RESEARCH ARTICLE

Defining Gene-Phenotype Relationships in *Acinetobacter baumannii* through One-Step Chromosomal Gene Inactivation

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ABSTRACT Rates of infection with hospital-acquired *Acinetobacter baumannii* have exploded over the past decade due to our inability to limit persistence and effectively treat disease. *A. baumannii* quickly acquires antibiotic resistance, and its genome encodes mechanisms to tolerate biocides and desiccation, which enhance its persistence in hospital settings. With depleted antibiotic options, new methods to treat *A. baumannii* infections are desperately needed. A comprehensive understanding detailing *A. baumannii* cellular factors that contribute to its resiliency at genetic and mechanistic levels is vital to the development of new treatment options. Tools to rapidly dissect the *A. baumannii* genome will facilitate this goal by quickly advancing our understanding of *A. baumannii* gene-phenotype relationships. We describe here a recombination-mediated genetic engineering (recombineering) system for targeted genome editing of *A. baumannii*. We have demonstrated that this system can perform directed mutagenesis on wide-ranging genes and operons and is functional in various strains of *A. baumannii*, indicating its broad application. We utilized this system to investigate key gene-phenotype relationships in *A. baumannii* biology important to infection and persistence in hospitals, including oxidative stress protection, biocide resistance mechanisms, and biofilm formation. In addition, we have demonstrated that both the formation and movement of type IV pili play an important role in *A. baumannii* biofilm.

IMPORTANCE *Acinetobacter baumannii* is the causative agent of hospital-acquired infections, including pneumonia and serious blood and wound infections. *A. baumannii* is an emerging pathogen and has become a threat to public health because it quickly develops antibiotic resistance, making treatment difficult or impossible. While the threat of *A. baumannii* is well recognized, our understanding of even its most basic biology lags behind. Analysis of *A. baumannii* cellular functions to identify potential targets for drug development has stalled due in part to laborious genetic techniques. Here we have pioneered a novel recombineering system that facilitates efficient genome editing in *A. baumannii* by single PCR products. This technology allows for rapid genome editing to quickly ascertain gene-phenotype relationships. To demonstrate the power of recombineering in dissecting *A. baumannii* biology, we use this system to establish key gene-phenotype relationships important to infection and persistence in hospitals, including oxidative stress protection, biocide resistance, and biofilm formation.

*A*cinetobacter baumannii is an increasingly problematic hospital-associated opportunistic pathogen. It rapidly acquires antibiotic resistance, leading to the spread of multidrug-resistant strains impervious to nearly all antibiotic treatments (1–3). Exacerbating this problem, *A. baumannii* shows a robust ability to persist on a variety of clinical surfaces despite desiccation and biocide treatment, which facilitates its transfer to new patients (4, 5). While a substantial amount of clinical data has documented the global rise of *A. baumannii* as an antibiotic-resistant pathogen, dissection of the molecular details of *A. baumannii* biology that could be used to combat its spread are lagging. Aside from antibiotic resistance, very little in known about basic *A. baumannii* biology or the role of specific biological factors and cellular processes that directly facilitate its infections and pathogenesis (6).

This discrepancy between the observed clinical impacts of *A. baumannii* and our mechanistic knowledge of its virulence is in part due to the lack of specific and rapid means to genetically manipulate *A. baumannii* to define and investigate precise gene-phenotype relationships.

Recombination-mediated genetic engineering (recombineering) is a quick and efficient way to perform rapid genome editing *in vivo*. Recombineering is mediated by phage proteins that belong to either the Red proteins of phage λ (Gam, Beta, and Exo) or the RecET proteins from the Rac prophage, which catalyze homologous recombination between short DNA segments. Both of these systems are functionally equivalent, with Exo/RecE exonucleases exposing 3′ single-stranded DNA that Beta/RecT bind and pair with the homologous genomic target region (7, 8). The Red sys-

tem also encodes a Gam protein that inhibits host nucleases and is absent from RecET systems. The *Escherichia coli* λ Red system is the most well-studied recombineering system (9, 10). The development of recombineering revolutionized *E. coli* genetics, allowing rapid gene substitutions and deletions in a single step without the need for restriction enzymes and building editing constructs *in vitro* (9, 10). Recombineering rapidly accelerated our genetic analysis of *E. coli*, solidifying *E. coli* as a model bacterium and facilitating the prominence of *E. coli* for use in synthetic biology and biotechnology. The development of recombineering for *A. baumannii* would dramatically advance our ability to genetically interrogate this emerging pathogen.

Here we exploited a RecET recombinase system to recombineer genes in the *A. baumannii* genome. We demonstrate the utility of this simple, one-step system for rapidly creating marked or unmarked deletion of genes and operons. Importantly, this system is portable, and with the appropriate choice of selection cassettes, we show that it can be used in different *A. baumannii* strains. We establish the utility of this system by exploring gene-phenotype relationships in *A. baumannii* biology that are important to infection and persistence in hospitals using recombineering. These phenotypic mechanisms include oxidative stress protection, biocide resistance, and biofilm formation. Interestingly, our analysis demonstrates that both the biogenesis and functional role of type IV pili play an important part in *A. baumannii* biofilm formation.

RESULTS

Construction of a recombineering system for *A. baumannii*. The purpose of this study was to develop a recombineering system in *A. baumannii* and show its utility in elucidating gene-phenotype relationships. We chose to begin with the ATCC clinical *A. baumannii* strain 17978 because of its wide use and well-annotated genome sequence (11). All strains and plasmids used in this study are listed in Table 1. Initially, we attempted to use the λ Red (*gam*, *beta*, and *exo*) recombineering system of *E. coli* (9). These genes were cloned into plasmid pMMB67EH, which we found allows for isopropyl β-D-1-thiogalactopyranoside (IPTG)-induced gene expression in *A. baumannii* (see Fig. S1A in the supplemental material). However, the *E. coli* λ Red system did not operate for recombineering in *A. baumannii*. This result is reasonable considering the organism-specific biases that have been observed for other phage recombinase systems (12, 13). Furthermore, we found that IPTG-induced expression of the *E. coli* λ Red system actually slowed the growth of *A. baumannii*, suggesting that it may be toxic when induced from pMMB67EH (see Fig. S1B).

