genes in serum resistance and investigate their potential as targets for vaccine or antimicrobial development.

Disclosures

The authors have no financial conflicts of interest.

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