Using the Basic Local Alignment Search Tool (BLAST) algorithm, we identified several RecT *A. baumannii* homologs, including a strong homolog to the *E. coli* λ Red Beta protein in the shotgun-sequenced *A. baumannii* strain IS-123 (gene ACINIS123_2461; E value 9e^{-75}). The adjacent gene in IS-123, ACINIS123_2462, was part of the *yqaJ*/*recE* superfamily of exonucleases and had sequence similarity to the Exo protein from the *E. coli* λ Red system (E value 2e^{-16}), suggesting that this gene pair may represent a recombination system in *A. baumannii*. We did not identify a Gam homolog in any *A. baumannii* strain. The RecET gene pair from IS-123 was cloned into the IPTG-inducible pMMB67EH vector for expression in *A. baumannii*. We call this the Rec$_{Ab}$ system. IPTG-induced expression of the Rec$_{Ab}$ system

did not affect growth of *A. baumannii* strain 17978 (see Fig. S1B in the supplemental material). We used *A. baumannii* 17978 expressing the Rec$_{Ab}$ system for the majority of these experiments.

Application of Rec$_{Ab}$ for gene replacement. Four regions of DNA that contain genes of various sizes and encoding different biological functions were selected from the *A. baumannii* 17978 genome for targeted deletion: A1S_2456 (LysR-type regulator), A1S_1750 (*adeB*), A1S_1030-A1S_1031 (*lon*), and A1S_2063 (*aceI*). We identified an error in the published *A. baumannii* 17978 genome that created an artificial frameshift in the gene encoding the Lon protease. This resulted in *lon* being annotated by two genes, A1S_1030 and A1S_1031. We chose this gene set because they represent genes of various lengths, location in the genome, and functions. A complete list of genes deleted in this study, along with their sizes and locations, is provided in Table 2. Through a commercial supplier (Integrated DNA Technologies), we synthesized oligonucleotides containing 125 bases flanking the coding sequence (CDS) of each targeted gene, including the ATG start codon or the stop codon (Fig. 1). We added an additional 18 to 25 nucleotides (nt) on the 3′ end of each primer to facilitate amplification of a PCR product with an antibiotic selection cassette flanked by the 125 bp upstream and downstream of the target coding sequence. A kanamycin resistance marker was amplified from TOPO TA 2.1 (Invitrogen) to create our recombineering PCR products.

Following transformation of the PCR product in *A. baumannii* cells expressing Rec$_{Ab}$, we successfully isolated colonies where each targeted gene was replaced with the kanamycin cassette (Fig. 2A). We did not identify any positive colonies if the Rec$_{Ab}$ system was not expressed. Eighty to one hundred percent of screened colonies had successfully recombined at the desired locus with some variation of efficiency among the target genes. The few false-positive colonies that grew on kanamycin but still carried the parental copy of the target gene were universally smaller than their true-positive counterparts. PCR screening showed that none of the false-positive colonies contained a kanamycin cassette. Furthermore, none of the false-positive colonies grew when streaked on fresh kanamycin agar plates.

Using primers that flank the target coding sequences, we sequenced the recombination site of two positive colonies for each gene target. The majority had the correct sequence; however, we did identify two colonies that had errors in the region of chemically synthesized homology, indicating that sequencing of the recombineering junction should be performed to validate fidelity of the synthesized DNA. These results indicate that the Rec$_{Ab}$ system is applicable genome-wide, efficient, and accurate.

The plasmid carrying Rec$_{Ab}$ is unstable in the absence of ampicillin selection. Colonies identified as positive for gene replacement with the kanamycin cassette were streaked on kanamycin plates overnight, and the resulting single colonies were patched to kanamycin and ampicillin plates. Fifty to seventy percent of the colonies tested had lost the Rec$_{Ab}$ plasmid, indicating that this plasmid can be easily cured after recombineering (data not shown).

To optimize the recombineering parameters in *A. baumannii*, we tested the length of homology required for efficient recombineering using the Rec$_{Ab}$ system. While PCR products with less that 75-bp regions of homology did not produce any recombinants under the conditions tested, PCR products with 100 bp of homology did produce recombinants. However, fragments with

TABLE 1 Strains and plasmids

Strain/plasmid	Relevant genotype and property	Source and/or reference
Strains		
A. baumannii strain 17978	Wild type	ATCC (39)
A. baumannii strain 19606	Wild type	ATCC
AT01	*A. baumannii* 17978 carrying pAT01	This study
AT02	*A. baumannii* 17978 carrying pAT02	This study
AT03	*A. baumannii* 17978 carrying pAT03	This study
AT04	*A. baumannii* 19606 carrying pAT04	This study
AT05	*A. baumannii* 19606 Δ*lon*::Kan^r	This study
AT06	*A. baumannii* 17978 Δ2456::Kan^r	This study
AT07	*A. baumannii* 17978 Δ*lon*::Kan^r	This study
AT08	*A. baumannii* 17978 Δ*lon*::FRT	This study
AT09	*A. baumannii* 17978 Δ*oxyR*::Kan^r	This study
AT10	*A. baumannii* 17978 Δ*oxyR*::Kan^r carrying pAT05	This study
AT11	*A. baumannii* 17978 carrying pABBR_MCS	This study
AT12	*A. baumannii* 17978 Δ*oxyR*::Kan^r carrying pABBR_MCS	This study
AT13	*A. baumannii* 17978 Δ*aceI*::Kan^r	This study
AT14	*A. baumannii* 17978 Δ*aceI*::Kan^r carrying pAT06	This study
AT15	*A. baumannii* 17978 Δ*aceI*::Kan^r carrying pABBR_MCS	This study
AT16	*A. baumannii* 17978 Δ*adeB*::Kan^r	This study
AT17	*A. baumannii* 17978 Δ*adeB*::FRT	This study
AT18	*A. baumannii* 17978 Δ*adeB*::Kan^r carrying pAT07	This study
AT19	*A. baumannii* 17978 Δ*adeB* Δ*aceI*::Kan^r	This study
AT20	*A. baumannii* 17978 Δ*adeB*::Kan^r carrying pABBR_MCS	This study
AT21	*A. baumannii* 17978 Δ*pilH*::Kan^r	This study
AT22	*A. baumannii* 17978 Δ*pilH*::Kan^r carrying pMMB67EH	This study
AT23	*A. baumannii* 17978 Δ*pilH*::Kan^r carrying pAT08	This study
AT24	*A. baumannii* 17978 carrying pMMB67EH	This study
AT25	*A. baumannii* 17978 Δ*pilG*::Kan^r	This study
AT26	*A. baumannii* 17978 Δ*pilUT*::Kan^r	This study
Plasmids		
pMMB67EH	Amp^r	ATCC (40)
TOPO TA 2.1	Kan^r	Invitrogen
pKD4	Kan^r	9
pWH1266	*Acinetobacter* plasmid	Lab stock
pBR322	General cloning plasmid	Lab stock
pABBR_MCS	Amp^r	This study
pAT01	pMMB67EH with *E. coli* λ-Red system	This study
pAT02	pMMB67EH with Rec$_{Ab}$ system	This study
pAT03	pMMB67EH with FLP recombinase	This study
pAT04	pMMB67EH with Rec$_{Ab}$ system, Tet^r	This study
pAT05	pABBR_MCS carrying *oxyR* with native promoter (p_*oxyR*)	This study
pAT06	pABBR_MCS carrying *aceI* with native promoter	This study
pAT07	pABBR_MCS carrying *adeB* with native promoter	This study
pAT08	pMMB67EH carrying *pilH* (p_*pilH*)	This study

125 bp of homology displayed a 1- to 2-log increase in recombination efficiency. Next, we tested recombineering efficiency using 100 ng, 500 ng, 1 μg, 5 μg and 10 μg of PCR product, but we did not observe any recombinants using less than 5 μg of PCR product. Using 10 μg of PCR product did not increase the efficiency of recombinant production compared with that with 5 μg. When using electrocompetent cells at a density of 10^{10} CFU/reaction and 5 μg of PCR product with 125 bp of flanking homology, we obtained approximately 100 colonies from each transformation, with a range of 20 to 200 colonies, depending on the gene being targeted for replacement.

Engineering of markerless mutants using an FLP/FRT recombinase system. The antibiotic resistance marker can be removed by incorporating FLP recognition target (FRT) sites that flank the antibiotic cassette and expressing the FLP recombinase (14). To do this, the FRT site-flanked kanamycin cassette from plasmid pKD4 is amplified and used for targeted gene replace-

ment as described above (9). After selection and isolation of an *A. baumannii* mutant that was cured of Rec$_{Ab}$, plasmid pMMB67EH carrying the FLP recombinase is transformed into the strain carrying the FRT-flanked gene replacement cassette. Colonies carrying the FLP recombinase are streaked on solid agar containing 1 mM IPTG, which induces expression of the FLP recombinase, to result in excision of the kanamycin resistance cassette and simultaneous loss of the unstable pMMB67EH plasmid. To demonstrate this technique, *adeB* and the *lon* genes were initially replaced with the FRT-flanked kanamycin cassette (Fig. 2B, lane 1 versus lane 2 and lane 4 versus lane 5, respectively). The kanamycin resistance cassette was removed by transformation of the pMMB67EH plasmid and activation of the FLP recombinase enzyme (Fig. 2B, lanes 3 and 6).

Application of Rec$_{Ab}$ in *A. baumannii* strain 19606. To determine if this system could be used in other *A. baumannii* strains, we attempted to delete *lon* in another widely used clinical *A. bauman*-

TABLE 2 Deleted genes in this study

Gene name	Gene location	Length (bp)	Locus tag	Accession no.
lon	1191086–1188755	2,331	A1S_1030	YP_001084064.1
			A1S_1031	YP_001084065.1
lon[a]	Contig sidl23403laccninz.GG704573	2,576	HMPREF0010_01568	GG704573.1
			HMPREF0010_01569	
LysR family transcriptional regulator	2844859–2845533	675	A1S_2456	YP_001085476.1
aceI	2406038–2406508	471	A1S_2063	YP_001085092.1
adeB	2039428–2036447	2,982	A1S_1750	YP_001084779.1
oxyR	1150365–1151153	789	A1S_0992	YP_001084026.1
pilUT	1036617–1038800	2,184	A1S_0896	YP_001083930.1
			A1S_0897	YP_001083931.1
pilH	3259867–3260118	252	A1S_2814	YP_001085822.1
pilG	3260253–3260606	354	A1S_2815	YP_001085823.1

[a] *A. baumannii* strain 19606.

nii strain, ATCC 19606. We observed that strain 19606 is resistant to the β-lactams ampicillin and carbenicillin. To overcome this, we replaced the *bla* gene on Rec$_{Ab}$ with *tet* for tetracycline resistance. Using this derivative, we easily isolated colonies with replacement of *lon* by the kanamycin cassette in strain 19606 (Fig. 2C). Thus, with the appropriate selection of resistance cassettes, this system appears to have far-reaching applications among different *A. baumannii* strains.

Deletion of an *A. baumannii oxyR* homolog results in increased sensitivity to hydrogen peroxide. To establish the relevance of this recombineering system in characterizing gene-phenotype relationships, we first focused on the well-studied response of bacteria to oxidative stress. Response to oxidative stress is important in order for bacteria to adapt and survive in the presence of biocides and antibiotics (15, 16). In *E. coli*, hydrogen peroxide triggers activation of the transcription factor OxyR, which induces expression of oxidative stress protection genes (17, 18). Deletion of *oxyR* in *E. coli* results in increased sensitivity to

hydrogen peroxide. The protein A1S_0992 from *A. baumannii* strain 17978 shows 29% identity (E value, $2e^{-34}$) to *E. coli* OxyR by BLAST bioinformatics analysis, suggesting that A1S_0992 may play an OxyR-like role in *A. baumannii*. Consistent with studies of OxyR in *E. coli*, deletion of A1S_0992 using our recombineering system produced an *A. baumannii* strain that displays increased sensitivity to hydrogen peroxide (Fig. 3, top). Ectopic expression of A1S_0992 (*oxyR*) from a plasmid complements the mutant phenotype, demonstrating that A1S_0992 is very likely an OxyR homolog that plays an important role in oxidative stress protection in *A. baumannii* (Fig. 3, bottom).

Application of Rec$_{Ab}$ to efflux pumps and biocide resistance. Recently, an efflux pump (encoded by *aceI*) responsible for resistance to the biocide chlorhexidine was discovered in *A. baumannii* 17978 (19). However, the authors were unable to delete *aceI* from the *A. baumannii* chromosome using traditional genetic tools. In contrast, we were easily able to replace *aceI* with a kanamycin cassette using the Rec$_{Ab}$ system (Fig. 2A). This demonstrates an
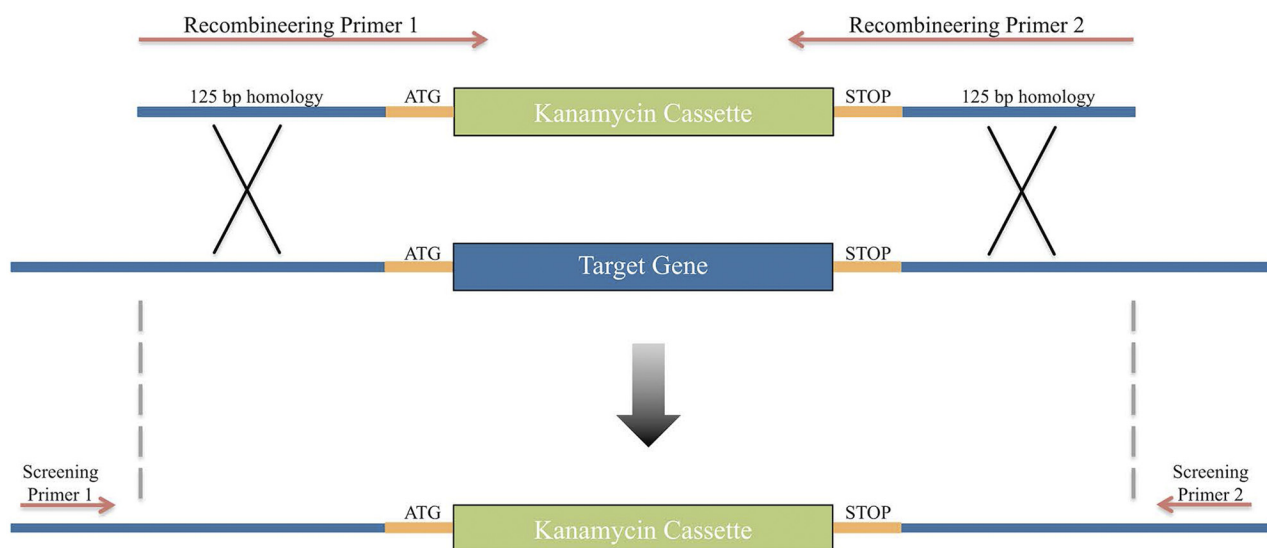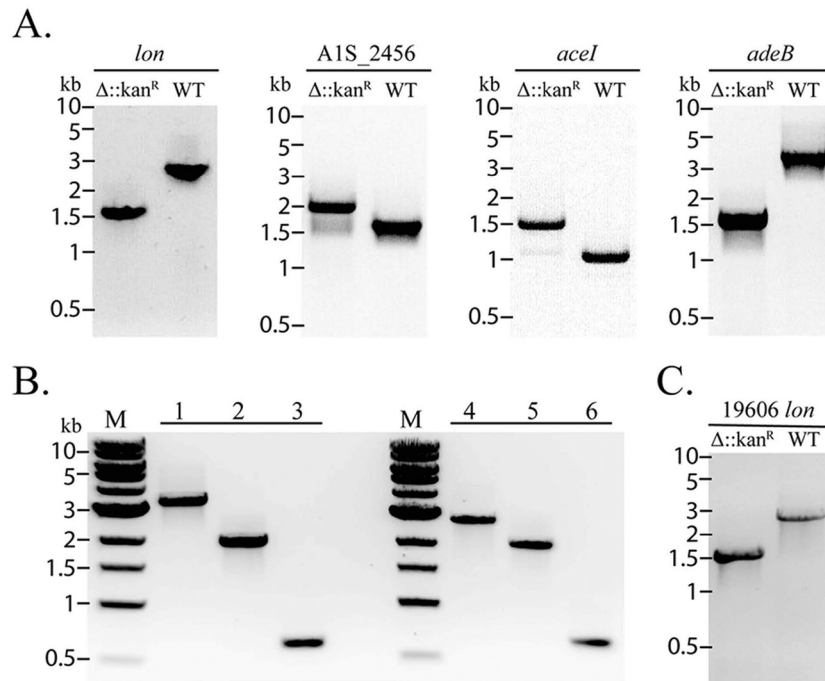

FIG 1 Schematic diagram of the one-step recombineering method. Primers containing 125-bp homology flanking the gene of interest are used to amplify the PCR product. The insert is then recombined into the genome using our Rec$_{Ab}$ system. Screening primers outside the region of homology are used to confirm insertion of the kanamycin cassette.

FIG 2 PCR verification of constructed mutants. (A) Following recombineering, PCR was used to verify the presence of the wild-type allele or the replacement of that allele with a kanamycin resistance cassette to create the mutant strain. The wild-type control is marked "WT." Shown are gene replacements with a kanamycin resistance cassette for targets *lon*, A1S_2456, *aceI*, and *adeB*. (B) Engineering of markerless mutants using the FLP-FRT recombination system. Lane 1, *adeB* wild type; lane 2, Δ*adeB*::FRT-Kan^r strain; lane 3, Δ*adeB* strain after excision of the kanamycin resistance cassette; lane 4, *lon* wild type; lane 5, Δ*lon*::FRT-Kan^r strain; lane 6, Δ*lon* strain after excision of the kanamycin resistance cassette. (C) Δ*lon*::Kan^r mutant of *A. baumannii* strain 19606. The 19606 strain wild-type control is marked "WT."

advantage in using the Rec$_{Ab}$ system over traditional mutagenesis strategies in *A. baumannii*. In their article, the authors demonstrated that removal of the *aceI* homolog in the closely related bacterium *Acinetobacter baylyi* resulted in a 2-fold decrease in resistance to chlorhexidine. In agreement with their data, we found a similar 2-fold decrease in resistance when *aceI* was deleted from *A. baumannii* 17978 (Table 3). Complementation of this mutant with expression of *aceI* from a plasmid restored chlorhexidine resistance to the parental level (Table 3).

In addition to *aceI* encoding a gene important for resistance to the biocide chlorhexidine, the AdeABC efflux pump is important for *A. baumannii* survival in the presence of chlorhexidine and has been shown to confer on *A. baumannii* resistance to a variety of other bactericidal compounds (20, 21). For example, our mutant strain carrying a deletion of *adeB* (A1S_1750) shows a 4-fold decrease in resistance to chlorhexidine. This efflux system has been previously shown to act synergistically with the AdeIJK efflux pump system in the presence of antibiotics (22). To investigate a possible relationship between AceI and AdeABC, we created a double *aceI adeB* mutant and tested its sensitivity to chlorhexidine. We observed an additive, not synergistic, effect between AceI and the AdeABC system for chlorhexidine resistance (Table 3). This suggests that these two systems act independently to mediate chlorhexidine resistance in *A. baumannii*.

Deletion of a type IV pilus retraction gene reveals importance for biofilm formation. Type IV pili were recently shown to be involved in natural transformation and twitching motility in *Acinetobacter nosocomialis* strain M2 (23, 24). The authors demonstrated that the genes involved in both assembly and the dy-

namics of this system played a role. Type IV pili have been shown to be important for biofilm formation in *Pseudomonas aeruginosa*; however, the role of this surface appendage in biofilm formation has not been studied in *A. baumannii* (25).

To explore the potential role of type IV pili in *A. baumannii* biofilm formation, we began by deleting the putative *A. baumannii* 17978 operon encoding homologs of the type IV pilus proteins PilU (A1S_0896) and PilT (A1S_0897) (see Fig. S2 in the supplemental material). PilU and PilT have been shown to be important for pilus function in *A. baumannii* and *P. aeruginosa* and have been implicated in pilus depolymerization and retraction (25). Using a crystal violet staining assay, we found that the *pilUT* mutant showed a marked decrease in biofilm formation compared to the parental strain (Fig. 4). Transmission electron microscopy showed type IV pili present on the surface of the parental *A. baumannii* 17978 strain but absent from our *pilUT* mutant (Fig. 5A and B). This supports the conclusion that fully functional type IV pili are important for biofilm production in this strain. Interestingly, a *pilT* mutant in *A. nosocomialis* strain M2 was shown to be defective for type IV-dependent natural transformation and twitching motility, indicating impaired pilus function, but it still produced visible pili on the cell surface (23). This suggests either strain variation in pilus formation between *A. baumannii* 17978 and M2 or that deleting both *pilU* and *pilT* is required to lose pilus formation on the cell surface.

Once assembled, pili undergo extension and retraction to mediate twitching motility. In *P. aeruginosa*, extension and retraction signaling is mediated by CheY-like response regulators encoded by *pilG* and *pilH*, respectively (26–28). To investigate the role of
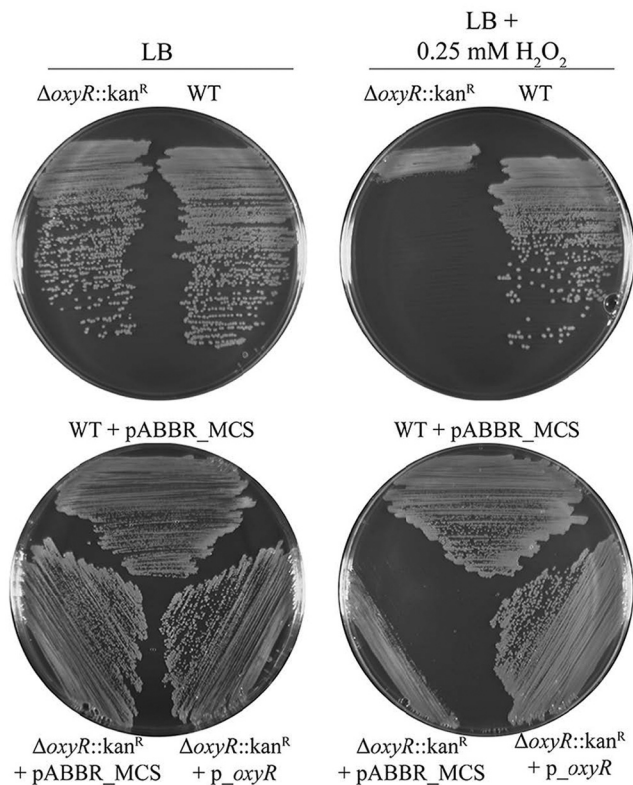
FIG 3 Deletion of *oxyR* homolog A1S_0992 from *A. baumannii* results in increased sensitivity to hydrogen peroxide (H_2O_2). The parental *A. baumannii* strain and *oxyR*::Kan^r mutant were struck on Luria-Bertani (LB) agar with or without 0.25 mM H_2O_2 (top). The *oxyR*::Kan^r mutant H_2O_2 sensitivity phenotype is recovered by ectopic expression of the *oxyR* allele from a plasmid (p_*oxyR*) but not by the empty plasmid (pABBR_MCS) (bottom).

pilus movement and signaling in *A. baumannii* biofilm production, we deleted the *pilH* (A1S_2814) and *pilG* (A1S_2815) homologs in *A. baumannii*. Deletion of the extension response regulator, PilG, had no effect on biofilm formation (Fig. 4). Consistent with this observation, pili were readily observed on the surface of the *pilG* mutant and were indistinguishable from those of the parental strain (Fig. 5D). Interestingly, deletion of the retraction response regulator encoded by *pilH* resulted in a severe decrease in biofilm formation that resembled findings for the *pilUT* mutant. Further supporting the role for type IV pili in biofilm

TABLE 3 Chlorhexidine MICs

Strain description	Chlorhexidine MIC (μg/ml)
Parental	4
$\Delta aceI$::Kan^r	2
$\Delta adeB$::Kan^r	1
Parental + empty vector	4
$\Delta aceI$::Kan^r + empty vector	2
$\Delta adeB$::Kan^r + empty vector	1
$\Delta aceI$::Kan^r complemented	4
$\Delta adeB$::Kan^r complemented	2
$\Delta adeB^a$	1
$\Delta adeB^a$ $\Delta aceI$::Kan^r	0.5

[a] *A. baumannii* 17978 carrying deletion of *adeB* after FRT-mediated excision of the kanamycin resistance cassette.
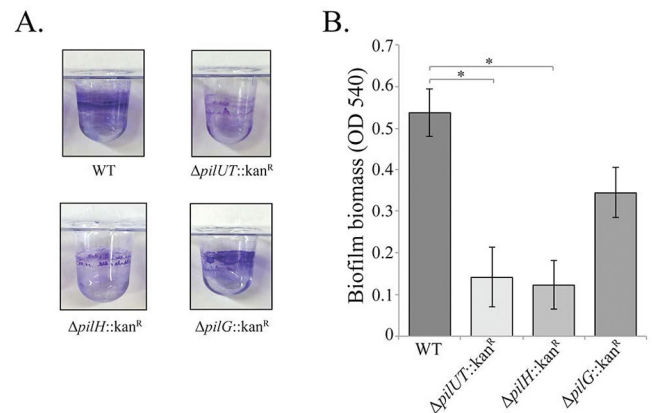
FIG 4 Biofilm formation assay of *A. baumannii* wild type, $\Delta pilUT$::Kan^r strain, $\Delta pilH$::Kan^r strain, and $\Delta pilG$::Kan^r strain, (A) Crystal violet staining of each strain in a PVC microtiter plate. (B) Biofilm formation measured by crystal violet staining for optical density at 540 nm (OD_{540}). Asterisks denote significant differences in biofilm formation (*t* test; *, $P < 0.0001$; $n = 8$).

formation, we could not identify any pili on the surface of the *pilH* mutant (Fig. 5C).

The growth rates of *pilUT*, *pilH*, and *pilG* mutants were similar to that of the wild type (WT) (see Fig. S3A in the supplemental material), showing that the observed alterations in biofilm formation were not due to growth defects. Complementation of the *pilH* mutant by ectopic expression of *pilH* from a plasmid restored biofilm formation to the same level as that of the parental strain carrying the empty vector (see Fig. S3B). Real-time quantitative PCR analysis of our complemented strain showed that ectopic expression of *pilH* closely matched endogenous expression (see Fig. S3C).

The gene downstream of *pilH* is another putative type IV pilus gene, A1S_2813, known as the *pilI* homolog. While *pilH* and *pilI* do not appear to be cotranscribed, it was also possible that replacement of *pilH* with a kanamycin cassette created a polar effect on *pilI* that could influence our results (see Fig. S2 in the supplemental material). To test this, we measured the transcript levels of *pilI* in our parental strain and *pilH* mutant and found them to be identical, indicating that our *pilH* mutation does not affect expression of *pilI* (see Fig. S3D).

DISCUSSION

While high-throughput "omic"-level approaches have dramatically increased our knowledge of *A. baumannii* genome content, we have lacked tools for rapid genetic manipulation to identify and confirm specific gene-phenotype relationships. In this study, we have identified a RecET recombinase system in the *A. baumannii* strain IS-123 that we have engineered as a tool for recombineering in *A. baumannii*. To illustrate proof of principle, we successfully deleted and replaced genes of various sizes that encoded different biological functions using PCR products containing an optimized homology of 125 bp. The ability of the Rec$_{Ab}$ system to function in two *A. baumannii* strains and the presence of homologous Rec$_{Ab}$ systems in other sequenced *A. baumannii* genomes suggest that it could be widely used in *A. baumannii*. Using the FRT-FLP system, we further demonstrated the feasibility of constructing markerless mutants, which allows stepwise deletion of multiple genes using Rec$_{Ab}$. Recombineering has catapulted the
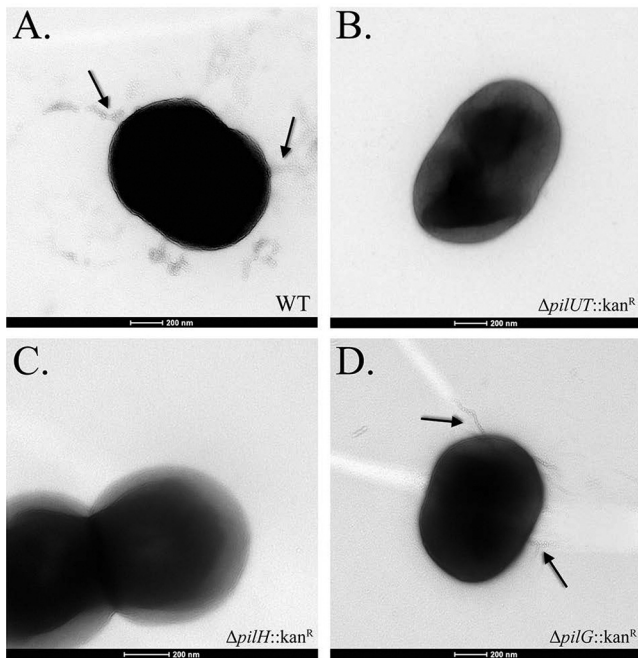
FIG 5 Transmission electron microscopy images of type IV pilus mutants of *A. baumannii* strain 17978. Black arrows specify pilus-like appendages. (A) Wild-type *A. baumannii* 17978; (B) Δ*pilUT*::Kan^r strain; (C) Δ*pilH*::Kan^r strain; (D) Δ*pilG*::Kan^r strain.

rate of genetic analysis of *E. coli*. It allowed for the creation of the widely used single-gene deletion Keio Library, solidified *E. coli* as the most tractable Gram-negative bacterium for studying genetics, and made *E. coli* the bacterium of choice for synthetic biology and biotechnology (29).

Aranda et al. recently described another method for creating stable gene replacements with PCR products (30). While similar in overall goal, our method diverges in several ways. To construct stable gene replacements, the method described by Aranda et al. uses PCR products with 500 bp of homology, which requires eight different primers and three separate PCR stages for synthesis. This method generates the desired mutants but is reported to be prone to significant illegitimate recombination between the PCR product and unknown locations in the genome. Incorporation of the PCR products at off-target locations could cause complications in evaluating phenotypes in subsequent studies. The Rec$_{Ab}$ system for stable gene replacement we describe here uses PCR products with 125 bp of homology, which requires two primers and one PCR stage for synthesis. The Rec$_{Ab}$ system has an apparent zero off-target rate. Furthermore, incorporation of the FRT sites allows for easy construction of unmarked mutants and subsequent construction of additional mutations in the same strain without the need for other selection markers.

Our understanding of *A. baumannii* biology lags far behind the clinical data outlining the emergence of this problematic hospital-associated pathogen. Genomic analysis indicates that even basic aspects of *A. baumannii* biology differ from that of model organisms like *E. coli* (31). Understanding how *A. baumannii* causes disease, persists in the clinical environment, and acquires antibiotic resistance is critical to preventing infections and managing disease. Adaptation of recombineering as a genetic technique to

quickly introduce genetic mutations into *A. baumannii* will allow a rapid expedition in the gene-phenotype analysis of this extraordinary multidrug-resistant pathogen.

Our analysis of oxidative stress protection and biocide resistance demonstrates the utility of our system for gene-phenotype analysis, especially for previously difficult genetic targets, such as *aceI*. Our analysis supports the predicted role for the OxyR homolog in *A. baumannii* oxidative stress protection and the function of AceI in chlorhexidine resistance in *A. baumannii*. Additionally, we utilized the FRT-FLP system to explore potential genetic interactions between AceI and the AdeABC efflux system by creating this double mutant. Our data suggest an additive effect, indicating that the efflux transporters play an independent, not synergistic, role in resistance to chlorhexidine.

Type IV pili are found on a plethora of Gram-positive and Gram-negative bacteria and are important for motility and attachment during biofilm development (25, 32). The ability to form a biofilm is recognized as a virulence factor in *A. baumannii* and may support its persistence in clinic (3, 33, 34). Here, we demonstrate that type IV pili are required for biofilm formation in *A. baumannii* strain 17978 and identify specify genetic determinates of pilus formation. Deletion of *pilU* (A1S_0896) and *pilT* (A1S_0897) or *pilH* (A1S_2814) results in dramatically decreased biofilm formation and an associated loss of visible pili on the cell surface. *pilU*, *pilT*, and *pilH* all encode proteins involved with pilus retraction, while *pilG*, which does not affect biofilm formation, encodes the signal for pilus extension (28). This suggests that pilus retraction may play a greater role then extension in forming pili and biofilm in *A. baumannii* 17878.

The pilus genes studied here were classified by comparison with their well-studied *P. aeruginosa* homologs (28). Comparing our results to those published for *P. aeruginosa*, we identify a similar correlation between pilus abundance and biofilm density but an opposite trend with respect to specific mutations. Agreeing with our results, PilT and PilU have been shown to impact biofilm formation in *P. aeruginosa* (23, 35–37). In contrast, however, *P. aeruginosa pilT* and *pilU* mutants present with a dense biofilm and hyperpiliated phenotype (23, 35–37). Additionally, we show that the response regulator required for pilus retraction, PilH, but not that for extension, PilG, is required for pilus and biofilm formation in *A. baumannii* 17978. Meanwhile, recent studies of *P. aeruginosa* type IV pilus mutants have confirmed that *pilG* mutants had no surface pilin, whereas *pilH* mutants were hyperpiliated (38). The correlation between pilus abundance and biofilm density agrees between *P. aeruginosa* and *A. baumannii*, but the opposite impacts of *pilU*, *pilT*, *pilG*, and *pilH* mutations suggest an important difference in pilus regulation and biofilm formation between these two bacteria.

Our dwindling supply of effective antibiotics requires development of new treatment options to combat the rapid emergence of multidrug-resistant *A. baumannii*. Information gained from the explosion in high-throughput sequencing offers many potential cellular functions that could be targeted. However, specific gene-phenotype relationships must be established to validate our choices and streamline our efforts considering the very long timeline to actual production of a therapeutic. The recombineering system here provides a previously unavailable rapid and specific means to verify gene-phenotype relationships, and successful application in oxidative stress management, biocide tolerance, and

biofilm formation demonstrates its applicability to a broad range of *A. baumannii* biology.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and antibiotics. The bacterial strains and plasmids used in this study are listed in Table 1. The *A. baumannii* Rec genes (Rec_{Ab}) were synthesized by Genewiz. The pABBR_MCS plasmid was generated as follows. The origin of replication from plasmid pWH1266 was PCR amplified and cloned into the PciI site of pBR322. We synthesized a multiple cloning site ending in a T7 transcriptional terminator and cloned this into the ZraI site of the above plasmid to create pABBR_MCS. Strains were grown in Luria-Bertani (LB) broth/agar at 37°C. The antibiotics carbenicillin (75 μg/ml) and kanamycin (25 μg/ml or 7.5 μg/ml) were added for selection as needed.

Gene inactivation using Rec_{Ab}. *A. baumannii* carrying Rec_{Ab} on pMMB67EH was inoculated into rich medium (LB) with carbenicillin to maintain the plasmid. IPTG was added to a final concentration of 2 mM, and the bacteria were grown for 3 h to mid-log phase at 37°C. After 3 washes in ice-cold 10% glycerol and concentrating 1,000-fold, 100 μl of bacteria (~10^{10} bacteria) were mixed with 5 μg of recombineering PCR product and electroporated in a 2-mm cuvette at 1.8 kV. After outgrowth in 4 ml rich medium containing 2 mM IPTG, the bacteria were pelleted, plated on 7.5 μg/ml kanamycin, and incubated overnight at 37°C. This method routinely yielded between 20 and 200 colonies.

PCR verification. Primers located outside the regions of homology were used to PCR amplify the genomic location of the genes of interest. These primers are listed in Table S1 in the supplemental material with the suffix "scr." Briefly, a freshly isolated colony was suspended in 100 μl of water with a plastic tip from which 2-μl portions were used in separate 20-μl PCRs following a 2-min preincubation at 95°C. Deletion of the gene and insertion of a kanamycin cassette subsequently yield a PCR product of a different size than when the gene is present.

Deletion of kanamycin cassette. Plasmid pKD4 was used as a template for generating a kanamycin cassette flanked by FRT sites. Gene inactivation was carried out as described above. Following confirmation and curing of the Rec_{Ab} plasmid, the mutant strains were transformed with pMMB67EH expressing the FLP recombinase and induced with 2 mM IPTG. Single colonies carrying this plasmid were isolated and cured. A loss of kanamycin resistance was observed in these colonies, which agrees with the PCR verification that the kanamycin cassette was lost.

Hydrogen peroxide sensitivity. The various strains were streaked on LB agar supplemented with 0.25 mM hydrogen peroxide. When necessary, carbenicillin was added at 75 μg/ml to agar plates to maintain plasmids; to maintain a control, plates containing only LB were also used. A fresh colony of the WT, a $\Delta oxyR$::Kan^r strain, and its complemented strain were streaked onto each plate. Plates were incubated at 37°C overnight.

MIC assay. MIC assays for chlorhexidine were performed in triplicate at least twice as previously described (39).

Biofilm assay. Biofilm formation by *A. baumannii* 17978 and mutant strains was tested by inoculating a 5-ml culture of Luria broth (LB) with a single colony and grown overnight to stationary phase. The overnight culture was then diluted with LB at 1:100, and 100 μl of the diluted culture was added to a polyvinylchloride (PVC) microtiter plate in replicates of eight. Strains were allowed to grow for 24 h at 30°C. Unbound cells were removed. Bound cells were washed twice with 200 μl sterile water and subsequently stained with 0.1% crystal violet for 15 min. Cells were then washed with 200 μl phosphate-buffered saline (PBS) three times and allowed to air dry for 30 min. Stain was then solubilized with the addition of 200 μl 95% ethanol. Next, 125 μl of solubilized stain was transferred to a new 96-well, polystyrene flat-bottom plate for optical density measurement at 540 nm using a SpectraMax Plus384 absorbance microplate reader with the software program SOFTmax Pro v. 6.2.2.

Genetic complementation of mutants. Complementation vectors for the $\Delta oxyR$::Kan^r, $\Delta adeB$::Kan^r, and $\Delta aceI$::Kan^r strains were constructed using primer sets listed in Table S1 in the supplemental material. The full-length genes with their native promoters were amplified, cloned into vector pABBR_MCS, and subsequently electroporated into their corresponding mutant strain. Complementation of $\Delta pilH$ was carried out by amplifying the full-length gene with a strong Shine-Dalgarno sequence with primer sets listed in Table S1. The gene was then cloned into pMMB67EH and electroporated into the $\Delta pilH$::Kan^r strain

RNA extraction and qRT-PCR analysis. RNAs from freshly streaked plates of wild-type and mutant strains were prepared using a Direct-zol RNA MiniPrep kit with TRI-Reagent (Zymo Research, Orange, CA). DNase treatment was carried out using a Turbo DNA-free kit (Ambion, Inc.) with RNA diluted to 10 ng/μl. Primers listed in Table S1 in the supplemental material were designed using the Primer3 software program (40) and synthesized by Integrated DNA Technologies, Inc. Final primer and RNA concentrations used were 200 nM and 30 ng, respectively. Reaction mixtures were prepared using the Kapa SYBR Fast quantitative PCR (qPCR) kit and monitored by using LightCycler 96 (Roche, Indianapolis, IN). Gene expression was normalized to *A. baumannii* 16S rRNA. Relative abundance was determined using the $\Delta\Delta C_T$ method. For each gene, reverse transcription-quantitative PCR (qRT-PCR) was performed for three biological replicates.

Transmission electron microscopy. Wild-type and *pilH*::Kan^r, *pilG*::Kan^r, and *pilUT*::Kan^r mutant strains were streaked on LB agar plates and incubated overnight at 37°C. Several bacterial colonies were transferred to 30 μl of 8% glutaraldehyde and swirled gently. Four microliters of the bacterial suspension was pipetted onto Formvar film 150 square mesh copper grids (Electron Microscopy Sciences). After the cells were allowed to adhere to the grids for 5 min, the liquid was wicked away with filter paper. The grids were then washed with molecular-grade water, and excess water was removed with filter paper. The grids were stained with 2% uranyl acetate for 1 min, and liquid was removed with filter paper. The grids were dried for 1 h before storing for later imaging. Images were obtained on an FEI Tecnai transmission electron microscope at the Microscopy and Imaging Facility at the University of Texas at Austin.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01313-14/-/DCSupplemental.

Figure S1, TIF file, 0.7 MB.
Figure S2, TIF file, 0.8 MB.
Figure S3, TIF file, 1.1 MB.
Table S1, DOC file, 0.1 MB.

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